Problems in the Chemical Diagnosis of Some Hereditary Metabolic Diseases

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The modern geneticist and his genetically oriented colleagues in clinical medicine and biochemistry now believe that all inherited metabolic variations arise from alterations in the biochemical composition of the organism; it is assumed that these alterations are the direct result of variations in the gene or genes which determine the synthesis of a specific protein molecule. Some well-known hereditary metabolic disorders, such as phenylketonuria and alcaptonuria, have been shown to be inherited as simple recessives, with the fundamental enzyme defect attributable to the lack or alteration of a single gene at a single locus. There are other metabolic diseases, however, in which the mode of inheritance is probably more complex. Here, a particular biochemical phenotype may be either under the combined genetic influence of several distinct genes, or controlled by one gene at any of several loci.

Because of the increasing clinical interest in the metabolic aspects of diseases, it is becoming more important for the clinical chemist to be aware of some of the problems associated with the diagnosis of hereditary diseases. This report will be primarily concerned with the significance and interpretation of variations in the urinary excre-
tion of phenolic acids, alpha-keto acids, and indolic acids which are known to be associated with some inherited metabolic disorders, in addition to those of less certain genetic status.

Phenolic Acids

In our laboratory, two-dimensional paper chromatograms of urine extracts were prepared according to the procedure of Armstrong et al. (1), and developed with diazotized sulfanilic acid, diazotized p-nitroaniline, or 2,6-dichloroquinone chlorimide. This technic was found satisfactory for the qualitative assay of phenolic acids in urine.

Phenylketonuria

The most outstanding hydroxy metabolite of phenylalanine found in the urine of phenylketonurics is o-hydroxyphenylacetic acid (o-HPAA). Figure 2 is a typical chromatogram of phenylketonuric urine, showing the greatly increased excretion of o-HPAA and the increased excretion of p-hydroxyphenyllactic acid. The detection of o-HPAA is an especially reliable diagnostic indicator for this disease since, in such cases, it is invariably excreted in the urine in increased amounts. o-Hydroxyphenylacetic acid is normally excreted at the

![Chemical diagram](image link)

Fig. 1. Scheme of alternate pathways of phenylalanine metabolism that may be involved in phenylketonuria. Dashed lines represent possible secondary blocks.
rate of 0.3-1.7 mg./24 hours (2) and increased to 100-400 mg./gm. of creatinine in phenylketonuria (3). If, as has been suggested (2), the main precursor of o-HPAA is phenylpyruvic acid then we might expect the presence of this relatively stable metabolite in increased amounts in urine even when phenylpyruvic acid can not be detected. The complete metabolism of phenylpyruvic acid to o-HPAA and other derivatives might occur when phenylpyruvic acid is formed in low concentrations as in the early stages of phenylketonuria. The pathways outlined in Fig. 1 show how this is possible. Because of the block in p-hydroxylation, phenylalanine is transaminated or oxidatively deaminated to phenylpyruvic acid, which is then subjected to hydroxylation. Although hydroxylation of phenylpyruvic acid in the meta or para positions is possible, it appears that ortho-hydroxylation is favored and that the o-hydroxyphenylpyruvic acid so formed is subsequently decarboxylated to o-HPAA. An alternate pathway to o-tyrosine is also possible, but there is no evidence, as yet, that this pathway is operating in phenylketonuria. When monoamine oxidase inhibitors have been administered to phenylketonurics, there is an increased excretion of phenylethylamine but not o-tyramine (4). If o-tyrosine was being formed in increased amounts, we would also expect an increased excretion of o-tyramine.

A rapid and accurate test for the presence of o-HPAA would be to run the urine* on a one-dimensional paper chromatogram in isopropanol-ammonia-water (8:1:1) and develop with 2,6-dichloroquinone chlorimide or diazotized sulfanilic acid (5). Figure 4 shows such a chromatogram of phenylketonuric whole urine on the first through the thirteenth day of a phenylalanine-free diet. The rapidly migrating o-HPAA spot can be easily qualified in a 4- to 5-hour run. In no other metabolic disease has o-HPAA been found to be excreted in increased amounts.

p-Hydroxyphenylpyruvic acid, p-hydroxyphenyllactic acid, and p-hydroxyphenylacetic acid have been reported to be excreted in somewhat increased amounts in phenylketonuria (6, 7). If this is true, however, it seems more likely that this tryosyluria reflects a liver enzyme dysfunction (inhibition of p-hydroxyphenylpyruvic acid oxidase?) rather than a direct anomaly of phenylalanine metabolism (8, 9).

