Phenylketonuria (phenylpyruvic oligophrenia) is an inherited condition in the human in which the ability of the liver to oxidize phenylalanine to tyrosine is impaired (2). Because this pathway represents the major, although not the only, manner in which phenylalanine is metabolized in mammals, considerable amounts of phenylalanine accumulate in the blood and tissues of affected individuals, and phenylalanine and abnormal metabolites derived from it are excreted in the urine. These metabolites are phenylpyruvic acid (3), β-phenyl-L-lactic acid (3), and phenylacetylglutamine (4), the conjugated form in which phenylacetic acid is excreted by the human. In addition to the biochemical abnormalities, most of the individuals who inherit the biochemical defect suffer a profound impairment of mental functioning. It has not been possible up to the present time to establish a connection between the biochemical abnormality and the development of the mental defect.

Experiments in which the phenylalanine intake of phenylketonuric children was restricted until normal blood levels of phenylalanine were obtained and phenylpyruvic acid excretion ceased were carried out in this laboratory (5). The results indicated that the mental defect does not occur as the result of a simple interference with the functioning of an otherwise normal central nervous system, but many observations were made which were in agreement with the idea that toxic metabolites might be responsible for some of the mental symptoms; similar results have been reported by Bickel, Gerrard, and Hickmans (6). In the course of the metabolic experiments, qualitative reactions were obtained with phenylketonuric urine which were not observed in the urine of normal individuals. Ex-
amination of the free organic acids of phenylketonuric urine showed that abnormal indole derivatives are excreted (7) and that some phenolic acids which are present in normal urine in small amounts occur in phenylketonuric urine in greatly increased amounts. The most prominent of these abnormal phenolic compounds has been isolated and has been identified as o-hydroxyphenylacetic acid, in confirmation of the initial chromatographic identification recently reported by Boscott and Bickel in a preliminary communication (8). It appears probable that the formation of o-hydroxyphenylacetic acid is not directly related to the development of the mental defect.

EXPERIMENTAL

O-Hydroxyphenylacetic acid was prepared by a modification of methods described in the literature (9, 10); the properties of the synthetic material compared favorably with those reported earlier. Phenylalanine was determined in fasting serum by the modification of the method of Kapeller-Adler described earlier (5).

O-Hydroxyphenylacetic acid (o-HPAA) may be detected by direct paper chromatography of urine of patients with phenylketonuria; it is visualized by spraying chromatograms with an alkaline solution of diazotized sulfanilic acid. It is more easily distinguished, however, if it is extracted from urine into an organic solvent before chromatography. Urine was acidified to pH 2 and extracted three times with 0.5 volume portions of ethyl acetate; the acids were taken into a small portion of saturated NaHCO₃ and then returned to a measured volume of ethyl acetate after acidification. Aliquots containing 2 to 6 γ of o-HPAA were chromatographed, and the intensity of the orange dye formed by coupling with diazotized sulfanilic acid was compared with the color produced by chromatography of known amounts of o-HPAA. Recovery experiments made by extracting urine to which had been added known amounts of o-HPAA were accurate to ±10 per cent by this procedure. The most satisfactory solvent system for the chromatography of o-HPAA has proved to be isopropyl alcohol—aqueous ammonia—H₂O = 8:1:1; Rₚ = 0.73, ascending chromatogram on Whatman No. 1 paper.

