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Research Article

A COMPARISON OF THE PHENYLALANINE CONTENT OF THE HEMOGLOBIN OF NORMAL AND PHENYLKETONURIC INDIVIDUALS: DETERMINATION BY ION EXCHANGE CHROMATOGRAPHY [1]

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INTRODUCTION

Despite extensive clinical and biochemical research, the relationship of the metabolic error in phenylketonuria to the clinical symptomatology is unknown (1, 2). The specific biochemical defect, inherited as an autosomal recessive gene, is the lack of phenylalanine oxidase in the liver (3, 4). This lack prevents the effective conversion of phenylalanine to tyrosine and results in high blood levels of phenylalanine and high concentrations in the urine of such metabolites of phenylalanine as phenylpyruvic acid, phenyllactic acid, and phenylacetic acid. How this biochemical defect produces mental deficiency is not known, but the apparent improvement in mental status of patients on low phenylalanine diets suggests a toxic action either of phenylalanine or of its derivatives (5, 6). One possible mode of phenylalanine toxicity would be through increased incorporation of phenylalanine in body proteins and consequent interference with the proper function of these molecules (7). The phenylalanine contents of body proteins of normal and phenylketonuric individuals have been compared but the results are equivocal. Block, Jervis, Bolling, and Webb (8), in the only properly controlled set of experiments, found no significant differences in the phenylalanine content of the serum proteins, erythrocytes, brains, livers, and kidneys of normal and phenylketonuric individuals. Schrappe (9) has claimed that blood proteins from phenylketonurics contain higher than normal amounts of phenylalanine (8.53 per cent) but Keup (10) was unable to confirm this finding.

None of these experiments was performed with sufficient accuracy to detect a difference of one or two phenylalanyl residues per protein molecule. Such a difference might influence the function of a protein if, for example, the phenylalanine replaced a tyrosyl residue. Hence, it was desirable to repeat these experiments with the necessary precision and accuracy so that a difference of only a single phenylalanyl residue might be detected. To achieve this end, a pure body protein and an accurate method for the determination of phenylalanine are required. In this research, the first condition was met by using carefully purified hemoglobin from both normal controls and well-documented phenylketonurics, and the second by using the techniques of ion-exchange column chromatography as developed largely by Moore and Stein (for a review see reference 11).

METHODS

Preparation of hemoglobin. Hemoglobin was prepared by the method of Drabkin (12) except for minor modifications, the most important of which was the conversion to the more stable carbonmonoxy form early in the purification.

This investigation used two preparations of hemoglobin from normal individuals and two from phenylketonuric patients. The first preparation of normal hemoglobin employed red cells from which the plasma had been removed; these cells were obtained from Hyland Laboratories, Los Angeles. The second sample of normal blood came from a known healthy donor. The possibility exists that the high levels of phenylalanine in the plasma of phenylketonuric patients may lead to adsorption of phenylalanine by the hemoglobin. In an attempt to rule out this possibility, phenylalanine was added to the whole normal blood to give a final concentration of 30 mg. per 100 ml. and the blood was allowed to stand at 20° C. for 24 hours prior to separation of the red cells. The specimens of phenylketonuric blood were provided by Drs. George Tarjan and Stanley W. Wright of the Pacific State Hospital, from patients who had been studied by them and who are described in their review (2). These patients are listed as Case Nos. 5 and 20.

Apart from the above difference, the subsequent prepa-

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ration of the four hemoglobin samples was identical. After the red cells had been separated from the plasma, they were washed twice with isotonic saline at 0° C. and CO was bubbled through the red cell suspension for 10 minutes. During the remainder of the purification all solutions were saturated with CO. The red cells were washed twice more with isotonic saline, hemolyzed with an equal volume of distilled water and 0.4 volume of toluene, and allowed to stand overnight at 1° to 2° C. The hemolyzed cells were then centrifuged at 18,000 G for 1 hour at 0° C., after which the hemoglobin layer was removed and recentrifuged in the same way. The hemoglobin solution was dialyzed against Drabkin's 2.8 M phosphate buffer initially at 37° C. and then at 1° to 2° C. After the crystals which formed overnight had been washed four times with 2.8 M phosphate buffer, they were dissolved in distilled water to give a volume equal to that of the original laked cell contents, and the crystallization was repeated. In the method of crystallization as here employed, almost no hemoglobin remains in the supernatant solution and any fractionation is thus avoided. These crystals were dissolved in distilled water and dialyzed against distilled water until the dialysate was negative for phosphates. The solution was finally dialyzed against glass distilled water, and the dialysate was negative for residue, phosphates, or ninhydrin positive material. The hemoglobin solution was then adjusted to a concentration of approximately 6 Gm. per 100 ml. Simultaneous aliquot portions were taken for determination of the concentration and for hydrolysis.

