conditions are sufficient to cause at least epimerization at C_{11} was shown by the following experiment. A mixture of 100 mg. of 6-epidesoxodesacetyldihydroisotenulin,[10] 3 ml. of ethanol and 0.1 ml. of 10% sodium hydroxide solution was heated on the steam-bath for 30 minutes. Removal of ethanol followed by cooling and careful dilution with water caused gradual crystallization of 6-epidesoxodesacetyldihydroalloisotenulin, m.p. 157–158°.

Allotetrahydrohelenalin.—A mixture of 0.15 g. of tetrahydrohelenalin,[4] 0.25 g. of sodium carbonate, 3 ml. of water and 0.5 ml. of ethanol was refluxed for 15 minutes. The solid tetrahydrohelenalin dissolved within 5–10 minutes. The alcohol was driven off and the solution was cooled and acidified with concd. hydrochloric acid. The semi-solid product was taken up in chloroform, the chloroform layer was washed, dried and concentrated *in vacuo*. The residual oil was crystallized from aqueous methanol to give 0.07 g. of colorless thick prisms, m.p. 161–163°, mixed m.p. with tetrahydrohelenalin depressed to 140°. Three additional crystallizations from aqueous methanol gave needles, m.p. 165.5°, $[\alpha]^{23}D$ 125° (95% ethanol, *c* 0.56); infrared bands at 3415 (bonded OH), 1760 (γ-lactone), 1730 (cyclopentanone, shoulder at 1720-bonded?) and 1412 cm.$^{-1}$.

Anal. Calcd. for $C_{15}H_{22}O_4$: C, 68.49; H, 8.03. Found: C, 68.64; H, 8.33.

Dehydroallotetrahydrohelenalin.—Chromic acid oxidation of 100 mg. of allotetrahydrohelenalin yielded 80 mg. of dehydroallotetrahydrohelenalin, m.p. 170°, $[\alpha]^{23}D$ 33.3° (95% ethanol, *c* 0.605), mixed m.p. with material from the oxidation of desacetyltetrahydrobalduilin A (m.p. 166°) 167–168°. The infrared spectra of the two samples were superimposable in every detail.

Desoxoallotetrahydrohelenalin.—A mixture of 0.1 g. of allotetrahydrohelenalin, 0.15 ml. of ethanedithiol and 0.25 ml. of boron trifluoride-etherate was allowed to stand for one hour, diluted with water and extracted with ether. The ether extract was washed with water, aqueous sodium hydroxide and again with water. Removal of solvent yielded an oil which refused to crystallize; wt. 0.11 g., infrared bands at 3450 and 1760 cm.$^{-1}$. The oil, wt. 0.31 g., was desulfurized with Raney nickel and worked up in the usual way to give 0.25 g. of an oil which refused to crystallize. It was purified by chromatography over alumina (solvent and eluent benzene), $[\alpha]^{22}D$ −20° (95% ethanol, *c* 0.53), infrared bands at 3450 and 1760 cm.$^{-1}$.

Anal. Calcd. for $C_{15}H_{24}O_3$: C, 71.39; H, 9.59. Found: C, 71.18; H, 9.47.

When the thioketal was chromatographed over alumina, there were some changes in the fingerprint region of the infrared spectrum. Desulfurization of 100 mg. of the thioketal, after chromatography and purification in the usual manner, yielded 80 mg. of a crystalline residue which was recrystallized from benzene–ligroin and ligroin. The felted needles melted at 134–135°, $[\alpha]^{24}D$ 15° (95% ethanol, *c* 0.28); infrared bands at 3570 and 3450 (non-bonded and bonded OH) and 1750 cm.$^{-1}$ (γ-lactone). There were significant differences in the fingerprint region of the two hydroxylactones.

Anal. Calcd. for $C_{15}H_{24}O_3$: C, 71.39; H, 9.59. Found: C, 71.96; H, 9.12.

Dehydrodesoxoallotetrahydrohelenalin.—Oxidation of 0.19 g. of the non-crystalline desoxoallotetrahydrohelenalin in 10 ml. of acetic acid with 5.2 ml. of a 1.33% solution of chromic acid in acetic acid was allowed to proceed at 5° overnight. Excess oxidizing agent was destroyed by adding methanol and the combined solvents were removed. The residue was diluted with water and extracted with ether. The ether extract was washed, dried and freed of solvents to give 0.155 g. of a viscous oil (isomer A) which refused to crystallize; $[\alpha]^{23}D$ −13.2° (95% ethanol, *c* 0.295), infrared bands at 1765 (γ-lactone) and 1700 cm.$^{-1}$ (cycloheptanone). The material gave a positive Zimmermann test.

