CXI. A NEW ERROR OF TYROSINE METABOLISM: TYROSINOSIS. THE INTERMEDIARY METABOLISM OF TYROSINE AND PHENYLALANINE[1].

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THE object of the present paper is to describe an addition to the few known errors of metabolism. That the condition here described for the first time is not common is shown by the following observation. The only way in which it may be routinely discovered is by following up urines reported as giving a doubtfully positive sugar test. Dr Blatherwick, the head of the laboratory of the Metropolitan Life Insurance Co., who was present when some of these findings were presented in Chicago in 1930, has since then followed up 14,753 urines which contained 0·3 % sugar according to Benedict's picric acid method. The test with Fiske's molybdic acid reagent described below was applied to these urines. No instance of our condition was found. One case of alcaptonuria was present.

I. THE HISTORY OF THE INVESTIGATION PRIOR TO THE PRESENT STUDY.

The subject (Max. M., Univ. Hosp. No. 41,154), afflicted with the metabolic disturbance here investigated, is a Russian Jew, aged 49, who during the first half of the year 1927 had been a patient in the Minneapolis General Hospital and had been diagnosed as a case of myasthenia gravis. The reasons for the diagnosis seem adequate and the patient still presents the ptosis, marked on one eye and somewhat less marked on the other, which appears as a characteristic of myasthenia gravis. For four years he has now been free from any acute symptoms of myasthenia gravis. During these years he has been helping with light work of various kinds and during the last year has been working as a repair tailor. He has however a constant creatinuria.

The patient was admitted to the University Hospital July 18, 1927[2], for

[1] Supported by grants from the Research fund of the Medical School of the University of Minnesota.

[2] Through his response to the interests of the Department of Medicine in making possible the uninterrupted hospitalisation of the patient, the Superintendent of the University Hospital, Mr Paul Fesler, has assured himself of our appreciation and demonstrated his realisation of the possibilities of his office in the promotion of clinical research.

the purpose of another investigation [Berglund, Medes and Lohmann, 1927]. During some phosphate determinations by the method of Fiske and Subbarow [1925] in the course of this study, his urine was found to reduce phosphomolybdic acid. This reducing power was further investigated and it was for a while thought of as a possible characteristic of myasthenia gravis [Medes, Berglund and Lohmann, 1927]. Later examinations of urines from more than half a dozen patients with myasthenia gravis have failed to support this assumption. On the basis of the whole behaviour of the anomaly as described in the following pages, it is now believed to be without connection with myasthenia gravis.

Considering the ability of this patient, under conditions described below, to excrete dihydroxyphenylalanine, it seems of importance to state that the patient shows no abnormal pigmentation; particularly that the parts exposed to light show no demarcation in colour from the rest of the body. On exposure of a skin area of the forearm to ultra-violet radiation no abnormal pigmentation developed. The cornea, lens and vitreous humour showed no abnormal precipitates.

II. Methods.

1. Isolation and purification of compounds.

Throughout the experiments, samples of the various compounds were isolated and identified, the methods being varied from time to time according to the conditions of the experiments. The following description applies to the isolation of tyrosine, *p*-hydroxyphenylpyruvic acid, *l*-*p*-hydroxyphenyllactic acid and *l*-3 : 4-dihydroxyphenylalanine which were all isolated from the urine after ingestion of 50 g. of tyrosine.

The urine was treated with an excess of lead acetate and the p_H adjusted to about 5·0 with dilute acetic acid. The precipitate was filtered off and discarded.

The further procedure followed the lines worked out by Guggenheim [1913] and modified by Raper [1926]. The p_H was adjusted to 7·0 with dilute NH_4OH and the precipitate allowed to settle—a process requiring about 3 hours. The clear supernatant liquid, which contained the tyrosine, was decanted and saved for recovery of the latter. The precipitate was centrifuged, washed twice with water and re-centrifuged after each washing. The washings were combined with the supernatant liquid obtained above. The precipitate was suspended in water and decomposed with an excess of H_2SO_4.

The resulting reddish-brown solution was extracted with ether for 24 hours in a continuous extractor, the ether removing the hydroxy- and keto-acids and leaving the dihydroxyphenylalanine. During the process hydrogen was bubbled in a slow stream through the solution to prevent oxidation of the dihydroxyphenylalanine.

Isolation of p-*hydroxyphenylpyruvic acid and* l-p-*hydroxyphenyllactic acid.* The ether extract obtained above contained the keto- and hydroxy-acids, the former highly soluble in the ether, the latter so slightly soluble that when present in large amounts, it crystallised on the sides of the ether chamber of the extractor and could be recovered merely by pouring off the ether. The ether was evaporated slowly, successive crops of hydroxy-acid being collected from time to time. Dilution of the syrupy residue with water caused the keto-acid containing traces of hydroxy-acid to crystallise immediately.

The crystals were removed by centrifuging and freed from hydroxy-acid by washing several times with cold water. The amount of material in the washings was comparatively small and usually no effort was made to recover it.

The *p*-hydroxyphenylpyruvic acid was purified by recrystallising repeatedly from hot water (charcoal), and finally recrystallising from dilute and from absolute alcohol. It separates according

to conditions in needles or rhombic or hexagonal plates. The hexagonal plates are the most stable as the other forms go over to them on standing, M.P. 219°. (Found C, 60·04; H, 4·57 %. Calc. C, 60·00; H, 4·47 %.)

The *p*-hydroxyphenyllactic acid was purified by several recrystallisations from water, followed by one or more from boiling ether. It separates as long silky needles; M.P. 168°. $[\alpha]_{5461}^{20°} = -20·56°$. (Found C, 59·32; H, 5·49 %. Calc. C, 59·30; H, 5·53 %.)

Both compounds give a strong Millon reaction, and were further identified by comparison with synthetic specimens prepared according to Plöchl [1883] and Neubauer [1909].

Through the courtesy of Dr E. C. Kendall I am indebted to Dr A. E. Osterberg of Dr Kendall's laboratory at the Mayo Clinic, Rochester, for elementary analyses of the various compounds isolated.

There is evidence that *p*-hydroxyphenylpyruvic acid exists in two tautomeric states analogous to those described by Bougault and Hammerlé [1915] for phenylpyruvic acid. Although the free acid is characterised by a high degree of insolubility it requires hours to precipitate out of solution when the sodium salt is acidified in the synthesis mentioned above. On the other hand, when the acid is recrystallised from hot water, precipitation occurs immediately upon the slightest cooling at the surface film, but if the acid is dissolved in alkali carbonate and the solution reacidified, precipitation of the acid then requires several hours.

Isolation of 1-3 : 4-*dihydroxyphenylalanine.* The reddish-brown solution remaining after extraction of the hydroxy- and keto-acids was saturated with SO_2 and concentrated in a vacuum desiccator over H_2SO_4 to a sticky mass containing crystals of uric acid and inorganic salts. This mass was extracted with cold water and filtered, by which process the amino-acid was separated from the insoluble residue. The filtrate was boiled with purified charcoal, cooled quickly, again saturated with SO_2 and re-concentrated. Upon repeating this process several times the dihydroxyphenylalanine finally crystallised out and was purified by repeated recrystallisation from hot water. If at any time the solution was left exposed to the air without SO_2, a rapid oxidation took place with the production of melanin. (Found C, 54·29; H, 5·53 %. Calc. C, 54·79; H, 5·62 %.)

The acid crystallises, as first observed by Guggenheim, in thick prisms or fine needles according to conditions, M.P. 270°. It gives a green colour with very dilute ferric chloride, changing to purple with ammonia. Contrary to the statement of Guggenheim, it does not produce a red colour with Millon's reagent but a faint pinkish coloration characteristic of dihydric phenols. I am indebted to Dr Raper for a sample of dihydroxyphenylalanine from *Vicia faba*; its properties were identical with those of the compound obtained from the urine. For the feeding experiments, about 6 g. were isolated from *Vicia faba* in this laboratory and smaller amounts were obtained by the oxidation of tyrosine by tyrosinase prepared from *Tenebrio molitor* [Gortner 1910, Raper 1926, 1927] and extracted and purified according to Raper.

Recovery of tyrosine. The filtrate from the second lead precipitation of the urine (p_H 7·0) contained tyrosine, which was precipitated as the lead salt by adjusting the p_H to about 9·5 with NH_4OH. The precipitate was removed by centrifuging, washed twice with water and the lead precipitate decomposed with H_2S. The PbS was filtered off with suction, the precipitate being washed several times with dilute HCl to dissolve any tyrosine which might have crystallised out. The solution was then boiled with charcoal, neutralised with Na_2CO_3 and concentrated by boiling under reduced pressure. From the resulting dark reddish-brown syrupy liquid impure tyrosine gradually precipitated. It was purified by the method described by Folin and Ciocalteu [1927].