Determination of concentration. The concentration of the hemoglobin solutions was determined both by dry weight and by the spectrophotometric methods of Drabkin and Austin (13).

The determination of the dry weight was performed in quadruplicate by heating the weighed 3-ml. aliquot portions first to 90° C. for two hours (to prevent boiling and spattering) and then to 105° C. for one hour. After cooling over "Drierite" and weighing, repeated heating to 105° C. for one hour produced no further loss in weight. Values for dry weight agreed within 0.5 per cent.

The concentration of the hemoglobin was also determined spectrophotometrically as carbonmonoxyhemoglobin and as ferrihemoglobin cyanide by the methods of Drabkin (13). The concentration using these techniques was 2 to 4 per cent less than that obtained by dry weight and agreement between the two spectrophotometric determinations was no better than 1 or 2 per cent.

The dry weight was considered to be the most reliable basis for calculating the amino acid composition of the hemoglobins prepared in identical fashion. The spectrophotometric determination as carbonmonoxyhemoglobin will be less reliable if a few per cent of ferrihemoglobin is present in variable amount in the different preparations. The spectrophotometric determination as ferrihemoglobin cyanide is unsatisfactory in our work because some difficulty was experienced in converting carbonmonoxyhemoglobin to ferrihemoglobin.

Hydrolysis. Four 1-ml. aliquot portions of each hemoglobin preparation were pipetted into long-neck hydrolysis flasks containing 10 ml. of doubly distilled 6 N HCl. Two hydrolyses were refluxed for 24 hours and two for 72 hours. Each hydrolysate foamed badly for the first hour and required frequent cooling of the neck of the flask to avoid loss of material; subsequently, there was no foaming over. Capryl or n-octyl alcohol was ineffective in preventing foaming at this stage as each distilled into the condenser and remained there. At the end of the hydrolysis, the HCl was removed by vacuum distillation with a rotary evaporator at 60° C.; one drop of capryl alcohol was an effective anti-foaming agent. In order to remove traces of HCl, the residue was taken up twice with distilled water which was removed by the same technique. Finally, the residue which was not completely soluble in 0.5 M acetic acid was transferred quantitatively with this solvent to a 10-ml. volumetric flask and the solution was brought up to mark. The insoluble material which probably consisted of heme degradation products was not removed before chromatography but, rather, 1 ml. of this suspension was pipetted directly on the column.

Chromatography. In these experiments, it was essential that the phenylalanine be completely separated from all other constituents of the protein hydrolysate. Because phenylalanine is the first amino acid to emerge from starch chromatograms under certain conditions (14) this method would have been convenient, but no developer which was studied would separate phenylalanine from traces of tryptophan which survive the hydrolysis.

Chromatograms on Dowex 50 require strict temperature control to separate phenylalanine from tyrosine (15) and suffer the disadvantage that phenylalanine emerges rather late in the chromatogram. However, Hirs, Moore, and Stein (16) describe the use of a 30-cm. Dowex 1-X8 column developed with 0.5 M acetic acid for separation of tyrosine, glutamic acid, and aspartic acid and it was known (17) that on this column phenylalanine precedes tyrosine but is incompletely separated from other amino acids. Therefore, it was hoped that a longer column would achieve a complete separation of phenylalanine.

Accordingly, a 0.9 × 100-cm. column of Dowex 2-X10 [3] was poured and found to separate phenylalanine from the remaining amino acids adequately for most purposes but not quite enough for these experiments. When the length of the column was increased to 200 cm., the phenylalanine separated completely from the remaining amino acids. The identity and purity of the zone of phenylalanine were proved by chromatographing a hemoglobin hydrolysate on the 200-cm. column. After those fractions which contained the phenylalanine were combined, the compound was dinitrophenylated and the DNP-derivative was chromatographed on silicic acid-Celite by the method of Green and Kay (19). The DNP-derivative was found to be pure DNP-phenylalanine in amounts equivalent to the

[3] The behavior of amino acids on Dowex 2-X10 is almost the same as that on Dowex 1-X8 and the former resin has been used for tyrosine determinations (18).

