PHENYLKETONURIA

and

allied

metabolic
diseases
The discovery that one form of mental retardation, phenylpyruvic oligophrenia, was in some way related to a genetic biochemical abnormality understandably became a remarkable scientific medical milestone. The hopes of all concerned were again raised when it was reported that a reduction in the elevated phenylalanine in the blood of phenylketonuric patients by dietary means may prevent the severe catastrophic retardation in mental, motor and psychosocial development. A critical and objective review of the experience of the past fifteen years now indicates that the problem may not be as simple as was originally thought. We have now found that the spectrum of the basic and clinical aspects of the disease is wide. We have found that certain children having transitory or permanent elevations in the blood phenylalanine may be confused with children who have classical phenylketonuria. These children may not require therapy.

Unfortunately the low phenylalanine dietary treatment does not provide assurance that mental developmental limitations can be prevented in all cases even though treated from early infancy. Further reports have appeared which indicate that inappropriately monitored dietary treatment leads to growth retardation, anemia, malnutrition and even death. It has been known for some time that certain phenylketonuric children in spite of persistence of their biochemical abnormalities have near normal or even normal intelligence and behavior. The results of more thorough studies on family constellations suggest that the number of these mild or untreated cases may be a far greater segment of the clinical spectrum of the disease.

Confronted with these problems it is now necessary to review the problem of phenylketonuria from the fundamental, the practical therapeutic, and the social and legislative aspects. The conference on Phenylketonuria and Allied Metabolic Diseases held in Washington, D.C. on April 6, 7, and 8, 1966 is an attempt to look critically at several important aspects of the problem. The reported variations in the clinical expression of the disease make it necessary to critically review the fundamental aspects of the primary hepatic enzyme limitation. The increasing number of reports concerning non-phenylketonuric causes of hyperphenylalaninemia make it imperative to redefine not only the basic variations in the primary enzymatic defect, but also make it necessary to clarify the significance of the biochemical screening procedures now so widely employed. It is essential to define more clearly the diagnostic criteria for the disease.

The conflicting and confusing aspect of the reports of the effectiveness of dietary treatment in the prevention of mental retardation, as well as the increasing number of reports of the dangers of such diets, make it necessary to objectively and critically review the value of the dietary treatment for phenylketonuria.

Our hopes concerning the effectiveness of treatment were raised so high as to encourage the development of widespread State legislative programs for diagnosis. Understandably our desires to prevent at least this one form of mental retardation led to nationwide action to implement diagnostic and therapeutic programs. In the face of conflicting evidence it is now imperative that all aspects of the problem be reviewed.

Hopefully this report of the proceedings will define more clearly the many aspects of the problem, will critically evaluate our present position and define directions to be taken for the future. Phenylketonuria represents only one disease which can be detected by biochemical procedures. The general principles evolved at this conference may serve to give direction to future programs concerning other biochemical disorders associated with faulty growth and development or with mental retardation in infants and children.

We are most grateful to the Children's Bureau for providing a grant to the Department of Pediatrics of the University of Minnesota to make this conference possible. Additional support was provided by the Minneapolis Association for Retarded Children (MARC). We are most appreciative for the splendid help provided by Miss Iris Goeschel, Principle Secretary, Department of Pediatrics, and by Mrs. Sigrid Willgohs for careful retyping and preparation of the discussions and manuscripts.

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PHENYLKETONURIA AND ALLIED METABOLIC DISEASES

Proceedings of a Conference held at
Washington, D.C. April 6-8, 1966

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I would like to begin with a short introduction concerning the biochemical aspects of phenylketonuria relating to phenylalanine metabolism. The finding of phenylpyruvic acid in urine and of phenylalanine in blood made it obvious that there was some defect in phenylalanine metabolism. About sixteen years ago we were able to demonstrate the presence of the enzyme phenylalanine hydroxylase in the liver of many animal species, including man. It soon became obvious that this was the enzyme responsible for the major route of phenylalanine metabolism and that the subsequent metabolism of phenylalanine depended on the prior conversion to tyrosine. It also became clear that the enzyme activity was found only in the liver.

Shortly thereafter, with Dr. Samuel Bessman we administered radioactive phenylalanine to phenylketonuric and nonphenylketonuric patients and to normal animals. As shown in Table 1, when phenylalanine-14C was administered to normal patients and animals there was appreciable conversion to tyrosine and the ratio of tyrosine to phenylalanine was relatively high. On the other hand, when phenylalanine-14C was given to phenylketonuric patients there was little conversion and the ratio of tyrosine-14C to phenylalanine was very low. However, there was one important observation which we subsequently verified, that there was some conversion of phenylalanine to tyrosine by the phenylketonurics. This was very small but definite. Nevertheless even without direct enzymatic analysis it was quite obvious that there was a considerable block in the conversion.

Following these studies Dr. Jervis managed to obtain liver biopsies from phenylketonurics and normals and showed that there was a marked diminution in the enzymatic conversion of phenylalanine to tyrosine. However, work in our laboratory showed that hydroxylation of phenylalanine to tyrosine required two enzymes and unknown cofactor(s). In subsequent studies in our laboratory and in the laboratories of Dr. A. Meister it was shown that only one of the two enzymes was lacking in liver from phenylketonurics. From these studies it appeared that cofactors and enzymes related to cofactors were adequate but that the liver phenylalanine hydroxylase was deficient. Shortly thereafter, Dr. S. Kaufman identified the cofactor as a reduced pteridine and showed convincingly that the pteridine was not missing from phenylketonuric liver.

From all these studies it was clear that in PKU there is a deficiency in liver phenylalanine hydroxylase. The question remained as to whether there was an absolute block in the liver enzyme.

Table 1.--Radioactivity in plasma protein phenylalanine and tyrosine following administration of phenylalanine-14C

<table>
<thead>
<tr>
<th>Patient</th>
<th>Phenylalanine c.p.m. per µ mole</th>
<th>Tyrosine c.p.m. per µ mole</th>
<th>Ratio Tyr/Phen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control - 1</td>
<td>7.8</td>
<td>2.2</td>
<td>0.29</td>
</tr>
<tr>
<td>Control - 2</td>
<td>21.0</td>
<td>4.7</td>
<td>0.22</td>
</tr>
<tr>
<td>PKU - 1</td>
<td>55.0</td>
<td>0.9</td>
<td>0.015</td>
</tr>
<tr>
<td>PKU - 2</td>
<td>33.0</td>
<td>0.7</td>
<td>0.021</td>
</tr>
</tbody>
</table>
or only a partial defect. Since there was a small conversion of phenylalanine to tyrosine we investigated the nonspecific microsomal aromatic hydroxylase in livers of patients with PKU and found this enzyme to be normal. However, since this enzyme could not hydroxylate phenylalanine it still didn't answer the question about the degree of the defect in the specific phenylalanine hydroxylase. I will return to this point later.

Since the primary lesion in PKU has turned out to be a liver defect and PKU involves defects in the central nervous system I should like to discuss some of the secondary effects that can arise from a diminution in the metabolism of phenylalanine, particularly those which can give rise to interaction with known biochemical mechanisms in the central nervous system. All of us are aware that in phenylketonurics, as a result of the inability to metabolize phenylalanine, the blood levels of phenylalanine are 10 to 50 times higher than normal and the tissue levels are correspondingly high. As a result of these very high concentrations phenylalanine can either inhibit certain enzymes in a competitive manner or itself become a substrate of enzymes designed for other substances. The significance of the last situation is important. The $K_m$ of phenylalanine for certain other reactions is so high as to have little significance under normal conditions. However, in patients with PKU phenylalanine concentrations in tissues become so elevated that reactions with such high $K_m$ values do take place. I will discuss a few of these, again keeping in mind the central nervous system.

The oversimplified diagram in figure 1 represents the blood-brain barrier. Most of you know about the barrier into the brain to nonessential, water-soluble, charged molecules. An acidic substance will not penetrate because it is charged. But this same area of the brain has within it specific catalytic mechanisms for taking up essential metabolites that otherwise have the same physical properties as the ones that are withheld. It is known that catalytic mechanisms are available for a variety of metabolites including L-amino acids.

Figure 2 summarizes the findings which prove that there is a catalytic mechanism for uptake of amino acids by brain. It is rapid and selective so that L-tyrosine is taken up, L-tyramine is not. It is stereospecific in that the D-isomers penetrate poorly, if at all, compared

Figure 1. Diagrammatic presentation of blood-brain barrier indicating its role in the regulation of amino acid uptake into the brain

Blood

Barrier to non-

essential water-
soluble, charged
molecules

Brain

Specific catalytic mechanisms for taking up L-amino acids and other essential metabolites
Figure 2. Evidence for catalyzed uptake of amino acids from blood into brain

1) Rapid  
2) Selective – Congeners not taken up  
3) Stereospecific – L-isomers much better than D-isomers  
4) Energy requiring – studies with brain slices  
5) Competitive – related amino acids inhibit uptake

with the L-isomers. Most important, for our immediate discussion, is that it is highly competitive and that related amino acids will compete with one another for uptake. There are groups of amino acids that apparently take part at the same transport site and there seems to be one site for the aromatic and long-chain aromatic amino acids and another for the neutral amino acids. The aromatic amino acids compete very effectively with one another. One can, by manipulating the concentration of leucine to make it 20 times higher than that of tyrosine, inhibit the uptake of tyrosine by over 90 percent. Phenylalanine inhibition studies present some problems because phenylalanine when administered to animals is converted to tyrosine by the liver. The effects of large amounts of circulating phenylalanine may be seen from the following experiments. When one administers tracer amounts of phenylalanine-14C and measures radioactivity in tyrosine the ratio of tyrosine in brain to tyrosine in plasma rises to high values. On the other hand, when one administers huge amounts of carrier phenylalanine along with the tracer dose of phenylalanine-14C there is still conversion of tyrosine but it is lower and the ratio of tyrosine in brain to plasma is much lower than without the carrier phenylalanine. One can inhibit tyrosine uptake with congeners of phenylalanine. These behave the same way as phenylalanine and have the big advantage in that they are not converted to tyrosine. With all of them the uptake of tyrosine into the brain is markedly inhibited. I don't mean to imply that competition affects only tyrosine transport. The same is undoubtedly true for leucine and tryptophan.

A pictorial presentation of competition for amino acid uptake into brain is shown in figure 3. To the left we see the situation for aromatic amino acid uptake into the brain under normal conditions. Under such conditions there are equivalent amounts of phenylalanine and other amino acids and each has an equal opportunity to be taken up. If, as shown on the right, the system is loaded with huge amounts of phenylalanine, this reduces the opportunity for uptake of other amino acids which utilize the same catalytic site. This effect has been well-documented in many studies on transport of amino acids into brain. Whether inhibition of uptake by brain secondarily limits the metabolism of tyrosine, tryptophan and other amino acids remains to be determined. It appears from the tissue levels of subsequent metabolites that this may be the case.

I'd like to proceed to other reactions and leave the area of transport. In figure 4 are shown two
Alternate routes of phenylalanine metabolism which increase when the major route, conversion to tyrosine, is blocked. Both alternate routes occur normally to a very minor extent because of the high $K_m$ values for phenylalanine for these enzymes and competition with tyrosine and tryptophan. As a result of the very marked elevation of phenylalanine in PKU these pathways now become of major significance. The one on the left, involving transamination to phenylpyruvic acid, is the one which is responsible for the product which is associated with the disease. This pathway gives rise first to phenylpyruvic acid then to phenyllactic acid, phenylacetic acid and ortho-hydroxyphenylacetic acid. Although these are interesting metabolites and can be detected in the urine they are to my knowledge without effect on the central nervous system. As a pharmacologist I don't consider them to be active compounds, although they could conceivably produce effects in some unknown manner.

The second metabolic route leads to phenylethylamine, which can be quite active on the central nervous system. It is formed by a nonspecific aromatic amino acid decarboxylase which is not only present in liver and kidney but also in the brain. The similarity between phenylethylamine and amphetamine is shown below.

![Diagram of alternate routes of phenylalanine metabolism]

Obviously the presence and continued synthesis of such a substance within the central nervous system could have pharmacologic significance. We have been able to show that greatly increased amounts of phenylethylamine are produced in phenylketonuria. As shown in Table 2 normals excrete less than 4 $\mu$g per hour and this is elevated 50- to 200-fold in phenylketonurics. It should be noted that the excretion of phenylethylamine in urines of patients is low unless one administers a mono-
amine oxidase inhibitor to prevent oxidation to phenylacetic acid (see fig. 3). Even with mono-
amine oxidase inhibitors the excreted values probably represent minimal values and con-
ceivably ten times as much may be formed in
the body of phenylketonurics, much of it within
the central nervous system. Admittedly, one
cannot say that phenylethylamine is the active
agent in PKU, but it certainly deserves serious
consideration and study.

I'd like to turn now to tyrosine metabolism
and consider the effects of phenylalanine thereon.
Figure 5 summarizes most of the known path-
ways of metabolism of tyrosine in animal tissues.
Quantitatively, conversion along the homogentisic
acid pathway is the major route of tyrosine

Table 2.—Urinary excretion of phenylethylamine
by normals and patients with PKU

<table>
<thead>
<tr>
<th>Patients</th>
<th>Urinary phenylethylamine µg per hour</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (6 subjects)</td>
<td>4</td>
</tr>
<tr>
<td>PKU - 1</td>
<td>190</td>
</tr>
<tr>
<td>PKU - 2a</td>
<td>450</td>
</tr>
<tr>
<td>PKU - 2b</td>
<td>810</td>
</tr>
</tbody>
</table>

All patients were given a long-acting monoamine
oxidase inhibitor 24 hours prior to testing.
metabolism. This system is limited to the liver. Tyrosine transaminase, which is involved in the first step in the pathway, is probably the same enzyme which forms phenylpyruvic acid from phenylalanine. Tyrosine may be considered the important branch point for a large number of synthetic reactions leading to thyroxine, proteins, melanin, etc. It should be noted that tyrosine is also derived from the proteins of the body and occurs in large amounts in the diet. There is normally sufficient tyrosine from these sources to make phenylalanine hydroxylase not essential as a source of tyrosine. However, it certainly is an essential enzyme from the standpoint of degrading ingested phenylalanine which otherwise accumulates to the extent observed in phenylketonuria.

I would like to discuss one particular pathway of tyrosine metabolism; the one leading to norepinephrine. The very first step in this pathway is hydroxylation to DOPA. The enzyme which catalyzes this step is tyrosine hydroxylase which occurs only in the sympathetic nervous system and in the brain. It is particularly active in brain. Phenylalanine can also be converted by this enzyme to tyrosine. This has been shown in vitro preparations. Therefore, although hydroxylation of tyrosine to DOPA is the major function of tyrosine hydroxylase, when phenylalanine levels are sufficiently high we may have conversion of phenylalanine to tyrosine in sympathetic tissue and brain. This conversion may actually represent the small amount of conversion of phenylalanine to tyrosine that is found in phenylketonurics. When phenylalanine is incubated with purified tyrosine hydroxylase, tyrosine and DOPA are formed. The K_m for phenylalanine is only one-third that for tyrosine but the rate is one-twentieth. So we have another interesting phenomenon. If we use tyrosine as substrate and phenylalanine as an inhibitor we find marked inhibition of the conversion of tyrosine to DOPA. With phenylalanine at 10^-3 M and tyrosine at 5 x 10^-5 M, concentrations which are observed in patients with phenylketonuria, we found as much as 80 to 90 percent inhibition of tyrosine hydroxylase. Interestingly enough, since phenylalanine is a substrate as well as an inhibitor, complete inhibition is not attained. Instead phenylalanine becomes limiting with respect to noradrenaline formation. This is true in vitro systems and must be investigated in vivo.

Two aspects of phenylalanine metabolism require investigation. First, does the small amount of conversion of phenylalanine to tyrosine, which occurs in phenylketonurics, take place through catalysis by tyrosine hydroxylase? In order to find that out we plan to administer a-methyl-tyrosine, a potent inhibitor of nervous system tyrosine hydroxylase, to phenylketonurics. Together with Dr. Albert Sjoerdsma we have already given a-methyl-tyrosine to other patients and have shown that it is possible to inhibit the formation of noradrenaline by as much as 75 percent. In one or two patients inhibition was as much as 90 percent. a-methyl-tyrosine does not inhibit liver tyrosine hydroxylase. In a phenylketonuric, therefore, inhibition of the residual conversion of phenylalanine to tyrosine (about 5 percent of normal) would prove that the conversion is due to the action of tyrosine hydroxylase in the peripheral and central nervous system. If so, then the genetic defect in the liver phenylalanine hydroxylase is absolute.

Secondly, does the inhibition by phenylalanine of tyrosine hydroxylase bring about a limitation of noradrenaline formation? The last few speculations concerning tyrosine hydroxylase were made possible because we recently isolated this enzyme and were able to characterize it. Tyrosine hydroxylase is interestingly similar to phenylalanine hydroxylase in its cofactor requirements and in terms of having an affinity for phenylalanine.

I want to emphasize though, that all the above observations are mere speculations at this time and are not presented as definite factors in the symptomology of PKU. However, it is worthwhile summarizing three of the metabolic effects because each has been shown to be capable of pharmacologic actions in in vitro or in whole animal studies.

1. Increased phenylalanine may inhibit uptake of other essential amino acids into the central nervous system. As a result, synthesis of protein, noradrenaline, serotonin and perhaps other substances may be reduced.

2. Increased phenylalanine is undoubtedly converted to phenylethylamine in the central nervous system. This compound produces amphetamine-like actions experimentally.
3. Increased phenylalanine may inhibit the formation of noradrenaline centrally and peripherally by inhibiting tyrosine hydroxylase. Inhibitors of tyrosine hydroxylase have been shown to be sedative in man and to produce withdrawal type symptoms of an excitatory nature when dosage is discontinued.

The three metabolic factors listed above and others may all contribute to the symptomatology of PKU. It is most likely that PKU is not the result of a single metabolic change. Probably many of the distortions in metabolism play a role in the overall disorder. Some may produce chronic and irreversible effects manifested by lowered IQ. Others may produce acute and reversible symptomatology. Recognition of the many possible causes for the symptomatology in PKU may help explain observed reaction to therapies and may suggest other approaches to the improvement and management of such patients.

REFERENCES


DISCUSSION

DR. WAISMAN: What is the effect of varying levels of tryptophan on tyrosine hydroxylase?

DR. Udenfriend: Tryptophan only minimally inhibits tyrosine hydroxylase. Phenylalanine has a much more marked effect. Interestingly enough—the tryptophan hydroxylase which occurs in many tissues and has been isolated from brain is a similar hydroxylase but our knowledge about its properties is not as great. However, Hayaisi in Japan and Gaull and Green in this country have shown enough of its properties so that we know that it requires a pteridine cofactor. Dr. Lovenberg, one of my former graduate students, isolated a tryptophan hydroxylase from mast cell tumors of mice and found to his surprise that it could hydroxylate both phenylalanine and tryptophan. Whether this is also characteristic of the brain enzyme we don't know. With the mast cell enzyme there was mutual inhibition of tryptophan hydroxylation by phenylalanine and of phenylalanine hydroxylation by tryptophan. Whether this will be true of the serotonin-forming enzyme we don't know, but if serotonin formation is blocked by phenylalanine we'd have another problem in phenylketonuria.

DR. WAISMAN: Were all of these experiments in vitro?
DR. UDENFRIEND: No, we have actually given phenylalanine to rats and removed the tissues and shown that the enzyme was inhibited when we administered about 300 mg./Kg of phenylalanine.

DR. WAISMAN: Was there any relation to blood values of phenylalanine?

DR. UDENFRIEND: Yes, definitely.

DR. ANDERSON: Did the parahydroxyphenolic acids have any action on the tyrosine hydroxylase?

DR. UDENFRIEND: No.

DR. SCRIVER: I want to ask if you thought the phenylethylamine could have come from the intestinal lumen.

DR. UDENFRIEND: No! This was studied quite carefully. First of all we can demonstrate phenylethylamine in the brain of animals. We can also show its presence in brain during administration of antibiotics. If one gives antibiotics to patients the amount of amines excreted in the urine increases. These amines are produced by the tissues and one can actually prepare enzymes from patient or animal tissues and show production of amines from amino acids. Phenylethylamine, tryptamine, tyramine; all the amines which are substrates of the decarboxylase enzyme, are found in the brain when a monoamine oxidase inhibitor is administered.

DR. WAISMAN: Do all these amines cross the blood brain barrier?

DR. UDENFRIEND: They don't have to because they are synthesized in the brain. However, a substance like phenylethylamine is structurally suited for penetrating into the brain. At the pH of the blood it is not charged. It is also lipid soluble so that it could penetrate. However, it is destroyed so rapidly in the absence of monamine oxidase inhibitors that only in the tissues where it is made could I conceive of its having any pharmacological activity.

DR. WOOLF: When you gave monoamine oxidase inhibitors to phenylketonurics, did you notice any pharmacological effect other than you would have seen in a normal?

DR. UDENFRIEND: There was nothing obvious that we could see during acute studies over a period of three days or so. We also attempted to detect increased amounts of these amines in heterozygotes. We thought this test might be more sensitive than the phenylalanine loading test. I would say it is no better and no worse. If phenylalanine accumulates amines also accumulate, but the same statistics result.

DR. KANG: In phenylketonurics obviously the transaminase is adaptively increased. Is this enzyme nonspecific for phenylalanine alone?

DR. UDENFRIEND: Recalling Dr. LaDu's work, it was quite apparent that phenylalanine is transaminated by the same mechanism normally responsible for tyrosine transamination. I think that the nonspecificity is very important. This situation pertains to all the enzymes involved in aromatic amino acid metabolism. Tyrosine transaminase can act on phenylalanine and related amino acids. The enzyme which forms ortho-hydroxyphenylacetic acid from phenylpyruvic acid is the same as the one which forms homogentisic acid from p-hydroxyphenylpyruvic acid. The interaction of phenylalanine and tyrosine on tyrosine hydroxylase is another example.

I think there is a great deal of enzymatic nonspecificity. In my generation we were taught that enzymes are highly specific substances. But as we look around more and more we find that even the enzymes involved in coding for protein synthesis are only specific insofar as they have a much higher affinity for one substrate than they do for others. If a disproportionate amount of a related substance is present it can utilize the enzyme. This can occur even in genetic coding.
AMINE METABOLISM IN PHENYLKETONURIA

SAMUEL P. BESSMAN, RAUL WAPNIR and DORA DUE
presented by SAMUEL P. BESSMAN

Thank you, Dr. Jervis. It is a pleasure to be here. May I take this opportunity to comment on the last paper?

Sid Udenfriend's story is neat
With amino compounds replete
But how can we tell
Within the brain cell
Which wins when they all must compete?

Disease is not a digital event. It is a distortion in a complex pattern of which we haven't yet understood the real import. We have all been interested in how this distortion peripherally manifests itself. This is the clinical syndrome. The problem is not simply that there is a focus of distortion. The widespread results of this distortion in the metabolic net tend to conceal the initiating cause. There are many individuals who have metabolic distortions which we can recognize, but they are not metabolically embarrassed by them, and this is the important point.

If we can find the reasons for the clinical difference between individuals who have the same chemical manifestations, we may understand the relevance of chemical manifestations to disease process.

Let us review briefly the metabolic abnormalities in PKU. Figure 1 shows the metabolism of the non-ring substituted acids, deriving from phenylalanine. Certain compounds are rather characteristic of human metabolism; for example, phenylacetyl glutamine is synthesized only by the human and the chimpanzee. We can find no particular significance in this, but we have always had the habit in biochemistry of assuming that unless we could see some obvious physiological role of a compound it must be vestigial or insignificant. Is phenylacetyl glutamine important to the mind of the human being and chimpanzee and not important to the mind of the rat, the monkey, and all other animals?

A more complicated picture emerges when the ring gets substituted, figure 2. Various compounds have been proposed as intermediates; for example, orthotyrosine. There are enzymes for a degradative pathway for orthotyrosine in the brain to produce various intermediates including orthotryptamine and orthohydroxyphenylacetic acid. But we find from Udenfriend's group and Armstrong's group that orthohydroxyphenylacetic acid can form from phenylpyruvic acid. This is an example of biochemical alternatives. There are three alternative pathways for the formation of phenylpyruvic acid; transamination of L (+) phenylalanine, oxidation of L (+) and of D (-) phenylalanine. Phenyllactic acid is formed by reduction of phenylpyruvic acid, probably by lactic dehydrogenase. This enzyme was the first to destroy the illusion that enzymes are usually specific. There are about 30 substrates for this enzyme, and it is not yet clear what physiologic role this enzyme may play.

The phenylalanine pathway, however, is relatively specific. It is directly related to the enzyme which is defective in PKU, phenylalanine hydroxylase. More recent findings suggest that perhaps the phenylalanine abnormalities are not the major problem in phenylketonuria. Dr. Tada, in our laboratory, found that PKU patients on a normal diet excrete excess amounts of indican. On a low phenylalanine diet they excrete less indican. Inability to oxidize phenylalanine, therefore, affects the excretion of indican, figure 3.

It was shown by Pare and Sandler that in PKU there is an abnormality in the 5-hydroxylation of tryptophan as shown by diminished excretion of 5-hydroxyindole acetic acid. These findings make it likely that there are a series of abnormalities in metabolism of tryptophan. In regard to the indican excretion it was said that children with PKU might have different intestinal activity. They might absorb tryptophan poorly or might synthesize more indole than normal from tryptophan because they have peculiar gut organisms, or they might be constipated.

There is no evidence that there are peculiar intestinal organisms in PKU, nor is there a
Figure 1. Metabolism of phenylalanine with unsubstituted benzene ring

A → B → C

PHENYLALANINE → PHENYLPYRUVIC ACID → PHENYLLACTIC ACID

D → E → F

PHENYLETHYLAMINE → PHENYLACETIC ACID

Figure 2. Pathways of metabolism of phenylalanine with substituted benzene ring

H → A → G

O-TYROSINE → PHENYLALANINE → TYROSINE

J → B → I → P → L

O-TYRAMINE → PHENYLPYRUVIC ACID → M-TYROSINE → P-HYDROXYPHENYL PYRUVIC ACID → TYRAMINE

M → N → K → R → Q → O

O-HYDROXYPHENYL ACETIC ACID → M-HYDROXY PHENYL ACETIC ACID → M-TYRAMINE → P-HYDROXYPHENYL LACTIC ACID → HOMOGENTISIC ACID → P-HYDROXY PHENYL ACETIC ACID
history of constipation in the cases we studied. We investigated the indican abnormality by studying the metabolism of indole administered orally. Figure 4 shows the normal pathway of formation of indican. Only the conversion of tryptophan to indole occurs in the gut. The subsequent steps all take place in the liver. We gave two children indole by mouth and the indican in their blood increased in a typical tolerance curve which has been repeated with a large number of children (figure 5).

The lack of appearance of indole in the blood in normals confirmed what had been known for a long time about adults, that the clearance of indole by the liver is almost 100 percent. This can be used very nicely, incidentally, as a test of whether or not there is a shunt in the hepatic circulation.

Figure 6 shows that phenylketonurics don't metabolize indole as efficiently as normals. When they are given indole by mouth, their liver cannot clear indole from the blood at the same rate as the normal child. This cannot be due to abnormal gut organisms, for the transport of indole through the gut continues but the liver just doesn't extract it, as shown by the rise in blood indole which they all manifest.

The second abnormality seen in these curves is that the indican level rises twice as high as in normal children. Apparently the clearance of
indican from the blood of phenylketonurics is also abnormal. This, too, has nothing to do with the gut organisms. The increased level of indican occurs even though the net conversion of indole to indican is smaller than normal.

Both of these phenomena suggest that the transport of indole compounds as exemplified by indole and indican, is perhaps abnormal in these children. Therefore transport not only of phenylalanine, as shown by Udenfriend's group, but also of indole and indole compounds is probably abnormal. We haven't completed experiments on the effect of phenylalanine on the transport indole, but there seems to be an abnormality here.

This abnormality of tryptophan metabolism warns us that in PKU the general metabolic network is distorted, remotely and remarkably.

These results with indole and the reports of excess indole acetic aciduria and indole lactaciduria from Armstrong's laboratory, and of excessive excretion of indole pyruvic acid made us investigate several other steps in the metabolism of tryptophan. It soon became clear that these children don't excrete as much of some intermediates of the degradation of tryptophan as normal people (figure 7).

Is the mental lesion in phenylketonuria a manifestation of excess or is it a disease of deficiency of some very important intermediates? The answer to this question is very important for therapy.

N-methylnicotinamide is on the pathway of synthesis of NAD, and NADP, co-enzyme I and co-enzyme II. This intermediate is diminished in the urine of patients with phenylketonuria. Kynurenic and xanthurenic acids are also diminished. Are these essential to normal brain growth and function?

I think the organism evolved over a longtime with these intermediates and they must have some significance. What the significance is I do not know. They do not comprise a major pathway of degradation, but degradation in biochemistry is negative. It is like heat production. It is something you don't talk about because it appears to represent a negative function.

The abnormalities of degradation of tryptophan which occur in PKU seem to be caused by phenylalanine metabolites which involve
The heavy bars indicate excretion and the light bars diminished excretion in phenylketonuria.

Figure 7. Pathways of tryptophan metabolism

The large number of compounds present in excess and in subnormal amounts in PKU might be insignificant individually but might have very large net effects. Occasionally an experiment of nature permits us some insight into a question of this kind. We found a family of five children of whom three had chemical phenylketonuria. All three had phenylpyruvic acid in the urine, high blood phenylalanine, and only one was mentally retarded.

Figure 11 shows phenylalanine tolerance tests on Owen, Brian, and Robert. Owen was found first at the age of 4, diagnosed as mentally retarded, and then tested for PKU. He is the youngest, about six years old now, and he has an IQ of 40 to 50.

Brian and Robert were found when they tested the whole family. Robert, at the age of 9,
has an IQ ranging between 90 and 97. Brian, now aged 13 always tested around 95-99.

The phenylalanine tolerance tests are based on an oral dose of 100 milligrams per kilo. There are four different tests for each child, figures 11 and 12. In general they have the same pattern for each child. Furthermore, the first two seem to cluster, and the second two seem to cluster. It is interesting that the three phenylketonuric children are boys, and the two normal siblings, both of them apparently heterozygotes, are girls. Only the father has a heterozygote type curve. This shows how unreliable the determination of heterozygosity may be, as many investigators are now finding.

These four controls revealed no trend in phenylalanine tolerance curves on treatment with folic acid for 8 months. The controls were studied simultaneously to show that there was no technical or procedural change which could account for the flattening out of the tolerance curves in the PKU patients. There was no change in IQ of the PKU patients during this study so we can say that a rather considerable change in tolerance to phenylalanine produces no change in IQ.
Figure 10. Additive effects of small concentrations of various indole materials which are inhibitors of tryptophan pyrrolose

ADDITIONAL EFFECT OF THE MIXTURE OF INHIBITORS ON TRYPTOPHAN PEROXIDASE

<table>
<thead>
<tr>
<th>INHIBITOR</th>
<th>CONCENTRATION</th>
<th>INHIBITORY EFFECT %</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADNDOLE</td>
<td>1.56 x 10^-4</td>
<td>11.0</td>
</tr>
<tr>
<td>B PHENYL-</td>
<td>1.56 x 10^-4</td>
<td>9.0</td>
</tr>
<tr>
<td>PYRUVIC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACID</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C OH PHENYL-</td>
<td>1.56 x 10^-4</td>
<td>6.5</td>
</tr>
<tr>
<td>ACETIC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACID</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D SKATOLE</td>
<td>1.56 x 10^-4</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MIXTURE OF A, B, C, D</td>
<td>6.25 x 10^-4</td>
<td>36.0</td>
</tr>
</tbody>
</table>

Figure 13 shows the phenylpyruvic acid excretion before and after phenylalanine loading. This perhaps explains what the folic acid did by showing that on prolonged treatment with folic acid the ability of the patient to excrete the dose as phenylpyruvate increases.

Each curve consists of four values. Minus two days, minus one day, one day after, and two days after the administration of a load of phenylalanine. These phenylpyruvate curves emphasize the lack of relation of the chemical test to IQ.

Valerie Cowie first reported normal mentality in an individual with phenylketonuria and said that the excretion of phenylpyruvic acid in this individual was lower than expected. Owen excretes less than the other boys even when given a load. Unfortunately, he is the one who has the IQ of 47. The second test on each boy shows an increased increment of phenylpyruvate after loading. The third test is also significantly higher.

At the same time we see no significant changes in excretion of phenylpyruvate by other members.

Figure 11. Phenylalanine tolerance curves on 3 siblings with PKU

The solid curves were done before, and one week after, folic acid therapy; the dotted curves after 1 and 6 months of therapy. In all three cases, the dotted curve which begins lowest in fasting value represents the last curve done.
Figure 12. Phenylalanine tolerance curves on two unaffected siblings, and the father and mother, under the same conditions as the affected siblings in Figure 11.

Note that there is no constant effect of folic acid and that each set of tolerance has a shape which is characteristic for the individual.
of the family. Apparently there is a progressive increase in the ability of PKU patients to excrete phenylalanine in the urine as phenylpyruvic acid. Prolonged folic acid therapy has made the children with PKU "tolerant" to phenylalanine by making them able to excrete phenylpyruvic acid better. There was no effect of this change in tolerance to phenylalanine on the IQ of any of the boys.

Figure 14 shows that indole lactic acid excretion is not affected significantly by phenylalanine, except in Owen, who excretes a little more than Brian and Robert. This agrees with other work which relates high excretion of indole lactic acid to phenylketonuria. The other boys show no effect of phenylalanine on indole lactic acid excretion.

Figure 15 shows 5-hydroxyindoleacetic acid excretion, which is a measure of serotonin metabolism. Owen has the highest control 5-hydroxyindoleacetic acid excretion and when given phenylalanine this drops and then goes up, which is what you'd expect, possibly because phenylalanine inhibits tryptophan metabolism. There is no effect on Brian and Robert.

On tryptophan dosage Owen excretes far more indoleacetic acid than Brian and Robert, who have a much more modest response. But Owen is the mentally retarded one. What role can serotonin play in brain function?

I wish, therefore, to add my confusion to Dr. Udenfriend's confusion. We have clearly not identified the chemical abnormality which is the necessary cause of the mental lesion. There are no grounds for certainty over any chemical data as correlates of clinical behavior in PKU.
Figure 15. 5-hydroxyindoleacetic acid excretion.

The first series was done during the first phenylalanine tolerance tests. The second set of curves was obtained several days before. The tryptophan dosage was 50 mg per kilo by mouth.

**DISCUSSION**

DR. WOOLF: I had the opportunity of studying Dr. Valerie Cowie's patient and, in addition to a relatively low phenylpyruvic acid excretion, this patient, now an adult, has low blood phenylalanine; the fasting phenylalanine is under 10 mg, percent. His IQ is about 98 on repeated testing by a battery of tests.

DR. UDENFRIEND: I want to ask about the patient that had the high 5-hydroxyindoleacetic acid level after loading. We have never seen this much conversion in any normal individual. If this is really 5-hydroxyindoleacetic acid I think...
you should examine that patient for malignant carcinoid.

DR. BESSMAN: We thought it was rather high. He is mentally retarded.

DR. UDENFRIEND: But there could be another explanation.

DR. BESSMAN: No, children with malignant carcinoid show more symptoms. He has no diarrhea. His blood pressure is normal. Furthermore, although the rate of conversion is high the total excretion is still within normal range.

DR. GAULL: Were there any EEG changes in these patients and were they affected by the folic acid?

DR. BESSMAN: Patients' EEG's were not affected by folic acid. The EEG's are abnormal in all of these children.

DR. W AISMAN: I think Dr. Bessman is becoming the great dissenter, and I think it would be worthwhile to perform similar studies on some other patients, not only on those patients who have such normal IQ's. I think it would also be important to study younger siblings with bona fide phenylketonuria.

DR. BESSMAN: These data represent about 5 percent of the total testings done on a number of other patients and we received the same kinds of answers. The reason we presented this particular material here is that we wished to compare the child with normal mentality, untreated, with the child with mental retardation, untreated. One could always say there are different kinds of phenylketonuria, as suggested by much of the work now being presented. These could be genetically different. We show that chemically in the same family there is nothing different, yet a wide variation exists in IQ among affected siblings.

This was suspected from previous work of a number of people with patients' siblings. It seems to us that perhaps the large majority of cases now called PKU may be children who might have manifested "peculiar behavior" in the past and have gone on to normal adulthood.

DR. BICKEL: We made a similar study on a limited scale some years ago with phenylketonuric child with normal IQ from a family where two other phenylketonuric siblings were severely mentally retarded. A phenylketonuric child of similar age with low IQ served as a control (Zscho, Kinderheilk. 79, 509, 1957). I sent urine to Dr. Jepson at the time and we found no difference in the phenylalanine blood level, the tyrosine blood level, or the excretion of phenylpyruvic acid and other phenolic compounds in the mentally normal and the idiotic child. I agree with you that the exact relationship between the metabolic error and the brain damage is still very obscure.

DR. UDENFRIEND: I think this probably will come up in the later discussions. Nobody is talking as a pharmacologist in terms of the reaction of a person in relation to a pharmacologically active agent. Assuming that in phenylketonurics that the same pharmacologic agent is responsible for a given effect, a neurological effect or the inhibition of learning, the patients will not all respond in the same way. That is a very important consideration. I can give you a few examples. We gave alpha methylparatyrosine to two types of patients, one group had essential hypertension, the other group (pheochromocytoma) over-produced noradrenalin. We noticed in most of them that alpha methylparatyrosine produced sedation. This was the most common effect. But the effect varied even with the same tissue level of alpha methylparatyrosine. With the same inhibition of tyrosine hydroxylase, there was a tremendous difference in the reaction of the patient. One patient fell asleep after having been given one or two doses of half a gram per day. Another one was given up to four grams per day. His enzyme was inhibited 75 percent yet he showed no such degree of sedation. I think we should keep normal variation very much in mind.

DR. BESSMAN: I want to point out that this is a most important point because of what I call our gun-barrel vision. All we can see is the bad angel, phenylalanine. We measure phenylalanine and say that as a result of changes in this bad angel we expect a pharmacological result. When we see a few "exceptions," we say Dr. Udenfriend was correct but let's sweep them under the rug. That's all right, but the rug is getting heaped and it's difficult to walk over now.

DR. KOCH: I had a question on Dr. Bessman's data showing an indole tolerance test, and I wondered if you had performed this same experiment on patients undergoing dietary

Provided by the Maternal and Child Health Library, Georgetown University
treatment, in other words, during period of serum phenylalanine levels.

DR. BESSMAN: We did not do indole tolerance tests on this family before and after giving folic acid. We have never treated these children in terms of lowering their phenylalanine.

The patient on whom I showed indican excretion, clearly handled indole differently before and after addition of phenylalanine to his diet. The phenylalanine appeared to interfere with both the conversion of indole to indican and the urinary excretion of indican.
I am not going to be able to quote Whitman or Browning or anybody else. I am going to relate our work on experimental phenylketonuria and I think this work has some validity in view of the discussions we have had thus far. This work should help in understanding the cause of mental retardation in phenylketonuria. We have induced phenylketonuria in a number of different animals and I would like to point out the opportunities for research which are available, using these models.

Our knowledge of this disease is not really as extensive as one might expect, despite its discovery in 1933 by Folling. This was even 25 years after Garrod first mentioned inborn errors of metabolism in 1908. Interestingly some of the original investigations by Thudicum many years earlier, in the 1860's and 1880's, hinted at this type of disease, namely an error of nature.

While we have a number of clinical conditions in which the accumulation of certain metabolites is part of the abnormality, we really should prefer not to be limited to working on patients alone, but to have animal models in which the chemical circumstances of the patients are simulated. About 10 years ago we first embarked on the problem of how we could reproduce or simulate the abnormal biochemistry and thus obtain experimental models which could be useful for biochemical experiments and for psychological procedures, and which would allow greater latitude than is possible with the human subject.

I think it is important to keep in mind that one must meet a certain number of criteria in order to claim that one has produced a successful animal counterpart. Now, one criterion would obviously be to obtain elevated blood levels of the substance; a second, to find appropriate metabolites of these amino acids in the brain and in the urine; and a third, to produce not only the chemical changes but perhaps also some pathological changes which might simulate findings in a patient. These changes might be brought about by altering enzyme systems in the liver or the brain, or by altering the absorption of the various amino acids as they cross the gut into the blood and from the blood across the blood-brain barrier. We also want to know something about how the various products of chemical reactions influence this absorption.

The results obtained from the use of animals will be valuable not only because we can obtain basic information on liver and brain, but we might also get some knowledge about the interrelationship between the two--I think there is a great interrelationship and we have been hinting at it already in this conference--namely, that the liver is very important for normal brain function. Needless to say, we would have an opportunity to correlate psychological and behavioral changes with the biochemical alterations.

In determining criteria for a successful model, we must also consider the research objectives. I think it might be appropriate to point out that changes which take place in one animal may not be biochemically reproduced in another, so the appropriateness of the model for the type of experiment desired is an important consideration. In addition, a model with satisfactory biochemical changes might be valuable for studying biochemical changes but it might not be suitable for psychological studies. The individual investigator must decide which model he can best use for his purposes.

Very early in our work we tried using dogs, and our goal was to obtain hyperaminoacidemias and hyperaminoacidurias, but the results were disappointing. We could not raise the blood level and could not get the dogs to take enough diet. It was problem of mechanics. The same was true of rabbits. Now that we have learned more, it might be possible to use these animals more successfully. We had already started work with
rats and wanted to see how other species performed. Hamsters, we found were as useful as rats, but recently one of my colleagues, Dr. Polidora, an experimental psychologist, has not been able to use hamsters because they are unable to solve the swimming maze which we have used in our psychological studies with rats. Nevertheless, hamsters were valuable for confirmation of our biochemical findings with rats.

How can we evaluate the models of inborn errors of metabolism? In order to illustrate how experimental models of hyperaminoaciduria and hyperaminoacidemia or ketonuria can be used to study enzyme relationships, I will present some studies with rats, hamsters and monkeys, and limit my remarks to phenylalanine since this is the purpose of the conference. But we have performed other experiments in which many other amino acids have been used in these animals. We have done less with hamsters but we have done a great deal with monkeys and we have done a great deal with rats.

I want to make clear at the outset that at no time in any of these experiments, or even now, am I claiming that we are reproducing any of the genetic relationships or reproducing the genetic situation. We are simply trying to demonstrate that it is possible to duplicate high amino acid levels in the blood, from which certain metabolites are subsequently found in the urine and tissues, and that we have an opportunity to obtain information on the entire subject of inborn errors by the use of such models. We might also get information which would be valuable for understanding some of the unknown mechanisms of brain damage that have already been discussed, and we will certainly talk about in the next few days.

Now, because we know most about the inborn error phenylketonuria, it is the best disease to duplicate.

In table 1 let me show you the liver phenylalanine hydroxylase activity in various animals. These data were summarized in a recent publication. If we assume the hydroxylase activity of the rat as 100, the mouse has about one-third as much, and the hamster has the least of all rodents studied. The hydroxylase activity of monkeys varies depending on the species. We haven't tried all types of monkeys but the liver of the rhesus monkey with which we have had the most experience is about equal in hydroxylase activity to that of the human liver. We have used many birds and fish, and data can be found in the publication cited.

We prefer to use animals with low hydroxylase activity for our phenylketonuria studies. Our purpose in using the rat is that we can get additional data from tissues as well as the blood. I don't want to kill many monkeys for this purpose yet.

We can raise the level of phenylalanine in the plasma significantly. Levels can be elevated as high as 30 to 80 mg. percent. It should be pointed out that these levels can be obtained despite the rat's high hydroxylase activity, and these high levels can also be obtained in the monkey. We have obtained levels in monkeys of 150 mg. percent when we force-fed the phenylalanine.

Another area that needed investigation was the effect of excess phenylalanine feeding on the enzyme phenylalanine hydroxylase, as our first experiments indicated that this activity was decreased. If low amounts are given, the amino acid acts as an inducer of the enzyme because this is an adaptive enzyme, but with excess substrate the amino acid does reduce the enzyme activity.

Table 1.--Phenylalanine hydroxylase activity in the livers of various species (as percent of rat livers) (1)

<table>
<thead>
<tr>
<th>Animal</th>
<th>Activity per 100g. body wt.</th>
<th>Activity per g. protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>100.</td>
<td>100.</td>
</tr>
<tr>
<td>Mouse</td>
<td>34.</td>
<td>22.</td>
</tr>
<tr>
<td>Hamster</td>
<td>18.</td>
<td>12.</td>
</tr>
<tr>
<td>Squirrel Monkey</td>
<td>37.</td>
<td>39.</td>
</tr>
<tr>
<td>Rhesus Monkey</td>
<td>3.</td>
<td>4.</td>
</tr>
<tr>
<td>Calf</td>
<td>9.</td>
<td>9.</td>
</tr>
<tr>
<td>Dog</td>
<td>--</td>
<td>43.</td>
</tr>
<tr>
<td>Pigeon</td>
<td>19.</td>
<td>47.</td>
</tr>
<tr>
<td>Ring Dove</td>
<td>3.</td>
<td>3.</td>
</tr>
<tr>
<td>White Bass</td>
<td>12.</td>
<td>57.</td>
</tr>
<tr>
<td>Bullhead</td>
<td>0.7</td>
<td>2.</td>
</tr>
</tbody>
</table>

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Table 2 illustrates that even if different strains of rats are used we get about the same effect. For example, the phenylalanine-fed rats of the Long-Evans strain have half the hydroxylase activity of the controls. If other strains are used, reduction of hydroxylase activity by one-half also occurs.

Table 3 shows that either 5 percent or 7 percent phenylalanine will reduce the hydroxylase activity. This figure also shows some additional data which my colleague Dr. Kerr has obtained. Rats fed a chow diet have a phenylalanine hydroxylase activity of about 300, and when phenylalanine is added to the diet the activity is reduced. When tryptophan is added to the phenylalanine, the activity remains low. With DL-phenylalanine and the combinations of DL- and L- at different amounts, one always gets a reduction in hydroxylase activity.

This model presents an opportunity to study the influence of maternal phenylketonuria on the developing fetus. Table 4 shows that the hydroxylase activity in pregnant rats can be significantly reduced by feeding a 7 percent phenylalanine diet. The level of phenylalanine is not as high as in younger rats. There is a change when older rats are used; the phenylalanine in the blood decreases from the levels of 30 to 50 mg. per 100 ml. to levels of around 15 to 25. The tyrosine level is also high, as is expected. So we can reduce the enzyme level in the liver and elevate the phenylalanine level in the blood at the same time, and study the effect of this change on the developing fetus.

This model should be useful in studying a problem which we are all going to face. What are we going to do with all these phenylketonuric patients whom we are treating and who have reasonably normal intelligence levels, or even some of Sam Bessman’s patients, who are normal without treatment? What is to be done to prevent retardation in children of these phenylketonuric patients when they marry and have children of their own?
Table 4.--Phenylalanine hydroxylase activity and plasma amino acid levels in pregnant rats fed excess phenylalanine

<table>
<thead>
<tr>
<th>Diet</th>
<th>(#)</th>
<th>Wt.</th>
<th>Hydroxylase per 100 g. body wt.</th>
<th>Phenylalanine mg./100ml</th>
<th>Tyrosine mg./100ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>(5)</td>
<td>426</td>
<td>41.6</td>
<td>2.7</td>
<td>1.3</td>
</tr>
<tr>
<td>7% L-Qal.</td>
<td>(15)</td>
<td>306</td>
<td>19.5</td>
<td>14.5</td>
<td>6.2</td>
</tr>
</tbody>
</table>

Table 5.--Rat Term Fetus: Phenylalanine and tyrosine plasma values

<table>
<thead>
<tr>
<th>Maternal diet</th>
<th>(#)</th>
<th>Fetal wt. grams</th>
<th>Phenylalanine mg./100ml plasma</th>
<th>Tyrosine mg./100ml plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>(5)</td>
<td>4.6</td>
<td>4.9</td>
<td>5.9</td>
</tr>
<tr>
<td>7% L-Qal.</td>
<td>(9)</td>
<td>4.9</td>
<td>30.5</td>
<td>10.7</td>
</tr>
</tbody>
</table>

Table 5 demonstrates that the weight of these fetuses is the same as those from normally-fed dams. Young rats do not lose much weight when fed a 5 percent phenylalanine diet. An older rat fed a 5 percent phenylalanine diet grows well and a 7 percent diet in an older rat causes little loss in weight. The animals are raised under alternate dark-light conditions in which the lights go on and off every three hours. This results in a more uniform food intake, since the rat is a nocturnal animal and will feed when the lights are out.

The fetus has marked elevation of plasma phenylalanine when it is born and the tyrosine is somewhat elevated. This demonstrates transplacental crossing of the phenylalanine into the young fetus.

Table 6 demonstrates that in the day-old pups from these pregnant females, the activity of the hydroxylase is somewhat elevated or nearly the same. Soon, if the mother's milk contains adequate phenylalanine, the hydroxylase activity will fall. Phenylalanine is transferred from the mother across to the baby through the milk, but the plasma levels are not as high as when the baby is directly fed a high phenylalanine milk.

To go to the serotonin story let me say I am not very much impressed by the role of serotonin in phenylketonuria. I am not unaware of the fact that there is a significant competition between phenylalanine and tryptophan for hydroxylation in phenylketonuria. We showed that tryptophan and phenylalanine were apparently acted upon by the same hydroxylase to convert these into 5-hydroxytryptophan, and tyrosine respectively. Udenfriend and his group later confirmed this but felt there were some differences in cofactor requirements. You remember he said it can now be found in brain as well.

So if we take the animal model and study the serotonin content--in this case it is 0.50 µg. per gm. (table 7). When phenylalanine is added, the serotonin content of the brain decreases. When tryptophan is added, it increases. When they are added in combination, less tryptophan is
Table 7.--Effect of phenylalanine and tryptophan on serotonin content of rat brain and liver (after one week)(4)

<table>
<thead>
<tr>
<th>No. of animals</th>
<th>Brain</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg/gm.wet/tissue</td>
<td>µg/gm.wet/tissue</td>
</tr>
<tr>
<td>Control</td>
<td>(9)</td>
<td>.50±.02</td>
</tr>
<tr>
<td>7% L-Qal.</td>
<td>(5)</td>
<td>.22±.03</td>
</tr>
<tr>
<td>5% L-Trypto.</td>
<td>(5)</td>
<td>.80±.06</td>
</tr>
<tr>
<td>5% L-Trypto</td>
<td>(5)</td>
<td>.70±.04</td>
</tr>
<tr>
<td>7% L-Qal.</td>
<td>(5)</td>
<td>.34±.01</td>
</tr>
</tbody>
</table>

Table 8.--Influence of dietary supplements on brain serotonin in rats(6)

<table>
<thead>
<tr>
<th>Diet</th>
<th>Brain serotonin (µg/g wet tissue) pair-fed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experimental</td>
</tr>
<tr>
<td>5% L-phenylalanine</td>
<td>.42</td>
</tr>
<tr>
<td>7% L-phenylalanine</td>
<td>.23</td>
</tr>
<tr>
<td>7% L-tyrosine</td>
<td>.51</td>
</tr>
<tr>
<td>5% Phenylacetic acid</td>
<td>.80</td>
</tr>
<tr>
<td>6% L-valine</td>
<td>.49</td>
</tr>
<tr>
<td>9% L-valine</td>
<td>.39</td>
</tr>
</tbody>
</table>

*Significant at P<0.01
Table 9.--Brain serotonin and liver phenylalanine pyruvate transaminase in Long Evans Rats(1)

<table>
<thead>
<tr>
<th>Age</th>
<th>Diet</th>
<th>Paired control</th>
<th>Ad lib. control</th>
<th>7 percent phenylalanine diet</th>
<th>Brain serotonin (µg/gm)</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>days</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Qal -pyruvate (activity/gm. protein)</td>
</tr>
<tr>
<td>52</td>
<td>(7) Diet</td>
<td>.29</td>
<td>.63</td>
<td>.73</td>
<td>33.9</td>
<td>733</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.9</td>
<td>792</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.5</td>
<td>663</td>
</tr>
<tr>
<td>67</td>
<td>(6) Diet</td>
<td>.36</td>
<td>.61</td>
<td>.65</td>
<td>26.0</td>
<td>695</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.9</td>
<td>767</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.0</td>
<td>694</td>
</tr>
<tr>
<td>82</td>
<td>(12) Diet</td>
<td>.61</td>
<td>.87</td>
<td>.84</td>
<td>9.4</td>
<td>483</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.8</td>
<td>564</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.1</td>
<td>567</td>
</tr>
</tbody>
</table>

Table 10.--Influence of excess phenylalanine diets on plasma amino acid levels and on enzyme activity in hamsters(1)

<table>
<thead>
<tr>
<th>Diet</th>
<th>Qαl (mg./100ml)</th>
<th>Tyro. (mg./100ml)</th>
<th>Phenylalanine hydroxylase per 100 gm. body wt.</th>
<th>Qαl pyruvate transaminase (µm/mg. protein/hr.)</th>
<th>Tyrosine α-keto glutarate transaminase (µm/mg. protein/hr.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chow</td>
<td>2.5</td>
<td>2.8</td>
<td>42.8</td>
<td>.85</td>
<td>2.39</td>
</tr>
<tr>
<td>+5% L-Qαl</td>
<td>11.2</td>
<td>9.3</td>
<td>31.6</td>
<td>.88</td>
<td>2.76</td>
</tr>
<tr>
<td>+7% L-Qαl</td>
<td>42.4</td>
<td>11.8</td>
<td>40.6</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

Table 10 points up some of the recent work we have done with hamsters. It will be seen that on 5 percent phenylalanine diets the plasma phenylalanine level is raised significantly, but with 7 percent phenylalanine is raised much higher. The plasma tyrosine level is also elevated.

The hamster's liver phenylalanine hydroxylase level is low in comparison with the rat, but adding 5 percent phenylalanine or 7 percent phenylalanine does not reduce the hydroxylase activity very much. The hamster model is therefore considerably different from the rat model, and might be preferred for some experiments.

Table 11 represents a study on hamsters in which we wanted to study the influence of various protein contents of the diet. The amount of protein fed to the animal also influences the hydroxylase activity of the liver. The normally-fed hamsters have a higher phenylalanine hydroxylase level than those fed a low protein diet; the casein diets with lower than optimum protein levels provide for less protein enzyme synthesis. Adding phenylalanine to the 9 percent casein diet did not affect the hydroxylase level. Addition of phenylalanine to the 25 percent casein caused a reduction in the phenylalanine hydroxylase
Table 11.--Effect of excess phenylalanine to various protein diets on plasma phenylalanine and tyrosine and on enzyme activity in hamsters(7)

<table>
<thead>
<tr>
<th></th>
<th>Plasma Gal. tyro. (mg./100ml)</th>
<th>Phenylalanine hydroxylase per 100 gm. body wt.</th>
<th>Phenylalanine pyruvate transaminase (μm/mg. protein/hr.)</th>
<th>Tyrosine -keto glutarate transaminase (μm/mg. protein/hr.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chow</td>
<td>2.9</td>
<td>2.8</td>
<td>42.8</td>
<td>.85</td>
</tr>
<tr>
<td>9% Casein</td>
<td>2.3</td>
<td>3.5</td>
<td>16.7</td>
<td>.61</td>
</tr>
<tr>
<td>+5% L-QL.</td>
<td>21.1</td>
<td>49.7</td>
<td>18.4</td>
<td>.64</td>
</tr>
<tr>
<td>18% Casein</td>
<td>2.6</td>
<td>4.2</td>
<td>38.5</td>
<td>.69</td>
</tr>
<tr>
<td>+5% L-QL.</td>
<td>10.4</td>
<td>15.7</td>
<td>28.5</td>
<td>.67</td>
</tr>
<tr>
<td>25% Casein</td>
<td>2.7</td>
<td>3.4</td>
<td>53.1</td>
<td>.82</td>
</tr>
<tr>
<td>+5% L-QL.</td>
<td>17.7</td>
<td>27.4</td>
<td>35.3</td>
<td>.81</td>
</tr>
<tr>
<td>60% Casein</td>
<td>2.9</td>
<td>4.6</td>
<td>42.6</td>
<td>--</td>
</tr>
</tbody>
</table>

activity. Little difference is demonstrated between a synthetic (casein) diet or a chow diet if the protein level is adequate.

Very high tyrosine levels were found in hamsters fed 9 percent casein plus phenylalanine. Similar levels of tyrosine and phenylalanine are found in some infants with "transient tyrosinemia." Many of these cases have phenylalanine levels of 14 and 15 mg. percent but they also have levels of tyrosine as high as 50 and 60 mg. percent. This elevation is transient because the tyrosine level decreases as the baby's liver function matures. The disparity between this condition and phenylketonuria is very obvious.

Figure 1 shows some of the data we obtained on monkeys, and I purposely did not pick the data showing the highest levels of phenylalanine. There are some levels here that are high and some that are low, but plasma levels do not appear to be correlated significantly with the intake. We feed our monkeys every four hours by hand, and under these circumstances, the growth of the monkeys is pretty good. Some of the animals who received phenylalanine grew better than the control animals, so it can be said that the growth of the monkeys fed 3 grams per kilogram per day of phenylalanine is not abnormal. Some of the monkeys were able to adapt to the behavioral tests, but they tested poorly and were, in fact, mentally retarded, as determined by testing and social behavior.

Figure 2 shows that, in fact, biochemical changes found in the patient can be duplicated and again I emphasize that we have not changed the genetics. We have no illusions about that. We are interested in duplicating the biochemical parameters which could be useful for studying an animal with phenylketonuria. The monkeys fed phenylalanine do in fact excrete the same compounds that the patient does.
Figure 2. Metabolites in monkey urine

Normal
Phenylalanine -- barely detectable
Phenylpyruvate -- absent

PKU
Phenylalanine †
Phenylpyruvate †
o-OH-Phenylacetate †
p-OH-Phenylactate †
Indolelactate †
Phenylacetylglutamine †

Table 12 just gives some quantitative data on the amount of phenylpyruvic acid formed and it is obvious the normal monkey does not excrete phenylpyruvic acid.

Table 13 shows that one finds decreased amounts of 5-hydroxyindoleacetic acid in the urine of phenylketonuric monkeys, and this metabolite is also decreased in the urine of phenylketonuric patients. (8) I am convinced from other data that the amount of 5-hydroxyindoleacetic acid excreted can be altered by changing the tryptophan and phenylalanine levels in the diet.

In a recent paper we were able to show that the placenta in a monkey does not differentiate between favorable or deleterious substances fed to the mother (table 14). (9) As far as the monkey is concerned, the placenta simply transfers to the fetus whatever is presented to the mother. For our purposes this is very good, because it will be seen that the ratio between the phenylalanine in the baby and in the mother is such that the baby is certainly exposed to a high concentration of phenylalanine in utero. Let me say in anticipation of some data we are going to publish soon, that the six monkeys obtained from phenylketonuric or hyperphenylalaninemic mothers do not function intellectually as do normal monkey babies. Our co-worker, Dr. Harlow, and his students have tested many normal monkey babies, and by contrast anyone who tests these phenylketonuric infants is struck by the fact that not only is social behavior abnormal but also learning performance is impaired. They huddle in the cage and don't present themselves to the testing situation. They do poorly on delayed response tests. They do poorly on discrimination reversal tests -- all the tests which the psychologists have performed on these animals from about 30 days of age.

What I have tried to do is to discuss phenylalanine-loaded animals in order to demonstrate that I believe that some of these questions can be answered by the study of animals under abnormal biochemical conditions, and that these animals

<table>
<thead>
<tr>
<th>Monkey</th>
<th>Mg. of phenylpyruvic acid in 24 hrs. urine</th>
</tr>
</thead>
<tbody>
<tr>
<td>108 (PKU)</td>
<td>257</td>
</tr>
<tr>
<td>118 (PKU)</td>
<td>465</td>
</tr>
<tr>
<td>109 (Control)</td>
<td>79</td>
</tr>
<tr>
<td>115 (Control)</td>
<td>79</td>
</tr>
</tbody>
</table>

Table 13.--Excretion of 5-hydroxyindolyl-acetic acid and indolyl-3-acids in normal and phenylketonuric monkeys per 24 H (9)

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of animals</th>
<th>No. of urine collections</th>
<th>5-HIAA (µg/day)</th>
<th>3-Indolyl-acids* (mg/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5</td>
<td>15</td>
<td>304±230*</td>
<td>1.48±2.03*</td>
</tr>
<tr>
<td>Experimental</td>
<td>5</td>
<td>10</td>
<td>47±26*</td>
<td>1.15±1.45*</td>
</tr>
</tbody>
</table>

*Standard deviations are reported for differences between collections.

†Total of indolyl-3-acetic, indolyl-3-lactic and indolyl-3-pyruvic.
Table 14.--Concentration of phenylalanine in maternal monkey serum and in fetal cord(§)

<table>
<thead>
<tr>
<th>Maternal diet</th>
<th>Number of animals</th>
<th>Phenylalanine mother mg.%</th>
<th>Phenylalanine cord mg.%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>(8)</td>
<td>1.26±.49</td>
<td>1.68±.28</td>
</tr>
<tr>
<td>L-Qal.</td>
<td>(1)</td>
<td>22.0</td>
<td>45.3</td>
</tr>
<tr>
<td>(0.2-1.4g/kg/day)</td>
<td>(1)</td>
<td>11.8</td>
<td>43.1</td>
</tr>
<tr>
<td></td>
<td>(1)</td>
<td>27.2</td>
<td>43.7</td>
</tr>
</tbody>
</table>

can be used to obtain data which are meaningful to answer some of the questions related to phenylketonuria. These results can be obtained without subjecting phenylketonuric patients to biopsy.

Now, I know that we do not have a complete absence of the enzyme as is found in a phenylketonuric patient, but I think the model as such has research opportunities; and I personally would be happier if more people used the monkey as a model in order to get data which I think would be meaningful, and which could provide further information on this key inborn error of metabolism.

REFERENCES


DISCUSSION

DR. GUTHRIE: What was the difference in behavior of the infant monkey who had been exposed to the high blood phenylalanine of the mother before birth and not afterwards as opposed to the behavior of the baby monkey exposed to high blood phenylalanine levels only after birth?

DR. WAISMAN: A monkey born of a mother fed high phenylalanine over a long period of time to produce blood levels equal to that found in a woman with phenylketonuria, is not damaged by obvious congenital abnormalities nor is it limited in size. Yet the blood level of this fetus is very high as shown by the 40 mg. percent in the cord blood.

In recent experiments we fed rats phenylalanine. The first ten days we fed phenylalanine, the next ten days and the third ten days we also fed phenylalanine. The next group we fed for the first period, and the last group the third. We alternated in order to get all the information we could on when damage might occur to rats.

It takes an exorbitant number of monkeys to do it in the same way so we had to compromise. We decided to let the brain be damaged in utero and then put the monkey on a normal diet. At a year of age we tested the monkey and I can tell you these monkeys do not perform normally. If an infant monkey is fed a high phenylalanine...
diet from birth to maturity, one still gets brain damage just like we might see from high levels present during pregnancy to birth. So I think we are able to get damage in both periods.

This is an important consideration, because as might be suspected, one of the problems that we must consider is the problem of the pregnant heterozygote. Does the insult of high phenylalanine during pregnancy after she takes a meal pose a threat to the fetus? Normals have variations in hydroxylase activity. Heterozygotes also have variation in hydroxylase activity. So with all these gradations, we must come to grips with the problem of how much damage there is in utero to the fetus of a heterozygote mother.

Most of the children born to heterozygote mothers are theoretically normal. But it is the patient while in utero and who is going to be a phenylketonuric who may suffer from high maternal phenylalanine levels. This might explain why we don't always get optimum IQ even though treatment is excellent.

DR. HSIA: Does the experimental production of phenylketonuria in any of these animal species have definite effect upon behavior regardless of pregnancy or age or anything.

DR. WAISMAN: I am not an experimental psychologist. Dr. Harry Harlow says, and I am convinced that these animals do not behave normally. I have been with the testers for long periods of time. I must say it takes a great deal of effort to test even one monkey 1200 to 1500 times. These animals don't learn. They behave abnormally. They don't discriminate. They do not have recall.

DR. SWAIMAN: Do you have any evidence bearing on the question of whether there is a crucial time before which high phenylalanine diet must be given to affect the developing brain?

DR. WAISMAN: We put some monkeys on the diet the first three months and then some on the diet, the first six months. Then we put them on normal diets. It looks as though the brain damaged is by high diets during the first three months or the first six months and they will be retarded. The monkeys fed diets after six months are not as retarded as those damaged early. If adolescent monkeys are fed high phenylalanine diets, at age 3 and 4, there are still behavioral effects, but it is mostly social and behavioral rather than learning.

DR. UNDENFRIEND: Permanent or reversible?

DR. WAISMAN: These are not reversible. I am glad you brought it up because in the paper in February 1966 in Science we showed it was reversible in the rat. You take away the phenylalanine from the rat and he performs the swimming maze more normally than before. So maybe this is just toxicity. That is why I say choose the model you want for your purposes.

DR. UNDENFRIEND: You mentioned phenylalanine hydroxylase. Did you measure it with the TPNH, using the two enzymes?

DR. WAISMAN: We did not use a sheep factor.

DR. UNDENFRIEND: How did you analyze it? The measured level of enzyme in different species may very well be an artifact of homogenization. If we assay the guinea pig heart for tyrosine hydroxylase, we can find a 100 units of activity. In the rat heart we find 2 units. It could be said that the guinea pig heart is much richer in tyrosine hydroxylase than the rat heart. However, they both form comparable amounts of noradrenaline when the whole organ is perfused with tyrosine. Unless corroborated by other methods enzyme assays on homogenates may be artifacts of the homogenization and are not to be taken as the content of the enzyme in vivo.

A better indication of activity is to give a radioactive dose of phenylalanine to your animals and see how much tyrosine is formed. This would be more meaningful. I don't think that in vitro enzyme activities are to be taken that seriously.

DR. WAISMAN: I realize different animals have different levels of co-enzyme I and co-enzyme II. What I am saying is the rat has everything, whereas the fish does not and the monkey does not.

DR. UNDENFRIEND: You were saying that one animal is a better one to use than another. The reasons you give may have little to do with it. What you measure in the homogenate as phenylalanine hydroxylase is just the residual activity.

DR. WAISMAN: I don't know what else to do except do it the best way we can.

DR. UNDENFRIEND: If you gave labeled phenylalanine to each species and found the same amount of radioactive tyrosine formed then you would know this is not to be taken seriously as quantitation of activity.
DR. BESSMAN: Do you use any other amino acids such as leucine or valine to produce these effects on mental function? Do the animals become retarded and is the retardation reversible?

DR. WAISMAN: We have fed monkeys tryptophan and they were not retarded. Tyrosine didn't have any effect when fed. When we added valine, isoleucine and leucine, they were retarded. When we fed methionine, the levels we used were toxic to the monkeys.

You are asking: Is this a generalized effect or a specific effect? I don't know. Maybe there is a unitarian mechanism by which damage to the brain occurs which influences intelligence.

DR. BICKEL: I am worried about your comparison of the young monkey fed a high phenylalanine diet in the first three months and its relationship to the human infant. It is my strong impression that if in the human infant we delay treatment until 2 months or even 2 1/2 months of age we can still expect absolutely normal children. Some of our best results so far have been in children whose treatment began at 6 or 8 weeks of age. I don't think that high phenylalanine levels in the first month or two do permanent damage to the human infant's brain.

DR. WAISMAN: I wouldn't say it as strongly as you. I wouldn't make a generalization because these patients vary so much. I have a patient I began on the diet at three weeks of age and she is very poor in her accomplishments although well-controlled. My best patient has an IQ of 117, and she was first treated at ten weeks of age. I don't make any comparison between monkeys and humans. However, remember one year in a monkey is equal to four years in a child.

DR. ROUSER: I was interested in the comparisons of different species, particularly in relation to the myelination process, because this is rather variable. In the studies that we have been doing on the human brain, using cerebroside and sulfatide levels, we find myelination to begin only shortly after birth. In some of the other animals myelination is very far along at birth.

I might add, in humans, using cerebroside and sulfatide levels as indicators, myelination goes on through about the tenth year of life as a nice steady curve but it is much more rapid during the first three years.

DR. JERVIS: What are the histological changes in monkeys?

DR. WAISMAN: We haven't sacrificed many of our monkeys intentionally. They are still very valuable to us. But of the two that died there was nothing gross. Electron microscopy also didn't show anything.
OVERALL VIEW OF OUR BIOCHEMICAL KNOWLEDGE OF PKU

GEORGE JERVIS

The task assigned to me by the organizers of this meeting is to summarize what we know and do not know on the biochemical aspects of phenylketonuria as seen from the viewpoint of a clinician rather than of a pure biochemist.

There is common agreement that these five are the metabolites of major importance in phenylketonuria: phenylalanine, phenylpyruvic, phenyllactic, phenylactic, and orthohydroxy-phenylactic acids. Phenylalanine increase in the blood, urine, spinal fluid and to a lesser extent in sweat and saliva, is the most significant biochemical alteration in the disease. Extensive data are at present available on the blood level of this metabolite at various ages and under various dietary conditions in PKU patients. However, information has not yet been obtained on the content of phenylalanine in various organs of patients and particularly in the brain. Information of this type would be of great value in assessing the several hypotheses which have been suggested in the attempt to explain the pathogenesis of mental retardation on the basis of phenylalanine excess. At present one must rely on data obtained from animals following administration of high doses of the amino acid, an experimental situation which is not entirely comparable to human phenylketonuria.