Extraction of melanin. Melanin was not present in the fresh urine of the subject of the present investigation but since dihydroxyphenylalanine and hydroxyphenylpyruvic acid are both potential precursors of melanin, its formation occurred under any condition permitting oxidation. Hydroxyphenyllactic acid is stable and does not oxidise to melanin. Dihydroxyphenylalanine takes up oxygen with such avidity that even saturating the urine with SO_2 does not prevent oxidation, and melanin formation occurred within a few hours after the urine containing this compound was voided. When hydroxyphenylpyruvic acid was present in urine oxidation could be prevented indefinitely by saturating the urine with hydrogen, but, if it were exposed freely to oxygen, melanin gradually separated out. The process required several weeks when the urine was merely left exposed to air, but when oxygen was bubbled through, precipitation was completed in 2–3 days. Warming or rendering alkaline further accelerated the reaction.

To separate the melanin the urine was evaporated and the melanin extracted by the method of Medes and Berglund [1928]. For its purification advantage was taken of the fact that melanin is insoluble in water at its isoelectric point (about p_H 4·0). The acidified methyl alcohol containing the extracted melanin was filtered and evaporated under reduced pressure to a thick syrup which was poured into 5 litres of water, the p_H adjusted to about 4·0, and the solution kept for several days. The melanin collected on the bottom of the flask and was recovered by decanting and centrifuging. It was washed with water, dissolved in anhydrous methyl alcoholic hydrogen chloride and again poured into water. The process was repeated until the melanin was ash-free. Yield, about 3 g. daily.

Products of intestinal decomposition. Search was made from time to time for the presence of other compounds known to represent products of intestinal decomposition of tyrosine. Hippuric acid crystals were observed occasionally, and varied roughly with the amount of tyrosine ingested. They disappeared entirely when the subject was on a tyrosine-free diet. No *p*-hydroxyphenylacetic acid or *p*-hydroxyphenylpropionic acid was found. There is a possibility that traces of some compounds may have been present in the acid lead fraction.

2. *Quantitative estimation of compounds.*

Copper reduction method. In early experiments, before the number and nature of the compounds had been determined, the reducing property of hydroxyphenylpyruvic acid was the outstanding characteristic by which quantitative estimations could be made, and an estimate of the relative amounts present under different dietary conditions was obtained by its reduction of the Folin-Wu [1919] sugar reagent, using the Folin and Berglund [1922] method for normal sugar and employing glucose as a standard. In later experiments the determinations with this reagent were continued in order to obtain an uninterrupted series of estimations made by one method, thereby providing a basis for interpretation of the early findings.

Both *p*-hydroxyphenylpyruvic acid and dihydroxyphenylalanine reduce this reagent, the equivalents (in aqueous solution) being:

1 mg. *p*-hydroxyphenylpyruvic acid = 0·45 mg. glucose;
1 mg. *l*-3 : 4-dihydroxyphenylalanine = 1·33 mg. glucose.

Since the latter, however, was absent except under high tyrosine feeding, the values were due to the keto-acid *plus* the normal urinary "sugar". In general, no attempt was made to convert the figures into terms of a keto-acid standard, since the error in this determination from the normal reducing substances of urine and from a slight loss of the keto-acid with Lloyd's reagent made it impossible to employ the method for other than relative values.

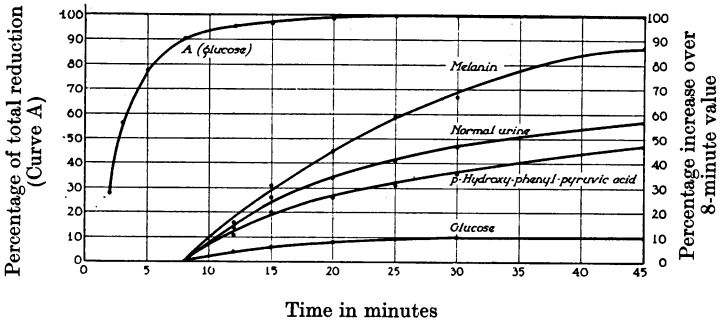


Fig. 1. Reduction of the Folin-Wu blood-sugar reagent. Reduction by glucose (Curve A). Percentage of total reduction at different time intervals. Reduction by melanin, normal urine, *p*-hydroxyphenylpyruvic acid and glucose. Values expressed in percentage increase above their 8-minute values.

The reduction of cupric oxide, while non-specific and hence capable of being used for estimation of other oxidisable substances, is particularly suited for the quantitative determination of glucose since the oxidation of glucose occurs with such rapidity that at 25 minutes it has reached approxi-

mately its maximum value. The percentages of the total reduction after various time intervals as found in one experiment are plotted in Fig. 1 (Curve A). It may be noted that at 8 minutes, the interval of heating as stipulated by Folin and Wu [1919], the curve is straightening. Later Folin and Svedberg [1926] recommended a period of 10 minutes and still later [Folin, 1929], 14 or 15 minutes. At 8 minutes, however, the rate of reduction has become retarded to such an extent that errors of determination due to slight differences in the time interval are only a small percentage of the total value. The same is not true of the various reducing compounds considered in this paper. Their comparative percentages of increase above the 8-minute values are also shown in Fig. 1. It may be seen that the rate of change is higher with *p*-hydroxyphenylpyruvic acid and the reducing substances of normal urine, and still greater with melanin so that a large error may be introduced with slight differences in time. Reduction of the reagent with dihydroxyphenylalanine reaches its maximum in 8 minutes.

Reduction of the Folin and Ciocalteu phenol reagent. This offered another method of estimating the daily output of tyrosine and of its three metabolites, but since the method was not specific it was not used in the metabolism experiments.

Reduction of phosphomolybdic acid. This was used in the form of the Fiske and Subbarow [1925] reagent for the determination of phosphorus. One cc. of urine was measured into a 50 cc. volumetric flask and 5 cc. of the molybdic reagent added. When the unknown was present in pure aqueous solution, 5 cc. of 3·5 % KH_2PO_4 were added to supply the phosphorus. The mixture was then made up to volume and read in the colorimeter against a standard of 5 mg. of *p*-hydroxyphenylpyruvic acid similarly treated with KH_2PO_4 and the molybdic reagent.

Both dihydroxyphenylalanine and *p*-hydroxyphenylpyruvic acid reduce this reagent. Table I shows the rates of colour development and the comparative reducing powers of the two compounds. As may be seen, the keto-acid developed its full colour by the end of 3 hours whereas the amino-

Table I. *Ratio between colour developed by* p-*hydroxyphenylpyruvic acid* (P_2) *with the molybdic acid reagent at various time intervals and the colour of the same compound* (P_1) *after 3 hours. Similar ratio between dihydroxyphenylalanine* (A) *and* p-*hydroxyphenylpyruvic acid* (P_1).

Time min.	$P_2/P_1 \times 100$	Time min.	$P_2/P_1 \times 100$	Time hrs.	$A/P_1 \times 100$
1	39·6	55	87·3	2·5	12·5
3	42·3	70	92·8	5	14·2
5	46·7	85	96·5	23	25·9
10	52·4	105	98·7	24	26·6
15	57·7	120	100·0	27	27·1
20	63·2	180	100·0	30	29·3
25	64·7			51·5	32·5
30	68·8			95·5	35·4
35	69·6			120	38·2
40	73·3			146	35·0
45	79·7			169	30·0

acid required 5 days; its reducing power was then equivalent to 38·2 % of that of the keto-acid. After 2·5 hours, the earliest time at which sufficient colour is developed to obtain a reading with the colorimeter, the colour development was only 12·5 % of the total keto-acid value. Since the amino-acid was present only during the three experiments given here in detail and then in comparatively small amounts, the colour developed at the end of 3 hours was taken to represent the amount of the keto-acid in the urine.

The greatest error in this procedure lay in slight variations in the degree of blueness or greenness of the final colour developed, variations dependent upon the relative amounts of ammonia and other substances in the urine under different dietary conditions. Apart from this difficulty, the determination by this method proved satisfactory since the proportionality of colour developed was good over a wide range.

Another potential source of error lay in possible variations in the relative amounts of the two

tautomeric forms of *p*-hydroxyphenylpyruvic acid and the rate of conversion to the enolic form which is responsible for its reducing properties. According to Bougault and Hammerlé [1915], heating the keto-form of phenylpyruvic acid in the presence of alkali transforms it to the enol derivative. Although heating the urine with alkali destroyed some of the compound, the rate of colour development with the molybdate reagent was unaffected, indicating that the hydroxyphenylpyruvic acid was present in urine almost exclusively in the enol form. These findings explain the failure of our early attempts to identify the reducing compound with a ketone. Repeated efforts failed to obtain a phenylhydrazone, an addition product with $NaHSO_3$ or a semicarbazone.