*Although whole urine may be used, ethyl acetate extracts of acidified urine are more reliable.
Galactosemia

The analysis in our laboratory of the urine of an untreated, 1-month old patient with galactosemia revealed a greatly increased excretion of p-hydroxyphenylpyruvic acid and p-hydroxyphenyllactic acid. This abnormal pattern is shown in Fig. 5A. On a galactose-free diet, these metabolites completely disappeared. As in phenylketonuria, the increased excretion of these compounds in all probability reflects the marked liver dysfunction and associated enzyme alteration encountered with this disease.

Infantile Tyrosinosis

Premature, or very young infants, occasionally exhibit a tyrosinuria which invariably returns to normal (10, 11). Figure 5B represents just such a condition. The patient was a 2-week old male with a diagnosis of hypoglycemia, jaundice, and convulsions. The chromatographic pattern is much the same as that of the galactosemic patient just discussed, with increased excretion of p-hydroxyphenylpyruvic acid and its derivatives. In addition, this patient excreted increased amounts of tyrosine. The abnormally increased concentrations of all these metabolites, however, returned to normal some 2 months later.

None of the 3 abnormally increased spots which appear to be common to these two chromatograms (Fig. 5A and 5B) has been identified. The lower spot appears to be spot 34 of Armstrong et al. (1) and the other 2 are arbitrarily designated as spots 44 and 45.

The increased excretion of tyrosine and p-hydroxyphenyllactic acid in a nonscorbutic individual presumably without liver damage has been reported in only one case (12). A genetic basis for this condition is therefore doubtful, and the tyrosinosis probably reflects some acquired alteration of tyrosine metabolism. It would not be surprising to find a similar biochemical picture in other hereditary or acquired diseases involving disorders of the liver.

Convulsive Disorders

Three cases of altered urinary phenolic acid patterns have come to our attention in the course of a survey of children with convulsive disorders. One case was an 18-month-old girl with myoclonic seizures and mental retardation. There were no siblings, but a maternal granduncle who began to have seizures at the age of 13 was placed in a state institution in his late twenties and died shortly thereafter.
Hereditary Metabolic Diseases

The phenolic acid chromatogram (Fig. 6A) of the propositus revealed an abnormal compound which migrated to a position which has not been previously reported. This substance may be the result of drug therapy (possibly Celontin, 150 mg. twice daily); the known hydroxylated products of the other administered drugs (Dilantin and phenobarbital) migrate to different positions. This spot will be designated as spot 46.

In the other two patients, 7- and 18-month-old girls with myoclonic seizures and mental retardation, an interesting alteration in phenolic acid excretion was encountered. This consisted in the greatly increased excretion, in both cases, of p-hydroxyphenylpropionic acid (Fig. 6B). Although no members of their immediate families were similarly affected, both families did have some history of central nervous system disorders. In one pedigree, a paternal uncle and paternal granduncle had childhood convulsions. In the other pedigree, one sibling died of an unknown central nervous system disorder at 10 months, a paternal aunt had seizures as a child, and a paternal great-aunt had grand-mal epilepsy. The increased excretion of p-hydroxyphenylpropionic acid was found in every urine sample examined over a 5-month period for one patient and in a high percentage of the urine samples of the other. The amount of p-hydroxyphenylpropionic acid also seemed to be closely correlated with the amount of p-hydroxyphenylacetic acid excreted, and although the intensities of these spots varied, their relative color intensities remained constant. The patient who excreted p-hydroxyphenylpropionic acid most persistently also seemed to excrete less than normal amounts of the 3-methoxy-4-hydroxy substituted phenylhydroacrylic, mandelic, and cinnamic acids.