Anal. Calcd. for $C_{15}H_{22}O_3$: C, 71.97; H, 8.86. Found: C, 72.42; H, 8.91.

Oxidation of 0.35 g. of the solid hydroxylactone in the same manner gave 0.33 g. of viscous ketolactone, $[\alpha]^{23}D$ 43.2° (95% ethanol, *c* 0.665). It gave a positive Zimmermann test. The infrared spectrum of this material (isomer B) did not differ significantly from that of the isomer A, the fingerprint region being rich in detail, although the rotation was quite different. In an attempt to prepare the oil for analysis, it was distilled in a high vacuum. Two fractions were collected: fraction 1, b.p. 135–143° (bath temp., 0.03 mm.), $[\alpha]^{24}D$ 42° (95% ethanol, *c* 0.3); and fraction 2, b.p. 155–160° (bath temp., 0.02 mm.), $[\alpha]^{24}D$ 75.8° (95% ethanol, *c* 0.545). It is not clear whether isomerization occurred during the distillation or whether the original material was a mixture. The infrared spectra of the two fractions were practically indistinguishable from each other and from the spectrum of isomer A.

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A Chromatographic Study of the Minor Components of Normal Adult Human Hemoglobin Including a Comparison of Hemoglobin from Normal and Phenylketonuric Individuals

BY MARGUERITE DAVIS CLEGG AND W. A. SCHROEDER

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An improved procedure is described for the separation of minor hemoglobin components in hemolysates of red blood cells and hence for the purification of the main component. A comparison of the minor components of hemoglobin from normal and phenylketonuric individuals has failed to reveal any significant differences in the number or amounts. Thus, the abnormal amounts of free phenylalanine in the organism have failed to influence the biosynthesis of the hemoglobin significantly.

Introduction

It is now well established that the hemoglobin from a hemolysate of normal adult human red blood cells is heterogeneous. This fact, although suspected earlier on the basis of experiments that did not permit the isolation of the components,[1–3] received its first unequivocal demonstration when Kunkel and Wallenius[4] separated minor fractions from human adult hemoglobin by starch block electrophoresis. At about the same time, by means of chromatography on the ion-exchange resin, IRC-50, Morrison and Cook[5] found a component (10%) that moved more rapidly and another (6%) that moved more slowly than the main component. Huisman and Prins[6] detected only the faster component with

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(4) H. G. Kunkel and G. Wallenius, *Science*, **122**, 288 (1955).

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different methods of chromatography on IRC-50. Allen, Schroeder and Balog,[7] again by chromatography on IRC-50, did not detect the more slowly moving component but were able to show that the faster component contained at least three heme proteins and one non-heme protein. None of these seemed to be identical to fetal hemoglobin.[7] Crystallized and non-crystallized hemoglobin were equally heterogeneous.

Huisman, Martis and Dozy[8] as well as Gutter, Peterson and Sober[9] have chromatographed adult hemoglobin on the cation exchanger, carboxymethylcellulose. Their results have much in common and superficially, at least, resemble those of Morrison and Cook.[5] Both groups of workers observed a more rapidly moving component that was not very well separated from the major component and a more slowly moving component that was well separated. The latter presumably corresponds to the more slowly moving component that Kunkel and Wallenius[4] isolated by starch block electrophoresis.

One may speculate as to the source and function of these minor components some of which may be present to the extent of only about 1% of the total hemoglobin. Certain possibilities are apparent. Inasmuch as there is a constant turnover of red cells, the minor components may simply trace the life history of the cell: some may be precursors of the mature hemoglobin molecule which is relatively stable for the most of the life of the cell and others may be the degenerate products of the aged cell. On the other hand, each component may be genetically determined. Whether the minor components arise from one source or the other, their relative quantity might be influenced by a stress to which the organism is subjected. The stress might be reflected either by increasing the production of one or more of the minor components or by interfering with the formation of the major component. One might expect to find an answer to these questions in individuals with hematological disorders, and, indeed, in thalassemia minor, there appears to be not only an increase in the amount of the minor components[10,11] but also an increase in their number.[12] Likewise, in cases of sickel-cell anemia,[13] the minor components are present to the extent of 10 to 27% as compared to 10 to 13% in normal individuals[7]; not only is there an increase in amount but also some difference in the kind of minor components as well as greater individual variation between sickle-cell anemics. It is not known whether these additional minor components are the result of the genetic abnormality which causes anemia, or whether they are formed as a result of the abnormal condition under which the protein is being synthesized.