The Millon reaction. The Millon reaction as developed by Folin and Ciocalteu [1927] proved of special value in both the metabolism studies and the isolation experiments, since molar equivalents of tyrosine, hydroxyphenylpyruvic acid and hydroxyphenyllactic acid all give rise to a similar red colour of about equal intensity. Since the phenols normally present in urine do not produce a red colour with Millon's reagent, this method permitted an estimate of tyrosine *plus* its two metabolites. In some of the experiments the reaction was employed to estimate the amount of tyrosine remaining in the urine after extraction of the ether-soluble compounds from an aliquot part. Thus by comcombining this method with the molybdate method, which was considered specific for *p*-hydroxyphenylpyruvic acid under the conditions of certain experiments, an estimation of each of the three compounds could be made. The procedure for carrying out the Millon reaction was as follows.

Into a 15 cc. centrifuge-tube were introduced 2·5 cc. urine and 2 cc. 15 % $HgSO_4$ in 6*N* H_2SO_4. This was allowed to stand one hour and centrifuged, after which the supernatant liquid was poured into a 50 cc. centrifuge-tube, the original tube drained carefully and its sides washed with a few drops of distilled water. 2·5 cc. of tyrosine standard containing 1 mg. per cc. in 2*N* H_2SO_4 were prepared in a second 50 cc. centrifuge-tube and 2 cc. of 15 % $HgSO_4$ in 6*N* H_2SO_4 added. To each of these two tubes containing standard and unknown, 10 cc. of 2*N* H_2SO_4 were added and the tubes heated in boiling water for 15 minutes. A second portion of 10 cc. of 2*N* H_2SO_4 was then added to each and the tubes allowed to stand for 30 minutes. If a slight cloudiness appeared in the unknown it was centrifuged again, after which the contents of the tubes were poured into 50 cc. volumetric flasks and 1 cc. of 2 % $NaNO_2$ added to each. They were diluted to volume and read at once in the colorimeter.

This modification of the Folin and Ciocalteu method [1927] was made for the sake of simplicity, and, while some errors were probably introduced, it was found that tyrosine added to normal urine could be determined within 3 %.

III. Metabolic studies[1].

1. Output during fasting.

At three different times the subject fasted for periods of 2 days, each period following a low protein diet. The results, recorded in Table II, show that during starvation, *p*-hydroxyphenylpyruvic acid is excreted to the extent of about 1·6 g. daily (column 4); that this is equivalent to the substances giving the Millon reaction; consequently no tyrosine or *p*-hydroxyphenyllactic acid is excreted, in confirmation of which neither of the two latter nor dihydroxyphenylalanine could be extracted from the urine. It may also be seen that although 3 years elapsed between the earliest and latest determinations there is a marked constancy in the amount of the *p*-hydroxyphenylpyruvic acid excreted. This amount then may be considered as of endogenous origin.

[1] The long metabolism experiments on a variety of diets were made possible only through the generous co-operation of Miss Gertrude Thomas, Head Dietitian of the University Hospital.

Table II. *Urinary output of various metabolites during graded levels of protein intake.*

Date	Total Millon g.	Millon after ether extraction g.	Phosphomol. reduction g.	Cu reduction g.	Creatinine g.	Creatine g.	Total N g.	Amino-N g.	
Fasting.									
Sept. 30, 1927	—	—	—	1·62	0·99	0·53	—	—	—
Oct. 1, 1927	—	—	—	1·56	1·00	0·52	—	—	—
July 8, 1929	1·58	—	1·63	1·63	1·15	0·49	—	—	—
July 9, 1929	1·47	—	1·57	1·36	1·12	0·56	—	—	—
June 3, 1930	1·68	—	1·79	1·57	1·21	0·67	4·07	—	—
June 4, 1930	1·58	—	1·53	1·47	1·25	0·64	3·81	—	—
Average	1·56	—	1·63	1·54	1·12	0·57	3·94	—	—
Mixed diet.									
Exp. 1 (15 days).									
Maximum value	3·04	—	—	3·33	1·35	—	—	—	—
Minimum value	1·89	—	—	1·33	1·02	—	—	—	—
Average value	2·49	—	—	1·97	1·18	—	—	—	—
Exp. 2 (12 days).									
Maximum value	3·28	0·45	—	2·82	1·26	0·87	—	—	—
Minimum value	2·12	0·37	—	1·33	1·05	0·52	—	—	—
Average value	2·74	0·40	—	1·75	1·12	0·64	—	—	—
Exp. 3 (3 days).									
Maximum value	4·68	—	3·28	2·33	—	—	—	0·22	—
Minimum value	3·70	—	2·50	1·76	—	—	—	0·21	—
Average value	4·15	—	2·78	2·02	—	—	—	0·22	—
Exp. 4 (5 days).									
Maximum value	4·08	0·50	3·57	4·01	—	—	—	—	—
Minimum value	2·50	0·37	1·91	2·12	—	—	—	—	—
Average value	3·13	0·44	2·75	2·89	—	—	—	—	—
High protein diet.									
Days of exp.					Cols. 3, 4				
1– 2 (Av.)	2·53	0·45	2·05	2·02	2·50	—	—	—	Hospital diet
3	2·10	0·39	1·75	1·62	2·14	—	—	—	Fasting
4	1·58	trace	1·62	1·56	—	—	—	—	Fasting
5	2·76	0·23	2·47	2·04	2·70	—	—	—	Meat diet
9	3·92	0·71	3·22	3·25	3·93	—	—	—	—
10–12 (Av.)	4·21	0·77	3·20	3·56	3·97—trace hydroxy-acid isolated				

2. *Output on a tyrosine-free diet.*

On a tyrosine-free diet adequate in calories (cooked starch, gelatin, tea and sugar with small amounts of lemon juice, about 2100 Calories) the subject excreted the same amount of *p*-hydroxyphenylpyruvic acid as during fasting. No tyrosine or other derivative of tyrosine could be isolated. In Table III, days 10 to 13 illustrate such a period; 1·64 g. of the pyruvic compound were found, which comprised all the substance giving the Millon reaction. In Table VI a similar period is recorded. The average output of *p*-hydroxyphenylpyruvic acid was 1·43 g., which corresponded closely with the total tyrosine value. As seen in the table, no hydroxy-acid or dihydroxyphenylalanine or tyrosine could be recovered.

3. Output on a mixed diet.

On a mixed diet the output of *p*-hydroxyphenylpyruvic acid rises from 1·60 g. daily to approximately 2·8 g. daily (Table II, column 4). This amount is no longer equivalent to the total Millon value, the latter showing a greater increase; in Exp. 4, which may be taken as an example, it has risen to 3·13 g. The difference, 0·38 g. represents tyrosine, determined as 0·44 g. on the ether-extracted fractions. There is no longer the same constancy in amount of the keto-acid, the output varying in this series of experiments from 1·91 to 3·57 g. (column 4). The total Millon value shows even wider variations, ranging from 1·89 to 4·68 g. The keto-acid is then both endogenous and exogenous in origin, the tyrosine exogenous.

4. On a high protein diet (Table II).

The output of *p*-hydroxyphenylpyruvic acid rises immediately from 1·62 g., its fasting level, to 2·47 g. and continues to increase to about 3·2 g, which output is maintained over a period of 5 days of the forced diet. The tyrosine, as determined by Millon's reagent on an ether-extracted portion, likewise gradually rose from a trace present on the second fasting day to 0·8 g. During the early days of the experiment, tyrosine and *p*-hydroxyphenylpyruvic acid constituted all of the substance giving Millon's reaction, as may be seen by comparing columns 2 and 4, but as the high protein diet continued a discrepancy between these values appeared, indicating the presence of some other compound included in the total Millon values. Beginning at the 6th day of the high protein diet, traces of *p*-hydroxyphenyllactic acid were isolated. No dihydroxyphenylalanine was present at any time.

5. Ingestion of tyrosine and phenylalanine.

It was shown early in the investigation that tyrosine increased the output of the reducing substance. Table III records a series of these experiments. In each case there was an immediate rise in the copper reduction value over the average value for the preceding days, followed by a falling off on the 1st or 2nd day following. In the first experiment 4·5 g. were given over a period of 4 days. As may be seen, the rise was less, 3·10 g. being the maximum value on the day following ingestion of 2·5 g., after which the output fell to its usual level. Even the ingestion of 0·2 g. produced a prompt elevation over the previous control period and with the low amounts employed here there was no evidence of a cumulative effect.