Precise methods to assay phenylalanine have now been developed which were not available to early investigators of the disease. The fluorometric method of McCaman-Robins seems to be preferred today by the majority of investigators because of its speed and accuracy. The method of LaDu, of comparable value, is used when a spectrofluorimeter is not available. In view of this methodological development, some of the old figures obtained with the Kappeler-Adler or the microbiological assay need perhaps to be revised.

That the increase of phenylalanine in the body fluids of the phenylketonuric is due to a defect of the liver enzymatic system hydroxylating phenylalanine to tyrosine is now well established. Workers in this field are indebted to Kaufman for a detailed knowledge of the mechanism of this complex system. It remains to be determined whether alternative pathways do exist for the hydroxylation of phenylalanine. The report of Udenfriend at this meeting that tyrosine hydroxylase present in sympathetic nervous system and in the brainstem can bring about the conversion of phenylalanine to tyrosine is of significance in this respect.

It is common clinical knowledge that remarkable variations to occur in fasting blood phenylalanine among phenylketonuric patients, particularly in infants and children, independently of the amount of dietary proteins. Moreover, during treatment with low phenylalanine diet there are wide variations in the utilization of phenylalanine. Some patients can tolerate only 25 mg/kg/day while others may ingest twice or three times that amount without showing an excessive level of blood phenylalanine. Finally, following phenylalanine load test in individuals either homozygous or heterozygous for the phenylketonuric gene, considerable variations may be observed in the blood curve of phenylalanine suggesting variation in the utilization of the amino acid. These observations seem to indicate varying degrees of activity of the hydroxylating enzymatic system. It would appear that there is a spectrum in enzymatic error from the almost complete absence of the enzyme seen in some patients to the mild insufficiency of some carriers. The sharp dichotomy, heterozygous-homozygous, would then be doubtful on the chemical level.

These interesting speculations need the support of biochemical procedures apt at measuring with reasonable precision the hydroxylase activity. Such procedures are still wanting. The phenylalanine loading test extensively used, measures roughly the rate of disappearance from the blood of an excess of the amino acid by several routes of which only one is the conversion of tyrosine. Measurement of blood tyrosine together with phenylalanine levels is an improvement of the
method but still not sufficiently sensitive. In my experience a similar lack of precision is seen with an intravenous administration of phenylalanine load. Determination of enzymatic activity in liver material obtained by biopsy is obviously possible only in exceptional cases. Even this assay is not strictly quantitative. Udenfriend and Bessman \(^{(4)}\) in their classical paper have used tagged phenylalanine and determined the amount of resulting tagged tyrosine. One would hesitate to recommend the extensive use of this method which uses \(^{14}\)C. Phenylalanine with tagged N, which would be less objectionable has not been used yet but deserves consideration. Unquestionably a precise method of measuring hydroxylase activity would be of great help to the physician in planning more efficiently a dietary regime, in recognizing "formes frustes" of the disease and in the detection of the carrier stage.

It has been demonstrated by Kaufman \(^{(4)}\) that in classical phenylketonuria the defective fraction of the hydroxylating system is the thermostable so-called mouse liver factor. The question remains whether other forms of phenylketonuria which deviate in some aspects from the classical clinical pictures are due to a defect in other constituents of the enzymatic system or to the action of inhibitors. This problem is of basic importance to the clinician and can be approached only by a close cooperation between physicians and biochemists. The discovery that in a dilute mutant of mice there is a partial defect of the phenylalanine hydroxylase due to inactivity of the thermostable so-called sheep liver factor, adds interest to the search of similar defect in the human.

The second major metabolite is phenylpyruvic acid. This is present in urine and blood and can be measured with various methods of which the enol-borate complex method of Knox is perhaps the most reliable. Again, its presence in tissue and particularly in brain has not been recorded as yet. The origin of phenylpyruvic acid from phenylalanine is well documented in vitro and in vivo. L-amino oxidase may play a part in this conversion but in all probability a much more important role is played by transaminase, a well known enzymatic system, which is present in many organs including the brain. The increased activity of this system in phenylketonurics would result in increased utilization of alpha ketoglutaric acid used in the exchange of the amino group. It is a fact that the phenylketonuric infant with a high phenylalanine blood level excretes a very small amount of ketoglutaric acid in the urine. The urinary output increases dramatically when the baby is placed on a phenylalanine restricted diet.

However, it is not known whether this depletion of alpha amino glutaric acid caused by excessive transamination implies a depletion in the brain with consequent disturbance in the Krebs cycle, a hypothesis suggested by Korey \(^{(2)}\) in the past.

The importance of transaminase pathways in phenylketonuria has been emphasized by the remarkable cases recently reported \(^{(6,9)}\) of very high phenylalanine blood levels without formation of phenylpyruvic acid, apparently because of lack of transaminase activity. These would be examples of hyperphenylalaninemia without phenylketonuria, a new condition to be considered in a differential diagnosis. The present information about the specificity of transaminase is still incomplete. Crude preparations, as first demonstrated by Knox, \(^{(10)}\) show that transaminase system is common to the three aromatic amino acids, phenylalanine, tyrosine and tryptophan. However, Canellakis and Cohen \(^{(11)}\) showed that a purified tyrosine-glutarate transaminase was not active on phenylalanine. There is a rough relation between blood phenylalanine and blood phenylpyruvic acid level and urinary output but individual variations in this relation are wide suggesting possible variations in transaminase activities. Phenyllactic acid, another major metabolite, is more imperfectly known. The amount excreted in the urine is less than phenylpyruvic. It can be demonstrated in the blood of animals injected with large amounts of phenylalanine but whether it is present in the tissues of phenylketonurics is still not known. The acid is decomposed in vitro by an enzymatic system present in the kidneys, \(^{(12)}\) Whether this reaction takes place in the phenylketonuric is not known. That phenyllactic acid may derive from phenylpyruvic acid by the activity of lactic dehydrogenase has been suspected for some time, but the reaction was always found to be very slow when compared to the rapidity of the conversion of lactic to pyruvic acid. Recent investigation has shown \(^{(14)}\) that this enzyme is responsible for not more than 20 percent of the amount converted, the remaining 80 percent is accounted for by an aromatic reductase present in many mammalian tissues \(^{(14)}\)
This discovery as well as the possibility of accurate measurement of the acid by gas chromatography will certainly lead to further study of this important metabolite.

Phenylacetic acid, the fourth major metabolite in phenylketonuria has received little attention although it has been known for over 50 years that it is excreted in conjugation with glutamine as phenylacetylglutamine. The condensation has been studied in vitro and found that it catalyzed by an enzyme of the human liver and kidneys. A small amount of the acid in phenylketonurics is excreted as glucuronide. Whether free acid is present in body fluids of patients is not known. One of the difficulties for such determination is the lack of a reliable quantitative method of determination.

The origin of phenylacetic acid from phenylpyruvic acid has been hypothesized but not extensively investigated. Oxidative decarboxylation is a possible pathway and another would be through phenylacetaldehyde which is then acted upon by an aldehyde dehydrogenase to the phenylacetic acid. Conversion of phenylpyruvic acid to benzaldehyde has been observed in bacteria but efforts to demonstrate this aldehyde in the urine of phenylketonurics have been unsuccessful. A last source of phenylacetic acid would be from phenylalanine through phenylethylamine by a well known metabolic route common to many biological amines. Since relatively large quantities of phenylethylamine have been demonstrated in phenylketonuric patients following monoaminoxidase blockade, the possibility of this alternative route should be considered.

The fifth major metabolite, orthohydroxyphenylacetic acid deserves a brief mention. It is present in the blood, but apparently not in the spinal fluid, of all phenylketonurics examined. It is constantly excreted in the urine in rather large amounts, usually somewhat less than phenylpyruvic acid. It may be present in the urine in the absence of phenylpyruvic acid when phenylalanine blood level is less than 10-12 mg. percent. The blood level of the orthohydroxyacid is grossly related to the level of the keto acid and the derivation of the former from the latter is now well established. Armstrong (10) has described in detail an enzymatic system effecting the conversion of phenylpyruvic to orthohydroxyphenylacetic acid, the enzyme being very similar, if not identical, with the well known para-hydroxyphenylpyruvate oxidase which catalyzes the formation of homogentisic acid. The reaction goes only in one direction since in no instance did the feeding of the orthohydroxy acid result in an increase of blood phenylpyruvic acid.

An alternate pathway from phenylalanine to orthohydroxyphenylacetic acid through orthotyrosine and orthotyramine has been suggested but not yet proved.

There are some minor metabolites in phenylketonuria deserving mention. Increased amounts of urinary orthohydroxypyrrolidin acids (pyruvic, lactic and acetic), and indole lactic and indoleacetic acid. Some of these compounds may originate from intestinal putrefactive phenomena, particularly the indole lactic may be of intestinal origin, since as Anderson will report at this meeting, tryptophan intestinal absorption is impaired in the disease. The reported increased indicanuria may have a similar origin, although Bessman (14) noted also a more rapid conversion of indole to indican due to a more rapid absorption, oxidation and sulfatation of indole than in the normal.

Hippuric acid urinary excretion is also increased in phenylketonuria apparently because of increased formation of benzoic acid from phenylalanine through a still unknown pathway.

New compounds derived from phenylalanine and pyridoxine were recently reported by Loo. (17) Finally the presence of a urinary hydantoin in phenylketonuric urine first reported by Dobriner some 25 years ago has been recently reinvestigated.

The significance of all these minor metabolites for a better understanding of the disease is still unknown.

The accumulation of various metabolites mostly derived from phenylalanine in the body of the phenylketonuric patient brings about abnormalities in the metabolism of other substances. Of these secondary metabolic defects the best known is an alteration of serotonin formation which was first demonstrated by British investigators (18) and repeatedly confirmed in patients and in the experimental phenylketonuria of various animal species. Decreased excretion of 5-hydroxyindoleacetic acid is associated with low serotonin blood level. It has been demonstrated that excess phenylalanine in vitro and in vivo inhibits the decarboxylase activity of decreased serotonin levels.

Lower levels than normal of norepinephrine and epinephrine together with decreased urinary output of 3-methoxy-4-hydroxy mandelic acid, have been repeatedly demonstrated in
phenylketonuria. These findings are apparently due to an inhibition of dopa-carboxylase by phenylpyruvic acid or other phenylalanine derivative.

Finally, some interference with melanin formation is probably present in phenylketonuric patient's, thus explaining the clinical observation of poor pigmentation. Tyrosinase activity is inhibited in vitro by excess phenylalanine. Consequently pigment formation is inhibited in vitro and probably in vivo.

From this brief survey, it appears that our present knowledge of the biochemical abnormalities in phenylketonuria is fairly extensive. Although several gaps are still to be filled, many of the basic biochemical facts are now well established.

In sharp contrast with this wealth biochemical information is the lack of satisfactory explanation on the mechanism by which biochemical abnormalities cause mental retardation. Many pathogenetic hypotheses have been proposed but thus far none has the support of incontrovertible facts. Our understanding of the disease in all its clinical and therapeutic aspects will be wanting until this basic problem is clarified.

REFERENCES


DISCUSSION

DR. ANDERSON: Dr. Jervis referred to a child in whom our studies suggested that a limitation of phenylalanine hydroxylase activity as well as a limitation of phenylalanine transaminase activity possibly was present. Using
quantitative chromatographic procedures we were unable to find any increase in the excretion of urinary metabolites formed along the transaminase pathway for phenylalanine, or any disturbance in the metabolic pathways for tryptophan. This child had blood phenylalanine levels maintained at 17 mg. percent for three weeks by increasing the diet, and by acute loading we induced a level of 52 mg. percent. No increase in the characteristic urinary metabolites of the phenylketonuric subject occurred.

It appeared that the child was a typical heterozygote; however, this conclusion depends upon the validity of changes in the blood phenylalanine and the tyrosine responses to oral loading. The child had what appeared to be a homozygous tyrosine response following phenylalanine loading. One of the parents fell into the category of our statistical group for normal adults. The other parent fell into the group closer to the heterozygote group.

Whether this is a new entity I don’t know. The child is now approximately 30 months of age. Our last observation was that on a normal diet from 14 to 21 months of age the phenylalanine level was about 5.5 mg. percent. Prior to that time values of 15 and 17 mg. percent were obtained on a general diet. Intelligence appeared normal. We came to the conclusion that the hyperphenylalaninemia present up to 3 months of age did not produce brain damage before dietary therapy was started.

DR. UDENFRIEND: Did you study tyrosine levels?

DR. ANDERSON: Yes.

DR. UDENFRIEND: What are the normal ones?

DR. ANDERSON: 1.5 mg. percent, 1.7 mg. percent.

DR. UDENFRIEND: They don’t go up?

DR. ANDERSON: No.

DR. JERVIS: We have similar cases from Syracuse University. Dr. Schneider will comment on it.

DR. EFRON: In an institution for the retarded in Massachusetts there is a child who was diagnosed at age 1-1/2 as having phenylketonuria on the basis of a serum blood phenylalanine by the LaDu test of 26 mg. percent and 119 mg. percent of phenylpyruvic acid in the urine. The child was severely retarded. The child is now 7 years old and has a fasting blood level of 2.7 to 4 mg. percent. Her phenylalanine level on loading does not go higher than about 15.8 mg. percent which would fall within the range of the heterozygote-type load, but she does not make tyrosine when given the load; the tyrosine drops a little as it does in PKU homozygotes. Yet this child is severely retarded with no other obvious symptoms. She also had eczema and seizures when she was young. So there can be retardation associated with the atypical PKU syndrome with declining blood levels.

DR. SCHNEIDER: We have a similar child 2-1/2 years old, begun originally at 10 weeks of age on a low phenylalanine diet.

In this case the father’s response to loading was even more clearly normal, and extensive blood typing indicated this was in fact the biological father of the patient. The child has been off the diet on a regular regimen since then and he still maintains a fasting blood phenylalanine level of 12 to 14 mg. percent. We have investigated his tolerance to a load four different times since the age of 10 months, most recently a week ago. He shows absolutely no rise in blood tyrosine on loading. In fact, his blood level drops a little bit in about two hours, for reasons I don’t understand. We didn’t perform the extensive studies Dr. Anderson did in regard to the transaminase activity. We were suspicious because we hadn’t seen any phenylpyruvic acid in the urine when in good health. This child came in several times with infection and at that time he would show a little reaction for phenylpyruvic acid in his urine. He almost always has orthohydroxyphenylacetic acid in his urine.

DR. UDENFRIEND: Is this child retarded?

DR. SCHNEIDER: No.

I would like to dwell on this point just one moment from the purely clinical point of view. We have in addition in the last year three more similar cases. The point I would like to stress here is that with all the mass screening going on, it’s fairly easy to distinguish the prematures and the occasional full-term baby with a very high tyrosine. It is not difficult to diagnose the baby who at 2 or 3 weeks of age has a blood phenylalanine of 65 mg. percent. What shall we do with these patients that are in the 20 mg. percent or under range?

DR. SCRIVER: We have been engaged in an experimental screening program using our chromatographic method and just recently we found
a Greek baby with persistent hyperphenylalaninemia (6-10 mg. percent) who did not have accompanying tyrosinemia. The phenylalanine loading done at 8 weeks of age showed some conversion of phenylalanine to tyrosine, but did not produce phenylalaninemia above 16 mg. percent, and the ferric chloride reaction and other such tests remained negative.

The things which interest us about this and other such babies are: (1) Do they have something like substrate inhibition of the hydroxylase? In this regard we have restricted substrate intake and seen plasma phenylalanine fall only to return to former levels with higher substrate intake?

(2) Do they have a pteridine cofactor deficiency or dependency? There is much interest in the cofactor requirements of enzymes and I'd like to hear from those who are more experienced with phenylalanine hydroxylase whether these babies should be treated with folic acid.

DR. UDENFRIEND: The question you raised can be answered in several parts. First, if there is a pteridine deficiency, we should find defects in other enzymes that require this pteridine. As an example one should see a comparable defect in the formation of noradrenalin. We should also get a similar defect in 5-hydroxyindoleacetic acid formation because tryptophan hydroxylase is a pteridine enzyme. There are some lipid enzymes involved also. This should be a rather generalized defect.

We don't know how the cofactor is formed in man. Folic acid which is related to it, because it is a pteridine, is not converted in microorganisms to the pteridine cofactor. Folic acid itself is a very poor cofactor. It may be that the animal makes its own cofactor or it may be that there is a new pteridine vitamin that is needed in the diet.

In bacterial systems the pteridines arise from guanidine triphosphate and there is a branching point where there is a common intermediate metabolism in one direction which yields the pteridine cofactor for hydroxylase type of reactions. In an alternate route of metabolism for the intermediate the side chain splits off and the pteridine combines with glutamic acid and parahydroxybenzoic acid to become folic acid. We don't know what happens in animals or man. It is difficult to conceive of folic acid giving rise to the pteridine cofactors so the alternative is that man can make the pteridine cofactor. This is interesting because he doesn't make folic acid. As stated above the cofactor may come from another vitamin we know nothing about.

DR. SCRIVER: May I say I wasn't referring solely to "deficiency" but also to dependency on cofactor. Bonner and his group found that tryptophan pyrrolase could be modified genetically in Neurospora not in reference to substrate binding, but in reference to its pyridoxal phosphate cofactor binding. As a result cofactor was not bound efficiently to the apoenzyme and therefore the enzyme did not function except at very high cofactor concentrations in the medium.

DR. UDENFRIEND: That is true. If a genetically modified enzyme is being made, its affinity for the co-enzyme may be different. That is always a possibility.

Dr. Jervis asked for an in vivo or simple test. I don't know why people don't use the conversion of radioactive phenylalanine to tyrosine as a simple test. I think the Atomic Energy Commission would give its approval, since one can now use tritium which is safe. We don't even have to worry about the degree of loading because we can take the ratio of phenylalanine to tyrosine, rather than the absolute conversion as Dr. Bessman and I did. We have studied two more patients since we published our first results and the results also seem to indicate 5 to 10 percent range of the activity of normals. We are planning to study two more patients. This could certainly be a very simple and standardized procedure without the necessity of biopsy.
SCREENING TESTS FOR THE DETECTION OF PHENYLKETONURIA: HOMOZYGOTES AND HETEROZYGOTES

DAVID YI-YUNG HSIA

Although nearly a million newborn infants have been screened for phenylketonuria over the past five years, we have accumulated only a limited amount of information on the genetic and non-genetic factors that influence the levels of serum phenylalanine in the newborn period. This presentation represents a summary of these data with the emphasis being placed on the observations themselves rather than on the fact that they were collected using the procedure described by Guthrie, LaDu, McCaman and Robins, or paper chromatography. It is hoped that by showing the paucity of data in this area, investigators on both sides of the Atlantic will be encouraged to add their experiences to this important area.

Values in Normal Newborns

There is general agreement that the serum phenylalanine level in the normal birthweight (NBW) newborn is about 2 mg. percent as shown in table 1. Using the McCaman and Robins fluorometric procedure, Hsia et al. reported 2.08 ± 0.70 mg. percent in 8490 newborns, and Hill et al. described 2.6 ± 0.3 mg. percent in 10 newborns. Similarly, in a survey of 210 newborns, Baker et al. have found that 121 infants had serum phenylalanine levels of 2 mg. percent or less, and 79 infants had levels between 2 and 3.9 mg. percent. Using their spectrophotometric method, LaDu et al. reported that all of some 30 full-term newborns aged 2 to 10 days had serum phenylalanine levels of 2.7 mg. percent or less, and using the bacterial inhibition assay, Guthrie has reported that only 0.07 percent of 400,000 newborn infants tested showed a blood phenylalanine level of 6 mg. percent or higher.

In NBW newborns, serum phenylalanine levels have been shown to be not appreciably influenced by maternal age, gravity, sex, race, age, birthweight or type of feeding. However, Kleinman et al. have shown that unrefrigerated samples sent through the mail will lead to higher values and a greater incidence of presumptive positive results as shown in table 2. This effect can be controlled by continuously holding the specimen frozen until tested.

Table 1.--Summary of reported phenylalanine levels in newborn infants

<table>
<thead>
<tr>
<th>Method</th>
<th>Authors</th>
<th>Nus tested</th>
<th>Results</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>McCaman-Robins</td>
<td>Hsia et al</td>
<td>3,934</td>
<td>2.08 ± 0.51mg%*</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Kleinman et al</td>
<td>8,490</td>
<td>2.28 ± 0.70mg%*</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Hill et al</td>
<td>10</td>
<td>2.6 ± 0.3mg%*</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Baker et al</td>
<td>210</td>
<td>121 &lt; 2mg%</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>79  2-3.9mg%</td>
<td></td>
</tr>
<tr>
<td>LaDu</td>
<td>LaDu et al</td>
<td>30</td>
<td>% &lt; 2.7mg%</td>
<td>9</td>
</tr>
<tr>
<td>Guthrie</td>
<td>Guthrie</td>
<td>400,000</td>
<td>0.07% &lt; 6mg%</td>
<td>10</td>
</tr>
</tbody>
</table>

*Mean ± Standard deviation.

These investigations were supported by grants from the Illinois Department of Public Health, the Illinois Mental Health Fund, and the United States Public Health Service.
Table 2.--Comparison of McCaman-Robins tests in two hospitals
(Based on data of Klienman et al(6))

<table>
<thead>
<tr>
<th></th>
<th>No.</th>
<th>Mean ± S.D.</th>
<th>% Positive*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Born in Hospital A</td>
<td>2349</td>
<td>2.01 ± 0.58</td>
<td>0.60</td>
</tr>
<tr>
<td>Born in Hospital B</td>
<td>1029</td>
<td>1.63 ± 0.47</td>
<td>0.09</td>
</tr>
<tr>
<td>Mailed to Hospital B</td>
<td>5112</td>
<td>2.53 ± 0.66</td>
<td>1.25</td>
</tr>
</tbody>
</table>

*Above 4 mg%.

Values in Phenylketonuric Infants (Homozygotes)

Serum phenylalanine levels have been recorded in 22 phenylketonuric infants as shown in figure 1.(5,11-16) In this group, the lowest recorded serum phenylalanine value obtained after birth was 5.5 mg. percent which is about seven standard deviations above the mean for NBW newborns. Thus, if one regards any value over 4 mg. percent as being "presumptive positive" and the test is an accurate representation of the true serum phenylalanine value, the probability of misclassifying a homozygous affected as being normal is minimal. Furthermore, because of the rapid and sharp rise of serum phenylalanine levels during the first week of life, it is difficult to confuse the hyperphenylalaninemia due to phenylketonuria from that due to other causes.

Values in Newborn Siblings of Phenylketonuric Children

A total of 87 observations have been made in the serum phenylalanine levels in 34 siblings of phenylketonuric children as shown in figure 2.(5,9,11,15) The mean and standard deviation of all the observations is 2.35 ± 0.57 mg. percent. These data would suggest that although one will occasionally see a significantly elevated serum phenylalanine level in a sibling such as Case 1 of Kang and Paine(11) where the levels ran over 6 mg. percent. Consistently, the average serum phenylalanine level of a group of siblings is only very slightly higher than that of a group of NBW infants. Assuming random distribution, one would expect that two out of three siblings in such families would be heterozygous carriers of the gene for phenylketonuria. If so, it would appear that heterozygosity for phenylketonuria does not play a major role in the etiology of hyperphenylalaninemia in the newborn.

Values in Newborn Infants with High Serum Tyrosine Levels

A number of reports(9,17,18) have appeared showing an increase of serum phenylalanine associated with the increase of serum tyrosine in the newborn infant. Human infants, particularly those with low birthweight (LBW) have a functional deficiency of both tyrosine transaminase and p-hydroxyphenylpyruvic acid oxidase.(19) The results in a marked accumulation of tyrosine and a moderate increase of phenylalanine as shown in table 3. When the serum phenylalanine and tyrosine levels of LBW infants are plotted against each other as shown in figure 3, it is apparent that although excessive tyrosine levels do not always result in an increase in the phenylalanine levels, almost every infant with a high phenylalanine level was shown to have an elevated tyrosine level. It was found that about one-third of the LBW infants showed serum phenylalanine
levels of 3.75 mg. percent or more or in excess of 3 standard deviations above the mean of NBW infants and in the latter group only two are within 3 standard deviations of the mean for serum tyrosine of NBW infants. From the studies we have done, it would appear that high tyrosine and phenylalanine levels also occur in NBW infants, but with far less frequency.
Hyperphenylalaninemia of Unknown Etiology

During the past year, several reports (20, 21) have appeared describing isolated cases of hyperphenylalaninemia where no etiological factor could be found. In some instances, the elevated serum phenylalanine levels persisted for weeks or months, in others, the levels returned to normal after a month or two. It is not unlikely that such infants have a transient or partial deficiency of phenylalanine hydroxylase, possibly related to an excess of phenylalanine as has been shown with the mutant mouse.(22)
Table 3.--Serum phenylalanine and tyrosine for infants of normal birthweight (NBW) and low birthweight (LBW) as compared with adult controls

<table>
<thead>
<tr>
<th></th>
<th>Phenylalanine (mg%)</th>
<th>Tyrosine (mg%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>Mean ± S.D.</td>
</tr>
<tr>
<td>Adult controls</td>
<td>90</td>
<td>1.55 ± 0.34</td>
</tr>
<tr>
<td>NBW infants</td>
<td>100</td>
<td>2.16 ± 0.49</td>
</tr>
<tr>
<td>LBW infants</td>
<td>50</td>
<td>4.29 ± 0.54</td>
</tr>
</tbody>
</table>

Figure 3. Comparison of serum phenylalanine and tyrosine levels in 48 LBW infants.
In such patients, phenylalanine tolerance tests\(^{(23)}\) should be undertaken. Allan et al.\(^{(16)}\) have shown that when 0.1 gm. per Kg L-phenylalanine is administered to newborn infants in the first few hours of life, a typical segregation takes place into those with phenylketonuria, those who are heterozygous carriers, and those who appear to be normal newborns as shown in figure 4. During the past two months, we have been referred two NBW infants with initial serum phenylalanine levels of 4.25 mg. percent and 13.5 mg. percent at about five weeks of age. After admitting the babies to the hospital, phenylalanine tolerance tests were performed with an oral load as shown in figure 5. It was felt that A showed a normal response and B may ultimately turn out to be a heterozygote.

Finally, in really doubtful cases, liver biopsy for phenylalanine hydroxylase activity should be considered. I would like to describe this simple procedure which was carried out in our laboratory in the past year. In figure 6A, liver obtained from a normal subject was incubated with DL-phenylalanine-\(^{14}\)C. On the upper half of the chart we see the results of the chromatography carried out on the material prior to incubation. Only a single peak for phenylalanine appears. In the lower half of the chart we see the results after incubation for one hour. We now see not only the peak for phenylalanine, but also a second peak for tyrosine showing satisfactory conversion of phenylalanine to tyrosine. In figure 6B, we performed the same studies on a punch biopsy specimen from a patient with phenylketonuria. Here we see no conversion of phenylalanine to tyrosine.

**Approach to Screening of Phenylketonuria**

All of us are aware of the fact that treatment with low-phenylalanine diet is not without danger. At this point, there is probably little choice between the two or three major approaches being used for the detection of elevated phenylalanine levels in the newborn period. In a comparative study between the McCam man and Robins and the Guthrie tests, Baker et al.\(^{(8)}\) felt that the Guthrie was somewhat quicker and gave fewer false positives. However, automation of the McCaman and Robins\(^{(7)}\) and more large scale use of the procedure\(^{(5,6)}\) have overcome both these objections in part. Rather, the critical question is the proper procedure that should be followed in a screening program when any of the tests shows a presumptive positive.

In view of the complexity of determining the cause of the elevated serum phenylalanine in the newborn infant, it is strongly recommended...
that all such infants should be referred to a recognized pediatric center where proper clinical and laboratory examination may be undertaken. At such a center, we would recommend that tests be carried out in the order shown in table 4. First, the serum phenylalanine levels should be repeated using a quantitative method such as McCaman-Robins or LaDu. Second, the serum tyrosine should be measured. If the serum phenylalanine is still elevated and the serum tyrosine is within normal limits, then an oral phenylalanine tolerance test using 0.1 Gm/Kg L-phenylalanine be used. If the results are still indecisive, a punch biopsy of the liver for phenylalanine hydroxylase activity should be considered.

Recently, screening tests for phenylketonuria have become somewhat in disrepute because a significant number of non-phenylketonuric infants with elevated serum phenylalanine levels have been placed on the low-phenylalanine diet without
Figure 6. Enzymatic hydroxylation of 1-phenylalanine-\(^{14}\)C to 1-tyrosine-\(^{14}\)C in liver from (A) normal control and (B) phenylketonuric patient.

**CONTROL**

![Graph showing enzymatic hydroxylation of phenylalanine to tyrosine.](image)

**PHENYLKETONURIC**

![Graph showing enzymatic hydroxylation of phenylalanine to tyrosine.](image)

Incubation was carried out with 0.02 ml 1.0 M phosphate buffer, pH 6.7, 0.02 ml 4 mN NAD, 0.02 ml 50 mM nicotinamide, 0.1 ml 2.5 mM DL-phenylalanine-\(^{14}\)C (Nuclear Accessories), and 0.1 enzyme in final volume of 0.26 ml at 37 \(^{\circ}\) C for one hour in air. 0.025 ml of reaction mixture before (above) and after (below) incubations was transferred to a paper chromatographic strip (Whatman No. 3) and developed in a solvent system of 2-butanol-3 percent acetic acid (5:2 v:v) by descending chromatography for 18 hours. The strips were counted in a strip counter.
Table 4.--Diagnostic tests to be undertaken for hyperphenylalaninemia

1. Repeat serum phenylalanine using quantitative method (either McCaman-Robins or LaDu).
2. Measure serum tyrosine.
3. Undertake oral phenylalanine tolerance test.
4. Consider liver biopsy for phenylalanine hydroxylase activity.

Proper diagnostic investigations. It is believed that by State and public health officials insisting upon a complete workup in questionable cases, this criticism can be answered, and that the interests of the community are best met by continuing routine screening.

REFERENCES

DISCUSSION

DR. UDENFRIEND: Dr. Berry stated that there was a problem of delineating carriers and a simple tolerance test was employed to verify that these individuals were carriers. I think this is a misleading statement. In fact, it is a very dangerous statement because this method labels individuals as carriers when all we can tell is that their phenylalanine tolerance curve is statistically high. Because of loose talk in the community of scientists, and loose writing of textbooks, it is now believed by many that we can detect individual heterozygotes by a phenylalanine tolerance test. This misconception has to be rectified and I think it should begin here. After all, can you take an individual and say to him, "You are a carrier?"

DR. HSIA: Where do we now stand in phenylalanine carrier detection in adults? A paper which I have permission to quote is that of Dr. Perry, who has recently collected data, on intravenous tolerances, oral tolerances, and he feels that one can delineate with somewhere between 70 to 80 percent certainty a heterozygous group of adults from a normal population. This was the experience that Dr. Steinberg and I had while studying a fairly large sample of patients. This is obviously not good enough in terms of classifying individuals permanently.

DR. UDENFRIEND: You read a curve to determine the carriers. What do the data look like when plotted?

DR. HSIA: We can get the mean and standard deviation of the two groups, the overlap, and at any given point we can calculate back what the probability of heterozygosity of that individual is in terms of the ratio. But we can shift as we move closer into the heterozygote range where many individuals can be said to have a 90 percent probability of being a carrier. I admit this method is imperfect, but this is the best that we can do at the moment.

DR. UDENFRIEND: What, did you say, about 90 percent?

DR. HSIA: We can establish a ratio of the probability at any given position on the curve where it overlaps. There is a formula for this function (Figure 7).

DR. UDENFRIEND: You mean that there is an exact quantitative relationship? Do you mean to say the higher the blood levels are the greater the possibility that the individuals are heterozygotes?

DR. HSIA: Yes.

DR. UDENFRIEND: I don't believe that there is an exact relationship.

DR. HSIA: I am not saying we can be absolutely certain but on a statistical basis we can be more certain.

DR. UDENFRIEND: Socially and legally you are treading in a very dangerous area.

DR. HSIA: I agree. I am just presenting what data is available. I think this is probably about as accurate as we are ever going to get using phenylalanine tolerance tests. I haven't done anything with labeled material. This may give us a little more information.

DR. WAISMAN: I don't know if this will answer Dr. Udenfriend's question or not, but I think we have to decide: Do we want to determine the conversion of phenylalanine to tyrosine? Is phenylalanine level by itself a criterion? Or do we want to measure both? One of the things we did is that we determined the range of the tolerance curve and picked out different points. The data were treated by the methods of discriminant
functions. The best discriminant was based on phenylalanine measurements of the fasting, 1-1/2 hour, 2 hour, and 3 hour levels. If the distribution of these discriminant scores is plotted, they fall into two separate groups (Figure 8) (Wang, H. L.; Morton, N. E.; and Waisman, H. S. Amer. J. Human Genet., 13:255, 1961). Now, the amount of overlap between the two groups is only 4 percent using this discriminant function method. Tyrosine levels do not contribute significantly to this discriminant, and use of phenylalanine levels was found to be adequate to discriminate between the normals and heterozygotes. This answers your objection that when we plot the curve it's only a qualitative evaluation of a curve. But if mathematics is utilized we can separate these two groups of individuals sufficiently so that we could say that if the test is done by the same people using the same method, and under the same conditions, we can begin to separate these two groups into carriers and normals.

Maybe that is good enough for you, but that is one thing we did.

DR. UDENFRIEND: All I can say is I have read many papers and they don't impress me that there are methods to distinguish with 96 percent certainty heterozygotes from normals without knowing that an individual had children who are phenylketonuric. You say that you can do it with 96 percent; I don't believe it.

DR. W AISMAN: I say the data of performing tolerance tests separates these two groups by discriminate function.

DR. HSIA: I showed you the slide of the data of Allen and I quoted it very clearly that this was Allen's data. In his slide he is able to distinguish between heterozygous siblings and non-heterozygous siblings. This is something I don't really think I can do. That is why I raised this question.

DR. UDENFRIEND: We are talking now about adults. I didn't ask this out of idleness. We have studied maybe two dozen patients with phenylalanine tolerance tests after fasting. We were in fact trying to add an additional discrimination to this test, leading to phenylethylamine. We thought that if there was one more hurdle to go over we might be able to improve the detection of heterozygotes. We didn't find that possible. But we measured phenylalanine
tolerances and we certainly couldn't have picked out the individual heterozygotes. We found several individuals that were known to be heterozygotes. I would say from my limited number of patients that I would be very careful before I would stick out my neck and say to any one, "You are a heterozygote."

DR. EFRON: This is obviously a real problem and the word "heterozygote" probably should not be used to apply to anything except parents or children of phenylketonurics.

DR. ANDERSON: We studied the plasma tyrosine responses following phenylalanine loading as originally done by Dr. Jervis. We then applied the same statistical approach to the tyrosine responses as did Drs. Wang and Waisman for phenylalanine. This gave us a discriminant function which permitted separation into two groups, normal and heterozygotes. In fact we used medical students as normals and found two of them in the intermediate group. We didn't tell them, however.

We must state clearly that we are talking about the separation of two populations, not individuals.

DR. BESSMAN: I would like some technical information. I think it is a good idea to perform radioactive phenylalanine hydroxylase assays. We have had a great deal of difficulty with what Dr. Hsia had done easily. I'd like to know the substrate assay you employed in this radioactive phenylalanine study.

DR. HSIA: The assays were done with incubation carried out with 0.02 milliliters 1.0 M phosphate butter, pH 6.7, 0.02 milliliters 4 mM NAD, 0.02 milliliters 50 mM nicotinamide, 0.1 milliliter 2.5 mM DL-phenylalanine-14C (S.A. 10 mc/mM) and 0.1 milliliter enzyme in final volume of 0.26 milliliter at 37 degrees Centigrade for one hour in air.

DR. BESSMAN: That was no tracer. Do you mean microcuries per milliliter?

DR. HSIA: No, this is the actual amount of DL-phenylalanine. I don't have here the exact concentrations.

DR. BESSMAN: I think that is a very important thing. We got into this trouble. There is much phenylalanine in this liver tissue. The phenylketonuria patient to whom you add a tracer dose of phenylalanine gets a diluted dose of phenylalanine-14C. If he has ten times as much phenylalanine in the cell as normal, one-tenth the apparent conversion of the tracer occurs. Actually, it is one-tenth the apparent conversion of the tracer so one-tenth of the activity appears to be present.

This has been a problem we have found extremely difficult to control and in fact it requires the initial measurement of the phenylalanine concentration.
LARGE-SCALE SCREENING FOR METABOLIC DISEASE IN THE NEWBORN IN GREAT BRITAIN

L. I. WOOLF

By 1956 it was becoming clear that the effectiveness of treatment of phenylketonuria with a diet low in phenylalanine depended on how early in life it was started. When treatment was started within a few weeks of birth the child developed normally; when treatment started after the age of one year there was often considerable neurological and intellectual improvement, but the child probably never realized its true potential and the more severely affected cases continued to pose serious social problems. For maximum effect treatment evidently had to start long before there was any clinical indication of phenylketonuria and it was therefore recommended that all infants should have their urine tested for phenylpyruvic acid a few weeks after birth.\(^{(1)}\)

Mass screening using ferric chloride or Phenistix\(^{®}\)

The first mass screening program was started in Cardiff in March, 1958.\(^{(2)}\) The mother of each newborn infant in the city was issued with a bottle and asked to collect a liquid specimen of urine which was tested by adding ferric chloride solution. The difficulty of collecting a liquid specimen of urine resulted in only 25 percent of the infants born during the year being tested in this way; the use of Phenistix\(^{®}\) as an alternative to ferric chloride was therefore investigated in Cardiff in 1958, both in the laboratory and under field conditions in the babies’ homes. Since March, 1959, Phenistix\(^{®}\) has been used in Cardiff and now over 99 percent of all newborn infants in the city are tested.

In February, 1959, Birmingham started its mass screening program, also using Phenistix\(^{®}\).\(^{(3)}\) Other cities and counties adopted the procedure until, by May, 1962, of the 145 local authorities in England and Wales, 131 were operating a mass screening program for phenylketonuria and 5 were actively planning such a program. By June, 1963, the urine of 2,400,000 newborn infants had been tested, using Phenistix\(^{®}\), and 104 cases of phenylketonuria has been discovered early enough for treatment to be maximally effective.*

In Great Britain every infant, almost without exception, is seen at regular intervals by a Health Visitor, a public health nurse. All births are notified to the local authority’s health department and the Health Visitor usually first visits the home when the baby is a fortnight old. From 50 to 80 percent of babies, depending on locality, are later brought to one of the local authority’s welfare centers (well-baby clinics) where they are again seen by a Health Visitor as well as by a physician. This is an ideal situation for mass screening of the newborn and, from the start in Cardiff in 1958, screening programs in Great Britain have relied mainly on the Health Visitors. On the first or second visit of the Health Visitor to the infant’s home, or of the infant to a welfare center, a Phenistix\(^{®}\) test strip is pressed against a diaper freshly wet with urine. The Phenistix\(^{®}\) strip is watched for the appearance of a green color for one minute after it is wetted by the diaper. Some local authorities, e.g. Cardiff, test each infant twice, at the ages of a fortnight and six to eight weeks, but most test only at three to four weeks or six to eight weeks.

For the test to be effective, the reactive end of the Phenistix\(^{®}\) strip must be wetted with urine. The strip must be pressed against a thoroughly wet diaper—a merely moist diaper is insufficient to wet the strip—but diapers are by their nature absorbent and urine quickly spreads by capillarity until no part of the diaper

*Data provided by Ames Co., Stoke Court, Slough, England.
is wet enough for a reliable test. This "dry nappy problem" was at one time the greatest obstacle in the British mass screening program; in some cases the Health Visitor would have to make repeated visits to the baby's home, or the mother would have to bring the baby repeatedly to the welfare center before a freshly wet diaper could be tested. At best this involved a considerable waste of time and money, at worst the test was abandoned after a few unsuccessful visits or an insufficiently wet Phenistix strip was accepted. Several ways of overcoming the "dry nappy problem" were investigated: the Phenistix® test was very briefly dipped in water then pressed on to the moist diaper, transferring the reagents like a rubber stamp; the Phenistix® test was very briefly dipped in water then pressed on to the moist diaper, transferring the reagents like a rubber stamp; a pad of paper tissues backed with a larger piece of polyethylene film was placed in the diaper—the paper tissues did not dry by capillarity and were kept wet in a plastic bag for testing; a gauze swab was substituted for the paper tissues in the last test. For various reasons, some technical, others psychological, none of these devices was very successful.

In 1963 reports began to appear of children, reputed to have been tested for phenylketonuria in infancy and found negative, who presented with the full clinical and biochemical picture of phenylketonuria at an age of nine months or later. These observations made it necessary to consider afresh every aspect of the screening program.

Biochemical basis of screening tests

The biochemical basis of the tests is that, in phenylketonuria, phenylalanine starts to accumulate in the tissues and body fluids immediately after birth. In normal individuals only a negligible proportion of the body's free phenylalanine undergoes transamination, but in phenylketonuria the high concentration of phenylalanine causes the rate of this reaction to be multiplied manyfold; the nature of the transaminase involved is unknown. Some of the phenylpyruvic acid is reduced to phenyllactic acid, a reaction catalyzed by lactic dehydrogenase, some is converted to \( \sigma \)-hydroxyphenylacetic acid, a reaction catalyzed by \( \sigma \)-hydroxyphenylpyruvate hydroxylase, and some is decarboxylated to form phenylacetylglutamine. Many of the factors determining the rates of these reactions of phenylpyruvic acid are unknown, as is what determines the rate of formation of phenylpyruvic acid from phenylalanine.

A high proportion of the phenylalanine accumulating in phenylketonuria is converted to phenylpyruvic acid; this acid, if it enters the blood, is rapidly cleared by the kidneys and appears in the urine. Ferric salts give a green color with phenylpyruvic acid (in the enol form); Phenistix® has buffered ferric ammonium sulfate as its active ingredient. The sensitivity of Phenistix® is the concentration of phenylpyruvic acid in urine which will give a just detectable green color with Phenistix®, is between 5 and 10 mg, per 100 ml, the same as the sensitivity of the ferric chloride test and the 2,4-dinitrophenylhydrazine test. It was believed that, if the concentration of phenylalanine in the blood exceeded 15 mg, per 100 ml, the urinary concentration of phenylpyruvic acid would exceed 10 mg, per 100 ml and that most phenylketonurics had reached these concentrations by a fortnight and all had done so by five weeks. The discovery of "occult" phenylketonurics, who excreted too little phenylpyruvic acid to give a positive reaction with Phenistix®(9,10) made it clear that these views were to some extent erroneous and that the prevalence of phenylketonuria could be far higher than had been suspected. The complexity of the problem is illustrated by the two sisters reported by Woolf et al.;(12) the clinically unaffected one and her brain-damaged sister had fasting blood phenylalanine concentrations of 8.5 and 9.9 mg, per 100 ml respectively, but the urine of the former almost always gave a clear positive reaction with Phenistix® while the urine of the latter sister was consistently negative. When a more sensitive technique (paper chromatography) was employed, the occult phenylketonuric was shown to excrete phenylpyruvic acid in amount just short of the concentration needed to give a positive reaction with Phenistix® and some orders of magnitude greater than the normal. This pair of sisters illustrates the general finding that, while the occult variety of phenylketonuria is correlated with milder clinical manifestations, it by no means follows that all occult phenylketonurics are unaffected. The temporary failure of some phenylketonurics to excrete sufficient phenylpyruvic acid for easy detection, e.g. after
fasting overnight or longer, is well known. While it is difficult to decide retrospectively, it seems probable that the phenylketonuric reported negative in infancy were temporarily of the occult type and that the Phenistix® tests were, on the whole, carried out conscientiously and efficiently.

Alternative tests for phenylketonuria

Whatever the cause, there was the danger that even a few missed cases would discredit the entire screening programme and cause it to collapse, particularly in view of the strain on the Health Visitors caused by the "dry nappy problem." Although use of Phenistix® had enabled many cases of phenylketonuria to be detected and treated, it was urgently necessary to try to find an alternative test that would not miss cases and would throw less strain on the Health Visitor. Factors that had to be considered were: (1) the reliability of the test, (2) the ease of obtaining specimens, (3) the cost in man-hours, materials, etc., and (4) the best age at which to test.

All mass screening methods for phenylketonuria effectively test for an abnormally raised concentration of some substance—phenylalanine, phenylpyruvic acid or o-hydroxyphenylacetic acid—in the blood or urine, i.e. all are to some extent quantitative as well as qualitative. The reliability of a test for a substance depends on (a) the difference between the highest level in normal individuals and the lowest level in untreated phenylketonurics, and, (b) the sensitivity, i.e. the threshold concentration at which a trained observer can detect a departure from normality. The simplest tests for phenylpyruvic acid—ferric chloride, Phenistix® and 2,4-dinitrophenylhydrazine—are too insensitive to detect some phenylketonurics. Paper chromatography for o-hydroxyphenylacetic acid is a far more sensitive test and has proved its ability to detect occult phenylketonurics. This is a laboratory test as opposed to a field test, i.e. the specimen of urine is sent to a central laboratory for testing by skilled personnel. An advantage is that the urine-impregnated filter paper can be used for screening tests for other inborn errors of metabolism.

Specimen collection

Collecting specimens of urine on filter paper is a practical procedure far simpler than attempting to collect liquid urine specimens. Since the Health Visitor merely leaves the strips of filter paper and instructions with the mother, the need for repeated visits to obtain a freshly wet diaper is obviated. It was decided to investigate this method of mass screening for phenylketonuria and some other inborn errors of metabolism on a pilot scale. The primary object of the investigation is to determine the feasibility of collecting specimens in this way and to estimate the expenditure of skilled man-hours and materials in testing specimens.

The areas chosen are the city of Cardiff, an industrialized seaport with a population of 240,000, the county of Oxfordshire, mainly rural with a population of 140,000, Bletchley and Chesham, two small but rapidly growing towns in Buckinghamshire. The total birth rate in the four areas is about 11,000 per year; a strong effort is made to obtain specimens from all these infants. The Health Visitors are responsible for collecting the urine-impregnated filter papers either by visiting the home, at the welfare centre, or by post. Each Health Visitor thus has a record of which mothers in the district have supplied specimens. The procedure of collecting the specimens and the wording of the instructions to the mother were worked out in consultation with the Medical Officers of Health and Health Visitors. The filter paper specimens, each in its serially numbered envelope, are posted to the laboratory in Oxford.

Laboratory methods

Preliminary investigations were aimed at finding laboratory techniques least expensive in skilled man-hours but still reasonably proof against human error. After some trial and error, chromatography is carried out as follows: sheets of Whatman No. 52 paper, 25.4 cm. square, are purchased with ten rectangular holes punched at one end and four round holes at the corners. A strip 6 mm by 3 cm, is cut (figure 1) from the
Figure 1. Small strips of urine-impregnated filter paper stapled across the holes in a ten inch square sheet; photographed after chromatography and color development.

The five specimens on the left are from Susannah, a known case of phenylketonuria; note the spots of o-hydroxyphenylacetic acid near the solvent front at the top of the sheet. The five specimens on the right are from two normal children. The round holes at the corners, by which the sheet is mounted in the frame, are partly visible. The sheets of Whatman No. 52 paper, ready punched with ten rectangular and four round holes, are obtainable from Reeve, Angel and Co., London.

urine-impregnated paper, the serial number of the envelope being first written on in pencil, and is fixed across one of the holes on the sheet by two small staples. When all ten holes are bridged in this way with urine specimens, the sheet is mounted in a frame and run overnight by onedimensional ascending chromatography in isopropanol--0.880 ammonia--water (8:1:1). The sheets are dried at room temperature and sprayed with a suitable diazonium salt reagent; both diazotized sulfanilamide and Brentamine Fast Red GG have been used. Every stage of the operation has been timed; the actual working time of a technician, from receipt of 50 specimens to examination of the sprayed sheets and recording results, is 23 minutes. Thus, if a technician spent two-fifths of his working time at this test alone, he could cope with at least 100,000 specimens annually.

If the specimen is from a phenylketonuric, o-hydroxyphenylacetic acid produces a characteristic orange spot (with diazotized sulfanilamide)
or purple spot (with Fast Red GG) of \( R_f \) value about 0.73. From time to time a filter paper strip soaked in a suitably diluted specimen of urine from a known phenylketonuric is introduced as a check on reliability and sensitivity. As well as phenylketonuria, the diazonium salt reagent will reveal tyrosuria (i.e. excessive excretion of \( p \)-hydroxyphenylactic acid, \( p \)-hydroxyphenylacetic acid and tyrosine) and histidinemia. There are two different causes of tyrosuria: late maturation of the enzyme \( p \)-hydroxyphenylpyruvate hydroxylase, causes "innocent" tyrosuria, a temporary phenomenon found in about 1 percent of all infants aged 2 weeks, highly correlated with prematurity, and apparently completely without clinical effect; \(^{17, 18, 19}\) on the other hand tyrosinosis (formerly termed "inborn hepato-renal dysfunction" or "congenital hepatic and renal dysfunction" \(^{20, 21, 22}\) ) is a rare disease, often fatal during early infancy, in which a defect of the same enzyme occurs. \(^{23}\) The two conditions are distinguished in the mass screening programme by the occurrence of glucosuria, generalized aminoaciduria and (sometimes) trace proteinuria in tyrosinosis but not in innocent tyrosuria.

Galactose in the urine is tested for with galactose oxidase followed by \( o \)-tolidine and peroxidase. One drop of galactose oxidase solution is applied to a 6 mm x 3 cm strip of urine-impregnated filter paper, bearing the serial number in pencil, and five minutes later one drop of chromogen is applied. Specimens are tested in batches of ten, a simple device of glass strips and rubber bands allowing high speed working.

Glucose is similarly tested for with glucose oxidase, peroxidase and \( o \)-tolidine, mixed freshly each day.

Protein is similarly tested for with an alcoholic solution of the potassium salt of tetra-bromophenolphthalein ethyl ester followed immediately by citrate buffer, pH 3.7. \(^{24}\) Tests with urine from a case showing "tubular" proteinuria, in which the predominant protein was an acid mucoprotein with the mobility of an \( \alpha_2 \)-globulin, showed that the reagent was sensitive to this protein though serum albumin produced a greater depth of colour.

Cystine and homocystine are similarly tested for by adding 1 drop of 5 percent potassium cyanide solution, covering to prevent evaporation, and ten minutes later adding 1 drop of freshly prepared sodium nitroprusside solution.

These four tests take, for 50 specimens, about 70 minutes of actual working time. It is planned to add a similar test for maple syrup urine disease. Secretarial work is kept to a minimum; the envelope in which each specimen came is rubber stamped with tests and results and returned to the appropriate local authority's health department which informs the Health Visitor of the results of testing. The only records kept in the laboratory are serial number, date of receipt, date of reporting and any positive results. A positive result of urgent significance is reported by telephone to the infant's family physician or pediatrician; in less urgent or doubtful cases the health department is asked for a second specimen of urine, either liquid or dried on filter paper.

The amino acids in the given area of filter paper, i.e. to a first approximation in a given volume of urine, can be isolated by one-step combined elution and ion-exchange desalting. Two-dimensional paper chromatography gives very clear amino acid patterns but, because creatinine cannot be determined on the very small volume involved, quantitation is difficult. This is a relatively time-consuming test used only in special cases where the other tests have indicated some abnormality and, either because of urgency or for some other reason, it is wished to examine the amino acids in the original specimen.

The best age at which to test

Two factors must be considered in choosing an age at which to apply a test for phenylketonuria: how quickly after birth the substance tested for reaches the threshold of sensitivity, taking into consideration the range of biological variation, and the ages at which appropriate specimens can be conveniently obtained using existing organizations. Data on the former point is inadequate; it is known that phenylketonuric urine sometimes contains chromatographically detectable \( o \)-hydroxyphenylactic acid as early as 24 hours after birth \(^{18}\) and is often positive by the age of 5 days \(^{11}\) but it is not known what proportion falls...
rapidly with increasing age. The same situation holds for the Guthrie test. From the organizational point of view 14 days is the ideal age for testing because this coincides with the first visit of the Health Visitor. It is believed that brain damage would still be absent or very slight if a phenylketonuric were not diagnosed before the age of 14 days and treatment were started at 3 weeks; this is based on the normal intelligence and absence of neurological abnormalities in phenylketonurics whose treatment started as late as this or later.

In maple syrup urine disease, galactosemia or tyrosinosis a fortnight after birth may not necessarily be early enough to prevent some degree of mental or physical disability. In these conditions, even more than in phenylketonuria, we are hampered by lack of data.

RESULTS

Since July, 1965, 6,565 infants' urines have been examined, in addition to a further eight from a special "high risk" group to be discussed below. No cases of phenylketonuria, galactosemia, homocystinuria, tyrosinosis or histidinemia have been found among the 6,565. Sixty-two infants had tyrosyluria; 68 infants had proteinuria, 13 infants gave a positive cyanide-nitroprusside test, 42 infants had glucosuria (>20 mg glucose per 100 ml urine) and 22 infants showed galactosuria; in all these cases further specimens of urine were obtained and examined. If apparently innocent tyrosyluria was the only significant finding, a second specimen on filter paper was obtained, in every other case a liquid specimen of urine was examined. If the second specimen was still positive, or if there was some other significant finding, either the family physician or pediatrician was alerted or further liquid specimens of urine were examined, depending on the nature of the finding.

The limit of sensitivity of the test for protein was about 1 mg of serum albumin per 100 ml of urine, but it was rather less sensitive to the proteins excreted in tubular proteinuria. Rough quantitation was achieved by comparison of the colors with those from standard strips prepared from normal urine to which had been added known amounts of (a) bovine serum albumin or (b) mixed proteins isolated from a case of tubular proteinuria. This is not a very sensitive test when the normal level of urinary protein is considered; it was all the more surprising that so many infants should apparently have protein in their urine. It seemed possible that the protein in these cases was not urinary but represented an exudate from say, inflamed skin; in most cases where protein was found on the first filter paper specimen, the liquid urine specimen obtained a few days later was free from detectable protein, but in several cases a very faint trace was present both on testing with tetrabromophenolphthalein ethyl ester and with salicylsulfonic acid, suggesting that the protein originally found was renal in origin. In one case the second specimen or urine contained 1.2 g of protein per 100 ml and some blood, suggesting a glomerular disorder.

In the 13 infants with a positive cyanide-nitroprusside test on the original filter paper specimen of urine, the follow-up liquid specimen of urine showed, in 3 cases, excessive excretion of cystine, lysine, arginine and ornithine in a pattern characteristic of cystinuria. These seem to be genuine cystinurics; in 2 cases other members of their families either have cystinuria or excrete amino acids in a pattern characteristic of the incompletely recessive variety of cystinuria. The other ten infants excreted excessive amounts of lysine and cystine, but not arginine or ornithine; most probably they have a temporary excessive excretion of these two amino acids, as described by Woolf and Norman. In some of these ten infants the urinary amino acid pattern has become normal after a few months; this may prove true of the remainder of some may be heterozygotes for the incompletely recessive type of cystinuria.

The test for glucose was roughly quantitated by adding, to each batch of specimens, standards in which glucose was dissolved in normal urine to give known concentrations. If a specimen gave less color than the 20 mg percent standard it was reported negative for glucose.

Galactose was roughly quantitated in the same way as glucose. Initially a concentration of 40 mg. galactose per 100 ml urine was taken as the upper limit of normal. However the number of infants with a higher urinary concentration of galactose was so large that now a second specimen is requested only if the concentration is above
SO mg per 100 ml; the pediatrician or family doctor is not alerted to possible danger of galactosemia unless the urinary concentration of galactose is above 160 mg per 100 ml. A few infants fell into this group and, in some cases, erythrocyte galactose-1-phosphate undytransferase was determined but found to be normal. One infant had, in the first filter paper specimen of urine, galactose equivalent to a urinary concentration of about 1000 mg per 100 ml. This specimen also had about 500 mg of glucose per 100 ml and there was a markedly excessive excretion of amino acids, as compared with a normal infant of this age, with a pattern suggesting renal tubular dysfunction. Yet the infant was thriving, the erythrocytes, obtained a few days after the filter paper specimen of urine, contained normal concentrations of galactose-1-phosphate undytransferase, and a second specimen of urine was normal. One feels this was a case of true transient galactosemia. It is perhaps significant that, although no member of the family had abnormal urinary findings, an elder sib was mentally retarded and behaved as if he had suffered some unspecified damage to the brain at or around birth.

COMMENTS

Tyrosyluria and the detection of phenylketonuria

In tyrosyluria the enzyme o-hydroxyphenylpyruvate hydroxylase is absent or inactive, but this is probably the enzyme which oxidizes phenylpyruvate to o-hydroxyphenylacetic acid, the substance tested for. It follows that if tyrosyluria is present the test for phenylketonuria fails; it is partly for this reason that, even when the tyrosyluria appears to be innocent, a second filter paper urine specimen is asked for.

The Guthrie inhibition assay in Great Britain

When alternatives to Phenistix® were first being sought, the Guthrie inhibition assay of phenylalanine in blood or urine was considered. Hudson tested filter paper urine specimens sent by post from the Liverpool area and North Wales. Only 67 percent of mothers returned the filter paper specimens; out of 23,000 infants so tested, 2 were positive by the inhibition assay and confirmed as having phenylketonuria.

Baker, Cheng, Liebeschutz and Sandler, working in Queen Charlotte's Maternity Hospital, tested 210 infants born there for phenylketonuria using both the Guthrie inhibition assay and the fluorimetric technique of McCaman and Robins. Blood was collected by heel-prick when the infant was seven days old. Scott has been testing the blood of infants born in Stobhill Hospital, Glasgow, a large general hospital with 200 maternity beds; infants born there remain in hospital until they are seven days old and a specimen of blood is obtained by heel-prick just before discharge.

Only 67 percent of infants are born in a hospital in Great Britain and many of these are discharged before they are seven days old. If testing by the Guthrie method is to be done at the best age for reliability, i.e. preferably not before 6 days, collection of blood from the 1,000,000 infants born annually must be done by Health Visitors or midwives. This has raised several problems and a number of local authorities are not at present prepared to have their Health Visitors collect blood. However, very recently in South-East Scotland, blood has been collected by the midwives attending domiciliary births, or the medical staff in hospitals, and sent to Dr. Scott for testing by the Guthrie inhibition assay; if this proves successful it may well influence the rest of the country.

The high-risk group

It was suggested by Professor Otto Wolff that, by examining specimens from the newborn siblings of known phenylketonurics at frequent intervals after birth, several aspects of tests for phenylketonuria could be compared with a group of which 25 percent, on average, would have phenylketonuria. The Medical Research Council's Working Party on Phenylketonuria decided to compare the Guthrie inhibition assay on blood and urine, paper chromatography, for o-hydroxyphenylacetic acid, Phenistix and accurately determined serum phenylalanine concentration in this way. In November, 1965, Dr. Hudson circularized all pediatricians in Great Britain asking that, if the birth of a sibling of a phenylketonuric was expected, they should
contact my laboratory and be issued with a "diagnostic kit" of filter paper for blood and urine, sterile lancets, stamped addressed envelopes and instructions. Three laboratories are collaborating: Dr. Hudson in Liverpool is examining filter paper urine specimens by the Guthrie inhibition assay, Dr. Scott in Glasgow is using the Guthrie test on blood, and in Oxford paper chromatography is employed. The nursing staff where the baby is born test the diapers with Phenistix® and a suitable laboratory estimates plasma phenylalanine level by, usually, the method of LaDu and Michael(22) after ultrafiltration. After three weeks the results are compared.

So far eight such infants have been investigated (including two in which investigations are incomplete); two have turned out to have phenylketonuria. Of these two, in one testing started at the age of nine days and all four tests were strongly positive by that time, the serum phenylalanine concentration being 43 mg per 100 ml. In the other infant, results were negative on the first day of life, specimens were not obtained on the second or third day, paper chromatography gave a clear spot of o-hydroxyphenylacetic acid on the fourth day urine specimen, while inhibition assay gave fourth day blood and urine phenylalanine concentrations of 8 to 12 and 15 mg phenylalanine per 100 ml, respectively and the Phenistix® test was negative. For some reason dietary treatment did not start immediately and further urine and blood specimens obtained every second day remained strongly positive to all three tests, but the first positive Phenistix® test was obtained on the 31st day of life.

As far as one can generalize on these two cases and the few in the literature, paper chromatography of urinary o-hydroxyphenylacetic acid, the Guthrie test on blood and the Guthrie inhibition assay of urine seem equally reliable tests for phenylketonuria and all three are more reliable than Phenistix® or ferric chloride. It seems that the first three tests probably all become positive at about the same time after birth.

The ethics of testing

Not all individuals with the genotype of one of these inborn errors of metabolism suffer actual disease. Thus several atypical phenylketonurics, with normal intelligence and, in many cases, no neurological abnormalities, have been reported in the world's literature. In such individuals phenylketonuria is discovered incidentally in the course of other investigations. Although such cases do not show the clinical features of phenylketonuria, the basic biochemical defect, absence or inactivity of phenylalanine hydroxylase, is the same as in typical phenylketonurics. Both groups have high concentrations of phenylalanine in the blood and tissues and, in consequence, both transaminate a very appreciable proportion of their phenylalanine to phenylpyruvic acid, some of which is converted further to phenyllactic acid, o-hydroxyphenylacetic acid and phenylacetylglutamine.

There is a tendency for clinically atypical phenylketonuria to be associated with relatively low plasma phenylalanine concentrations, e.g. 6 to 10 mg per 100 ml(12) but other factors almost certainly enter. This is illustrated by a pair of twin boys in whom phenylketonuria was diagnosed by Dr. Laurance at the age of 11 months.* Blood grouping and other investigations made it virtually certain that they were monozygotic, yet one was of normal intelligence (IQ about 100) and the other severely retarded (IQ about 55). The retarded twin had a very abnormal EEG with frequent paroxysms of high voltage delta activity with some spiking; the other twin had a normal EEG apart from very occasional brief paroxysms of high voltage delta activity. Biochemical investigations gave results as follows:

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<tr>
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<th>Twin P</th>
<th>Twin K</th>
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<tr>
<td>Plasma phenylalanine</td>
<td>26.6</td>
<td>26.0</td>
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<tr>
<td>tyrosine</td>
<td>0.95</td>
<td>1.0</td>
</tr>
<tr>
<td>Urinary phenylalanine</td>
<td>28.4</td>
<td>27.5</td>
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<tr>
<td>o-hydroxyphenylacetic acid</td>
<td>14</td>
<td>18</td>
</tr>
<tr>
<td>phenylpyruvic acid</td>
<td>99</td>
<td>87</td>
</tr>
</tbody>
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*I am indebted to Dr. B. Laurance for permission to quote these cases.
An earlier investigation found 41 and 39 mg of phenylalanine per 100 ml of Prs and Krs plasma, respectively. The close similarity of the figures for the two twins is consistent with their monozygosity, but cannot be correlated with their mental or neurological status. The difference in clinical manifestations cannot be genetic in origin if they are monozygotic.

We do not know what proportion of phenylketonurics are clinically unaffected, but these atypical phenylketonurics will be picked up by mass screening procedures and treated unnecessarily with a low-phenylalanine diet. This is the only course open to us since we cannot distinguish in early infancy those who, if untreated, become mentally retarded or show some of the other clinical features of phenylketonuria. The twins described illustrate that clinical differences are not necessarily related to genetic or biochemical differences. The only advantages of treating all newborn phenylketonurics with the low-phenylalanine diet are the cost, the relatively unpalatable nature of the diet and the danger of dietary deficiencies. There is evidence from several sources that the number of atypical phenylketonurics does not exceed the number clinically affected; treating up to twice as many patients as may be necessary seems a small price to pay for preventing the mental deterioration otherwise inevitable in at least half of them.

In cystinuria the position is different. Lewis found the prevalence of cystinuria to be 1:2,600 and this high figure is supported by the results of mass screening reported here and by other investigations. On the other hand, Renander found that in Sweden only 1 person in 230,000 was operated on for cystine stones. It seems that only about 1.1 percent of individuals with the genotype and biochemical features of cystinuria suffer from cystine stones at any time in their lives. At present it would seem that cystinurics detected by mass screening in infancy should remain untreated except, perhaps, for a recommendation that the intake of water should not be allowed to drop too low; the family physician should be alerted to the danger of urolithiasis.

We know little about the relationship of most other inborn errors of metabolism to clinical disease. In tyrosinosis and histidinemia, for example, it seems possible that some, perhaps the majority, of those with the genotype for the condition are unaffected clinically. Only by continuing and extending mass screening programs will we discover the true prevalence of these conditions.

REFERENCES

DISCUSSION

DR. BESSMAN: I am delighted to hear Dr. Woolf say these things because he has the scientific conservatism that we have come to expect from our much more solemn and thoughtful British brethren. I think he cast a note of real seriousness. He asked a question. We already have been fooled by cystinuria, and know that it would be a mistake to treat all of these patients that we think may have it. The question he makes us ask is: Are we making another mistake? The mistake we may be making is the belief that treating these children is harmless.

DR. WOOLF: Well, I tried not just to ask a question but to answer it. I tried to say that the phenylketonuric should be treated at birth, at detection, because we don't know if he will be enzyme deficient states. II. Relation to the clinical diagnosis of phenylketonuria. Pediatrics, 33:512, 1964.


27. Hudson: Personal communication.


30. Scott: Personal communication.


mentally retarded or not if he's untreated. He stands at least a 50 percent chance of being mentally retarded so we have no option but to go ahead. After the first few months we can start investigating and see what happens biochemically when he is taken off the diet. In some of these patients the blood phenylalanine stays well down when they are untreated. In those cases I think one should certainly consider very seriously whether to continue treatment or not. Of course young phenylketonuric infants can have higher blood phenylalanine levels than later in life. So I think there is no option but to treat even if there is some waste of money.

DR. BESSMAN: This is a serious assumption that you make, that you are not doing any harm. This is the mistake we make in medicine many times.

We assume that this diet is harmless, and I think the assumption is very dangerous. I think this is why we have to find evidence that it's true, because if we inadvertently cut the mental function of a child from 110 down to 90 by a harmful diet we may still claim success of treatment. These numbers go into the statistics mill, and we never know that we have damaged the child. I submit, however, that a loss of 20 points in this way is more damaging to the child and the community than the putative benefit of changing an IQ of 40 to 70.

DR. WOOLF: That's fair enough. The answer is we never assumed it was harmless; we always assumed that it was dangerous. It is dangerous. Helen Berry emphasized some of the dangers of overtreatment, of insufficient monitoring of treatment, of giving too little phenylalanine. These are the dangers. We were studying this problem as far back as 1949 and we realized the need for phenylalanine because the patients couldn't make their own. We said they'd need some but didn't know how much then. I think we underestimated in some cases, but we didn't do harm in most cases. In some cases we did; we learned by our mistakes. I don't know if I misunderstood you but I gather you think the whole diet may be harmful quite apart from the phenylalanine.

DR. BESSMAN: Exactly.

DR. WOOLF: You could regard phenylketonuria as a disease of amino acid imbalance. The normal individual has evolved over countless millions of years to thrive on the proteins he finds about him, which contain about 5 or 6 percent phenylalanine. Since it is quite toxic, the normal individual has evolved an enzyme system that disposes of phenylalanine quickly. The phenylketonuric person is so efficient in his use of phenylalanine that he almost kills himself. We can restore the amino acid balance by giving him, not a normal diet, but a diet in which the amount of phenylalanine in relation to the other amino acids is such that he metabolizes and gets rid of his phenylalanine by all the other pathways—transamination, excretion in the urine, and other pathways—at about the same rate as he gets rid of all the other amino acids fed to him. This produces a normal blood level of phenylalanine.

DR. BESSMAN: The difficulty with this suggestion is: What is the effect of a diet that has been heated, hydrolyzed, passed through charcoal, and then supplemented with what we think we have removed?

We make the assumption that we are not destroying any growth factors, any factors necessary for mental function, and we assume that there are no essential peptides in a normal diet, which may be destroyed on hydrolysis. Yet, if you give a "total" hydrolyzed amino acid diet to a child, he doesn't grow exactly as well as normal, and we don't know about the effect on mental growth.

DR. WOOLF: I can give you a practical answer. These children grow naturally. They are healthy and well. We have a famous pair of twins picked up at 17 days of age. The treated twin is taller than her non-phenylketonuric twin. So there is normal physical growth. We know she has better mental health than without the diet.

DR. BESSMAN: How do you know that? What data do you have to compare?

DR. WOOLF: The pair of twins I just referred to have an older phenylketonuric sister who was not diagnosed until she was 2½ years old. She was put on a low phenylalanine diet at that time but her IQ has improved hardly at all, it is still under 20, she continues to have epileptic seizures as she had before treatment started and her EEG is still very abnormal. This may be contrasted with her younger sister who was diagnosed as phenylketonuric at the age of 17 days and in whom treatment started effectively
at the age of 5 weeks; her IQ has been steady at about 90 for the past 9 years or so, she has never had epileptic seizures and her EEG is normal.

Dr. Snyderman: A newborn baby can grow for 6 months perfectly normally on a completely synthetic diet. At about that time we see evidence of a lack of something still unidentified which is necessary for human growth. We have been able to demonstrate this time and time again.