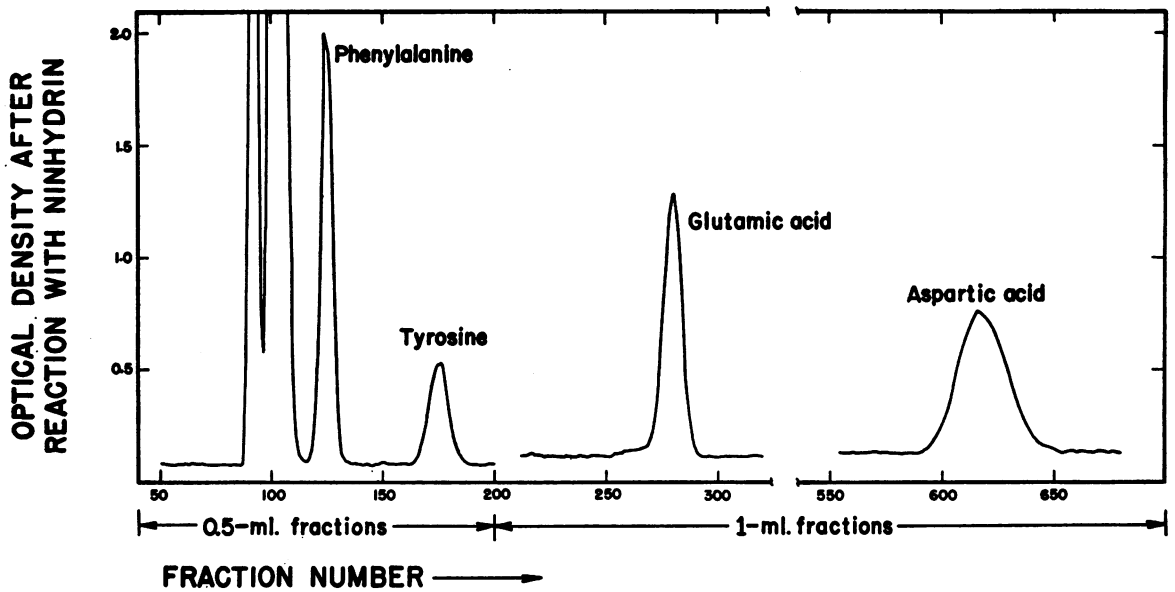

FIG. 1. SEPARATION OF SOME AMINO ACIDS IN A COMPLETE HYDROLYSATE OF ADULT HUMAN HEMOGLOBIN BY CHROMATOGRAPHY ON DOWEX 2-X10 WITH 0.5 M ACETIC ACID AS DEVELOPER

phenylalanine determined by a duplicate chromatogram on the Dowex 2-X10 column.

The Dowex 2-X10 which was used for these columns had been purified essentially in the manner described by Hirs, Moore, and Stein (16) for Dowex 1-X8 and had been passed wet through a 200-mesh sieve.

As mentioned above, a 1-ml. portion of hydrolysate was placed on the column. After this sample had penetrated into the column, it was rinsed in with 3×0.5 ml. of 0.5 M acetic acid. Development was carried out with 0.5 M acetic acid. At the beginning of the chromatogram, the flow rate of developer was set at 3.5 ml. per hour by adjusting the position of the solvent reservoir in order to apply the necessary hydrostatic pressure. After 200 0.5-ml. fractions had been collected (well past the tyrosine peak) 18 cm. of mercury pressure was applied to increase the flow rate to 7 ml. per hour and 120 1-ml. fractions were collected until emergence of the peak of glutamic acid was complete. The next 230 ml. of effluent were not collected in fractions because of the absence of zones and finally 120 more 1-ml. fractions were taken to collect the aspartic acid. After the completion of the chromatogram, the upper 2 cm. of the column, which contained the brown products of the hydrolysis, were removed and replaced with fresh resin. Then, 100 to 200 ml. of 0.5 M acetic acid were passed through the column before its next use.