In order to determine the level of tyrosine intake at which each compound becomes excreted, the subject was placed on a tyrosine-free diet as described above and increasing amounts of tyrosine administered (Table III). During the tyrosine-free period the output of tyrosine *plus* its monohydroxy derivatives fell from an average of 3·21 g. to 1·60 g. (column 2), and of *p*-hydroxyphenylpyruvic acid from 2·75 g. to 1·64 g. (column 4). Tyrosine disappeared from

Table III. *Effect of ingestion of tyrosine.*

Day of experiment	Total Millon g.	Millon after ether extraction g.	Phosphomol. reduction g.	Cu reduction g.	Creatinine g.	*p*-Hydroxyphenyllactic acid (Recovered)	*p*-Hydroxyphenyllactic acid (Computed)	Total N g.	
Exp. 1.									
1– 6 (Av.)	—	—	—	2·97	1·05	—	—	—	Hospital diet
7–15 (Av.)	—	—	—	2·32	1·07	—	—	—	—
16	—	—	—	2·55	1·13	—	—	—	0·2 g. tyrosine
17	—	—	—	2·46	1·05	—	—	—	1·0 g. tyrosine
18	—	—	—	2·96	1·12	—	—	—	0·8 g. tyrosine
19	—	—	—	2·99	1·16	—	—	—	2·5 g. tyrosine
20	—	—	—	3·10	1·17	—	—	—	—
21–23 (Av.)	—	—	—	2·78	1·13	—	—	—	—
24	—	—	—	3·66	1·14	—	—	—	5·0 g. tyrosine
25	—	—	—	2·98	1·05	—	—	—	—
26–32 (Av.)	—	—	—	2·62	1·06	—	—	—	—
Av. control periods	—	—	—	2·52	1·08	—	—	—	—
Exp. 2.									
1– 5 (Av.)	3·21	0·50	2·75	2·89	—	—	—	—	Hospital diet tyrosine-free
10–13 (Av.)	1·60	—	1·64	1·74	—	—	—	6·29	Diet started day 6
14	1·72	—	1·70	1·74	—	—	—	5·94	2 g. tyrosine
15	1·79	Trace*	1·91	2·02	—	—	—	4·28	2 g. tyrosine
16	2·66	—	3·11	2·46	—	—	—	3·62	2 g. tyrosine
17	2·78	Trace*	2·73	2·74	—	—	—	5·12	2 g. tyrosine
14–17 (Av.)	2·24	—	2·36	2·24	—	—	—	4·74	—
18	2·94	—	2·92	2·70	—	—	—	4·90	5 g. tyrosine
19	3·31	†	2·59	2·80	—	—	—	3·19	—
18–19 (Av.)	3·12	—	2·76	2·75	—	—	—	4·05	—
20	5·24	0·74	3·79	3·45	—	—	—	5·40	10 g. tyrosine
21	5·33	0·75	3·83	2·95	—	—	—	3·95	—
22	4·12	0·48	2·67	3·29	—	1·2	—	5·09	—
23	1·98	0·37	1·63	1·46	—	0·6	—	4·92	—
24	2·00	†	1·80	1·73	—	0·3‡	—	5·58	—
25	1·57	†	1·27	1·98	—	Traces	—	4·11	—
26	1·57	—	1·59	2·06	—	—	—	4·22	—
27	1·36	—	—	—	—	—	—	—	—
Exp. 3.									
1– 3 (Av.)	4·21	0·75	3·23	2·61	—	—	—	—	Hospital diet (80 g. protein)
4	7·24	2·14	4·64	3·20	—	0·32	—	—	15 g. tyrosine
5	8·84	2·44	5·20	4·63	—	0·74	—	—	(Urine darkened
6	6·22	1·32	3·26	2·18	—	0·92	—	—	on standing. 30
7	4·13	0·92	4·09	3·28	—	0·20	—	—	mg. dihydroxy-
8	4·28	0·82	3·15	2·50	—	—	—	—	phenylalanine isolated impure)
Exp. 4.									
1– 3 (Av.)	4·15	0·85	2·79	3·02	—	—	0·45	—	Hospital diet (80 g. protein)
4	6·58	1·21	3·95	3·57	—	0·52	1·41	—	10 g. tyrosine
5	7·85	2·16	4·83	3·95	—	0·41	0·86	—	10 g. tyrosine
6	8·83	2·52	5·17	4·82	—	0·73	1·14	—	15 g. tyrosine
7	10·85	3·45	6·43	5·42	—	0·70	0·98	—	15 g. tyrosine (Urine darkened di-
8	8·48	1·00	5·20	5·11	—	1·10	2·28	—	hydroxyphenyl
9	4·58	0·67	2·64	3·09	—	0·80	1·27	—	alanine isolated)

* It is not clear whether the colour produced was due to tyrosine or to unextracted *p*-hydroxyphenylpyruvic acid.

† Tyrosine present in small amounts, but the solution cloudy and colorimeter readings could not be obtained. A few crystals were isolated.

‡ Concentration too low to determine accurately.

the urine except for questionable traces. The administration of 2 g. (14th day) produced an immediate rise, which became progressive as similar doses were given on successive days. During this period the *p*-hydroxyphenylpyruvic acid probably constituted all of the substances determined with Millon's reagent as shown by a comparison of columns 2 and 4. The traces of some compounds reacting with Millon's reagent in the ether-extracted fractions were not considered significant, as minute amounts of *p*-hydroxyphenylpyruvic acid could easily have remained unextracted. No crystals of tyrosine or the hydroxy-acid could be isolated.

On an intake of 5 g. of tyrosine there was an immediate increase in the Millon-reacting constituents with a considerable discrepancy on the 2nd day (19th day of exp.) between those and the *p*-hydroxyphenylpyruvic acid. At this time crystals of tyrosine were isolated from the urine.

On the next day 10 g. of tyrosine were administered, with an immediate jump in the *p*-hydroxyphenylpyruvic acid to 3·8 g. and in the total monohydroxy-derivatives to 5·2 g., a level which remained practically constant for 2 days. On each of these 2 days 0·74 g. of tyrosine was excreted. Tyrosine *plus* *p*-hydroxyphenylpyruvic acid amount to about 4·5 g. leaving an unexplained discrepancy between them and the "total Millon." No hydroxy-acid could be isolated. On the 2nd day following the ingestion of 10 g. tyrosine, however, hydroxy-acid began to appear in the urine in amounts large enough to be determined by the optical rotation method. Traces were isolated also on the following day.

The results of this experiment are in agreement with those of the high protein diet, that when the output of *p*-hydroxyphenylpyruvic acid approximates 3 g., tyrosine appears in the urine, and that when the output of the keto-acid is pushed still higher, *p*-hydroxyphenyllactic acid also appears. In the case of the latter, there is a lag in its appearance, in this experiment 48 hours after the ingestion of the tyrosine, a behaviour at variance with that of the other compounds, all of which are excreted at their maximum on the first 2 days.

In Table III Exp. 3 is recorded in which the intake of tyrosine was pushed still higher, 15 g. being fed during a period on high protein diet. The maximum output of "total Millon" reached 8·8 g. on the day following ingestion. The hydroxy-acid, which was possibly present in traces on the 3rd day, was isolated to the amount of 0·32 g. on the day of the tyrosine ingestion, and increased in amount on the 2 following days, recalling a previous experiment in which there was a lag in its maximum excretion. The 3rd day following feeding (day 7 of the experiment) it was noted that the urine was darkening and that the reducing properties also were greater than those of the urine of the previous day. From the urine of this day 30 mg. of dihydroxyphenylalanine were isolated. The dihydroxyphenylalanine, then, is excreted at a still higher level of tyrosine intake than is required for elimination of either tyrosine or *p*-hydroxyphenyllactic acid, and is excreted after a still greater delay.

One further experiment with tyrosine feeding was carried out (Table III, Exp. 4), principally for the purpose of investigating the conditions attending the elimination of dihydroxyphenylalanine as well as for the purpose of securing sufficient crystals for identification. While on a mixed diet somewhat high in proteins, the subject was given 10 g., 10 g., 15 g. and 15 g. of tyrosine on 4 successive days. From the urine of the first day tyrosine, *p*-hydroxyphenylpyruvic acid and *p*-hydroxyphenyllactic acid were all isolated. The last, in this instance, was already present possibly in minute amounts owing to the rather high protein diet which had been maintained for 10 days previously. On the 3rd and 4th days after the first tyrosine administration rapid darkening of the urine occurred, and on these two days 100 mg. dihydroxyphenylalanine were isolated. From the combined urines of the entire experiment the following were obtained in highly purified form:

p-hydroxyphenylpyruvic acid	4·217 g.
l-*p*-hydroxyphenyllactic acid	3·162 g.
l-tyrosine	2·086 g.
l-dihydroxyphenylalanine	0·109 g.

Tyrosine and *p*-hydroxyphenyllactic acid can be isolated in pure form almost quantitatively, whereas the purification of the keto-acid is attended with such losses that the final product represents only a small percentage of the amount initially present.