This does not apply to the phenylketonuric child under treatment. He is not, in the present treatment regimen, being treated with a completely synthetic diet. Only part of the diet is synthetic. A number of natural foods are included in the diet, and these children will grow normally for an unlimited period of time with small supplements of natural foods. I would like to ask Dr. Bessman what evidence he has that a child who is properly maintained and monitored on a low phenylalanine diet is being harmed?

Dr. Bessman: I can only say I have looked over all the data that has been published and the average growth of children on these low phenylalanine diets is not normal. Each investigator says, "I have a couple of examples which are doing very well because they are well monitored."

I have seen people treating many cases and they each claim their patients are doing well and they only show their selected data. If you look at all the data the patients are not growing well.

Dr. Bickel: I strongly disagree with Dr. Bessman. I am a clinician and a pediatrician. I have responsibilities toward the parents and the child. If I allow damage to occur to the brain of a child which I can't later undo because I have been too generous with phenylalanine intake, I have not acted properly. I think it is much worse to have brain damage than a little less growth or some transient bone disease. I agree that in the course of the diet we may occasionally overtreat. We may even treat one patient too many, but I think that in this session so far the danger of the diet has been grossly exaggerated and the harm done to an untreated child has receded out of sight.
SUITABLE AND PREFERRED METHODS OF CHROMATOGRAPHY FOR THE STUDY OF URINE AND BLOOD IN INBORN ERRORS OF METABOLISM

MARY L. EFRON

It is generally agreed that the preferred method for quantitation of most amino acids present in biological fluids is the technique of ion-exchange chromatography. A major reason for the 1 to 3 percent accuracy of this method is that amino acids are separated from each other and from almost all interfering substances, as they are quantitatively eluted one by one from a column of resin. The chemical reagent which produces a visible color with the amino acid is thus applied to a relatively pure substance.

No matter how accurately a chemical method measures the amount of a pure compound, the method is subject to error when applied to the measurement of one compound in a mixture of many other substances, e.g., when the compound to be measured is present in blood or urine.

Ideally, therefore, the diagnosis of amino acid disorders and the measurement of amino acids in biological fluids during therapy should be based on ion-exchange chromatography. Unfortunately, this technique is at present much too expensive to be applicable to mass screening and treatment programs. For this reason, simpler and cheaper "second best" methods must be sought for detecting and monitoring therapy of phenylketonuria and other amino acid disorders.

Several excellent chemical methods for determination of blood phenylalanine in blood and urine are now available and have proved their usefulness. The bacterial inhibition assay of Guthrie has also proved to be surprisingly quantitative; at least for the measurement of blood phenylalanine concentrations of about 15 mg per 100 ml or less. This method has the great advantage that samples of blood can be collected on filter paper and sent through the post to a central laboratory, thus allowing for very frequent monitoring of blood levels and for acceptance by physicians of the small effort involved in collection of samples for screening programs. The automated McCaman-Robins method in use in the North Carolina PKU screening program takes advantage of the simplicity of collection of blood samples on filter paper. Studies are underway to determine whether quantitation of circles of blood punched out of a standard filter paper may be as accurate as the quantitation of very small samples measured from liquid serum by pipetting.

Paper chromatography has the same advantage as ion-exchange chromatography in that the amino acid to be quantitated is first separated from other amino acids and from most other compounds before application of the chemical reagent. Since the color value of the spot on paper is compared visually with the color of standard spots, the method is not as accurate as ion-exchange chromatography. Like the bacterial inhibition assay, however, the blood phenylalanine can be estimated within 2 mg per 100 ml if enough standards are applied to the same paper for comparison. This technique is very simple and quite inexpensive. One needs only glass jars, a few inexpensive reagents and filter paper. A technician can be instructed in a few hours in the methodology.

When the mass screening program for phenylketonuria was first instituted in Massachusetts, it was decided that all specimens which had any elevation of blood phenylalanine by inhibition assay should be tested, not only by a confirmatory chemical test, but also by paper chromatography before the diagnosis of phenylketonuria was made. It was appreciated (1) that certain infants would have and elevated phenylalanine concentration secondary to the accumulation of tyrosine because...
of a transient deficiency of para-hydroxyphenylpyruvic acid oxidase. These infants should not be diagnosed as phenylketonuric. (2) That infants with liver disease would show an increase, not only of phenylalanine but also of many other amino acids in the blood. The bacterial inhibition assay and chemical tests would be unable to distinguish this condition from phenylketonuria and these infants might be subjected to a low phenylalanine diet. (3) That previously undescribed abnormalities of amino acid metabolism might be detected in the entire population of newborn infants screened for elevated blood phenylalanine. (4) That a dehydrated or a premature infant on a very high protein diet might have an increase in the blood phenylalanine concentration, as well as in the concentration of other amino acids.

In retrospect, we are certain that the decision to do a paper chromatogram on every blood sample which has an elevated blood phenylalanine was a wise one. On three occasions, we have been consulted by physicians in other States which were not then doing routine chromatography. In each case, the infant had been found to have an elevated blood phenylalanine concentration by means of the Guthrie Inhibition assay. The enzymatic assay of LaDu was then performed and was reported as indicating a very much higher blood phenylalanine concentration. The three infants were then put on Lofenalac®. In two of the States, the infants did poorly on the low phenylalanine diet and this was the reason for consultation. We asked for the original blood spot which was collected in the newborn period for the bacterial inhibition assay. Paper chromatography showed a marked increase in the tyrosine concentration with only a moderate elevation of blood phenylalanine. It may be that the technicians performing the test were not experienced enough to distinguish tyrosine from phenylalanine by means of this chemical method. The infants were immediately taken off the low phenylalanine diet. In the third State, the infant was the very first one detected with a markedly elevated blood phenylalanine. The physicians in charge of the State screening program were concerned about the diagnosis, because of the discrepancy between the results of the inhibition assay and the LaDue test and because the infant was premature. Paper chromatography was hastily set up and (with such motivation) the technique of paper chromatography of blood spots was mastered within two days. Again, the blood tyrosine concentration was much greater than the blood phenylalanine concentration. The infant was taken off Lofenalac® but it was an unfortunate beginning for a screening program which had already met some resistance in that state.

We have encountered on several occasions in apparently healthy and thriving infants, an elevation of tyrosine, phenylalanine, valine, leucine, isoleucine and methionine in the blood. In each instance, the infant has been followed without dietary therapy and the amino acid pattern has returned to normal within two or three months. Without paper chromatography, these infants might well have been put on a low-phenylalanine diet.

Methods

Blood Chromatography.--Paper chromatography of blood amino acids has only recently been widely applied. Until a few years ago, it was thought that blood must be deproteinized before chromatography. The reason for this is that Dent (1) who first applied paper chromatography to the study of amino acids in physiological fluids, used phenol as the first chromatographic solvent; in phenol, protein streaks so that chromatography of blood in this solvent is impossible without prior deproteinization. Most investigators, therefore, confined their efforts to the study of urine.

In 1962, Culley et al. (2) observed that serum could be chromatographed directly, without deproteinization, if an acid-alcohol solvent, itself an excellent protein precipitant, were used as the chromatographic solvent. With the advent of screening programs which utilized blood collected on filter paper, it seemed desirable to develop a method for chromatography which took advantage of this simple collection system. (3) In our experience, chromatography of blood spots is as satisfactory or more so than serum chromatography. Indeed, when a hemolyzed serum sample arrives at the laboratory, we promptly spot it on paper, autoclave the specimen and treat it exactly as a whole blood spot.

Figure 1 shows the semi-quantitation of blood phenylalanine in serum. Standards with phenylalanine concentrations of 2.5 to 30 mg percent per 100 ml are spotted on the same paper as the
serum. Two samples are applied, one containing 5 microliters of serum and the other 10 microliters. The size and color of the spot is then compared with that of the standard solution and a reasonably accurate estimate of the serum phenylalanine concentration made. It is quite easy to detect 2.5 mg per 100 ml of phenylalanine in this manner; therefore if no detectable phenylalanine spot is present, we would consider an increase in the phenylalanine intake in a child on dietary therapy. If the spot is greater than the 7.5 mg per 100 ml standard, the phenylalanine intake may need to be decreased. This degree of accuracy is quite sufficient for monitoring blood phenylalanine concentration during therapy.

Figure 2 shows the chromatography of blood spots punched out of filter paper. The phenylalanine concentration can be estimated within about 2 mg per 100 ml.

This method is in daily use in monitoring several patients on therapy. In addition, we have detected actual patients with the following inborn errors using the blood spot method: hyperglycinemia, hydroxyprolinemia, hyperprolinemia, citrullinemia, hypermethioninemia, maple syrup urine disease and ornithinemia (a previously undescribed inborn error of metabolism). Samples are sent to the laboratory from great distances. We confirmed a case of phenylketonuria in Puerto Rico and we receive many samples from Singapore, Lebanon, etc. The advantages of a method which enables samples to be sent for only the price of a postage stamp are obvious. About one-third of all newborn infants in Massachusetts are now being tested by this method, in an attempt to determine the incidence of amino acid disorders and to detect treatable disorders early.

Chromatography of Urine.--Urine amino acid chromatography has been less helpful in the study of phenylketonuria. This is necessarily the case for all disorders of those amino acids which are efficiently reabsorbed by the renal tubule. These amino acids do not appear in great excess in the urine unless the concentration in blood is very high. Disorders such as maple syrup urine disease are easily missed if only urine is tested. In contrast, disorders with accumulation of amino acids which are not reabsorbed by the renal tubule (e.g. cystathioninuria and argininosuccinic aciduria) as well as defects in a renal tubular "transport system" (e.g. cystinuria and Hartnup Disease) are much more easily detected in urine than in blood.

In PKU programs, urine phenol chromatography is useful for monitoring the excretion of
p-hydroxyphenylacetic acid (PHPLA), p-hydroxyphenylpyruvic acid (PHPPA) and o-hydroxyphenylacetic acid (OHPPA). O-hydroxylacetic acid is quite stable and is easily separated and roughly quantitated by paper chromatography. The chromatographic method of Berry \(^{(4)}\) is a simple and reliable one for separation of these phenols.

It is recommended that this technique be available in all laboratories concerned with the diagnosis of and monitoring of therapy in patients with phenylketonuria and tyrosinemia.

Thin Layer Chromatography.—This relatively new technique is almost as simple as paper chromatography and is more rapid. In addition, quantitation can be obtained by scraping off the spots into a test tube and measuring the ninhydrin color. It requires very little blood; 1-2 microliters is a suitable quantity for a serum amino acid chromatogram. Thin layer plates can be used for two dimensional chromatography in the same solvents which are in common use for paper chromatography. The technique is not as useful for urine chromatography because salt produces more streaking on thin layer plates than on paper. A method which desalts the sample is in use in Gainesville, Florida;\(^{(5,6)}\) this method uses the DNP derivatives of amino acids which are extracted into organic solvents, leaving most of the salt behind (figures 3, 4). The DNP amino acids can be eluted from the thin layer chromatograms and quantitated spectrophotometrically.

Ion-Exchange Chromatography.—Much research is at present underway to simplify and make less expensive the technique of column chromatography. The amino acids in protein hydrolysates can now be separated and quantitated in three hours or less. The cost of an amino acid analyzer suitable for measuring amino acids in physiological fluids is still of the order of $8000 to $15,000.

On a standard Technicon amino acid analyzer using a technique designed for physiological fluids, the complete amino acid analysis requires 17 hours. Obviously the number of analyses which can be done in this fashion is limited.

In our laboratory, we have recently adapted a method developed by Dr. Gerald Mechanic of the Massachusetts General Hospital, which gives an accurate measurement of tyrosine and phenylalanine in about 30-45 minutes. The method uses a small column 0.9 by 20 cm with a water jacket kept at 65°C. It requires only one buffer, a sodium citrate buffer which is 0.38M with respect to sodium and has a pH of 4.30. When this buffer
Figure 3. Thin layer chromatography of the "ether soluble" DNP amino acids according to the method of Sunderman, et al.

Figure 4. Thin layer chromatography of the "acid soluble" DNP amino acids according to the method of Sunderman, et al.

Solvent Front

Solvent Front

2,4-DNA

2,4-DNA

Solvent Front

Solvent Front

Starting Point

Starting Point

Tolune - Pyridine - Ethylenechlorohydrin - NH₄OH

(100:30:60:60 v/v)

CHCl₃ - Benzel - HAc

(70:30:3 v/v)

n-Propanol - NH₄OH

(70:30 v/v)

n-Butanol - NH₄OH

(80:20 v/v)

is pumped through the column at 60 ml per hour, the acid and neutral amino acids are eluted in a single peak, followed by a peak composed of the leucines and then by distinct peaks of tyrosine and phenylalanine, which can be accurately quantitated (figure 5). The appropriate amount of plasma for analysis on our Technicon amino acid analyzer is 0.1 ml to 0.25 ml. With two columns, one regenerating while the other operates, at least 15 samples plus a standard can be analyzed each day. With higher flow rates and a new commercially-available resin with small particle size (Spherix XX-909-10, Phoenix Precision Instrument Co.), the speed of elution of the amino acids can be increased and the column length decreased so that a run is completed in 15 minutes (17) (figure 6). This system has now been set up in the Massachusetts State Diagnostic Laboratory and will be used as a confirmatory test for phenylketonuria to replace or supplement chemical methods. It has the great advantage of accurately quantitating tyrosine as well as phenylalanine. We have consulted one of the manufacturers of amino acid analyzers and are now testing the method on an analyzer which could be marketed for about $3500. This is competitive with the cost of spectrophotometers and fluorometers, and might soon be practical for laboratories involved in PKU screening programs. For any laboratory which now operates an amino acid analyzer, this method can be set up at virtually no additional expense.

It is anticipated that future developments in column chromatography, with improved resins and methods, will enable us to use this technique both for monitoring blood levels in treated patients with amino acid disorders, and possibly even for screening for metabolic disorders.
A 10-13 micro particle size resin Spherix xx-8-60-0, (Phoenix Precision Instrument Co.) was used in an 0.9 x 20 cm column. The amino acids were eluted with a pH 4.30 sodium citrate buffer, 0.38M with respect to sodium ion at a flow rate of 60 ml per hour.
Figure 6. Ion exchange chromatography of a standard solution of tyrosine and phenylalanine, 0.1 μ mole of each, using a Phoenix automatic amino acid analyzer, model VG 8000.

SUMMARY

1. Paper chromatography of amino acids is recommended as an adjunct to the methods now available for screening and for monitoring of blood levels both in phenylketonuria and in other inborn errors of amino acid metabolism.

2. No patient should be diagnosed as phenylketonuric without demonstration by some method that phenylalanine is the only amino acid which is markedly elevated in the blood. At present, paper and ion-exchange chromatography are the methods of choice for this purpose. Paper chromatography is less accurate but also much less expensive and it is practical for mass analysis.

3. Thin layer chromatography offers another possible approach for quantitation of phenylalanine and other amino acids in blood and urine.

4. Ion-exchange chromatography is the best method available to date, but is now too expensive for routine use. A simplified method for column chromatographic quantitation of tyrosine and phenylalanine in physiological fluids has been presented. It is hoped that better techniques will soon enable us to use column chromatography routinely in programs designed to detect and treat PKU and other disorders of amino acid metabolism.

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DISCUSSION

DR. SCRIVER: I want to make two comments. A Spinco Model 120 amino acid analyzer may be run using 20 cm of the spherical resin for basic amino acids in physiological fluids in a 0.9 cm diameter column to separate tyrosine and phenylalanine. The elution conditions are: buffer flow rate 50 ml/min; column temperature 55°C or 60°C; and buffer pH 4.25 (0.2N sodium concentration). Elution occurs in about 45 minutes.

DR. EFRON: Anybody who has an amino acid analyzer can set this up at very little expense. There are several new resins which have excellent properties for this separation.

DR. UDENFRIEND: How about the cleaning out that goes on afterwards?

DR. EFRON: We use two columns. One is cleaning up while the other is running.

DR. SCRIVER: My other comment concerns our experimental screening program for amino-acidopathies; we collect the bloods in capillary tubes, an initial sample being collected shortly after birth and a follow-up sample at 4-8 weeks of age. We have examined about 20,000 children now and a rough time and motion study shows that we are almost as efficient as Dr. Woolf's procedure. We can run through 250 samples a day without too much difficulty, and this includes the clerical time. The interesting thing is that this type of screening program proves the newborn infant is a very diverse organism. He may appear to have a great number of aminoacidopathies, including maple syrup urine disease, phenylketonuria, homocystinuria, homocitrullinemia, etc. on the basis of the screening test; a few days later on repeat study, he is normal. The use of this investigation of the human infant has underscored the "normal" diversity of the organism at that age. It is an interesting screening program and there is a good deal to be learned about adaptation at the time of birth. Our experience would be that any positive test must be confirmed by a subsequent follow-up.

DR. UDENFRIEND: When we collect blood on paper we dry it and therefore enzymes are probably inactivated. It is my experience that if a liquid sample of blood is collected, changes occur. After all, huge amounts of protein are present. In measuring tyrosine I find that allowing a blood sample stand in the icebox for several days, the value gradually rises as you showed. So I wonder about the capillary samples that are sent through the mail. Don't these levels gradually increase?

DR. SCRIVER: In the technique Dr. Efron developed and the technique we developed the rise is small and not significant. We sent known samples through the mail, summer and winter and no significant change occurred.

There is one disadvantage to capillary tubes in Canada. When sent in the mail, a certain number of them will freeze.

DR. BICKEL: I was very interested in your methods but I was always worried about one problem with chromatography. There are a lot of the amino acids in the blood, and they run close together, and I was worried about overlapping and I often found myself repeating the study two-dimensionally. I am a little worried using your method because I fear overlapping.
DR. EFRON: This is a good point. We have picked up an example of the most difficult disorder to detect, hyperglycinemia. Glycine overlaps several other amino acids but if elevated it produces a large spot. In order to determine which amino acid is elevated we can cut out another blood spot and chromatograph it in a different solvent system which will separate the areas that are overlapped in the first solvent system. We also use special stains to detect selected amino acids. Each time a new amino acid disorder comes along, we must change the system a bit to be sure that we can detect the new abnormality.
The concept that the problem of meeting protein requirements is basically that of providing the minimal requirements of the essential amino acids has prevailed for over fifty years. In 1914 Mendel(1) wrote "The possibility of growth and the extent to which it is accomplished are limited by the supply of each individual amino acid. It matters not whether this is exhibited as such or in the guise of protein, in either event, the law of the minimum is exemplified". This view, which led to a great deal of effort to determine the amino acid requirements of man (that of Rose for men, of Leverton for women, of our group for infants, and most recently that of Nakagawa for older children) has succeeded in providing a large body of basic information. However, it should be pointed out that these requirements are not rigid ones which pertain to all circumstances but are related and influenced by a number of other factors.

The greatest problem in ascertaining the adequacy of protein has been what criteria to use. In adults, the maintenance of nitrogen equilibrium has been used widely; however such equilibrium can be attained with different intakes of nitrogen since the body adjusts itself to higher intakes with an increase in both turnover rate and in excretion. There is a belief that nitrogen equilibrium at a low level is not as healthy a state as nitrogen equilibrium at a higher level, but results obtained from studies with different types of stress have not demonstrated any advantage of higher nitrogen intake.

The normal state in the growing child is that of nitrogen retention, the younger the child the greater the retention in relation to body weight with the premature infant having the greatest nitrogen retention. The definition of "normal" nitrogen retention is also complicated by the fact that it varies directly with the intake. Infants given high intakes of protein or nitrogen will retain correspondingly large amounts. This increased retention with intake seems to be a function of age, occurring in the infant but not in the older child. The explanation seems to be that higher protein intakes permit more rapid chemical maturation of the body. Direct analysis of the fat-free body(2) has demonstrated a nitrogen content of 3.3 percent in the adult human while the newborn baby has just under 2 percent and the premature infant even less. It is presumed that the adult composition is much more rapidly attained when more protein is fed. Animal work has confirmed that this does occur(3). Whether there is any advantage to chemical maturation such as more rapid functional maturation remains to be demonstrated.

The most practical measure of the adequacy of the diet in the growing child is the weight gain. Standards of weight gain are documented much better than those of nitrogen retention. Weight gain due to fluid accumulation or to excess fat deposition becomes readily apparent. Increase in length or height is a useful criterion in long term studies. The increase in bone length over short intervals can be measured accurately by the X-ray technique developed by Day and Silverman(4).

A number of chemical measurements have been recommended to ascertain the state of protein nutrition. The serum albumin is regularly depressed, but it may take a prolonged period of inadequate protein intake before a significant drop becomes apparent. A more sensitive test, but one not readily available, is the measurement of the albumin pool of the body by the use of labelled albumin. Although a number of plasma enzymes fall with insufficient protein intake, such measurements are not specific enough and have not proved to be of any more value than the level of the serum albumin. Plasma urea levels tend to fall as the protein intake becomes deficient,
however a nutritionally inadequate protein may result in high blood urea levels because of deamination of amino acids which cannot be utilized. The plasma alpha amino nitrogen has also been suggested as an index of the adequacy of protein intake; it does tend to fall in cases of severe and prolonged protein depletion. However, in less severe depletion, it may fail to reveal the deficiency state since the unessential amino acid levels of the plasma tend to rise at the same time that the essentials fall, thus the sum total may remain within the normal range. The free amino acids of the plasma are a very sensitive indication of the adequacy of the protein intake. We have observed significant changes in the plasma aminogram as early as 48 hours after the reduction in intake of a protein of good quality, and marked changes may be observed in true deficiency states such as kwashiokor.(5) (figure 1).

The exact protein requirement of the infant is not known at present. Newborn infants and even premature infants apparently do well over a wide range of protein intake. For some years we have been interested in the effects of extremes of protein intake on the premature. Clinically, all but a few of the smallest prematures (those with a birth weight under 1200 grams) seem to do as well when fed 2 grams of protein as when given 9 grams of protein per kilogram per day (figures 2 and 3). These two diets are carefully constructed so that they are similar in all respects except for the protein content. Both groups of babies conform to the same standards of weight gain and both grow at the same rate by the X-ray bone growth technique. There are however, a number of chemical differences. The high solute load imposed by the high protein diet on the immature kidney reduces the margin of safety against dehydration. The blood urea nitrogen of the high protein group tend to be extraordinarily high—often in the range of 100 mg percent while the levels of the low protein group remain in the normal range. We have not been able to demonstrate any untoward effects that may be attributed to this chemical derangement. Others(6) have shown that the blood urea nitrogen of premature infants tends to rise with intakes of over 3.5 grams of protein per kilogram. There is also a great difference in the amount of nitrogen retained, the high protein group retaining two to three times as much as the low protein group. Since only a small fraction of this retained nitrogen can be accounted for by the increase in the non-protein nitrogen constituents of the plasma, the presumption is that chemical maturation is proceeding more rapidly. In addition there are a number of distinct differences in the plasma aminograms of the two groups (figures 4 and 5).

Of equal importance as the quantity of protein in the diet is the quality. The deficits of some poor quality proteins may be compensated for by feeding more of the protein. However, it is possible for the quality to be so poor that good growth and nitrogen retention cannot be attained regardless of how much protein is fed. Such a situation is demonstrated in figure 6.

There may be much more subtle manifestations of amino acid imbalance than the failure to grow and retain nitrogen. An intake of one essential amino acid just slightly below its requirement while the remainder of the diet is adequate in every way may result in very unusual changes in the plasma aminogram. Such changes occur even when weight gain and nitrogen...
Figure 2. Protocol of a premature infant fed 2.0 grams of cow's milk protein per kg.

COW'S MILK
2.0 GM PROTEIN/KG

The weight gain conforms to normal standards (Bellevue Hospital Premature Unit) and the nitrogen retention is approximately 200 mg/kg/day.
Figure 3. Protocol of a premature infant fed 9.0 grams/kg of cow's milk protein.

COW'S MILK
9.0 GM PROTEIN/KG

The weight gain also conforms to the normal standard but the nitrogen retention is much greater.
retention are still in the normal range; but there must be significant alterations in the amino acid metabolism to result in such marked deviations in the plasma (figure 7). Similar very striking changes in the plasma aminogram are seen after inadequate intake of the other branched chain amino acids (figure 8) and have been observed as early as three hours after the dietary shift. The response to complete withdrawal of phenylalanine from the diet of a normal infant differs from branched chain amino acid withdrawal. There is a depression in the levels of a number of other amino acids, the most marked deviation being in the tyrosine level (figure 9).

Not only is there a need for protein to fulfill the requirement of all the essential amino acids, but there is, in addition, a need for a certain amount of unessential nitrogen. We were able to demonstrate this in full term infants in whom we were trying to ascertain the minimal protein requirement. The intake of cow's milk protein was reduced while all the other dietary components were kept constant. Nitrogen retention fell and then there came a point when the weight gain ceased. At this point, there were two possibilities: (1) that there might be a deficiency of one or more essential amino acids that was limiting in milk protein, and (2) that there might be a deficiency of "unessential" nitrogen. We tested the
Figure 6. Response of a premature infant to a protein of poor quality.

COTTONSEED FLOUR

Increasing the quantity fed does not result in adequate weight gain.
Figure 7. Alterations in the plasma aminogram resulting from a leucine intake just below the requirement.

PLASMA AMINOGRAM
1 WEEK PARTIAL LEUCINE DEFICIENCY (M.S.)

The dotted line is the average of 14 infants receiving the full quota of leucine and the shaded area represents one standard deviation above and below the average.

Figure 8. Alteration in the plasma aminogram resulting from the withdrawal of isoleucine for 4 days.

PLASMA AMINOGRAM
IN ISOLEUCINE DEFICIENCY (TA)

Figure 9. Alteration in the plasma aminogram resulting from the withdrawal of phenylalanine for 1 day (average of 4 subjects).
second possibility first by adding a supplement of a very simple nitrogenous compound, urea; and weight gain and nitrogen retention were resumed at their previous rates (figure 10). Similar studies were performed on four other subjects with the same results. Evidence that this material was indeed being utilized for new tissue production was obtained from studies with tagged urea. It was incorporated into both serum protein and hemoglobin.

The factors which influence protein requirement also influence amino acid requirements; hence they may be valid only under the circumstances in which they were determined. However, when amino acid requirements are determined with all other dietary factors similar to natural ones, the result is then a set of values with a wider area of usefulness. The studies of the amino requirements of infants, carried out by our group at New York University, made use of a synthetic diet which is based on the composition of human milk. A synthetic diet was necessary in order to control accurately and at will the intake of the amino acid under study. The protein moiety was a mixture of 18 L-amino acids (both essential and unessential) in the same proportion as they occur in human milk. Adequate calories were provided, and in addition a mineral and a vitamin mixture, containing all the known factors were included (Table 1). Before the studies were initiated, the diet was given the most severe test; it was fed to a number of premature infants and good weight gain did occur (figure 11).

All of the amino acid requirement studies were carried out in the same manner, all were performed in male infants one to three months of age. After a suitable control period on the complete amino acid diet in which the infant's growth rate and nitrogen retention were ascertained, the amino acid under study was then completely withdrawn from the diet. The diet was kept isonitrogenous at all times by the substitution of an appropriate amount of nitrogen. The amino acid under study was then introduced in a stepwise fashion until both weight gain and nitrogen retention were in the same range as they were on the full amino acid diet. Such a study of the phenylalanine requirement is shown in figure 12. The phenylalanine requirement determined for six infants was between 47 and 90 mg/kg/day\(^{6}\). These studies were performed with a full quota of tyrosine. It is to be presumed that the phenylalanine requirement would be considerably higher in the absence of adequate tyrosine.

Similar studies have now been performed for all of the essential amino acids. Of particular interest has been the demonstration that the young baby, unlike the adult, has a definite requirement for histidine\(^{6}\). Withdrawal of this amino acid also produced the only specific clinical picture seen in the whole series of studies. Babies not only failed to gain weight and retain adequate

---

**Figure 10. Demonstration of the requirement for "unessential nitrogen"**

**BABY RI 2 WEEKS OLD**

<table>
<thead>
<tr>
<th>TOTAL SERUM PROTEIN</th>
<th>SERUM ALBUMIN</th>
<th>SERUM GLOBULIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>5</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>NITROGEN RETENTION (MG/KG/DAY)</th>
</tr>
</thead>
<tbody>
<tr>
<td>300</td>
</tr>
<tr>
<td>200</td>
</tr>
<tr>
<td>100</td>
</tr>
<tr>
<td>30</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>WEIGHT IN KILOGRAMS</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.0</td>
</tr>
<tr>
<td>5.5</td>
</tr>
<tr>
<td>5.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PROTEIN INTAKE (GM/KG/DAY)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0</td>
</tr>
<tr>
<td>4.0</td>
</tr>
</tbody>
</table>

The infant failed to gain weight and retain adequate nitrogen when 1.1 grams/kg of cow's milk protein was provided. Normal weight gain and nitrogen retention resulted when a supplement of urea was added.
<table>
<thead>
<tr>
<th></th>
<th>Gm.</th>
<th>% of Total Calories</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>L-Amino acid mixture</strong>*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Alanine</td>
<td>2.67</td>
<td></td>
</tr>
<tr>
<td>L-Arginine</td>
<td>4.58</td>
<td></td>
</tr>
<tr>
<td>L-Aspartic acid</td>
<td>8.78</td>
<td></td>
</tr>
<tr>
<td>L-Cystine</td>
<td>2.14</td>
<td></td>
</tr>
<tr>
<td>L-Glutamic acid</td>
<td>17.56</td>
<td></td>
</tr>
<tr>
<td>L-Glycine</td>
<td>2.06</td>
<td></td>
</tr>
<tr>
<td>L-Histidine</td>
<td>1.76</td>
<td></td>
</tr>
<tr>
<td>L-Isoleucine</td>
<td>6.11</td>
<td></td>
</tr>
<tr>
<td>L-Leucine</td>
<td>11.76</td>
<td></td>
</tr>
<tr>
<td>L-Lysine hydrochloride</td>
<td>7.10</td>
<td></td>
</tr>
<tr>
<td>L-Methionine</td>
<td>1.68</td>
<td></td>
</tr>
<tr>
<td>L-Phenylalanine</td>
<td>4.89</td>
<td></td>
</tr>
<tr>
<td>L-Proline</td>
<td>6.11</td>
<td></td>
</tr>
<tr>
<td>L-Serine</td>
<td>5.34</td>
<td></td>
</tr>
<tr>
<td>L-Threonine</td>
<td>4.58</td>
<td></td>
</tr>
<tr>
<td>L-Tyrosine</td>
<td>4.58</td>
<td></td>
</tr>
<tr>
<td>L-Tryptophan</td>
<td>1.68</td>
<td></td>
</tr>
<tr>
<td>L-Valine</td>
<td>6.64</td>
<td></td>
</tr>
<tr>
<td><strong>Corn oil</strong></td>
<td>160</td>
<td>43%</td>
</tr>
<tr>
<td><strong>Dextrin-Maltose</strong></td>
<td>375</td>
<td>45%</td>
</tr>
<tr>
<td><strong>Mineral mixture</strong>*</td>
<td>22.3</td>
<td></td>
</tr>
<tr>
<td><strong>B vitamin mixture</strong>*</td>
<td></td>
<td>10.0 ml. per day</td>
</tr>
<tr>
<td><strong>Vitamins A, C, and D supplied as TriVisol</strong></td>
<td></td>
<td>0.6 ml. per day</td>
</tr>
</tbody>
</table>

*The composition of the mineral mixture was as follows NaCl 18.9%, CaHPO₄ (anhydrous) 25.4%, MgSO₄ (anhydrous) 6.8%, KHCO₃ 44.4%, KCl 2.88%, Fe₂ Citrate 2.21%, CuSO₄ (anhydrous) 0.24%, MnSO₄ (anhydrous) 0.15%, KI 0.015%, NaF 0.03%.

**The composition of the B vitamin mixture was as follows: thiamine 0.38, riboflavin 2.0, niacinamide 0.85, calcium pantothenate 3.5, pyridoxine 0.67, hexahydroxycyclohexane 180, para-aminobenzoic acid 0.5, folic acid 0.05, choline chloride 147, biotin 0.03, cyanocobalamin 0.015 mg.
nitrogen, but those under 4 months of age developed a skin rash clinically and pathologically similar to that of infantile eczema.

The amino acid requirements of infants are summarized in Table 2. There is the question of how valid these results are, obtained with synthetic diets instead of natural diets. They are, however, in the same range as those which infants fed either human milk or cow's milk receiving 2 grams of protein/kg would receive. Since all of these studies were carried out in relatively short periods of time the question also arose of how adequate they were for prolonged periods of time. All continued to gain weight well and retain adequate nitrogen for periods as long as four months.

Confirmation of the validity of the phenylalanine requirement figures has come from the requirement of the phenylketonuric infant under treatment, who is receiving a different type of diet. If one considers that the minimal requirement of an amino acid is that amount which provides for normal growth and tissue repair without any excess to be processed through other channels,
TABLE 2.--The Essential Amino Acid Requirements of Infants Compared to Intakes on Low Protein Diets Compatible With Health

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Minimum Requirement Mg/Kg/Day</th>
<th>Human Milk (155 MI/Kg)</th>
<th>Cow's Milk Fed at Level Providing 2 Gm. Protein per Kg.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histidine</td>
<td>34 (16&lt;34)*</td>
<td>32</td>
<td>45</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>119 (102&lt;119)</td>
<td>123</td>
<td>128</td>
</tr>
<tr>
<td>Leucine</td>
<td>150** (76&lt;229)</td>
<td>230</td>
<td>216</td>
</tr>
<tr>
<td>Lysine</td>
<td>103 (88&lt;103)</td>
<td>112</td>
<td>156</td>
</tr>
<tr>
<td>Methionine (in presence of cystine)</td>
<td>45 (33&lt;45)</td>
<td>73</td>
<td>52</td>
</tr>
<tr>
<td>Phenylalanine (in presence of tyrosine)</td>
<td>90 (47&lt;90)</td>
<td>92</td>
<td>104</td>
</tr>
<tr>
<td>Threonine</td>
<td>87 (45&lt;87)</td>
<td>89</td>
<td>92</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>22 (15&lt;22)</td>
<td>31</td>
<td>30</td>
</tr>
<tr>
<td>Valine</td>
<td>105 (85&lt;105)</td>
<td>128</td>
<td>138</td>
</tr>
</tbody>
</table>

*The higher requirement for each amino acid is qualified by the symbol "less than" because the minimum requirement must lie somewhere between that which is adequate and a lower figure which was shown to be inadequate.

**With one exception the requirement for leucine did not exceed 150 mg. per kg.: The subject who required 229 mg. per kg. may have represented an anomaly.

then the phenylketonuric infant is an ideal subject for ascertaining phenylalanine requirements. Thus far, all of the young phenylketonuric infants that we have observed, have, when free of infection, gained weight normally and maintained plasma phenylalanine levels in the normal range when receiving 70 to 85 mg/kg of phenylalanine per day. These figures are valid only for the first months of life; there is then a gradually decreasing requirement when expressed on a weight basis.

Tyrosine has usually been considered to be an unessential amino acid, enough can be produced from phenylalanine when the intake of the latter amino acid is adequate. However, the nature of the metabolic defect in phenylketonuria makes tyrosine an essential amino acid and hence it must be provided as such in the diet. This fact became quite apparent when we treated our first patient in 1954. The only dietary preparation available at that time was low in tyrosine as well as phenylalanine and accordingly we added a supplement of tyrosine. The effect of omitting the tyrosine supplement for one week on hair pigment is demonstrated in figure 13.

We have not determined the tyrosine requirement, but it would seem very unlikely that the amount provided by 150 cc/kg of human milk or 2.0 gm/kg of cow's milk protein would be more than the requirement. This is between 93 and 116 mg/kg/day; it is of interest to note here that we have recently obtained some information that suggests that tyrosine seems to be an essential amino acid for some premature and newborn infants(16). A number of such infants fail to gain at a normal rate when tyrosine is withdrawn from the diet. This is presumably the result of a maturation defect in the same enzyme system.
SUMMARY

Although there are a number of gaps in our knowledge of both protein and amino acid requirements, certain practical recommendations can be made. Protein intakes of 3.0 gm/kg for the premature and of 2.0 to 2.5 grams for the full term infant are compatible with good growth, eliminate the chemical alterations which occur with higher intakes and provide a margin of safety. The phenylalanine requirement of young infants does not exceed 90 mg/kg/day; intakes ranging from 47 to 90 mg/kg/day have been accompanied by normal weight gain, adequate nitrogen retention, and ever clinical evidence of good health.

REFERENCES


DISCUSSION

DR. WAISMAN: Did you have a chance to study the blood amino acid concentrations when a PKU child was maintained on 25 mg per kilo per day of phenylalanine or any other levels? Did the other amino acids change in any way?

DR. SNYDERMAN: We have had the opportunity to study only a few phenylketonuric children on inadequate intakes of phenylalanine because our aim has been to provide enough to maintain normal plasma levels and to insure normal growth. The plasma levels of other amino acids
did not markedly deviate. Restriction of the intake of branched-chain amino acids causes the greatest alteration in the plasma aminogram.

DR. SASS-KORTSAK: When treating phenylketonuria, our aim is to keep the patient on a diet which contains the minimal amount of phenylalanine necessary for normal growth. If then the patient is provided with amounts of Lofenalac\textsuperscript{8} to satisfy these requirements in respect to amino acids other than phenylalanine and the phenylalanine intake is adjusted by adding to the diet pure phenylalanine to achieve acceptable blood levels of phenylalanine, will this child grow normally? Is there any other factor missing?

This question refers also to Dr. Berry's remarks yesterday. She maintained that milk or some other natural foods should be fed to these children to provide some hypothetical essential food constituent.

DR. SNYDERMAN: The situation is complicated. It has been our feeling after our work some years ago in which we demonstrated still unidentified factors in normal diets, that it is best to include normal foods as early as possible in the diets of these children. Our own experience has been that if we begin giving necessary phenylalanine in the form of milk that we avoid growth failure.

Brewer's yeast can be given also to prevent growth failure in this situation. We have demonstrated this phenomenon in normal children.

DR. SASS-KORTSAK: May I rephrase my question? Is it always possible to correct failure of growth in a patient with phenylketonuria who is on a diet consisting of Lofenalac\textsuperscript{8} plus phenylalanine by increasing the phenylalanine intake only?

DR. SNYDERMAN: I don't have the answer for phenylketonurics, because we have not fed phenylketonurics completely synthetic diets. I think I have some information in maple syrup disease which would be similar.

The first child we treated for this disease was kept on a completely synthetic diet in order to maintain strict dietary control. That child had growth arrest at about 7 months of age on the completely synthetic diet. Since then we have provided the necessary amounts of the branched-chain amino acids in the form of milk. Children treated in this way have not had growth arrest.

DR. GAULL: We have been making the tacit assumption that the compound distal to the metabolic block becomes essential, but because it's made available in the diet the need has been met and the only significant problem is the accumulation of the metabolites proximal to the block.

If a homocystinuric patient is put on a synthetic diet and cystine is omitted, he falls into profound negative nitrogen balance within 24 hours.

I don't know whether this occurs in homocystinuria alone or in all of the metabolic errors. Is the deficiency distal to the block as unimportant as we have assumed?

DR. SNYDERMAN: A normal young baby given a deficient diet goes into less positive nitrogen balance readily. If an essential amino acid is withdrawn from a young baby a decrease in retention results almost immediately. This response is not different than what is seen in the cases with a metabolic error.

If leucine is removed from the diet of a normal infant the plasma level of leucine will drop considerably within three hours.

DR. GAULL: This seems to be of more than theoretical interest. These problems arise during maturation. This type deficiency may have permanent effect although occurring only intermittently during periods of critical maturation of the brain.

DR. BICKEL: We had trouble with the treatment of maple syrup disease. After some months the patients developed severe dermatitis though we closely followed the suggestions from your group to give all the necessary nutrients. We found out afterwards that for a longer period the yeast which had been given to the children was cooked because the children didn't take the yeast uncooked. Do you have any information about a thermo-labile factor in yeast necessary to prevent such serious outcome? Unfortunately, both children died of septic dermatitis.

You stated that you found a decrease of blood amino acids when a normal child was suddenly given a low phenylalanine intake. Have you any data that if you prolonged the experiment you would find an increase of the other amino acids as we have found in phenylketonuria?

DR. SNYDERMAN: In answer to the first question, we just don't know where this essential factor resides in yeast. We don't have a good test situation to try different factors. We have not been troubled with dermatitis in any of our children with maple syrup urine disease.
The second question concerned amino acid blood levels. This was a fairly acute study. If phenylalanine deficiency is allowed to persist, the child breaks down tissue and there will be an increase in other blood amino acids as well.

DR. ROUSER: I was wondering about the possibility that some essential fatty acid factor may be involved, particularly in view of the dermatological problems that appear.

I also want to ask if you have followed blood lipids in these children, and, more particularly have you noted any type of unusual fatty acid patterns?

DR. SNYDERMAN: The diet is not deficient in fats or fatty acids. The children are getting corn oil, which should provide adequate fat.

Earlier, when searching for this unidentified factor we tried other fats and combinations of other fats. They appear to have no bearing on the situation.

DR. SWAIMAN: To what length have you gone to document in those newborns who needed tyrosine, a relative phenylalanine-to-tyrosine block?

DR. SNYDERMAN: We are working on the question of essentiality of tyrosine for the newborn. We have noted that the child stops gaining weight when tyrosine is taken from the diet and when tyrosine is re-introduced into the diet normal weight gain is resumed.

It seems that there is enough of deficiency in the enzymes so that the infant can't make enough tyrosine.

DR. WAISMAN: Dr. Sass-Kortsak's point about providing phenylalanine in pure form in addition to the Lofenalac® presents a problem, because I think one important thing we must remember is that all of these patients are different. All the phenylketonuric patients do not fall into one mold and their requirements vary. I think that it is more important to provide intact protein in some form.

This unknown essential factor has been recognized by nutritionists for a long time. As Dr. Snyderman said, it is not easy to identify it. It might be a small peptide. It might be an unknown amino acid.

I would also say that Lofenalac® is a poor diet for older children, but younger infants can get along on Lofenalac® until 2, 3 or 4 months of age. When I fed Lofenalac® to monkeys they failed in about two months.

If they are then fed phenylalanine in small amounts that are similar to an infant's needs, they do respond. If they are then given more phenylalanine because the first amount might have been too little, they respond again. After that, no matter how much phenylalanine is provided, the infant monkey will not grow unless a very small amount of intact protein either in the form of powder or liquid skim milk or any other source of protein is added to the diet. This might be an animal-method assay procedure by which this factor can be found.

DR. SNYDERMAN: I don't believe that this factor is a protein. Our original work in which we first demonstrated the existence of the factor was performed with children on casein which is a whole protein. We found the same difficulty. At that time we didn't add B₁₂, until we added it. We used at least half a dozen factors in the intervening years and have not come upon the exact substance. But it is present in natural foods.

DR. BESSMAN: I'd like to ask two short questions. One, were any psychological tests done during the time when you deprived infants of important amino acids for relatively long periods of time?

Two, do you think that it's dangerous to deprive an infant of an important amino acid in such a critical period in their early part of life?

DR. SNYDERMAN: I don't know how we can perform psychological tests and show any difference in a 2-month-old child in one week's time. Certainly the behavior of the child toward nurses who are specially trained and know these children very well did not change in the least.

Until somebody can demonstrate any long-term difficulties in children deprived for very short periods of time, I don't think that this is a procedure I should worry about. I wouldn't deprive the child of an amino acid for longer than a week.

DR. UMANSKY: The question I have refers to whether other possible indices of nutritional status may be affected, for example, antibody responses to antigenic stimuli and the ability to withstand later nutritional stresses of other types, such as caloric deprivation.

In other words, do these infants exposed to deficient protein intakes shown any indication that physiologic responses other than linear growth and weight gain may be affected later on?
DR. SNYDERMAN: I do not think that this question can be answered at present. I do want to emphasize that these infants were receiving adequate protein. We are also engaged in a study in premature infants in which we are trying to determine if the protein intake has any effect on the development of enzyme systems known to be deficient in the premature infant.

There is a good deal of similar animal work. Dr. Halac from our group did a number of such studies, and he was unable to demonstrate that rats fed high protein intakes previous to stress were able to withstand stress any better than rats fed a low intake of protein.

DR. BICKEL: I wonder how much overlap there may be in the phenylalanine requirement of the normal child and of the phenylketonuric child.

DR. SNYDERMAN: I think the phenylketonuric child is the ideal child in whom to determine the requirement of phenylalanine.

The phenylketonuric child has the same requirements for tissue repair and growth as the normal child. The requirement of an essential amino acid is the least amount that will take care of body needs without any excess to be taken care of by other metabolic pathways and this, of course, is the aim of treatment in phenylketonuria. We have been very interested to find that all of the young babies we have treated have required phenylalanine intakes in the same range as those determined in our requirement studies for the normal infant. Fourteen infants under 3 months of age have all required 70-80 mg per kilogram per day of phenylalanine.

DR. SCHNEIDER: I would just like to cite a pilot study I did a couple of years ago that will somewhat pique your curiosity, and indicate perhaps that the phenylketonuric baby is not the ideal subject in which to study the minimum requirement.

Now, I have to hedge my comments by saying that I don't think the baby has phenylketonuria, I think he is heterozygous, whatever that means. At any rate, what was done was the following: He was placed on Lofenalac® at that time, about 40 mg of phenylalanine per kilogram per day. His blood level was running around 2 to 3 mg percent of phenylalanine. A synthetic diet was made up to provide over five days the same calories as his previous diet, the same total nitrogen and the same phenylalanine intake. The object was to see whether Lofenalac® might be keeping his blood level down for reasons besides a diminished phenylalanine intake.

I tried five different combinations, changing the amino acid mixture around but always providing at least one and a half times the upper limits of requirement of the essential amino acids and adequate tyrosine. I haven't found a mixture yet that would keep his blood phenylalanine level down as well as Lofenalac® even though the phenylalanine content was the same, the calories were the same, and the total nitrogen was the same.

Even after adding more free amine and several of the other essential amino acids, I still was unable to keep his blood level down.

Yet when I would put him back on Lofenalac® it came right down again. This was a very quick change in a matter of 12 to 24 hours. By three days the level would usually plateau about as high as it was going.

I think there is something about the balance here. I don't know the answer by any means. If one were going to use the phenylketonuric child to gauge the minimum phenylalanine requirement I have the impression that one would have to define very carefully the rest of the diet.

DR. SNYDERMAN: No, I think I attempted to make the point that there were many factors that influence amino acid requirements as well as protein. Without knowing how you juggled your other components of the diet, it would be very difficult to ascertain what was wrong.

If some other imbalance were caused the youngster may go into negative nitrogen balance and his phenylalanine would rise.

DR. HSIA: I want to ask the question as to whether you had any opinion on the use of differing phenylalanine requirements as a means of segregating separate types of phenylketonuric infants?

DR. SNYDERMAN: Well, certainly we have a fair variation in the so-called "normal". Indeed, we have a variation between 47 and 90 mg percent. Perhaps the only thing we can say is this: That if you take a normal infant and give him 90 mg phenylalanine per kilo, he maintains a normal phenylalanine level. If you give him 180...
mg per kilo. he still will have normal level, and if you give him 270, he still will have a normal level.

However, in phenylketonuria, perhaps we can give 100 to 120 mg per kilogram, but when we get to levels present in normal milk, the plasma phenylalanine level is going to rise. Perhaps if the blood level doesn't rise the patient is one of these other types, but we do not have any definite information about this point at present.
METHODS AND PROBLEMS OF ADMINISTRATION
OF THE LOW PHENYLALANINE DIET:
GUIDES TO BIOCHEMICAL AND DIETARY CONTROL

HELEN K. BERRY

It is essential that the diagnosis of phenylketonuria be confirmed before therapy is begun. Biochemical criteria which we require for making an unqualified diagnosis of phenylketonuria are as follows: \(^1\)

1. Serum phenylalanine over 15 mg percent
2. Urine phenylalanine over 100 μg/ml
3. Urine orthohydroxyphenylacetic acid over 10 μg/ml
4. Serum tyrosine below mg percent

The first three measurements form the basis for the biochemical monitoring system. One objective in use of the low-phenylalanine diet is to reduce serum phenylalanine levels. Phenylalanine in the diet which is not used for protein synthesis and which is not further metabolized is excreted in the urine. Orthohydroxyphenylacetic acid is considered the single most characteristic metabolic byproduct in phenylketonuria. \(^2\) It is present in urine from phenylketonuric patients at concentrations of serum phenylalanine at which no other abnormal metabolites of phenylalanine are present. It has been shown to be formed by a specific liver enzyme rather than a non-specific hydroxylation system. \(^3\) It has not been found in urine specimens from patients with fasting elevations of phenylalanine up to 20 mg percent from causes other than phenylketonuria.

The treatment of phenylketonuria consists of providing an intake of phenylalanine high enough to meet the nutritional needs of a growing child without exceeding his limited capacity to utilize it. The minimum amount required is determined by the amount needed to support optimal growth of the treated child. The maximum amount of phenylalanine which can be tolerated depends on the renal clearance of any excess of phenylalanine above that required for growth. The necessary balance can be achieved by considering serum phenylalanine levels, urinary phenylalanine excretion and urinary orthohydroxyphenylacetic acid excretion in relation to the dietary intake of phenylalanine.

Methods

Early in treatment the parents are taught to collect blood from a finger or heel puncture. They are provided with microhematocrit tubes, sealing material, and a protective package in which to mail blood specimens to the laboratory. Blood is obtained daily during the first week of treatment, weekly until the end of the second month, and thereafter at intervals of two weeks until one year of age. Specimens are obtained at monthly intervals thereafter, except during illness or deficiency states, when they may be requested more frequently. Blood specimens were usually obtained about 2 hours after a meal.

Filter paper urine specimens are collected daily during the first month of treatment, twice weekly to the end of the first 6 months, and weekly or every two weeks afterward, except daily during periods of illness.

The low-phenylalanine diet is prescribed as a combination of the following items:

- Lofenalac®
- Natural foods with phenylalanine content to make up the difference between the amount of phenylalanine supplied by the protein substitute and that required to support optimal growth
- Vitamin and mineral supplements
- Sufficient fluids

This work was supported in part by Grant HD00324 from the National Institutes of Health,
The dietary prescriptions for the individual infant are calculated on the basis of his protein, caloric, and fluid requirements. Phenylalanine content of the diet is adjusted according to individual needs as determined by the results of blood and urine testing. The parents are supplied with a list of a wide variety of foods for which the phenylalanine content is given for one tablespoonful of each food. Records are kept by the mother of daily intake of the amount of Lofenalac® and the type and amount of other foods and liquids which are actually consumed. The records are mailed with the daily urine specimens.

Initially serum phenylalanine was measured by paper chromatography. When the more sensitive fluorimetric method became available we used both procedures in determining serum phenylalanine levels of patients under treatment for phenylketonuria. Comparable results were obtained with the two methods. In a series of 130 determinations carried out with both methods the standard error of the paper chromatographic procedure was 1.4 mg percent, of the fluorimetric procedure, 1.2 mg percent. Standard error of duplicate determinations by the fluorimetric method was 0.6 mg percent. Subsequently the methods have been used interchangeably. The paper chromatographic procedures for measuring phenylalanine and orthohydroxyphenylacetic acid in urine specimens obtained on filter paper have been described in detail. Strips of standard size are cut from the dry paper specimen. These are used to prepare chromatograms for measurement of the appropriate substance.

It is not feasible to obtain many 24 hour specimens from young infants; obtaining a liquid specimen of any kind may be difficult. In earlier work urinary excretions were reported in terms of creatinine as a reference substance to take into account individual differences in urine volume. This was a useful correction in comparing excretion values from adults. Changes in muscle mass, on which creatinine excretion is dependent, are so great in infants and young children that creatinine is not useful as a reference. In a study of 2250 subjects ranging in age from birth to 16 years correlation of creatinine excretion with age was .45. After statistical analysis of data on excretion of amino acids and other urinary constituents by 2250 subjects were concluded that concentration per unit volume of urine was less variable than any other factor we could measure in a random urine specimen. Fluid intake of infants and young children is fairly uniform from day to day. Urinary excretion levels are expressed simply as μg/ml.

Results

Dietary control and biochemical control are of equal importance if the diet is to be properly designed and adequately managed. It is on the basis of the biochemical results that dietary changes are made. The following examples illustrate the relations between dietary phenylalanine intake, serum phenylalanine concentration, and urinary excretion of phenylalanine and orthohydroxyphenylacetic acid. The periods are arbitrarily chosen to facilitate interpretation of the graphs.

Figure 1 represents an infant who had a positive blood screening test of 20 mg percent at 3 days of age. Repeat serum phenylalanine at 11 days showed a level of 25 mg percent. The baby was first seen by us at 3 weeks of age. The biochemical findings represented in period 1 confirmed a diagnosis of phenylketonuria: serum phenylalanine was 33 mg percent, urine phenylalanine was over 100 μg/ml, orthohydroxyphenylacetic acid was present. The urine was also positive for phenylpyruvic acid. On the basis of those data a decision was made to treat the child.

Period 2 shows the first step in treatment. As a general rule the phenylalanine intake must be decreased to an amount 1/6 or 1/7 that usually supplied by an infant formula. This was done by substituting Lofenalac® formula for the regular evaporated milk formula. Lofenalac® is never given as the sole source of nourishment for more than 24 to 48 hours. By the second day serum phenylalanine had decreased to 3 mg percent. Urine phenylalanine decreased and orthohydroxyphenylacetic acid was no longer detected. Phenylalanine was added to the diet in the form of milk, 50 mg phenylalanine per ounce. On the third day serum phenylalanine was below 1 mg percent and the phenylalanine intake again was raised to a total of nearly 400 mg/day until the serum phenylalanine level rose above 3 mg percent.

Initially during the first stages of treatment (period 2) increases in phenylalanine intake were made rapidly until serum level reached 3 mg percent; then additional phenylalanine was added more cautiously until the phenylalanine concentration in serum approached 7 mg percent. However, serum levels continued to fall, as in period
3, and rapid adjustments in intake were again necessary.

Phenylalanine intake is adjusted with milk during the first few weeks of life. The phenylketonuric baby is introduced to strained infant foods at the usual time. By age six weeks Lofenalac® is offered as a semi-solid food, somewhat the consistency of a baby cereal.

Figure 2 shows a portion of early treatment of another infant. Treatment was begun elsewhere at 3 weeks of age (period 1). Serum phenylalanine dropped from 47 mg percent to 3 mg percent over a period of 12 days during which Lofenalac® alone was given. The family moved to Ohio when the baby was 5 weeks old (period 2). The private physician to which she was referred was familiar with our biochemical monitoring procedure. Frequent blood tests were continued and urine testing began. No change in the diet was made. The beginning of period 3 the child was first seen in our clinic. Serum phenylalanine was less than 1 mg percent, urine phenylalanine excretion was low, and orthohydroxyphenylacetic acid was negative. The intake was increased to 300 mg/day immediately in order to raise serum phenylalanine levels above 3 mg percent. Urine
phenylalanine excretion increased to 30 to 50 µg/ml, and serum phenylalanine responded to the increased intake. A brief period of increased phenylalanine excretion is shown at the end of period 3, followed by a decreased excretion to very low levels and accompanied by a drop in appetite. This pattern usually warns us of phenylalanine deficiency.

If the phenylalanine intake is not increased to meet the needs of the rapidly growing infant during the first months of life the resulting deficiency causes growth failure, anemia, rachitic bone changes, and death may occur. Figure 3 shows data from a child who had some of these symptoms. Treatment was begun at age 2 weeks. The diet consisted of Lofenalac® alone for the first 3 months. By that time the child was severely anemic with hemoglobin of 8 grams percent. She was transfused and one ounce of milk was added to the daily diet. Weight dropped from 75th percentile at the time treatment was started to 3rd percentile by 4 months. Height decreased more slowly. The patient was referred to our clinic at 5 months of age, having spent most of the prior 2 months in a hospital. Hemoglobin was 9 gm percent. Height and weight were below 10 percent. There were minimal bone changes suggestive of rickets. Hair was sparse. Serum phenylalanine was below 0.5 mg percent. This patient presented the characteristic picture of phenylalanine deficiency. Phenylalanine intake was immediately increased to 250 mg/day. Attempts to raise it still higher were initially unsuccessful because the child had never had solid food and first had to be taught to eat. Three months were required to correct the phenylalanine deficiency and raise serum phenylalanine levels into the range of 3 to 7 mg percent. The growth rate increased, weight improving more rapidly than height. She was near the 50th percentile in weight by one year of age and 37th percentile in height. The effect of phenylalanine deficiency and its subsequent correction on growth is shown in figure 4 in which length of the humerus at several stages is shown. The present patient is case 2. Compared to normal children of the same age the humerus length was below the 3rd percentile at 6 months of age; by 5 years humerus length was 90 percent. Other phenylketonuric patients whose treatment began in infancy at our clinic are shown.

Figure 3. Graphic presentation of height and weight, serum phenylalanine concentrations and phenylalanine intake in patient PM during first year of life.

The period during which the diet is administered principally as a liquid formula is the most difficult portion of treatment if properly carried out. During this time phenylalanine requirements are increasing most rapidly, and the supposedly normal baby is developing at normal rates. This early period is critical in establishment of feeding patterns. Careful attention must be paid to stages
of eating development to ensure that the child acquires eating practices and habits which make management of this severely restrictive diet possible over a long period. If a phenylalanine deficiency is allowed to develop as in this child, weeks or months may be required to correct it.

Figure 5 shows a somewhat later stage in the treatment of a child with phenylketonuria who was now 3 years old. The earlier years of treatment had not been eventful and the mother had been quite successful in making the necessary dietary adjustments. Period 1 shows a period of several weeks characterized by elevation of serum phenylalanine in the range from 15 to 25 mg percent increased excretion of urinary phenylalanine and of orthohydroxyphenylacetic acid. No marked alterations had been made in the total phenylalanine intake, and there was no evidence that the child was obtaining food without the mother's knowledge. However, she had never become accustomed to taking Lofenalac® in the "paste" or semi-solid form. She consumed approximately 30 ounces Lofenalac® formula daily. The total protein intake, that supplied by Lofenalac® formula with the small amount of natural foods, was judged less than the optimum for a child of this age. During the 6 month period from July to December there was 1 lb. weight gain, the weight percentile dropping from 50 percent in July to 10 percent in December. During period 2 the mother succeeded in increasing the total amount of Lofenalac® first by using double strength formula and then gradually increasing the amount of Lofenalac® in the paste or pudding form. No change was made in the total phenylalanine intake. However, the serum phenylalanine decreased to less than 7 mg percent and the urinary excretions decreased. It is not possible to supply sufficient protein to promote optimal growth in Lofenalac® as given as a liquid formula alone. A child cannot be expected to consume more than a quart of liquid daily including formula. By 3 years of age nearly 2 quarts of Lofenalac® (normal dilution) would be required to furnish the protein requirement.

It is particularly difficult to maintain biochemical control during illness. Figure 6 illustrates the effect of an acute episode.

Period 1 showed serum phenylalanine concentration between 3 and 7 mg percent; urine phenylalanine was 20 to 40 µg/ml, orthohydroxyphenylacetic acid was negative. Phenylalanine intake during period 1 was 400 mg/day. During period 2 there was elevation of urinary phenylalanine to 80 µg/ml and traces of orthohydroxyphenylacetic acid were excreted. A brief period of refusal to eat was followed by an increase in serum phenylalanine to 11 mg percent in spite of a decrease in phenylalanine intake. Period 3 includes an episode of diarrhea, vomiting and refusal to eat.

Mothers are instructed routinely to increase the total phenylalanine intake by 50 percent during an acute illness; they may use small quantities of high protein foods to achieve this if necessary, e.g. egg yolk or egg nog, milk, jello, broth, soups. Blood
Figure 5. Monitoring of treatment of SO at age 3 years.

Note decrease in serum phenylalanine levels and decrease in urinary excretions when total protein intake was increased, although no changes were made in total phenylalanine intake.

and urine specimens are requested more frequently during an illness.

During the two days following the sharp drop in food intake shown in period 3 serum phenylalanine remained at 6 mg percent. However, although the mother attempted to maintain a sufficiently high intake of phenylalanine as instructed, the child continued to be ill and the total phenylalanine intake remained low. During the illness serum phenylalanine rose to 37 mg percent, urine phenylalanine excretion was between 250 and 500 μg/ml and orthohydroxyphenylacetic acid excretion was 80 to 220 μg/ml. During period 4 the phenylalanine intake was increased to 500 mg/day for a week. Orthohydroxyphenylacetic acid excretion dropped rapidly to zero while urine phenylalanine excretion decreased more slowly. Serum phenylalanine dropped to 3 mg percent.

While phenylalanine deficiency represents the greatest potential during the first year of life, the management of illnesses remains a problem as long as treatment is continued.

Figure 7 shows the effect of several illnesses in a 5 year old. By the 5th year of treatment the frequency of blood tests has declined to one per month; urine specimens are still tested at weekly or twice weekly intervals. The child shown in figure 7 was in nursery school, experiencing his first contacts with other children. The winter was a stormy one from the standpoint of illness of one sort or another. During period 1 serum phenylalanine was 5 mg percent, urine phenylalanine excretion between 10 and 70 μg/ml,
Note periods of increased urinary excretion of phenylalanine and orthohydroxyphenylacetic acid characteristic of acute illness. Although, serum phenylalanine was not measured, elevated urinary excretion pattern warns that serum levels have increased.

Orthohydroxyphenylacetic acid was negative; phenylalanine intake was 350 to 425 mg/day, including 250 to 300 mg contributed by Lofenalac® in the form of 24 to 30 ounces liquid Lofenalac® and 22 to 26 tablespoons as semi-solid paste. Period 2 shows a decrease in the phenylalanine intake to 200 mg/day. The mother reported the child had a cough and fever. Urinary phenylalanine increased to 150 µg/ml and orthohydroxyphenylalanine intake was restored to 400 mg/day as soon as possible (period 3) by increasing natural foods in the diet and then, as the child's appetite returned, by increasing the Lofenalac®. Serum phenylalanine was in the control range between 3 and 7 mg percent when measured several days later and urinary excretions were normal. Period 4 shows another appetite failure with total intake of 200 mg/day, again accompanied by increased excretion of urinary phenylalanine to 300 µg/ml and...
orthohydroxyphenylacetic acid present. This represented chickenpox. In period 5, the acute phase over and the balance restored, serum phenylalanine was in the range between 3 and 7 mg percent, urine phenylalanine between 20 and 60, and no orthohydroxyphenylacetic acid was excreted. The mother was instructed to obtain blood and urine specimens at more frequent intervals to check dietary control following the chickenpox. Serum phenylalanine was less than 1 mg percent at the end of period 5; on the same day there was a decrease in phenylalanine intake. This time the child had a runny nose, fever and symptoms of a cold or flu. Following the low serum phenylalanine level, urine phenylalanine excretion increased, orthohydroxyphenylacetic acid appeared in the urine briefly. Several days later (period 6) the minor illness over, serum phenylalanine was in the control range. Intake of phenylalanine varied around 400 mg/day. This series shows how urinary excretions are used to follow dietary control even though serum phenylalanine values are not available. It also illustrates why isolated and infrequent measurements of serum phenylalanine levels, unless accompanied by other biochemical and dietary data, are of little value in interpreting the day-to-day problems which arise in treatment of phenylketonuria.

The repeated testing of blood and urine specimens and recording of food intake which we recommend may seem tedious. The micro-methods for urine and blood analysis are quite adequate to furnish the information necessary to monitor the diet. Neither parent nor child are unnecessarily burdened by the collection of specimens. There are two significant advantages to be gained from the frequent testing. It is possible to maintain continuous control over the diet rather than only during the few days preceding collection of a blood specimen. When serum specimens are obtained at infrequent intervals there is no assurance that the measured level of phenylalanine reflects the degree of dietary control being exercised. Parents may easily withhold foods from a phenylketonuric child for several days before a clinic visit to bring blood phenylalanine levels to the low value acceptable to their physician while being permissive or careless in the interim. It is less easy to juggle the records of dietary intake of protein substitute, natural foods and urinary excretions to produce misleading results.

Urinary phenylalanine serves as an indicator both of excessive phenylalanine in the diet, in which case the excretion rises above a maximum level, and of deficient phenylalanine intake, in which the excretion of phenylalanine falls below a minimum level. The maximum and minimum levels are determined empirically for each child during the early stages of treatment and are related to serum phenylalanine concentrations. Urinary orthohydroxyphenylacetic acid serves as a further indicator by warning that the capacity of the child to utilize phenylalanine for protein synthesis or to eliminate it in the urine has been exceeded and serum phenylalanine levels have increased.

A second advantage is that it is virtually impossible to treat a non-phenylketonuric child with the low-phenylalanine diet for more than a short time without recognizing the error. It is difficult to raise phenylalanine levels above 3 mg percent in a non-phenylketonuric child who is fed Lofenalac® even if additional natural foods are provided.

The effect of administration of phenylalanine-limited diet to a child who had elevated blood phenylalanine without other biochemical characteristics of phenylketonuria is shown in figure 8. A preliminary screening test at 4 days of age was positive. Several repeat tests were done, all showing moderate elevation of serum phenylalanine. The baby was sent to us when he was 6 weeks old. Serum phenylalanine rose from 8 mg percent to 16 mg percent within 5 days (period 1). Urine phenylalanine was 120 μg/ml. Urine was negative for orthohydroxyphenylacetic acid and for phenylpyruvic acid. Serum phenylalanine dropped to 7 mg percent while the baby was still taking his regular formula and urinary phenylalanine also decreased. This shows the effect of a lag of even 24 hours in obtaining laboratory results, for treatment was undertaken (period 2). Following withdrawal of milk and substitution of Lofenalac® serum phenylalanine dropped to less than 1 mg within 24 hours. Additional phenylalanine was added to furnish a total of 250 mg phenylalanine on the third day of treatment, 400 mg by the fourth day and for the next week. Urinary phenylalanine excretion remained low and serum phenylalanine was less than 3 mg percent. During the next three weeks phenylalanine intake was gradually increased to 600 mg/day. Serum phenylalanine rose from 3 mg percent but urinary...
After little more than a month of carefully controlled administration of low-phenylalanine diet, it was discontinued (period 3). The biochemical monitoring of blood and urine specimens continued as before and records of food intake were kept. Serum phenylalanine ranged from 9 to 1.5 mg percent on a phenylalanine intake of 900 to 1100 mg/day; urinary excretion of phenylalanine was above the range for normal children, but not over 100 μg/ml, the minimum excretion found in phenylketonuria.

This child is now 18 months old and has been carefully followed in our clinic while on a normal diet. Serum phenylalanine levels have ranged from 2 to 10 mg percent, mean 6 mg percent; urinary phenylalanine excretion ranged from 20 to 150 μg/ml, and recently orthohydroxyphenylacetic acid has been detected intermittently in amounts ranging from 10 to 20 μg/ml.

There are no neurologic abnormalities. Intelligence quotient was 110 using the Cattell Infant Scale; Vineland Social Maturity Scale showed a social quotient of 130. Language development was normal for his age. We can find no evidence that the moderate elevation of phenylalanine levels in this range need to be corrected by diet. Tolerance testing indicated that both parents were carriers. We cannot explain the low phenylalanine levels if this is phenylketonuria,
unless it is an example of the early history of those children who later will be recognized as having normal intelligence with classical biochemical features of phenylketonuria.

SUMMARY

The preceding examples illustrate how blood and urine tests may be used to guide dietary management in the treatment of phenylketonuria. Serum phenylalanine may be elevated in a specimen for a number of reasons: transient phenylalanine deficiency with tissue breakdown releasing excess phenylalanine; illness associated with increased protein catabolism, again releasing excess phenylalanine; protein deficiency, in which other essential amino acids are missing and even small amounts of phenylalanine in the diet cannot be utilized; as well as because of an excessive quantity of phenylalanine in the diet. The total picture obtained from urine testing and dietary intake permit informed interpretation of serum phenylalanine levels. Our program may be said to have the following distinguishing characteristics: Precise and frequent monitoring using microtechniques for measuring serum phenylalanine concentration; prompt dietary alteration based on results of blood and urine tests and on information on dietary intake supplied routinely by the parents; establishment of ranges of dietary requirements and tolerances for phenylalanine determined empirically for each child; creation of the most normal eating atmosphere possible under an unusually restrictive regimen.

REFERENCES


DISCUSSION

DR. SASS-KORTSAK: Would you agree that there may be an alternate explanation for your findings? Could it be that during these periods of illness there was breakdown of tissue proteins which provided phenylalanine in excess of dietary intake and that this could be responsible for the rise in blood phenylalanine levels during these periods. Following recovery, the high phenylalanine blood levels dropped not because of the increased food intake but because the illness was over and the patient went into a positive nitrogen balance.

DR. BERRY: The problem which presents itself in management is that as the phenylalanine intake drops, protein catabolism does occur. This effect is accelerated during an illness. We have found empirically that we can restore the phenylalanine levels from the high to the low range more rapidly by increasing the intake than by decreasing it.

DR. BESSMAN: Advice has been given people on the basis of evidence which I have not yet seen that 5 mg percent is a proper serum level of phenylalanine and 6 mg percent obviously by exclusion must be bad. We have had a moderate number of years of suffering with this problem ourselves and we can't find these nice numbers.
What leads you to believe that 5 mg percent is the critical level?