The original ninhydrin method of Moore and Stein (20) was used to determine the amino acid content of the effluent fractions after they had been brought to pH 5 with N NaOH (0.1 ml. or 0.2 ml., depending on the size of the fraction). The color yields of phenylalanine, tyrosine, glutamic acid, and aspartic acid were redetermined and found to be 0.92, 0.93, 1.04, and 0.97, respectively. Four chromatograms with known phenylalanine in amounts of the same magnitude as was present in the hydrolysates yielded quantitative recovery with ± 3 per cent precision. The tyrosine and glutamic acid were also recovered quantitatively, but only about 85 per cent of the aspartic acid was recovered from known amounts of amino acid, or when added to a hydrolysate in which the aspartic acid had previously been determined.

A typical chromatogram is illustrated in Figure 1. The optical density of the individual fractions after reaction with ninhydrin is plotted against the fraction number. The fractions with optical density above 1.00 were read at an appropriate dilution, but, before plotting, the optical density was calculated to what would have been observed in the same volume as the blank fractions. The peak of the phenylalanine appears at 60 ml. of effluent volume, tyrosine at 83 ml., glutamic acid at 176 ml., and aspartic acid at 517 ml. Tryptophan is found in 24-hour hydrolysates as a small zone with its peak at 151 ml. As it produces only a slight increase of optical density readings above the blank an estimate of the amount of tryptophan could rarely be obtained, although generally there was evidence for its presence as in the figure.

Determination of serum phenylalanine. The phenylalanine content in the phenylketonuric serum was determined in the following way: A 2-ml. sample of the patient's serum was diluted to 10 ml. with distilled water and the proteins were precipitated with 10 ml. of 10 per cent trichloracetic acid. The precipitate was washed twice with 5 ml. of 5 per cent trichloracetic acid, and the combined supernatant solution and washings were extracted five times with an equal volume of ether to remove the trichloracetic acid. The aqueous layers were then concentrated to dryness at 50° C. in a rotary evaporator and

TABLE I

Individual values for the contents of several amino acids in normal and phenylketonuric hemoglobin
(Gm. of amino acid per 100 Gm. of hemoglobin)

Type	Normal										Phenylketonuric									
Individual	1. Bank blood						2. M. C.				3. J. G.				4. G. M.					
I.Q.											49				8					
Serum phenylalanine											29 or 39 mg./100 ml.*				13 or 21 mg./100 ml.*					
Case number											20				5					
Hours hydrolysis	24				72		24		72		24		72		24		72			
Hydrolysate no.	1		2		3		1		3	4	1		2	3	1		2		4	
Chromatogram no.	A-27	A-30	A-28	A-31	A-29	A-32	A-33		A-34	A-35	A-36	A-39	A-37	A-38	A-43		A-44		A-45	
Phenylalanine	7.56	7.39	7.75	7.43	7.39	7.51	7.69		7.46	7.46	7.53	7.85	7.42	7.42	7.62		7.44		7.58	
Tyrosine	3.08	3.50	3.37	3.39	3.15	3.14	3.15		3.22	3.00	3.21	3.39	3.30	3.11	3.24		3.13		3.37	
Glutamic acid	7.06	7.06	7.37	7.00	7.09	7.18	7.03		7.06	7.10	7.16	7.27	6.80	6.88	7.09		7.16		7.25	
Aspartic acid	9.82	9.56	9.83	9.70	9.36	9.79	9.41		9.99	9.57	10.11		9.37	9.78	9.61		9.77		9.99	

* The lower value was obtained by the procedure described in Methods, whereas the higher value was obtained by Dr. Marvin Armstrong (6) by the Kapeller-Adler method.

dissolved in 10 ml. of distilled water which was removed by the same technique. The residue was dissolved in 2 ml. of 0.5 M acetic acid and 1 ml. of this solution was pipetted on the 100-cm. Dowex 2-X10 column and the determination completed as above.

RESULTS AND DISCUSSION

The individual results of amino acid analyses from all chromatograms of hemoglobin hydrolysates are shown in Table I. The number of the preparation and hours of hydrolysis are shown. The case number in Wright and Tarjan's review (2), the intelligence quotient, and the serum phenylalanine level of the two phenylketonuric patients are also given.