Phenylalanine also increases the output of *p*-hydroxyphenylpyruvic acid. In Table IV are recorded two such experiments. The ingestion of 10 g. of

Table IV. *Effects of phenylalanine ingestion compared with that of tyrosine.*

Date (1928)	Total Millon g.	Millon after ether extraction g.	Phospho-mol. reduction g.	Cu reduction g.	Hydroxy-acid (isolated) g.	
Av. May 26 to 30	—	—	—	2·45	—	Hospital diet
May 31	—	—	—	2·87	—	5 g. phenylalanine
June 1	—	—	—	4·58	—	10 g. phenylalanine
„ 2	—	—	—	3·50	—	—
„ 3	—	—	—	2·67	—	—
„ 4	—	—	—	2·33	—	—
(1930)						
Av. June 2 to 4	3·27	0·47	2·74	2·59	—	—
June 5	3·83	0·51	2·86	2·91	—	10 g. phenylalanine
„ 6	4·52	0·79	3·67	3·23	—	—
„ 7	4·02	0·66	3·04	2·63	Trace	—
„ 8	3·31	0·53	2·58	2·43	0·32	—
Av. June 9 to 12	3·37	0·42	2·59	2·67	—	—
June 13	4·94	0·83	3·85	3·90	—	10 g. tyrosine
„ 14	5·15	0·63	4·41	3·54	—	—
„ 15	3·82	0·31	3·03	2·67	0·41	—
„ 16	3·24	0·41	2·90	2·59	—	—

phenylalanine causes an immediate rise of monohydric phenols as shown by the total Millon, a rise somewhat slower and more prolonged than that pro-

duced by tyrosine under similar conditions as shown later in the table. The same holds true for the excretion of tyrosine (column 3). Tyrosine causes a somewhat greater rise in the *p*-hydroxyphenylpyruvic acid, this increase also occurring on the first 2 days. *p*-Hydroxyphenyllactic acid is excreted only on the 2nd day following tyrosine ingestion, whereas in the phenylalanine experiment it appears only in traces on the 2nd day and is excreted in maximum amount on the following day.

A calculation of the excess of these three substances excreted above the expectancy, as estimated in each control period, shows that following phenylalanine feeding an excess of 4·37 g. was excreted, whereas, after tyrosine ingestion, an excess of 8 g. was excreted, *i.e.*, in the former case 5·63 g. were unaccounted for and in the latter 2 g. It is possible that part of the phenylalanine was excreted unchanged.

Glycine, tryptophan and creatine do not increase the output of the reducing compound in the urine (Table V).

Table V. *Effect of ingestion of various metabolites on the output of* p-*hydroxyphenylpyruvic acid.*

Volume cc.	Creatinine g.	Sugar g.	
2155	1·10	2·64	Hospital diet
2395	1·08	2·37	—
1985	1·05	2·25	—
1940	1·10	2·42	—
2585	1·02	2·64	5 g. glycine
2940	1·00	2·12	—
2240	1·13	2·64	—
1880	1·11	2·58	—
2400	1·10	2·48	—
2150	1·14	2·49	5 g. glycine
2325	1·01	2·34	—
2250	0·97	2·21	—
1760	1·07	2·26	—
2270	1·21	2·35	15 g. tryptophan
1950	1·13	2·61	—
1920	1·26	2·49	—
1970	1·09	2·66	—
1975	1·07	2·60	5 g. creatine
1660	1·25	2·46	5 g. creatine
1820	1·33	2·54	10 g. creatine
2350	1·40	2·38	10 g. creatine
2500	1·19	2·61	—

6. *Response to the various tyrosine derivatives.*

In the following series of experiments the three metabolites of tyrosine, *p*-hydroxyphenyllactic acid, *p*-hydroxyphenylpyruvic acid and dihydroxyphenylalanine were fed. The results are given in Table VI.

(*a*) p-*Hydroxyphenyllactic acid.* 2 g. of *p*-hydroxyphenyllactic acid were fed on each of 2 successive days, the subject being on a tyrosine-free diet. None of the compound was recovered the first day, a fact in accord with the good agreement between the "total Millon" and the *p*-hydroxyphenylpyruvic

Table VI. *Effects of ingestion of* p-*hydroxyphenyllactic acid,* p-*hydroxyphenylpyruvic acid and dihydroxyphenylalanine.*

Day of experiment	Total Millon	Phospho-mol. reduc-tion	Cu reduc-tion	Millon after ether extrac-tion	Recovered (impure) Keto	Hy-droxy	Dihy-droxy phenyl-alanine	Tyro-sine	Diet
1	2·15	1·94	1·66	0·85	1·03	—	—	Trace	Tyrosine-free
Maximum (4–8)	1·81	1·49	2·79	0·30	0·73	—	—	—	—
Minimum (4–8)	1·40	1·33	1·15	—	0·56	—	—	—	—
Average (4–8)	1·53	1·43	1·68	—	—	—	—	—	—
9	1·30	1·31	1·64	—	0·67	—	—	—	2 g. *p*-hydroxyphenyllactic acid
10	2·00	1·39	2·06	—	0·62	0·82	—	—	2 g. *p*-hydroxyphenyllactic acid
11	1·71	1·59	2·17	—	0·71	0·64	—	—	—
12	1·17	1·03	2·75	—	0·69	0·22	—	—	—
13	1·37	1·36	1·70	—	0·76	0·10	—	—	—
14	1·19	1·20	1·44	—	0·62	—	—	—	—
15	2·18	2·20	2·14	—	1·09	—	—	—	4 g. *p*-hydroxyphenylpyruvic acid
16	3·85	2·62	2·09	—	1·47	1·04	—	—	4 g. *p*-hydroxyphenylpyruvic acid
17	1·56	1·42	1·33	—	1·21	0·21	—	—	—
18	1·83	1·67	1·72	—	0·96	0·36	—	—	—
19	2·03	1·79	1·94	—	0·97	0·33	—	—	—
20	1·81	1·70	2·34	—	1·20	0·11	—	—	—
21	2·08	1·71	2·34	—	1·15	—	—	—	—
22	1·69	1·67	2·29	—	0·64	—	—	—	—
23	1·00	1·17	1·75	—	0·50	—	—	—	—
24	1·10	1·50	1·56	—	0·52	—	—	—	—
25*	1·20	1·37	2·22	—	0·54	—	46	—	2 g. dihydroxyphenylalanine
26*	1·18	1·39	1·60	—	0·60	—	141	—	—
27*	1·93	1·76	1·79	—	1·20	—	529	20	—
28*	4·09	3·15	3·31	—	1·76	—	101	57	—
29†	2·19	1·92	1·40	—	—	—	—	+	—
30	1·75	2·25	2·55	—	1·46	—	—	43	—
31	2·51	2·19	2·62	—	1·04	—	—	—	—
32	1·57	1·56	2·94	—	0·61	—	—	—	—
33	1·43	1·69	2·45	—	—	—	—	—	—
34	—	1·43	2·00	—	—	—	—	—	—
35	—	1·30	1·44	—	—	—	—	—	—

* Urine blackened on standing. † Collection incomplete. No darkening.

acid as determined by the molybdate reagent. This agreement no longer existed on the following day, when 0·82 g. of the hydroxy-acid was isolated. Its excretion continued over a period of 4 days, during which time 1·78 g. were recovered. No increase in the *p*-hydroxyphenylpyruvic acid occurred except for a doubtful elevation on the 3rd day (column 4). No tyrosine could be detected.

(*b*) p-*Hydroxyphenylpyruvic acid.* 4 g. of this compound were administered on each of 2 successive days. There was an immediate increase in the "total Millon" value, all of which was due to the excreted pyruvic acid. On the second day the output rose to 3·9 g., approximately 1 g. of which consisted of the hydroxy-acid. The latter was recoverable for 5 days, while the increased output of the keto-acid continued over a period of 8 days. No tyrosine could be detected.

(*c*) *Dihydroxyphenylalanine.* On the 25th day of the experiment 2 g. of dihydroxyphenylalanine were administered. There was an immediate excretion of the compound, 46 mg. being eliminated on the 1st day, increasing to 529 mg. on the 3rd, and falling to 101 mg. on the 4th. None could be isolated on the

5th day and there was no tendency for the urine to darken. During the first 2 days the excretion of *p*-hydroxyphenylpyruvic acid was within the limits of the control period, and constituted the whole of the total Millon-reacting substance. On the 3rd day however there was a sudden increase in the keto-acid followed by a still greater increase on the 4th day and a slow decline during the 3 following. On the 3rd–6th days tyrosine was excreted. No hydroxy-acid was detectable at any time. This experiment demonstrates that the oxidation of tyrosine to dihydroxyphenylalanine is a reversible reaction.

7. *Homogentisic acid feeding.*

(*a*) *Comparison of urines containing homogentisic acid and* p-*hydroxyphenylpyruvic acid.* Attempts were made from time to time during the course of these studies to isolate homogentisic acid, but at no time could crystals of the latter be found nor could any reactions typical of homogentisic acid be elicited. Some time later urine from an alcaptonuric was obtained and direct comparisons could be carried out[1]. In Table VII are noted some differences in the reactions of urines containing homogentisic acid and *p*-hydroxyphenylpyruvic acid.