I'd like also to ask: What is meant by the statement "protein requirement" when in reality a patient is given a deficient hydrolyzate that is unutilizable as protein as long as there is no phenylalanine in it. To me this is a very dangerous kind of therapy to give to a child. It is dangerous to give to an animal. We can make a runt out of an animal feeding him this stuff. We are a little more sophisticated about diet. I'd like to understand on what basis you make these statements.

DR. BERRY: I think it's well-recognized that if amino acids are given, more nitrogen may be required to promote growth.

One of our criteria for following the diet has been that we require adequate growth, and if we do not get adequate growth, we are not doing a good job.

We selected 3 mg percent as a lower figure because we found if we maintained phenylalanine levels below 2 mg percent, growth ceased in a child. If we raised it above that, growth was sustained.

I could show you growth curves of children, some of them up to 3 or 4 years of age, whose treatment was started in our clinic or elsewhere in infancy. During the first year the growth proceeded at above the 50th percentile, in some cases up to the 90th percentile. Height was similar. Between 3 and 4 years, growth rate of one child began to decline. For some reason we are not meeting his nutritional needs, whether they be for phenylalanine or for protein. Whether it is possible to completely fulfill protein requirements, I am not prepared to say.

In the case of one child I talked about in the initial presentation, she was below the 10th percentile when we began treatment at age 6 months. Now at 5 years of age she is growing at about the 90th percentile.

Another factor we consider essential in the treatment is normal bone development. All of our children have been followed routinely with X-ray examination of the wrist, the long bones, the bone density, and various other things. All of the bone densities were normal. The long bone lengths were normal—perhaps a little high in some cases. We had no wrist changes in children treated at less than 24 months of age. We treated some children who were 4 and 5 years of age early in our experience, and they had diminished bone density; they had the wrist changes; they had retarded growth.

You may also wonder what happens to the children on psychological testing. Their IQs are generally in the normal range. Our point is that if we are not improving the children, we are not hurting them.

DR. ANDERSON: I wonder where all that phenylalanine is coming from.

DR. BERRY: I am not sure where phenylalanine is coming from. I only know this happens during illness. Of course, like most of you, when we first noticed this phenomenon, we restricted the intake, and we usually developed a phenylalanine deficiency; the child became lethargic and sometimes would require two or three weeks before he was straightened out.

We thought that during the illness there might be an increased need for protein so we increased the phenylalanine intake in a number of patients, and to our surprise the blood phenylalanine level dropped.

DR. FISCH: Dr. Berry mentioned growth changes in phenylketonurics and I don't think we can say this is necessarily present because these changes are present in untreated phenylketonuria.

The other point is your use of bone age as criteria of development. We have had the opposite experience that you have had and our patients on the diet are way below normal in weight and length and they have normal bone age development. So I don't think this normal bone age need reflect good development.

I wonder if you can make the statement that your treatment leads to a normal rate of development. Are they normal in weight and height compared with normal children?

DR. EFRON: In your series, what are your figures for growth? How do you evaluate the growth of your treated babies?

DR. BERRY: We have been using height and weight as one criterion for growth. For example, we have data on seven children treated from infancy, some for four or five years. These children are growing normally compared to the Harvard grid standards, in the 90th percentile.

The other statement that I am perfectly willing to make is that children who are treated beginning at 9 months of age may have growth failure.
Children who are treated at 2 years vary a great deal. We have not restricted the phenylalanine intake as severely as many people. We have found that the few children we have who have been treated for four or five years, have IQs above 100. These are children who have retarded phenylketonuric siblings. We are not prepared to say one way of treatment is better than the other. We are not even prepared to say that these children at 5 or 6 years who have normal growth and apparently normal intelligence are going to be normal children, because they may not be.

DR. UDENFRIEND: You said you compared your patients to the Harvard scale. Did you also measure normal children in your hospital?

DR. BERRY: Yes. These have been worked out with the Silverman standard.

DR. UDENFRIEND: I just wondered if maybe in your community they grow bigger normally.

DR. BERRY: I don't think so.

DR. UDENFRIEND: I mean have you run controls and measured all these things with control children in your community to make sure your areas aren't larger than expected from the Harvard scale?

DR. BERRY: No. As a matter of fact, if we ran the population in Cincinnati I think we'd find them a little below normal. And in some cases we find the treated phenylketonurics are a little bigger than siblings.
A CRITICAL ASSESSMENT OF THE DIETARY TREATMENT OF PHENYLKETONURIA.
EXPERIENCES WITH 45 CASES OVER THE LAST 6 YEARS

HORST BICKEL

This account is limited to the treatment of phenylketonuria in the years 1960 to 1965, as our earlier results with a phenylalanine-restricted diet have already been published.\(^1\)\(^-\)\(^7\) The limitation to this 6-year period has the advantage that the criteria of treating the patients, assessing their progress and controlling their biochemical adjustment were relatively uniform. Of a total of 68 phenylketonuric children admitted during this period to our hospital 45 were treated in a systematic way and are still under observation while in 13 cases treatment was discontinued for various reasons and in 10 cases no therapy was started because of the severity of the brain damage or the advanced age of the patients. Thirty-six of the 45 patients were treated for periods of 10 to 58 months; the average was 30 months.

Composition and Control of the Diet

In composing the diet the phenylalanine-restricted casein hydrolysate predominantly used was "Cymogran\(^\circledR\)" through Lofenalac\(^\circledR\) as well as a very similar Japanese product "Lopheemilk\(^\circledR\)," "Phentol\(^\circledR\)," another Japanese hydrolysate similar to "Ketonil\(^\circledR\)" and "Albumaid\(^\circledR\)," a British protein hydrolysate derived from oxen blood, were also given to several patients for trial periods. The composition and the manufacturers of these foods are summarized in table 1. It may be noted that Cymogran\(^\circledR\) and Albumaid\(^\circledR\) have a relatively high protein and low phenylalanine concentration. This renders them especially suitable for the composition of a mixed diet for children beyond infancy, because the hydrolysate with its

Table 1.--Composition of various phenylalanine-restricted foods

<table>
<thead>
<tr>
<th>Phenylalanine-restricted protein hydrolysate, 100 g powder</th>
<th>Phenylal. mg</th>
<th>Protein g</th>
<th>Fat g</th>
<th>Carbohydrate g</th>
<th>Calories</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cymogran</td>
<td>10</td>
<td>29</td>
<td>9</td>
<td>38.5</td>
<td>400</td>
<td>Glaxo-Allenburys Ltd., London</td>
</tr>
<tr>
<td>Lofenalac</td>
<td>80</td>
<td>15</td>
<td>18</td>
<td>57</td>
<td>443</td>
<td>Mead-Johnson, Evansville, Indiana</td>
</tr>
<tr>
<td>Lopheemilk</td>
<td>67</td>
<td>16</td>
<td>18</td>
<td>59</td>
<td>443</td>
<td>Snow Brand Milk Products, Tokyo</td>
</tr>
<tr>
<td>Phentol</td>
<td>100</td>
<td>62</td>
<td>0</td>
<td>0</td>
<td>250</td>
<td>Take-da-Jakuhin-Koggo KK., Osaka</td>
</tr>
<tr>
<td>Albumaid</td>
<td>0</td>
<td>33</td>
<td>0</td>
<td>50</td>
<td>370</td>
<td>Scientific Hospital Supplies Ltd., Liverpool</td>
</tr>
</tbody>
</table>

Provided by the Maternal and Child Health Library, Georgetown University
unpleasant taste takes up only a small volume and the phenylalanine requirement of these patients can be supplied in the form of normal proteins in milk, vegetables and fruit. The taste of Lofenalac® and Lophemilk®, though still unpleasant, is better than that of the other hydrolysates, but this advantage is practically cancelled out by the necessity of giving a double quantity of these foods for the same protein intake. 30 g protein daily is provided by 100 g Cymogran® or Albumaid® with 10 mg or no phenylalanine respectively, or by 200 g Lofenalac® or Lophemilk® with 160 or 135 mg phenylalanine respectively. Judging from the clinical and biochemical response, good results can be achieved with any of these preparations, though none of them is yet really satisfactory, mainly because of the offensive taste common to all of them. The ideal product should have a high protein and a low phenylalanine concentration and a pleasant or at least not repulsive taste.

Specimen diet sheets for a 10-week and a 13-month-old patient are shown in tables 2 and 3. For each patient the daily requirement not only for protein, but also for fat, carbohydrate and calories was carefully calculated. The phenylalanine requirement has to be assessed during a clinical adjustment period which in young infants may take 3-4 months, in older infants and children 1-2 months. During this period the children are under close medical and biochemical control. They become accustomed to the unpleasant taste and the rigidity of the diet, while their phenylalanine blood level is checked twice or three times weekly by Guthrie's microbiological method and their urinary amino acid excretion at longer intervals by paper chromatography. Baseline psychometric and electroencephalographic examinations are performed and sufficient time is spent with the parents to instruct them carefully about the nature of the disease and the management of the diet. After sending the children home, the Guthrie test is performed at monthly intervals on blood specimens sent in by post, and the clinical and biochemical check-ups with further psychometric and electroencephalographic tests and dietetic counseling of the parents are undertaken during a 24-hour hospital admission every 2-3 months in infants and every 5-6 months in older children.

In the patients, as in normal children, the daily phenylalanine requirement varies widely at different ages and from case to case. Figure 1 shows the requirement of some of our patients who during the time of the assessment were clinically and biochemically well controlled, gained weight satisfactorily and had a phenylalanine blood level of 1-4 mg per 100 ml, as measured by column chromatography or the Guthrie test. The diagram shows that the requirement was especially varied during the first year of life, when the intake ranged from 30 to over 50 mg phenylalanine per kilogram per day, while in the second and third year it was between 20-30, thereafter under 20 mg per kilogram per day.

As stated before, the Guthrie test was used widely for the biochemical control of the dietary adjustment. In our experience this test, though not strictly quantitative, is sufficient for this purpose. It is highly specific and very convenient, as the blood drops from a finger puncture can be

Table 2.--Specimen diet for a 10 weeks old patient with phenylketonuria

<table>
<thead>
<tr>
<th>Phenylketonuria</th>
<th>10 weeks, 5 100 g BW</th>
</tr>
</thead>
<tbody>
<tr>
<td>60 g Cymogran</td>
<td></td>
</tr>
<tr>
<td>110 g milk</td>
<td></td>
</tr>
<tr>
<td>35 g sugar</td>
<td>44 mg phenylalanine</td>
</tr>
<tr>
<td>15 g cornflour</td>
<td>4.3 g protein</td>
</tr>
<tr>
<td>150 g carrots</td>
<td>3.6 g fat</td>
</tr>
<tr>
<td>25 g margarine</td>
<td>17 g carbohydrate</td>
</tr>
<tr>
<td>100 g apple juice</td>
<td>126 Cal</td>
</tr>
<tr>
<td>30 g carrot juice</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.--Specimen diet for a 13 months old patient with phenylketonuria

<table>
<thead>
<tr>
<th>Phenylketonuria</th>
<th>13 months, 10.7 kg BW</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 g Cymogran</td>
<td></td>
</tr>
<tr>
<td>100 g milk</td>
<td></td>
</tr>
<tr>
<td>30 g sugar</td>
<td>29 mg phenylalanine</td>
</tr>
<tr>
<td>5 g cornflour</td>
<td>3.2 g protein</td>
</tr>
<tr>
<td>50 g potatoes</td>
<td>3.2 g fat</td>
</tr>
<tr>
<td>25 g margarine</td>
<td>15.1 g carbohydrate</td>
</tr>
<tr>
<td>150 g apples</td>
<td>108 Cal</td>
</tr>
<tr>
<td>50 g cornflour bread</td>
<td></td>
</tr>
<tr>
<td>20 g marmalade</td>
<td></td>
</tr>
<tr>
<td>100 g apple juice</td>
<td></td>
</tr>
<tr>
<td>30 g orange juice</td>
<td></td>
</tr>
</tbody>
</table>

Provided by the Maternal and Child Health Library, Georgetown University
taken at short intervals even at home, enabling the doctor to keep a close control of the patient's phenylalanine blood level. To convince us of the reliability of the test, we have compared the results of Guthrie's method with those of LaDu's method and of the column chromatographic technique of Moore and Stein. Figures 2a and 2b show that there is a reasonable agreement between these methods, especially in the important range between 1-8 mg per 100 ml, though the Guthrie test tends to give somewhat lower values than the other methods. For the measurement of phenylalanine concentrations below 1 mg per 100 ml the Guthrie test is not suitable, but values of 1 and 2 mg per 100 ml are easily distinguishable if a 1 mg standard is added to the test plate. Figure 3 demonstrates the suitability of the test for the biochemical control of the diet, though at the time of preparing this test plate we did not yet use the 1 mg standard.

Figure 1. Phenylalanine requirement of patients with phenylketonuria at different ages (see text)

Figure 2a. Comparison of phenylalanine estimations in the blood by Guthrie's and LaDu's method.
Psychometric Results

The behavior of the children was analyzed before and at intervals during the treatment by the Bühler-Hetzer developmental test, which was nearly always carried out by the same psychologist. It is obvious that a single test like this one or even a battery of tests are not sufficient to characterize fully a child’s development, behavior, statural and mental functions, but better means of reaching a short objective statement are lacking. Tables 4–7 summarize the results of the test prior to therapy, the last test under therapy, the duration of the therapy and gains or losses of IQ points (more correctly named DQ = developmental quotient). They also give an overall statement of the quality of the biochemical control, "very good" control standing for phenylalanine blood values practically always between 1-3 mg per 100 ml, "good" for values between 1-4, rarely up to 6 mg per 100 ml, "moderate" for occasional values up to 6-8 mg per 100 ml, "bad" for repeated values up to 8 mg per 100 ml, "very bad" for values repeatedly above 8 mg per 100 ml. The quality of the dietary control was strongly influenced by the child’s social background, the competence of the mother and the child’s temperament and eating habits, severely damaged children being generally much more difficult to handle than children with better mentality.

Summarizing the data from these tables, it is obvious that the best results are achieved when the diet is started within the first three months of life. A delayed start between the fourth and sixth month or in the second half year of life...
Figure 3. GUTHRIE’S test used for the control of the diet.

Row 7: Standard row showing the growth zones of bac. subtilis around blood discs containing 2-2-4-4-6-6-8-12-20 mg percent L-phenylalanine.

Row 1: Blood specimens of normal newborns aged 4-7 days.

Row 2-4: Phenylalanine blood levels of a phenylketonuric infant, rising under a normal diet from < 2 to > 20 mg percent and under a phenylalanine-restricted diet to < 2 mg percent.

Row 5-6: Decrease of high phenylalanine blood levels of another patient to normal levels under a phenylalanine-restricted diet.

shows increasingly low IQ levels at the onset and insufficient though still marked recovery under treatment. The second half of this table as well as the next tables bear out the observation that as a rule better results can be expected from good than from bad biochemical control. One patient even lost IQ points under a very badly controlled diet.

The results of treatment starting in the second and third year show a great variety of responses with recoveries from IQ levels of 50 to 83, 30 to 60, or only from 31 to 35 despite good biochemical control. Even when started after four years of age the diet may still occasionally raise an IQ level from 60 to 75, which is an important gain for the child, but the very limited number of cases and the shortness of the observation period still prohibit any generalized conclusion as to the prognostic value of the diet in this age group. It seems that the older the child is at the beginning of therapy, the more limited but also the more varied is the response. It cannot be predicted but has to be assessed by a therapeutic trial period of 1/2-1 year, though in
Table 4.--IQ development of patients whose diet was started during the 1st - 3rd month of life

<table>
<thead>
<tr>
<th>Case</th>
<th>Duration of therapy (months)</th>
<th>First and last IQ</th>
<th>IQ gain or loss</th>
<th>Biochemical control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>57</td>
<td>88-95</td>
<td>±</td>
<td>good</td>
</tr>
<tr>
<td>2</td>
<td>50</td>
<td>92-105</td>
<td>±</td>
<td>good</td>
</tr>
<tr>
<td>3</td>
<td>25</td>
<td>76-100</td>
<td>+24</td>
<td>good</td>
</tr>
<tr>
<td>4</td>
<td>23</td>
<td>95-96</td>
<td>±</td>
<td>very good</td>
</tr>
<tr>
<td>5</td>
<td>17</td>
<td>96-90</td>
<td>±</td>
<td>very good</td>
</tr>
<tr>
<td>6</td>
<td>11</td>
<td>101-96</td>
<td>±</td>
<td>moderate</td>
</tr>
<tr>
<td>7</td>
<td>10</td>
<td>107-97</td>
<td>±</td>
<td>very good</td>
</tr>
<tr>
<td>8</td>
<td>10</td>
<td>82-91</td>
<td>±</td>
<td>moderate</td>
</tr>
</tbody>
</table>

Table 5.--IQ development of patients whose diet was started during the 4th - 6th month and during the 7th - 12th month of life respectively

<table>
<thead>
<tr>
<th>Case</th>
<th>Duration of therapy (months)</th>
<th>First and last IQ</th>
<th>IQ gain or loss</th>
<th>Biochemical control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>58</td>
<td>40-62</td>
<td>+22</td>
<td>bad</td>
</tr>
<tr>
<td>2</td>
<td>47</td>
<td>73-80</td>
<td>+7</td>
<td>moderate</td>
</tr>
<tr>
<td>3</td>
<td>14</td>
<td>81-80</td>
<td>±</td>
<td>good</td>
</tr>
<tr>
<td>4</td>
<td>13</td>
<td>82-80</td>
<td>±</td>
<td>moderate</td>
</tr>
</tbody>
</table>

Table 5.--IQ development of patients whose diet was started during the 7th - 12th month of life respectively

<table>
<thead>
<tr>
<th>Case</th>
<th>Duration of therapy (months)</th>
<th>First and last IQ</th>
<th>IQ gain or loss</th>
<th>Biochemical control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>48</td>
<td>64-53</td>
<td>-11</td>
<td>very bad</td>
</tr>
<tr>
<td>2</td>
<td>34</td>
<td>54-63</td>
<td>+9</td>
<td>moderate</td>
</tr>
<tr>
<td>3</td>
<td>32</td>
<td>45-77</td>
<td>+32</td>
<td>very good</td>
</tr>
<tr>
<td>4</td>
<td>25</td>
<td>67-77</td>
<td>+10</td>
<td>moderate</td>
</tr>
<tr>
<td>5</td>
<td>22</td>
<td>68-75</td>
<td>+7</td>
<td>good</td>
</tr>
<tr>
<td>6</td>
<td>10</td>
<td>45-50</td>
<td>+5</td>
<td>bad</td>
</tr>
</tbody>
</table>
Table 6.—IQ development of patients whose diet was started during the 2nd year of life

<table>
<thead>
<tr>
<th>Case</th>
<th>Duration of therapy (months)</th>
<th>First and last IQ</th>
<th>IQ gain or loss</th>
<th>Biochemical control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>52</td>
<td>50-83</td>
<td>+33</td>
<td>good</td>
</tr>
<tr>
<td>2</td>
<td>49</td>
<td>57-88</td>
<td>+31</td>
<td>very good</td>
</tr>
<tr>
<td>3</td>
<td>48</td>
<td>35-53</td>
<td>+18</td>
<td>very good</td>
</tr>
<tr>
<td>4</td>
<td>47</td>
<td>31-60</td>
<td>+29</td>
<td>very good</td>
</tr>
<tr>
<td>5</td>
<td>41</td>
<td>30-60</td>
<td>+30</td>
<td>good</td>
</tr>
<tr>
<td>6</td>
<td>36</td>
<td>14-23</td>
<td>+9</td>
<td>bad</td>
</tr>
<tr>
<td>7</td>
<td>28</td>
<td>35-50</td>
<td>+14</td>
<td>moderate</td>
</tr>
<tr>
<td>8</td>
<td>21</td>
<td>44-50</td>
<td>+6</td>
<td>good</td>
</tr>
<tr>
<td>9</td>
<td>14</td>
<td>44-60</td>
<td>+16</td>
<td>good</td>
</tr>
</tbody>
</table>

Table 7.—IQ development of patients whose diet was started during the 3rd and during the 4th - 6th year of life respectively

<table>
<thead>
<tr>
<th>Case</th>
<th>Duration of therapy (months)</th>
<th>First and last IQ</th>
<th>IQ gain or loss</th>
<th>Biochemical control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>49</td>
<td>31-41</td>
<td>+10</td>
<td>bad</td>
</tr>
<tr>
<td>2</td>
<td>46</td>
<td>44-56</td>
<td>+12</td>
<td>moderate</td>
</tr>
<tr>
<td>3</td>
<td>29</td>
<td>22-35</td>
<td>+13</td>
<td>moderate</td>
</tr>
<tr>
<td>4</td>
<td>27</td>
<td>31-35</td>
<td>+4</td>
<td>good</td>
</tr>
<tr>
<td>5</td>
<td>13</td>
<td>34-37</td>
<td>+3</td>
<td>very bad</td>
</tr>
<tr>
<td>6</td>
<td>10</td>
<td>35-40</td>
<td>+5</td>
<td>bad</td>
</tr>
</tbody>
</table>

PKU diet started during the 3rd year of life

PKU diet started during the 4th - 6th year of life

<table>
<thead>
<tr>
<th>Case</th>
<th>Duration of therapy (months)</th>
<th>First and last IQ</th>
<th>IQ gain or loss</th>
<th>Biochemical control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>36</td>
<td>35-45</td>
<td>+10</td>
<td>moderate</td>
</tr>
<tr>
<td>2</td>
<td>21</td>
<td>20-23</td>
<td>+3</td>
<td>good</td>
</tr>
<tr>
<td>3</td>
<td>11</td>
<td>60-75</td>
<td>+15</td>
<td>very good</td>
</tr>
</tbody>
</table>
patients beyond the age of 2 with an IQ of 30 or less such a trial period is probably not worthwhile in view of the limited success to be expected. Figure 4 further illustrates the importance of starting the treatment early, but also the great variety of IQ values in the various age groups, which is not only due to the influence of the diet but also to the extent of brain damage before therapy was started.

Figure 5 visualizes schematically the IQ changes in untreated and treated patients. The drawn-out descending line for the IQ of untreated cases is based on very limited data of longitudinal studies of phenylketonuric cases and on single test results of untreated patients at different ages. The dotted lines suggest the gains to be expected from therapy starting at different ages, based on our own experience and on the results of other workers in this field.\(^{(7,9)}\) Of special interest is the lag period of the first three months, when the patients, born with a normal

![Figure 4. Relation between the highest IQ reached during therapy and the age at the start of the phenylalanine-restricted diet.](image)

![Figure 5. Schematic illustration of the mental development of phenylketonuric patients in relation to the start of therapy (---untreated cases, --treated cases) (see also text).](image)
IQ, do not seem to become damaged yet to any noticeable degree. This is borne out by the data in table 4, where some cases with normal development were at the start of therapy in their third month of life. It is possible that the brain at that early period is not yet susceptible to biochemical damage, perhaps because myelination is not yet properly underway. Another implication of the diagram is the conclusion that any IQ gain under therapy should be assessed not against a stationary, but against a progressively falling IQ without treatment, so that even the prevention of a further IQ decrease may be of great importance, especially during the period of rapidly progressive brain damage between the ages of 6 and 18 months.

"Over"-- and "Under"--Treatment

The clinical and biochemical control of our patients has by no means always been optimal, and we, as I believe every worker in this field, have had a difficult time finding a reasonably satisfactory approach and technique of managing these patients. One important point still under discussion is the question as to whether the dietetic control should aim at normalizing the metabolic deviation, and especially the phenylalanine blood level, as far as possible or if a somewhat increased blood level can be tolerated without damage to the child, for instance 8 mg per 100 ml and above (normal range 0.8-2 mg per 100 ml). As it has not yet been proven that such a considerable rise of the phenylalanine concentration over longer periods of time is harmless to the patients' brain we have from the beginning attempted to keep the phenylalanine blood levels between 1-2 mg per 100 ml, with a maximal and only temporary upper limit of 4 mg per 100 ml. Figure 6 shows that on such a regimen the other aminoacids in the plasma can also be kept within the normal range (estimation done by column chromatography, measurement of basic aminoacids is not yet concluded).

If the dietary supply of phenylalanine is too low or if the diet is taken badly or vomited, the phenylalanine plasma level may drop to concentrations below 0.5 mg per 100 ml, which leads to an increase of various other aminoacids in the plasma, resulting in an "aminoacid imbalance" (figure 7). Phenylalanine blood levels above 8 mg per 100 ml (figure 8) are generally due to an excessive phenylalanine intake, but in the presence of severe feeding problems, loss of weight and breakdown of body proteins they may occasionally be the result of a marked phenylalanine deficiency. Contrary to our expectation we have so far not been able to distinguish these opposite states by the behavior of the other aminoacids in the plasma. The expected increase of these aminoacids in phenylalanine starvation and their decrease on a too liberal phenylalanine intake was not substantiated by our data. It seems that the generalized aminoaciduria developing in states of phenylalanine deficiency is still the best biochemical criterion for overtreatment (figure 9), while an excessive phenylalanine intake is associated with an isolated phenylalaninuria without increased excretion of other aminoacids. There is, however, a tendency toward a moderate increase of various aminoacids in the urine even on a well-balanced phenylalanine intake. This is probably due to aminoacid imbalances in the
protein hydrolysates and to the unphysiologically fast absorption of these hydrolysates, which offer all the amino acids to the intestinal wall at once instead of slowly and intermittently during the usual digestion of intact proteins.

During a period in which we tended to over-treat our patients and did not yet fully realize the high phenylalanine requirement of phenylketonuric infants, the gain in length and weight was retarded in many of our patients as compared with figures of untreated patients (figure 10). The retardation of growth in length was statistically significant. There was also some retardation of bone age as measured by ossification centers of these children at the left wrist.

A peculiar osteodystrophy first described by Feinberg and Fisch was observed in several patients during infancy (figure 11). It was more pronounced than that observed during the following months. We assume that these changes were due to protein starvation while vitamin D deficiency did not seem to be an etiological factor. Since we keep our patients on a somewhat higher phenylalanine intake, with the range of phenylalanine blood levels mentioned above, these side effects on the skeletal system were nearly completely prevented and are now only observed in cases with severe feeding difficulties. Osteodystrophy has not been encountered for the last two years despite careful roentgenological control of our patients.

It may be expected that a deficient phenylalanine intake might also delay normal brain development, but for this supposition we have no indications in our case material, probably because the starvation periods were not long and severe enough. One of our patients, who as an infant...
in 1960 was noted repeatedly to have a phenylalanine blood level below 0.8 mg per 100 ml, generalized aminoaciduria, osteodystrophy and delayed physical growth, has now at the age of 5 1/2 an IQ of 100 (figure 12) and is of normal height and weight. Two other patients, who were overtreated during infancy, have also attained normal height and weight. We feel that short periods of overtreatment, though disadvantageous to the child's physical development, are of a smaller risk to the mental development of these patients than phenylalanine blood levels rising repeatedly and considerably above the normal range.
Figure 10. Mean length and weight and its deviation in untreated (above) and treated (below) phenylketonuric children in percent of normal values in healthy children of the same age.

Figure 11. Osteodystrophy in a 5 month old infant with phenylketonuria treated since the age of 6 weeks.

Electroencephalographic Changes Under Treatment

In over half of the patients whose treatment was not started until 6-18 months we found seizure patterns in the EEG with or without clinically apparent convulsions, the severely affected children showing more regular and pronounced changes than the more mildly affected patients. While in our early therapeutic trials we have very occasionally observed a deterioration on EEG changes soon after introducing a phenylalanine restricted diet, this has not been encountered during the present observation period. In some of our patients the improvement of the EEG abnormalities was as good as that published by Low et al. in his figure 3, while in others it was slower and less striking. Though it is known that the seizures and EEG changes of phenylketonuria, if present at all, tend to improve spontaneously with advancing age, the diet seems to accelerate this natural course and to be of definite help in managing this complication of the disease. In several cases additional antiepileptic drugs such as mylepsine and hydantoin derivatives were needed to prevent seizures and improve hypsarrhythmia and other seizures potentials in the EEG. ACTH was repeatedly administered, but in no instance was the therapeutic response convincing.
CONCLUSIONS

These conclusions are based on our experience with the management of 45 cases of phenylketonuria over a limited period and are, therefore, somewhat tentative:

1. Every child with phenylketonuria diagnosed in the first and second year of life should be treated with a phenylalanine-restricted diet. "Formes frustes" with phenylalanine blood levels above 8 mg per 100 ml should also be treated so long as there is no agreement on the "danger level" of phenylalanine or the other metabolites for the brain. Children with blood levels below 8 mg per 100 ml should not be treated but followed up carefully.

2. Patients in the third or fourth year at their discovery should nearly always be given the benefit of the doubt and have a trial period of about one year's treatment. Severely damaged patients with IQ levels below 30 should not be treated.

3. Patients in the fifth year or older should only be treated if the IQ is above 40. Again a trial period of one year is recommended.

4. The age at which treatment can safely be terminated is still uncertain. The transition from strict phenylalanine reduction to a mixed diet of low protein content may be attempted at the age of 8 or soon after, but a careful follow-up is suggested.

5. The biochemical control of the diet should aim at phenylalanine blood levels of 1-3 mg, maximal 4 mg per 100 ml. It has not yet been proved that higher levels are harmless in the long run. During fever and inflammatory diseases milk addition of 30-50 g daily is advisable.

6. "Overtreatment" can be recognized by phenylalanine estimation of the blood in combination with paper chromatographic analysis of the aminoacid pattern in the urine.
Figure 13. Phenylalanine loading test in an infant with the "forme Fruste" of phenylketonuria (3 m, 4050 g), and a healthy infant (2½ m, 4850 g).

7. Good control of the diet will become easier when better phenylalanine-restricted protein hydrolysates are available. Especially the taste, but also the aminoacid composition, protein concentration and absorption properties in the gut leave scope for improvement.

8. The diagnosis of phenylketonuria should be based on repeated blood phenylalanine estimations with two independent and reliable methods, and a chromatographic analysis of the total aminoacid pattern of blood or urine. If the FeCl₃ reaction in the urine is negative at phenylalanine blood levels above 15 mg per 100 ml beyond the sixth week of life, phenylalanine loading tests may be required to help establish the diagnosis. The diagnosis of infants put on dietary treatment early should be repeatedly checked by phenylalanine tolerance tests with loads of milk (400–500 g daily for 3–6 days) and phenylalanine blood estimations at the end of the loading period.

REFERENCES


DISCUSSION

DR. GUTHRIE: I agree with Dr. Bickel’s comments about the inaccuracy of our test when the phenylalanine blood levels are high. We have known of this problem since we developed the test. The remedy is extremely simple: dilute the liquid serum sample.

Once the concentration of phenylalanine is below 20 mg percent, there is excellent agreement with all other methods. Of course, a dried blood spot can’t be diluted. But this is not a problem because if the level is over 20 mg percent the exact figure is not important.

I think your use of this method is extremely important as a very helpful way of monitoring children on the diet. If the family lives some distance away, this method still permits assay of weekly specimens, since the mother can be trained to spot the blood.

DR. HSIA: I want to compliment Dr. Bickel on his very interesting and very excellent study. We have had the same experience both in terms of the dietary therapy, the ultimate IQ of children, the complications, and the growth curves.

We have placed 34 children on the diet over the last seven or eight years. Thirteen children were under two months of age. Essentially we have the same results as Dr. Bickel.

I think this is the time when there should arise a consensus which will be useful to people who are involved in the practical aspects of this problem. Should the next phenylketonuric infant, not a heterozygote, be treated with a diet or not? I think the value of a conference of this type is to arrive at some consensus on the matter.

DR. UDENFRIEND: I can speak freely because I am not a psychologist. From your data one must conclude that the intelligence of no phenylketonuric child has ever been improved materially above where it was initially. In other words you say there is a natural decline in intelligence in phenylketonuria if one doesn’t institute treatment. If treatment is begun, you say, you keep them from degenerating further. Is this essentially what you are doing? You are not improving any child to any great extent. There is no rise from IQ 40 to IQ 100. Is this correct?

The second thing you are saying is that all children at birth, whether phenylketonuric or not, are absolutely normal. That is, all phenylketonuric children test with an IQ of 100 at birth. Is that so?
DR. BICKEL: No.

DR. UDENFRIEND: Your falling IQ curve begins at 100 at birth. I can hardly believe such uniformity nor the shape of that beautiful curve.

DR. BICKEL: Like almost every beautiful and elegant curve, it isn't quite true. It represents a broad generalization. I made this broad generalization and afterwards went into details.

I think most people agree that there is a variation of intelligence at birth in phenylketonurics, just as there is in normal children. Some may have an IQ of 80 and some 120.

DR. UDENFRIEND: Your conclusion then is that the diet prevents deterioration? You have no data to show that there is an improvement in IQ?

DR. BICKEL: You are right to some extent. I think the most important point is that brain damage is prevented. If brain damage has once occurred there is only a limited time span in which the damage that has been done can be to some extent reversed. It is, however, quite important if a child with an IQ of 40 can be brought to an IQ of 65 or 70. The one child belongs in an institution, and the other can live a somewhat limited life outside the institution.

DR. UDENFRIEND: If that is so, then one has to be extremely cautious in evaluating therapy because one cannot use this same child as a control. When there is a distinct improvement, then obviously something has changed. However, when the argument is used that all that is accomplished is maintenance of intelligence then we don't have a simple experiment where each child is his own control. We then must speak only about changes in the general population.

DR. BICKEL: To some extent you are right, though in many patients there is distinct improvement.

DR. SWAIMAN: Have the children with the electroencephalographic pattern of hypsarrythmia been given pyridoxine before they were placed on the diet?

DR. BICKEL: No.

DR. SWAIMAN: I would like to point out that we see children who have hypsarrythmic patterns that can be reversed by the administration of pyridoxine. Of course, there is reason to believe that perhaps pyridoxine requirements in individuals with phenylketonuria might be greater on the basis of greater transamination needs. The low phenylalanine diet per se may not cause the record to return to normality; but rather allows more pyridoxine to become available.

As a pediatric neurologist, I find the accurate clinical assessment of motor and intellectual development during the first six months of life and even sometimes during the first year of life difficult and arbitrary. Before these children have any degree of verbal ability assessment of intellect is very difficult. The difficulty in assessment makes me skeptical of any data that closely defines normal ranges of intelligence during this period of life.

In the infant we are forced into the classical posture of assessing a child's motor abilities and attempting to extrapolate this data to prognosticate future intellectual ability. The correlation is fairly good, but it is far from perfect. There is real question as to how precise this type of data is before the first year.

DR. BICKEL: I am fully aware of the difficulty of testing infants. I don't know any better method of determining in an easily demonstrable manner if the children are improving or deteriorating. I haven't heard of anyone who has a better means.

DR. ANDERSON: We have submitted a report (Pediatrics, June, 1966) concerning a child who at three months of age had an EEG diagnosis of hypsarrythmia, was treated with ACTH with fairly satisfactory response, and then later was found to have phenylketonuria. So ACTH can also modify this electroencephalogram.

DR. FISCH: I don't know that 100 IQ is the average of phenylketonuric children at birth, because many people have noted that a number of patients have low IQ levels even if the treatment is begun very early.

The EEG changes usually improve, but not as uniformly as you pointed out. We have idiots with normal EEG records, and we have seen very severe, markedly abnormal EEG's of successfully treated children with normal intelligence.

On the other hand, we have noticed a shift in the EEG to the normal direction following dietary restriction.

DR. WOOLF: Concerning the effect of over-treatment, Dr. Platt has shown some time ago that if growing animals are deprived of adequate protein, they suffer neurological damage which is reflected in abnormal behavior patterns during
life. At death the brain is abnormal in structure; it is deficient in neurons. So we must recognize that overtreatment also may produce the mental retardation we are trying to prevent by treatment. This is a danger. I don't suppose you have met it very often because you control your patients very well.

We have measured the IQ's of children started late on therapy. One was started at two years and four months with an IQ of 42. Over the years her IQ rose steadily. There was a steady rise up to about IQ 70. At the age of about seven or eight years it seemed to level off.

The second case was begun at age two years and 11 months with an IQ of 71. There was a steady, slow rise to IQ 103.

So we haven't merely maintained IQ, we have noted improvement even in an older child.

DR. BICKEL: I think you put your finger on an important point. I can only say that overtreatment in our hands has occurred primarily in the very young infant. These infants have developed mentally normal despite these periods of overtreatment which may have lasted weeks.
OBSERVATIONS OF PHENYLKETONURIA

RICHARD KOCH, PHYLLIS ACOSTA and KAROL FISHLER

presented by RICHARD KOCH

The disease phenylketonuria was described over 30 years ago, but treatment aimed at preventing the neurologic sequelae of the disease is recent. Institution of a low phenylalanine diet mitigates or actually seems to prevent the occurrence of mental retardation when the diagnosis is made in early infancy. The recent development of screening programs utilizing techniques applicable to the newborn nursery has provided the clinician an unusual opportunity to obviate or minimize the occurrence of mental retardation in affected infants.

On the basis of casefinding in newborn screening programs the estimate of the incidence of phenylketonuria has been doubled from a high of 1 case in approximately 20,000 births to 1 in 10,000 births. Some questions have been raised as to whether all of the cases thus detected are truly classical cases. On the basis of the evidence thus far presented it would appear that in most, if not practically all, cases continuously elevated blood phenylalanine levels would lead to mental retardation unless treatment is initiated early, while serious consequences may follow the administration of dietary therapy without appropriate supervision (due to excessive phenylalanine deprivation, etc.). When treatment is properly supervised, little or no detrimental effect is apparent. None the less, there is need for a definitive diagnosis with the potential of differentiating those conditions in which phenylalanine elevations may be transient for one reason or another and where phenylalanine elevations are likely to persist. This paper presents pertinent findings from our experience at the Childrens Hospital of Los Angeles in the diagnosis and treatment of 90 cases seen between 1958-1965. Only 57 were treated with the low phenylalanine diet.

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Diagnosis

Prior to widespread screening it was accepted that a child with an elevated serum phenylalanine was phenylketonuric. Children were diagnosed clinically on the basis of mental retardation, fair complexion, blond hair, eczema, convulsions, and family history or on urine screening tests for phenylalanine metabolites in groups of retarded individuals. It is now quite clear that other factors are involved in evaluating infants with hyperphenylalaninemia identified by newborn screening techniques. As a result of these new data the care of presumptive positive infants has been changed considerably. At present presumpive positives in our area are retested with the same test as quickly as possible. When two consecutive tests reveal blood levels of phenylalanine exceeding 4 mg percent, the infant has been referred to a phenylketonuric diagnostic center. Nine such centers have been established in California and provide medical supervision and dietary supplies.

The typical case of phenylketonuria was usually referred with significantly elevated blood phenylalanine levels by the McCaman and Robins method (over 20 mg percent). In these infants low serum tyrosine levels sometimes associated with the usual phenylalanine metabolites in the urine served to confirm the diagnosis. The use of the ferric chloride urine test or the Phenistix® test alone is not adequate for judging the presence or absence of phenylalanine metabolites in the urine. Chromatography of serum and urine was the technique utilized for confirmation of a diagnosis of phenylketonuria in our case material.

At present the clinician faced with a confirmed positive blood phenylalanine test must consider several possibilities.

Recently we have identified a baby with tyrosinosis due to a "presumptive positive" PKU test. In this case serum phenylalanine levels ranged between 5-15 mg percent and serum tyrosine...
levels exceeded 20-30 mg percent. The presence of typical urinary tyrosine metabolites served to confirm the diagnosis. It has been known for some time that premature babies are likely to exhibit a slightly elevated serum phenylalanine due to late development or inactivation of the tyrosine oxidizing system. These infants will show a positive screening test. In such cases, abnormality of tyrosine metabolism can be further documented by finding tyrosine metabolites in the urine. Administration of ascorbic acid corrects this defect promptly. The possibility of maternal phenylketonuria must also be considered. Recently such a case (No. 75) was found by testing the mother of one of the presumptive positive cases. The mother’s sister (Case No. 30) was subsequently found to be phenylketonuric and severely retarded (IQ 48). Follow-up studies on the presumptive positive case revealed an IQ of 78 at age two years.

The recent report by Anderson et al. of an atypical phenylketonuric who in all likelihood is a heterozygote for PKU is another possibility to be considered. In view of the several diagnoses that may be associated with elevated serum phenylalanine levels during the newborn period, it is now apparent that serum and urine amino acid chromatography are necessary for establishing a diagnosis of phenylketonuria. A family history of phenylketonuria and a low tolerance for phenylalanine documented during dietary treatment serve as further confirmatory evidence for the diagnosis. Phenylalanine loading tests may also be useful. Certainly liver biopsy would not be a justifiable procedure to be performed in my judgment.

Present Method of Treatment

At the time of referral, the infant is hospitalized. Serum and urine amino acid chromatography, electroencephalogram, blood count, and routine urinalysis are obtained in addition to the usual history and physical examination. Particular attention is paid to the family history. With confirmation of diagnosis the infant is arbitrarily started on 30-40 mg of phenylalanine per pound of body weight per day allowing for adequate protein and caloric intake. Serum phenylalanine levels are determined daily until normal levels are achieved. Serum levels are also obtained on all siblings, both parents, and any retarded relatives. The parents are taught the essentials of the low phenylalanine diet by the nutritionist utilizing Lofenalac® as the base formula. The family structure is evaluated by the social worker; a developmental or psychological assessment is performed by the psychologist; and arrangements are made for the public health nurse to visit the home one week following discharge from the hospital. The public health nurse advises the parents on health practices, reinforces the physician’s instructions, assists with management problems and demonstrates the method of obtaining finger tip blood on filter paper for the inhibition assay test which the parent obtains for monitoring the infant’s diet after discharge. The samples, initially obtained every week, are mailed to the clinic laboratory along with a three day diet history preceding the procurement of blood for phenylalanine determination. After six months of age the frequency of monitoring is decreased to monthly intervals. The infant continues to receive regular well baby care from the family physician who is kept informed of dietary changes and phenylalanine levels. Several pamphlets on phenylketonuria are also furnished to both the family physician and the parents. The infant is seen in the PKU clinic at monthly intervals initially, then two month, and finally three-four month intervals until five years of age. After this age, if dietary control is good, they are seen at intervals of six months. An attempt is made to treat all new patients with the low phenylalanine diet. In some of the older children we have been unsuccessful, but it is surprising how easily most children accept the low phenylalanine diet. Naturally the older the child, the more difficult it is to initiate treatment due to the unusual taste of Lofenalac®. Some of the older children will starve themselves for several days before accepting this formula. Parents need reassurance that this is not harmful. Serum phenylalanine levels remain elevated during this period even though oral intake of protein is at a minimum. This is probably due to tissue protein catabolism.

Case Material

Of the ninety phenylketonuric subjects forty-six were female, forty-four male. All were Caucasian; their ages ranged from newborn to
26 years. The oldest (age 26 years) was a housewife with three mildly retarded children. Two exhibited other unrelated diseases, one scleroderma, and the other cystathioninuria.

The ethnic backgrounds were predominantly Irish, English, Scotch, and German. Two patients were of Caucasian origin with admixture of Jewish and oriental stock. There were no Negroes.

Unfortunately, mental retardation was the presenting symptom in 50 of our 90 cases. Forty PKU individuals were identified through various screening programs; 12 before three months of age. Thirteen were identified in public school programs, 12 by sibling screening of index cases, 9 by newborn blood tests, 2 by urine diaper tests in well baby clinics, 2 in the screening program for out-patients at Children's Hospital of Los Angeles, 1 by Phenistix testing in a private physician's office, and 1 during intake evaluation at a State hospital for the retarded.

The young PKU children in our sample were not "typically" blond or blue-eyed. Irritability, hyperactivity, and slow development were the common presenting symptoms. Eczema occurred in 12, convulsions in 5, and autistic symptoms in 6. An unusual urine odor was rarely reported.

Results of Dietary Therapy

Table 1 lists the age of diagnosis, initial DQ/IQ*, DQ/IQ change, dietary duration, and present age of 24 phenylketonuric children whose diet was under excellent control. While 14 of these infants were evaluated quite early, when tests may not be precisely predictive as could be desired, the experiment does indicate the effectiveness of dietary control in maintaining mentality and even in permitting improvement. The average age of diagnosis in this group was 15 months. The average initial DQ/IQ was 75; each child's DQ/IQ rose an average of 23 points; and their present average age is 3 years 3 months.

Table 2 summarizes the data on 21 PKU children with good dietary control. The average age of diagnosis was 1-3/4 years with an initial average DQ/IQ of 58. Each child's average DQ/IQ rise was 17 after an average of 3 years 3 months diet duration. The present average age of this group is 4-3/4 years.

Table 3 details data obtained from 12 PKU children with poor dietary control. The average age at time of diagnosis was 2-1/4 years with an average initial DQ/IQ of 41. Even so, the average rise in DQ/IQ points was 21 for this group after an average of 4-1/6 years of treatment.

Table 4 presents data on the 23 individuals who were not treated with the low phenylalanine diet. They were in general older (average age 12 years) and more retarded. Dietary therapy was attempted in a few without success. Two of this group are unusual. One child has an IQ of 115 at age 10 years without therapy (Case No. 84). He was first diagnosed at age 3(34) at which time his IQ was 122. He is healthy, his EEG is normal, and serum phenylalanine values range from 12 to 17 mg percent. The other individual is a PKU mother (Case No. 75) with normal IQ. The ten individuals who were subsequently retested have all shown a decline in their IQ measurements but due to the profound degree of mental retardation this data is questionable. Nine have been institutionalized. Ten patients have been lost to follow-up.

Table 5 summarizes the data obtained on the 80 patients. The relationship of dietary control to age of diagnosis, initial DQ/IQ, increase in DQ/IQ with treatment, treatment period, and present age of sample are presented. The obvious critical factors are the age of diagnosis, early initiation of therapy, and the degree of dietary control.

Normal IQ potential seems attainable with early diagnosis and proper dietary management. In contrast, late diagnosis and no therapy usually results in severe mental retardation. Figure 1 dramatizes the importance of early identification. During the first year of life it appears that about 50 IQ points are lost or about one IQ point per week.
Table 1.--Data on twenty-four PKU children with excellent dietary control

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Age Dxd. Yrs.-Mos.</th>
<th>Initial DQ/IQ</th>
<th>DQ/IQ Change</th>
<th>Diet Duration Yrs.-Mos.</th>
<th>Present Age Yrs.-Mos.</th>
<th>Present Disposition</th>
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<tbody>
<tr>
<td>1</td>
<td>3 - 11</td>
<td>56 DQ</td>
<td>+4</td>
<td>1 - 1</td>
<td>5 - 0</td>
<td>EMR Class*</td>
</tr>
<tr>
<td>2</td>
<td>0 - 9</td>
<td>84 DQ</td>
<td>+19</td>
<td>3 - 11</td>
<td>4 - 8</td>
<td>Kindergarten</td>
</tr>
<tr>
<td>4</td>
<td>0 - 5</td>
<td>75 DQ</td>
<td>+26</td>
<td>3 - 9</td>
<td>4 - 2</td>
<td>Home</td>
</tr>
<tr>
<td>12</td>
<td>0 - 7</td>
<td>59 DQ</td>
<td>+30</td>
<td>0 - 6</td>
<td>1 - 1</td>
<td>Home</td>
</tr>
<tr>
<td>16</td>
<td>4 - 0</td>
<td>42 SB</td>
<td>+37</td>
<td>4 - 0</td>
<td>8 - 0</td>
<td>EMR Class (Moved '65)</td>
</tr>
<tr>
<td>18</td>
<td>0 - 6</td>
<td>67 DQ</td>
<td>+38</td>
<td>1 - 0</td>
<td>1 - 6</td>
<td>Home</td>
</tr>
<tr>
<td>21</td>
<td>1 - 9</td>
<td>37 DQ</td>
<td>+35</td>
<td>3 - 3</td>
<td>5 - 0</td>
<td>TMR Class</td>
</tr>
<tr>
<td>22</td>
<td>0 - 1</td>
<td>90 DQ</td>
<td>--</td>
<td>0 - 6</td>
<td>0 - 5</td>
<td>Home</td>
</tr>
<tr>
<td>31</td>
<td>0 - 1</td>
<td>100 DQ</td>
<td>+15</td>
<td>1 - 0</td>
<td>1 - 1</td>
<td>Home</td>
</tr>
<tr>
<td>34</td>
<td>1 - 6</td>
<td>36 DQ</td>
<td>+19</td>
<td>0 - 6</td>
<td>2 - 0</td>
<td>Home</td>
</tr>
<tr>
<td>42</td>
<td>1 - 5</td>
<td>60 DQ</td>
<td>+41</td>
<td>2 - 8</td>
<td>4 - 1</td>
<td>Nurs. Cl. (Moved '64)</td>
</tr>
<tr>
<td>43</td>
<td>3 - 0</td>
<td>40 DQ</td>
<td>+42</td>
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<td>5 - 2</td>
<td>EMR Class</td>
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<td>1 - 0</td>
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<tr>
<td>48</td>
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<td>+13</td>
<td>1 - 7</td>
<td>1 - 8</td>
<td>Home</td>
</tr>
<tr>
<td>49</td>
<td>1 - 5</td>
<td>97 DQ</td>
<td>+13</td>
<td>1 - 8</td>
<td>3 - 1</td>
<td>Home</td>
</tr>
<tr>
<td>52</td>
<td>0 - 1</td>
<td>100 DQ</td>
<td>-4</td>
<td>3 - 2</td>
<td>3 - 2</td>
<td>Moved 1964</td>
</tr>
<tr>
<td>53</td>
<td>6 - 4</td>
<td>56 DQ</td>
<td>+11</td>
<td>2 - 10</td>
<td>9 - 2</td>
<td>EMR Class (Moved '64)</td>
</tr>
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<td>0 - 6-1/2</td>
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</tr>
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<td>0 - 6</td>
<td>0 - 7</td>
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</tr>
<tr>
<td>69</td>
<td>2 - 1</td>
<td>48 DQ</td>
<td>+52</td>
<td>5 - 3</td>
<td>7 - 4</td>
<td>EMR* Class</td>
</tr>
<tr>
<td>70</td>
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<td>100 DQ</td>
<td>+29</td>
<td>0 - 9</td>
<td>0 - 10</td>
<td>Home</td>
</tr>
<tr>
<td>72</td>
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<td>100 DQ</td>
<td>--</td>
<td>0 - 7</td>
<td>0 - 9</td>
<td>Home</td>
</tr>
<tr>
<td>82</td>
<td>1 - 7</td>
<td>66 DQ</td>
<td>+20</td>
<td>3 - 7</td>
<td>5 - 2</td>
<td>Kindergarten</td>
</tr>
<tr>
<td>83</td>
<td>0 - 1/4</td>
<td>100 DQ</td>
<td>+14</td>
<td>2 - 1</td>
<td>2 - 1</td>
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</tr>
</tbody>
</table>

*EMR refers to an educable mentally retarded children's class in public school which accepts individuals with IQs ranging from 50-70.
Table 2.--Data on twenty-one PKU children with good dietary control

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Age Dxd. Yrs.-Mos.</th>
<th>Initial DQ/IQ</th>
<th>DQ/IQ Change</th>
<th>Diet Duration Yrs.-Mos.</th>
<th>Present Age Yrs.-Mos.</th>
<th>Present Disposition</th>
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<tr>
<td>6</td>
<td>5 - 6</td>
<td>34 DQ</td>
<td>+15</td>
<td>5 - 2</td>
<td>10 - 8</td>
<td>TMR* Class</td>
</tr>
<tr>
<td>8</td>
<td>0 - 1</td>
<td>100 DQ</td>
<td>0</td>
<td>0 - 11</td>
<td>1 - 0</td>
<td>Parents disbelieve Dx.</td>
</tr>
<tr>
<td>11</td>
<td>1 - 1</td>
<td>60 DQ</td>
<td>+10</td>
<td>1 - 0</td>
<td>2 - 1</td>
<td>Home</td>
</tr>
<tr>
<td>13</td>
<td>3 - 0</td>
<td>60 SB</td>
<td>+10</td>
<td>4 - 6</td>
<td>8 - 6</td>
<td>EMR** Class, off diet 1 year</td>
</tr>
<tr>
<td>14</td>
<td>2 - 0</td>
<td>39 DQ</td>
<td>+49</td>
<td>4 - 0</td>
<td>8 - 0</td>
<td>TMR Class - off diet 2 years - hyperactive</td>
</tr>
<tr>
<td>29</td>
<td>1 - 0</td>
<td>61 DQ</td>
<td>+28</td>
<td>4 - 4</td>
<td>5 - 4</td>
<td>Kindergarten</td>
</tr>
<tr>
<td>36</td>
<td>0 - 10</td>
<td>47 DQ</td>
<td>+23</td>
<td>1 - 3</td>
<td>2 - 1</td>
<td>Home</td>
</tr>
<tr>
<td>37</td>
<td>0 - 8</td>
<td>37 DQ</td>
<td>+62</td>
<td>3 - 11</td>
<td>4 - 7</td>
<td>Nursery Class</td>
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<td>45</td>
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<td>+10</td>
<td>6 - 6</td>
<td>9 - 0</td>
<td>Foster Home: TMR Class</td>
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<td>4 - 5</td>
<td>Home</td>
</tr>
<tr>
<td>55</td>
<td>4 - 8</td>
<td>46 DQ</td>
<td>-1</td>
<td>1 - 4</td>
<td>6 - 0</td>
<td>Moved 1963</td>
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<tr>
<td>57</td>
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<td>36 DQ</td>
<td>+3</td>
<td>2 - 2</td>
<td>3 - 0</td>
<td>Seizure disorder</td>
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<td>5 - 1</td>
<td>7 - 1</td>
<td>Hyperactive</td>
</tr>
<tr>
<td>63</td>
<td>0 - 2</td>
<td>100 DQ</td>
<td>+11</td>
<td>1 - 0</td>
<td>1 - 2</td>
<td>Home</td>
</tr>
<tr>
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<td>50 DQ</td>
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<td>1 - 6</td>
<td>2 - 10</td>
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</tr>
<tr>
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<td>1 - 5</td>
<td>49 DQ</td>
<td>+23</td>
<td>4 - 1</td>
<td>5 - 6</td>
<td>EMR** Class</td>
</tr>
<tr>
<td>77</td>
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<td>104 DQ</td>
<td>-1</td>
<td>3 - 8</td>
<td>3 - 8</td>
<td>Home</td>
</tr>
<tr>
<td>80</td>
<td>0 - 6</td>
<td>50 DQ</td>
<td>+20</td>
<td>3 - 10</td>
<td>4 - 4</td>
<td>Training Class for retarded children</td>
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<tr>
<td>81</td>
<td>1 - 10</td>
<td>98 DQ</td>
<td>+11</td>
<td>3 - 3</td>
<td>5 - 3</td>
<td>Kindergarten</td>
</tr>
<tr>
<td>89</td>
<td>1 - 0</td>
<td>65 DQ</td>
<td>+21</td>
<td>4 - 0</td>
<td>9 - 0</td>
<td>EMR Class, off diet 4 years</td>
</tr>
<tr>
<td>90</td>
<td>0 - 9</td>
<td>50 DQ</td>
<td>+15</td>
<td>2 - 9</td>
<td>3 - 6</td>
<td>Home (Moved 1965)</td>
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</table>

*TMR refers to a classroom in the public school for trainable mentally retarded children ranging in IQ from 30-50.

**EMR refers to public school class for IQ range 50-70.
Table 3.--Data on twelve PKU children with poor dietary control

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Age Dxd. Yrs.-Mos.</th>
<th>Initial DQ/IQ</th>
<th>DQ/IQ Change</th>
<th>Diet Duration Yrs.-Mos.</th>
<th>Present Age Yrs.-Mos.</th>
<th>Comment</th>
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<tbody>
<tr>
<td>7</td>
<td>1 - 1</td>
<td>31 DQ</td>
<td>+12</td>
<td>5 - 2</td>
<td>6 - 3</td>
<td>Child Development Center - Mother retarded, IQ 70. Home.</td>
</tr>
<tr>
<td>15</td>
<td>3 - 0</td>
<td>27 DQ</td>
<td>-1</td>
<td>2 - 0</td>
<td>5 - 0</td>
<td>Transient family. Home.</td>
</tr>
<tr>
<td>20</td>
<td>2 - 6</td>
<td>30 DQ</td>
<td>--</td>
<td>5 - 6</td>
<td>8 - 0</td>
<td>Also has cystathioninuria. Father unstable; mother IQ 81. Home.</td>
</tr>
<tr>
<td>24</td>
<td>0 - 6</td>
<td>57 DQ</td>
<td>+13</td>
<td>2 - 6</td>
<td>3 - 0</td>
<td>Family difficult. Home.</td>
</tr>
<tr>
<td>28</td>
<td>1 - 11</td>
<td>48 DQ</td>
<td>+12</td>
<td>4 - 4</td>
<td>6 - 3</td>
<td>Hyperactive. Home.</td>
</tr>
<tr>
<td>35</td>
<td>8 - 0</td>
<td>51 SB</td>
<td>+1</td>
<td>1 - 9</td>
<td>9 - 9</td>
<td>Transient family. Home.</td>
</tr>
<tr>
<td>41</td>
<td>0 - 8</td>
<td>37 DQ</td>
<td>+34</td>
<td>7 - 0</td>
<td>7 - 8</td>
<td>Mother retarded, IQ 72. Diet initially good - now poor. EMR* Class. Home.</td>
</tr>
<tr>
<td>54</td>
<td>1 - 8</td>
<td>66 DQ</td>
<td>+20</td>
<td>1 - 10</td>
<td>3 - 6</td>
<td>Mother retarded, IQ 70. Child has scleroderma. Home.</td>
</tr>
<tr>
<td>60</td>
<td>0 - 10</td>
<td>20 DQ</td>
<td>+45</td>
<td>3 - 0</td>
<td>3 - 10</td>
<td>Unstable family. Home.</td>
</tr>
<tr>
<td>67</td>
<td>1 - 6</td>
<td>46 DQ</td>
<td>+38</td>
<td>4 - 4</td>
<td>5 - 10</td>
<td>Chronic staphylococcus infection. Home.</td>
</tr>
<tr>
<td>78</td>
<td>1 - 2</td>
<td>62 DQ</td>
<td>+16</td>
<td>6 - 5</td>
<td>7 - 7</td>
<td>Mother unable to keep child on diet. EMR* Class. Home.</td>
</tr>
<tr>
<td>79</td>
<td>3 - 2</td>
<td>10 DQ</td>
<td>+66</td>
<td>6 - 5</td>
<td>9 - 7</td>
<td>Mother unable to keep child on diet. EMR Class. Home.</td>
</tr>
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</table>

*EMR refers to public school class for IQ range 50-70.
Table 4.--Data on twenty-three untreated PKU individuals

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Age Dxd. Yrs.-Mos.</th>
<th>Initial DQ/IQ</th>
<th>DQ/IQ Change</th>
<th>Time Involved</th>
<th>Present Age Yrs.-Mos.</th>
<th>Comment</th>
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</thead>
<tbody>
<tr>
<td>3</td>
<td>4 - 7</td>
<td>20 DQ</td>
<td>--</td>
<td>--</td>
<td>7 - 5</td>
<td>Institutionalized Pacific State Hospital</td>
</tr>
<tr>
<td>5</td>
<td>13 - 0</td>
<td>Not done</td>
<td>--</td>
<td>--</td>
<td>15 - 0</td>
<td>Fairview State Hospital</td>
</tr>
<tr>
<td>9</td>
<td>13 - 2</td>
<td>54 DQ</td>
<td>--</td>
<td>--</td>
<td>15 - 1</td>
<td>TMR* Class - home, Pacific State Hospital</td>
</tr>
<tr>
<td>17</td>
<td>4 - 6</td>
<td>29 DQ</td>
<td>NM**</td>
<td>4 - 0</td>
<td>10 - 1</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>13 - 0</td>
<td>65 SB</td>
<td>--</td>
<td>--</td>
<td>14 - 0</td>
<td>EMR*** Class - home, Home - day care.</td>
</tr>
<tr>
<td>25</td>
<td>14 - 0</td>
<td>31 Leiter</td>
<td>--</td>
<td>--</td>
<td>16 - 0</td>
<td>TMR Class - home.</td>
</tr>
<tr>
<td>27</td>
<td>13 - 0</td>
<td>46 SB</td>
<td>--</td>
<td>--</td>
<td>14 - 0</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>19 - 0</td>
<td>48 WAIS</td>
<td>--</td>
<td>--</td>
<td>22 - 0</td>
<td>Home - day care.</td>
</tr>
<tr>
<td>32</td>
<td>2 - 5</td>
<td>18 DQ</td>
<td>NM</td>
<td>8 - 0</td>
<td>10 - 5</td>
<td>Pacific State Hospital</td>
</tr>
<tr>
<td>33</td>
<td>7 - 0</td>
<td>10 SB</td>
<td>NM</td>
<td>11 - 0</td>
<td>18 - 0</td>
<td>Pacific State Hospital</td>
</tr>
<tr>
<td>46</td>
<td>18 - 9</td>
<td>74 WAIS</td>
<td>--</td>
<td>--</td>
<td>20 - 0</td>
<td>Day care - home.</td>
</tr>
<tr>
<td>50</td>
<td>17 - 0</td>
<td>77 WAIS</td>
<td>--</td>
<td>--</td>
<td>18 - 0</td>
<td>EMR - home.</td>
</tr>
<tr>
<td>51</td>
<td>13 - 0</td>
<td>72 WISC</td>
<td>--</td>
<td>--</td>
<td>14 - 0</td>
<td>EMR - home.</td>
</tr>
<tr>
<td>56</td>
<td>12 - 0</td>
<td>10 SB</td>
<td>NM</td>
<td>4 - 0</td>
<td>16 - 0</td>
<td>Private institution.</td>
</tr>
<tr>
<td>61</td>
<td>14 - 0</td>
<td>49 SB</td>
<td>--</td>
<td>--</td>
<td>15 - 0</td>
<td>Day care - home.</td>
</tr>
<tr>
<td>65</td>
<td>9 - 0</td>
<td>14 DQ</td>
<td>NM**</td>
<td>4 - 0</td>
<td>13 - 0</td>
<td>Day care - home.</td>
</tr>
<tr>
<td>71</td>
<td>3 - 0</td>
<td>NM</td>
<td>NM</td>
<td>9 - 0</td>
<td>12 - 0</td>
<td>Day care - home.</td>
</tr>
<tr>
<td>74</td>
<td>15 - 6</td>
<td>80 WISC</td>
<td>--</td>
<td>--</td>
<td>16 - 0</td>
<td>EMR*** Class - home,</td>
</tr>
<tr>
<td>75</td>
<td>26 - 0</td>
<td>96 WAIS</td>
<td>--</td>
<td>--</td>
<td>28 - 0</td>
<td>Home - housewife.</td>
</tr>
<tr>
<td>76</td>
<td>18 - 0</td>
<td>28 MP</td>
<td>NM</td>
<td>2 - 0</td>
<td>21 - 0</td>
<td>Private institution.</td>
</tr>
<tr>
<td>84</td>
<td>4 - 0</td>
<td>122 SB</td>
<td>-7</td>
<td>6 - 0</td>
<td>10 - 0</td>
<td>Regular school - home.</td>
</tr>
<tr>
<td>85</td>
<td>1 - 5</td>
<td>38 DQ</td>
<td>NM</td>
<td>6 - 0</td>
<td>8 - 6</td>
<td>No response to diet (2 years) - Pacific State Hospital.</td>
</tr>
<tr>
<td>87</td>
<td>18 - 0</td>
<td>NM</td>
<td>NM</td>
<td>2 - 0</td>
<td>20 - 0 (Expired)</td>
<td>Private institution</td>
</tr>
</tbody>
</table>

*TMR refers to a classroom in the public school for trainable mentally retarded children ranging in IQ from 30-50.

**NM refers to a child with a non-measurable IQ.

***EMR refers to public school class for IQ range 50-70.
Table 5.—Summary of results of dietary therapy

<table>
<thead>
<tr>
<th>Control</th>
<th>Excellent</th>
<th>Good</th>
<th>Poor</th>
<th>None</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average: Age of diagnosis</td>
<td>15 mons.</td>
<td>18 mons.</td>
<td>27 mons.</td>
<td>144 mons.</td>
</tr>
<tr>
<td>Average: DQ/IQ Initially</td>
<td>75</td>
<td>58</td>
<td>41</td>
<td>43</td>
</tr>
<tr>
<td>Average: Increase in DQ/IQ</td>
<td>+23</td>
<td>+17</td>
<td>+21</td>
<td>-19*</td>
</tr>
<tr>
<td>Average: Treatment Period</td>
<td>2 yrs.</td>
<td>1.75 yr.</td>
<td>4 yr.</td>
<td>--</td>
</tr>
<tr>
<td>Average: Age of Sample Now</td>
<td>3.3 yrs.</td>
<td>4.75 yr.</td>
<td>6.67 yr.</td>
<td>15 yr.</td>
</tr>
<tr>
<td>Number of PKU Children</td>
<td>24</td>
<td>21</td>
<td>12</td>
<td>23</td>
</tr>
</tbody>
</table>

*IQ estimates rather than formal testing due to profound degree of mental defect.

The present status of these 90 individuals is summarized in Table 6. Note that the nine children who were institutionalized were all in the non-treatment group.

Table 6.—Present status of 90 PKU subjects in community

<table>
<thead>
<tr>
<th>Home</th>
<th>Too young for school</th>
<th>Regular school</th>
<th>Educable Mentally Retarded Class (50 - 70 IQ)</th>
<th>Trainable Mentally Retarded Class (30 - 50 IQ)</th>
<th>Child Development Center (0 - 30 IQ)</th>
<th>Housewife</th>
<th>Foster Home</th>
<th>Institutional Care</th>
<th>Lost to follow-up</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>26</td>
<td>7</td>
<td>15</td>
<td>10</td>
<td>11</td>
<td>1</td>
<td>1</td>
<td>9</td>
<td>10</td>
<td>90</td>
</tr>
</tbody>
</table>

All of the cases under treatment have been maintained on a low phenylalanine diet. None have been deliberately taken off therapy although most of the 12 children listed in Table 3 are youngsters who are difficult to control. Discontinuance of the low phenylalanine diet is still an unsettled question.

Recently we saw an eight year old phenylketonuric patient (Case 14) who was treated elsewhere from 16 months to 4 years of age by the low phenylalanine diet. Her developmental quotient at the time of diagnosis was 39. An IQ of 89 (Stanford Binet L-M) is recorded for age 4 when dietary therapy was discontinued. Subsequently there was no obvious intellectual deterioration, however, she was more restless, irritable, and sleepless than when she had been on diet. At age 6-1/2 years she began to experience psychomotor seizures. An electroencephalogram revealed a diffuse abnormality with spike foci. Her serum phenylalanine at that time was 34 mg percent. Her growth pattern was normal (height and weight) and no eczema was noted, but her hair color was lighter than when the diet was discontinued. Intelligence testing revealed an IQ of 88 on the Stanford-Binet Scale at age seven years. She has improved on anti-convulsant medication and is in a special class for retarded children.

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Most of the large surveys of PKU children in the literature are based upon institutionalized populations. The most frequent signs and symptoms mentioned in previous reports are mental deficiency, blond hair, blue eyes, and seizures but these signs are not common in infancy. Recently there has been a greater emphasis on vomiting, eczema, and urine odor as early signs in infancy. Twelve of our patients had been diagnosed incorrectly as severe eczema unresponsive to usual therapy before a diagnosis of PKU was established. In each, the onset of eczema preceded the development of mental retardation. Obesity, in our experience, is a frequently associated finding in the infant with untreated PKU and sometimes leads to a mistaken diagnosis of hypothyroidism because of the accompanying mental retardation. Three had been so treated in spite of normal blood protein-bound iodine values.

The PKU child is usually hyperactive and exhibits unpredictable, erratic behavior. Excessive rocking movements, grinding of teeth, arm waving, and overall aimless behavior occasionally are misdiagnosed as early childhood schizophrenia. Six of our patients were considered autistic until the identifying urine test for phenylpyruvic acid revealed the true basis for their behavior. Only five of our patients manifested seizures, three myoclonic, one petit mal, and one psychomotor. The marked contrast in the signs and symptoms observed in our patients and those reported in the literature is undoubtedly due to their young age. The experienced clinician can avoid making errors in diagnosis only by making full use of results from routine screening tests and other confirmatory laboratory procedures. This points up the value of continuing routine testing in various clinics such as child guidance, epilepsy, eczema, cerebral palsy, metabolic, etc.

It has been our practice to obtain a serum chromatogram and phenylalanine level on all newborn siblings of known PKU patients. The infant is placed on a low phenylalanine diet at five days of age until the serum chromatogram and phenylalanine level have been reported. If the chromatogram and serum phenylalanine are normal, a normal diet can be safely resumed. Guthrie tests are performed again at two, four, and six months of age to confirm absence of PKU.

On the other hand, if the serum value for phenylalanine is greater than 5 mg. percent, the infant is considered potentially phenylketonuric and further confirmation of the diagnosis is obtained by paper chromatography of serum and serial McCaman-Robins determinations.

The decision as to whether an older child should be treated with the low phenylalanine diet is difficult. General agreement exists among physicians that all PKU patients less than three years of age should be treated. Centerwall points out, however, that older children deserve a clinical trial of dietary therapy and that improvement in older children may only become apparent after months to years of careful treatment. Our experience substantiates his observations.