Isolation of o-Hydroxyphenylacetic Acid from Phenylketonuric Urine—40 liters of urine (containing 30 gm. of creatinine) from a patient with phenylketonuria were acidified to pH 2 and extracted in portions with 3 × 0.5 volume portions of ethyl acetate. The pooled ethyl acetate solutions were extracted with small portions of saturated NaHCO₃ solution to separate the organic acids, and the combined bicarbonate extracts were acidified and extracted with smaller volumes of ethyl acetate. The organic acids were finally obtained in 1 liter of ethyl acetate; this extract contained approximately 2.4 gm. of o-HPAA, as estimated by paper chromatography.
The organic acids in this solution were subjected to a modified counter-current distribution procedure in which six 1 liter portions of 1.0 M citrate buffer, pH 3.60, were passed through eight 1 liter portions of ethyl acetate. Most of the o-HPAA remained in the first portion of ethyl acetate, but a small amount passed into the second and third. The first portion was then subjected to another similar distribution with buffer of pH 3.6. The acids in the combined second and third ethyl acetate portions from both distributions were extracted into a small portion of NaHCO₃ solution and saved. The first ethyl acetate portion remaining after the second treatment was then subjected to a similar procedure in which six 1 liter portions of 1.0 M citrate buffer of pH 5.20 were passed through eight portions of ethyl acetate. With this system, most of the o-HPAA distributed in Portions 1 to 8, the highest concentration occurring in Portions 2 and 3. Portion 1 was again treated with buffer at pH 5.2, Portions 2 to 8 from both distributions were pooled, and the acids were extracted into a small volume of NaHCO₃ solution and combined with Portions 2 and 3 from the distributions at pH 3.6. The combined bicarbonate solutions were acidified to pH 2, saturated with salt, and extracted thoroughly with ethyl acetate, to give a total volume of 150 ml. This solution was again treated by passage of 6 X 150 ml. portions of buffer of pH 5.2 through 8 X 150 ml. portions of ethyl acetate. Portions 2 to 8 were pooled, and the acids were extracted into bicarbonate and then back into a small volume of ethyl acetate. This solution contained approximately 1.0 gm. of o-HPAA.

The ethyl acetate was concentrated to a low volume by evaporation at room temperature under a stream of nitrogen. The thick syrup was allowed to stand overnight at room temperature, and was filtered to remove the β-phenyl-L-lactic acid that crystallized. The phenyllactic acid was washed on the filter with a small portion of cold ethyl acetate, the ethyl acetate was again blown off the combined filtrate and washings, and another crop of phenyllactic acid was collected. This procedure was repeated until no more phenyllactic acid crystallized. A total of 2.3 gm. of crude phenyl-L-lactic acid was obtained. This material was recrystallized from hot water (Norit) to yield a nitrogen-free product melting at 122-124°, [α]₁₀ -21.4° (c 1, H₂O) (phenyl-L-lactic acid, m.p. 124°, [α]₀ -20.4° (c 1.2, H₂O), (11)).

The oil that remained after the removal of the phenyllactic acid was dissolved in 30 ml. of 50 per cent acetic acid, and the solution was clarified with a small amount of Norit and treated with 15 ml. of 10 per cent mercuric acetate in 50 per cent acetic acid. The precipitate, which contained the mercuric complexes of indole acids contaminated with small amounts of phenolic acids, was separated and worked up for indole compounds; the identification of β-3-indolyllactic acid as the major component of this fraction has been reported elsewhere (7). The acetic acid solution was di-
luted with 10 volumes of water, the aqueous solution was extracted exhaustively with ethyl acetate, and the acids were then extracted into a small volume of bicarbonate and finally returned to ethyl acetate. The ethyl acetate was evaporated in a stream of nitrogen to yield a semicrystalline residue; this was suspended in benzene and filtered, and the crystalline precipitate was washed with benzene. This material (390 mg.) had the chromatographic behavior and qualitative reactions of one of the polar phenolic constituents of normal urine; the characterization and identification of this material will be presented elsewhere.

The residue from the benzene filtrate was dissolved in 50 ml. of 6 N HCl, and the HCl was removed by distillation in vacuo at 80°. Crystals of coumaran-2-one, the lactone of o-HPAA, began to condense on the walls of the condenser as the last of the acid was removed; the residue had at this time a strong odor of coumaran-2-one. The residue from the distillation was dissolved in ether, and the acids were extracted into bicarbonate. The ether solution of neutral substances was evaporated to dryness, the residue was treated with a small amount of 1 N NaOH to hydrolyze the coumaran-2-one, and the o-HPAA formed by this treatment was extracted from the acidified solution with ethyl acetate. The 14 mg. of crude product that was obtained were recrystallized from chlorobenzene to yield 10 mg.