Table VII. *Comparison of reactions of urines containing homogentisic acid and* p-*hydroxyphenylpyruvic acid.*

	Homogentisic acid	*p*-Hydroxyphenylpyruvic acid
Effect of keeping	Darkens	Does not darken
Effect of alkali	Darkens immediately	Darkens slowly
Benedict's qualitative reagent	Reduces immediately to a brownish colour	Reduces slowly to a greenish colour
Phosphomolybdic acid	Reduces at once blue in acid sol. green in alkaline sol.	Reduces slowly dark bluish green
Alkaline silver lactate	Reduces immediately in the cold	Reduces but slightly to a light gray
Ppt. with lead acetate	Ppt. at $p_H = 5{\cdot}5$	Ppt. at $p_H = 6{\cdot}5$

(*b*) *Reactions of homogentisic acid.* Walkow and Baumann [1891] showed that homogentisic acid reacts with Millon's reagent to give a red colour, since prolonged heating of the acid induces lactone formation between the carboxyl and the *o*-phenolic group. Since none of the reactions used above effects a differentiation between homogentisic acid and the compounds present in the urine of our patient, the simplest tests were employed: the phosphomolybdic acid reduction and the Folin and Wu sugar reagents.

With the phosphomolybdic acid reagent, homogentisic acid develops full

[1] I am indebted to Dr K. K. Sherwood of Redmond, Washington, formerly of this hospital, for first having brought the existence of this case to our attention and to Dr John W. Stuhr of Stillwater, Minnesota, for having brought us in contact with this patient. The patient is a German farmer from near Stillwater, Minnesota.

colour in 4 minutes. The following table gives the percentage of the total reduction effected after various time intervals:

Time in min.	1	1·5	2	2·5	3	3·5	4
Percentage reduction	62	77	85	92	97	99	100

The comparative reducing power of homogentisic acid (4 minutes' standing) and *p*-hydroxyphenylpyruvic acid (3 hours' standing) is:

1 mg. *p*-hydroxyphenylpyruvic acid = 1·33 mg. homogentisic acid.

Homogentisic acid produces its maximum reduction of the Folin and Wu sugar reagent in 8 minutes. The following table gives the percentage of the total reduction effected and the glucose equivalent (mg. glucose = 1 mg. homogentisic acid) after various time intervals. In all cases the glucose standard was heated 8 minutes.

Time in minutes	1	2	3	4	5	6	7	8
Percentage reduction	59	73	81	87	94	98	99	100
Glucose equivalent	0·50	0·62	0·69	0·74	0·80	0·83	0·85	0·86

Table VIII. *Reducing value of urine following ingestion of homogentisic acid.*

	Day of experiment	Volume cc.	Phosphomol. reduction g.	Cu reduction g	
Dec. 28, 1931	1	1080	2·09	1·73	Hospital diet
,, 29 ,,	2	795	2·15	1·59	—
,, 30 ,,	3	1505	2·23	1·78	—
,, 31 ,,	4	840	2·04	1·65	2·5 g. homogentisic acid in 3 doses from 10 to 11 a.m.
Jan. 1, 1932	5	920	2·16	1·70	—
Jan. 13, 1932	1	1055	1·99	1·62	Fasting
,, 14 ,,	2	840	1·48	1·48	—
,, 15 ,,	3	890	1·48	1·19	—
,, 16 ,,	4	390	1·57	1·15	—
,, 17 ,,	5	750	1·65	1·24	Low protein diet. 5·2 g. homogentisic acid in 4 doses from 10 a.m. to 4 p.m.
,, 18 ,,	6	700	2·17	1·99	—
,, 19 ,,	7	1040	1·73	1·27	—
Jan. 21, 1932	1	650	1·50	0·77	Low protein diet
,, 22 ,,	2	750	1·61	0·92	—
,, 23 ,,	3	745	1·66	1·11	—
,, 24 ,,	4	880	1·58	1·01	—
,, 25 ,,	5	550	1·69	0·99	8 g. homogentisic acid in 8 doses from 9 a.m. to 7 p.m.
,, 26 ,,	6	1060	1·65	0·98	—
,, 27 ,,	7	1230	1·60	1·02	—

(*c*) *Feeding of homogentisic acid.* In the observations recorded in Table VIII, homogentisic acid was administered in increasing doses in three separate experiments. The reduction of the molybdic acid and the sugar reagents was followed, darkening of the urine looked for, and isolation of homogentisic acid attempted. In the first experiment, 2·5 g. homogentisic acid were fed with negative results: no increased reduction of either reagent occurred, no darkening of the urine took place on standing and no homogentisic acid could

be recovered. In the second experiment 5·2 g. of the acid were administered during a period of fasting. On the day following the ingestion there was a slight rise in the reducing power of the urine and, on attempted isolation of lead homogentisate, a few crystals of characteristic shape were observed under the microscope. They were too few for positive identification. A third experiment was made with 8 g. homogentisic acid, an amount which has been found to be slightly more than the maximum completely assimilable by a normal individual. No increase in the reducing power of the urine occurred and no homogentisic acid was recovered. This experiment furnishes convincing evidence that this subject is able to oxidise 2 : 5-dihydroxybenzene derivatives, with the same ease as can normal individuals.

IV. Melanin ingestion by a normal individual.

As has been mentioned before, the isolated keto-acid can be oxidised to melanin. An experiment has been performed to see whether this melanin when ingested is metabolised or is excreted unchanged. Accordingly, 0·5, 3 and 5 g. of melanin were fed to a normal person.

As a preliminary experiment it was determined that the melanin is not digested by trypsin. The amount of reducing substance in the urine was estimated by the Folin-Wu sugar reagent and the subject was put on a diet approximately carbohydrate-free to reduce the normal "sugar" value of the urine to the lowest limit. The tubes were kept in the boiling water-bath for 20 mins. At this interval, the reducing power of the purified melanin compared with that of glucose is: 1 mg. melanin = 0·047 mg. glucose. The colour of the urines was dark and exact matching of standard and unknown was impossible. Table IX gives the result of the experiment.

The glucose values in column 4 of the table show that there is an increased output of reducing substances following the ingestion of 3 and 5 g. of melanin

Table IX. *Estimation and recovery of melanin from the urine of a normal person fed 0·5, 3 and 5 g. melanin.*

Day of experiment	Melanin fed g.	Volume of urine cc.	Glucose g.	Increase in reduction 0·047	Melanin recovered g.
1	—	855	0·43	—	—
2	—	730	0·50	—	—
3	0·5	720	0·44	—	—
Av.	—	—	0·46	—	—
4	3·0	1120	0·53	2·60	2·01
5	—	730	0·51		
Av.	—	—	0·52	—	—
6	—	740	0·38	—	—
7	5·0	965	0·68	4·94	4·23
8	—	1110	0·46		
Av.	—	—	0·57	—	—
9	—	800	0·33	—	—

from *p*-hydroxyphenylpyruvic acid. This increased output is distributed over 2 days following its ingestion and may be determined almost quantitatively by the Folin and Wu sugar method. Deducting the normal output for 2 days from the 2 days' output after feeding and dividing by 0·047 to convert it to its true melanin value, we find an output of 2·60 and 4·94 g. melanin respectively; 2·01 and 4·23 g. were recovered. Therefore melanin formed from *p*-hydroxyphenylpyruvic acid cannot be metabolised, but is excreted unchanged and may be recovered almost quantitatively.

V. Effects of tyrosine feeding to normal individuals.

Against the background of this knowledge of the intermediary metabolism of tyrosine, a renewed inquiry into tyrosine metabolism in normal individuals was made. The result of feeding of massive doses (50 g.) of tyrosine is shown in Table X.

Table X. *Results of feeding 50 g. of tyrosine to a normal individual in ten 5 g. doses administered between 12.30 a.m. Dec. 1 and 9.30 p.m. Dec. 2.*

			Millon		Sugar		Amino-N	
No. of sample	Period (1930)	No. of hrs.	Total mg.	Per hr. mg.	Total mg.	Per hr. mg.	Total mg.	Per hr. mg.
1	10.30 p.m. Nov. 30–6.30 a.m. Dec. 1	8	—	—	105	13	22	3
2	6.30 a.m.–2.30 p.m. Dec. 1	8	168	20	156	20	37	5
3	2.30 p.m.–10.30 p.m. Dec. 1	8	424	53	145	18	34	4
4	10.30 p.m. Dec. 1–6.30 a.m. Dec. 2	8	266	33	73	9	40	5
	Total	24	858	—	—	—	—	—
	Isolated 0·7 g. *p*-hydroxyphenyllactic acid. 2·1 g. hippuric acid.							
5	6.30 a.m.–4.30 p.m. Dec. 2	10	266	27	185	19	37	4
6	4.30 p.m.–10.30 p.m. Dec. 2	6	391	65	122	20	25	4
7	10.30 p.m. Dec. 2–6.30 a.m. Dec. 3	8	340	43	146	18	26	4
	Total	24	997	—	—	—	—	—
	Isolated 1·6 g. *p*-hydroxyphenyllactic acid. 2·65 g. hippuric acid.							
8	6.30 a.m.–2.30 p.m. Dec. 3	8	269	34	120	15	26	3
9	2.30 p.m.–10.30 p.m. Dec. 3	8	173	22	176	22	41	5
10	10.30 p.m. Dec. 3–6.30 a.m. Dec. 4	8	—	—	115	14	26	3
	Isolated 2·5 g. hippuric acid.							
11	6.30 a.m.–2.30 p.m. Dec. 4	8	—	—	—	—	31	4

A question not touched upon heretofore concerns the degree of absorption of tyrosine when ingested. The question has not seemed of striking importance in the previous case where the results indicated that at least with small amounts the absorption was fairly complete. The degree of intestinal decomposition after ingestion of large amounts by normal individuals is indicated by the isolation of 7·25 g. of hippuric acid (equivalent of 7·25 g. tyrosine) from the urine of the normal individual who took 50 g. of tyrosine in 10 doses over a period of 2 days. The feeding of the tyrosine gave no increase in the amino-acid content of the urine as determined by Folin's amino-acid reagent [1922]. The

urine gave a red colour with the Millon reagent and no colour with the molybdic acid reagent, indicating the presence of hydroxy-acid. From the urine 2·3 g. of *l-p*-hydroxyphenyllactic acid were isolated. No tyrosine could be recovered.