Whether to treat PKU children with normal intelligence with dietary therapy is another difficult problem. Horner has reported on three patients in whom dietary therapy was discontinued at age four years. His report suggested that the children seemed to be doing well following discontinuance of dietary therapy, although the observation period was admittedly short in one patient. Occurrence of seizures in children following cessation of dietary treatment, as observed by us and also by Langdell, is an actual and potential hazard. This conclusion is supported by the observations of Bickel who suggests that therapy should be continued at least until adolescence.

Every effort is made to maintain the serum phenylalanine content below 6 mg percent after treatment has been initiated. Maintenance of a level below 3 mg percent, as recommended by Bickel and Kretchmer, has been difficult with older children in our experience. When too many restrictions are placed on their diet, the older children generally do not cooperate readily and may obtain forbidden foods from neighbors, schoolmates, and the family refrigerator.

Phenylketonuria is a disease requiring the skills of various disciplines including a nutritionist, biochemist, social worker, public health nurse, and psychologist, for a well-rounded treatment program. The nutritionist is very helpful in making the diet more acceptable, especially to the older child. She can also help parents in the day-to-day management of the diet, not only in the regulation of phenylalanine intake, but also in insuring a proper balance of all other

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essential factors. In case of dietary indiscretion, the nutritionist's special knowledge is needed in readjusting the diet or in calculating intake of phenylalanine and other essentials.

The social worker is helpful in family counseling, maintaining good rapport with the family, and aids in interpreting the physician's findings to the parents. Public health nursing is very useful to parents in the day-to-day problems of habit training, discipline, and feeding routines. The nurse is especially valuable for home visits and follow-up where patient contact has been lost. The role of the psychologist is self-evident. The biochemist working with the nutritionist and clinician provides the absolutely necessary laboratory data in cases of uncertain diagnosis and where patients are difficult to control on a theoretically good dietary regimen. It is through the use of such additional skills that a pediatrician finds himself in an optimal position to provide coordinated care for the child and his family.

CONCLUSIONS

Our data suggests that infants with hyperphenylalaninemia should be referred to a pediatric center capable of providing optimal diagnostic evaluation and therapy. The physician should avail himself of the allied health disciplines in such a medical setting to assist him in treatment. Nutrition, public health nursing, social work and psychological assistance are particularly helpful. The State of California has established a PKU diagnostic and evaluation center to accept such referrals.

The therapeutic results obtained in the cases reviewed reveal that the best result in terms of optimal intellectual development were obtained with diagnosis and treatment instituted as early as possible. Caution is advised as to discontinuation of dietary therapy. Our experience suggests that discontinuance of the low phenylalanine diet early in childhood may be harmful.

More phenylalanine should be prescribed than is usually recommended in order to allow adequate growth and prevent bone changes. Additional information is required to determine the optimum blood phenylalanine levels required to prevent damage to the brain. Our experience further suggests that the low phenylalanine diet is nutritious and supports normal growth and development. Improper dietary management, however, can be dangerous due to phenylalanine and protein deficiency. The treatment of phenylketonuria may be a lifetime need. Finally treatment should be attempted even in the older child.

REFERENCES


The assistance of other members of the staff of the PKU clinic are acknowledged in the report of this material, and Kenneth N. F. Shaw, Ph.D. for his laboratory resources.

DISCUSSION

DR. BESSMAN: The problem here is the interpretation of numbers. First Dr. Udenfriend and Dr. Swaiman expressed some reservation. Many of us believe that there is something wrong with the quantitative measurements of intelligence. But let's accept the quantitative measurement.

I would like to discuss the little boy who developed so beautifully from IQ 48 to 93. I think Dr. Koch's data teaches us a lesson about the meaning of control. (Fig. 1).

Let us look at the graph in detail. We see that this patient was put on therapy for a year and during this first year the therapy had no effect. Next comes a sudden rise from 51 to 68 in six months of the second year of therapy.

The IQ stabilized on the same therapy again, a carefully controlled therapy, for about two and a half years. All of a sudden, another spurt in IQ occurred. I think that the interpretation of this is very important. Dr. Koch interprets this change as showing a clear effect of the diet. It seems to me that except for two sharply defined six month periods there was no change in IQ during several years on the diet. Two events seem to have occurred in this period which affected the measured IQ. In other words, because the patient was on the diet all during his life we credit it with any change which occurred, even though only two out of about ten month intervals showed any "effect" at all. The second "jump" in IQ seems to have occurred when the child would have gone to nursery school. I can't account for the first "jump" in IQ but again it seems like some environmental or technical effect.

I think that we ought to look at our data very carefully before we grab at every positive change and call it success, post hoc propter hoc.

DR. GERSHENSEN: I would assume that you probably used something like the Gesell during the early phase and switched into another test later. At what age was there a switch in the testing in reference to this chart?
DR. KOCH: Let me answer the question in regard to the chart first. We did use Gesell testing in our clinic, and at age 3 we switch to Stanford-Binet, for LM. So that the round dots represent Gesell developmental quotients and the square for the IQ data.

Dr. Bessman suggested that perhaps the IQ rise from 72 to 93 might be due to school attendance. This child did go to nursery school at age 4, at which time he had an IQ of 67. He continued in nursery school for 2 years, and at the present time he is in a regular second-grade class. There is no question that environmental stimulation is important. We would not want to minimize the importance of any program for these children. For example, when he went to nursery school, even though we didn't demonstrate an IQ rise, there certainly was a behavioral change. He was a hyperactive youngster. The mother was unable to control him well. This was a cooperative nursery school, and she matured with the process as well as the child.

All of us in this room know that IQ is influenced by many, many variables, and I am sure that I didn't say in my presentation that his progress is all due to the diet. It's due to many, many factors, of which the diet is perhaps the most important.

DR. GUTHRIE: Behavior is a lot more important than IQ in actual daily life, and behavior is what Dr. Bickel used to first demonstrate the beneficial effect of this diet.

The only way he was able to demonstrate this effect was with motion pictures. With his motion pictures of a child who was 2 years old, he demonstrated the remarkable change in this child's behavior as influenced directly by the phenylalanine levels in that child's blood. The mother didn't know when he had lowered the blood phenylalanine and when he had increased it. Nobody knew but Dr. Bickel.

This behavioral change is important in family life. Dr. Bruhl has shown in a five-year, carefully controlled study in the same environment with older people in an institution, with controls and experimental patients in the same institution, the behavior can be objectively and remarkably improved.

DR. KOCH: I think Dr. Guthrie's comment is valid. Behavior has been neglected because of the absence of the necessary psychological skills to measure it.

I did see a manuscript of Dr. Fisch in which he observed patients in a clinical research center and did some blind observations. Perhaps you may wish to comment on that.

DR. FISCH: I am impressed by some changes but we cannot measure them. Our psychologists have spent a year's period of time with the most delicate instruments, and can show very little difference of changes in behavior while we put the child on and take the child off the diet.

We have the impression there are changes, but this is really very hard to document. I agree with you it is very important, but I don't know how we can at this moment establish proof.

DR. WAISMAN: I think we also have to face up to the fact that the people we are treating are not all alike. These are biological phenomena. Individuals differ so much, perhaps impressed by prenatal influences.

Now, I would like to discuss some data and you may make any interpretation you wish. Figure 2 depicts that which many of us agree upon, that there is a drop in IQ if we delay the treatment. These treated PKU children are compared with their own normal siblings, and to their untreated PKU siblings. Now, further studies (Figure 3, Berman, P. W.; Waisman, H. A.; and Graham, F. K.: Child Development, accepted for publication) supports a concept which we talked about before, namely, that maybe we are preventing degeneration, so that the earlier we treat, the less fall there is in IQ. If we look at the chart we see that there is some increase in IQ between the first and the last test but it is not very impressive in the treated children.

---

**Figure 2**

<table>
<thead>
<tr>
<th>INTELLIGENCE TEST SCORE</th>
<th>NORMAL</th>
<th>TREATED</th>
<th>UNTREATED</th>
</tr>
</thead>
<tbody>
<tr>
<td>120</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>80</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>60</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>40</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>AGE AT WHICH TREATMENT WAS BEGUN</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 MO.</td>
</tr>
<tr>
<td>10</td>
</tr>
</tbody>
</table>

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Curiously for unknown reasons, and I must say that these are averages, some will not respond as well, and some will respond like Dr. Koch's patient, whose IQ increased from 48 to 93. Not all will respond. If we look at his data you will see that some of the individuals simply didn't do well.

The next chart (figure 4) does not represent a typical case. This is my best case. I think it demonstrates that on a low phenylalanine diet a child may attain good height and good weight. Now, the phenylalanine levels were maintained within reason. There were occasional times when it went up to 12 mg percent. But, by and large, it was kept below 8 mg percent, and we are beginning to agree that this is a safe level.

Now, what does this little girl's IQ show? I think we must be clear that it is better to chart the developmental rate* rather than IQ by itself. I have evidence that the mental age increases at a slow rate before treatment, which is due to the effect of environment and everything else, and then, when a diet is begun mental age increases abruptly. Then, when the child is taken off the diet or poorly controlled, the rate of gain levels off again.

Now, the next chart (figure 5) illustrates the variation in response to treatment. K.M. is the same girl who is the best case. L.L. is her cousin who was diagnosed at one year of age, and her IQ stayed about the same all the way through. She is educable and I want to say this is important, a fact no one has stressed. If a child can be changed from severely retarded to trainable or educable, then we have accomplished something. Don't you think for a moment that this isn't important. It improves all the problems that we are talking about. The improvement in walking, for example, is very important. The child is easier to care for, is easier to feed, and the behavioral problems are all improved. Those are important gains.

Then we have also shown a typical case or better yet, average case. This girl, J.D., was

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*This is based on mental age gain, and if converted to a per month rate of gain, it is independent of the length of time the child was studied. It is also an age-independent measure (Berman, Waisman, and Graham, loc. cit.)
very well-controlled, maybe over-controlled. I don't know. But she simply grew at a minimum rate. Her IQ fell. Her rate of gain was very slow for reasons that we don't know, maybe it was prenatal damage. Her control was reasonably good, except there were a number of low blood levels.

So what is the conclusion? I have pointed out that there are individual variations. I have pointed out that some patients do very well and some do not and that averages perhaps mean little, and that whatever gain we get, at any age, is significant, and I don't think we should underestimate this.

DR. KOCH: I feel that an intimate experience with these families and children biases me to the conclusion that the diet is beneficial. I recognize this, but all we can do is present the data we have.

DR. UDENFRIEND: I think that the impression that Dr. Bessman made that there is complete confusion about the possible etiology of PKU is really an over exaggeration. I think that while there can be factors other than phenylalanine involved in producing the behavioral changes one sees in phenylketonuria, one has to remember that if one draws a metabolic map showing where phenylalanine can interact there are many sites. It can get involved in protein synthesis. We all know of the current studies relating macromolecular biochemistry and memory. By producing an amino acid imbalance either by over-treatment or under-treatment, one certainly could interfere with protein synthesis and therefore change behavior. If one examines other portions of the metabolic map one finds interaction with humoral agents such as noradrenalin. If one looks at phenylalanine as an inhibitor of noradrenalin biosynthesis, we can compare its action with those of another well-known inhibitor, \( \alpha \)-methyltyrosine. Such compounds can influence mood and general behavior. If one gives \( \alpha \)-methyl-tyrosine to patients—and this has been done in about 30 or 40 patients—they show the following symptoms:

The patients seem to be partially sedated, and yet, peculiarly, they are somewhat agitated. Dr. Sjoerdsma has published some of this data. The most interesting thing about them is that when one discontinues \( \alpha \)-methyl-tyrosine after long treatment, the patients all undergo a withdrawal type of effect where two things are observed. The patients all say they feel remarkably better, yet they undergo a short period of sleeplessness. This is not disturbed sleeplessness. They say they are feeling much better but just don't need to sleep. \( \alpha \)-methyl-tyrosine may produce effects resembling one aspect of the symptomology of phenylketonuria, if we want to look at it that way. We may be able to divide up the effects on thought and memory, from those on mood and general behavior.

This goes back to the criticism of so called experimental phenylketonuria in animals. It may well be that the use of phenylalanine to produce hyperphenylalaninemia in an animal that doesn't have the phenylalanine hydroxylase block is not a true model.

We may have to divide up the symptomology of PKU and explore them each individually. One might study the effects of an inhibitor of protein synthesis or an amino acid analog on memory, perhaps in monkeys. Statistically, goldfish are better subjects for behavioral studies, as was shown by Agranoff. Another group might work on

Figure 5

COMPARISON OF 3 TREATED PHENYLKETONURIC CHILDREN

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the humoral aspects of PKU and get some interesting data of another sort. We know that marked changes in behavior can be produced by withdrawing or adding an inhibitor of such important humoral agents as noradrenalin or adrenalin.

DR. KOCH: There is some suggestion that some of the IQ rise that we see actually is related to a better ability of the patient to relate to the test material and to mature and interact with his environment so that the beneficial effect actually of the low phenylalanine diet may not be in improving the IQ per se but rather in improving the environmental milieu so that the patient can learn as he grows older.

DR. ANDERSON: I wanted to ask about your homozygous mother who gave birth to the child with the high serum phenylalanine. Dr. Fisch has a report now in press in Pediatrics concerning a homozygous mother who gave birth to three children. One died, one proved to be phenylketonuric. The other was not phenylketonuric and according to the 'tolerance test' is a heterozygote. Both children according to gestational age (using Lubchenko's published reference chart) were small in weight and length for the gestational age. Both had microcephaly at birth. The phenylketonuric now 3-1/2 years old continues to have slow growth perhaps predetermined by the gestational development limitation. The 5 year old, a non-phenylketonuric child, also has slow growth, about 6 or 7 standard deviations below the mean of the height and weight curves for normal children. A review of the literature revealed mention of birth weight in only one report where it was stated that the child was small. No other mention of birth weight is present in the reports concerning offspring of about 13 homozygous mothers.

DR. KOCH: This mother has three children who are heterozygotes. She is married to a schizophrenic father. All three children are retarded. Their IQs are between 25 and 75. The children are very slightly microcephalic in comparison to normal standards.

DR. BERRY: I want to remark on Dr. Waisman's data which showed IQs of treated infants, unaffected siblings, and older treated phenylketonuric siblings of the same group. While he didn't emphasize it, the older siblings, I presume, of these younger infants were the ones in the lower right-hand corner with low IQ.

DR. KOCH and DR. BICKEL must have in their groups young infants born into families already having an older phenylketonuric child. What are the differences in the same family between the older treated and the younger treated children?

DR. KOCH: That's a good point. I must admit I haven't studied this. I would say they are more intelligent, but I don't have the specific data.

DR. BERRY: I think this is the kind of data we need to decide whether or not treatment is going to be effective at a given age.

DR. BICKEL: We have quite a few of those cases. Actually, the first patient which we diagnosed in early infancy was out of a family where another child had been severely affected without being treated. This infant yielded one of our best results. Another little boy with an IQ of now 104 started on the diet when he was 2 months of age. I can't see any defect in him whatever now. He has two siblings who are in mental institutions with very low IQs.

DR. BERRY: Isn't this the kind of information we need to gather and pool to determine whether or not treatment has any effect on IQ?

DR. BICKEL: We were repeatedly struck by the fact that in families where the children were not yet treated you can have one child with a near-normal IQ and others severely defective in institutions.

I think there is a great variety of brain involvement in patients of the same family, which makes me wonder if this range of intelligence is really genetically determined.

DR. MCPHERSON: In Dr. Koch's tables showing the excellent results of the excellent management I discerned a possible statistical flaw that might answer Dr. Udenfriend's skeptical question about what babies' IQs are at birth and how you measure them.

It appears that there are a number of children here diagnosed quite early, and for these babies Dr. Koch has assigned a value of 100, obviously not measured.

These are included in the average value of IQ at time of diagnosis which was 75 for the group. There is an average rise of 22.8 points for the whole group. But many of these children are listed as having very significant rises. One
had 15, another 7, another 13, another -4. Another one shows a gain of 50 points over an initial assigned value of 100.

DR. KOCH: Yes.

DR. MCPHERSON: The child ended up a near-genius.

DR. KOCH: Yes, a bright child.

DR. MCPHERSON: The very bright rather skew the figures for the group. I wonder if we shouldn't assume by retrospection that this child had 150 IQ at birth and we didn't actually improve him by treatment with the low phenylalanine diet but merely maintained his brain in an undeteriorated state.

DR. KOCH: That's an excellent point, Dr. McPherson. I appreciate your pointing that out.

The assignment of 100 DQ to the week old or one month old is an arbitrary decision.

DR. FULLER: In answer to Dr. Berry, we had 18 sib pairs or triads with one of each group beginning the diet earlier, since the diagnosis was usually made on the basis of an older sib.

Of the 20 younger siblings, 12 go in the expected direction. In other words, the one diagnosed earlier is doing better. But 8 go in the other direction, that is the child diagnosed later is performing better than the one diagnosed earlier.

DR. KOCH: Were the 8 that went in the other direction also on the diet?

DR. FULLER: Yes. They were all on the diet.

DR. BRUHL: I would like just to remark about the behavior improvement we see in long-range studies in our institution. We have published a control study on a dozen or more PKU patients. We have seen in time after lowering the phenylalanine blood level a definite change in behavior. The patients are less irritable. They are easier to manage. Very interestingly, we saw the most dramatic changes within the first 3 months. Afterward those behavioral changes leveled off. We had the definite impression that there was a sort of detoxification.

We have in our institution two daughters of two PKU mothers. They are both heterozygous according to phenylalanine loading tests. They are mentally retarded. They are illegitimate children; in all probability the father was normal. They are heterozygous without doubt. They are definitely not PKU, but one is microcephalic.

DR. BICKEL: I have always been impressed with two actions on the phenylketonuric brain. The first is an immediate toxic action which you can introduce by giving phenylalanine and which may quickly subside by introducing a low phenylalanine regimen.

The other action is the long-standing damage which is much more difficult to remove, if at all. It may be important to determine the biochemical basis of these two different actions.
PHENYLKETONURIA: PSYCHOLOGICAL AND DEVELOPMENTAL EVALUATION

RENEE FULLER

During the past six years, 112 phenylketonuric outpatients were given periodic psychological examinations to ascertain (a) developmental changes in phenylketonuria, and (b) to quantify the long-term effects of a diet of restricted phenylalanine intake.

Subjects and Procedure

Participants in this New York State study on phenylketonuria were asked to come to the clinic for psychological testing at the following intervals depending on the age of the child.

Prior to 1 year of age, every 6 weeks,
from 1-2 years of age, every 2 months,
from 2-3 years of age, every 3 months,
and so on, up to the age of 6 years. After the age of 6 the testing was once a year. The frequent examinations during the early years was to observe the more rapid development of that period. The age range of the study was from zero to 12 years, the range of dietary initiation from a few days old to 5 1/2 years.

Two types of tests were given. These were the Gesell and the Stanford-Binet, form L. In Gesell testing, separate scores were maintained for the four Gesell categories. Binet testing was done as soon as both adaptive and language categories of the Gesell Developmental Schedule were above 24 months. As soon as the child was ready for Binet testing an attempt was made to give the test every six months until the child reached a mental age of 5. Thereafter Binet testing was restricted to approximately once every year.

Attempts were made to monitor the children biochemically every six weeks. Blood phenylalanine levels were determined by the LaDu method and more recently by the McCaman-Robins fluorimetric method. If the parents were not able to come to the clinic, they were asked to send the blood.

For 97 of the children we came fairly close to the above schedule for psychological testing, at least while they were under our care. Usually it was easier to get cooperation for the less frequent psychological examinations than for the more frequent blood analyses.

Two comparison groups were run in order to gain a wider scope of information. These were: (a) a comparison group of outpatients of the same age range as the phenylketonurics who also came to the clinic, but for reasons other than phenylketonuria, N = 80, and (b) normal siblings of the phenylketonurics, N = 75.

The diseased comparison group was run in order to set off test patterns peculiar to phenylketonuria as exhibited on the Gesell. Children with sensory defects or obvious motor impairment were excluded from the comparison group, since their selective impairment would as a matter of course differentiate them from the phenylketonurics. In addition the number of Negroes and Jews was limited to one each to approximate the phenylketonuria sample. Actual matching was not possible because the population from which the comparison group was drawn was not large enough to permit this. But the two groups had the same age range and had the similarity of having sought help at the same clinic.

The second comparison group, the normal sibs, were Binet tested in order to establish a baseline of maximum IQ expectation as a result of dietary treatment. Since IQ correlations between sibs are usually high, an average or below average IQ from a normal sib would presumably place a lower ceiling on results from dietary treatment than if the IQ of the normal sib was superior.

Results

Separate averages were taken for the four Gesell categories: motor, adaptive, language, and personal-social. In tables 1 and 2 the results of each category are ranked as to: (a) the most affected category, (b) the second most affected
category, (c) the second least affected category, and (d) the least affected category.

In table 1, the 102 phenylketonurics on the diet who were old enough to be ranked in the four categories are shown. The same thing is done for the diseased comparison group in table 2. As can be seen on table 1, neither motor or personal-social development is ever the most affected category in the phenylketonurics. On the other hand for 18 of the comparison group, table 2, motor was the most affected category, and for 5 cases personal-social was the most affected. Diagnosis does not appear to be a factor in the distribution of the comparison group, except in cases for which personal-social was the most affected category. This last was associated with possible schizophrenia. At the other end of the categorization, there were no cases among the phenylketonurics for whom language was the least affected category, although for 16 of the comparison group it was.

The same distribution held for the phenylketonurics prior to dietary treatment. The pre-diet sample is limited, however, to 26, since many of the cases were started prior to 1 year of age.

Table 2. The four categories of development on the Gesell scale are shown in a four-way classification for the diseased comparison group.

<table>
<thead>
<tr>
<th>Physical</th>
<th>Mental</th>
<th>Motor</th>
<th>Language</th>
</tr>
</thead>
<tbody>
<tr>
<td>Most Affected</td>
<td>2nd Most Affected</td>
<td>2nd Least Affected</td>
<td>Least Affected</td>
</tr>
<tr>
<td>Motor</td>
<td>18</td>
<td>18</td>
<td>20</td>
</tr>
<tr>
<td>Personal-Social</td>
<td>5</td>
<td>20</td>
<td>25</td>
</tr>
<tr>
<td>Adaptive</td>
<td>29</td>
<td>26</td>
<td>15</td>
</tr>
<tr>
<td>Language</td>
<td>28</td>
<td>16</td>
<td>20</td>
</tr>
</tbody>
</table>

and some of the patients had been placed on the diet before admission to our outpatient clinic. The same distribution held for the earliest group placed on the diet, prior to 2 months of age, N = 19. Phrased more simply, this same distribution held for all the multiple Gesells given to all the phenylketonurics during this study. Because this finding held for all chronological and mental age levels, actual matching for age between phenylketonurics and their comparison group was not necessary.

Among the phenylketonurics then, the language and adaptive categories were invariably more affected than motor and personal-social. The extent of this difference for language motor and personal-social between the phenylketonurics and their comparison group can be seen in figures 1 through 4.

In figures 1 and 2, language age is plotted against motor age irrespective of chronological age. If language were at the same stage as motor behavior, the plot would be on the diagonal. When motor behavior is superior to language, the plot is below the diagonal, whereas when language is superior to motor it is above.

In figure 1 the scores for all the phenylketonurics are below the diagonal, in many cases
Figure 1. Motor scores for the phenylketonurics are plotted against language scores.

The diagonal represents a hypothetical line of equal development for the two Gesell categories.

considerably so, showing the extent of the superiority of motor over language development. For the comparison group appearing in figure 2, however, almost as many cases appear above the diagonal as below. For the comparison group then there is no pattern, while for the phenylketonurics there is.

The same is done for personal-social development in figures 3 and 4 as was done for motor on the preceding two figures. Figure 3 shows the extent of superiority of personal-social over language in the phenylketonuric. The comparison group does not show this pattern. As can be seen in figure 4, there
Figure 2. Motor scores for the diseased comparison group are plotted against language scores.

The diagonal represents a hypothetical line of equal development for the two Gesell categories.

are cases with marked superiority of language over personal-social among the comparison group—although the frequency is less than for motor development. Further detail concerning the Gesell findings and also the comparison group were covered in a previous paper.\(^{(1)}\)

Because of the selectivity of the impairment in the phenylketonuric, no Developmental Quotients (DQs) were used. Doing so would have required the averaging of the frequently widely disparate categories of motor and personal-social on the one hand and language and adaptive on the
Figure 3. Personal-social scores for the phenylketonurics are plotted against language scores.

The diagonal represents a hypothetical line of equal development for the two Gesell categories.

other. The resultant average would not have been descriptive of the child.

To make comparisons with later Binet results, language and adaptive scores (these two categories having similar results) were averaged. By dividing chronological age into this average, a pre-Binet quotient was obtained for those children with too low a mental age for the latter test. This pre-Binet quotient was found to be surprisingly consistent with later Binet scores, and avoided the over-rating of the pre-Binet child which occurs when early motor and personal-social development are included in the intelligence ratings of phenylketonurics.
Figure 4. Personal-social scores for the diseased comparison group are plotted against language scores.

The diagonal represents a hypothetical line of equal development for the two Gesell categories.
In figures 5 through 11, this language-adaptive/CA quotient is used prior to the mental age of 2 and the Binet IQ thereafter. The Gesell quotient is represented by a filled circle, the Binet by a cross. The plots are joined by different types of lines representing various phenylalanine averages between test periods. Phenylalanine averages below 2 mg/100 ml are indicated by alternate horizontal and vertical dashes; levels between 2 and 5 mg/100 ml by a thin unbroken line (and arbitrarily called good dietary control); levels from 5 to 9 mg/100 ml by a broken line (equally arbitrarily called fair dietary control) and above 9 mg/100 ml by a cross-hatched line (called poor dietary control). Off diet altogether appears as a thick black line.

Figure 5 shows those children started on the diet prior to 2 months of age on whom there is extensive test data. Contrary to some theoretical speculation, not all of this group are performing in the normal or dull normal range. Of the 19 children started on the diet at this early age (not all shown on graph), 8 are performing below a quotient of 70. Yet of these, as can be seen in figure 5, several were maintained on good dietary control. At the same time there are some children performing in the normal or dull normal range who were under poor dietary control. It should be pointed out that TEST RESULTS OF THE FIRST FEW MONTHS OF LIFE DO NOT HAVE THE RELIABILITY OR VALIDITY OF LATER EXAMINATIONS. Furthermore, early language and adaptive evaluations do not contain the numerous test items that exist for the older child.

Figure 6 includes 7 children placed on the diet from 2 to 6 months who have been followed for an extensive period of time. Of the 9 belonging to this age group, 5 are performing below a quotient of 70. Yet of these, as can be seen in figure 5, several were maintained on good dietary control. At the same time there are some children performing in the normal or dull normal range who were under poor dietary control. It should be pointed out that TEST RESULTS OF THE FIRST FEW MONTHS OF LIFE DO NOT HAVE THE RELIABILITY OR VALIDITY OF LATER EXAMINATIONS. Furthermore, early language and adaptive evaluations do not contain the numerous test items that exist for the older child.

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Figure 6 includes 7 children placed on the diet from 2 to 6 months who have been followed for an extensive period of time. Of the 9 belonging to this age group, 5 are performing below a quotient of 70. Examples of dietary control below 2 mg/100 ml can be seen in the first three IQ figures, 5 through 7. Several of these children show a definite drop in quotients.

The rest of the IQ figures include all those children in a particular age range on whom there are three or more tests encompassing at least a year. Figure 7 shows 11 of the 16 phenylketonurics who were placed on Lofenalac® between 6 to 12 months of age. Of the 16, 11 are at present functioning at a quotient below 70.

In figure 8 are shown 13 of the 15 children who started the diet from 12 to 18 months. Of the 15 started at this age, 11 show a quotient below 70. It appears from this data that with progressively later onset of dietary treatment the groups show progressively lower quotients. Taken as a whole the graphs, 5 through 11, show increasing impairment. But taken singly, there are patients in the later graphs that perform better than those in the early graphs.

Eleven of the 13 children placed on the diet between 18 to 24 months are shown in figure 9. Of these, 11 have a quotient below 70. The next graph, figure 10, covers dietary initiation between 2 and 3 years. Of the 16 children placed on the diet during this period, 12 test with a quotient below 70.

Twenty-three children were placed on Lofenalac® after 3 years of age, 20 have a quotient below 70. Nine of the 19 shown in figure 11 are now off the diet. It should be pointed out that in the later graphs, the diagnosis was almost invariably made because of mental deficiency. In the earlier graphs, on the other hand, especially the one showing children started before 2 months of age, the diagnosis was either made as a result of mass screening or because of a known PKU sib.

Taken as a group, the older a child is when placed on the diet, the lower will be his IQ expectancy. Expectancy, however, is not the same as certainty. Children started on the diet later sometimes perform better than those started earlier. This becomes very apparent in figure 12.

In figure 12 are shown 18 of the 19 phenylketonuric sibships in this study. Sib pairs are plotted according to age at the start of dietary treatment on the abscissa and the latest Binet or Gesell quotients on the ordinate. Sib pairs are connected by a dotted line.

Of the 21 possible relationships between the sibs, 12 were in the expected direction, i.e. progressive impairment with later onset of dietary treatment. But in 8 cases the converse was true — that is, the sib who was placed on the diet earlier in life performed less well than the one placed later. Because of the same home environment, degree of dietary control was similar for the sib pairs.

The four arbitrary divisions of dietary control used previously appear on table 3, as well as the additional one of off diet. The percentage of those showing an increase in rate of development

Provided by the Maternal and Child Health Library, Georgetown University
Figure 5. Phenylketonurics started on the diet prior to 2 months of age.

The Gesell language-adaptive CA quotient is represented by a filled circle, the Binet by a cross. Phenylalanine averages below 2 mg/100 ml are indicated by alternate horizontal and vertical dashes, levels between 2 and 5 mg/100 ml by a broken line, above 9 mg/100 ml by a cross-hatched line, off diet is a thick black line.

Provided by the Maternal and Child Health Library, Georgetown University
Figure 6. Phenylketonurics started on the diet between 2 to 6 months of age.

The Gesell language-adaptive/CA quotient is represented by a filled circle, the Binet by a cross. Phenylalanine averages below 2 mg/100 ml are indicated by alternate horizontal and vertical dashes, levels between 2 and 5 mg/100 ml by a thin unbroken line, from 5 to 9 mg/100 ml by a broken line, above 9 mg/100 ml by a cross-hatched line. Off the diet is a thick black line.

Provided by the Maternal and Child Health Library, Georgetown University
Figure 7. Phenylketonurics started on the diet between 6 to 12 months of age.

The Gesell language-adaptive/CA quotient is represented by a filled circle, the Binet by a cross. Phenylalanine averages below 2 mg/100 ml are indicated by alternate horizontal and vertical dashes, levels between 2 and 5 mg/100 ml by a thin unbroken line, from 5 to 9 mg/100 ml by a broken line, above 9 mg/100 ml by a cross-hatched line, off the diet is a thick black line.
Figure 8. Phenylketonurics started on the diet between 12 to 18 months.

The Gesell language-adaptive/CA quotient is represented by a filled circle, the Binet by a cross. Phenylalanine averages below 2 mg/100 ml are indicated by alternate horizontal and vertical dashes, levels between 2 and 5 mg/100 ml by a thin unbroken line, from 5 to 9 mg/100 ml by a broken line, above 9 mg/100 ml by a cross-hatched line, off the diet is a thick black line.
Figure 9. Phenylketonurics started on the diet between 18 and 24 months of age.

The Gesell language-adaptive/CA quotient is represented by a filled circle, the Binet by a cross. Phenylalanine averages below 2 mg/100 ml are indicated by alternate horizontal and vertical dashes, levels between 2 and 5 mg/100 ml by a thin unbroken line, from 5 to 9 mg/100 ml by a broken line, above 9 mg/100 ml by a cross-hatched line, off the diet is a thick black line.

Provided by the Maternal and Child Health Library, Georgetown University
Figure 10. Phenylketonurics started on the diet between 2 and 3 years of age.

The Gesell language-Adaptive/CA quotient is represented by a filled circle, the Binet by a cross. Phenylalanine averages below 2 mg/100 ml are indicated by alternate horizontal and vertical dashes, levels between 2 and 5 mg/100 ml by a thin unbroken line, from 5 to 9 mg/100 ml by a broken line, above 9 mg/100 ml by a cross-hatched line, off the diet is a thick black line.
Figure 11. Phenylketonurics started on the diet after 3 years of age.

The Gesell language-adoptive/CA quotient is represented by a filled circle, the Binet by a cross. Phenylalanine averages below 2 mg/100 ml are indicated by alternate horizontal and vertical dashes, levels between 2 and 5 mg/100 ml by a broken line, above 9 mg/100 by a cross-hatched line, off diet is a thick black line.
Figure 12. Phenylketonuric sibs plotted according to age of dietary initiation on the abscissa and most recent Binet or Gesell quotient on the ordinate.

The sib pairs are joined by dotted lines.
for each of the four Gesell categories is shown as a function of dietary control. There appears to be little or no difference between the 2 to 5 mg/100 ml and the 5 to 9 mg/100 ml group. These two groups were therefore combined for statistical purposes and compared with the other three types of dietary control.

Comparing the 2 to 9 mg/100 ml groups with those below 2 mg/100 ml produced a $X^2$ which was significant beyond the .001 level. The comparison of the 2 to 9 mg/100 ml and the above 9 mg/100 ml levels was significant at the .01 level, and the comparison between 2 to 9 mg/100 ml and the off diet group at the .001 level. When a two-way classification is made of Binet scores, the results are similar, except there are not enough cases to form the below 2 mg/100 ml group. The significance is not altered if we let $N =$ the number of children in a particular dietary classification instead of the number of tests. The average increase or decrease in the developmental categories per type of dietary control is taken for each child. $N$ is thereby reduced, but the difference between types of dietary control increased and the level of significance remains the same.

A two-way classification of all the children taken off the diet appears on table 4. Altogether there are 29 children on whom there are post diet results. Again the most recent Binet is used when this is available. In those cases where the development is below the two year basal of the Binet the Gesell quotient is used instead. The most recent test is compared to the last test prior to discontinuation of the diet. Because of the limited sample, neither the extent of increase or decrease in quotients nor age of discontinuation could be analyzed. Results for the off diet group on table 4 are more pronounced than they were on the previous table. The difference lies in the fact that table 3 shows results for all off diet Gesell tests, whereas table 4 only gives the most recent test results. If deterioration after discontinuation of dietary treatment takes place over time, it would be more evident on table 4 than on 3.

The last figure concerns the normal siblings. The dotted line in the background of figure 13 represents the empirical frequency distribution of Binet IQs. The regular black line represents the observed frequencies for the normal siblings. There are many more high IQs than one would expect by chance. A similar observation was made by Munro in 1947.\(^{(2)}\)

### Table 3. The percentage of Gesell subtests showing an increase in quotients when compared to the preceding examination.

<table>
<thead>
<tr>
<th>Type of diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>GOOD D</td>
</tr>
<tr>
<td>FAIR D</td>
</tr>
<tr>
<td>POOR D</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>% OF SUBTESTS SHOWING INCREASED QUOTIENT</th>
<th>PHENYLALANINE AVERAGE BETWEEN TEST SESSIONS</th>
</tr>
</thead>
<tbody>
<tr>
<td>motor</td>
<td>adaptive</td>
</tr>
<tr>
<td>53</td>
<td>51%</td>
</tr>
<tr>
<td>47</td>
<td>45%</td>
</tr>
<tr>
<td>49</td>
<td>46%</td>
</tr>
<tr>
<td>92</td>
<td>52%</td>
</tr>
<tr>
<td>22</td>
<td>27%</td>
</tr>
</tbody>
</table>

The dietary categories on the right indicates the phenylalanine averages between test periods.

### Table 4. Phenylketonurics taken off the low phenylalanine diet.

<table>
<thead>
<tr>
<th>RISE IN IQ or DROP IN IQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>LANGUAGE-ADAPTIVE/CA RATIO</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>GOOD D</th>
<th>FAIR D</th>
<th>POOR D</th>
</tr>
</thead>
<tbody>
<tr>
<td>GOOD D</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>FAIR D</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>POOR D</td>
<td>6</td>
<td>13</td>
</tr>
</tbody>
</table>

Type of dietary control prior to discontinuation is shown. Good dietary control represents phenylalanine levels from 2 to 5 mg/100 ml, fair dietary control levels from 5 to 9 mg/100 ml, poor dietary control levels above 9 mg/100 ml. The most recent test since discontinuation is compared with the last test prior to discontinuation. Whenever Binet scores are available these are used.
Figure 13. Frequency distribution of Binet IQs of the normal siblings of the phenylketonurics.

The dotted line in the background represents the expected distribution.
SUMMARY

1. There is a pattern of impairment among the phenylketonurics that becomes apparent when they are compared to a heterogeneous group of mental retardates.
2. Taking all the phenylketonurics as a group, the earlier they are placed on the low phenylalanine diet, the better is their test performance.
3. However, taken singly, some phenylketonurics started on good dietary control shortly after birth show marked retardation.
4. On the other hand, some phenylketonurics started on the diet after 3 years of age show great improvement to the point of little or no retardation.
5. Phenylalanine levels below 2 and above 9 mg/100 ml as well as off the diet are reflected by poorer test performance than phenylalanine levels ranging from 2 to 9 mg/100 ml (significance between the .01 and .001 level).

REFERENCES


DISCUSSION

DR. KENNEDY: I would like to ask if you determined phenylalanine blood levels of these children on the day they were tested. Perhaps there is a possibility that a high or low blood phenylalanine level on a particular day would influence the test. Do you have this data?

DR. FULLER: Yes, in almost all our cases, the blood levels were done on the same day as the psychological examination.

The question has been raised in this meeting whether parents prior to testing feed their children a more restricted diet. This may be possible in a few of our cases, I suspect it is unusual.

It is my clinical impression that on days when the children have high phenylalanine levels their performance is poorer. Certain of the subtests appeared to reflect motoric changes following high levels.

DR. BESSMAN: I have asked Dr. Grassi of The Children's Bureau to comment on Dr. Fuller's paper.

DR. GRASSI: First, I would like to compliment Dr. Fuller on an excellent study.

Usually, we criticize studies because they have no control groups; she had two control groups. I think this is very important because often we see studies without control groups. I think we have all seen examples of this type of study. Someone does a study by obtaining some scores before treatment and then some scores afterwards and makes a comparison without controls. Controls are particularly important when dealing with so-called IQs because an increase of 10 or 15 points may not be significant. The standard deviation involved may be 10 points or more; therefore, a 10 point increase may be completely insignificant.

I think the fact that Dr. Fuller found that under one month of age the reliability and validity are poor makes us stop to think because we have been putting IQ labels on children of 2, 3, 4, and 5 weeks of age. I would agree with her, too, that we need to look into the situation and consider what we are measuring.

I am very much concerned about this business of infant testing. I think psychology has very little to offer in this respect, because we (psychologists) have been so busy doing other things in other areas that we haven't paid too much attention to infant testing. Our infant testing procedures, our instruments, or whatever you want to call these things, leave something to be desired.

Unfortunately, we use the Cattell and the Gesell and the other tests of this type. Clinicians often call results of these tests intelligence, and then proceed to put IQ labels on children based on these results.

Someone said the other day, "We don't even know what this IQ is." And someone else challenged
the use of IQs. He said, "If you can give me something better to use, I will use that." Maybe we should look for something better because what is IQ anyway? I imagine there aren't many people here who could define what is an IQ. Many would say it deals with intelligence, and others would say it deals with achievement. Actually, IQ means the rate of mental development (RMD). It has little to do with intelligence, and only indicates the rate at which the individual is developing.

In other words, if a 4 year old has a mental age of 3, this means he is developing at a 75 percent rate of the development of a normal or average child at that age. I was very happy that Dr. Fuller kept referring to developmental quotient and not IQs.

The point I am making is that we need to look for new and different procedures for evaluation of these babies. I think maybe we might find the answers in the experimental laboratory.

As you know, we had some discussion on work with animals. I am not sure I completely agree with labeling animals as mentally retarded, but this is another point. Pavlov, for example, could tell which dogs were intelligent, which were not. He was able to tell which dogs had personality disturbances and which were fairly well adjusted. Maybe, then, we should begin by using some of these experimental techniques. In fact, I am very much interested in this possibility. After our discussion yesterday, I contacted some clinicians and found that there are some experimental psychologists who are performing developmental measurement experiments on babies using experimental procedures such as reaction time, conditioning, etc.

I am interested in gathering a group of experimental psychologists to start exploring their way of evaluating these children. I don't think any of us are satisfied with the way we are doing it now. We need to look into new and different procedures before we can establish reliable methods for valid assessments of infants.

DR. SOLOMONS: What percentage of these children were institutionalized?

DR. FULLER: They were all outpatients. Three have since been institutionalized.

DR. UDENFRIEND: Is all that variation of measured IQ also true of normal children or other abnormal children? In other words, is this the degree of fluctuation that occurs if children are tested who are not on a diet or are not phenylketonurics? Do comparable amounts of fluctuation occur in the measured IQ if a population of other types of mentally retarded children are tested?

DR. FULLER: In a normal population, unless a child is sick or something else is acutely wrong, we expect pretty good repeat reliability on the Binet. That's how the test was designed. Actually, that's what I found in some of the phenylketonurics when someone else, or myself gave them the alternate Stanford-Binet form within a short span of time. The scores were always very close. When a period of time elapsed the fluctuations appeared.

DR. UDENFRIEND: What about another form of mental retardation without treatment?

DR. FULLER: That may depend on the form of mental retardation. Usually we expect little fluctuation. These tests are pretty stable.

DR. UDENFRIEND: This isn't unusual for PKU, then, this fluctuation of the psychometric scores of an individual from month to month.

DR. FULLER: It may be. It might be also true of other particular forms of mental retardation. Perhaps some day we'll have enough data and can make a generalization on this point.

DR. FISCH: You have 112 phenylketonuric children and you tested them very often. I am wondering how many psychologists performed these tests?

DR. FULLER: One.

DR. LaDU: Do you intend to continue following this group as long as they don't go into institutions for a longer time?

DR. FULLER: I am afraid this is the end of the study.
Phenylketonuria is an inherited metabolic disorder which may lead to severe degrees of mental retardation. (1) Although the condition was described as early as 1934 by Folling, a Norwegian biochemist, no simple means of large scale screening or suitable treatment was discovered until recent years.

Just as with most of the disorders of the human body, phenylketonuria has degrees of severity. Varying degrees of brain damage and mental retardation result from this primary genetic defect and the gradation in severity may cause the actual diagnosis to be overlooked unless screening is done as a routine part of infant and child health supervision.

Interest in phenylketonuria coupled with vigorous research has brought simplified methods for the detection of the condition, as well as the actual treatment. A commercial laboratory has developed a simple method of testing urine with a test tape impregnated with ferric chloride. A less expensive method exists in which 10 percent ferric chloride is dropped onto the wet diaper of an infant or into a test tube of urine. In both of these procedures a definitive blue-green color change indicates a positive test. (2) The main drawback of the urine screening procedure is that it is not reliable before the infant reaches 4 to 6 weeks of age. Therefore, brain damage may have already occurred to some extent before treatment is begun.

In recent years, the bioassay screening procedure has been developed by Guthrie which semi-quantitatively determines the blood level of phenylalanine. (3, 4) This procedure can be performed on a drop of blood from the heel of the infant, and may be used with reliable results when an infant is as young as 2 to 3 days of age. Obviously, this procedure is highly desirable, if we believe in the premise that the earlier the treatment of phenylketonuria can be initiated, the greater the possibility that mental retardation will be prevented.

On the basis of our present inconclusive knowledge, if the so-called "true" phenylketonuria is found early in infancy and is treated properly, irreparable brain damage and mental retardation can either be prevented or the degree of severity lessened. (5)

Thus far, the only feasible means of treatment of phenylketonuria has been to remove excess phenylalanine from the diet. A commercial preparation is available which, when given along with selected foods, accomplishes this purpose. The diet still supplies the patient with the phenylalanine necessary for normal growth and tissue repair, as well as adequate protein. (6) Further interest in the development of suitable diets is evidenced by current work directed at developing special recipes which are designed to be more palatable, as well as inserting more variety into the diet for older phenylketonuric children.

The economic impact of failure to prevent the aftermath of untreated phenylketonuria is a very important factor. In most States the cost of maintaining a severely retarded child in an institution is between $2,500 and $3,000 a year. The cost of maintaining a child at home on the special low phenylalanine diet is less than one-fourth this cost.

The question has been raised frequently as to whether or not mass screening programs are truly justified, since phenylketonuria is such a rare condition. It is interesting to remember, however, that in the past ten years, the incidence of "true" phenylketonuria and/or hyperphenylalanemia has increased from an estimated 1 in 40,000 live births to an estimated 1 in 10,000 births at the present time. Perhaps, the incidence will prove to be higher.

With the simplicity and relatively low cost of mass screening programs and with the current evidence that treatment is effective in preventing serious damage, early casefinding must be recognized as the humanitarian approach and
basically a public health responsibility. Mass screening among newborn infants has already pointed up the variations in serum phenylalanine levels which heretofore were not recognized. Certainly, this recognition is quite important since it has precipitated more careful evaluation before treatment is begun and has lead to more careful follow-up.

Screening with the Guthrie test has now become a routine procedure in most of our hospitals but, because of the rarity of phenylketonuria the importance of the procedure is not often reinforced by actually finding a case. Therefore, a continuing process of motivating hospital personnel to carry out the procedure adequately and responsibly is required. Public health agencies can accomplish this through providing continuing education to hospital personnel through the use of films and actual demonstrations on the most effective time-saving methods. Periodic communications, such as a monthly newsletter on the overall problem, is an effective motivation device also.

Phenylketonuria lends itself to the philosophy and practice of public health perhaps more than any other condition, disorder, or disease. Certainly, the principles of preventive medicine can be applied fully through early casefinding, treatment, follow-up, health education, continuing laboratory services and genetic counseling. Public health agencies must go beyond simple casefinding provided through mass screening programs. To this end, participation in the preventive aspects of total case management is imperative.

In most instances, it is impossible for medical centers to offer the necessary follow-up services in the home of phenylketonuric patients, yet it is here that the effectiveness of dietary treatment faces the true acid test. Experience has shown that most mothers of young infants, in whom phenylketonuria has been diagnosed, are frightened and bewildered. In order to overcome these obstacles to proper management, continuing support is needed. This support is rarely provided through a monthly or even weekly visit to a clinic or hospital setting. Further, the occasional pediatrician in private practice who chooses to treat his own cases, can rarely give this continuing support which is needed to assure successful treatment.

Public health agencies must be accepted as an important part of a multidisciplined team approach. They can provide effectively the continuing support to the family, through broad services which are not limited to the indigent. The success of these services, however, are dependent upon an intensive education of professional public health personnel as to the nature of phenylketonuria and how they can give assistance to the physicians and medical centers, as well as to the parent in the home. In most instances the public health nurse, with proper orientation to the condition, can give guidance to the parents of phenylketonuric children. They can very effectively maintain a continuing liaison with the medical center and practicing physician, keeping them informed of specific problems, illnesses, family crises, or any other deterrents to effective treatment and individual case management. Public health agencies should be prepared also to provide nutritional consultation services through the medical center or physician managing the case. Such consultation may be in the form of direct service to the family of the patient and to the public health nurse who will make frequent visits to the home.

To go beyond mass screening of infants, further casefinding can be provided through family studies, and it is in this area that public health can make a major contribution. Again, it is difficult, if not impossible, for medical centers and private physicians to go beyond the immediate family of the patient so affected in further screening for phenylketonuria. Because of its genetic pattern, the high-risk group to contact and study not only includes the parents and siblings, but relatives as well. (7)

This point was demonstrated by a public health agency two years ago when it undertook a survey of families and near relatives of known cases of phenylketonuria in the State. Parents, siblings, first and second cousins and, in a number of instances, aunts and uncles were screened using the ferric chloride urine test. After screening 258 individuals in this high-risk population, 16 additional cases of phenylketonuria were confirmed. (8)

Six of these cases had never been diagnosed and all six individuals were severely mentally retarded. It was difficult to understand why a simple ferric chloride screening procedure had never been carried out on any of these children. In any case, the results of this survey were sufficient to convince one that screening of
relatives of known phenylketonuria cases should be an integral part of public health phenylketonuria detection programs.

The survey brought out another important aspect which must receive emphasis in program planning, and that is the need for health education. A questionnaire prepared to find out just how much the parents of the confirmed cases of phenylketonuria knew about the condition revealed that:

1. 61 percent did not know the disorder was inherited.
2. 58 percent did not understand the importance of early diagnosis.
3. 56 percent said that they had never discussed the condition with a professional source.
4. 56 percent did not know that the condition can be treated with a special diet.

Casefinding through screening procedures should be further expanded by physicians and public health agencies to include all patients with neurological difficulties, such as convulsive disorders. It has been stated that seizures occur in 25 percent of phenylketonuric patients. Hence, the diagnosis of epilepsy may lead one to overlook the possibility of phenylketonuria. Further, since 79 percent of phenylketonuria cases are said to have abnormal electroencephalograms, this finding should certainly lead one to rule out the condition. (9)

Finally, public health agencies should assume the responsibility for screening all mentally retarded children in the special classes of public and private schools. Such a practice would uncover other high-risk populations, many of whom are in the childbearing age and might benefit from genetic counseling, along with health education.

While this paper concerns itself with casefinding as it relates to phenylketonuria, it is not intended to overlook the possibilities of screening for other metabolic diseases. The public health application of broader screening procedures awaits only the proven results of research. The future of this entire field represents a true challenge and only through a close cooperative relationship with research centers, medical centers, and practicing physicians can public health contribute to its fullest potential. Its success will be dependent upon an open line of communication between all concerned. Without this we may expect a failure in fulfilling our obligation to humanity which is in reality the prevention of diseases whenever possible, but when not possible, the prevention of severe handicap conditions which are frequently the aftermath of the disease.

REFERENCES


DISCUSSION

DR. GUTHRIE: A year ago we began to screen the patients in hospitals for the mentally ill because of the well-known fact that a large fraction of untreated phenylketonurics are...
behavior problems. The children are not rarely diagnosed as childhood schizophrenics before they are found to be phenylketonurics.

Five phenylketonurics were found in the first 10,000 mentally ill patients in Buffalo State Hospital, Gowanda State Hospital, and Binghamton State Hospital. At least one patient was not retarded but really a public nuisance. He is in his 50's.

Dr. Denniston in Pennsylvania studied one of this man's three sisters who was phenylketonuric and who was clinically normal. She had one retarded child and one child who died ("maternal PKU").

Another one of the five individuals is a woman in the Binghamton State Hospital who was reported to me in writing by the staff as having graduated from high school and a few years after having graduated from high school was admitted to the Binghamton State Hospital as a psychotic individual and has been there ever since. She is now in her 50's, too.

The other three individuals were grossly mentally retarded and could well have been found in an institution for the mentally retarded, but were placed for reasons of convenience in institutions for the mentally ill.

In the next 10,000 tests, we only found one case and it is not confirmed yet. So that would dilute the figures down to 5 in 20,000 or about 1 in 4,000, which is not much higher in frequency in casefinding than if mentally retarded cases with an IQ from 70 to 75 are screened.

DR. UDENFRIEND: Have any of these PKU children ever been treated in an institution, or are any of them being treated in institutions? How does the dietary therapy in an institution compare to the same type of low phenylalanine diet in the home?

DR. JERVIS: I would like to point out that in some States, the percentage of retarded children in State psychiatric hospitals is pretty high because patients are admitted with the diagnosis of psychosis with mental deficiency. The psychosis may improve but because of mental deficiency they remain in the hospital. It is not surprising therefore that some PKU patients will be found in psychiatric hospitals. Incidentally, the Guthrie test is the ideal test for State institutions because one can collect the spots rapidly in a large number of patients.

As far as treatment is concerned, the State institutions for retarded children are not suited for experimental purposes because with few exceptions children below one year of age are not admitted. In our institution we treat the institutionalized PKU children below the age of 5. There are few such cases and unfortunately they are already quite defective on admission.

DR. UDENFRIEND: What about some of the training schools that get younger children?

DR. BRUHL: We have had 17 patients on a low phenylalanine diet over the past five years. We began in January 1958 on patients, and we had paired samples controls. The older patients, ages 15 to 40, have only been treated for about five years. We are continuing to treat the younger ones.

Among that group are several which I took over from Dr. Fisch because there were difficulties in the families. We institutionalized them and continued to keep them on the low phenylalanine diet.

Dr. Jervis already pointed out that the majority of cases we see have permanent brain damage already. Consequently, any improvement we see is not in the sphere of intelligence.

For instance, two boys were placed on the diet when they were 7 and 9 years old. They were not able to walk, were only able to crawl. They are walking now. We had cared for them over several years and they just did not walk. They did not do anything. After dietary treatment they became more alert, they started out to walk around the bed. Within a few months, they were walking freely. Eczema disappeared, and rigidity disappeared. In the older ones improvement in intelligence doesn't occur.

Treatment in a State institution lends itself to longitudinal studies in many areas including growth.

DR. SOLOMONS: At the AAMD meeting in Winnipeg last year, Dr. Frank Menolascino of the Nebraska Psychiatric Institute reported on a case in a project yet unpublished. This was in an institution for the retarded.

They took several PKU children who were behavior problems, and they put them on the diet, and there was no doubt about it that the behavior improved.

However, unbeknown to the staff, they put the children back on a regular diet that looked exactly the same. And the behavior continued to improve.
DR. ASHLEY: I would like to ask Dr. White in view of several of the speakers mentioning the importance of hospitalizing newly found cases of phenylketonuria whether he also does this.

We have felt that we can get our confirmatory tests, get the mothers involved in the diet at an early stage of the diagnosis and that we were better off to have the diet managed at home. We have then used the Guthrie test for monitoring.

DR. WHITE: Of course, you understand that the only infants that we have much to say about are those who qualify for State assistance in terms of treatment and hospitalization.

Certainly, we have recommended hospitalization for the initial regulation of the diet and confirmation of the diagnosis. Our State laboratory at the moment is not in a position to do the confirmatory test. So, we do recommend hospitalization. We also suggest readmission intermittently for phenylalanine loading to be sure that the diagnosis is correct.
The public health screening program in Massachusetts for the detection of phenylketonuria and other inborn errors of metabolism is based on the bacterial inhibition techniques developed by Dr. Robert Guthrie, and the paper chromatography technique devised by Dr. Mary Efron. It is a joint project of the diagnostic Laboratories and the Division of Maternal and Child Health working closely with the Children's Hospital in Boston, and aided by support from the Children's Bureau. Since Dr. Efron's presentation covered the paper chromatography part of the screening program, this paper is confined to our experiences with the bacterial inhibition screening methods for detecting phenylketonuria and two other related metabolic disorders.

First, we find that the bacterial inhibition screening test for phenylketonuria is economically and effectively performed on heel blood samples collected from newborn babies using the technique developed by Guthrie.\(^1,2\) We request daily mailing from the hospitals of the filter paper blood spots, which should be taken shortly before the baby's actual discharge. Punched out discs are planted for overnight incubation on Demain's medium, which has been previously seeded with B subtilis spores, and into which has been incorporated B-2-thienylalanine—an inhibitor to B subtilis, but which is itself counteracted by phenylalanine.

Figure 1 shows a disc from a phenylketonuric baby in the horizontal row just below center right. The increased phenylalanine has diffused out into the medium, counteracting the otherwise inhibiting B-2-thienylalanine, resulting in the large halo of B subtilis growth. By comparing this halo with the growths surrounding the discs in the control row just above, the level is seen to read close to 50 mg percent. Such tests are, of course, followed routinely by paper chromatography, and also by ion exchange chromatography, and chemical tests performed on repeat specimens to confirm the diagnosis before treatment is begun. The tyrosine level is also carefully checked, a ferric chloride or Phenistix\(^\circledR\) done on the urine and the patient's clinical picture appraised.

Some inadequate specimens will be noted on this plate. These are reported as unsatisfactory, and repeats requested, but we run them anyway because they can give important tentative information. Note also the small halos of growth around each of the non-elevated bloods. These represent normal phenylalanine levels, and should be present in a properly adjusted test plate. They are useful as points of reference for elevations above the normal, and, if absent in a monitor blood of a phenylketonuric baby under treatment, as a signal of alarm that the phenylalanine level is dangerously low.

While we have on rare occasions been alerted by early intermediate elevations, it is possible also that a phenylketonuric baby might go home at too early an age to reveal any significant phenylalanine elevation. A repeat filter paper blood specimen, which we find more reliable than a filter paper urine specimen, is therefore recommended as a routine at the baby's 4- to 6-weeks' visit to his doctor or clinic. One newborn with an initial 4-6 mg percent heel blood reading was detected on the 4-weeks' follow-up blood spot by a reading greater than 20 mg percent. Because of this event, the cutoff point in our laboratory and generally over the country was reduced from 6 to 4 mg percent. Interestingly, this baby appears now to be one of the atypical phenylalaninemas at present requiring less than the usual dietary restriction. As a matter of policy we ask for an immediate repeat specimen if the level is even close to 4 mg percent. Among several States phenylketonuric babies missed on the initial blood have been detected because of the 4- to 6-weeks' follow-up specimen.
The screening program for phenylketonuria in Massachusetts was begun in July, 1962, and has enjoyed virtually 100 percent coverage of newborns since the spring of 1963, with 80 percent to 90 percent response on the recommended follow-up specimens. At the end of the first week in April, 1966, about 365,000 newborns have been tested with the detection of 36 cases with persisting blood levels about 20 mg percent. In the discussion immediately following this paper, Dr. Joseph Kennedy of the Children's Hospital in Boston, will add a few cases not quite meeting these criteria. Twenty-nine of the positive cases ranging in age from 1 month to 3½ years are now under treatment by Dr. Kennedy at the Children's Hospital. The median of the IQs done is close to 100 with a low of 80 and a high of 138.\(^3\) As we have previously reported, two early cases of the total series moved out of State to be treated elsewhere, and died. The incomplete information we have strongly suggests, now, that the death of one of these may have been associated with too low a phenylalanine dietary level.

Intermediate elevations, i.e., levels from 4 mg percent to but not including 20 mg percent do on occasion occur—in our series about once in 800 in newborns. In our opinions these should not be called "presumptive positives," but they do indicate the need each for a repeat specimen. Frequently, they represent transient elevations due to immaturity in the production of the phenylalanine hydroxylase enzyme; they are fairly common in prematures. Such elevations, are, however, also seen in true phenylketonurics, who have not yet been ingesting protein long enough to produce a significant elevation, but will on the repeat specimen. In our experience the occasional intermediates have not been troublesome.
in the screening program, but they do require further study and judgment in interpretation.

While it is not the purpose of this paper to discuss the therapy of phenylketonuria, since our laboratory only looks over the fence at the treatment clinic, we should nevertheless like to share some observations that have emerged out of the series as a whole. One of our early cases, a 6-weeks' premature baby with an initial Guthrie assay level of greater than 20 mg percent of blood phenylalanine, who was subsequently confirmed beyond doubt as a phenylketonuric, including positive ferric chloride tests, exhibited nevertheless an unusually high requirement for phenylalanine. Indeed, his blood level under treatment at one time became dangerously low, and he developed a bone-marrow vacuolization requiring a transfusion of packed red cells. Later, because of a persisting low phenylalanine serum level in spite of a considerably increased dietary phenylalanine, he was put on 100 percent whole milk feedings, whereupon his serum phenylalanine level rose to 60 mg percent. Readjusted finally on a moderately restricted phenylalanine intake, his level returned to acceptable levels, and he has continued to do very well. This and other cases amply demonstrate that considerable variation in phenylalanine requirements do exist among phenylketonurics, and thus necessitate individualized treatment for each. Interestingly, the 17-month-old sister of this newborn, previously unsuspected as a phenylketonuric although exhibiting the typical odor and autistic movement and still retarded even in walking attempts, was demonstrated also to be a phenylketonuric.

Again, although caution must be exercised in the diagnosis of phenylketonuria in prematures, we have detected two additional premature babies confirmed as phenylketonurics. They constitute two of triplet brothers; the third brother is normal. The point is that premature babies can be true phenylketonurics.

While in our series fairly high elevations that are transient are not uncommon in prematures, we have on at least one occasion encountered a level above 20 mg percent in a full-term baby, which soon was normal on a non-restricted diet, and consequently was excluded as a phenylketonuric. A second possible such circumstance is under study.

In our total series, as will be discussed by Dr. Kennedy immediately at the close of this paper, roughly ten of the cases belong to the atypical phenylalaninemic group. Often beginning with a delayed phenylalanine rise, they frequently have levels up to and above 20 mg percent, but rarely exhibit markedly high levels. Usually, they show negative urinary ferric chloride tests. Under conventional phenylalanine restriction regime the serum phenylalanine levels tend to subside very rapidly to normal levels or even below. Consequently, they require special care in treatment if the hazard of too low a phenylalanine level is to be avoided. In any case, we feel that the atypicals should be detected, carefully observed, and conservatively treated, each one on an individual basis.

While the inhibition test is not technically difficult, problems not infrequently do arise. The screening is therefore best performed in central laboratories that are doing large numbers of screening assays. Otherwise it is very difficult for a laboratory to realize sufficient interest and experience to achieve optimal results. The laboratory serving a hospital maternity service, for example, of even 5,000 babies a year, can hardly become very experienced with the detection of a phenylketonuric baby about once in two years. We would feel that the problems of transient and intermediate elevations of atypical phenylalaninemias and the general paucity of understanding about the phenylalanine disorder in toto, all speak eloquently for the need not only of centralized screening laboratories, but even more for pediatric team consultation centers for confirmatory diagnosis and treatment. These must work hand-in-hand with the screening center, for without expert treatment facilities, hasty and superficial, or even ill advised treatment is bound to occur. Further, because so little real information on the treatment of phenylketonuria is readily available at present, it is hoped that from this conference will be published responsible guidelines reflecting the common denominator of enlightened thinking in this somewhat baffling, but very treatable disorder.

Good therapy requires frequent monitoring of the patient's phenylalanine blood levels. Frequent testing of blood glucose levels has long been a sine qua non in diabetes, but a comparable monitoring of phenylalanine levels has not so far been used nearly as much in the treatment of phenylketonuria. There is nevertheless just as certainly the similar hazard of either too low or
too high a level of phenylalanine in the blood. We highly recommend the bacterial inhibition test on filter paper blood as very easily and effectively used for this purpose. We have already referred to the absence of a growth halo in a monitor blood of a baby under treatment as a useful signal of alarm. Parenthetically, not only is the monitoring of great value to the doctor treating the case, but also quite as a by-product it stimulates the morale and efficiency of the screening laboratory, which becomes increasingly knowledgeable as it sees its part in the whole picture of the metabolic disorders.

Mabry et al. have published a study of three phenylketonuric mothers with a total of seven living children all of whom are mentally retarded.([t]) Since these children are all heterozygotes rather than phenylketonurics, it is reasonable to assume that their retardation is associated with the in utero exposure of each to the increased phenylalanine level of the mother's blood. The possibility of such an exposure is consistent with Kerr and Walsman's experiments in monkeys showing that phenylalanine elevations in the mother are actively transported across the mammalian placenta.([t]) Further, we have in our State encountered two phenylketonuric mothers, all four children of whom are in institutions as severe retardates; three are reported as microcephalics. Therefore, the phenylketonuria treatment center should carefully maintain an up-to-date registry on all the phenylketonurics it detects and treats, so that the possible later resumption of treatment for the female phenylketonuric automatically will come up for consideration during her pregnancies as indicated. Otherwise, we shall have the hazard of possible mental retardation in the mother's heterozygote offspring from exposure in utero to her elevated phenylalanine. Indeed, we are here confronted with a peculiar responsibility, because our very screening programs--valuable as they are--nevertheless will greatly increase the number of phenylketonuric girls who are going to be normal enough mentally to marry and have children. Because there is still so grave a danger that this information may not promptly reach the obstetrician in charge, we should like to see at least the extremely simple ferric chloride test routinely performed on each expectant mother at her first visit to the doctor. Otherwise, there will certainly be born in the future retarded children whose retardation very likely could have been prevented. Furthermore, if damage to the infant brain can take place even before birth from a phenylketonuric mother, then it follows that we cannot be sure that the damage to a phenylketonuric baby does not start even from the early weeks of life. Therefore, the aim should be to discover and begin treatment as early as the diagnosis can be firmly established.

Passing now to other inborn errors of metabolism, the galactosemia inhibition test has not in our hands so far demonstrated sufficient reliability to recommend it, at least as yet, for routine screening of newborns for galactosemia. One case has been detected among 166,000 tested in our program in Massachusetts, but through an unfortunate delay the information failed to reach the physician in time for treatment and the child died on the sixteenth day of life. In addition, a case with a normal level at 3 days, has been detected in California on the follow-up 4 week specimen.([t]) In our pilot study we encountered a large number of babies with transient galactose elevations, which on further testing proved not to be galactoseemics.

An effective public health screening procedure for galactosemia is greatly to be desired. For best reliability a test for the absence of the enzyme rather than for the elevation of blood galactose is to be preferred. Further, since it is necessary to begin treatment at the earliest possible moment, a test that could use cord blood would certainly constitute a very great advantage.

In 223,789 newborns tested in Massachusetts for maple syrup urine disease by the Guthrie bacterial inhibition technique, one case has been detected--unfortunately, and through no basic fault in the inhibition test itself--too late to save the life of the child. One case also has been detected in Oregon out of about 36,500 newborns tested, and at last report was progressing satisfactorily under treatment.([t]) No important technical difficulty has been uncovered in the test itself. The method is, therefore, worth continued further trial for screening detection of the maple syrup urine metabolic disorder.

Returning now to the diagnosis and treatment of phenylketonuria, one closing word about the monetary cost. In the screening and treatment for just this disorder, we have estimated ten thousand dollars ($10,000) as the total cost of the program per baby discovered and treated, which compares with an estimated one hundred thousand dollars.
($100,000) for the severely retarded undetected-in-time phenylketonuric in an institution for his lifetime. The human aspect, of course, is much more important, but let it be emphasized that this program from the start has been a saving rather than an expense.

SUMMARY

Our public health screening program in Massachusetts for the detection of phenylketonuria and other inborn errors of metabolism uses both the bacterial inhibition techniques by Dr. Robert Guthrie, and the paper chromatography technique devised by Dr. Mary Efron. The paper chromatography screening has been covered by Dr. Efron's discussion at an earlier session. For the bacterial inhibition screening methods for detecting phenylketonuria and two other allied metabolic disorders, the main points and conclusions are as follows:

1. The Guthrie bacterial inhibition screening test for phenylketonuria, the technique of which is briefly reviewed, is effectively performed on heel bloods collected from newborn babies.

2. A phenylketonuric baby may on a rare occasion be discharged from the hospital at too early an age to reveal a phenylalanine elevation. A repeat filter paper blood specimen is therefore recommended as a routine at the baby's 4- to 6-weeks' visit to his doctor or clinic.

3. The screening program for phenylketonuria in Massachusetts was begun in July, 1962, and has enjoyed virtually 100 percent coverage of newborns since the spring of 1963, with 80 percent to 90 percent response on the recommended follow-up specimens. At the end of the first week in April, 1966, over 365,000 newborns have been tested with the detection of 36 phenylketonurics. Important details of the results are discussed.

4. While the inhibition test is not technically difficult, it is best performed in central laboratories that are doing large numbers of screen-assays. Otherwise it is difficult for a laboratory to realize sufficient interest and experience to achieve optimal results.

5. Expert pediatric consultation facilities for diagnosis and treatment must be available, and there must be hand-in-hand cooperation between the screening laboratory and the treatment facility. Aside from the much more important human aspect, the cost per phenylketonuric child detected and treated is much less than the lifetime maintenance in an institution as a severe retardate.

6. Frequent monitoring of phenylalanine levels during treatment is extremely important. The bacterial inhibition test on filter paper blood is quite easily and effectively used for this purpose.

7. A registry should be carefully maintained and kept up to date on all the phenylketonurics detected and treated, so that the later resumption of treatment for the female phenylketonurics will come up automatically for consideration during pregnancies as indicated. Otherwise, there will exist the hazard of mental retardation in the heterozygote offspring, because of the exposure in utero to the mother's elevated phenylalanine.

8. Although one case of galactosemia has been detected in Massachusetts and one case in California, the galactosemia inhibition test has not in our hands so far demonstrated sufficient reliability to recommend it, at least as yet, for routine screening of newborns for galactosemia.

9. An effective public health screening procedure for galactosemia is greatly to be desired. For best reliability a test for the absence of the enzyme rather than for the elevation of blood galactose is to be preferred. Further, since it is necessary to begin treatment at the earliest possible moment, a test that could use cord blood would be a great advantage.

10. In 223,789 newborns tested in Massachusetts for maple syrup urine disease by the Guthrie bacterial inhibition technique, one case has been detected. In Oregon, one case also has been detected out of about 36,500 newborns tested, and at last report was progressing satisfactorily under treatment. No important technical difficulty has been uncovered in the test itself. The method is, therefore, worth continued further trial at this time for screening detection of the metabolic disorder--maple syrup urine disease.
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DISCUSSION

MR. HORMUTH: Dr. Kennedy, you were going to elaborate on some of these cases that Dr. MacCready mentioned.