Fig. 1. Absorption spectra of o-hydroxyphenylacetic acid
of pure o-HPAA. This material had physical and chemical properties identical with those of authentic o-HPAA; m.p. 144-145° (authentic 144-146° (10); no depression of the melting point upon admixture with the isolated material). Both authentic and isolated materials had the same crystal form when crystallized from chlorobenzene and showed the same behavior on the micro melting point stage of subliming to form a mixture

Table I

Excretion of o-Hydroxyphenylacetic Acid by Phenylketonuric Patients

<table>
<thead>
<tr>
<th>Name</th>
<th>Age</th>
<th>Intelligence quotient</th>
<th>Fasting serum phenylalanine (mg. per 100 ml.)</th>
<th>Urinary o-hydroxyphenylacetic acid (γ per mg. creatinine)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clifford W.</td>
<td>48</td>
<td>70</td>
<td>22</td>
<td>80</td>
</tr>
<tr>
<td>Dean D.</td>
<td>18</td>
<td>22</td>
<td>37</td>
<td>100</td>
</tr>
<tr>
<td>David R.</td>
<td>14</td>
<td>3</td>
<td>38</td>
<td>175</td>
</tr>
<tr>
<td>James A.</td>
<td>11</td>
<td>9</td>
<td>37</td>
<td>135</td>
</tr>
<tr>
<td>Linda B.</td>
<td>9</td>
<td>22</td>
<td>40</td>
<td>210</td>
</tr>
<tr>
<td>Gary L.</td>
<td>9</td>
<td>40</td>
<td>40</td>
<td>250</td>
</tr>
<tr>
<td>Judy D.</td>
<td>8</td>
<td>14</td>
<td>40</td>
<td>235</td>
</tr>
<tr>
<td>Allan D.</td>
<td>6</td>
<td>3</td>
<td>38</td>
<td>130</td>
</tr>
<tr>
<td>Ann L.</td>
<td>6</td>
<td>3</td>
<td>44</td>
<td>130</td>
</tr>
<tr>
<td>David J.</td>
<td>3</td>
<td>50</td>
<td>50</td>
<td>250</td>
</tr>
<tr>
<td>Jill D.</td>
<td>3</td>
<td>63</td>
<td>40</td>
<td>300</td>
</tr>
<tr>
<td>Robert D.*</td>
<td>1</td>
<td></td>
<td>63</td>
<td>400</td>
</tr>
<tr>
<td></td>
<td>&quot;</td>
<td></td>
<td>17</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>&quot;</td>
<td></td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>&quot;</td>
<td></td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>&quot;</td>
<td></td>
<td>2</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Mary J.*</td>
<td>1</td>
<td></td>
<td>52</td>
<td>300</td>
</tr>
<tr>
<td></td>
<td>&quot;</td>
<td></td>
<td>10</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>&quot;</td>
<td></td>
<td>2</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

* The first value listed for each of these infants was obtained under natural conditions. They were given a diet deficient in phenylalanine (5); the succeeding values were measured at times when the blood phenylalanine was lowered.

The bicarbonate solution obtained in purifying the coumaran-2-one was acidified, and the acids were extracted into ethyl acetate. An additional amount (160 mg.) of crude phenyllactic acid was obtained when this solution was concentrated. The filtrate from the phenyllactic acid was again concentrated to a low volume. A different crystalline compound sep-
arated; this was suspended in benzene, collected on a filter, and washed with benzene. This substance (145 mg.) was impure o-HPAA. Ultraviolet spectrophotometry and paper chromatography showed it to contain approximately 65 per cent of o-HPAA contaminated with about 4 per cent of p-hydroxyphenylacetic acid and 30 per cent of phenyllactic acid. This material was not purified further.