In view of the evidence that p-hydroxyphenyllactic acid can serve as a precursor of p-hydroxyphenylpropionic acid through p-hydroxy-cinnamic (p-coumaric) acid in the rat (13), it is possible that p-hydroxyphenylpropionic acid is formed by a similar pathway in man. However, since p-hydroxyphenylpropionic acid is not normally excreted in man, it appears that this compound is efficiently metabolized under normal conditions. Therefore, in order to explain the observed increased excretion of p-hydroxyphenylpropionic acid in the cases described, we could postulate some block in the dehydrogenation of p-hydroxyphenylpropionic acid to p-hydroxycinnamic acid. The decreased excretion of the 3-methoxy derivatives of p-hydroxycinnamic acid might then result from the increased concentration of p-
hydroxyphenylpropionic acid secondarily inhibiting the O-methylation of p-hydroxyphenylacetic acid. A proposed scheme for the metabolism of these tyrosine derivatives is outlined in Fig. 7. However, because our knowledge of the intermediary metabolism of phenolic acids is still fragmentary, in addition to the fact that these variations could arise as secondary effects, one must be cautious in relating these abnormally excreted metabolites to a specific clinical state.

**Dietary and Drug Effects**

An important precaution in the interpretation of phenolic acid chromatograms is to be on the lookout for urinary drug metabolites in any patients undergoing therapy. Both phenobarbital and Dilantin can appear as their hydroxylated derivatives. These are p-hydroxydilantin and p-hydroxyphenobarbital which appear on Armstrong’s (1) chromatographic map as spots 25 and 26, respectively. The p-hydroxyphenobarbital spot can be seen in Fig. 8, which is the chromatogram of a seizure patient on phenobarbital sedation (32 mg./t.i.d.). It is interesting that other convulsive patients of equal size and weight receiving the same dose of phenobarbital may not show p-hydroxyphenobarbital on their chromatograms. It is possible that variation in the metabolism of this drug may in itself be under genetic control. In this respect, succinylcholine (14) and isoniazid (15) are two compounds which have been shown to be metabolized differently in individuals of different genetic constitutions.

Since it is likely that other drugs could be similarly excreted, it is imperative that drug therapy be stopped or strictly regulated before an unknown spot can be ascribed to an endogenous origin. In addition, the intake of such substances as aspirin, coffee, bananas, and vanilla must be carefully controlled as they are known to alter phenolic or indolic acid patterns significantly (16-19).

**α-Keto Acids**

**Phenylketonuria**

In this disease, phenylpyruvic acid is usually excreted at the rate of 0.3-2.0 gm./24 hours, and in this range of concentration can be readily detected by the standard ferric chloride reaction. However, it is possible to get a negative ferric chloride reaction with phenyl-

*The identification of p-hydroxydilantin and p-hydroxyphenobarbital was made by Dr. Marvin D. Armstrong of the Fels Research Institute, Yellow Springs, Ohio.*
ketonuric urine (20) especially when phenylpyruvic acid is being excreted in very low concentrations. Some factors which may be contributing to this negative test are the transitory nature of the reaction, the masking effect of phosphates, and the effects of light and temperature. Therefore, in those cases of suspected phenylketonuria which give a negative ferric chloride reaction either in solution or on reagent paper strips, further testing should be instituted employing technics which would minimize the above factors. Saifer and Harris (21) have recently developed a technic in which the ferric chloride color reaction is stabilized by low temperature, omission of light, the introduction of ferrous ions, and precipitation of the phosphates by uranyl nitrate. The use of a mixture of magnesium chloride, ammonium chloride, and ammonia has also been shown to be effective in removing phosphates (22). The ether extraction method of Berry and Woolf (23) is highly satisfactory for routine quantitative determinations of phenylpyruvic acid. False positive color reactions with ferric chloride can result from such urinary compounds as salicylic acid (purple), chlorpromazine derivatives (green) (24), and bilirubin (green) (20); the latter can be removed from the urine by absorption with calcium carbonate.

As will be seen in the discussion of the following metabolic disorder involving keto-aciduria, the increased excretion of phenylpyruvic acid alone may not always be pathognostic for phenylketonuria.