DR. KENNEDY: These are cases with persistent blood phenylalanine elevation over 12 mg percent who had no tyrosine by chromatography. There were 44 such infants studied in the three and one-half years of the program. Twenty-two of these, or half, we feel have typical PKU with high phenylalanine blood levels, no tyrosine, and a positive urinary ferric chloride test. Nine infants cannot yet be classified because they are young. Thirteen appear to have a milder form of PKU characterized by lower initial blood levels, although they do rise over 20 mg percent. It takes longer to get there, and they do not go up as high eventually.

All but one of these children when tested in the newborn period had a negative urinary ferric chloride test. They have in general been able to tolerate more phenylalanine. At age one and one-half years, most of these infants have been able to take over 500 mg per day of phenylalanine without raising their blood levels unduly. None of our so-called severe PKU children have been able to take this much phenylalanine at this age. Another difference between the infants in each group becomes apparent when their siblings are considered. The severe cases have a total of 35 siblings, 9 of whom have PKU. The milder cases have 19 sibs, only one of whom has PKU.

Both groups have been treated with the exception of those in the milder group who maintained a blood level under 12 mg percent on a low protein diet.

The results of treatment are about the same. All of these children appear normal except there may be some small statistical decrease in their IQ.

The other major difference has been that the milder cases have grown better on treatment. They are taller, heavier, and they are darker.

We wondered whether these milder children were able to make phenylpyruvic acid. Their diet protein intake was gradually increased over several months until their blood phenylalanine rose and their urines became positive for phenylpyruvic acid. One child's blood level never rose, but instead dropped to 6 mg percent where it has remained ever since.

We have one other similar 3-1/2 year old child who initially had similar values, who is also maintaining a blood phenylalanine level which is just above normal.

DR. BERRY: I would like to ask Dr. MacCready the frequency of tests which have to be repeated because the mother or the baby has been given an antibiotic.

DR. MACCREADY: We have had very few that have come to our attention for that reason.

DR. UDENFRIEND: Could you identify them from the tests?
DR. MacCREADY: Well, perhaps if there was an area of inhibition around the disc, we would at least be suspicious. We would then on general principles have it repeated. But as far as we know, it hasn't been an important problem.

DR. BERRY: If a phenylketonuric baby has been given an antibiotic, would you expect a negative test?

DR. MacCREADY: I don't know.

DR. BERRY: Shouldn't this be investigated?

DR. GUTHRIE: We would see this in two ways.

In the first place, the test plates will eventually show some background growth all over, $\beta$-2-thienylalanine is not a complete inhibitor, and often when the tests are read, we can see faint turbidity. An antibiotic would produce a definite clear inhibition zone.

A second possibility occurs if the baby has a high phenylalanine concentration, and also high antibiotics in blood. Then the inhibition zone would be inside the growth zone. And the phenylalanine responses would be seen as a halo.

DR. MacCREADY: I might add that routinely when we see anything out of the ordinary around that disc, we perform paper chromatography. So we would have that additional check.

DR. WOOLF: I would like to ask Dr. MacCready to clarify one point about the urine test which compares so badly with the blood follow-up test.

Am I right in thinking this is a Guthrie inhibition test on the urine? Because, if so, this is a test for phenylalanine. We know that looking for phenylalanine in urine is not as good as looking for it in blood because of variability in renal clearance and urine concentration.

As far as I know, the paper chromatographic test for orthohydroxyphenylacetic acid in urine has not yet been fully evaluated against the blood test.

DR. MacCREADY: Well, that was the reason that we shifted from the follow-up urine to the follow-up blood test. We have never had any experience with routine follow-up ferric chloride test. I would like to point out that the Guthrie test will react either to phenylalanine or phenylpyruvic acid. We have had some cases where ferric chloride tests have been performed and have been negative when the phenylalanine blood test has been positive.

We have had no experience with doing any other type of tests on the urine except ferric chloride or phenistix.

DR. SCRIVER: I would like to ask Dr. Kennedy the ancestry of his patient with the atypical hyperphenylalaninemia and whose one sibling had classical phenylketonuria.

DR. KENNEDY: A family of Italian origin.

It is actually not quite clear whether the sibling has a mild or more severe type. It seems to be more severe.

DR. SCRIVER: Our experience with atypical hyperphenylalaninemia in Montreal occurred in a Greek family. Dr. Efron tells me that many of the atypical cases that you are finding in your community are of Mediterranean origin.

DR. KENNEDY: I am not following this particular family, but I can say that this is an unusual family because the mother of the two children runs a blood level consistently between 6 and 8 mg percent.

DR. ANDERSON: Did you perform phenylalanine loading tests on the mothers and fathers in these atypical cases? The reason I ask is I believe our parent was of Italian descent, and it was the father who was the heterozygote.

DR. KENNEDY: We have data on ten parents and five families, but are not prepared to interpret it yet.

DR. EFRON: In answer to the loading question, it is very clear that at least one of the parents had a normal response.

We have also seen an instance in which both parents had an abnormal load test.

DR. BESSMAN: I am concerned about the discussion by Dr. MacCready because I agree with him that we have to be very careful. I would like to know why 20 mg percent is the concentration chosen to establish the diagnosis of phenylketonuria?

The second question I would ask concerns the pregnant mother suspected of being phenylketonuric. These women with phenylketonuria who bore children were never treated. These mothers have been phenylketonurics all their lives, and we are seeing them now only because we are seeing their abnormal children. Do you intend to carefully plan the care of these mothers during pregnancy? I would like a protocol of what you would consider optimum care of a pregnant woman without causing possible damage to her fetus by your treatment.
DR. MacCREADY: Yes, why 20 mg percent? Now, that figure is empirical. So was the figure that was employed before, 15 mg percent, which was used largely because that is the concentration at which the urine ferric chloride test is likely to be positive. We just felt that perhaps it was a little bit more conservative to use 20 mg percent.

In no sense have we eliminated the specimen that is under 20 mg percent. The ones that are between 4 and up to 19 mg percent inclusive, we classify for the present as intermediates. They are followed very closely at the clinic. We feel that is the job of the pediatrician. In other words, screening points out that further study is needed. We think beyond that point the pediatric consultant must pursue the problem.

I would be pleased if anyone wished to comment, if he thinks that some other figure than 20 mg percent would be better.

With regard to the second question concerning the management of mothers during pregnancy, I tried to be very careful in what I said. The obstetrician and the pediatrician working together may believe that some form of phenylalanine restriction is indicated or may think that restriction is not the proper treatment for these people. In either case, these mothers should be under careful observation. If the decision is to treat, it certainly must be done with a great deal of skill.

We do not know the phenylalanine requirements of the fetus. However, this does not mean that we are not going to investigate the situation. It seems to me we are in much the same situation that we were when we first discovered that we had the disorder of phenylketonuria. We did not know how to treat it; we worked to find out. So my reply is that this problem is something we should be very much aware of, and we have to work it out.

DR. EFRON: Dr. MacCready mentioned one patient who was over 20 mg percent. When he was brought into the hospital, his phenylalanine concentration came down to lower levels and he was not treated.

One does not pick these in an arbitrary fashion. But the patients who are between 15 and 20 are watched for a little while to see if they are going to come up or go down. They are brought in and evaluated by Dr. Kennedy. After evaluating them for a few days, if they come down, they are not put on the diet. If they go up, he becomes concerned about them.

DR. BESSMAN: If you eat, the phenylalanine level goes up. Do you say the diagnostic level is over 20 mg percent if they eat? I am asking questions because we are dealing with rigid rules to which people are forced to comply by popular demand, I am asking people here who are recommending these rigid rules to say publicly that they are not rigid. You have used such expressions as "careful observation" and "skill", but you have not indicated what to observe or what skill to exercise.

DR. EFRON: But the 20 mg percent is not taken as a rigid rule in Massachusetts.

DR. BESSMAN: But it is taken in your publications as a rigid rule.

DR. FISCH: Does anyone know how many of the so-called negative tests turn out to be positive?

DR. MacCREADY: No babies have as yet come to our attention as phenylketonurics, who were screened as negative in the program. We are saving all our filter paper bloods, nevertheless, because they can be re-run.
ORGANIZATION OF COMMUNITY SERVICES
IN PHENYLKETONURIA

RUDOLF HORMUTH

Thus far in this conference we have considered many aspects of the condition we call PKU. We have shared some knowledge and some of our ignorance with one another. We have ranged in our dialogues from explanations of existing biochemical knowledge of the disease through discussions of how to maintain a patient on the diet.

In all of these discussions, amid the welter of speculations and the pursuit of research interests, it is comparatively easy to lose sight of our primary objective, the delivery of a preventive health service to a child and his family.

All of our formulas and our technical concepts need to be superimposed on a picture of a child and his family. All of the data and knowledge presented at this conference must ultimately be fitted into an equation which provides an answer as to how we can deliver meaningful, efficient and effective service.

It is quite obvious that as yet we have not evolved such a satisfactory equation in regard to PKU. We do not seem to be completely certain of what to deliver, nor do we seem to be quite certain as to how or when to deliver what we have available.

Obviously more basic information about the condition of PKU would help tremendously. We don't fully understand the condition. There appear to be some questions about the efficiency of our casefinding mechanism. There does not always appear to be complete certainty of everything that our instruments are detecting when we make a diagnosis. We are not completely confident of the outcome of our treatment approaches. There is admittedly a great deal of uncertainty.

On the other hand, our knowledge of PKU, its detection and treatment is not quantitatively different from the basic understanding we have of many other medical conditions. In these other conditions we diagnose and we treat on the basis of the best available knowledge. We don't seem to hesitate. We use what we have. Many times, based on experience and new findings we subsequently redefine our concept of a condition. We change our diagnostic and management approach. We seldom express guilt about such shifts and changes resulting from new findings.

In the area of PKU, however, I am acutely aware of an unusual amount of guilt and apology for what we don't know. An uncertainty, a hesitation, which interferes with and detracts from our ability to devise a mechanism through which we can deliver the best service we know in as effective and efficient a way as we can with the knowledge and resources available.

I submit that we now know enough - not everything - but enough to devise such a mechanism and to deliver meaningful service to most of the children whom we label as PKU and their families.

What goes into any such service that we hope to be able to deliver should include all of the elements of casefinding, diagnosis, evaluation, treatment and follow-up care. The depth and extent to which actual services are delivered to a particular patient and family in any or all of these areas will vary considerably. A prime requirement for any mechanism we devise to deliver service is that it be sufficiently flexible so that we can individualize the service package to fit in not only with abilities and peculiarities of the agents who are dispensing service but also with the individual differences of the consumers who receive them, and the communities and settings in which such services are provided and utilized.

It is most important to keep in mind that we are attempting to deal with children who don't live in a test tube or in a vacuum. They live in families, and these families in turn live in our society and communities. Objectives and goals for children in different families vary considerably. In our culture we are not attempting to reproduce exact duplicates functioning alike on similar levels. Different child and family oriented goals dictate the use of multiple choices of course, of action, and the use of different resources to achieve ends.
The "package" we are attempting to design is one that is to be delivered to every newborn infant and his family. It must be designed so as to assure most families that their newborn infant does not have PKU, or if the infant does, that our confirmatory procedures are sound and not open to question. It must be designed so as to give the assurance to parents that early detection is important and worthwhile and that an artificial diet can be provided which can prevent mental retardation and allow the infant to grow and develop normally. The "service" that we are attempting to provide therefore is designed to reach about 4 million babies and their families each year through thousands of maternity facilities and hundreds of medical specialists, laboratories, technicians and elaborate channels of communication. From this our package should select out approximately 400 infants, label them, provide the dietary product and necessary management and counseling that presumably allow the patient to develop normally.

From the point of view of sheer numbers to be served, therefore, this program assumes rather large proportions. As with any large program, extra care must be exercised to develop the kinds of standards that will assure good quality and at the same time can reasonably be implemented with some uniformity in all of the States and by all of the different people required for such an operation.

An even greater problem in the delivery of such a service are some missing pieces and some unanswered questions in areas which from my point of view should be vital parts of any service package. For example, I am certain that I do not have to remind you that in many areas of medical care we do not always succeed in achieving a complete cure by intervention. We sustain life, but some of the lives that we sustain are no longer perfect. Sometimes the intervention itself creates distortions in the pattern of life and in the pattern of family living or in the ability of the individual and his family to utilize existing resources.

A diagnostic medical label of and by itself can completely destroy or distort relationships within a family. It can close some doors in terms of the normal channels and outlets people seek and choose to meet their needs.

In planning a PKU program we cannot make the assumption that having diagnosed the condition as best we can, having interpreted the findings to the family, having placed the infant on a diet and having set up procedures to monitor this diet, that we have fulfilled our responsibility or that we have an adequate service. We have intervened and we have a responsibility to follow through and to provide assurances to ourselves, the family, and society that this intervention of and by itself will not in the end create a worse distortion of the patient's and the family's life than without such intervention. Some of the necessary ingredients for such assurances as of now are lacking and have not yet been faced.

We need to consider the fact that by the diagnosis we are in effect saying to the families, "Your baby is doomed to severe mental retardation, but there is a means of possible prevention through a very special kind of diet." We can prescribe the diet and monitor the blood levels, but in effect we are saying to these families, success or failure, normal development or severe retardation, depends on you and how you administer the diet. We are placing these families in a continuous anxiety state, if you wish to approach it from this point of view. How much of what we are recording in terms of success or failure in treatment is a reflection of what might be a chronic anxiety state? What are we really doing to this home environment? Can we cope with it and how do we cope with it? What psychological side effects is the treatment having on the families and in turn on the child? How and by what means can we assure families that if the blood levels of an infant go up they have not failed and caused irreparable damage?

The index of success in treatment that has usually been proposed for inclusion in our service package rests much too heavily on measured intelligence. The attempt to utilize an IQ as the major index of success or failure in treatment is one of the greatest problems in structuring an acceptable program. Both the consumers of our proposed program and the array of individuals and agencies we are asking to deliver services are not used to judging the success of medical treatment on the basis of high levels of intelligence, I know of no other medical treatment which is judged by such a criterion. It appears to me that in PKU such an index is equally inappropriate.

It likewise seems inappropriate to judge success of treatment on the basis of achieving...
admission of a child to a school program. This has comparatively little meaning. Perhaps if school success is an important measure of adjustment in our society we ought to begin to look at what constitutes school readiness. Might it be possible to break down school readiness into smaller dimensions - perhaps 20 areas? Could we score achievement in each of these areas and perhaps evolve a profile that might be one part of an index of treatment success? We need more and better answers here in order to construct our service program.

We likewise need more and better answers to other problems as the PKU children grow up. How long do we treat? How long do we monitor? What do we do with adolescent PKU's? What kind of emotional problems will they have? What are their problems in terms of parenthood? What type of counseling do they need and when and how do we provide it?

Beginning with screening, we have a responsibility to follow through on all of these areas and to include answers and provision of services for these questions and contingencies.

As I have stated before, there are many ingredients which are lacking or inadequately defined in this PKU service package we are attempting to put together. Despite these lacks we can deliver what we have in the best way we know how. In order to deliver this service, however, it should be clear that we require a tremendous network of organized resources ranging from the primary physician through an array of public health personnel, laboratory and hospital and school personnel, all related through a functional communications system through which responses can be made to all types of emergency and changing situations.
THE ROLE OF GOVERNMENT AND LEGISLATION IN MANAGEMENT OF PROBLEMS IN MEDICINE

JOSEPH D. COOPER

The orientation I have as a political scientist who seeks an understanding of the means by which advances in phenylketonuria and other allied metabolic diseases may be implemented is necessarily much different from that which most of you have. Few political scientists have thus far given attention to the medical sector of our political system.

While I have tried to grasp what might be called the technology of phenylketonuria, my interest in doing so has been both guided and constrained entirely by a need to understand logical sequencites in program planning.

More importantly, I have been concerned with the emerging problems of government in medicine. The Federal Government, at least, has only recently begun to assume an activist role in the conduct of medical programs affecting the general public. It would be unfair to expect it to inaugurate major new programs without committing some errors.

Of course scientists know very well that error and misstep are part of the development of normal science. We should have a high tolerance for unpredictable and unavoidable error. But we should have a low tolerance for errors which can be avoided or minimized through adherence to well-established doctrines and methodologies.

In the phenylketonuria program, I believe we (society) have committed several errors.

First, we allowed a tentative advance to form the basis for national service programing before we had sufficiently well elucidated both the relevant scientific techniques of diagnosis and management.

Secondly, mandatory legislation has been chosen as the medium for achieving program acceptance without regard to sensitive questions which concern the future practice of medicine.

Third, we have failed to see to it that when a government bureau assumes an active role in program leadership as well as in program finance, it also assumes responsibilities for rendering itself subject to being monitored. In other words, who guards the guardian?

The history of phenylketonuria is a classical example of a brick-by-brick building of a diagnostic therapeutic model. Only three decades ago, the disease was given a definite identity by Dr. Fölling. We have come very fast in a short time. In rapid succession, the mechanics of the disease were elucidated, diagnostic techniques were developed and improved, and methods for treating this metabolic disease through dietary management were also developed and continually improved.

To a considerable extent, this chain of development in the United States was advanced through research funding by the National Institutes of Health, the National Foundation, the National Association for Retarded Children and perhaps others.

The advances in phenylketonuria excited two types of interest. Scientists sought to widen their understanding of metabolic and other inheritable diseases. The continuing study of the biochemical phenomena of phenylketonuria and other diseases is expected to provide important new insights into clinical pharmacology. Benefits which may be expected to accrue include increased drug and therapeutic efficacy and safety.

The second category of interest has been the programmatic one. The mental retardation movement, dominated in large part by commendably active parent groups, together with organizations such as the National Association for Retarded Children saw in the phenylketonuria program both a real and a symbolic opportunity for advances in the preventive control and amelioration of retardation.

Important pediatric personnel joined in the efforts to secure widespread adoption for the
diagnostic-therapeutic model of phenylketonuria. In this endeavor, they were given leadership by
the Children's Bureau of the Department of Health, Education and Welfare which adopted
an official policy that every newborn child be
given a PKU test.

This programmatic approach as evidenced by
the number of legislative enactments has been
eminently successful. At least as of mid-1965,
32 States had enacted PKU screening laws of
which 27 were mandatory.

To a great many people, this must seem
commendable progress. One reads many stories
in public print about the great breakthroughs in
PKU. PKU has become something of a household
word. For a relatively rare disease, it has
become one of the best known. Unfortunately,
this flurry of legislation cannot be viewed as
progress by many social scientists.

Even if the PKU program were ready for
widespread adoption, the use of compulsory legis-
alation is highly questionable.

One of the most striking aspects of the entire
program from a scientific standpoint is the
apparent absence of well-controlled testing. The
PKU program has been brought to its present
state of widespread adoption on the basis of
scattered clinical observations of populations
which have been statistically insignificant.

The effort to universalize the program will
make it even more difficult in the future to obtain
control groups for scientific evaluation of diag-
nostic and therapeutic techniques.

I do not suggest thereby that the PKU pro-
gram is entirely without merit. Rather, I suggest
it is not without substantial risk to children
whose symptoms might falsely suggest that they
have phenylketonuria when, in fact, they do not
have this disease or, at least, do not have low
mentalities.

Far from the PKU program serving as a model
for others to come, it seems in need of a great
deal of genetic repair of its own. The unknowns
are many. They embrace both diagnostic and
management techniques.

At the May, 1965 meeting of the Society for
Pediatric Research in Philadelphia, Dr. Bessman
brought up his famous false syllogism: "If one
were to assume a woman is a person with two
eyes and, therefore, a person with two eyes is a
woman, one would be laughed out of this august
assembly; however, if one says that a mental
defective who has phenylketonuria has a high
blood level of phenylalanine . . . and therefore a child
with a high phenylalanine blood level . . . is a phen-
ylketonuric with mental retardation in the future--
is just as silly as the primary syllogism." (4)

In the absence of controlled studies, there is
no evidence that a low phenylalanine diet is not
potentially harmful to a child of normal mentality
who happens to have biochemical symptoms of
phenylketonuria, but who actually may not have
this affliction.

Furthermore, because 15 percent or less of
cases with a positive Guthrie test are found to
have a disease suggestive of phenylketonuria,
there is a great deal of room for possible error
despite the use of confirmatory tests. (4) One
theory is that PKU is not a single disease as
first believed, but may be two or more different
diseases. Either different treatment or no treat-
ment may be indicated. (4)

At the Rosewood seminar, Dr. Jervis cited
two untreated phenylketonurics, sibs, one of whom
was imbecile, the other an Oxford University
student. The literature has reference to many
other normal mentality or high mentality cases
who are untreated phenylketonurics. (4)

Many people are of the opinion, particularly
among the general public, that a simple diet is
all that is needed to prevent retardation. This
simple diet, in truth, calls for continued obser-
vation of the infant as the discussions this
morning brought out. The diet must be modified
so that nutrient balance is maintained as require-
ments undergo the sensitive changes found in
a growing infant.

Induced dietary deficiency can and has caused
deaths. Somehow, these do not come to the
attention of the medical profession generally or
even to the pediatric sector of the profession.
Causes of death may be recorded as suffocation,
pneumonia, crib death, and so on. Whereas in
an unknown number of cases the ultimate causal
factor was induced dietary insufficiency. (5)

I could go on with other examples of the
state of unknowns in this exciting new area of
metabolic disease, but you know much more than
I about them. My charge to you is that you should
have had many more conferences and symposia
such as the present one before you allowed the
PKU program to become so broadly disseminated.

One of the first things I learned years ago
as a systems analyst is that you do not commit
any major endeavor on a broad scale without first having undergone pilot testing. Inevitably, such testing leads to correction of assumptions and procedural techniques; such testing we have not had in the PKU program. Rather, the cry went out that society owed it to every child to have a PKU test at birth and that it would be unethical to deprive any child of the dietary treatment once he had been diagnosed and confirmed as phenylketonuric.

The claim has been made that the physician is still free to decide the course of treatment for the infant. Theoretically this may be so. But the spectre of the malpractice suit must exert a powerful stimulus to prescribe in accordance with the cultural mores. For these are what influence the court.

As a model for the future, may I suggest that protracted pilot tests should be conducted in one or more States under controlled scientific models planned in order to derive sufficient experience before entering upon the next major phase. That phase, in turn, should be the conduct of wider field trials under varying conditions. Then, assuming that the program demonstrates its worthiness, it should be expanded at a rate consistent with the development of adequate facilities and operating manpower. Under no circumstances should new programs which affect the destinies and well-being of people be put into effect without first having established the availability of necessary and competent manpower, laboratory facilities and laboratory techniques. I submit to you that if you do subject children to that type of program in the absence of these components, that practice is malpractice.

Looking back on the PKU program, I recall visiting the Children's Bureau's program officer who happened to have in hand at the moment I arrived a copy of a letter from a State health department which reported that it had a law on the books for compulsory screening, but it did not have adequate facilities with which to carry out screening and subsequent monitoring and dietary management.

I also recall Dr. Jervis at Rosewood telling us that in New York State, a State which is at once both rich and tax-poor, no two hospitals necessarily have the same procedures. Each has its own laboratory facilities. There are no central facilities to assure a concentration of skill and techniques of the highest standards. We were told here this morning such facilities are very important.

Which brings me to the legislative matter. In our great country, one of our cultural mores which has served us well in the past, but which is stumbling somewhat in the medical area, has been the belief that we have but to spend money and to employ vast numbers of people, and we can work almost any miracle. Well, we haven't done it in the area of phenylketonuria even though we assume for a moment that mandatory legislation is a good idea. One would think that there would first be an inventory of the type that I have mentioned before putting a law on the books.

One reason I oppose the use of legislation to prescribe medical practice is that despite tremendous advances which have been made in the past two or three decades, medicine is still more art than science. It is beset with unknowns and variations at every turn. We are far from that stage when we can begin to put things in pigeon holes or even really to automate them. Again, when we read the Sunday supplements, it appears we are going to put disease profiles into computers. Then we will walk through long buildings. As we go through, transducers will be strapped on us and body fluids extracted. When we emerge there will be a print-out for a physician which will have a spectrum of diagnoses and prognoses and probable treatments and whatever. I don't know whether any of us will see that occurrence in our lifetimes.

The fashions and fads of medicine continue to undergo change. I would feel much more comfortable if the profession itself would assess diagnostic and therapeutic advances and put them in perspective for the benefit of both the profession and the public rather than to have people from the social sector come in and determine what the programs ought to be at this stage. And I am from the social sector.

Besides, an eager public presses upon its practitioners to make available to them the latest of medical miracles. You know the patient who comes into the doctor's office with a newspaper clipping and says, "I have read and can I have." If he can't get it, he will go out and get a commercial grade of dimethyl sulfoxide and treat his own arthritis or bursitis.

I have little doubt from observing recent phenomena that if there is something really important that has been developed, it will find
its way very quickly to practicing physicians. We need not stimulate inordinately this pressure to bring the latest in laboratory advances to the physician and the public. Somehow, it gets there. Our public is eager for the latest.

The second reason for avoiding legislative stricture is that social choice becomes preempted by such legislation. The demand for medical service is insatiable. It has not been met by existing pools of medical manpower. If anything new puts demands upon the manpower pool, it will put it to a greater strain. As we develop new techniques, the resources required to deliver them to the individual seem to vary inversely with the incidence of the disease. This phenomenon contributes to the squeeze upon manpower. It will be a limiting factor in the future.

Increasing attention is being given to preventive medicine in the hope that this will lighten the load. This approach too, may prove to be a delusion. Mounting experience begins to suggest that deferred demands are created that are of greater magnitude.

Most of you are pediatricians. What happens to the incidence of disease in what were childhood diabetics who have been allowed to survive through medical management into later years? For example, their vascular disorders are about double the rate of those of other people. How much does this cost society? I do not thereby suggest we do not treat these children. I think it raises deferred problems. We enable more people with genetic defects to survive into adulthood and to reproduce; these also are deferred problems with which society must deal. I don't know how we can cope with them.

If I were the father of a child with a genetic defect I would want it to survive. But society as a whole has a broader problem. At any given time, society may have to judge where best to employ its resources. It might decide, for example, to put more effort into providing better diagnostic facilities for the 99 out of 100 children who are admitted to institutions for the retarded who do not have PKU as compared to the less than 0.8 percent of those who are phenylketonurics. At least some people are alarmed at the number of misdiagnoses of children admitted to institutions for the retarded. Should we not have a law for them as well? Should we have a law for every conceivable metabolic defect as has already been suggested; for example, maple syrup urine disease which may have an incidence of one in a quarter-million?

Perhaps we need laws to quarantine people when they are likely to spread infection. But phenylketonurics do not spread infection. While we are on the subject of the incidence of phenylketonurics we perhaps need some new surveys which would find out the incidence of true phenylketonuria, so we might have new statistical guidelines. The prevailing incidence was cited as about 1 in 20,000. It has risen to 1 in 10,000. Perhaps that figure needs again to be reduced. Perhaps proceedings such as this will help provide the improved insights which will lead to improved casefinding.

What is the role of the government bureau? How much active leadership does the program leader provide? In fact, should the government itself be program leader as has been largely the case with the PKU program and the Children's Bureau.

It is a simple truism to say that the Federal Government wields tremendous power. It is also a simple truism to say that money and power are synonymous. Even when a government agency leans as far back as it can without toppling, it exerts a power over its beneficiary constituency. How many are likely to speak up with impunity and immunity to criticize the sponsoring government agency when they are financially dependent upon that agency or an associated agency?

Time was when the men of academia would not allow themselves to become seduced by government funds, not just in medicine, but throughout because they felt this would impair their academic freedom. Times are different. No one else can really support the vast endeavor of science any more. Occasionally, one hears that the government really doesn't influence academic freedom or impair it. Academicians and their government sponsors are found sometimes on the same platform, both saying science is being advanced while at the same time freedom is being preserved. I wish it were so.

There may be exceptions, but I can think of hundreds of people who have said, "Don't quote me, but..." or, "Off the record..." I participated in a little colloquium a couple of weeks ago where there were some deans who let their hair down. I suggest you ask some medical school deans how free they feel in making academic decisions when Federal funds are involved and
really how free they feel to provide leadership for their own faculties who walk all around them; flow around them as a stream around a stone. I don't think anyone can say precisely how serious this situation is. One just gets a feeling that it is more serious that it should be. Moreover, much of it is self-induced by grant recipients rather than by sponsoring government agencies. We must recognize that, too.

My point, however, is that the government agency itself must do whatever it can to rise above its own power.

In this respect, I believe the Children's Bureau has been in error. The error began when it wholeheartedly embraced the dissemination of a service program prematurely. Committed to its position, then, it tended to become selective in the dissemination of scientific information. Voices of opposition have been identified as minority viewpoints which already are well known and need not be further disseminated.

Adverse commendation by well-credentialed bodies of medical scientists have been kept from view on the grounds these are views in contradiction with other views already expressed. At least, these are things that were said to me.

In the jargon of the stock market, bullish information is disseminated widely while bearish information is pooh-poohed or submerged. This is not in the interests of science nor in the interests of the public.

I bring these out because I think they are part of our learning curve. The lessons from them must be worked into the slope of the curve.

In sum, who guards the guardians?

REFERENCES


4. See references in the bibliographies of phenylketonuria published by the Children's Bureau.


DISCUSSION

DR. KOCH: In some respects, I am in sympathy with some of Dr. Cooper's viewpoints, but I would like to correct several errors in his presentation.

Most of the money that supported the PKU screening program did not come primarily from the National Association for Retarded Children, National Foundation, or NIH, but primarily came from the Children's Bureau for support of the 400,000 newborn study trial.

Secondly, the statement referring to an absence of tightly controlled studies isn't quite accurate either. I feel that the Massachusetts experience in which they have had statewide screening since 1962 is a very solid program and information regarding it has been disseminated throughout the United States. I feel that the suggestion that the Massachusetts group has withheld knowledge or data or have not reported deaths is a gross exaggeration. I think Dr. MacCready was the first one to alert pediatricians and physicians around the country to the need for guidelines and to the need for us, as physicians, to develop these guidelines so that we can instruct and assist our confreres who are faced with an infant with a presumptive positive serum phenylalanine test.

The third thought I have is that Dr. Cooper used the term "mandatory" in terms of 27 State laws of the 32 that have been passed. I have seen most of those State laws, and they allow for the parent to object to the use of the test. Mandatory means "must" and "must" does not allow for
exception. So that they are not mandatory in this respect. So I don't think this term is accurate.

Fourthly, I was somewhat shocked to hear Dr. Cooper insinuate that perhaps we shouldn't treat the juvenile diabetic. I know he didn't really mean that.

DR. COOPER: I didn't say that.

DR. KOCH: But that's the way it sounded to me. I would not want a social scientist to advise what the physician should or should not treat. Certainly the physician must retain the right of deciding therapy in spite of governmental infradiction of the rights of physicians. Pediatricians by and large have not surrendered this right. The patients are still under our care, and we have the right to treat.

I think lastly that the point Dr. Cooper made about the physician's right to treat or not to treat the PKU child at the present time is a moral judgment is incorrect. I don't think there is any answer except to treat with the present body of knowledge that we have at our disposal.

DR. COOPER: On the first point, on the funding, I had reference in my remarks to really the initial stages, the research stages, which were certainly followed by the Children's Bureau with greater funding for the dissemination of the program. I don't think there are any conflict. There were early phases in which NIH and National Foundation supplied funds.

DR. GUTHRIE: No. It was really National Association for Retarded Children. The grant from the National Foundation was for a different and unrelated thing.

DR. COOPER: On the controls, Dr. Koch, I would be very much interested in seeing literature citations on the control groups as compared to the Massachusetts experience. My impression is that it is still an impressionistic finding and simply to have a well-performed program in Massachusetts does not in itself say that this is a scientifically modeled program with experimental controls.

I read Dr. MacCready's 1964 report on that program. I read his admonitions, many which he repeated here today, which I think substantiate some of the alarms that I have expressed.

I noted there are two deaths cited in that paper. But one was cited as suffocation. One was cited as pneumonia. Subsequently, I believe Dr. MacCready has found that they are perhaps attributable to other causes, including induced dietary insufficiency as later data came in.

They may not have occurred under the management of the Massachusetts General Hospital at the time, but there were two deaths which were reported at the time in the literature, not attributed to induced dietary deficiency. I don't think we have had improper reporting.

On the matter of parent consent, Dr. Koch, do you think that the parent can make a judgment? Is this really the kind of judgment that the medical profession largely has to make. Perhaps the parent can veto it, but he can't really make that judgment.

DR. LADU: I would like to defend the Children's Bureau and some of the organizations that you have mentioned specifically.

It is good to have a critic of these programs. I think we should be our own best critics. But I think it is important to think about the way this program did develop and particularly the Children's Bureau's role in the development of the PKU program.

As I remember my presence on the technical committee must go back to 1957 or so, Dr. Koch was on it at that time and Mrs. Berry was on it. The program was explored as a possibility and the problems were discussed. If you would read the minutes of some of those meetings, I think you would find a different impression than you have now of some of these things because the publicity at that time was designed to make known this disease to the scientific community and also the problem of inadequate methods for detection of blood levels of phenylalanine.

Through the Children's Bureau, stimulation was carried out. The methods that have been developed, at least two of them, were a direct result of the need being voiced for general methods to detect phenylketonuria and to measure blood phenylalanine.

The aim was also to collect what information was known and make it available to interested people. The problem at that time was whether the reports of the beneficial effects of the diet were really true. It was clear that there were not enough cases in any one local area to make a statistical evaluation.

It meant that the solution had to depend upon a collection from different investigators of a large enough number of cases. It meant having some uniformity in testing phenylalanine blood
levels and testing of development and intelligence. Any other parameters that were needed would be developed in such a way that this data could be compared within a reasonable time.

So the Children's Bureau undertook to do as much as they could to see that these needs for collecting the data were made known and that some uniformity was encouraged without imposing restrictions.

The pressure for legislation has certainly not come directly from the Children's Bureau. Talking with their people I know that they realize this was coming. They have done what they could to make what legislation has gone into effect as reasonable as possible. But we cannot blame them for initiating State laws.

It seems to me that we are looking back at a program that is moving in the right direction. I am quite sure that 20 or 30 years from now when we look at the programs on cancer, we are going to see much of wasted money, a number of stupid things that were done, and probably have some relatively harsh criticisms to make about that whole program. But the research and development and final solutions of diseases are not like building a bomb or building a bridge. These are the sequence of developments to which scientists are accustomed. I think we accept the inadequacies in our abilities to apply research information as fast as we would like to see it done. The lag in both the public and the physicians in taking up this information and our inadequacies in seeing the whole story at one time is due to our specialized interests. I think we accept the inadequacies in our abilities to apply research information as fast as we would like to see it done.

DR. MACCREADY: I think that Dr. Cooper is quite correct in saying that we need treatment facilities along with the screening program. As a matter of fact, I brought that out in my presentation. I would like to point out that when the National Association for Retarded Children recommended that screening first be as widespread as possible, at the same time it recommended that funds be appropriated for proper treatment facilities. I would like to point out also that the Children's Bureau has made funds available for supporting treatment facilities in a number of the areas in this country.

Now, I want to say just a word or two about the two deaths that occurred. As Dr. Cooper said, in the original publication in 1964, we mentioned both deaths which have occurred, one baby in New Hampshire, and the other a baby who had moved to Rhode Island. We mentioned then the only information that was available to us at that time.

The New Hampshire baby experienced a very sudden death. The only information I obtained was from the medical examiner. He made the diagnosis of pneumonia, which is certainly not a very complete diagnosis. We have no more information, but hope some day that we shall be able to obtain it.

In the other case, we have some additional indirect information which suggests, as I said, that death may have been associated with a dietary deficiency. But in the early days, there was very little monitoring. So again, the information is incomplete. This, however, is all the information that we have.

DR. GUTHRIE: I don't think it is possible for you as a political scientist to discuss the scientific evidence that is available concerning the dietary aspects of PKU.

But I do want to address myself to your criticism of society as a whole which is the only criticism that I can discuss with you as a member of society, as a taxpayer, as a parent of a retarded child, and as the uncle of a phenylketonuric child. My niece, who is now 8 years old, gave me impetus to develop the screening test.

I feel if I were to sit silent and allow the others around me to defend the program that I would be irresponsible because, in fact, the test I was fortunate enough to develop has led to the problems that you have raised.

In the 30 years to which you alluded, the present evidence would indicate that 12,000 phenylketonuric infants have been born in the United States without considering the other countries who have been waiting, so to speak, for the results of Fölling's discovery. In subsequent years after 1953, the dietary treatment has been employed and assessed on these children.
I fully agree with you it is very difficult when any new progress is made in medicine or science to use new knowledge properly. I agree with Dr. MacCreary that there is a lag in this country between government supported research and government aid for application of resultant information for the welfare of the citizens. This is partly because of socioeconomic reasons, but it is also the distribution of medical aid to our citizens which is at fault. Many of us are very much interested in seeing to it that new knowledge about PKU as an example of medical genetics is applied as quickly as possible.

I am a member of the Public Health Committee of the National Association of Retarded Children. I am one of the parent members of that committee who voted to advise the 50 chapters of the Parents' Association. We were responsible enough to give the Parents' Association all the guidance of which we were capable to define the type of legislation for which we felt they should press.

A number of the laws that were passed in the States almost coincided with the type of law that was recommended by the National Association of Retarded Children. This recommendation was that it would be extremely desirable that the law would leave decisions concerning all regulations involved to the State health department, such as the type of specimen to be collected, when it was collected, the test that was to be carried out, and what laboratories were to be approved.

There was no treatment prescribed in the law. The laws as far as I know all included the provision that parents could refuse testing of their child on the basis of their conscience or religious grounds. The effect was that the laws compel the collection of a specimen. The details concerning the specimen are left up to the State health department in those laws with which I am familiar.

I feel that the knowledge of technique of identification of individuals who are carriers and subsequently giving them this information for their own protection through the tests of infants is important new knowledge which will then be subject to the judgment of the individual and judgment of society. It is that judgment that you are really discussing, not the scientific evidence concerning the treatment.

I feel as a taxpayer that too little of our tax money is being used to quickly apply the results of medical research for the benefit of individuals. We need programs in which the information can be collected as rapidly as possible so that we can come to conclusions as to how to apply these results more quickly.

DR. WOOLF: Dr. Cooper said, or implied, that by treating phenylketonurics, we were laying up an extra genetic load for future generations, if I understood you correctly.

The magnitude of this load can, in fact, be calculated reasonably accurately by the principles of population genetics.

If you assume every treated phenylketonuric grows up normally, marries and has the average number of children, then in somewhere between 50 and 51 generations, the gene frequency will double itself. That's quite a long time.

DR. COOPER: Dr. Koch, I didn't suggest, and I really want this to be in the record, that we not treat the childhood diabetic. And, again, if I were the parent of a diabetic child, I would want that child treated.

I am simply saying that society is building deferred problems. I don't know how we are to cope with them, but they are problems which are emerging. The very success of medical progress is what is creating the more difficult area of treating constitutional disorders, degenerative disorders of all kinds to enable people to live to older ages. I simply alluded to that generalized problem.

On the matter of pressure for legislation, we have to go back to those sequential acts which have an effect. When the Children's Bureau took the position that every newborn child should be given a PKU test it paved the way and opened the door for the National Association for Retarded Children to take that Children's Bureau policy and to build upon it a legislative program.

Although the Children's Bureau was in the wings and did not come forth saying, "We are in favor of legislation," it did have a hand in recommending this universal screening approach. It participated to that extent. Whether silently it had some participation which is not on the record, I do not know. Government agencies have not been reluctant to participate in the politics of their own affairs.

Dr. Guthrie, I must apologize to you, but I raise the personal question of your dual hat
wearing. You said that you have problems of retardation in your immediate and related family.

I think in some respects and with my apologies, it makes you a party at interest. I think that you cannot disassociate yourself from your subjective interest as it pertains to your scientific interest. I would find it very difficult. Perhaps you can, but there is the possibility that you cannot. It is very difficult to separate the two. This is why we have all kinds of qualifying procedures in the courts of law, particularly as to who are competent witnesses. I could understand in some proceedings where you would be dismissed as a prejudiced witness. That is, you have a stake in your own conclusions.

DR. GUTHRIE: Excuse me. That was my point. I wanted you to know my prejudice. Otherwise, I wouldn't have told you.

DR. COOPER: But it is there, nevertheless.

DR. GUTHRIE: Of course it is. I am glad of it in this case.

DR. COOPER: All right, but I am simply underscoring the fact.

On the matter of wasted money, I think I did say in my paper that we must expect error. Error is in the nature of normal scientific progression. But we can avoid error if we follow some normal procedures in experimental design.

On the matter of the pilot testing and additional testing, I made a distinction between the research phases and the application phases. The point that I tried to make is that we jumped too quickly into application. We did not know enough. This Conference as a whole, I think, is testimony to that statement.

In the long reach of time, in the hundreds of thousands or millions of years people have had phenylketonuria. Does it really matter whether we wait a decade longer or two or three to assure ourselves that the old dictum of medicine is not dishonored -- "Above all, do no harm"? I think that we have to take a little more time to let these problems work themselves out.

The question is still open, do we need legislation at all? Do we need legislation as Senators Kennedy and Prouty have proposed in a bill for the research funding and promotion and facilitation in any metabolic disease area which results in mental or physical defect? Would we be justified socially in having a nationwide screening program for maple syrup urine disease where there is so low an incidence?

The question has been put to me, what price human life? One of the NIH directors raised that question with me in this area. I think it is a proper question, but we must consider it relative to the deployment of all of our resources. The National Foundation for Neuromuscular Disease wants a national genetic alert for agammaglobulinemia, 6 glucose dehydrogenase--I can't pronounce them all. The incidence of them has not yet been established. How many of these shall we grind into law? To what extent should society encumber itself with these laws?

I am confused because in the State of Massachusetts, we had almost a complete voluntary programing, but we had to have a law.

In my own State of Maryland, we had almost complete voluntary programing, but we were forced into a law.

I think perhaps I have covered it sufficiently.

DR. ANDERSON: I want to make one comment perhaps and that is that in our design of this conference, what has transpired was exactly what Dr. Swaiman and I hoped would transpire. We did not want Dr. LaDu to agree with Dr. Kaufman, nor did we want Dr. Bessman to agree with Dr. Udenfriend.

We picked those people whom we felt were on certain sides of certain fences. We were asked if we would be willing to consider the development of a Conference of this nature. We wanted it to be a Conference that we felt would be an effective one to bring together the best disciplinarians that must relate to this total problem.

One of the finest things about submitting a request to the Children's Bureau, particularly this one, was that we did not have to define the experimental protocol for a committee review. And I think perhaps, Dr. Cooper, if it had been subjected to committee review, the protocol might not have been approved.

So I am only trying to express the freedom that the Children's Bureau has given us to bring together what everyone has long felt necessary--this kind of exchange of opinion.
I have been asked to say a few words about the implications of the legislation on PKU. The scope of this drive for compulsory testing with all that it entails is far wider than its well-meaning proponents envision. I think we are on the verge of a very important finding in mental retardation by improper emphasis. We are skirt-ing an issue of enormous import which might have 50 to 100 times the beneficial impact of any contemplated good that could come out of this program for phenylketonuria. If we continue with our present strategy we will necessarily divert our eyes from the vast opportunity now available.

It might be instructive to review the natural history of disease. For example, diabetes was known until about 1920 as a fatal disease, as a severely emaciating, metabolically crippling disease. When insulin was discovered, all of these people no longer were doomed.

Then a remarkable thing began to happen. As we studied diabetes it appeared as if there was an epidemic because a great many more cases of diabetes were found than had ever been suspected. This epidemic has continued. In a meeting in Washington, D.C. about a month ago, sponsored by the American Diabetes Association, the remarkable statement was made that one out of two people over the age of 60 has diabetes. There weren't that many diabetics in 1920.

Why did this happen? It was a question of diagnosis. Our criteria of diagnosis of diabetes changed as our knowledge grew. We no longer call diabetes severe ketosis, emaciation, acidosis and death. We diagnose diabetes on the basis of normal behavior of the blood sugar. We have learned to detect the subtle manifestation which only rarely progresses into the serious clinical entity which was the first observed form of the disease.

Another example is cystic fibrosis. The first cases I saw of cystic fibrosis were invariably fatal. It was also an extremely rare disease. The diagnostic signs of cystic fibrosis were pulmonary disease, cardiac disease, mal-absorption syndrome, and death.

Now, what is the diagnostic basis of cystic fibrosis? It is an abnormality in the electrolyte content of the sweat. When a patient has such an abnormality together with minimal signs of gastrointestinal, pulmonary, cardiac, or hepatic disease, we call it cystic fibrosis. As a result of the change in diagnostic criteria, there is a verifiable epidemic of cystic fibrosis. We seem to have become very expert in curing, or at least "caring for," cystic fibrosis. The mortality has diminished remarkably.

But, are there fewer children dying today of cystic fibrosis in proportion to the population than died 20 years ago when we first found out about the disease? Or is it possible that the kind of children who were never considered to have cystic fibrosis in the past are not dying today as they didn't die before? Are we "curing" mild cases?

This is true of every disease known to man. A doctor has to observe something seriously wrong to discover disease. He discovers a new disease, then further study reveals minor variations which do not affect living or life.

Why can't this be true of phenylketonuria? In fact, we are finding that it is true.

The question then arises as to how much credit we are taking for curing or preventing mental retardation in children who would never have been retarded? Nobody knows. In fact, if we continue to treat every child, we will never know whether PKU is a disease which manifests only rarely a mental lesion.

The personality lesion in PKU is another story. It was alluded to by Dr. Fuller and we have seen this in untreated and treated children. The Toronto Children's Group have also observed the personality abnormalities.

We have a case, which was reported as success of treatment in one of the more exuberant series, 135 IQ. Unfortunately, this child is a severely abnormal person. Many adult patients
with PKU have been found by accident in mental institutions. They are really psychotic with normal IQs. Many of the PKU patients treated or untreated with normal IQs are semi-psychotic. When we record a diagnosis of PKU on the basis of a blood test, are we recording a prognosis of insanity? If we are we had better be more sure of our diagnostic criteria than we now are.

Discoveries in medicine go through a regular pattern. The normal turnover in the marketplace of ideas in medicine is very rapid. The Nobel prize was given recently for a phenomenon shown on a movie film. This was the famous cortisone treatment of arthritis. Arthritis was cured, and you could see it in the movies. The cortisone turned out to be a psychic energizer. It turned out to be a pain killer. The disease progressed. There was no prevention of the degeneration due to arthritis.

Each new discovery reminds us of the great stone face in Washington Irving's famous story. Every new highly publicized dramatic cure that comes along is the philosopher's stone, the elixir of life. Then, we review and refine our ideas in the light of the cold realities of medicine.

This has been the usual interplay in the medical market. There is no other way it can happen. The innovator must have the initial enthusiasm to carry him through the wall of resistance and ignorance and refusal to change. Every new idea is an insult to those who were working on the same problem and missed the idea. And so they strive to kill it. This is a fact.

The individual who has a new idea will fight to preserve it and must have the enthusiasm to carry it through. And, don't let anybody tell you science is cold and objective. If it is, it is dead.

Let us now recall the sulfanilamide prevention of gas gangrene. I think there are people in this audience old enough to remember what happened during World War II. At Pearl Harbor, there was a vast number of casualties - somewhere around 25,000 - many compound, comminuted fractures. What was done? No medical team in the world could handle that many casualties. They put sulfanilamide in the wounds of these casualties because it was a miracle drug. That's all they could do. Although they had many complications, one complication they did not see in Pearl Harbor was gas gangrene. Almost none of the severe casualties got gas gangrene.

What was the experience in the past? The experience in World War I was 50 to 70 percent. Apparently a dread complication was prevented just by pouring sulfanilamide in the wounds. A war was going on and with military dispatch things happened. In the breast pocket of each soldier's shirt he had to carry a little paper salt shaker with a string attached to it. And, if you were wounded in the military service, you could reach in if you had a hand free - left, I might say - and stick it into the pocket and take out the little envelope. If you had teeth, you could pull off the string. And, then you could pour this sulfanilamide on the wound, if you could reach it.

Procedures for putting crystalline sulfanilamide into surgical wounds were developed, in some cases by using shakers suspended from the ceiling of operating rooms. The intern would hold the abdomen open at the end of the operation with a couple of retractors, they would pull a string, and a fine cloud of sulfonamide would float down into the wound.

Dr. Robert Elman, my professor of surgery, expressed concern at a public meeting, saying that controlled studies should be done to validate the observation. An important man in our medical society stood up at a meeting and said, "Dr. Elman, if you withhold this lifesaving therapy from any patient and I know of it, I will volunteer to testify against you in a malpractice suit. I am tired of all this guinea pig business with people. You have no heart. Twenty-five thousand patients are enough", and the numbers reverberated from the rafters.

Dr. Elman didn't do the controlled experiment but some people in England did. They randomly chose patients as they moved into the operating room and treated some of them with sulfanilamide and some did not get the crystals in the wound. They found what is now obvious. There was less infection, and lower morbidity in the untreated group of patients. Everyone then realized what they had always known - that the crystals acted like a foreign body.

The little salt shakers were unscrewed from the ceilings in the hospitals. The little packets of sulfanilamide came out of the pockets. How did all of this confusion develop? It was very simple. The pineapple plantations of Hawaii are not the manure-covered fields of France. There were no bacteria in the dirt in Hawaii to cause gas
gangrene. That is very simple and the physicians who first reported the sulfonamide therapy and those who followed them should have known it.

They were very important people, dedicated men. They had the interest of our army and our people at heart. But they were wrong and their good intentions cannot absolve them of the onus of uncontrolled ill-conceived reporting.

During World War II another therapeutic irrationality was ballyhooed into common usage, the treatment of liver failure with amino acids. It is possible during times of war that we develop what may be called the "war on" philosophy.

The war on cancer, the war on crime, the war on mental retardation, the war on dope addiction. These neat phrases assume there is a simple problem and a simple, usually violent, or magical solution. The "good guys" will destroy the enemy, and that will be the end of it. But unfortunately these simple models rarely conform to reality.

During World War II, there were many healthy young fellows in the armed forces who got hepatitis. A few physicians in charge of the hepatitis program either refused to read the literature or didn't bother. They didn't know that amino acids could not be metabolized adequately by diseased liver and ammonia would accumulate to toxic levels, although much had been written about this. It was well known that the blood amino acid concentration was elevated in liver disease, so they gave large doses of amino acid parenterally, literally to force the liver to use amino acids. This type of treatment had been tried and abandoned in the early part of the century. Most of the soldiers with hepatitis, given this regimen survived. There were no adequate controls so the occasional deaths were ascribed to other causes. Probably the treatment was withheld by these young soldiers because they were initially healthy. When it was used on civilians many died. I realize now that the amino acid therapy I used on several children with liver disease in the early 40's either killed them or hastened their demise.

I can talk about the X-ray therapy for large tonsils—a popular therapy ten years ago. We thought we were doing a wonderful thing. We avoided surgery, and probably sowed the seeds of cancer.

Medicine stumbles along through trial and error. Ideas which seem ingenious and irresistible have their vogue and are subjected to careful scrutiny, either by competitors or by disappointed practitioners. This has been the story in the past, until a couple of years ago. Then we became very wise. We decided our knowledge was so certain that we began to legislate medicine.

There is a new kind of law appearing. The law has nothing to do with the payment for medical care. The law has to do with the nature of medicine. The law says, "This is a test for phenylketonuria" and every State law on the books today except one, one State, mentions a test for phenylketonuria—not "a test for the amount of phenylalanine in the blood," it says—"a test for phenylketonuria." By law we have defined the disease. This is incredible. Impatiently the law says "We know the definition of a disease."

Let us consider the dimensions of this certainty. We have sat here for three days, unable, within wide limits, to come to agreement as to the lower limit of the phenylalanine level in the blood diagnostic for phenylketonuria. And yet we make it compulsory in 27 states that a patient take a test, the answer to which is equivocal.

This is how we have made a definition of the practice of medicine.

As Dr. Cooper pointed out, we have another problem. When you define the practice of medicine by prescribing the basis for the diagnosis of a disease, you are not defining just a diagnosis; you are implying the treatment. And, when you imply the treatment, it is a bold man who does anything but that. Looming over him is the force of public policy. Even the PKU laws which are not compulsory say it is a matter of public policy. And, if you defy the laws of public policy, I would like to know what happens in a malpractice suit.

These are the problems we have created for ourselves. Perhaps it is too late to do anything about phenylketonuria. The details of whether we are right or wrong in phenylketonuria mean nothing.

The question is: Is this a model to be repeated again and again? Should we forge a straitjacket of law so that each new rare disease, that some individual finds, defines the future diagnosis and care of all such cases? If we make screening laws, the first publications, which always report success, will become the basis of medical practice, and woe to him who tries anything else.

Where does this frenetic haste for legislation stem from? It stems from our tremendous
eagerness to get cases to study. This is the most charitable definition that can be put on it--that we were eager to find children who had the disease. We were impatient with voluntary use of screening, even though it was extending rapidly through the country. We made a legal vacuum cleaner to find these children. We justified it on several different bases, depending on the audience. To each other we said that screening and therapy are experimental and must be tested. But I would like to see a single law in the text of which, or the preamble of which, it is said, "This is a law to compel people to take part in a research project." Yet this is how we justified it to each other. I have never seen evidence of testimony to a legislative committee in any State--that we were not sure about the screening tests, or about the therapy.

They had a test for phenylketonuria. That's all we wanted to talk about. The uncertainty of the test, the questionable data on therapy, these were not discussed. Now, after more than two years of compulsory testing, these are the very problems we are discussing. And we can't agree at this time on the values which should have been established before any laws were put on the books.

The thought I want to leave with you is this. You may not agree with it. You may not think there is any sense at all to it. But I want to point out that if the current drive for compulsory screening goes on for very much longer, there will be no orderly and intelligent progress in the practice of medicine, there will be no investigation. The man with the loudest mouth with the closest access to publicity will determine diagnosis and therapy and the laws he encourages the layman to write will make certain that no one does anything different.
Membrane transport plays a complex role in the total display of the phenylketonuric phenotype. Specific interactions during membrane transport may contribute significantly to the genesis of the mental retardation of this disease, although the final evidence for this interpretation is still forthcoming. For purposes of the present discussion, transport functions in phenylketonuria may be considered under three headings: 1. Membrane transport of amino acids. 2. Membrane transport of non-amino organic acids. 3. Non-ionic diffusion of weak organic acids. Each transport function will be discussed in turn; general considerations will precede specific reference to transport phenomena in phenylketonuria.

I AMINO ACID TRANSPORT

General Comments

Membrane transport of amino acids is a catalytic process of particular significance in the intermediary metabolism of these compounds; the process is of general biological importance. The recent introduction of non-metabolizable amino acid analogues has allowed more specific investigation of cellular transport as a function distinct from cellular metabolism of the solute. Several extensive reviews have explored the ever-increasing body of information on biological transport which has become available in the past decade. 

Transport mechanisms are analogous to enzyme-catalyzed reactions in many ways, and may be described in the terms used traditionally to describe enzyme systems. Intact cellular transport systems for amino acids exhibit substrate specificity, an ability to establish concentration gradients, and a dependence on metabolic energy.

Solute contact with the access system apparently occurs at a "reactive site" wherein resides the specificity of substrate transport. Saturability is a characteristic of membrane transport, implying a finite limit to the access function available to a particular solute. Optical configuration, position of the amino group, R-group configuration and ionization of carboxyl and amino groups are each determinants of the individual characteristics of different solute transports. Solutes with closely related chemical and molecular characteristics can be competitive inhibitors of each other's transport.

The nature of the reactive site is unknown, but it is presumably a function of the protein layers of the membrane. Separate access systems for influx and efflux transports probably exist and modification of either can affect the equilibrium state with respect to intracellular accumulation. The specificity of a transport system is most easily visualized in terms of the surface conformation of the protein in the membrane, implying that transport processes should be subject to genetic regulation.

After contact with the membrane, solute transfer across the membrane occurs without metabolic conversion of solute. Temperature and pH optima for transfer are exhibited. Centrifugal transport is both energy and ion (Na\(^+\), K\(^+\)) dependent. The initial rate and equilibrium kinetics of the total process can be described in terms which can be depicted by conventional Lineweaver-Burk plots.

The majority of investigations have described cellular transport function in the tumor cell or in micro-organisms. However, there is increasing interest to describe transport in vitro in...
normal multicellular tissue such as intestine, kidney, and brain. One of the logical extensions of such investigation of amino acid transport is to study transport characteristics in living human subjects. Here too, the transport function exhibits saturability and competitive inhibition both in the intact subject and in biopsy tissue.

The functional organization of a membrane system for amino acid transport can be depicted by the simplified scheme of figure 1. This scheme serves to define in general terms the mechanism by which hyperaminoaciduria, for example, could occur in the human subject where this phenomenon is a reflection of modified amino acid transport in the renal tubule. The mechanisms underlying abnormal aminoaciduria are described in table 1.

**Table 1.** --Mechanisms of membrane transport impairment underlying hyperaminoaciduria

1. Increasing saturation of the system by its substrate ([S] in figure 1).
2. Competitive inhibition of substrate transport by another molecule ([I] in figure 1), with overlapping affinity for the system.
3. Acquired or hereditary modification of the reactive site (dotted outline; viz. 2 in Figure 1).
4. Acquired or hereditary impairment of the transfer process (viz. 3, Figure 1).

There is abundant evidence for genetic regulation of membrane transport of amino acids. Specific mutations affecting amino acid transport in microorganisms can be produced. There is also very provocative information indicating that some microorganisms transport particular amino acids by two systems with differing characteristics, one having high affinity for a specific substrate but low capacity, and the other system exhibiting lower affinity usually with overlapping (group) specificity, and high capacity. Only one type of transport system may be affected by mutation. Whether evidence will be found in man for multiple access systems with substrate and group specificity is an interesting challenge for the investigator. Compilation of existing data concerning multiplicity of transport system in man indicates that in renal tubular and intestinal transport cells there are at least five major transport systems exhibiting group specificity (table 2).

**Transport of L-phenylalanine**

The equilibrium kinetics of L-phenylalanine accumulation by mammalian tissue in vitro have been investigated using hamster intestinal preparations, rat kidney cortex slices, and mouse brain slices. Concentration against a gradient was observed in each experimental system. Equilibrium is achieved quickly and the capacity for phenylalanine transport is limited when compared with that for small neutral aliphatic amino acids. The equilibrium Km value is $1.8 \times 10^{-3} \text{ M}$ for intestinal sacs, and $0.4 \times 10^{-3} \text{ M}$.
Table 2.—Membrane transport systems in man

<table>
<thead>
<tr>
<th>Major System (Group specificity)</th>
<th>Amino Acids</th>
<th>Specific Systems (Substrate specificity implied)</th>
<th>Disease *</th>
</tr>
</thead>
<tbody>
<tr>
<td>-amino</td>
<td>β-ala, Tau, βAla</td>
<td></td>
<td>Hyper-β-alaninemia* (Scriver et al, 1966)</td>
</tr>
<tr>
<td>-NH² Systems</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acidic</td>
<td>Glu⁺, Asp⁺</td>
<td></td>
<td>None known (viz. Webber, 1963; Scriver, 1965)</td>
</tr>
<tr>
<td>Basic</td>
<td>Lys, Arg, Orn, Cys (S-S)</td>
<td></td>
<td>Cystinuria (Bartter et al, 1965) (3 phenotypes)</td>
</tr>
<tr>
<td>Neutral 1</td>
<td>Pro, Hypro, Gly</td>
<td></td>
<td>Hyperprolinemia* (Scriver et al, 1964) Familial glycinuria? (deVries et al, 1957)</td>
</tr>
<tr>
<td>Neutral 2</td>
<td>The remaining neutral a.a.</td>
<td></td>
<td>Hartnup Disease (Scriver, 1965) &quot;Blue diaper&quot; syndrome (Drummond et al, 1964) Methionine Malabsorp. (Hooft et al, 1965)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Disease elucidating transport system.

"Saturation-competition" mechanisms for aminoaciduria.
when plasma phenylalanine concentration approaches 2 mM. A maximum rate of transport for L-phenylalanine has not yet in fact been defined in man, but it is probably in excess of 20 μM/min/1.73M².

Absorptive transport of phenylalanine from the intestine, in phenylketonuria and allied diseases is presumably normal. The time-response for phenylalanine appearance in plasma after oral loading of L-phenylalanine is comparable to the control subjects. This suggests that there is no delay in absorption. Fecal phenylalanine is apparently normal in untreated patients.

Phenylalanine concentration of cerebrospinal fluid is elevated in phenylketonuric patients. Assuming that the CSF concentration reflects cerebral intratransport of 5-OH tryptophan (5-HTP), consideration of both is necessary to evaluate tryptophan aberrations in phenylketonuria.

Augmented urinary excretion of indole derivatives and diminished circulating 5-HT and 5-HIAA excretion constitute the major aberrations in the untreated patient. Correlative studies in experimental phenylketonuria in the rat, mouse, and monkey reveal an indole defect similar to that in man. Normal circulating indole levels are restored by feeding precursor (L-tryptophan or 5-HTP) in the presence of phenylalanine loading; all indole abnormalities are overcome by reducing the phenylalanine intake. The ameliorative effects have been documented both in man and in the experimental animal.

The indole defect is associated with a reduction in brain 5-HT concentration in experimental phenylketonuria. Temporary or permanent behavioral deficits are thought to be associated with the 5-HT abnormality but the actual significance of such observations in the experimental animal is open to discussion.

Transport of L-tryptophan. Active transport of this amino acid has been studied in intestine. Competitive inhibition between phenylalanine and tryptophan during transport has been documented. This phenomenon probably explains the abnormal urinary indole pattern which has been observed in phenylketonuria. Oral ingestion of tryptophan augments the abnormal indoluria and restriction of phenylalanine decreases it. Significant amounts of free tryptophan are found in the feces of untreated patients.

The last-mentioned investigators proposed that phenylalanine competes with tryptophan for absorption, thereby causing intraluminal accumulation of tryptophan (figure 2 and Section III). Phenylalanine must be present intraluminally in elevated concentrations to be an effective inhibitor of intestinal transport. Christensen has demonstrated that equilibration will occur between the intestinal lumen and plasma. Therefore, elevated levels of plasma phenylalanine will achieve a higher steady-state distribution with regard to the intestinal lumen and produce elevated levels of phenylalanine therein.

Whether competition for tryptophan transport at gut and tissue levels actually plays any role in the mental retardation of phenylketonuria is unsubstantiated. It is probably indicative that defects in tryptophan transport in other diseases

Figure 2. Tryptophan metabolism in PKU

Try. → gut degradation

(1) *Absorption (gut) → IAA etc.

(2) Try → 5-OH Try → (3) 5-HT (4)

etc.

CNS Transport

* Transport steps

The possible impairments of tryptophan metabolism in phenylketonuria include inhibition of membrane transport and of enzyme catalyzed reactions.

Steps 1 and 2 refer to membrane transport of tryptophan and 5-HTP respectively. Inhibition of step 1 is operative in the intestine in phenylketonuria and accounts for the abnormal excretion of indolic acids. Whether significant inhibition of cerebral transport of tryptophan also occurs, is still undecided. Transport of 5-HTP (step 3) was formerly believed to be critical for cerebral 5-HT synthesis (step 4), however tryptophan hydroxylation (step 2) has now been demonstrated in brain tissue.
Transport of 5-Oh tryptophan. Although no direct demonstration of active transport of 5-HTP has been made with intestinal preparations, there is evidence for mediated transport in brain in vitro and in vivo. L-phenylalanine competitively inhibits this transport. Yuwiler and coworkers reviewed the origins of brain serotonin depletion in experimental phenylketonuria, and proposed that inhibition of 5-HTP transport was probably one of the important causes.

Inhibition of 5-HTP transport would be important if there were no cerebral synthesis of 5-HTP as precursor to 5-HT in that organ. 5-HT itself is not transported by brain tissue and until recently there was no evidence that brain could hydroxylate tryptophan. Now, however, it has been shown that brain can synthesize 5-HTP from L-tryptophan by a specific hydroxylase. Therefore, the decrease in brain 5-HT, circulating 5-HT and 5-HIAA excretion in natural and experimental phenylketonuria cannot be a reflection solely of impaired 5-HTP transport particularly in brain would have to be the critical step, if transport reactions play any role in phenylketonuria.

Transport of L-tyrosine

Mediated transport of L-tyrosine has been observed in intestinal segments, and in kidney in vivo and in brain both in vitro and in vivo. L-phenylalanine is a competitive inhibitor of L-tyrosine uptake by these tissues. Guroff and Udenfriend suggested that by this mechanism phenylalanine might impair biogenesis of neuroregulatory catecholamines in phenylketonuric brain.

The experiments by Yuwiler's group have not provided any new information in this area. Their observation that brain norepinephrine levels were unaffected by phenylalanine loading, cannot be evaluated since phenylalanine loading in the normal animal will also produce an elevation of tyrosine in plasma, brain and other tissues. Boylen and Quastel could not demonstrate inhibition of catecholamine synthesis in guinea pig adrenal medulla slices when L-phenylalanine was present in high concentrations in the medium. Synthesis was impaired only when phenylpyruvate was used, and the effect involved inhibition of dopa decarboxylation. Fellman and Devlin found normal catecholamine concentration in post-mortem phenylketonuric adrenal glands. Thus it seems that any invocation of impaired transport as the underlying mechanism of catecholamine depletion in phenylketonuria is still in need of further proof.

Effects on the Intracellular Pool of Free Amino Acids

Interest has recently been directed to the effect of phenylalanine on the intracellular amino acid pool in brain and other tissues. Following loading in vivo the free pool was altered more in liver than in brain in the mature rat. The fetal rat does not exhibit any response to maternal injection with L-phenylalanine until late in pregnancy. The free amino acid pool is disturbed when brain slices are incubated with phenylpyruvate, but not with L-phenylalanine. The in vivo effects are therefore probably more dependent on transamination reactions with phenylpyruvate than on L-phenylalanine competition with transport functions. The significance of these observations and their relation to brain growth will undoubtedly be pursued in the future.

II MEMBRANE TRANSPORT OF NON-AMINO ORGANIC ACIDS

Considerable transport specificity exists within major classes of chemical compounds; strict transport specificity is found between different classes of compounds. This is clearly the case with reference to transport of an amino acid and its equivalent keto acid. Lin and coworkers observed that hamster intestinal sacs actively transported L-phenylalanine, but not phenylpyruvic acid. Mutant transport phenotypes which abolish amino acid transport (e.g. tryptophan transport in Hartnup disease) do not impair transport of the equivalent keto acid.
The excretion patterns of two non-amino organic acids (phenylpyruvic acid (PPA) and 5-hydroxyindoleacetic acid (5-HIAA)) have received considerable attention in phenylketonuria. Most of the investigations dealt with the altered metabolic origins of these two compounds in phenylketonuria. The role of transport in the excretion pattern should also receive some consideration.

**Phenylpyruvic Acid**

Renal excretion of PPA has been investigated in man. Large amounts of PPA were found in urine at low plasma levels, even though approximately 90 percent of PPA is protein-bound in plasma. Renal excretion by a tubular mechanism is strongly suggested by these findings. High renal clearance rates of PPA were found in untreated human phenylketonuric subjects at endogenous PPA levels. The urinary excretion rate of PPA was reduced with benemid (p-di-n-propylsulfamylbenzoic acid). Alkalinization of urine (pH 7-8) had no effect on PPA excretion (see Section III). These observations indicate mediated tubular secretion of PPA.

**5-Hydroxyindoleacetic Acid**

Renal handling of 5-HIAA has been investigated in vivo using the living dog as a model system. Because 5-HIAA is significantly ionized at neutral pH and has a polar group in the ring, it cannot be excreted by simple diffusion mechanisms (see Section III). Eighty percent or more of 5-HIAA is protein-bound in dog plasma. Renal clearance of 5-HIAA exceeds the glomerular filtration rate at lower plasma values; a maximum secretion rate is reached when the plasma level is approximately 35 mg percent. Urinary excretion of 5-HIAA is inhibited by probenecid. These findings indicate that a mediated tubular secretion accounts for 5-HIAA excretion.

Evidence for mediated transport of 5-HIAA was also obtained in vivo in the rat and in vitro using rat, guinea pig and rabbit kidney slices. Concentrative uptake of 5-HIAA in vitro is dependent on aerobic metabolism and is inhibited by probenecid.

**III NON-IONIC DIFFUSION OF WEAK ACIDS**

Weak organic acids of small molecular size, and with a large unionized fraction in the neutral pH range, can diffuse in the lipid component of biological membranes. Excretion of the free unionized acid fraction from peritubular plasma into urine will be maximal into alkaline urine. Free indole-3-acetic acid (IAA) is a weak organic acid (pKa = 3.8). It may circulate in the body either as protein-bound free acid or as a conjugate of glutamine. Urinary excretion of IAA has been studied in normal man and in the rat. At low urine pH (4.5) man excretes 40 percent of total IAA (free + conjugate) in the free form; at pH 8.0 90 percent is excreted in the free form. Similar observations were made in the rat. Following injection in the rat, IAA diffuses down a concentration gradient into liver, muscle, kidney, but not into brain. IAA is found in increased amounts in the urine of untreated phenylketonurics and this abnormality is dependent upon the activity of normal gut flora, which convert unabsorbed tryptophan into indolic acids. IAA excretion in phenylketonuria is therefore analogous to the situation in Hartnup disease, in sprue and in the blue diaper syndrome. In each of these disorders, intestinal absorption of L-tryptophan is impaired for different reasons; but regardless of initial cause, the final urinary excretion of IAA observes universal diffusion laws. Excretion of IAA by the phenylketonuric patient will be no different.

\[
R = \frac{1 + 10(pHu - pKa)}{1 + 10(7.4 - pKa)}
\]

*The equation describing excretion of a weak acid into renal tubular fluid is:* 

\[
R = \text{concentration ratio} \\
pHu = \text{tubular urine pH} \\
pKa = -\log_{10} \text{dissociation constant of the weak acid}
\]

Provided by the Maternal and Child Health Library, Georgetown University
Phenylacetic acid is a weak acid and should diffuse easily. However, it is effectively conjugated with glutamine in man; excretion of the free form has not been studied specifically in normal or phenylketonuric subjects.