The next question was the determination of the minimum amount of tyrosine required for the appearance of the hydroxy-acid. Table XI records

Table XI. *Excretion of* p-*hydroxyphenyllactic acid by a normal person following ingestion of tyrosine.*

Period of collection	No. of hrs.	Millon: Total output mg.	Millon: Output per hr. mg.
2·5 g. Tyrosine at 8, 9, 10, 11 a.m. (Oct. 6)			
Oct. 4, 8 p.m.–Oct. 5, 8 a.m.	12	66·4	5·5
Oct. 5, 8 a.m.–8 p.m.	12	52·2	4·4
Oct. 5, 8 p.m.–Oct. 6, 8 a.m.	12	62·4	5·2
Oct. 6, 8 a.m.–8 p.m.	12	110·0	9·2
Oct. 6, 8 p.m.–Oct. 7, 8 a.m.	12	150·0	12·5
Oct. 7, 8. a.m.–8 p.m.	12	64·8	5·4
2·5 g. Tyrosine at 9.30 a.m. (March 30)			
2·5 g. Tyrosine at 10.30 a.m.			
March 30, 1931. 6.30 a.m.–9.30 a.m.	3	18·0	6·0
9.30 a.m.–1.30 p.m.	4	28·6	7·2
1.30 p.m.–3.30 p.m.	2	15·4	7·7
3.30 p.m.–6.30 p.m.	3	18·8	6·3
6.30 p.m.–10.30 p.m.	4	20·1	5·0
10.30 p.m.–March 31, 6.30 a.m.	8	48·4	6·1

two experiments in which 10 and 5 g. were taken by the same individual as above. In these experiments the procedure of estimation was varied to increase the delicacy of the Millon test. The subjects were placed on a restricted fluid intake to reduce the volume of the urine and the latter was shaken twice with Lloyd's reagent to remove pigment (Lloyd's reagent does not remove *p*-hydroxyphenyllactic acid). A series of standards was set up with tyrosine in concentrations of 0·1, 0·3, 0·5 and 1 mg. per cc., the procedure being as previously described. The estimations could probably have been made more accurately with the phenol reagent but this procedure was employed for the sake of continuity to obtain comparative results. After the 10 g. ingestion there was an increase in the output of the Millon-reacting substances and traces of the hydroxy-acid were isolated. No rise occurred following ingestion of 5 g.

The regularity of this rise after ingestion of 10 g. tyrosine was investigated by the feeding of this dose to normal individuals. The results are presented in Table XII. A rise in the output of the colour-producing substances invariably occurred. The average Millon value preceding the ingestion of tyrosine was 65 mg. per 12 hour period. In some instances there was no immediate increase, in other cases the output rose to 200–350 % of its original value. In most instances, by the third period following ingestion, values had again fallen to normal, but considerable irregularity was displayed throughout. It is difficult to decide whether these variations are caused by differences in rate of absorp-

Table XII. *Millon reaction of urine of normal persons after ingestion of 10 g. of tyrosine, taken in four 2·5 g. doses at intervals of 1 hour. First dose at beginning of period marked (*). Urine collected in 12-hour periods.*

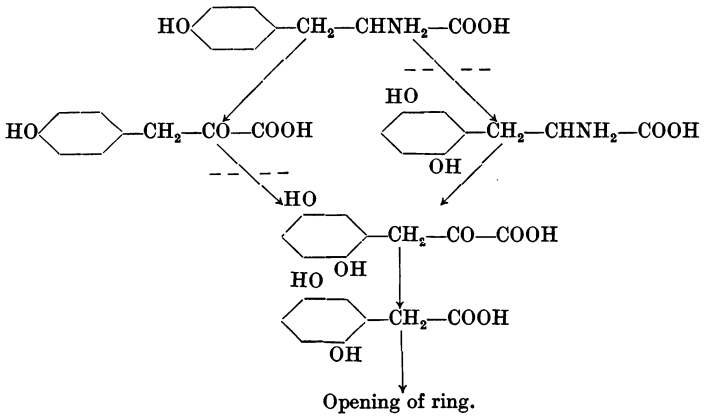
	Millon reaction (tyrosine equivalent of urine)								
	mg.	mg.	mg.	mg. (*)	mg.	mg.	mg.	mg.	mg.
G.M.	67	52	62	110	300	65	—	—	—
R.	75	71	80	150	200	52	—	—	—
R.J.	—	24	75	67	98	—	—	—	—
L.F.	71	63	60	75	182	—	—	—	—
V.	63	50	47	67	143	—	—	—	—
L.	63	22	77	216	342	—	—	—	—
H.	—	—	60	80	81	455	130	—	—
T.B.	—	63	118	135	200	56	—	—	—
H.N.	—	—	47	127	140	91	63	65	50
H.	—	—	26	95	105	77	78	62	57
M.F.	—	—	50	135	141	80	65	—	—

tion or produced by differences in rate of metabolism. That they tend to be characteristic of the individual was shown by repetition of the test in several instances.

With these easily obtainable results in normals it was natural that the ever-recurring search for chemical tests of hepatic function should present itself. Twenty patients with a variety of liver diseases have been tested but no characteristic difference from normal behaviour has been found.

Discussion.

According to Neubauer [1928, p. 862], two paths of oxidation of tyrosine are open to the normal individual, one leading through *p*-hydroxyphenylpyruvic acid and the second through 2 : 5-dihydroxyphenylalanine. Both lead to the production of 2 : 5-dihydroxyphenylpyruvic acid and thence to homogentisic acid.



According to Dakin [1922], the only path open for the oxidation of tyrosine in the normal individual lies through *p*-hydroxyphenylpyruvic acid, in which compound opening of the ring occurs.

(*a*) In the subject of this investigation there is a block in the series of reactions after formation of *p*-hydroxyphenylpyruvic acid. According to the first hypothesis, the error of metabolism involved is an inability to perform 2 : 5-oxidation of the benzene ring; specifically, he cannot oxidise tyrosine to 2 : 5-dihydroxyphenylalanine or the monohydroxyphenylpyruvic acid to the 2 : 5 dihydroxy-derivative. According to Dakin's theory, his anomaly would consist in his inability to open the ring. To test this point he was fed 2·5 g. of homogentisic acid as recorded above. On the two days following its ingestion no darkening of the urine took place on standing or on being rendered alkaline; no significant increase in the reducing power of the urine occurred, and no homogentisic acid could be recovered, demonstrating beyond doubt that our subject is able to open the benzene ring when oxidised in the 2 : 5-position. This experiment leans strongly towards the hypothesis of Neubauer and others, that normal oxidation of tyrosine includes introduction of a second hydroxyl in a position leading to the production of homogentisic acid as a preliminary to disruption of the ring.

The inability of the patient to dispose normally of *p*-hydroxyphenylpyruvic acid is complete, since during fasting his output drops to a constant level of about 1·6 g. which may be regarded as a reasonable figure for the total endogenous tyrosine catabolism. If the block is complete in alcaptonuria, an equivalent amount of homogentisic acid should be excreted under similar conditions. It is with difficulty that from the mass of literature on alcaptonuria, figures for the absolute output of homogentisic acid under fasting conditions can be obtained. Isolation figures of unstable compounds are liable to wide error and in many of the studies of alcaptonuria quantitative estimations have not been corrected to terms of absolute homogentisic acid values. In one of the few such records, Mittelbach [1901] gives the daily output of a fasting male alcaptonuric as 1·7 g. which is the equivalent of 1·8 g. of tyrosine or of the keto-acid, a figure in close agreement with that obtained on this patient.