Before attempting to decide whether differences exist in the amino acid contents of the two hemoglobins, let us consider the reproducibility of determinations of this type. Comparisons may be made of the results from duplicate determinations on the same hydrolysate, from hydrolysates of the same preparation of hemoglobin, and from different preparations of hemoglobin. Thus, we may compare, respectively, the results of Chromatograms No. A-27 and A-30 (Table I), of these and Chromatograms No. A-28 or A-31, and finally, of all the foregoing with Chromatogram No. A-33. It is apparent that duplicate determinations on the same hydrolysate agree no better than duplicate determinations in which still other variables are present. These three variables, then, have no specific influence on the results. The individual determinations of phenylalanine and glutamic acid agree within the range of ± 3 per cent usual in column chromatography. The error for tyrosine exceeds this. Part of the error in the tyrosine determinations may be due to the smaller quantities of this amino acid, which make any error in the blank more significant. The variation in the values for aspartic acid is large and is quite likely related to its destruction on the column.

In Table II are shown the results of a statistical study of the data [4] (21). An analysis of variance

[4] In Chromatogram No. A-39, a further attempt to assess the extent of the destruction of aspartic acid was made. For this experiment, a portion of the same hydrolysate that was used for Chromatogram No. A-36 was placed on the column and followed immediately by a known amount of aspartic acid solution. The resulting chromatogram was unusual in that the optical density was abnormally high between phenylalanine and the pre-

TABLE II

Statistical study of results
(Gm. amino acid per 100 Gm. hemoglobin)

Amino acid	Type of hemoglobin: Normal Mean	Normal Standard deviation of mean	Phenylketonuric Mean	Phenylketonuric Standard deviation of mean	Difference in means	95% Confidence limit of diff. in the means	Residue weight	Residues in doubt
Phenylalanine	7.52	±0.05	7.50	±0.03	−0.02	0.09	0.25	0.36
Tyrosine 24 hours	3.30	±0.08	3.22	±0.04	0.08	0.25	0.27	0.93
72 hours	3.13	±0.05	3.24	±0.13				
Glutamic acid	7.11	±0.04	7.06	±0.06	0.05	0.17	0.22	0.77
Aspartic acid	9.67	±0.07	9.77	±0.12	−0.10	0.14	0.20	0.70

for one variable of classification reveals no evidence of inhomogeneity of the four hemoglobin preparations and allows us to pool the data of the different preparations from the normal and phenylketonuric individuals for all the amino acids studied. Except for tyrosine, the results from the 24-hour and 72-hour hydrolysates do not differ. A "t" test on the combined data from both types of hemoglobin reveals that there is less than a 20 per cent probability that the smaller value of tyrosine in the 72-hour hydrolysates is due to chance alone. Hence, although the data do not reveal a significant difference by usual standards, it is unjustifiable in the case of this amino acid to pool the results on 24-hour and 72-hour hydrolysates in the light of other evidence that tyrosine is lost on prolonged hydrolysis (22).

In order to test the hypothesis that phenylketonuric hemoglobin has an increased amount of phenylalanine, and, as a corollary, a diminished amount of other amino acids, we take the difference between the means, subtracting the mean value of phenylalanine content for normal hemoglobin from that of phenylketonuric hemoglobin, and, in the case of the other amino acids, subtracting the phenylketonuric amino acid content from the value for normal hemoglobin (see column 5, Table II). The differences are small, and, taking into account the variation of the individual measurements, the hypothesis would probably be rejected without any statistical treatment. However, to evaluate more closely the effect of variation of individual measurements, the 95 per cent one-sided confidence limit of the difference in means was obtained. This difference, taken only in the direction of our hypothesis, will be exceeded by chance alone once in 20 times (21). Thus, in Table II are found not only the means, the standard deviation of the means, and the difference between the means, but the 95 per cent one-sided confidence limit of the difference. For comparison, in the next column the weight of each amino acid in Gm. per 100 Gm. of hemoglobin equivalent to one residue per molecule is given, and finally, in the last column, the quotient of the confidence limit of the difference by the residue weight is given. This quotient provides an estimate of the number of residues of amino acid in doubt. There is less than one chance in 20 that phenylketonuric hemoglobin exceeds normal hemoglobin by as much as 0.36 residue. At the 99.5 per cent level of significance there is less than one chance in 200 that phenylketonuric hemoglobin exceeds normal hemoglobin by as much as 0.72 residue. It is also evident that there is less than one chance in 20 that phenylketonuric hemoglobin has one residue less of tyrosine, glutamic acid or aspartic acid.