Phenylethylamine is a weak base and it will therefore accumulate in acid media. The excretion of this compound in phenylketonuria and its uptake by brain (which is acid relative to plasma) should warrant study.

**COMMENT**

**Implications for Treatment of Phenylketonuria**

If impairment of transport or metabolism, of tryptophan and tyrosine (or of other amino acids) is of any importance in phenylketonuria, then specific counteraction of these abnormalities should be beneficial. Unfortunately, at the present time there is no indication that impaired transport plays a single decisive role in human phenylketonuria. Nor can we expect the experimental models of phenylketonuria (obtained by loading with phenylalanine) to provide unequivocal evidence in this direction, because in all such models there is an associated disturbance in tyrosine metabolism. Assessment of reports by workers who have tried dietary supplementation with tryptophan derivatives in man and in the experimental animal is difficult because of the lack of precise techniques to allow an evaluation of improvement in behaviour, which can be correlated with a single pharmacological event such as restoration of 5-HT levels in brain. An objective unequivocal correlation has never been obtained in the human patient and, as mentioned above, associated metabolic abnormalities cloud the issue in the experimental models. The issue is of some importance because tryptophan and tyrosine supplementation in addition to the low phenylalanine diet during the early days of life could be attempted. It would be essential to prove, however, that neurohormonal imbalance existed and was detrimental to the patient, and that restoration of normal balance could be achieved by dietary means without producing additional amino acid imbalance.

**REFERENCES**

41. Woolley, D. W., and van der Hoeven, Th.: Prevention of a mental defect of phenylketonuria with serotonin congeners such as melatonin or hydroxytryptophan. Science, 144:1503, 1964.
DISCUSSION

DR. ANDERSON: Dr. Scriver was kind enough to refer to the preliminary work on competitive absorption by Dr. Yarbro and me, and I would like to ask Dr. Yarbro to briefly discuss this work.
As I see it, the problem hinges on the following: Competitive inhibition of phenylalanine transport by L-tryptophan, and of tryptophan transport by phenylalanine has been demonstrated. I think quite satisfactorily and elegantly in intestinal sacs and brain slices. The question is, does this inhibition have any bearing on the pathogenesis and treatment of this disease?

Dr. Udenfriend: We all admit transport is not an end in itself. It is a mediation of processes leading to something else so that we consider it one of the intermediate catalysts in metabolism. The idea then is that we should think in terms of what can happen to tryptophan and tyrosine. I think we should place leucine and isoleucine in the same category, because we can obtain just as much competition between these particular amino acids and the aromatic amino acids. One must consider disturbances not only in serotonin and noradrenaline metabolism, but in other metabolic processes including protein synthesis. It may be that at certain stages of brain development incorporation of amino acids into protein is more important than their incorporation into noradrenaline and serotonin. Later, the reverse may be true.

In the case of a humoral agent like serotonin, whatever it does, I don't think that a slight change in its concentration in brain is an indication of an important change in its utilization or metabolism. We now know that the rate of turnover of many substances can be considerably altered without changing the concentration. This is particularly true of noradrenaline. One can alter the rate of turnover, as much as threefold in many tissues including brain, as measured by incorporation, without changing the absolute amount present. It appears as though there is present some regulatory mechanism which may be an end-product inhibition that sees to it that the more agent that is released by stimulation, the more is made, and vice versa.

The idea of giving more tyrosine, tryptophan or other amino acids to phenylketonurics has entered many people's minds. One of the things that interested me is Dr. Waisman's statement that of the many amino acids he has given to animals in large amounts, tyrosine and tryptophan are the least toxic. That is they don't alter the behavior of monkeys or rats. If he can assure us they are not toxic then perhaps they can be given to patients in large doses to overcome an apparent decrement in noradrenaline and serotonin concentration. Of course, one may run into the problem of amino acid imbalance.

The idea that two transport systems may be available to a single amino acid is really one of the more intriguing aspects of transport at the present time. Although I think one should be cautious before applying this idea to man, we do have data in our laboratory which suggest that amino acids in man may be transported by two systems. In bacteria, on the other hand, amino acid transport is organized in such a way that a transport system with high specificity may be used for protein synthesis. Another type of transport system, which operates at a high capacity but with much lower affinity, can be used for energy metabolism. Therefore the organism can respond differently to its environment. Kepes has discussed this phenomenon in relation to adaptive significance for the organism.

Dr. Bickel: Linneweh and coworkers (Klin.Wschr., 41:253, 1963) have done some tracer work with arginine and leucine, and have shown that in rat ganglion cells there was a decreased uptake of labelled leucine when the phenylalanine level was increased.

So I agree that brain damage may not only be due to a deficiency of serotonin or tyrosine, but also of some other amino acids which are important for brain metabolism. If this were so, one wonders if supplementation of the diet with the deficient amino acids might not be another approach to preventing brain damage. But I think it is really easier to lower the phenylalanine blood level than to supply other amino acids in unknown quantities.

Dr. LaDU: You were asking about some experiments that other people might have done concerning transport in competition with phenylalanine. It reminds me of a rather na"ive series of experiments that we carried out quite a few years ago when I was still at NIH. The reabsorba-bility in the phenylketonuric seemed to be unchanged. This is really unfortunate. If we have the equivalent of a renal glycosuria where the ability to reabsorb phenylalanine were impaired, we might have a somewhat advantageous stage in controlling the blood level and we wouldn't have to be so fussy about the diet.

We fed guinea pigs phenylalanine and then injected over a period of time amounts to keep the blood level 40 to 60 mg percent. It was easy to
maintain, and the micro method was employed so that we knew the plasma concentration. We attempted to find any other substance injected intraperitoneally that would compete with phenylalanine reabsorption in the kidney. We would then expect to find extra phenylalanine excreted in the urine.

As you can guess, we tried a number of amino acids and phenylalanine analogs, the D forms and so forth. We really found nothing that was an effective competitor, enough at any rate to cause significant increased excretion or poorer reabsorption of phenylalanine.

DR. SCRIVER: This is just the sort of work we are doing on some phenylketonurics at the moment. It is Quastel I believe who had the idea that the ideal answer for this disease is a non-metabolizable synthetic analog of phenylalanine which would partially block the transport of phenylalanine at the gut level and all you would have to do would be to give a dose of this compound daily in the diet and forget about the phenylalanine restriction.

DR. ROUSER: One phenomenon with regard to changing levels of amino acids in blood might be significant in phenylketonuria. In earlier studies in our laboratory, the levels of free amino acids in plasma, leukocytes, and erythrocytes were determined in animals and in humans in a variety of situations. Of particular interest was the observation of a phenomenon that we call "chasing out" since when one amino acid is presented to the cells at very high concentration, it is taken up by the cells in relatively large amount and other amino acids are released. We observed this in particular with glutamine and Roberts and coworkers observed this earlier with ascites tumor cells when glutamine was administered intraperitoneally. The characteristic levels of free amino acids may return to the cell when the large elevation of one amino acid is abolished as by metabolism of glutamine first to glutamic acid and then to other products. I therefore wonder if a high level of phenylalanine may not result in a change in the levels of amino acids of tissue cells. Such changes could alter intermediary metabolism. The leukocyte may be taken as an example of a more or less typical tissue cell while the erythrocyte is relatively atypical and does not show this type of phenomenon.

DR. SNYDERMAN: We find that there is a constant ratio between plasma and red blood cell concentrations of amino acids. If the plasma level is raised by administering a load of an amino acid, the red cell level also rises and the ratio remains constant. Conversely, when the intake of an amino acid is reduced, both the plasma level and the red cell level fall and the ratio is unchanged. There seems to be a process resembling membrane diffusion.

DR. SCRIVER: I want to pick up the use of the word "diffusion." The red cell has a concentrative transport mechanism like any cell, but because it is such a fragile cell, good concentration by a membrane transport process does not occur. The cell ruptures first. Wilson and I are studying the effect of protecting red cells with a sucrose medium, to see whether we can find concentrative transport processes like those present in kidney tissue.

DR. WOOLF: Dr. Jepson some years ago did feed neomycin to a phenylketonuric. I don't know whether he ever published this work, but he told me about it. He found the excretion of indole acetic acid fell to normal but the excretion of indole lactic acid was unaffected and it still remained high.

DR. SCRIVER: Was this a patient receiving the special diet at the time?

DR. WOOLF: I gather not. I gather this was an untreated phenylketonuric.

It looks as though we have two different mechanisms, as you might expect, because I don't know that the bacteria in the gut would produce indole lactic acid.

DR. SCRIVER: One of the things to remember is that indole lactic acid will come from D-tryptophan. There is a paper (Drummond et al., C.M.A.J., 94:834, 1966) in which this artefact has been recognized. Changes were present because the patient was receiving D-tryptophan in the diet. The early diets for phenylketonuria contained D-tryptophan as well as the L-tryptophan.
INHIBITION OF PHENYLALANINE HYDROXYLASE IN LIVER
BERT N. LaDU and VINCENT G. ZANNONI
presented by BERT N. LaDU

In spite of extensive investigations over the past few years to characterize the enzymatic system which catalyzes the oxidation of phenylalanine to tyrosine in mammalian liver, there are still difficulties encountered when one attempts to determine the activity of phenylalanine hydroxylase in a variety of tissue preparations. Even though all of the problems are not solved at the present time, some progress is being made, and it is appropriate to review the current status of the enzyme assay at this conference.

For several years we have been interested in the enzymatic hydroxylation of aromatic compounds and in the biochemical alterations in phenylketonuria. During the course of both of these studies it became evident that the assay of phenylalanine hydroxylase in mammalian tissues is complicated because of the complex enzymatic and cofactor requirements. Not only are there problems in providing the necessary constituents in optimal concentrations, but there are also inhibitors of the hydroxylase in the particulate fractions of liver (and other tissues), as well as inhibition by some metabolites of phenylalanine. It has become apparent that few, if any of the reported values for phenylalanine hydroxylase activity, including assays of human liver, have been obtained under adequate or optimal conditions.

Assay for Phenylalanine Hydroxylase

Dr. Kaufman will discuss the detailed mechanism and the individual components of the enzyme system involved in the hydroxylation of phenylalanine in another session; for the purpose of this discussion we will consider the three main components, as established by the studies of Mitoma(1) and Kaufman:(2-6)

1. The primary enzyme (phenylalanine hydroxylase)
2. The cofactor (a reduced pteridine)
3. The accessory enzyme systems - which reduce the pteridine cofactor to its active (tetrahydro) form.

The first enzymatic assay for phenylalanine hydroxylase was developed by Udenfriend and Cooper(6) in 1952. From our present understanding of the reaction, it is clear that the assay depends upon the presence of an adequate amount of pteridine in the tissue under study (no supplement is added), and upon the enzymatic reduction of DPN to supply sufficient reduced pyridine nucleotide for reduction of the pteridine cofactor. Many studies during recent years have employed this assay (or minor modifications). For example, it was used to show that the liver of phenylketonuric patients lacks the hydroxylase component.(7,8) This finding was supported by Kaufman's demonstration that phenylketonuric liver contains adequate pteridine.(9)

During studies on the purification of the enzymatic components of the overall hydroxylation system from rat and sheep liver, Kaufman developed a more elaborate assay system for the hydroxylase component; the assay requires the addition of TPN, glucose, glucose dehydrogenase, 2-amino-4-hydroxy-6,7-dimethyl-tetrahydropteridine (DMTHP)* and dihydropteridine reductase (sheep liver enzyme).(4) With this method, higher values for liver phenylalanine hydroxylase activity are obtained in several mammalian species (rat, guinea pig, and rabbit). Higher values were noted particularly in measurements of the hydroxylase activity in the liver of newborns.

* DMTHP = 2-amino-4-hydroxy-6,7-dimethyl-tetrahydropteridine

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rats since the pteridine cofactor levels are low in these tissues.

Theoretically, it should be possible to develop a more direct, simpler assay for phenylalanine hydroxylase by supplying optimal concentrations of reduced pyridine nucleotide and reduced pteridine. This would avoid the need for complex enzymatic systems to generate the reduced forms of the cofactors. Such an assay would simplify the problem of establishing maximal activity so that the rate limiting component is the concentration of phenylalanine hydroxylase.

Studies in our laboratory with rat liver homogenates have shown that both the amount of homogenate used, and the amount of reduced pteridine added, are critical variables in the assay for phenylalanine hydroxylase (Table 1). Without added pteridine, the low hydroxylase activity observed (calculated per ml of homogenate) is relatively higher when larger amounts of homogenate are used. Obviously, more pteridine is provided by the larger amounts of homogenate. Maximal activity (6.0 μmoles of tyrosine formed/45 min/ml enzyme preparation) was obtained when 0.05 ml of homogenate was assayed with small amounts of pteridine (0.26 μmole DMTHP). When larger aliquots of homogenate were assayed, e.g., 0.1 ml, the pteridine had to be increased to 2.30 μmoles to obtain maximal activity (Table 1). Thus, by using relatively small aliquots of homogenate with adequate pteridine concentrations, values for rat liver phenylalanine hydroxylase activity are obtained which are nearly four times as high as previously reported. Similar determinations with homogenates of rabbit liver showed very low hydroxylase activity (less than 0.1 μmole of tyrosine/45 min/ml of homogenate) without the addition of reduced pteridine. A higher concentration of pteridine was required for maximal activity with rat liver than with rabbit liver homogenates (Table 2). With adequate amounts of pteridine the values are three times higher than previously reported for rabbit liver. From the data presented in Table 2, it might appear that the affinity of rabbit liver hydroxylase for reduced pteridine is higher than rat liver hydroxylase based upon the pteridine concentration needed to give half

Table 1.-Phenylalanine hydroxylase activity in rat liver homogenates with various concentrations of homogenate and 2-amino-4-hydroxy-6,7-dimethyl-tetrahydropteridine

<table>
<thead>
<tr>
<th>Aliquot</th>
<th>Hydroxylase activity*</th>
<th>μmoles of tyrosine formed/45 min/ml enzyme preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05 ml in assay</td>
<td>μmoles</td>
<td>0.24</td>
</tr>
<tr>
<td>0.10 ml &quot; &quot;</td>
<td>μmoles</td>
<td>0.30</td>
</tr>
<tr>
<td>0.20 ml &quot; &quot;</td>
<td>μmoles</td>
<td>0.67</td>
</tr>
<tr>
<td>DMTHP added, μmoles</td>
<td>0.0</td>
<td>0.07</td>
</tr>
</tbody>
</table>

Phenylalanine hydroxylase activity was assayed with various amounts of the 15,000 x g supernatant fraction prepared from a 25 percent crude homogenate according to the method of Udenfriend and Cooper. The reaction was carried out at 25°C with 2.0 μmoles of L-phenylalanine. The flasks contained 5 μmoles nicotinamide, DMTHP as indicated in the table, 0.8 μmoles DPNH (except that 1.5 and 2.5 μmoles were added with 1.4 and 2.3 μmoles of DMTHP, respectively) and phosphate buffer, 0.2M, pH 7.0 to give a final volume of 1.5 ml. Incubation time was 45 minutes.

*Activity values are all calculated per ml of 15,000 x g supernatant fraction of liver homogenate.

For the sample of 2-amino-4-hydroxy-6,7-dimethyl-tetrahydropteridine used in these experiments, we are indebted to Dr. E. M. Gal.

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Table 2.—Phenylalanine hydroxylase activity in rat and rabbit liver homogenates with various amounts of 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine

<table>
<thead>
<tr>
<th>DMTHP added, μmoles</th>
<th>Hydroxylase activity*</th>
<th>DMTHP added, μmoles</th>
<th>Hydroxylase activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>--</td>
<td>0.3</td>
<td>--</td>
<td>0.04</td>
</tr>
<tr>
<td>0.26</td>
<td>3.0</td>
<td>0.22</td>
<td>0.75</td>
</tr>
<tr>
<td>0.52</td>
<td>3.4</td>
<td>0.44</td>
<td>1.6</td>
</tr>
<tr>
<td>0.78</td>
<td>4.1</td>
<td>0.88</td>
<td>2.4</td>
</tr>
<tr>
<td>1.40</td>
<td>5.0</td>
<td>1.32</td>
<td>2.5</td>
</tr>
<tr>
<td>2.30</td>
<td>6.4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*μmoles of tyrosine formed/45 min/ml of enzyme preparation (15,000 x g supernatant fraction of liver homogenate).

Phenylalanine hydroxylase activity was determined using 0.1 ml of rat or rabbit liver supernatant fractions prepared from a 25 percent homogenate by the assay method of Udenfriend and Cooper. The reaction was carried out at 25° with 2.0 μmoles of L-phenylalanine; incubation volume was 1.5 ml. The incubation time was 45 minutes.

maximal activity. However, it is apparent from the data in Table 1 that such an interpretation would be incorrect. Obviously, factors such as the instability of the reduced pteridine and competitive reactions contribute to the amount of pteridine required in these crude enzyme preparations.

The effect of added pteridine on phenylalanine hydroxylase activity in various mammalian species is summarized in Table 3.

Table 3.—Liver phenylalanine hydroxylase activity of various species

<table>
<thead>
<tr>
<th>Phenylalanine hydroxylase activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without DMTHP</td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td>Dog</td>
</tr>
<tr>
<td>Rat</td>
</tr>
<tr>
<td>Guinea Pig</td>
</tr>
<tr>
<td>Rabbit</td>
</tr>
<tr>
<td>Human</td>
</tr>
</tbody>
</table>

*μmoles tyrosine formed/45 min/ml of 15,000 x g supernatant fraction.

Phenylalanine hydroxylase activity was determined with 0.10 ml of 15,000 x g supernatant fraction prepared from a 25 percent crude homogenate according to the assay method of Udenfriend and Cooper. Incubation conditions and flask contents were the same as described in Table 1.

†The amount of DMTHP added to assure excess pteridine in each species was as follows: dog, 1.32 μmoles; rat, 2.30 μmoles; guinea pig, 1.32 μmoles; rabbit, 1.32 μmoles; human, 1.40 μmoles.
Human liver phenylalanine hydroxylase shows a closer similarity to rabbit than rat hydroxylase. The maximal activity (μmoles tyrosine/45 min/ml homogenate) is in the same order of magnitude as rabbit liver and high pteridine concentrations are required to reach maximal activity (figure 1).

Kaufman found normal human liver phenylalanine hydroxylase to be considerably more active when supplemented with pteridine cofactor.(9) The low activity he found without added cofactor agrees well with the results of Wallace, Moldave and Meister(7) from three normal human liver specimens, assayed by a modification of the Udenfriend and Cooper method. These results, and data obtained with the method developed in our laboratory are shown in Table 4.

Mitoma, Auld and Udenfriend(8) found no hydroxylase activity in normal liver samples obtained at autopsy 4 hours after death. Our experience with autopsy specimens has also shown that activity decreases rapidly. For this reason liver biopsy samples are required if phenylalanine hydroxylase is to be measured. Furthermore, analyses should be made as soon as possible, preferably within hours, since the activity of frozen human liver biopsy samples gradually deteriorates.

Particulate Fraction Inhibitor of Phenylalanine Hydroxylase

Phenylalanine hydroxylase activity of crude liver homogenates decreases rapidly even if the homogenates are frozen. Little activity remains upon reassyay of the frozen sample several days later. However, hydroxylase activity is well preserved in 100,000 x g supernatant fractions and little reduction occurs when these preparations are frozen for 2 weeks. The differences in stability of the hydroxylase under these conditions suggest that either the particulate fraction contributes to the denaturation and autolysis of the enzyme during storage, or that the particulate fraction contains an inhibitor of the hydroxylase and gradually inactivates it.

Evidence for a particulate fraction inhibitor was obtained by Coleman(11) in studies on phenylalanine hydroxylase activity in mice. He compared the hydroxylase activity of crude liver homogenates with 15,000 x g supernatant fractions of mice carrying the dilute-lethal gene. Animals homozygous for this gene were said to have "phenylketonuria" since they showed some of the biochemical disturbances and neurological symptoms characteristic of this metabolic disorder. Coleman attributed their reduced ability to metabolize phenylalanine to the presence of a potent particulate fraction inhibitor of the hydroxylase rather than to an inherited deficiency of the enzyme. Rauch and Yost(12) presented further evidence for a particulate fraction inhibitor of phenylalanine hydroxylase in dilute-lethal homozygotes and suggested that the progressive neurological symptoms and death at about 3 weeks of age were related to the altered metabolism of phenylalanine secondary to the inhibited enzyme.

Studies in our laboratory(13) have shown that mice homozygous for the dilute-lethal gene have the same level of liver phenylalanine
Table 4.--Phenylalanine hydroxylase activity of normal human liver biopsies

<table>
<thead>
<tr>
<th>Investigators</th>
<th>Activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without cofactor</td>
</tr>
<tr>
<td>Wailace, Moldave and Meister†</td>
<td>0.18</td>
</tr>
<tr>
<td></td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td>0.32</td>
</tr>
<tr>
<td>Kaufman‡</td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td>0.15</td>
</tr>
<tr>
<td>LaDu and Zannoni§</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>0.03</td>
</tr>
</tbody>
</table>

* Recalculated as µmoles tyrosine/45 min/250 mgm wet weight of liver, at 25.0.
† Wailace, et al.,(7) three patients average 10.7 percent conversion of 0.4 µmoles of L-phenylalanine in 30 min at 370 with 0.1 ml of a 33 percent liver homogenate. Assuming a temperature coefficient of 2; 0.5 x 0.107 x 0.4 x 45/30 x 250/33 = 0.243 µmole tyrosine/45 min/250 mgm of liver.
‡ Kaufman,(9) two patients average 0.064 µmole of tyrosine/60 min at 250 with 0.3 ml of a 25 percent liver homogenate. Recalculation of the data: 0.064 x 45/60 x 10/3 = 0.16 µmole tyrosine/45 min/250 mgm of liver.
§ LaDu and Zannoni data; first patient, from Table 3 and figure 1. The second patient was a 34 year old male without liver disease. Enzymatic assays were made within one hour after removal of liver biopsy.

hydroxylase activity as those of other genotypes. The neurological disturbances and death occur without elevation in the blood concentration of phenylalanine or the excretion of phenylpyruvic acid in the urine. Assays of hydroxylase activity in mouse liver homogenates (crude and 15,000 x g supernatant fraction) are consistently lower in the crude homogenate preparations from all genotypes. Furthermore, a similar difference is also found in liver fractions from other species (Table 5).

Further experiments with the supernatant fraction of rat liver homogenates have shown that the addition of mitochondria reduces hydroxylase activity (Table 6). These results made it seem reasonable to look for an inhibitory factor in mitochondria which could be characterized more specifically.

After acidification and extraction with ether, aged mitochondria, stored at 40 for several days, yield an inhibitory factor extracted into the organic solvent. The spectrophotometric properties of the ether extract suggested that the inhibitor was a naphthoquinone-like compound. For this reason, a series of naphthoquinones were tested as inhibitors of phenylalanine hydroxylase (Table 7). The most active inhibitor of the series was 1,2-naphthoquinone. It produced 98 percent inhibition at 3 X 10^{-4}M and 50 percent inhibition at 8 X 10^{-5}M. Menadione (vitamin K,) was less potent and it produced 50 percent inhibition of the hydroxylase activity at approximately 3 X 10^{-4}M. None of the naphthoquinone inhibitors were effective in their reduced (diol) forms.

Inhibition of phenylalanine hydroxylase by naphthoquinones was not influenced by changes in substrate (phenylalanine) concentrations, and the inhibition was clearly not competitive with substrate. Further studies were undertaken to find whether the inhibitor acted upon the primary
Table 5.—Liver phenylalanine hydroxylase activity in crude homogenates and 15,000 x g supernatant fractions of various species

<table>
<thead>
<tr>
<th>Species</th>
<th>Crude homogenate</th>
<th>15,000 x g supernatant fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µmoles tyrosine formed/hr/gm liver</td>
<td>µmoles tyrosine formed/hr/gm liver</td>
</tr>
<tr>
<td>Mouse (Homozygous (dilute-lethal))</td>
<td>0.8</td>
<td>1.5</td>
</tr>
<tr>
<td>Heterozygous</td>
<td>0.8</td>
<td>1.4</td>
</tr>
<tr>
<td>Homozygous (dilute non-lethal)</td>
<td>0.8</td>
<td>1.7</td>
</tr>
<tr>
<td>Rat</td>
<td>1.8</td>
<td>3.2</td>
</tr>
<tr>
<td>Dog</td>
<td>1.0</td>
<td>1.7</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>1.9</td>
<td>1.6</td>
</tr>
</tbody>
</table>

*Phenylalanine hydroxylase activity was assayed with 0.5 ml of 20 percent crude liver homogenate or 0.5 ml of 15,000 x g supernatant fraction. The incubation period was for one hour at 25°C with 2.0 µmoles of L-phenylalanine. Flasks contained DPN, nicotinamide, phosphate buffer, 0.2M, pH 7.0 as described by Udenfriend and Cooper. Values given for mice were obtained from a pooled sample of 6 to 8 livers.

Table 6.—Inhibition of rat liver phenylalanine hydroxylase by rat liver mitochondria

<table>
<thead>
<tr>
<th>Liver enzyme preparation</th>
<th>Phenylalanine hydroxylase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µmoles tyrosine formed/hr per ml of enzyme preparation</td>
</tr>
<tr>
<td>100,000 x g supernatant fraction</td>
<td>0.78</td>
</tr>
<tr>
<td>Crude liver homogenate</td>
<td>0.54</td>
</tr>
<tr>
<td>15,000 x g supernatant fraction</td>
<td>0.63</td>
</tr>
<tr>
<td>&quot; with 0.2 ml mitochondria</td>
<td>0.53</td>
</tr>
<tr>
<td>&quot; 0.4 ml mitochondria</td>
<td>0.26</td>
</tr>
<tr>
<td>&quot; 0.8 ml mitochondria</td>
<td>0.14</td>
</tr>
</tbody>
</table>

Phenylalanine hydroxylase activity was determined with 0.5 ml of the various rat liver preparations prepared from a 25 percent crude homogenate by the assay method of Udenfriend and Cooper. Incubation conditions and flask contents were the same as described in Table 5.

Mitochondria were isolated by centrifugation of the 3,000 x g supernatant fraction for one hour at 15,000 x g. The particles were washed once and suspended in isotonic KCl so that 1.0 ml of the final mitochondria suspension was equivalent to 1.0 g of liver.
Table 7.—In vitro inhibition of rat liver phenylalanine hydroxylase by naphthoquinones

<table>
<thead>
<tr>
<th>Active inhibitors</th>
<th>Inhibition</th>
<th>Inactive compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,2-naphthoquinone</td>
<td>98%</td>
<td>1,4-naphthalenediol</td>
</tr>
<tr>
<td>1,4-naphthoquinone</td>
<td>70%</td>
<td>1,5-</td>
</tr>
<tr>
<td>Menadione</td>
<td>56%</td>
<td>1,3-</td>
</tr>
<tr>
<td>1,3-dichloro-1,4-naphthoquinone</td>
<td>55%</td>
<td>Menadiol diphosphate</td>
</tr>
<tr>
<td>2-amino-1,4-naphthoquinone</td>
<td>25%</td>
<td>Hydroquinone (1,4-benzenediol)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Quinone (1,4-benzoquinone)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Coenzyme Q_{10}</td>
</tr>
</tbody>
</table>

The final concentration of the compounds tested was $3.0 \times 10^{-4}$M. They were preincubated with enzyme and other flask constituents for 3 minutes prior to the addition of L-phenylalanine.

Phenylalanine hydroxylase was determined with 0.5 ml rat liver supernatant fraction prepared from a 25 percent crude liver homogenate. The incubation period was for 30 minutes at $25^\circ$ with 2.0 μmoles of L-phenylalanine. Flasks contained DPN, nicotinamide, phosphate buffer, 0.2M, pH 7.0 as described by Udenfriend and Cooper.\(^6\)

hydroxylase enzyme or the cofactor generating system (Table 8). Preincubation of 1,2-naphthoquinone with purified hydroxylase enzyme (Fraction I) followed by addition of purified generating system (Fraction II) showed marked inhibition; the reverse experiment showed no inhibition when the quinone was preincubated with purified generating system (Fraction II). In other experiments it was found that the addition of DMTHP did not prevent inhibition by naphthoquinones.

Further experiments on the inhibition by the particulate fraction components are in progress, and a few reasons why these studies are pertinent to our conference topic might be mentioned. It would be of considerable value if a potent non-competitive inhibitor of phenylalanine hydroxylase were available so that a biochemical model of phenylketonuria could be induced in experimental animals. Furthermore, the demonstration of low hydroxylase activity in patients with the biochemical features of phenylketonuria does not distinguish between an inherited deficiency in enzyme synthesis and an inhibited enzyme due to compounds of endogenous or exogenous origin. At present, we can not exclude the possibility that some phenylketonuric patients have inhibited phenylalanine hydroxylase secondary to another metabolic disturbance in which metabolites inhibitory to the enzyme accumulate in abnormal amounts in liver.
Table 8.--Inhibition of purified rat liver phenylalanine hydroxylase by 1,2-naphthoquinone

<table>
<thead>
<tr>
<th>Phenylalanine hydroxylase activity</th>
<th>μg tyrosine formed/hr per 0.2 ml enzyme fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction I (hydroxylase)</td>
<td>4</td>
</tr>
<tr>
<td>Fraction II (cofactor reduction system)</td>
<td>0</td>
</tr>
<tr>
<td>Fraction I + Fraction II</td>
<td>36</td>
</tr>
<tr>
<td>(Fraction I with 1,2-NQ)* + Fraction II</td>
<td>6</td>
</tr>
<tr>
<td>Fraction I + (Fraction II with 1,2-NQ)*</td>
<td>32</td>
</tr>
</tbody>
</table>

Fraction I and Fraction II were purified from rat liver according to the method of Mitoma and 0.2 ml of each fraction used in the assays.

* Fractions I or II were pretreated with 1,2-naphthoquinone (at a final concentration of 7.0 x 10⁻⁵M) overnight at 4°C. Fractions I and II not treated with inhibitor were stored under the same conditions.

Phenylalanine hydroxylase activity was determined with 2.0 μmoles of L-phenylalanine by the assay method of Udenfriend and Cooper. The incubation period was for one hour at 25°C. Flasks contained DPNH, nicotinamide, phosphate buffer, 0.2M, pH 7.0.

Inhibition of Phenylalanine Hydroxylase by Phenylalanine Metabolites

In contrast to the noncompetitive inhibition of the naphthoquinones, several metabolites of phenylalanine act as competitive inhibitors. Excess phenylalanine is well known to inhibit the hydroxylase, but it has not been emphasized that phenylpyruvic acid is even more potent as an inhibitor. The affinity of the hydroxylase for the keto acid is 15 times greater than for phenylalanine (calculated from the $K_\text{m}$ and $K_\text{p}$ data for phenylalanine and phenylpyruvic acid). Other metabolites (phenyllactic acid, orthohydroxyphenylacetic acid and phenylacetic acid) are much less active as competitive inhibitors (Table 9).

Experimentally, we have been able to demonstrate the inhibition of the liver hydroxylase in vivo by the administration of phenylpyruvic acid to mice (Table 10). Rather marked hydroxylase inhibition following phenylpyruvic acid was observed in livers of mice homozygous for the dilute-lethal gene, but heterozygous animals (and non-lethal homozygotes) showed practically no reduction in enzyme activity. It is of interest that the effectiveness of the inhibitor depended upon the genetic background of the test animals. Possibly analogous genetic factors in man contribute to the biochemical alterations characteristic of phenylketonuria and other hereditary metabolic disorders.

Either a hereditary reduction in phenylalanine hydroxylase or environmental factors such as an excessive intake of phenylalanine might lead to an accumulation of phenylpyruvic acid and other metabolites in the liver sufficient to inhibit hydroxylase activity. A partial hereditary block in phenylalanine metabolism might thus appear to be complete because of this mechanism.
Table 9.--In vitro inhibition of rat liver phenylalanine hydroxylase by some aromatic metabolites

<table>
<thead>
<tr>
<th>Active inhibitors</th>
<th>Inhibition</th>
<th>Inactive compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenylpyruvic acid</td>
<td>55%</td>
<td>Phenylacetic acid</td>
</tr>
<tr>
<td>p-Hydroxybenzaldehyde</td>
<td>20%</td>
<td>o-Hydroxyphenylacetic acid</td>
</tr>
<tr>
<td>DL-p-Hydroxyphenyllactic acid</td>
<td>14%</td>
<td>p-Hydroxyphenylpyruvic acid</td>
</tr>
<tr>
<td>DL-Phenyllactic acid</td>
<td>12%</td>
<td>Phenylacetaldehyde</td>
</tr>
</tbody>
</table>

The compounds tested were added to the incubation flasks at the same time as L-phenylalanine and the final concentration of the inhibitors was 2.0 x 10^{-4} M.

Phenylalanine hydroxylase activity was determined with 2.0 μmoles of L-phenylalanine by the assay method of Udenfriend and Cooper. The incubation period was for one hour at 25°C. Flasks contained DPNH, nicotinamide, phosphate buffer, 0.2 M, pH 7.0.

K_I for phenylpyruvic acid = 2.0 x 10^{-6} M
K_M for L-phenylalanine = 1.4 x 10^{-4} M

Table 10.--Inhibition of liver phenylalanine hydroxylase in mice after intraperitoneal injection of phenylpyruvic acid

<table>
<thead>
<tr>
<th>Phenylalanine hydroxylase activity†</th>
</tr>
</thead>
<tbody>
<tr>
<td>After I.P. phenylpyruvic acid</td>
</tr>
<tr>
<td>µmoles tyrosine formed/hr/gm liver</td>
</tr>
<tr>
<td>Homozygous (dilute-lethal) (8)</td>
</tr>
<tr>
<td>Heterozygous (8)</td>
</tr>
</tbody>
</table>

* The animals received an intraperitoneal injection of sodium phenylpyruvate (10 mg in saline/10 gm body weight) one hour before sacrifice.
† Phenylalanine hydroxylase activity was determined with 0.5 ml of 20 percent liver homogenates at 25°C with 2.0 μmoles of L-phenylalanine according to the method of Udenfriend and Cooper. The flasks contained 0.8 μmole DPN, 5.0 μmoles of nicotinamide, phosphate buffer, 0.2 M, pH 7.0; total volume was 1.5 ml.

Values are given as means ± standard deviation.
Number of animals in parentheses.
Summary

Since we can not assume that the biochemical defect is the same in all patients or families with phenylketonuria, it is essential to investigate, in detail, the properties of the phenylalanine hydroxylase enzyme system and to define the biochemical basis for any observed reduction in its activity more precisely. We must consider not only hereditary deficiencies in the quantity of hydroxylase, but also qualitative changes in its structure which make it a less efficient catalyst. Other possibilities to consider would be: a deficiency of the accessory systems required to reduce the pteridine cofactor, a deficiency of the cofactor and inhibition of the enzyme by the accumulation of metabolites. Further refinements in the assay for phenylalanine hydroxylase and the components of the enzyme system should aid in defining the defects and may be of practical value since special therapeutic measures may be appropriate for particular variant types of phenylketonuria.

REFERENCES

7. Wallace, H. W.; Moldave, K.; and Melster, A.: The enzymatic conversion of phenylalanine to tyrosine in phenylpyruvic oligo-

DISCUSSION

DR. BESSMAN: This is a very interesting paper from many standpoints. One, it shows a very thoughtful approach to an enzyme assay, and it should give us pause when we try to interpret biopsy data or animal experiments.

We can confirm a great deal of what Dr. LaDu has found about concentrations and assays. We have developed an assay that can detect the enzyme in milligrams of liver tissue but it manifested these concentration problems. Maybe we are fortunate because we don't have enough inhibitor and the mixture is dilute enough that we add a big excess of reduced cofactor.

In most studies one of the basic tenets of enzyme assay has not been observed. If we perform the assay with one amount of tissue, we don't know whether we have reached the maximum activity that tissue could demonstrate. Whenever we do an assay, it is necessary to use several different quantities of the extract. Only where there is a linear relationship between tissue
concentration and activity can there be a reliable quantitative value for tissue content of enzyme. If we find a non-linear relation between tissue extract added and activity, the calculated data are not reliable. I think this could give us a great deal to think about and help us design better assays.

One other thing. It might be a help if Dr. LaDu has done this—he mentioned that in liver tissue, he doesn't have the cofactor problem or probably it doesn't exist in vivo. I wonder if he has tried to assay a little piece, five or ten cubic millimeters of liver—this is about the size of sample a punch biopsy would provide.

DR. LaDU: I know exactly what you are thinking of, but the rate-limiting step may still be the concentration of pteridine. Obviously, the concentration is relatively much higher in the liver than it is in our assay.

It would be of interest to calculate, if we had accurate assays, the pteridine concentration in liver. We might be able to make some calculations of how this would limit the rate. We haven't studied the effect of adding pteridine to slices or small pieces of tissue and I don't know quite how we would solve the problems of diffusion, etc.

But I am sure that in enzyme assays using homogenates we are diluting the endogenous pteridine, and the dilution of pteridine is much more critical than dilution of the enzyme. There is a need for a method of measure of the pteridine as well as the hydroxylase. This is the only way I can see that we can determine whether some variants of phenylketonuria are due to a deficiency of the cofactor system rather than the hydroxylase. These unusual types of phenylketonuria should be studied very carefully with this possibility in mind.

DR. SCRIVER: Dr. LaDu has brought up the problems of cofactor availability and also the problem of apoenzyme inhibition. We usually think about developmental genetics and developmental changes in terms of the amount of apoenzyme present to perform the reaction. Is there any possibility that statements which have been made about functional development of phenylalanine hydroxylase apoenzyme in normal human infants are now suspect, because in fact most human infants receive vitamin K at birth and this may inhibit the assay if done at this time? Do you think there is need to re-do these neonatal studies in order to assess the effect of vitamin K in vivo.

DR. LaDU: I think that is a possibility, and I think that another, more important problem is we do analogous studies to those already done in rats on developmental changes of phenylalanine hydroxylase activity. The first studies seemed to show a reduction of the hydroxylase and later this was corrected by Dr. Kaufman. He showed that it was really a limitation in the cofactor which accounted for the lower activity in newborn rats. This type of limitation may well occur in newborn infants.

It may not be the hydroxylase activity which is so much lower but a reduction in the cofactor level. The cofactor has a great deal in common with the cofactor for hydroxylase of p-hydroxyphenylpyruvic acid. In fact, the pteridine can be used in place of reduced 2,6-dichlorophenolindophenol. It is quite possible that in tyrosinemia and the increased level of phenylalanine we see in some newborn infants could both be due to a lower level of the cofactor pteridine.

DR. GAULL: Have you re-examined brain? Have you examined brain mitochondria in your active liver supernatant?

DR. LaDU: You bring up the very interesting problem of whether other tissues really have some phenylalanine hydroxylase activity, which might be accessible for testing heterozygotes, etc. We haven't examined other tissues carefully yet. We have looked at rat kidney and rat brain. Using the assay just described here, we haven't found any significant activity in either of these tissues.

Homogenates of rat brain, however, do have inhibitory activity. Brain mitochondria added to the liver system do inhibit phenylalanine hydroxylase.

DR. UDENFRIEND: I merely wanted to point out there are general considerations for all oxygenases which should be kept in mind. There are now half a dozen oxygenases, all of which use pteridine as a cofactor. If there is an entity that we pick up as disturbed phenylalanine metabolism which is related to faulty pteridine biosynthesis or faulty pteridine content, this should show up in other parameters of oxygenase activity, in the formation of noradrenalin, the formation of serotonin, the hydroxylase of phenylalanine,
and in the biosynthesis of lipids. It is quite conceivable that faulty pteridine metabolism could be responsible for hyperphenylalanemia.

There is also the problem of intracellular inhibitors that foul up in vitro assays. Most hydroxylases are partly inhibited in crude homogenates due to unknown substances in the homogenate. This is particularly evident with the hydroxylase responsible for the final step in noradrenalin formation, dopamine-β-hydroxylase. Kaufman and one of my former students have reported on this inhibition. It is an amazing situation. When you start to purify dopamine-β-hydroxylase from adrenal gland the fractionation step gives an apparent purification of a thousand percent apparent yield. This happens because one leaves behind inhibitory substances. It turns out that part of this inhibition, a good deal of it, is in mitochondria, as Dr. LaDu pointed out. Dopamine-β-hydroxylase is not a pteridine-requiring enzyme but is an ascorbic acid hydroxylase. A good deal of this inhibitory activity is in mitochondria. Part is organically extractable. Part is associated with protein. The same thing is true for tryptophan hydroxylase in brain. Professor Hayaishi has told me he has to go through extensive purification before he can detect it.

I think that these inhibitors are not necessarily functioning in vivo. In perfusion studies in animals or in vivo biosynthesis, one doesn't find evidence for such inhibitors. This is what I was referring to yesterday when Dr. Waisman spoke about enzymic activity varying from species to species. I don't know but Dr. Waisman was probably using Dr. Kaufman's earlier assay. What was measured there did not parallel what one would find from perfusion studies or following in vivo administration of labeled precursor.

It is true that if one takes a biopsy sample that is fresh, immediately after excision, and not stored too long and assays it under optimal conditions, one can get meaningful data. But sometimes this is not achieved.

So I think I would be concerned, no matter how well the assay is developed, unless biopsies are well planned some types of inhibitory factors come into play. I don't know how one can interpret casual autopsy or biopsy materials in terms of genetic problems.

DR. WAISMAN: I must say that all assays we did on our animals of various species were all fresh. But I think even beyond that, the addition of pteridine would boost up those values.

DR. BESSMAN: Just one point about what Dr. Udenfriend just mentioned. I don't think it's possible to reason that if pteridine is deficient it will reveal itself as a general lesion, general abnormality. This is not true, for example, for the pyridoxine systems in which very remarkable apparent blocks in certain pathways occur and normal activity in other pathways remain. This phenomenon is dependent upon the affinity or Km of the cofactor.
UNANSWERED QUESTIONS IN THE PRIMARY METABOLIC BLOCK IN PHENYLKETONURIA

SEYMOUR KAUFMAN

There are two types of talks that one could give on the metabolic block in phenylketonuria: the first would be entitled "The Answered Questions in the Primary Metabolic Block"...and would take about 1 or 2 minutes to deliver. The second would be entitled the "Unanswered Questions"...and would take at least 30 minutes to deliver. This afternoon I will give the longer lecture on the unanswered questions.

Many of these questions and some of the answers have come from work done on the normal mammalian enzyme system that catalyzes the conversion of phenylalanine to tyrosine, the phenylalanine-hydroxylating system. We call it an enzyme "system" to indicate that it is more complex than a single enzyme. It has been known since the work of Jervis in 1953 (1) that this enzyme system is not functional in phenylketonuria.

It is perhaps not unexpected that the more we have learned about this system, the more complex it has become.

The earliest indication that the conversion of phenylalanine to tyrosine is a complicated reaction was the finding by us (2) and by Mitoma (3) that at least two enzymes and reduced pyridine nucleotide are involved in the hydroxylation reaction. We called it an enzyme "system" to indicate that it is more complex than a single enzyme. It has been known since the work of Jervis in 1953 (1) that this enzyme system is not functional in phenylketonuria.

It is perhaps not unexpected that the more we have learned about this system, the more complex it has become.

The earliest indication that the conversion of phenylalanine to tyrosine is a complicated reaction was the finding by us (2) and by Mitoma (3) that at least two enzymes and reduced pyridine nucleotide are involved in the hydroxylation reaction. We purified one of these from rat liver and the other from sheep liver extracts and, until we knew which reactions these enzymes catalyzed, we referred to them as the rat and sheep liver enzymes.

With these enzymes, TPNH was far more active than DPNH. (2) We showed that the overall reaction involved in tyrosine formation could be described by equation 1.

$$\text{TPNH} + \text{H}^+ + \text{phenylalanine} + \text{O}_2 \rightarrow \text{TPN}^+ + \text{tyrosine} + \text{H}_2\text{O} \quad (1)$$

There was no detectable reaction if either the rat or sheep liver enzymes was omitted. The first goal was to find out what each of these two enzymes and the TPNH were doing in the system.

Before we were able to make any progress in this direction, it became apparent that the system was still more complicated. We obtained evidence that another coenzyme, besides TPNH, was an essential component of the system. (4)

We purified this new cofactor from boiled extracts of rat liver. Many years before we knew what the structure of this compound was, we had evidence indicating that it might be a pteridine. The only pteridine that was then known to be involved in metabolism was the reduced form of the vitamin, folic acid. We tested tetrahydrofolate and found that it had some cofactor activity in our phenylalanine hydroxylating system.

We had evidence which ruled out the possibility that our cofactor was identical with tetrahydrofolate. This evidence, in fact, pointed to the likelihood that the cofactor, if it was a pteridine, did not have the PABA-glutamate side-chain of folic acid, i.e., the cofactor was probably an unconjugated pteridine.

We prepared several unconjugated pteridines and found that they were considerably more active than tetrahydrofolate. (5)

Figure 1 shows the structure of tetrahydrofolate and of one of the more active synthetic, unconjugated pteridines. The most active synthetic compound that we tried was the 6-methyl tetrahydro pteridine.

Figure 2 shows a comparison of the relative activities in the hydroxylating system of tetrahydrofolate, the 6,7-dimethyl compound and the 6-mono-methyl compound.

With the use of these unconjugated pteridines, we were able to learn something about the role in the reaction of all of the components. We found that in the presence of these tetrahydro pteridines, TPNH and the sheep liver enzyme were no longer essential. For the first time we could demonstrate...
tyrosine formation with an aerobic mixture of phenylalanine, the tetrahydropteridine and the rat liver enzyme. On the other hand, although no longer essential, TPNH and sheep liver enzyme still were able to stimulate the reaction.\(^6\)

An experiment that illustrates these facts is shown in figure 3 where we compared the time-course of tyrosine formation in the presence of the 6,7-dimethyltetrahydropteridine with and without TPNH.

In the absence of TPNH, tyrosine formation leveled off after a short time and the amount of tyrosine formed at the plateau was almost equal to the amount of tetrahydropteridine added; that is, under these conditions, the reduced pteridine was functioning stoichiometrically.

In the presence of TPNH, by contrast, tyrosine formation continued essentially linearly for as long as we had the patience to measure it. This meant that the pteridine was functioning catalytically under these conditions—each mole of pteridine supporting the formation of many moles of tyrosine.

Based on this experiment, and others, the hydroxylation reaction was formulated as shown in equations 2 and 3 where \(XH_4\) stands for the
tetrahydropteridine and XH₂ for its oxidized product, a dihydropteridine. The latter compound was shown to be a new type of dihydropteridine with a quinonoid structure.(7)

\[ \text{XH}_4 + \text{phenylalanine} + \text{O}_2 \rightarrow \text{XH}_2 + \text{tyrosine} + \text{H}_2\text{O} \] (2)

\[ \text{XH}_2 + \text{TPNH} + \text{H}^+ \rightarrow \text{XH}_4 + \text{TPN} \] (3)

According to this scheme, the rat liver enzyme catalyzes the hydroxylation reaction in which both phenylalanine and XH₄ are oxidized. The rat liver enzyme, therefore, is the hydroxylase. This is the stoichiometric, non-catalytic function of the tetrahydropteridine in the absence of TPNH.

The sheep liver enzyme catalyzes the reduction, by TPNH, of the quinonoid dihydropteridine, thus allowing the pteridine to function catalytically. The sheep enzyme can be called dihydropteridine reductase.

The role of TPNH is to keep the pteridine in the active, tetrahydro form. The role of the tetrahydropteridine is to donate an electron pair ultimately to oxygen to reduce it to the oxidation level of a hydroxyl group.

We finished our identification of the naturally occurring cofactor after this work had been completed. The structure of the cofactor is shown in figure 4. It is an unconjugated pteridine called 7,8-dihydrobiopterin.(8)

We think that the cofactor functions in the hydroxylase system in the same way as do the synthetic tetrahydropteridines. The only difference is that the cofactor, as isolated, being in the 7,8-dihydro configuration, is not active in the hydroxylation system until it is reduced to the tetrahydro form. The enzyme that catalyzes this initial reductive reaction is dihydrofolate reductase.(8) Once this initial reaction has taken place, this enzyme is no longer needed since the pteridine then shuttles back-and-forth between the tetrahydro and the quinonoid-dihydro forms. Incidentally, it is the reaction catalyzed by dihydrofolate reductase that is by far the most sensitive one to anti folic drugs such as amionopterin; the rest of the hydroxylation system is two to three orders of magnitude less sensitive to this type of inhibitor.(8)

A scheme in which these pteridine transformations are summarized is shown in figure 5, where 7,8-XH₂ stands for 7,8-dihydrobiopterin.

Figure 5

This then, in bare outline, is the enzyme system that is affected in phenylketonuria. Naturally, it was of interest to try to delineate which component was not functional in the disease. After Jervis's study, Meister and Udenfriend and their coworkers presented evidence that the more labile of the two enzymes was missing in the disease.(9,10) This work, however, was done before the cofactor had been described and it left open the possibility that it was the cofactor that was actually missing. Indeed, under certain conditions the cofactor is by far the most labile component in the whole system.
Eight years ago we assayed biopsy liver samples from normal and phenylketonuric patients and found that the cofactor was present in at least the normal concentration in the phenylketonuric liver samples.\(^{(1)}\) We also showed that phenylalanine hydroxylase was almost completely inactive, whereas dihydropteridine reductase was active in the phenylketonuric liver samples.\(^{(1)}\) This was the first proof that an enzyme was missing or non-functional in this disease.

What then is unknown about the nature of the metabolic block in phenylketonuria? Our ignorance can be divided into three parts or three questions.

1. How certain is it that it is phenylalanine hydroxylase that has impaired function in classical phenylketonuria?
2. If it is phenylalanine hydroxylase, how has it been affected? Is the protein not synthesized? Is an altered protein made in its place?
3. What are some possible variants of the disease?

The answer to the first question can be quite positive but not absolutely so. The degree of uncertainty is related to the possibility that an unknown enzyme may still be involved in the hydroxylation reaction and that this hypothetical enzyme is present as a contaminant in the rat-liver phenylalanine hydroxylase. The degree of uncertainty then is related to the purity of the phenylalanine hydroxylase that was used to restore activity to the phenylketonuric liver homogenates. Until this type of experiment can be repeated with pure hydroxylase, some slight uncertainty exists.

The answer to the second question is that there are obviously many ways in which a mutant enzyme can have an impaired function. It could have a very poor affinity for the cofactor or for its substrate. Since there is evidence that the rat liver hydroxylase is inhibited by excess phenylalanine in vitro, there is the possibility that an altered enzyme could have an enhanced sensitivity to excess phenylalanine.

(I will return to some of these points later.)

The final question is in many ways the most intriguing one and is clearly related to the other two questions.

One can conceive of variants of phenylketonuria that involve any one of the other components of the system: the cofactor, dihydrofolate reductase and dihydropteridine reductase. Also, if the hydroxylase is affected, it can be changed in many ways, some of which I indicated above.

Since our work established the cofactor role of unconjugated pteridines in the phenylalanine hydroxylating system, many additional oxygen-requiring reactions have been shown to need a pteridine cofactor. So far, none of these reactions appears to be vital for the survival of the organism. As the list of pteridine-requiring reactions grows, however, the possibility will increase that the cofactor may play a role in a reaction which is necessary for life. In that case, obviously, a mutation that led to the complete absence of the cofactor might be a lethal one. At the moment, a variant of phenylketonuria in which the cofactor is missing is a real possibility. I should say that in our early study we examined liver samples from five or six phenylketonuric patients and the cofactor was present in every one.

Dihydrofolate reductase can be ruled out as the missing enzyme in a variant of the disease because this type of mutation would almost certainly be a lethal one in view of the key role played by this enzyme in one-carbon metabolism.

The lack of dihydropteridine reductase is a possible variant. Presumably, the consequences of lacking this enzyme might be the same as a lack of the cofactor.

We have recently examined two liver biopsy samples from patients who do seem to have a variant of phenylketonuria-hyperphenylalaninemia. These samples were obtained from Dr. Joseph Kennedy of the Children's Hospital Medical Center in Boston.

What can be said about the phenylalanine hydroxylating system in this disease is still limited, but one thing is clear—the amount of hydroxylating activity is very low compared to our old series of controls. There are indications that they may have somewhat more activity than true phenylketonurics, but the series is too small to say this with any degree of certainty.

Although we have not done direct cofactor assays on these liver samples, lack of cofactor cannot be the sole defect. We know this because we could not restore their hydroxylase activities to normal with cofactor addition.

As I mentioned earlier, there are several possible ways in which an altered protein could have lower enzyme activity. Some of these are: (a) poor affinity for the cofactor; (b) poor affinity for the substrate; and (c) inhibition by excess substrate.
The first possibility is unlikely in the case of these patients because quite large amounts of cofactor could not restore the enzyme activity.

We invested a large fraction of one of the biopsy samples to test the last two possibilities. The results need confirmation because we are dealing with exceedingly low levels of enzyme activity, but we could not detect any inhibition by excess phenylalanine. Moreover, the Km for phenylalanine is not too different from the Km observed with the purified enzyme from rat liver. Of course, we do not know the Km of the enzyme from normal human liver, but as an educated guess we can say that there is nothing seriously wrong with the affinity for the substrate in this case.

Based on this extremely limited study, we can say that the hepatic phenylalanine hydroxylase activity in hyperphenylalaninemia is much lower than in normals. This decreased activity cannot be due to a lack of the phenylalanine-hydroxylation cofactor as the sole defect. It is also unlikely that the low activity is due either to a poor affinity for phenylalanine or to an abnormal sensitivity to excess phenylalanine.

Further studies will be required in order to distinguish, at the enzyme level, this disease from "classical" phenylketonuria.

REFERENCES


DISCUSSION

DR. GAULL: Dr. LaDu discussed the relative inhibition of phenylalanine hydroxylase in the whole homogenate versus the particulate free supernate. Could you give us just a few details on your assay system and any comments you might have about the inhibition of the supernatant hydroxylase.

DR. KAUFMAN: We haven't really studied whole homogenates versus supernatants in any detail.

In one of our old series of normal controls, dating back, now, eight years, the sample was large enough so that we divided it in half. On one half, we studied the supernatant fraction, and on the other half, the homogenate. We could detect no serious inhibition due to the homogenate at the levels which we were using.

Our assay conditions with the purified enzymes are such that all components in the system are in excess. That is, the cofactor and the enzymatic equipment for keeping the cofactor in the reduced active form. This is also true with the crude homogenates that we have used in our
human liver work. It should be emphasized that these were not high-speed supernatant fractions.

I think as soon as one varies from this standard assay system, it would be hard to predict what would happen. And, of course, this lack of serious inhibition is based on only one experience on one human liver homogenate.

DR. UDENFRIEND: You indicated that you were measuring phenylalanine-hydroxylase activity in these phenylketonuric livers. This is rather interesting. Could you give us an idea about how high this activity is compared to normal controls?

DR. KAUFMAN: I would hesitate to put a figure on it. But my guess is it is somewhere between 3 to 6 percent.

DR. UDENFRIEND: In other words, it is detectable. Are you sure it is phenylalanine-hydroxylase and not the sympathetic nervous system enzyme in that same tissue? You would also have sympathetic innervation in the liver and may be measuring tyrosine hydroxylase with phenylalanine as a substrate. Have you thought about that?

DR. KAUFMAN: Yes. All we can say is that we are measuring the enzymatic conversion of phenylalanine to tyrosine. That's phenylalanine hydroxylation. Whether that is a function of another protein, I don't think our studies are in a position to say.

DR. UDENFRIEND: I think it would be important to use an inhibitor of tyrosine hydroxylase when measuring phenylalanine-hydroxylase activity in PKU livers.

DR. LaDU: I would like to ask how the tissue was stored and how long it was preserved before the assays. This seems to be very critical because of the instability of this particular enzyme.

DR. KAUFMAN: Perhaps Dr. Kennedy could tell me how soon after the biopsy they were shipped to us.

DR. KENNEDY: They were shipped down by air an hour or two after they were taken and they were placed on dry ice immediately.

DR. KAUFMAN: In other words, they were frozen immediately on removal from the patient and assayed between two or three days of receipt, kept frozen all that time. We have done literally thousands of assays on liver samples frozen for that long a time. Under our assay conditions with the enzymatic equipment for keeping the cofactor reduced, we never detected more than 10 or 20 percent loss in activity over a period of two or three weeks.

DR. LaDU: Do you find human liver has about the same activity as rat or some other species?

DR. KAUFMAN: No. In our experience, human liver homogenates are, as I recall, perhaps about half as efficient as the rat.

DR. LaDU: Of your reported values for the rat?

DR. KAUFMAN: Yes. We have one other bit of pertinent experience. In the old study, on some of the normal human liver homogenates, we had sufficient amount of material to do re-assays after rather extended periods of storage. So we do have some experience on the stability of the human enzyme.

Again, that experience is not too different from what we found with rat liver homogenate. A very slow decrease in activity that might amount to 20 percent in two weeks on frozen storage. This finding is practically important because it means one doesn't have to complete the assay work on the first day.

DR. LaDU: We have found in several species, including rat, that the activity can be increased from 10 to 30-fold by adding much more pteridine than the 0.2 or 0.22 micromoles you have usually used with the generating system. If, instead, one uses an excess of TPNH or DPNH with liver homogenates from various species, we find 0.7 micromole or more is really needed to prevent pteridine being a limiting factor.

Your published values for rat liver phenylalanine hydroxylase activity, I believe, have ranged from 0.5 to 1.0 micromole tyrosine formed per 45 minutes per cc of 25 percent homogenate. We find about 6 micromoles. Of course, without added pteridine, the values are much lower.

The published data so far on human liver phenylalanine hydroxylase activity have been in the order of 0.2 μ moles of tyrosine formed/45 min/250 mgm. of liver. Using more pteridine (without the generating system) we find about 2.0 μ moles of tyrosine.

DR. KAUFMAN: Under your conditions, does the activity ever level off with excess pteridine?

DR. LaDU: It does. I suspect that in your assays, even with the generating system, the pteridine is still rate limiting.
DR. KAUFMAN: On several occasions, including this recent study, we tested the activity of several different pteridine concentrations. We could find no difference on going from the lower to the higher value which gives us some assurance that we are at the lower value working with saturated amounts.

DR. LaDU: I think you would find an increase in activity with more pteridine if you also added more TPNH.

DR. KAUFMAN: I doubt that.

DR. BESSMAN: Well, it is a clear difference of opinion.

DR. KAUFMAN: I would like to ask one question of Dr. LaDu. Has he made non-enzyme activity corrections under these conditions in each case?

DR. LaDU: Yes.

DR. UDENFRIEND: I think I could act as mediator. What Dr. LaDu is saying is that he has modified the test. Instead of using the generating system, which is a normal constituent, he has left it out completely and is doing the assays for phenylalanine hydroxylase the same as we do our assays for tyrosine hydroxylase. In this case one adds excess pteridine, so that reduced pteridine becomes a co-substrate. One needs only enzyme, reduced pteridine and phenylalanine, and can leave out all the other ingredients. With this assay system Dr. LaDu says he gets much higher activity and he gets no blank values. Is it really necessary if one merely wants to assay phenylalanine hydroxylase to add all these other substances?

DR. KAUFMAN: No, it certainly isn't.

DR. UDENFRIEND: Why can't one simplify the assay and add the reduced pteridine?

DR. KAUFMAN: Certainly that's a proper thing to do. We actually described this type of assay in our publications seven years ago.

Of course, we will not detect the abnormality if these other components are affected in a disease, We would be looking at one aspect only.

DR. UDENFRIEND: One would look at it in another assay.

DR. KAUFMAN: Right. One would have to do separate assays for the other enzymes. There is certainly nothing wrong with that, but I would like to say that the thing I do find surprising is that we use a certain amount of the enzymatic generating system and we have titrated this so that the system levels off as demonstrated in those curves. No matter how much further addition of what we call the sheep liver enzyme we add at that level of pteridine, we cannot increase the activity. Although I certainly believe the results that you present but I am just wondering if you have any explanation of what is happening at the enzyme level.

DR. LaDU: Yes, I think we do.

If we use different amounts of pteridine, the activity goes up. But an excess of DPNH or TPNH is needed to protect the reduced pteridine.

If we use 0.2 micromoles of the pteridine, we get the same values as you report with 0.2 micromoles with the generating system. It appears that the level of TPNH or DPNH limits how much active pteridine is available.

DR. KAUFMAN: Why can't one increase that by doubling the level of TPNH?

DR. LaDU: Because it is important to keep the pteridine reduced and this depends upon the level of DPNH or TPNH that has been added. If we add only 0.2 micromole of TPNH and add increasing amounts of pteridine, we don't get a further increase in activity.

It doesn't seem to depend upon a continued generation of TPNH, but rather the concentration of TPNH present which limits how much reduced pteridine that is protected.

So, with 0.25 micromole of TPNH, that's the maximum concentration we can have at any one time, And this seems to prevent the ability of additional pteridine to stimulate the reaction.

In other words, if you would increase the concentration of TPNH with your generating system, I think you would see a further boost in activity with additional pteridine.

DR. KAUFMAN: Is this, now, with crude systems, or is it with purified material?

DR. LaDU: This is using a 15,000 x g sample, as you have described.

DR. BESSMAN: May we make a conclusion from this, at least, that anybody who has the temerity to come up with an absolute statement about enzyme levels is a pretty heroic fellow.

DR. GUTHRIE: Would Dr. Kaufman have an opinion concerning the question that was asked before about the efficiency or possible value of 14C-Phenylalanine administration and measurement of labeled tyrosine in study indirectly of enzyme activity?

DR. KAUFMAN: I think they would certainly be of value. We had some experience with trying to correlate enzyme levels with this kind of
in vivo test where we were studying the activity of this system in newborn rats. We found, in contrast to work of others, that there was a slight deficiency of the system at birth in the rat but very slight compared to some earlier reports.

Let's say it was approximately 40 or 50 percent less than the adult level. And we wanted to try to see how this would correlate with the in vivo test that was just outlined. We could detect no differences between newborn and adult rats under conditions of the in vivo test when the results at the enzyme level indicated perhaps a 40 or 50 percent deficiency in the newborn animals.

So based on this experience, I would guess that certainly the in vivo test is useful for detecting very big deficiencies in the enzyme. But I would guess that it would not be too good at detecting minor differences. Perhaps as much as a 50 percent difference could be missed that way.

(Because Dr. Kaufman was not present at the time of Dr. LaDu's presentation, the editors felt that further clarification of the problem would come from a brief note written by Dr. Kaufman after he had read the transcript of Dr. LaDu's paper and discussion. Dr. Kaufman's note follows.)

In view of the fact that I was not present at the Conference on the day of Dr. LaDu's presentation, I welcome this opportunity to comment on some of the points that were raised both in Dr. LaDu's talk and in the ensuing discussion.

There is no doubt that the problem of assaying an enzyme activity in crude tissue extracts is a formidable one, and that these problems may be compounded with an enzyme system as complicated as phenylalanine hydroxylase. In spite of these difficulties, however, valid assays have been described for all of the known components of this complex enzyme system. Furthermore, there is not a single case where these assay methods have failed to give an accurate in vitro reflection of a suspected physiological change in the level of the hydroxylase. These in vivo variables include changes in enzyme activity in newborn animals vs. adults, phenylketonurics vs. normals, and aminopterin-treated animals vs. controls.

While Dr. LaDu's paper contains much useful and interesting information, it also could lead to serious confusion of some fundamental issues. Although not stated explicitly, it is clear that throughout Dr. LaDu's paper he has equated maximum in vitro enzyme activity with "true" or "correct" activity; the implication is that any value less than the maximum one is necessarily untrue or incorrect. A corollary of this implication is the conclusion that an enzyme working at less than its maximum rate cannot be the rate-limiting component in an enzyme assay (see page 194). I seriously question the validity of both of these viewpoints.

As indicated by Dr. Bessman in the discussion following Dr. LaDu's paper, there is only one criterion for the validity of an enzyme assay: the rate of the reaction should be proportional to the concentration of the enzyme. It is a simple laboratory exercise to show that this criterion can be met with less than saturating concentrations of cofactors or substrates if initial rates are measured. The rate of the reaction will be less than the maximum one, but this lower rate is still a valid measure of the enzyme activity.

With regard to the second point, there are many variables (temperature, pH, concentration or nature of ions, concentration of substrates and cofactors) that can increase the rate of a reaction by increasing the catalytic efficiency of an enzyme. If under these varied conditions, the rate of the reaction is still proportional to enzyme concentration, the enzyme-catalyzed step must still be the rate-limiting one, even if this enzyme is not functioning at its highest rate. There is, therefore, no justification for the conclusion that maximum rates are the only ones that measure an enzyme's "true" activity. We have, for example, reported that the activity of the phenylalanine hydroxylating system is about 3 times higher when the 2-amino-4-hydroxy-6-methyl tetrahydropteridine is used in place of the 6,7-dimethyl compound. It certainly does not follow that the higher rates achieved with the more active pteridine are a more correct measure of the enzyme's activity than those observed with the less active pteridine. Indeed, there may be disadvantages to the use of unphysiologically high concentrations of the tetrahydropteridines to achieve higher rates. Pertinent to the theme of this conference is the possibility that a variant of PKU may exist in which an altered phenylalanine-hydroxylase is produced which has a poor affinity for the pteridine cofactor. In this situation, an essay performed with relatively enormous concentrations of reduced pteridine could give a misleading
result. It should be clear that there is no single 
assay condition that will cover every conceivable 
physiological variable.

I would also like to comment on a question 
raised by Dr. Udenfriend in the discussion follow-
ing my paper. He raised the possibility that 
the low, but significant, hydroxylation of phenyl-
alanine that we observed in the two liver samples 
from patients with phenylalaninemia might be 
due to tyrosine hydroxylase rather than to phenyl-
anine hydroxylase. From Dr. Udenfriend's pub-
lished data on the hepatic activity of tyrosine 
hydroxylase, (and on the rate of phenylalanine 
hydroxylation catalyzed by this enzyme), it can 
be calculated that the hydroxylation rates that 
we observed in these two liver samples were ap-
proximately 30,000 times higher than could be ac-
counted for by the tyrosine-hydroxylase activity.
Chemical analysis of lipid composition is the first step in the sequence leading to the understanding of disorders of lipid metabolism. By chemical analysis, a number of inherited disorders have been shown to be defects of lipid metabolism. Following the demonstration of abnormal concentrations, usually abnormally high levels, of lipids, in vitro assay procedures have pinpointed more precisely the enzymatic defect in some cases. The group of disorders known as the sphingolipidoses (Tay-Sachs, Niemann-Pick, Gaucher's and Fabry's diseases and metachromatic leucodystrophy) are now rather firmly established as defects of sphingolipid metabolism, probably dependent upon deficiencies of degradative enzymes for the particular lipids involved. Neurological involvement is seen in some sphingolipid disorders. Some less well defined disorders with neurological changes appear to be sphingolipid disorders related to the classical and rather well defined sphingolipidoses. Familial elevations of blood triglycerides and cholesterol are well known clinically although the nature of the disorders at the molecular level is not known. The brain may be affected by defects in carbohydrate and amino acid metabolism. The effects on the brain seen in the uncontrolled patient with galactosemia can be prevented by reduction of the amount of galactose in the diet. Galactosemia thus provides an example of a defect in carbohydrate metabolism that can produce changes in the brain. Phenylketonuria is commonly thought to provide a similar example of a defect of amino acid metabolism associated with neurological problems. The nervous system is affected in other conditions in which some metabolic abnormality seems probable but the defects at the molecular level have not been defined. New procedures for chemical analysis offer new possibilities for exploration to determine the metabolites involved.

Lipids play an important role in the structure and function of the brain as components of cellular membranes. Most of the lipid in brain is present in myelin, the membrane wound about the axons of the neurones. Abnormalities occurring during the developmental period of brain can interfere with myelination and give rise to structural and functional defects. This appears to be the case in the sphingolipidoses. The nonmyelin lipid occurs in other cellular membranes (microsomal, nuclear, mitochondrial, etc.). Abnormalities of lipid metabolism can thus affect the structures and functions of all subcellular organelles.

In this report the status of the general methodology for lipid analysis of brain is considered first, the lipid composition changes taking place during normal development are then described, abnormalities found in the sphingolipidoses are defined, and reference is made to findings in cerebral cortical atrophies. The possibility that other disorders of phospholipid and glycolipid metabolism exist and how these may be searched for is then considered.