Excretion of o-Hydroxyphenylacetic Acid by Phenylketonurics—Samples of urine from seventeen patients with phenylketonuria have been examined; all showed the excretion of relatively large amounts of o-HPAA. The rate of excretion of o-HPAA by these patients has been expressed conveniently with respect to the creatinine content of spot collections of urine; the close agreement which has been observed among different samples obtained from a given individual at different times of collection has indicated that this represents a satisfactory measure of the excretion and provides an excellent means for comparing the excretion of individuals of different ages and sizes and with differing degrees of food intake, physical activity, and mental impairment. Table I lists semiquantitative approximations of the rate of excretion of o-HPAA by several patients with phenylketonuria.

Excretion of o-Hydroxyphenylacetic Acid by Normal Individuals—All of the normal individuals so far investigated have been found to excrete small, but definite amounts of o-HPAA. The amount excreted has been estimated to be 0.3 to 0.5 per mg. of creatinine; for a normal adult, this would amount to about 1 mg. of o-HPAA daily.

DISCUSSION

o-Hydroxyphenylacetic acid has been identified as the abnormal phenolic metabolite present in greatest amount in the urine of patients with phenylketonuria. This was accomplished by isolation of the metabolite and by a direct comparison of the properties of the isolated material and synthetic o-HPAA. It should be noted that the poor recovery of o-HPAA in the isolation was made because the procedure was aimed primarily at the isolation of other compounds. In an earlier report from this laboratory (1) it was noted that material isolated from phenylketonuric urine did not have the physical properties recorded for o-HPAA. Previous workers (9, 12) were able to recrystallize o-HPAA from chloroform; we have not been able to use chloroform as a solvent for this purpose. It is possible that the chloroform used some time ago may have been impure and was different in solvent action from reagent grade chloroform now in use. Also, the first small amount of o-HPAA that was obtained in preliminary experiments crystallized in flat plates and melted at 162°, rather than in prisms melting at 145°. Occasionally, synthetic o-HPAA likewise has been obtained in this
high melting form. It is interesting in this regard to note that Blum (12) recorded the isolation of o-HPAA which had the properties of o-HPAA, but not its melting point. He was able to convert his material into lactone and back into o-HPAA having the proper melting point. It seems probable that he may have obtained the higher melting form in his experiment.

The most important question posed by the identification of o-HPAA as a metabolite in phenylketonuric urine concerns a possible relation between its formation and the development of the mental defect. The data of Table I indicate that the production of o-HPAA is in some manner directly related to blood levels of phenylalanine in the patients. This is shown more clearly when the data are presented graphically, as in Fig. 2. Levels for serum phenylalanine and urinary excretion of o-HPAA are reported for thirteen patients, in which blood levels of phenylalanine ranged from 22 to 63 mg. per 100 ml. In addition, comparable data are reported which were obtained with two of the patients who were receiving phenylalanine-restricted diets at times when they had different serum levels of phenylalanine. Borek et al. (13) have shown that there is no significant correlation between levels of serum phenylalanine and the degree of mental defect in patients with phenylketonuria; hence it seems reasonable to conclude that the production of o-HPAA cannot be related in any direct manner to the occurrence of mental defect.
We are indebted to Dr. V. F. Houston, Director of the Utah State Training School for making available to us the facilities of that institution, to Mr. Mark Allen for the psychometric evaluation of the patients, and to Mrs. Betty Fernelius for her aid in carrying out experiments with the patients at the Training School. We wish to thank Patricia Wall for the analytical work reported here.

SUMMARY

1. o-Hydroxyphenylacetic acid has been identified as a constituent of the urine of patients with phenylketonuria. The amount excreted ranges from 100 to 400 mg. per gm. of creatinine. Normal individuals also excrete o-hydroxyphenylacetic acid, but at the level of less than 1 mg. per gm. of creatinine.

2. The amount of o-hydroxyphenylacetic acid excreted by phenylketonurics is directly related to the phenylalanine levels in the blood. It is probable that the excretion of increased amounts of this metabolite is not related in any direct manner to the occurrence of the mental defect.

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