These results must not be interpreted to mean that the absolute amount of phenylalanine in hemoglobin is known with such accuracy because it does not take into account the possibility of constant errors such as errors in determination of the concentration of hemoglobin, the color yields by the ninhydrin procedure, and so forth. However, because two materials prepared and analyzed in the same way are compared, such constant errors are cancelled and this precision is possible in the comparison.

A comparison of the amino acid content of normal hemoglobin with the results of other workers

ceding peak. Because of the unusual procedure and result, the determinations of this chromatogram have been omitted from the statistical data of Table II.

TABLE III

Comparison of results with those of previous workers (Gm. of amino acid per 100 Gm. of hemoglobin)

	This paper	Schroeder, Kay and Wells (23)	Van der Schaaf and Huisman (22)	Rossi-Fanelli *et al.* (24)
Phenylalanine	7.52	7.66	7.93	9.62
Tyrosine 24 hours	3.30	2.99	4.40	2.90
72 hours	3.13			
Glutamic acid	7.11	7.17	7.20	7.41
Aspartic acid	11.40*	11.02	10.60	9.99

* Corrected for 15 per cent loss on the column.

is seen in Table III (22–24). Aside from the divergent reports of Van der Schaaf and Huisman (22) for tyrosine, and of Rossi-Fanelli, Cavallini, and DeMarco (24) for phenylalanine and aspartic acid, the results are in good agreement. Schroeder, Kay, and Wells (23) report a slightly higher value for phenylalanine than was found in the present study, but their phenylalanine was contaminated with tryptophan which was not removed by starch chromatography. If the amount of tryptophan that survived acid hydrolysis and is present in Chromatogram No. A-33 is calculated as phenylalanine, its quantity is 0.13 Gm. per 100 Gm. of hemoglobin. If this amount is subtracted from Schroeder, Kay, and Wells' value for phenylalanine content of 7.66, the result, 7.53, is in exceptional agreement with the present value of 7.52.

The aspartic acid has been corrected for the loss that is known to take place on the column, but the result reported here is less reliable than that of other workers.

CONCLUSIONS

It may be concluded from the results of the present work that, if normal and phenylketonuric hemoglobin are of uniform phenylalanine content, they do not differ by as much as one residue of phenylalanine per molecule. It is possible that some of the molecules of the phenylketonuric hemoglobin have one or more extra residues of phenylalanine but the accuracy of the present technique is unable to detect a difference in the average phenylalanine content of much less than one residue per molecule.

Further work is being carried out in these laboratories on the phenylalanine content of other body proteins. If such investigations yield results similar to the above, it would render unlikely the possibility that phenylalanine acts as a toxic substance by accumulating to an abnormally large extent in proteins. This indicates that either the cells can exclude from sites of protein synthesis the high levels of phenylalanine found in body fluids, or that the gene-controlled protein synthesis is remarkably efficient.

SUMMARY

1. One possible mechanism for phenylalanine toxicity in phenylketonuria is the abnormal accumulation of phenylalanine in body proteins and the displacement of other amino acids. A vital protein containing a single extra residue of phenylalanine might function abnormally and produce the clinical symptomatology.

2. The phenylalanine, tyrosine, glutamic acid and aspartic acid content of two samples of hemoglobin from normal individuals and two samples from phenylketonuric patients have been compared. The amino acid determinations were made chromatographically on a 200-cm. column of Dowex 2-X10, an anion exchange resin, with 0.5 M acetic acid as a developer.

3. The results show no significant difference between normal and phenylketonuric hemoglobin. Moreover, there is less than one chance in 200 that the phenylalanine content of phenylketonuric hemoglobin exceeds that of normal hemoglobin by as much as one residue of phenylalanine, and less than one chance in 20 that phenylketonuric hemoglobin has one residue less of tyrosine, glutamic acid, or aspartic acid.

4. From the experimental evidence, the phenylalanine toxicity in phenylketonuria is not associated with any abnormal accumulation of phenylalanine in hemoglobin.

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REFERENCES

1. Jervis, G. A., Phenylpyruvic oligophrenia (phenylketonuria). Genetics and the inheritance of integrated neurological and psychiatric patterns.