**SAMPLING PROCEDURES FOR BRAIN LIPID ANALYSIS**

Different regions of the brain have different lipid compositions and it is thus essential to obtain specimens that are to be compared from exactly the same site. This is difficult to achieve in practice as pointed out in detail elsewhere. Analysis of small amounts of grey or white matter from the frontal lobe, a common procedure, is not satisfactory owing to the variability of both grey and white matter samples from...
different regions of brain. Specimens exposed to formalin or other preservatives (rather than preservation by freezing) are unsatisfactory since lipid composition is altered by chemical preservatives.

When the brain is cut in half longitudinally, homogenized and an aliquot removed, a sample representative of whole brain is obtained that is useful for analysis of changes affecting the brain as a whole. With such samples, the difficulty of variations in composition of small samples is overcome and the total amount of lipid in brain can be determined accurately. This sampling procedure may not be satisfactory where morphological changes are confined to small areas of the brain and the large mass of presumably normal tissue would be expected to mask the changes. In such cases smaller anatomical units must be analyzed. When improved procedures are developed for isolation of each subcellular structure, analysis of these structures may be expected to disclose abnormalities that cannot be defined as well by analysis of other types of samples.

ANALYTICAL PROCEDURES

Modern procedures for precise quantitative analysis are based upon chromatographic separation of intact lipids. The deficiencies of earlier procedures employing solvent partition, solvent precipitation, and analysis of water soluble hydrolysis products have been considered in detail recently and will not be considered here. Precise quantitative analysis is accomplished by column or thin layer chromatography or combinations of the two procedures.

Thin Layer Chromatography. Following separation by two dimensional thin layer chromatography, phosphorus analysis of spots provides a means for precise determination of the molar ratios of phospholipids. The other lipids of brain (cholesterol, free fatty acids, ceramide, cerebrosides and sulfatides) can be determined by quantitative thin layer chromatography using charring and transmission densitometry. Charred (black) spots are produced by spraying with a mixture of sulfuric acid and potassium dichromate and heating. The char intensity determined by transmission densitometry is used for quantitative analysis. The values for cholesterol, free fatty acids and ceramide by the charring procedure are rather precise. Values for cerebrosides and sulfatides, particularly of fetal and infant brains where glycolipid levels are low, are less accurate with thin layer chromatography alone than values obtained by column chromatography followed by quantitative thin layer chromatography. Thin layer chromatography alone does not provide a satisfactory means for determination of gangliosides.

Quantitative Removal of Nonlipid Contaminants. Lipid extracts contain nonlipid contaminants including protein, salts, free amino acids, etc. The solvent partition procedure of Folch et al. for removal of nonlipid contaminants is not suitable for precise quantitative analysis since some lipid is lost into the nonlipid phase and some nonlipid remains in the lipid phase. The procedure is least reliable and predictable when applied to samples from pathological states. Cellulose column chromatography is useful for separation of lipids from nonlipids but Sephadex column chromatography is the best available quantitative procedure. By Sephadex column chromatography nonlipid contaminants are quantitatively separated from lipids, and gangliosides are separated from other lipids. Sephadex column chromatography thus provides a means for accurate determination of total lipid and when combined with quantitative thin layer chromatography, accurate values can be obtained for the major gangliosides. The combination of Sephadex column chromatography with quantitative thin layer chromatography provides the simplest means for obtaining relatively accurate values for total lipid and most of the phospholipids and glycolipids of brain.

Column Chromatography on Diethylaminoethyl (DEAE) Cellulose. The use of DEAE cellulose was introduced for brain lipid analysis in 1961. DEAE column chromatography provides a means for separation of polar lipid classes by ion exchange combined with partition chromatography. The elution sequence for DEAE columns is quite different from the sequence with silicic acid columns so commonly used for lipid separations. A major difference between the two are the complete separation of acidic and nonacidic lipids with DEAE whereas some acidic lipids are eluted with nonacidic lipids from silicic acid. Also, the
choline lipids (phosphatidyl choline, lysophosphatidyl choline, sphingomyelin) are eluted before phosphatidyl ethanolamine from DEAE. These differences and the relatively mild conditions used in DEAE chromatography make DEAE the preferred type of column for quantitative analysis. The combination of Sephadex and DEAE column chromatography with quantitative thin layer chromatography provides a method of analysis with several advantages compared to thin layer chromatography alone or in combination with Sephadex column chromatography. Minor components such as phosphatidyl glycerol, diphosphatidyl glycerol, and phosphatidic acid are concentrated in a separate fraction by DEAE column chromatography and are then more accurately determined by quantitative thin layer chromatography. Analysis of fatty acid composition of minor components is greatly facilitated by DEAE column chromatography prior to thin layer chromatography. DEAE column fractions are composed of a smaller number of components and quantitative one-dimensional thin layer chromatography with charring or phosphorus analysis is possible since spot overlap is eliminated and the improved resolving power of two dimensional chromatography is less essential.(8)

Silicic Acid Column Chromatography. Although silicic acid column chromatography has been widely used for lipid separation, it has not found extensive application for brain lipid analysis. A combination of silicic acid and silicic acid-silicate columns was used for determination of phosphatidyl ethanolamine and phosphatidyl serine(13) and silicic acid column chromatography following DEAE column chromatography provided a means for quantitative column chromatographic separation of several brain lipids.(12) Recently, however, a new procedure was developed(14) that extends the usefulness of silicic acid column chromatography for brain lipid analysis. With this procedure a short column (5 cm) is eluted with chloroform (8 column volumes), acetone (40 column volumes), and methanol (10 column volumes) to provide fractions composed of cholesterol, cerebrosides plus sulfatides (accompanied by two trace components, probably ceramide dihexoside and diglycosyldiglyceride), and phospholipids. Elution with chloroform, acetone, and methanol separates brain lipids into sterol, glycolipid and phospholipid fractions that are readily determined by quantitative thin layer chromatography. Some decomposition of phospholipids takes place as a result of adsorption onto silicic acid and thus the procedure is most useful for glycolipid determinations. The very low levels of glycolipids in fetal brain as well as in some types of neuropathology can be determined with ease and accuracy by the silicic acid column-thin layer chromatography procedure.

Additional Types of Columns. Two other column chromatographic procedures deserve mention. Silicic acid-silicate column chromatography provided the first means for quantitative separation of phosphatidyl ethanolamine from phosphatidyl serine(12) and is still the only column procedure reported for quantitative separation of phosphatidyl choline from sphingomyelin.(15) Florisil, a synthetic magnesium silicate, was introduced by Radin et al. (16) for column chromatographic determination of cerebrosides and the procedure was subsequently modified.(12) The Florisil procedure is not, however, completely reliable for quantitative analysis. Since elution of silicic acid with acetone provides the same separation into cholesterol, glycolipid and phospholipid fractions obtained with Florisil, use of silicic acid rather than Florisil is indicated.

POSTMORTEM AUTOLYSIS

While of obvious importance, the extent to which postmortem autolysis affects lipid analysis has not been considered in any systematic manner by previous investigators. We therefore began a study of the effects of autolysis on organ lipid composition. A summary of the findings with bovine and human organs extracted at various times after death are presented here. The results with organs other than brain are important since examination of other organs is essential for definition of the changes in lipid metabolism.

The autolytic rates of organs differ. Whole brain shows little change even after standing for 48 hours at 24°C, but this is related to a considerable extent to the fact that myelin is relatively stable and contributes most of the lipid. Heart and lung undergo relatively slow autolysis while liver autolizes very rapidly. Kidney and spleen are intermediate. Autolysis is extensive and rapid at room temperature but very slow at 4°C, almost
no change being detected, e.g., in heart or lung lipids after standing for as long as one week at the reduced temperature.

The lipid composition changes in heart, an organ relatively stable to postmortem changes, are shown in Table 1. At 24°C for 24 hours all glycerophospholipids decreased in amount while at 4°C even for 7 days there was practically no change in lipid composition. At 24°C the total decrease in phosphorus represented by phosphatidyl choline, phosphatidyl serine, phosphatidyl inositol, phosphatidyl glycerol, and diphosphatidyl glycerol (7.3 percent) is close to the increase in phosphorus at or near the origin (6.0 percent) where most of the corresponding lysophospholipids appeared but were insufficiently separated for individual determination. The decrease at 24°C in phosphatidyl ethanolamine (4.0 percent) is almost equal to the measured increase in lysophosphatidyl ethanolamine (4.3 percent) that migrated with sphingomyelin. Evidently glycerolphosphatides were degraded to the corresponding lysophospholipids. The control values in Table I do not represent accurately the phospholipid content of heart in vivo since the specimen was obtained 7 1/2 hours postmortem.

During postmortem autolysis the glycerolphospholipids decrease in amounts, but sphingomyelin of normal organs and cerebrosides in Gaucher's disease undergo little or no change. Autolysis therefore results in a relative increase in the amount of sphingolipid in organs. The levels of cholesterol and triglyceride remain substantially the same through prolonged periods of autolysis. Free fatty acid increases steadily during autolysis and the level of free fatty acid is the best single indicator of the extent of autolysis.

Table 1.—Lipid Changes in Human Heart at 4°C and 24°C

<table>
<thead>
<tr>
<th>Lipid Class</th>
<th>Control</th>
<th>24 hrs at 24°C</th>
<th>2 days at 4°C</th>
<th>7 days at 4°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphatidyl choline</td>
<td>39.8</td>
<td>34.5</td>
<td>40.3</td>
<td>39.3</td>
</tr>
<tr>
<td>Phosphatidyl ethanolamine</td>
<td>25.1</td>
<td>21.1</td>
<td>25.5</td>
<td>24.9</td>
</tr>
<tr>
<td>Phosphatidyl serine</td>
<td>1.8</td>
<td>0.9</td>
<td>1.4</td>
<td>1.5</td>
</tr>
<tr>
<td>Phosphatidyl inositol</td>
<td>5.4</td>
<td>4.9</td>
<td>4.7</td>
<td>4.6</td>
</tr>
<tr>
<td>Phosphatidyl glycerol</td>
<td>0.3</td>
<td>0.0</td>
<td>0.2</td>
<td>0.4</td>
</tr>
<tr>
<td>Diphosphatidyl glycerol</td>
<td>10.8</td>
<td>10.5</td>
<td>11.2</td>
<td>11.1</td>
</tr>
<tr>
<td>Sphingomyelin plus lysophosphatidyl ethanolamine (^2)</td>
<td>5.6</td>
<td>9.9</td>
<td>6.0</td>
<td>6.5</td>
</tr>
<tr>
<td>(X_1) (^3)</td>
<td>0.6</td>
<td>0.4</td>
<td>0.3</td>
<td>0.4</td>
</tr>
<tr>
<td>(X_2) (^4)</td>
<td>0.9</td>
<td>2.1</td>
<td>0.7</td>
<td>1.5</td>
</tr>
<tr>
<td>Uncharacterized (^5) at or just off the origin</td>
<td>9.8</td>
<td>15.8</td>
<td>9.8</td>
<td>9.6</td>
</tr>
</tbody>
</table>

\(^1\)Values expressed as percentage of the total phosphorus. Analysis performed by phosphorus assay of spots after two dimensional thin layer chromatography with chloroform/methanol/28 percent (by wt) aqueous ammonia followed by chloroform/acetone/methanol/acetic acid/water 5/2/1/1/0.5.

\(^2\)The lysophosphatidyl ethanolamine spot overlapped with the sphingomyelin spot making separate analysis impossible.

\(^3\)\(X_1\), an uncharacterized phospholipid, migrated well ahead of phosphatidyl ethanolamine in both solvents.

\(^4\)\(X_2\), another uncharacterized phospholipid, migrated slightly less than phosphatidyl choline in the first solvent and with it in the second solvent making \(X_2\) appear just below phosphatidyl choline on the chromatogram.

\(^5\)Phosphorus at or near the origin included lysophosphatidyl ethanolamine of phosphatidyl choline, phosphatidyl serine, and phosphatidyl inositol.
In general, autolysis is rapid at room temperature, very slow near 0°C, and prevented almost completely for prolonged periods at or below -20°C. It is clear that organs should be obtained as fresh as possible and frozen until analyzed. The post mortem autolysis period should be specified when values for glycerol-phospholipids of human organs are reported. Values for sphingolipids when expressed as percentages of the wet or dry weight are not affected to any appreciable extent by autolysis.

**BRAIN LIPID COMPOSITION CHANGES DURING NORMAL DEVELOPMENT**

The brain undergoes extensive changes in lipid composition after birth and these changes must be determined accurately if deviations from normal are to be clearly defined. Fortunately modern chromatographic methods are suitable for this purpose and the major changes in composition have been defined in a relatively precise manner recently.\(^{(6, 17)}\)

During development, brain mass and total lipid increase, water content decreases, and the relative amounts (proportions) of the different lipids change markedly. The relative proportion of gangliosides decreases while cerebrosides and sulfatides increase. These changes are readily understood when it is appreciated that cerebrosides and sulfatides are components of myelin that increases in amount during development while gangliosides occur largely in nonmyelin structures. Changes in phospholipid composition also reflect the large increase in myelin during development. The phospholipid values in Table 2 show that phospholipids occurring largely in myelin (sphingomyelin and phosphatidyl serine) increase in amount while phosphatidyl choline that is more abundant in nonmyelin structures decreases. The proportions of phosphatidyl ethanolamine and phosphatidyl inositol undergo little change since both of these lipids occur to very nearly the same extent in myelin and nonmyelin structures.

| Table 2.—Phospholipid Composition of Normal Whole Brains at Different Ages* |
|-----------------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
|                 | Fetal  | 1 d    | 3 w    | 5/2 m  | 8 m    | 22 m   | 6 y    | 8 y    | 33 y   |
| Phosphatidyl choline | 51.6   | 49.7   | 47.5   | 40.7   | 38.5   | 35.7   | 32.7   | 32.8   | 30.4   |
| Sphingomyelin     | 1.9    | 3.7    | 4.0    | 7.4    | 9.3    | 11.2   | 12.9   | 13.6   | 14.8   |
| Phosphatidyl serine| 11.9   | 11.8   | 12.6   | 13.9   | 14.5   | 15.4   | 16.8   | 16.8   | 17.1   |
| Phosphatidyl ethanolamine | 31.1  | 31.5  | 32.3  | 35.3  | 35.0  | 34.9  | 34.6  | 33.8  | 35.1  |
| Phosphatidyl inositol | 2.9  | 3.2  | 3.3  | 2.3  | 2.6  | 2.8  | 3.2  | 3.0  | 2.2  |

*Expressed as percent of the phospholipid phosphorus of the lipids shown; d, w, m, y: days, weeks, months, years. Values from reference 17.
LIPID COMPOSITION CHANGES IN THE SPHINGOLIPIDOSES

The group of hereditary diseases known as the sphingolipidoses are disorders of sphingolipid metabolism. The classical types are rather well understood and appear to be the result of hereditary deficiencies of degradative enzymes for sphingolipids. There is at least one known disorder for each of the different sphingolipids. Involvement of sphingomyelin in Niemann-Pick disease, ganglioside in Tay-Sachs disease, cerebroside in Gaucher's disease, sulfatide in metachromatic leucodystrophy, and ceramide polyhexosides in Fabry's disease has been demonstrated. In each disorder accumulation of a sphingolipid in one or more organs is observed. Organ involvement varies and depends upon the characteristic lipid distribution of each organ to a great extent. Thus sulfatide accumulation in metachromatic leucodystrophy occurs in brain and kidney, the only organs containing more than traces of sulfatide in normal individuals. Since sphingomyelin is found in all organs, it is not surprising to find that sphingomyelin accumulates in brain and other organs in Niemann-Pick disease. In Tay-Sachs disease ganglioside accumulation is marked in brain, but an increased ganglioside level is found in other organs and in erythrocytes that normally contain gangliosides.

The accumulation of a sphingolipid in brain interrupts the normal maturation process and the other lipids of brain are present in amounts characteristic for an immature brain regardless of the age at the time of death.

Quantitative differences in enzyme deficiencies are indicated in particular in Gaucher's disease. The rate of deposition of cerebroside is quite variable as judged by the wide variations in the clinical picture. In the chronic forms of the disease, the brain is not involved and the extent of hepatosplenomegaly varies widely, in some cases being large in childhood and in other cases becoming prominent much later. In the acute (infantile) form of Gaucher's disease the brain is involved and recently an increase of both cerebrosides and sulfatides in brain in this disorder has been found. Liver and spleen in acute Gaucher's disease show the same large accumulation of glucocerebroside found in the chronic forms of the disease. This variable picture in Gaucher's disease could arise from differences in the level of the deficient enzyme, the enzyme level being lowest in the acute form of the disorder. Quantitative differences of degradative enzymes also appear to exist in other sphingolipid disorders.

From the practical standpoint it is to be stressed that thin layer chromatography is a relatively simple procedure that can be used as an aid in diagnosis of disorders of lipid metabolism. Altered proportions of lipids are easily detected and measured by thin layer chromatography. From the standpoint of understanding the nature of brain disorders, the fact that accumulation of a sphingolipid in brain is associated with an overall lipid pattern generally typical of an immature brain is important. We may expect to find the immature brain lipid pattern in metabolic disorders of carbohydrate, amino acid, etc. metabolism when the disorders are associated with abnormal development of the brain. Determination of brain lipid composition with modern chromatographic techniques in galactosemia, phenylketonuria, and disorders of amino acid metabolism generally should be very instructive.

LIPID CHANGES IN SENILE AND PRESENILE CEREBRAL ATROPHY

Lipid composition of brain in the cerebral cortical atrophies has been investigated to a very limited extent. Senile atrophy with little or no decrease in brain mass was not found to be associated with any distinct deviation from normal brain lipid composition. A decrease of sulfatide was found in one case but was not observed in two additional cases. In an hereditary form of presenile atrophy (Alzheimer's disease) with a large decrease (about 42 percent) in brain mass, no evidence for primary lipid changes was obtained, although a decrease of sulfatide was observed in one case but not in another. The findings in Alzheimer's disease can be explained as being related to a degeneration of the brain from an unknown cause with death and degeneration of cells leading to reduction in mass but with retention of a relatively normal amount of lipid expressed on a wet or dry weight basis of the brain.
remaining tissue and with normal proportions of the lipid classes.

The study of degenerative changes of adult brain presents a major difficulty that must be clearly recognized. If a change in the biosynthetic or degradative rates of one or more lipid classes should occur in later life, the large mass of myelin present could mask relatively small changes occurring outside of myelin. Detection of such relatively minor changes might thus best be accomplished by analysis of isolated subcellular organelles or organs other than brain. These possibilities are presently under investigation.

AREAS FOR FUTURE STUDY

The availability of relatively rapid, simple and accurate procedures for analysis of phospholipid and glycolipid composition of organs makes possible a systematic examination of the roles of polar lipids in human disease. Quantitative thin layer chromatography is particularly suitable for such studies. The polar lipids are components of cellular membranes and thus changes in polar lipid composition may be reflected in alterations of cellular functions (such as permeability, excitability, and contractility) and the course of biosynthetic reactions may be influenced by virtue of the structural roles the lipids have in the cellular apparatuses performing the functions and chemical reactions. Since lipid chemistry generally and quantitative methodology in particular have progressed to the point where broad based studies are possible, it seems appropriate to consider where and how the procedures may be applied.

The sphingolipidoses provide important general information on the role of polar lipids in disease. A disorder for each type of sphingolipid is known and the diseases appear to arise from deficiencies of degradative enzymes. The enzyme deficiencies appear to vary quantitatively, thus giving rise to conditions of varying severity. Such a gradation is most apparent for Gaucher's disease. Juvenile and adult forms of Niemann-Pick disease (involving sphingomyelin) have been described and disorders of ganglioside and sulfatide metabolism appear to exist in both infantile and juvenile forms. Stanbury et al.\(^1\) have considered these variations in detail. Knudson et al.\(^2\) encountered a disorder with brain involvement beginning during the second year of life that appears to be related to accumulation of sphingomyelin in organs but to a lesser extent than in Niemann-Pick disease where neurological involvement is apparent at an earlier age.

The gradation in the sphingolipidoses leads to the expectation that more acute forms of some sphingolipid disorders will be found and that less complete enzyme deficiencies will be found in other cases. A profitable general approach involves the following sequence: First, selection for study of hereditary disorders where derangement of a process, e.g. brain development or renal function, is well documented; second, chemical analysis of polar lipid composition of brain and other organs to disclose abnormalities; and third, in vitro assay to pinpoint the enzymatic defects.

Many interesting possibilities exist. Fabry's disease occurring in adults may appear in more acute forms not presently recognized as defects of sphingolipid metabolism. A search for a more acute form should begin with studies of conditions with renal involvement. Hereditary disorders involving the kidney offer interesting possibilities in general since the kidney contains sphingomyelin, ceramide polyhexosides, and sulfatides. Some hereditary disorders of renal function are known at present only as processes associated with abnormal excretion of one or more substances in a more or less characteristic manner. The Fanconi syndrome is a good example. The molecular basis for such abnormal processes is not known, but the possibility that some are disorders of sphingolipid metabolism must be examined. Disorders such as phenylketonuria and maple sugar urine disease in which the central nervous system is involved and abnormal metabolites are excreted in the urine should be examined with the same thought in mind.

The processes for removal of triglycerides and cholesterol from blood are known to be defective in familial disorders (hypertriglyceridemia and hypercholesterolemia). The processes have not been defined at the molecular level, but it is apparent that the defects may be related to altered membrane function and perhaps due to abnormalities of sphingolipid metabolism.
The sphingolipidoses illustrate clearly that lipid composition may be altered in several organs but the clinical problems may be referable primarily to one organ. Thus, the brain is involved in Tay-Sachs disease, hepatosplenomegaly is prominent in chronic Gaucher's disease, and renal abnormalities are important in Fabry's disease. The age of onset is an important factor in organ involvement. Brain may be involved when a disorder appears during the developmental stages. Degradation of gangliosides and ceramide polyhexosides involves splitting of several different types of chemical bonds, probably by different enzymes, and a series of related but distinctly different clinical conditions are thus to be expected. Such variants may find preferential expression in different organs.

One of the most interesting possibilities is that relatively mild enzymatic deficiencies of degradative enzymes for sphingolipids may exist and cause clinical problems later in life. It seems quite reasonable to suppose that relatively mild enzymatic deficiencies (perhaps best thought of as "latent") may become more important in middle or old age as a result of changes in overall metabolic status and that these changes could produce severe clinical problems. Following this line of reasoning we are presently investigating organ polar lipid composition in cases of cerebral cortical atrophy, particularly of the Alzheimer's type. Perhaps these conditions are related to changes in lipid metabolism that are difficult to detect in brain specimens by lipid analysis due to the large mass of myelin but that may be more readily detected by analysis of other organs.

It seems probable that any of the sphingolipids could be involved in disorders with expression in later life. A milder disorder related to failure to degrade sulfatide as in metachromatic leucodystrophy might lead to renal problems since only minute traces of sulfatide are present in organs other than brain or kidney. Ceramide polyhexosides when affected may also be expected to cause renal problems by analogy to the changes in Fabry's disease and the occurrence of ceramide polyhexosides in lens(21) suggests the possibility of a defect in this organ. Changes in sphingomyelin metabolism could affect any organ, while changes in ganglioside metabolism could affect brain, spleen, liver, and the lens, all known to contain gangliosides, and perhaps other organs as well.

Although distinctly different from the hereditary disorders under discussion, lipid abnormalities are generally considered to be important components of vascular disease. While clearly a complex subject not to be grouped with the hereditary disorders under discussion, some comment seems appropriate since large differences in phospholipid composition have been found in diseased vessels and a relatively small deficiency of an enzyme concerned with sphingomyelin metabolism might be an important factor in vascular disease. Buck and Rossiter(22) first reported an increase of sphingomyelin in atherosclerotic aortas and this was confirmed by column chromatography by Böttcher.(23) We have confirmed this increase of sphingomyelin in the diseased aorta using thin layer chromatography. Fetal aorta was found to contain much less sphingomyelin than adult aortas without extensive atherosclerotic change while grossly atherosclerotic aortas were found to contain still more sphingomyelin. Interpretation of these changes is complicated by the fact that the same general picture is observed as a result of autolysis (see above), although the picture cannot be attributed entirely to postmortem autolysis. In the present context it seems sufficient to point out that the phospholipid changes in atherosclerosis are in keeping with autolysis studies in that the degradative enzymes for sphingolipids appear to be less active than those for glycerol phospholipids and that an increased level of sphingomyelin in vessels and perhaps other organs could be produced by cell degeneration and death with less complete hydrolysis of sphingomyelin leading to its accumulation.

Several obstacles will probably be encountered in broad based studies designed to disclose additional disorders of sphingolipid metabolism. It is apparent that lipid metabolism is quite generally influenced by abnormalities of metabolism not involving lipids directly, e.g., in glycogen storage disease and diabetes, while other disorders may depend upon primary defects of lipid metabolism. Both primary and secondary changes should, however, be defined carefully for better understanding of disease processes. Comparison of lipid changes in known nonlipid disorders with suspected disorders of lipid metabolism may provide a valuable means for establishing standards by which primary metabolic derangements can be distinguished from secondary effects.
accounts of various inherited metabolic diseases are presented and lipid changes associated with some nonlipid disorders are described.

Sampling of organs can present numerous problems. Thus, study of whole brain may not disclose a defect expressing itself in only one area of the brain. Studies of samples that are not representative of the brain and for which true controls are not available may account, along with analytical difficulties, for the variable findings in phenylketonuria. Conditions first arising after full development of the brain, despite the fact that they occur throughout the brain as a whole, may be masked by the large amount of myelin lipid. It will be necessary to study the affected areas of brain in some cases and isolated pure preparations of subcellular structures in others. Similarly, in studies of other conditions, e.g., renal tubular dysfunction, examination of the isolated structures such as tubules may prove to be essential. Procedures for isolation of such structures should therefore be developed.

REFERENCES

DISCUSSION

DR. SWAIMAN: I was interested to find when we invited Dr. Rouser that he had never examined a phenylketonuric brain. Maybe I should not have been. But the fact remains that our interest in phenylketonuria includes the derangement of brain structure and we must at some point obtain adequate lipid analysis of PKU brain.

DR. ROUSER: I might add that we don't need much tissue for analysis.

DR. MENKES: I think I should like to extend Dr. Rouser's methods to phenylketonuria. We have examined three phenylketonuric adults, one phenylketonuric child, and also one very interesting case of a severely retarded non-phenylketonuric heterozygote of a phenylketonuric mother.

Our conclusions are as follows: In all three adult phenylketonurics there was a reduction of cholesterol and of cerebrosides to the lower limits of normal or just beyond. A fair number of control specimens were used.

Secondly, as far as the structure of the cerebrosides and sulfatides were concerned—and these were the only ones we could determine because we had three autopsy specimens and we could not on those specimens determine the structure of the more labile phospholipids—we found that in one case there was a very striking reduction of the kerasin cerebroside. This was present in at least two of the sections of this phenylketonuric patient, but is not a specific finding in phenylketonuria.

In all three adult phenylketonurics we have seen a reduction in kerasin sulfate and a reversal of the normal ratio of kerasin to cerebron sulfate. Normally kerasin sulfate and cerebron sulfate are present in a 4 to 1 ratio. In the PKU specimens the ratio is reversed.

Again I would wish I could say this finding is specific for PKU. Unfortunately, we have noted the same finding in demyelinating disorders.

In the biopsy material we obtained on the phenylketonuric child, we isolated the proteolipids by the methods that Folch and Lees used. The fractionation of the proteolipids on silica was perfectly normal by our standards and also normal when compared to Folch's data.

Finally, in the specimen from a child who was not a phenylketonuric but was exposed to high phenylalanine environment during gestation, we noted that the cerebrosides were reduced to the level seen in the PKU specimens, but that the concentration of sulfatides was normal, as indeed it is in all the phenylketonurics we have studied up to now.

I should say a few words on Maple Syrup disease. In untreated Maple Syrup disease there is no cerebroside or sulfatide and very little sphingomyelin.

DR. ROUSER: Were the specimens put into formalin?

DR. MENKES: Two were. Three were not. It made no difference.
DR. ROUSER: You mean it makes little difference with the cerebrosides. Because it makes a vast difference in some of the other lipids.

DR. MENKES: Of course it does in proteolipids.

DR. ROUSER: One of the things that has been very bothersome to us is the habit of the pathologist of placing all of the brain into formalin. Studies of formalin preserved brain has led to erroneous descriptions of lipid change in disease.

One thing more. How big were the samples?

DR. MENKES: They varied. The biopsy weighed one gram. The other ones were fairly large.

DR. ROUSER: This problem of sample size again leads to difficulty. I always like to see findings with small samples confirmed with a larger specimen or at least studies reproduced exactly in the normal and the pathological state. Those are some very important considerations.

I don't want to deny that these changes occur. I am just saying that there are difficulties. I think there are a great deal of secondary changes.

We think the changes in phenylketonuria may not be related to primary effects on lipid abnormalities. In that sense our findings with brain lipids in Alzheimer's disease are interesting.

In this disease we found a large reduction of sulfatide in some, but not all specimens. The reason for this variation is not known. These may be interesting secondary effects, but they may very well have a good deal to do with the neurological problem in a particular patient.

I still look forward to examining PKU specimens myself.

DR. WOOLF: I have had the opportunity to examine the brains of four phenylketonuric children aged from one year to fourteen years. We didn't use Dr. Rouser's very elegant techniques because this was some years ago. We performed quantitative lipid galactose, cholesterol and cholesterol ester determinations.

Lipid galactose was markedly reduced. Cholesterol was also markedly reduced in all four brains as compared with age-matched controls.

This checked pretty well with what Dr. Menkes says. The findings were more marked in our cases because ours were younger.

Cholesterol ester was not present in three cases, as in normal. In one case there was cholesterol ester in some parts of the brain; those were the parts of the brain which the pathologist found to be undergoing demyelination by histological examination. A fourteen year old was thought to be a case of Schilder's disease plus phenylketonuria. Now, this is really a very worrying and important observation. At least it worries me. Did this child undergo demyelination because of a high phenylalanine content in the blood?

We know that cholesterol ester doesn't stay in the brain for any length of time. It is pretty rapidly removed during demyelination. We feel this cholesterol ester I found, and the pathologist also found, was there for only six months or a year before death. Would Dr. Rouser agree with us?

DR. ROUSER: I don't know anything about cholesterol esters in brains because we have never found any. But it sounds reasonable.

DR. WOOLF: This was an acute lesion happening not too long before death. Is it a coincidence that this child had phenylketonuria?

I may say the same pathologist has found the same thing in four other phenylketonurics in the late adolescent or early adult group who also show the clinical signs of Schilder's disease. I haven't looked at those brains yet. But this possibility worries me; if we are going to take the phenylketonurics off the diet and put them on a normal diet, are they going to undergo demyelination in late adolescence?

DR. SWAIMAN: I think one of the obvious points to be emphasized is that adequate study of the lipid composition of brain can be carried out only if the brain is frozen immediately after removal and not put into formalin.

DR. SCRIVER: Can I draw a comment from Dr. Rouser? Earlier in this session he discussed the increasing interest in membranes, and there are now techniques which can inform us about the morphology of membranes. We are paying more attention particularly to the protein and lipid components of the membrane and attempts are being made to define them more specifically.

There is a third component which we have to think about, and that is the water in the membrane. The point I am referring to specifically is the emerging theoretical and practical implications about the structure of water in the membrane and the fact that water is a highly organized structure in reference to its contact with lipids.
One of the things that I would like Dr. Rouser to comment upon, is the impact of molecules with a-polar side chains such as phenylalanine, valine, leucine upon the structure of water in membranes. Does he think this has any bearing in the study of diseases such as phenylketonuria, and maple syrup urine disease?

Would these molecules have any structure-forming effect on water in membranes? And if so, is there merit in investigating the impact of this on sodium and potassium transport and conductance by membranes?

DR. ROUSER: Naturally I don't know how to answer the questions. And whatever one says in this regard must be, of course, based on interesting speculation.

There are some approaches, however, to some of these things, and I have been trying to encourage people to take these approaches. One of the most interesting approaches with regard to the water structure has been: What can you build, given what we know about the composition of a membrane?

I challenged Dr. F. A. Vandenheuvel with that problem and made sure that he would get to work right away by putting him as a key speaker on a symposium. And he came up with some interesting things. Perhaps some of you have read his paper on the structure of myelin produced in response to that challenge.

Several other people have attempted to do much the same thing. And in building these structures they all end up with the fact that they have to have a well-organized water layer regardless of the assumptions as to type of protein and the configuration of water, about which there is some very vigorous discussion I might add.

Water is very highly organized in these membrane structures. When molecular models of membranes are built according to scale with the known lipids and the apparent sizes of the proteins, there isn't much space for water. The spacings are appropriate for a certain type of water, but I don't want to get into the type since this is a point of much discussion. The spacings are adequate for water to reach across polar groups. Perhaps there are two layers of water in between a lipid protein layer, or perhaps one.

Now, the next part of your question. Could amino acids change the organization? I really don't know how to think about this problem in detail except with molecular models, but I would say this: Membranes are not stable, they are subject to thermal agitation, to many possible derangements, and, therefore, it certainly is quite conceivable that any kind of substrate, any kind of substance present in abnormally large amounts, could shift the normal status of this membrane, change the pores, change even the composition of the membrane.

In the future we hope to isolate the subcellular organelles from brain specimens to determine whether changes in lipid composition are present. Some very strange particles can be seen by electron microscopy. We would like to know something about their composition. But that's all in the future.
It is painfully apparent that although almost twenty years have passed since the elucidation of the enzymatic block in phenylketonuria the basic causes of the mental retardation that accompany this disease remain unknown. The consistently noted microcephaly of these patients draws attention to the various synthetic pathways of developing brain. Although other broad areas of metabolism are justly suspect, the possibility of derangement in amino acid and protein metabolism leading to protein synthesis must be considered. Unfortunately, the basic processes leading to protein synthesis in developing brain have not been explored in great detail. The recent demonstration of relationship between nucleic acid metabolism, learning and memory make this area more intriguing. It is the intent of this presentation to briefly review pertinent studies relating to protein synthesis in developing brain; and to point out the meager experimental data available concerning the relationship of phenylalanine and its metabolites to the various steps involved in protein synthesis.

The steps at which accumulation of phenylalanine or its metabolites most likely could interfere with protein synthesis include: (1) blood brain barrier exchange or transport; (2) synthesis of non-essential amino acids in brain; (3) interference in the sequence of reactions leading from amino acid activation to incorporation into protein.

**FREE CEREBRAL AMINO ACIDS**

**Blood Brain Barrier System**

The likely importance of the various aspects of the blood-brain barrier system in the regulation of protein synthesis must be emphasized. (Under no circumstances should the blood-brain barrier be equated with the blood-spinal fluid barrier.) The rather common assumption that the blood-brain barrier does not exist or is faulty in the neonatal period is doubtful. Rather, it is more likely that the increased net uptake of various substances from blood to brain in the neonatal period is due to a dynamic system actively supporting such transport. The modulation of amino acid transport effected by the barrier system varies with age, amino acid, pH, plasma and cerebral concentration of that amino acid, and the region of brain under scrutiny. The blood-brain barrier is really a mechanism of control of net uptake by brain. The "barrier" allows free interchange between many amino acids of plasma and brain even when there is no net brain or plasma uptake. Studies have demonstrated that amino acids can be transported in or out of brain against a concentration gradient. The rate of exchange of amino acids is increased if the cerebral concentration is increased.

**Common Transport Mechanisms**

It is likely that certain amino acids share common blood-brain transport systems or carriers. This hypothesis would best explain the findings of Guroff and Udenfriend that stereospecificity and blood-brain barrier function for tyrosine was as pronounced in newborn animals as in adults. They found that the cerebral uptake of L-tyrosine was as rapid as in adult rat brain and that more intracellular concentration took place in newborn brain. They concluded that the transport mechanisms for various ions and metabolites are more active in newborn brain while the blood-brain barrier remained intact. There is competition for cerebral uptake among aromatic amino acids and, therefore, good reason to believe that aromatic amino acids share a common transport pathway for entry into the immature brain. This type of competition suggests that high phenylalanine concentration results in lower...
concentrations of other aromatic amino acids in brain. Studies of adult rat brain in-vivo and in-vitro, (9) and studies of amino acid transport in other tissues (10) tend to confirm this common transport hypothesis.

Evidence for specific and non-specific interference with amino acid transport in the presence of high plasma levels of phenylalanine was reported by Carver. (11) Carver studied adult male rats who had received 100 mg. of L-phenylalanine and noted changes of free amino acids of brain at 15, 30 and 120 minutes. Significant amino acid changes occurred in brain of aspartic acid at 120 minutes, of glutamine at 15 and 120 minutes, of isoleucine at 15 minutes, of leucine at 15 minutes, of tyrosine at 30 and 120 minutes, of phenylalanine at 15, 30 and 120 minutes and of gamma aminobutyric acid at 15 and 30 minutes. He noted more drastic changes in the liver amino acid pool and speculated that the blood-brain barrier system serves to protect the amino acid pool in brain from large deviations in plasma amino acid concentration. It should be pointed out that changes in the non-essential amino acid concentrations may well stem from other difficulties than impairment of transport.

Noall (12) and co-workers demonstrated that uptake of alpha amino isobutyric acid by rat tissues, including brain, was enhanced by epinephrine, hydrocortisone and growth hormone.

**Cerebral Synthesis of Amino Acids**

Studies have demonstrated that the carbon of the following amino acids was interchangeable with the carbon of glucose in one day old mouse brain: aspartate, serine, glutamate, proline, glycine, alanine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, histidine, lysine and arginine. (13, 14) The labeling of the carbon of essential amino acids in newborn brain has not been confirmed by other workers.

Over 50 percent of the alpha amino nitrogen in brain is present as glutamate, glutamine and gamma aminobutyric acid. It has been demonstrated by Roberts, Flexner and Flexner that most of the cerebral glutamic acid and glutamine carbon in immature and mature mouse brain is derived from glucose. (15) Further evidence of the lack of net glutamate uptake by adult brain was reported by Schwerin, Bessman and Waelsch. (16) They found that cerebral net uptake of glutamine occurred but there was no net uptake of glutamate. Exchange of glutamate between blood and brain took place freely.

In contrast to the findings in adult brain, Himwich, Petersen and Allen demonstrated net brain uptake of glutamate in newborn albino rats. (17) Similarly, increases in uptake in immature brain versus mature brain have been shown for lysine and leucine. (18, 19) Alanine and aspartate are found to be readily labeled when brain is incubated with radioactive glucose and there is good reason to believe that the carbon of these substances is also derived principally from glucose via pyruvate and Kreb's cycle intermediates. (20)

### CEREBRAL PROTEIN SYNTHESIS

Lajtha studied flux of lysine from plasma to non-protein nitrogen pool to proteins in adult brain and also in ten day old rat brain. (21) By utilizing specific activity-time curves, he was able to construct charts of flux and half life of the labeled lysine in the non-protein nitrogen pool in brain and in the brain protein. He found that the average half life of proteins of brain in the ten day old animals was less than the half life of proteins at similar elapsed times after injection in adult animals. Flux of lysine in the young brain from the non-protein nitrogen pool to protein was greater at longer elapsed time after lysine administration than in adult brain. This data supports the hypothesis that a greater average turnover rate of proteins as well as greater flux of lysine into protein occurs in developing brain.

Kelley, in her study of nitrogen content of developing rabbit brain, noted that nitrogen content increased until the fiftieth day of life. (21) She also noted that cerebellar areas contained more nitrogen than cerebral and brainstem areas. Generally, phylogenetically older areas of the brain reached their maximum percentage nitrogen content before phylogenetically younger areas.

### Hormonal Affects

Over the past few years, evidence has been amassed that thyroxin administration causes in-vitro and in-vivo increase in amino acid

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Provided by the Maternal and Child Health Library, Georgetown University
incorporation into protein. (22) Studies of rat liver by Sokoloff and Kaufman (23) demonstrate that the increase in activity following thyroxin treatment is localized to the step involving the transfer of sRNA bound amino acid to microsomal protein. Gelber, Campbell, Deibler and Sokoloff (24) in their studies, found L-thyroxin to increase amino acid (L-leucine) incorporation into protein in immature rat brain as compared to inhibition under the same circumstances in adult brain. This particular phenomenon is present in liver of any age animal. It has been known for many years that thyroxin administration does not cause increase of cerebral oxygen consumption in adult brain. In contrast, in the developing brain the rates of both lipid and protein synthesis are higher than in adult brain and thyroxin does, indeed, increase oxygen uptake. (25)

Studies by Wagle (26) in adult rats reveal that hypophysectomized animals show decreased incorporation of alanine, glutamic acid, methionine and phenylalanine into protein in vivo. Incorporation is increased to a near normal rate by the administration of growth hormone or insulin and fully restored by giving both growth hormone and insulin. The administration of cortisol to intact rats increases incorporation of amino acids into protein. It is thus clear that there are several hormonal factors that modify the rate of protein synthesis. It is also becoming increasingly clear that these factors are of greater importance in immature tissue including brain than in mature brain. Although it is thus theoretically possible that hormonal abnormality could influence protein synthesis in phenylketonuria, there is no substantiating evidence that this is true. It must be pointed out that studies of hormonal concentration and/or activity in young phenylketonuric patients are not available.

Amino Acid Composition of Cerebral Protein

Clouet and Gaitonde (27) determined arginine and histidine concentrations in protein of developing rat brain by means of colorimetric methods. No other amino acids were studied. Studies of nitrogen content, water content and lipid of brain were carried out at the same time. They found that the protein nitrogen content of the brain of rats of various ages from fetal life to adulthood revealed a regular increase with age on a wet-weight basis. The protein nitrogen content on a wet-weight basis of six regions of the brain also varied with the age of the rat. In young rats, regional protein nitrogen content was greatest to least in the following order: medulla, hypothalamus, cerebellum, midbrain, olfactory bulb, cortex. In the older rats, the order was midbrain, cortex, cerebellum, hypothalamus, olfactory bulb, medulla. The concentrations of the amino acids arginine and histidine were significantly lower in the brain proteins of the younger rats. The arginine/histidine ratio showed progressive decrease from birth to old age, due to a greater increase in concentration of histidine over the increase in arginine.

The work of Wender and Waligora (28, 29) demonstrates changes in the content of amino acids in the proteins of developing nervous tissue. These authors utilized the guinea pig in their study. The guinea pig is mature by a large number of standards at time of birth. Nevertheless, they noted significant changes in the amino acid content of protein in their developing nervous system. They substantiated the earlier findings of Clouet and Gaitonde that the concentration of arginine and histidine are significantly lower in the proteins of younger rats. Wender and Waligora found that after birth the concentration in dry white matter protein of glutamic acid, arginine, tyrosine, phenylalanine and leucine increased and the content of histidine, serine and valine decreased. Study of gray matter, however, revealed values of all the protein amino acids to be greater on a dry weight basis than in white matter. They thought, however, that only the differences in histidine, serine, arginine and tyrosine were statistically significant. They also noted early in development that the white matter protein composition changes, whereas the gray matter of protein amino acid composition appears to be stable. Later in development, white matter protein composition remains fairly constant, while there are significant changes in gray matter protein amino acid composition.

Incorporation of Amino Acids into Protein

There is little reason to believe that the process of amino acid incorporation into brain protein is different from amino acid incorporation in other tissues. It is known that the two high
energy compounds, adenosine triphosphate (ATP) and guanosine triphosphate, are necessary to these reactions. Interference with energy production could lead to disruption of the mechanisms of incorporation of amino acids into protein. (30) Studies by Swaiman and Milstein, (1) Swaiman, Lemieux and Milstein (2) of oxygen uptake and oxidation of glucose carbon to carbon dioxide in developing rabbit brain reveal no impairment in brain slices by high concentrations of leucine, valine, isoleucine, phenylalanine, phenylpyruvic acid, o-tyrosine, alpha ketoisocarproic acid or alpha ketoisovaleric acid.

The first step of protein synthesis entails the activation of individual amino acids by means of their specific activating enzymes and ATP. A complex consisting of the enzyme, the amino acid and adenosine monophosphate is formed. Wender and Hierowski (33) studied this step in developing guinea pig brain by means of hydroxamate assay. They found cysteine, histidine, glutamic acid, glycine, tyrosine, proline and tryptophan to be activated in fetal white matter. They also reported that activation of glutamic acid was much higher than the other amino acids. In three day old animals they noted the same situation to exist. In nine day old guinea pigs, the activation of cysteine was not present. The only significant change in the nine day old animals from the three day old animals was the suggestion of some increase in glutamic acid activation. The 2-month-old animals and 6-8-month-old animals manifested no different pattern than the 9-day-old guinea pigs, except that there appeared to be a continued increase in the rate of cerebral glutamic acid activation.

In the fetal gray matter, the activation of glutamic acid was again much increased over that of cysteine, histidine, glutamic acid, glycine, tyrosine, proline and tryptophan. After birth the activation of cysteine in gray matter disappeared early. The rate of activation of glutamic acid continued to increase during development. It appears that the activation of amino acids in the gray matter is very similar to that in the white matter, both quantitatively and qualitatively. Comparing this study with their previous work, the investigators concluded that there was no correlation between the amino acid content of the protein in cerebral white and gray matter and the rate of amino acid activation. This finding was most pronounced with reference to glutamic acid which had very high activity in both gray and white matter, but is not found in high concentration in cerebral proteins. These studies point to the fact that the dynamics of protein formation are not necessarily related to absolute amino acid concentration. In a similar vein, Flexner, Flexner and Roberts (34) concluded after studies of glucose and amino acids in newborn and adult brain, "the concentration ratios of amino acids in tissue protein is not consistently paralleled by the ratios of apparent rates of incorporation of these amino acids into tissue protein."

In the next step, the amino acid group is shifted from the activating enzyme complex to an amino acid-specific transfer ribonucleic acid (sRNA). After formation of the aminoacyl complex with sRNA the amino acids are transferred to the ribosomal fraction of the microsomes. (Ribosomal fractions have been reported to be found to a much lesser degree in mitochondria and cell nuclei.) The transfer includes the arrangement of the amino acids on the high molecular weight ribonucleic acid template of the ribosome. The crucial sequence of the ribosomal nucleic bases is thought to be mediated in some manner by the genetically determined desoxyribonucleic acid (DNA) of the cell nucleus.

Recent studies by Murthy and Rapaport (35) demonstrate that pH 5 enzymes secured from developing rat brain are as efficient as pH 5 enzymes secured from adult rat brain in their ability to enhance leucine incorporation into brain microsomes. The rate of leucine incorporation, however, was inversely proportional to the age of the animal from which the brain microsomes were obtained. This group of experiments suggest that there may be crucial times during early development when amino acids are incorporated more rapidly into brain microsomes. Other recent studies have demonstrated that incorporation of amino acids into brain ribosomes is inhibited by free fatty acids. (36) The investigators speculated that this inhibition may indicate one of the mechanisms involved in the regulation of in vivo protein synthesis. It is thus apparent that the maturational changes in lipid synthesis may well have affect on protein synthesis in developing brain.

After formation of the proper peptide bond sequence the finished protein is released by a poorly understood mechanism. There is
obviously little or no information on these various individual steps in developing brain.

The problems of protein catabolism and qualitative protein composition of various regions of the brain at various ages are undoubtedly important in the understanding of the process of brain growth and development. Although metabolic diseases may well have their affect on these important phases of protein metabolism information on these subjects is scarce.

**SUMMARY**

1. Amino acid transport from plasma to brain is dependent on animal age, amino acid plasma and cerebral concentration of that amino acid and the region of brain under scrutiny. The blood brain barrier system allows and supports free exchange of plasma and brain amino acids and active transport is most likely the mechanism underlying greater uptake by brain of numerous amino acids. There is little evidence that the blood brain barrier is incompetent in the developing animal.

2. Compromise of common transport carrier systems make it likely that a specific amino acid, including phenylalanine, influences flux of other amino acids into the amino acid pool of brain.

3. There is good evidence that certain circulating hormones (i.e. thyroxin, epinephrine, hydrocortisone, insulin and growth hormone) can significantly affect both amino acid transport into brain as well as amino acid incorporation into cerebral protein.

4. Net protein synthesis increases in brain with age. The older phylogenetic areas reach their steady adult protein concentration earlier than the newer phylogenetic areas. Little is known of the qualitative protein changes on a regional basis in developing brain. The whole brain content of arginine and histidine increases with maturity. The arginine/histidine ratio decreases because histidine concentration increases at a more rapid rate than arginine concentration.

5. Protein amino acid concentration in developing brain appears to be unrelated to rate of specific amino acid activation in the process of protein synthesis. It appears that the average protein turnover rate is more rapid in developing brain than mature brain.

6. Further studies of affect of high phenylalanine and phenylalanine-metabolites on the various steps of protein synthesis in developing brain are sorely needed. The recent studies relating cerebral protein synthesis to learning and memory make this area an exceedingly important one.

**REFERENCES**


DISCUSSION

DR. LADU: The work of Ryan and Carver was mentioned earlier on the antibody synthesis in animals with levels of phenylalanine on the order of 8 mg per cent. Perhaps some of the experts here would volunteer some opinions as to whether antibody formation is really depressed in untreated phenylketonurics, whether there is any evidence of decreased protein synthesis of this kind.

DR. SWAIMAN: The work that I had reference to, of course, was in relationship to the non-protein nitrogen pool of brain.

DR. LADU: Yes. But this work is of interest in terms of protein synthesis in general as influenced by levels of phenylalanine. One example is reported in Science, and I wondered whether anyone has really seen this to be true in phenylketonuria.

The levels, as I remember again, were around 8 mg percent, which isn't too high, and yet they seemed to show decrease in antibody synthesis. Whether this has any bearing on protein synthesis in phenylketonuria would be of some interest.

DR. WAISMAN: I don't have any answers. I think that there are two ways to examine this problem. One is if the albumin and globulin are evaluated by electrophoresis, there is no difference in the globulin. Now, that is too gross a determination of antibody formation. The clinical experience of most people is that these patients are not more susceptible than other children to the usual childhood diseases. That is indirect evidence.

DR. UDENFRIEND: The experiments Sam Bessman and I ran many years ago during which we gave labeled tyrosine to phenylketonurics and normals, thus bypassing the block, show that tyrosine was incorporated into albumin and globulin at a comparable rate. That is, we could see no disturbance in the incorporation of tyrosine into the plasma protein.

DR. SWAIMAN: This was plasma protein.

DR. UDENFRIEND: Serum albumin and serum globulin. There could have been minor changes in composition, but certainly the absolute amount of incorporation and rate of turnover, as measured by the disappearance of radioactivity, were exactly comparable.

DR. BESSMAN: The composition of hemoglobin was studied by Schroeder, and there is no difference in phenylalanine and tyrosine content of hemoglobin from normals and PKU's. We studied tryptophan content and it was normal. We looked for ortho- or meta-tyrosine, and it wasn't present in the concentration of one molecule per mole.

We have been interested in Winzler's work. He has never been able to reproduce it. That doesn't mean it's not valid. It just means that this is a very difficult area to study.

The problem of pools is very frightening, and most work present in the literature concerning rate of protein synthesis is compromised by the question of the intracellular specific activity of the presumed tracer.

If there is a high plasma concentration of phenylalanine and we give a tracer dose of radioactive phenylalanine to an animal, the rate of incorporation of radioactivity from phenylalanine to an animal, the rate of incorporation of radioactivity from phenylalanine will be low. One could say protein synthesis is depressed, but this is only a simple example of the problem.

In all of these experiments one must be extremely careful to measure the pool size, and specific activity, as well as incorporation.

DR. SWAIMAN: That's right. In some of the earlier work the radioactivity was all that was determined. Specific activity of amino acids was approximated by use of tables to estimate amino acid concentration.
DR. MENKES: There are several problems that one encounters in trying to characterize cerebral proteins and trying to determine whether indeed there is an abnormality.

The first type protein that one would like to examine are the protein or proteins of myelin, the proteolipid aggregation. Now, we can isolate these. The methods for isolation and preparation are simple at first glance. We can do this by Dr. Lees' method, as we are doing now in our laboratory. We get a preparation which varies from patient to patient, from brain to brain in terms of hexose content, protein content and phosphorus content.

The second thing one could try is study the proteolipid fraction. If we cleave proteolipid we get very nice white precipitate which can be hydrolyzed and analyzed for the various constituent amino acids. We did this in several normal brains and the results are the same as have been reported for beef brain by Folch and his group.

However, analysis for amino acids is so rough, even using the Moore and Stein column, that we could not possibly determine one amino acid out of place as could be the case in phenylketonuria.

We attempted a controlled hydrolysis of proteolipid protein but this material is insoluble in every solvent we have used so far. Because of this difficulty there is no way we can get a fingerprint.

DR. GAULL: Any reason to believe it is a single protein?

DR. SWAIMAN: This, of course, poses the real question. How many single proteins have we isolated from brain and followed them through development?

DR. GAULL: It is impossible to carry out accurate studies until there is a protein in brain that we can isolate and characterize.

DR. SWAIMAN: The reports of Hyden's work with McEwen in isolating a particular protein fraction would suggest that by repeated high-voltage electrophoresis we may be coming to the era where these things are possible.

DR. UDENFRIEND: Do you think that essential amino acids are made from glucose in animals? I don't think that has ever been corroborated.

DR. SWAIMAN: There has never been any corroborated. I am not sure I believe it. We have been unable to repeat the work in our laboratory.
SUMMARY

JOHN A. ANDERSON, M.D., Ph.D.,
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In presenting a summarization of the essential areas discussed, an attempt will be made to report as accurately as possible the context of the presentations and discussions. No doubt our personally colored interpretations have been interjected but an objective evaluation of the presentations and ideas has been attempted.

It was apparent that many differences of opinion would arise because the planners of the conference purposely selected contributors who were known from their publications to differ in their approach or interpretation of the basic and clinical aspects of the disease. It is obvious that the wide spectrum of opinion concerning the basic and clinical aspects make it almost impossible to accurately reflect all of the ideas and information presented and discussed.

The following objectives were established in the selection of the respective discussion areas. A clear understanding of the basic genetic enzyme deficiency was necessary. It was necessary to define more clearly all of the known secondary metabolic aberrations present in the disease. It was essential to more clearly understand the related neonatal adaptive enzyme processes. The knowledge concerning the specific and multiple metabolic disturbances which could be responsible for faulty development of the central nervous system needed to be evaluated. The specific amino acid needs for infants and children during early development needed to be reviewed. Finally, it was necessary to define the most effective therapy, the role of the practicing physician and local, state and federal health agencies.

Phenylalanine Hydroxylase Activity

A clearer but as yet incomplete understanding of the basic biochemical enzyme defect in phenylketonuria is presented in this conference.

The direct proof of the diagnosis of this enzymatic deficiency disease must ultimately rest upon demonstration of a limitation in tissue enzymatic activity. Several means of assay are being employed. The direct assay for phenylalanine hydroxylase activity on liver biopsy tissue is a method that is clinically possible but certainly not without difficulty and a small degree of risk to the patient. Evidence is presented that a simplified incubation system could be utilized and such specific simplification may lead to further standardization. However, a simplified system may overlook unusual forms of the disease or related problems due to deficiency in the generating system for the pteridine cofactor. Other evidence suggests that hydroxylase activity is lost upon storage of the frozen tissue and frozen tissue homogenates, while the frozen supernate prepared from the homogenate is relatively stable. This may be related to evidence that an inhibitor of phenylalanine hydroxylase activity is present in liver mitochondrial fractions and appears to be a naphthoquinone-like compound. This inhibition is not competitive with the substrate phenylalanine but operates to disturb the rate of formation of the active reduced form of the pteridine cofactor. Differences attributed to variations in the amount of phenylalanine hydroxylase may in part reside in differences in the rate of cofactor formation. It appears that species differences in hepatic phenylalanine hydroxylase activity may be in part due to the presence of natural inhibitors which reduce the rate of formation of the reduced pteridien cofactor.

The presence of enzymatic activity in phenylketonuric patients which catalyzes the transformation of a small amount of phenylalanine to tyrosine has been well documented. The suggestion is made that this hydroxylation is the result of the action of tyrosine hydroxylase on a high concentration of phenylalanine. It is obvious that
knowledge of enzymatic reactions that may substitute for enzymatic deficiencies may have great implication in the therapy of these diseases if methods of enzyme stimulation or induction prove feasible.

It is clear that further observations in human tissues may provide a more complete explanation for the following clinical situations in which dysfunction of phenylalanine hydroxylase activity may be present:

1. Neonatal delay in phenylalanine hydroxylase activity.
2. Variations in phenylalanine hydroxylase activity in homozygous, heterozygous as well as in normal subjects.
3. Idiopathic non-phenylketonuric subjects manifesting transitory or even persistent hyperphenylalaninemia.

Biochemical Abnormalities - Relation to Central Nervous System Development and Dysfunction

It appears from discussions at this conference that our knowledge of the specific biochemical factors responsible for faulty growth, development and differentiation of the central nervous system during fetal and postnatal life is exceedingly limited. There appears to be no good evidence that abnormal proteins are synthesized or that an impaired rate of protein synthesis takes place in developing brain in the presence of high concentrations of phenylalanine. The frequency of mental retardation and evidence of intrauterine growth retardation and microcephaly in non-phenylketonuric offspring of untreated phenylketonuric mothers provides rather strong indirect assumptive evidence that the elevated phenylalanine, the low tyrosine and the associated phenylalanine derived metabolites present in the mother may be harmful to the fetus. The studies on the detrimental influences of increased concentrations of L-phenylalanine in pregnant animals, particularly monkeys, tend to support these clinical observations. These observations do not exclusively incriminate phenylalanine. On the other hand the presence of normal development and mentality in certain children with delayed neonatal development of hepatic hydroxylase, normal development in certain cases of so-called idiopathic hyperphenylalaninemia, and normal development in certain cases of phenylketonuria indicate the possibility that phenylalanine itself may not be harmful under certain circumstances. There is no doubt that the central nervous system in phenylketonuric children usually does not develop properly. However, it appears that there is a great reluctance on the part of the contributors to this conference to conclude that phenylalanine is primarily responsible for both fetal and postnatal developmental limitations.

The excellent presentations and summaries concerning our present knowledge of the importance of secondary metabolic mechanisms involving the formation of neuro-humoral amines do not permit clear definition of the specific roles they may play in central nervous system development and function in phenylketonuria. It is, however, clearly evident that this aspect of the problem needs far more intensive study in both untreated and treated subjects. In animal experiments the observed limited synthesis of serotonin, most likely due to a competition by excess phenylalanine and its metabolites for the tryptophan hydroxylase enzyme, no doubt has an important role in deranged neural function. Pharmacologic approaches to this problem have been exceedingly limited and are most important areas for further investigation. Considerable emphasis is placed on the need to explore the metabolic pathways for synthesis of other important neural amines in untreated and dietary treated phenylketonuric patients as well as in normal children.

Cerebral Lipids

Meager data is available on alteration in lipid composition of the phenylketonuric brain. There appear to be changes in lipid composition of the brain present in phenylketonuria which are common to certain demyelinating diseases. No specific abnormalities are noted in this disease to date. Unfortunately, data obtained from formalin preserved specimens does not provide reliable information concerning important brain lipid
components. In view of the reports of abnormalities in myelination in phenylketonuria further information concerning lipid metabolism would be of great importance.

Amino Acid Transport

Transport of aromatic and other amino acids across membranes of the gut wall in phenylketonuric patients or into the brain in experimental animals is compromised by high concentrations of phenylalanine in the blood. This disturbance could be of considerable importance during periods of rapid growth of body tissues when the rate of protein synthesis is great. A chronic amino acid imbalance could have a detrimental effect on the rate of protein synthesis in developing brain, particularly during that period when the process of functional differentiation of the central nervous system is most rapid. Transport limitations at the gut wall could reduce the availability of substrates necessary for synthesis of catecholamines and serotonin.

Screening Tests

Screening tests that have been employed to date have consisted of studies of the blood phenylalanine concentration, the urine phenylketones, and occasionally urine ortho-hydroxyphenylacetic acid. The large scale screening tests carried on in the United States and in other countries, particularly in Europe, utilize a bacterial inhibition test. This test, however, yields a number of false positive reactions and reflects only an elevated blood phenylalanine. Further definitive diagnostic studies must be carried out in all suspect cases. Unquestionably chromatography of amino acids should be used as an adjunct to methods now available for screening for phenylketonuria as well as for other inborn errors of amino acid metabolism. Paper chromatography is only roughly quantitative and suspect observations must be followed by more precise quantitative ion exchange chromatography for definitive evaluation of the amino acid disturbance. Caution must be exercised in the interpretation of results obtained by chromatographic screening for amino acid disorders. Faulty diagnosis of metabolic diseases in newborn infants may occur when in reality the abnormal amino acid pattern may be only reflection of transitory neonatal renal and enzyme limitations. The more biochemical procedures required for screening and definitive diagnosis of phenylketonuria and other inborn errors of metabolism are too expensive and complex for general use. These more accurate quantitative biochemical procedures should be done in medical centers where qualified scientific staff and techniques can be developed and maintained.

Diagnosis of Phenylketonuria

It is difficult to extract a consensus from this conference of criteria for diagnosis of phenylketonuria. The blood phenylalanine concentration determined by accurate biochemical methods should be at least above 15 to 20 mg percent when the patient is maintained on a general diet. The plasma tyrosine in the presence of an elevated phenylalanine should be significantly reduced below the normal concentration values. The excretion of urinary phenylketones under the above circumstances should be significantly increased. Confusion may exist because certain normal as well as phenylketonuric infants may have transitory delay in the development of the phenylalanine transaminase activity required for the conversion of phenylalanine to phenylpyruvic acid. The presence of measurable amounts of ortho-hydroxyphenylacetic acid in the urine when the plasma phenylalanine is above 10 to 12 mg percent even in the absence of significant increases in urinary phenylketones indicates that increased phenylpyruvic acid formation is occurring.

It is imperative that the above observations be repeated during the early infancy period to be assured that the plasma phenylalanine remains elevated in relationship to be increased dietary protein intake and that the plasma tyrosine concentration is below the normal range. Treatment should not be instituted unless confirmatory biochemical observations are made. In treated children provocative protein or phenylalanine loading should be periodically utilized to be assured that the biochemical disturbances persist.
In general the hyperphenylalaninemia of premature and certain normal infants is not above 15 to 20 mg percent and is usually associated with an elevated tyrosine value indicative of transitory limitation in the development of the tyrosine transaminase activity. These transiently elevated phenylalanine and tyrosine situations are not associated with significant and persistent increased excretion of phenylketones or orthohydroxyphenylacetic acid. In general the non-phenylketonuric cases of hyperphenylalaninemia which may persist for months or years have blood phenylalanine levels which do not rise above 15 to 20 mg percent and may also have an essentially normal blood tyrosine concentration. These children in response to an added phenylalanine load may have a phenylalanine tolerance curve and tyrosine response which resembles that found in the phenylketonuric homozygous subject.

Identification of Phenylketonuric Heterozygotes

Although it is simple enough to state that no patient is absolutely known to be a heterozygote until he or she has become a parent of a phenylketonuric child, more practical means of distinguishing these individuals from the normal population must be sought. The evidence presented at this conference suggests that the vast bulk of heterozygote and normal populations can be separated into two groups by mathematical evaluation of the blood phenylalanine and/or the tyrosine responses following phenylalanine loading. However, this evaluation indicates only the probability that an individual who is not the parent of a phenylketonuric child is a heterozygote. The practical question is not whether we can separate populations one from another, but whether we can with absolute certainty separate a normal from a heterozygous individual. Our ability to perform this task has great social, psychologic, moral and legislative implication. It may well be that other studies utilizing tissue or blood assays not yet feasible may answer this problem. Certainly further studies in terms of the individual's ability to transform phenylalanine to tyrosine are necessary to further clarify this problem. It is not advisable to implement genetic counseling for individuals on the basis of the present biochemical tests.

Dietary Management

There are a number of questions concerning the use of the low phenylalanine diet in the prevention of mental retardation in phenylketonuria. There is evidence from animal as well as from clinical studies that essential factors found in natural foodstuffs may be lost during protein hydrolyzation. Certain observers point out that a fair amount of natural protein is included in the diet and that the diet of any one patient never entirely consists of synthetic foods alone. The excellent, though as yet limited studies concerning the specific amino acid requirements for infants in the early months of life, are not as yet adequate to permit us to structure a complete table concerning the ranges of needs for specific amino acids for growing infants. There are no controlled studies on the growth of normal infants maintained on low phenylalanine diets which assure us of nutritional adequacy. Questions concerning the possibility that the diet may indeed be harmful to mental growth in normal, in non-phenylketonuric retarded and in the phenylketonuric patient are unanswered.

The participants at this conference who have had most experience in the utilization of the diet all attempt to maintain blood phenylalanine concentrations between 3 and 6 mg percent. All express concern that if the blood concentration remains persistently below 2 mg percent, phenylalanine deficiency syndrome may occur. There are apparently no good data defining minimal phenylalanine needs of infants. The assumption that a blood phenylalanine concentration above 6 mg percent is harmful is not justified by existing evidence.

While the evidence concerning dietary treatment is not as conclusive as desired, the data presented by the discussors does suggest that the diet properly prescribed and monitored and begun within the first six months of life is of value in the prevention of mental retardation in
some cases of phenylketonuria. Some children manifest only improved sociability and manage-
ability. Present data concerning the effectiveness of dietary treatment leaves unsolved many very
important problems. Which patients do not need the diet and would remain normal? Which patients
will not be benefited from the diet? If we are to gain insight and information necessary to answer
these questions, we cannot insist that every patient diagnosed as having phenylketonuria be
treated in early infancy.

If dietary management is to be employed, certain principles must be followed. There is
need to monitor plasma phenylalanine and tyrosine concentration in the blood, and also those param-
eters which assure that the requirements for all other amino acids are met. Bacterial assay
for blood amino acid concentrations should be used only as a temporary expedient while prefer-
able methods are being instituted. The develop-
ment of a competent scientific staff and ade-
quate instrumentation within medical centers
responsible for the administration of the
therapeutic program is essential. Manage-
ment of the therapeutic program for phenyl-
ketonuria must remain at the study level before
 general recommendations for therapy can be
made.

Evaluation of Development

The success of any therapy for phenyl-
ketonuria will depend upon preservation of in-
tellectual potential and continued intellectual
acquisition. Hence, the instruments necessary
for evaluation of these characteristics become
all important. It is quite clear from the dis-
cussions that accurate measurement of intel-
llectual ability and capability in infants is difficult.
We have no way of measuring the level of intelli-
gence at birth. The instruments used to
evaluate rate of intellectual development during
the first years of life are not quantitative, and
cannot be used in a longitudinal manner on
a single child or on a group of children. This
problem at present poses the major obstacle to
the scientific investigator who wishes to employ
each patient as a self control.

Legislation

Legislation has been enacted in a large
number of States to screen newborn infants for
phenylketonuria. The general public, and indeed
most of the medical profession were led to
believe that screening tests for hyperphenyl-
alaminemia are indeed tests for phenylketonuria.
This is not true. Furthermore a great deal of
impetus for the passage of legislation came
from preliminary medical data which indicated
favorable effects of the low phenylalanine diet.
We now know a child fulfilling the criteria for the
diagnosis of phenylketonuria may benefit from
dietary therapy. There are, however, a number of
patients who do well without the diet, who may be
endangered by the diet and who in general do not
benefit from the diet. Although legislation in effect
stipulates screening not therapy, we have already
experienced the practical effect of such legislation
which has included public pressure for a specific
therapy at a time when competent medical investi-
gators are not in agreement on criteria for diag-
nosis, natural course of the disease or optimal
therapy or therapies. It appears that legislatures
have seen fit to pass laws authorizing screening
for a disease in newborns which is not communi-
cable, not fatal and for which there is no clearly
established therapy. We have not established legis-
lation for a number of diseases which are poten-
tially as handicapping as phenylketonuria, in which
criteria of diagnosis and method of therapy is rela-
tively uniform but have successfully relied upon the
processes of education of the public and the medical
profession to implement these important health
needs.

The Future

There are still many important questions
that must be answered concerning the disease
phenylketonuria. Further carefully designed stud-
ies conducted by both basic and clinical scientists
must be done. The accumulation of sound scientific
knowledge concerning phenylketonuria, as is true
for other diseases, is often a slow and tedious
process. When more complete understanding of
the basic processes present is available we are
likely to be in a more secure position to define
practical approaches to accurate diagnosis and

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possibly prevention and management. We have moved too rapidly and have encouraged the development of general widespread management programs based on rather limited knowledge. Early results of therapy were uncritically accepted with considerable enthusiasm, but we now find that such enthusiasm was not entirely justified. It is doubtful that our present knowledge is sufficiently adequate to embark upon a general widespread application of a uniform management program. At least at this conference it was difficult to extract a general agreement concerning the kind of general management program that should be applied to all cases of phenylketonuria. Dietary management if attempted should assure that all nutritional and growth needs of the treated children are met. We must avoid superficial dietary approaches which, if poorly monitored, we now know can do serious harm. We must search out the reasons for success of treatment in some patients and equally important the reasons for failure in other patients.

It is obvious that treatment programs for phenylketonuria must continue to remain at the clinical investigative level, pursued in a scientific manner and approached from a variety of standpoints. The approaches should be multi-disciplinary, utilizing the talent and resources of highly qualified biochemists, physiologists, nutritionists, pharmacologists, psychologists, neurologists, pediatricians and others. Such inter-disciplinarity of efforts will help define to basic scientists areas of limited knowledge and hopefully will encourage them to more specifically direct their research efforts on the unsolved areas.