(*b*) When tyrosine is introduced into the diet at approximately 3 g. daily, tyrosine also appears in the urine. Such an event does not occur in normals. Abderhalden [1912] ingested 150 g. of tyrosine without recovering any, and numerous instances of feeding smaller amounts are on record. The experiments of Kotake *et al.* [1922] indicate that tyrosine in normal rabbits goes over with ease to the keto-acid. The experiments on feeding tyrosine to normals recorded in this paper also show no recovery of tyrosine. With our patient this is not the case, and whenever the intake of tyrosine is slightly elevated, *e.g.* when it is between 2 and 5 g. daily, the capacity of the tissues to convert tyrosine completely to the keto-acid is exceeded and tyrosine itself is excreted. But that this limitation of the conversion capacity is not absolute, but affected by mass action, is shown by the continuous increase in the keto-acid output.

(*c*) At such a time *p*-hydroxyphenyllactic acid also appears in the urine. Dakin [1922, p. 68] is inclined to question Blendermann's [1882] identification

of this acid in the urine of rabbits fed large quantities of tyrosine, but accepts it [p. 90] as the substance found by Schultzen and Riess [1869] in pathological urines. It seems, in fact, to be accepted that this compound occurs rather rarely and usually under pathological conditions.

The experiments with our patient, as well as those with normals recorded above, while confirming the derivation of this compound from *p*-hydroxyphenylpyruvic acid, indicate that the reaction takes place with comparative ease whenever, by mass action effects, the capacity of the tissues to oxidise tyrosine approaches its limit.

The time relations of the appearance of this compound bear out this supposition. In Table IV, following feeding of 10 g. of tyrosine, while the output of the keto-acid and even of tyrosine rose immediately, there was a lag of two days in the hydroxy-acid output, which finally occurred at a time when the peak of excretion of tyrosine and keto-acid had passed. That this lag may be partially due to a certain slowness of the organism in excreting the hydroxy-acid is indicated by the delay in its appearance in the urine after feeding the latter acid itself. These findings bear out Dakin's conclusion that this compound is not an intermediary product in the normal oxidation of tyrosine.

(*d*) The path of oxidation of phenylalanine has been an unsolved question, although evidence has been accumulating that at least one route passes through the tyrosine stage. This evidence consists largely of the findings of Embden and Baldes [1913] that phenylpyruvic acid did not yield acetoacetic acid in the surviving liver and that following perfusion with phenylalanine, traces of tyrosine were isolated. Dakin [1922, p. 86] was unable to confirm this latter finding. In the experiments with our patient, recorded in Table XI, the rise in both the reducing value of the urine and the Millon-reacting substances following administration of phenylalanine was clear cut, although not so prompt or so marked as that following tyrosine ingestion. This evidence strongly supports the theory that phenylalanine is converted at least partly into tyrosine.

(*e*) In our patient, under high tyrosine feeding 3 : 4-dihydroxyphenylalanine appears in the urine. The theoretical importance of this finding is twofold: first, the establishment of the fact that 3 : 4-oxidation of the benzene ring takes place in the body, and second, the isolation of a proved precursor of indole derivatives and of melanin.

From the findings in this investigation, oxidation in the 3 : 4 positions offers some striking contrasts to 2 : 5-oxidation. The latter must take place readily in the normal individual the former with difficulty. There is also a marked difference in the case of the further oxidation of the products, homogentisic acid being broken down completely by the normal individual when ingested in moderate amounts, the 3 : 4-compound appearing in the urine when fed in similar quantities. Guggenheim [1913], for instance, ingested 2·5 g. of the amino-acid and within 5 hours excreted it in sufficient amounts to produce characteristic darkening of the urine and to yield a positive test for catechol

with ferric chloride. Fromherz and Hermanns [1914] believed that they demonstrated that *p*-hydroxyphenylpyruvic acid and dihydroxyphenylalanine were burned in the body to about the same extent. From our own experiments however, it seems probable that Fromherz and Hermanns were dealing with excreted *p*-hydroxyphenyllactic acid rather than the pyruvic acid. Since the lactic acid represents only a small portion of the pyruvic acid produced by mass action effects, this experiment cannot well be used to compare the relative capacity of the organism to deal with the two substances. The experiments with our subject show difficulty in oxidising 3:4-dihydroxyphenylalanine, which not only fails to be further oxidised to any great extent, but becomes partly reduced to tyrosine. The production of the 2 : 5-compound may be looked upon as a purely catabolic process, while that of 3 : 4-dihydroxyphenylalanine might serve certain specific anabolic purposes.

Tyrosinosis.

The essential feature in our patient, then, consists in a complete stoppage of the oxidation of tyrosine at the stage of *p*-hydroxyphenylpyruvic acid, together with a slowing of every step theretofore. Thus results an excretion not only of *p*-hydroxyphenylpyruvic acid and its reduction product *l*-*p*-hydroxyphenyllactic acid, but also of tyrosine and its oxidation product, *l*-3 : 4-dihydroxyphenylalanine. For this condition, the name tyrosinosis is proposed.

The two errors of tyrosine metabolism now known, alcaptonuria and tyrosinosis, both involve blocks in the oxidation of tyrosine, but at different stages. In tyrosinosis the block occurs following formation of *p*-hydroxyphenylpyruvic acid and consists essentially in the inability of the subject to oxidise the *p*-monohydroxy-compound to the 2 : 5-dihydroxy-derivative. He can, however, open the ring previously oxidised in the 2 : 5 position. In alcaptonuria, the subject is able to bring about 2 : 5 oxidation but cannot further metabolise this compound. He can, on the other hand, oxidise the benzene ring by some other route.

Garrod [1909] suggests that inborn errors are probably caused by "the congenital lack of some particular enzyme, in the absence of which one step is missed, and some normal metabolic change fails to be brought about." It is not easy to explain the slowing of all successive reactions involved in tyrosinosis on this assumption. The formation of *l*-*p*-hydroxyphenyllactic acid is clearly enzymic in character.

It is known that the conversion of tyrosine into dihydroxyphenylalanine is brought about by the action of tyrosinase. Raper states that the enzyme acts only in this first step of the reaction and the further production of melanin takes place spontaneously in the presence of oxygen. In test-tube experiments, the production of the red quinone stage takes place with much greater difficulty in pure solution than when the enzymes from the meal-worm are present and it seems probable that more than one type of enzyme is involved and that the

later stages are also catalysed. According to Bloch [1917] the enzyme present in the melanin-producing cells of the epidermis catalyses only the oxidation of dihydroxyphenylalanine (Dopa). No evidence has been presented as to the method or site of production of the dihydroxyphenylalanine.

Summary.

Under the name of tyrosinosis a new error of tyrosine metabolism in man is described which gives information as to several stages of the intermediary metabolism of tyrosine. Briefly, the condition consists in a slowing of the first steps and a complete stop at the stage of *p*-hydroxyphenylpyruvic acid, but with preserved ability to oxidise homogentisic acid.

The endogenous tyrosine metabolism in this individual results in the daily elimination of about 1·6 g. of *p*-hydroxyphenylpyruvic acid. This compound is excreted entirely in the enol form. As the level of tyrosine metabolism is raised by ingestion of the amino-acid either as protein or in pure form, the *p*-hydroxyphenylpyruvic acid increases and in addition the following substances also appear in the urine: first tyrosine, later *l*-*p*-hydroxyphenyllactic acid and when the tyrosine metabolism is raised still further also *l*-3 : 4-dihydroxyphenylalanine.

Dihydroxyphenylalanine, when fed, is excreted partly unchanged, partly causes elimination of tyrosine and increased elimination of *p*-hydroxyphenylpyruvic acid.

Phenylalanine, when fed, causes elimination of tyrosine, increased elimination of *p*-hydroxyphenylpyruvic acid and excretion of traces of *l*-*p*-hydroxyphenyllactic acid. Whether phenylalanine also appears in the urine was not determined.

p-Hydroxyphenylpyruvic acid, when fed, reappears unchanged and also causes elimination of *l*-*p*-hydroxyphenyllactic acid, but does not give rise to tyrosinuria.

The *l*-*p*-hydroxyphenyllactic acid, when fed, reappears unchanged.

Homogentisic acid, when fed, does not reappear in the urine nor is there any indication of its influencing the elimination of any of the compounds previously discussed.

During no experimental condition did melanin appear preformed in the urine of this individual.

Nitrogen-free melanin prepared by oxidation of the *p*-hydroxyphenylpyruvic acid and ingested by a normal individual was quantitatively recovered from the urine. It was not fed to the individual with tyrosinosis.

This condition is taken to prove the assumed positions of *p*-hydroxyphenylpyruvic acid as one early step in normal tyrosine metabolism, and of *l*-*p*-hydroxyphenyllactic acid as a side-product outside the direct path of reactions. The latter acid is readily formed in normal individuals; its optical activity indicates its enzymic production. The preserved power of the patient

to oxidise homogentisic acid might suggest the 2 : 5 oxidation of tyrosine as a normal intermediary step, impossible for this individual to take—while the next step is impossible for the alcaptonuric.

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