Proc. A. Research Nerv. & Ment. Dis., 1954, 33, 259.
2. Wright, S. W., and Tarjan, G., Phenylketonuria. Am. J. Dis Child., 1957, 93, 405.
3. Jervis, G. A., Phenylpyruvica oligophrenia deficiency of phenylalanine-oxidizing system. Proc. Soc. Exper. Biol. & Med., 1953, 82, 514.
4. Udenfriend, S., and Bessman, S. P., The hydroxylation of phenylalanine and antipyrine in phenylpyruvic oligophrenia. J. Biol. Chem., 1953, 203, 961.
5. Bickel, H., Gerrard, J., and Hickmans, E. M., Influence of phenylalanine intake on chemistry and behaviour of a phenylketonuric child. Acta paediat., 1954, 43, 64.
6. Armstrong, M. D., and Tyler, F. H., Studies on phenylketonuria. I. Restricted phenylalanine intake in phenylketonuria. J. Clin. Invest., 1955, 34, 565.
7. Pauling, L., The probability of errors in the process of synthesis of protein molecules *in* Festschrift Prof. Dr. Arthur Stoll. Basel, Birkhäuser, 1957, p. 597.
8. Block, R. J., Jervis, G. A., Bolling, D., and Webb, M., Chemical and metabolic studies on phenylalanine. III. The amino acid content of tissue proteins of normal and phenylpyruvic oligophrenic individuals. A note on the estimation of phenylalanine. J. Biol. Chem., 1940, 134, 567.
9. Schrappe, O., Zur pathologischen Physiologie des Phenylbrenztraubensäure-Schwachsinns. Nervenarzt, 1952, 23, 175.
10. Keup, W., Die Amino säure-Bausteine körpereigener Proteine bei Oligophrenia phenylpyruvica. Monatsschr. f. Psychiat. u. Neurol., 1955, 130, 299.
11. Schroeder, W. A., Column chromatography in the study of the structure of peptides and proteins. Progr. Chem. Org. Nat. Prod., 1954, 11, 240.
12. Drabkin, D. L., A simplified technique for a large scale crystallization of human oxyhemoglobin. Isomorphous transformations of hemoglobin and myoglobin in the crystalline state. Arch. Biochem., 1949, 21, 224.
13. Drabkin, D. L., and Austin, J. H., Spectrophotometric studies. I. Spectrophotometric constants for common hemoglobin derivatives in human, dog, and rabbit blood. J. Biol. Chem., 1932, 98, 719.
14. Stein, W. H., and Moore, S., Chromatography of amino acids on starch columns. Separation of phenylalanine, leucine, isoleucine, methionine, tyrosine, and valine. J. Biol. Chem., 1948, 176, 337.
15. Moore, S., and Stein, W. H., Chromatography of amino acids on sulfonated polystyrene resins. J. Biol. Chem., 1951, 192, 663.
16. Hirs, C. H. W., Moore, S., and Stein, W. H., The chromatography of amino acids on ion exchange resins. Use of volatile acids in elution. J. Am. Chem. Soc., 1954, 76, 6063.
17. Hirs, C. H. W., Personal communication.
18. Schroeder, W. A., and Kay, L. M., The amino acid composition of *Bombyx mori* silk fibroin and of Tussah silk fibroin. J. Am. Chem. Soc., 1955, 77, 3908.
19. Green, F. C., and Kay, L. M., Separation of sixteen dinitrophenylamino acids by adsorption chromatography on silicic acid-Celite. Anal. Chem., 1952, 24, 726.
20. Moore, S., and Stein, W. H., Photometric ninhydrin method for use in the chromatography of amino acids. J. Biol. Chem., 1948, 176, 367.
21. Dixon, W. J., and Massey, F. J., Jr., Introduction to Statistical Analysis, Second Edition. New York, McGraw-Hill Book Company, Inc., 1957.
22. Van der Schaaf, P. C., and Huisman, T. H. J., The amino-acid composition of human adult and foetal carbonmonoxyhaemoglobin estimated by ion exchange chromatography. Biochim. et biophys. acta, 1955, 17, 81.
23. Schroeder, W. A., Kay, L. M., and Wells, I. C., Amino acid composition of hemoglobins of normal negroes and sickle-cell anemics. J. Biol. Chem., 1950, 187, 221.
24. Rossi-Fanelli, A., Cavallini, D., and DeMarco, C., Amino-acid composition of human crystallized myoglobin and haemoglobin. Biochim. et biophys. acta, 1955, 17, 377.