Smith-Strang Syndrome

The only other metabolic disorder in which phenylpyruvic acid has been found to be excreted in increased amounts is that described by Smith and Strang (25) and Jepson et al. (26). The 9-month-old child they studied was mentally deficient, had completely white hair, and excreted phenylpyruvic acid, $p$-hydroxyphenylacetic acid, $p$-hydroxyphenyllactic acid, indoleacetic acid, and $\alpha$-hydroxybutyric acid in the urine. Phenotypically, this case might easily have been classified as phenylketonuria. However, the fact that no $o$-HPAA could be detected in the urine, which as mentioned earlier is diagnostic for phenylketonuria, implicated a block in $\alpha$-keto acid metabolism rather than a defect in the para-hydroxylation of phenylalanine. This syndrome is a good example of a biochemical phenotype which is similar to that associated with phenylketonuria, but undoubtedly under the control of a separate genetic mechanism.
Branched-Chain Ketonuria (“Maple-Syrup Urine” Disease)

In this condition, characterized by marked loss of all integrated central nervous system functions, the branched-chain α-keto acids (α-ketoisovaleric, α-ketoisocaproic, α-keto-β-methyl-n-valeric) and probably their α-hydroxy acids are excreted in the urine in increased amounts (27, 28). The disease appears to be related to the block in the metabolism (decarboxylation?) of the α-keto acids encountered in the Smith-Strang disease described above. In one 20-month-old patient examined by Dancis et al. (27), α-ketoisocaproic acid and α-ketoisovaleric acid were found to be excreted at the rate of about 250 mg./24 hours.

Although the branched-chain α-keto acids give a different color reaction with ferric chloride (blue-black) than does phenylpyruvic acid (blue-green), some confusion in the qualitative interpretation of the keto acids could result. Because of this factor, in addition to the relative instability of α-keto acids, it would seem best to convert them to their 2,4-dinitrophenylhydrazones and examine them chromatographically. For this purpose, the Menkes modification (29) of the method of Seligson and Shapiro (30), or the methods of Biserte and Dassonville (31) would appear satisfactory.

Alterations of Less Certain Status

Menkes (29) has reported two children with severe cerebral dysfunction who possessed abnormal excretion patterns of α-keto acids. One had greatly increased amounts of α-ketoglutaric acid (320 mg./sq. meter/24 hours) and the other showed the presence of 2 abnormally increased α-keto acids, which were not indentified.

Indolic Acids

The intermediary metabolism of tryptophan appears to be especially sensitive to metabolic disturbances which are not primarily related to tryptophan metabolism. In all of the diseases which have been discussed here there is generally an increased urinary excretion of indoleacetic acid, indolelactic acid, and indican, and decreased excretion of 5-hydroxyindoleacetic acid. It has been suggested that perhaps these alterations in indole excretion may be due in part to an inhibition of tryptophan or 5-hydroxytryptophan decarboxylase brought about by the increased production of abnormal metabolites associated with these disorders (32). That these alterations in tryptophan metabolism represent separate mechanisms has been
Fig. 2. (top) Two-dimensional chromatogram of urinary phenolic acids from a phenylketonuric patient. Note increased excretion of o-hydroxyphenylacetic acid (13) and p-hydroxyphenyllactic acid (14). Fig. 3. (bottom) Two-dimensional chromatogram of urinary phenolic acids from a normal human subject. Spot 1 is p-hydroxyphenylacetic acid; 2, m-hydroxyphenyllactic acid; 3, m-hydroxyhippuric acid; 4, p-hydroxyhippuric acid; 5, 5-hydroxyindoleacetic acid; 6, salicyluric acid; 7, homovanillie acid; 13, p-hydroxybenzoic acid; 13, o-hydroxyphenylacetic acid; 17, vanillyl glycine; 19, vanillie acid.
Fig. 4. (top) One-dimensional chromatogram of urinary phenolic acids from a phenylketonuric patient on the first through the thirteenth day of a phenylalanine-free diet. Arrows point to o-hydroxyphenylacetic acid spots.

Fig. 5. (bottom) Two-dimensional chromatograms of urinary phenolic acids from patients with galactosemia (A) and infantile tyrosinosis (B). Arrows point to spots showing combined increased excretion of p-hydroxyphenylpyruvic acid and p-hydroxyphenyllactic acid. Spots 34, 44, and 45 have not been identified.
Fig. 6. Two-dimensional chromatograms of urinary phenolic acid from patients with myoclonic seizures and mental retardation. Note increased excretion of p-hydroxyphenylpropionic acid (22), p-hydroxyphenyllactic acid (14), and unknown (46). Spot 26 is p-hydroxyphenobarbital.
Fig. 7. Scheme for the intermediary metabolism of some tyrosine derivatives.

Fig. 8. Two-dimensional chromatogram of urinary phenolic acid from a patient with myoclonic seizures on phenobarbital sedation (32 mg./i.d.). Arrow points to p-hydroxyphenobarbital spot.
largely discounted by the fact that dietary control of the abnormally increased primary metabolite results in the normal excretion of indoles (33, 34).

Indoleacetic acid has been demonstrated to be excreted in increased amounts in alkaline urine (33). However, it does not seem likely, in view of the acid urines found in ketoacidurias, that urinary pH would be an important contributing factor to the increased excretion of indoleacetic acid. Moreover, only indoleacetic acid excretion would be expected to be affected in this manner, as the polar effect of hydroxyl substitution of the 5-hydroxyindoleacetic acid and indoleacetic acid molecules would probably make these compounds less likely to be affected by urinary pH (34).

It is of interest that the oral administration of indoleacetic acid and certain phenylalanine metabolites in man can result in an increased excretion of 5-hydroxyindoleacetic acid (30, 35). These experimental conditions, however, may result from the short-term release of tissue serotonin and its subsequent oxidation to 5-hydroxyindoleacetic acid, in which case an attempt should not be made to relate these findings to the chronic clinical state.

Which of these above-mentioned alterations in the excretion of indoles can be attributed to metabolic variation and which to renal factors (36), has not been fully resolved.

**Hartnup Disease**

This metabolic disorder (37) appears to be one inherited disease in which the primary error may involve tryptophan directly, either through altered transport (38) or metabolism. In this disease, indoleacetic acid, tryptophan, and indican are excreted in increased amounts, whereas 5-hydroxyindoleacetic acid and indolelactic acid are supposedly normal. However, Weyers and Bickel (39) have described a case of Hartnup disease in which indolelactic acid is also excreted in increased amounts.

**Summary and Conclusions**

Some hereditary or acquired, qualitative or quantitative differences in the urinary excretion patterns of certain phenolic acids, \( \alpha \)-keto acids, and indolic acids have been reviewed and discussed. The ketoacidurias are summarized in Table 1. As can be seen, there is considerable overlap in the excretion of primary, and presumably secondary metabolites. From these comparisons together with the
Table 1. Alterations in the Urinary Excretion of Certain Organic Acids Associated with Three Ketoacidurias

<table>
<thead>
<tr>
<th>Urinary metabolites</th>
<th>Phenylketonuria</th>
<th>Smith-Strong syndrome</th>
<th>Branched-chain ketonuria</th>
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<td><strong>Phenolic acids</strong></td>
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<td>g-Hydroxyphenylacetic acid</td>
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<td><strong>Indolic acids</strong></td>
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Key: N represents normal excretion; +, slight to moderately increased excretion; and ++, moderate to greatly increased excretion.

Other disorders discussed throughout this presentation, the following conclusions might be drawn:

1. Phenylketonuria can be readily separated from the other ketoacidurias by the presence of increased amounts of o-hydroxyphenylacetic acid.

2. The increased excretion of α-ketoisocaproic acid and α-ketoisovaleric acid should be definitive for branched-chain ketonuria.

3. Secondary disturbances in tyrosine and tryptophan metabolism, which are associated with many of these disorders, are often expressed in an increased production of the tyrosine metabolite p-hydroxyphenylpyruvic acid and its derivatives (which appear to be characteristic of liver dysfunction in both hereditary and acquired diseases) and in an increased excretion of the tryptophan metabolites
of indoleacetic acid, indolelactic acid, and indican, and a decreased excretion of 5-hydroxyindoleacetic acid.

4. Because of the biochemical or symptomatic similarities between many metabolic disorders, it is sometimes difficult to make an accurate diagnosis unless variations in the excretion patterns for each disease entity are thoroughly understood, so that overlapping factors or variations in one factor will not confuse the diagnosis.

5. The dietary and drug intake of the patient under observation must be assiduously controlled before an abnormal, or abnormally increased, metabolite can be assigned an endogenous metabolic origin.

Keeping these facts and precautions in mind should reduce the likelihood of reporting a variant of a known hereditary metabolic disease as a new disorder. Nevertheless, since the study of metabolic diseases is so new, it is important at this stage to report all significant variations of even the well-established diseases.

References