One may ask whether the presence of abnormal metabolites during protein biosynthesis will influence the molecule that is being formed. This problem might be resolved in a hematologically normal condition in which a different stress, genetic or acquired, is present during the biosynthesis of hemoglobin. Such a situation obtains in patients suffering from phenylketonuria. In these individuals, a biochemical defect, inherited as an autosomal recessive gene, prevents the effective conversion of phenylalanine to tyrosine and results in blood levels of phenylalanine as high as 40 mg. per 100 ml., compared to 1 to 2 mg. per 100 ml. in the normal individual. The apparent improvement in mental status of patients on diets low in phenylalanine,[14,15] and the normal development of infants who were born with this deficiency and reared on low phenylalanine diets suggest a toxic action of the phenylalanine or of its metabolites. One possible mode of toxicity would be through increased incorporation of phenylalanine into body proteins and the consequent interference with the proper function of these molecules. If this is the mode of action, however, it must have altered the hemoglobin very little because the phenylalanine content of whole hemoglobin of normal and phenylketonuric individuals is not significantly different.[16] However, as noted,[16] an amino acid analysis is unlikely to detect a difference in a small percentage of the molecules. At the time of this analysis, the full extent of the heterogeneity of whole hemoglobin was not realized but, as it became more apparent, an investigation of the minor components in hemoglobin from phenylketonuric individuals was clearly indicated. If the large amount of phenylalanine alters the biosynthesis of normal protein by increased incorporation, then minor components other than those normally present might be found. If it impairs normal protein biosynthesis, then the relative proportions of the different components might be changed. If no difference is observable, the changes are either below the levels of detection, or the biosynthesis of hemoglobin is not influenced by such abnormal conditions.

This investigation was begun to determine whether or not differences could be detected in the amount and kind of hemoglobin components in the blood of normal individuals and of phenylketonurics. It has led to an extension of the methods of hemoglobin chromatography that were in use in these laboratories and to the detection of additional minor components in hemolysates of normal red cells. Differences in hemoglobin from normal and phenylketonuric individuals have not been found.

Experimental

Preparation of Hemoglobin Solutions.—Blood, freshly drawn from laboratory workers, was mixed with 3.2% sodium citrate dihydrate (one ml. of citrate solution to 5 ml. of blood). The cells were separated from the plasma by centrifugation for 5 min. at 6000 r.p.m. in a refrigerated centrifuge (0–5°), then stirred with an equal volume of 0.9% saline solution and centrifuged again as above. The supernatant saline solution and white cells were carefully

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removed. This washing was repeated four times before hemolysing by frequent shaking with an equal volume of distilled water and 0.4 volume of toluene for 15 minutes at room temperature. After the hemolysate had been centrifuged for 1 hr. at 25,000 × *g* at 0–5°, the middle layer of oxyhemoglobin solution was drawn off and again centrifuged at 25,000 × *g* to remove any remaining cell debris. The hemoglobin was used for chromatography after it had been dialyzed for at least 12 hr. at 0–5° against 100 times the volume of the developer that was to be used for subsequent chromatography.

The samples of phenylketonuric blood were obtained from patients at Pacific State Hospital, Pomona, California. These samples were packed in ice and delivered to these laboratories within 4 hr. after having been drawn.

Preparation of Chromatographic Columns.—Amberlite IRC-50 synthetic cation-exchange resin was prepared and columns were poured according to the method of Allen, *et al.*[7] In these experiments only 1 × 35-cm. columns were used.

Developers.—The developers used were those described by Allen, *et al.*,[7] with one exception. This new developer, termed No. 5, was prepared by dissolving 16.56 g. of sodium dihydrogen phosphate monohydrate, 7.1 g. of anhydrous disodium hydrogen phosphate and 2.6 g. of potassium cyanide in 4 l. of water. The *p*H at room temperature was 6.85 ± 0.05 and the sodium ion concentration was 0.055 *M*.

Operation of Columns.—Prior to starting a chromatogram, the top 3 cm. of the resin in a column was stirred and allowed to settle. The hemoglobin to be chromatographed was diluted to a concentration of 50 to 60 mg. in 3 ml. of developer and run slowly onto the column with a bent-tip dropper without disturbing the surface of the resin. The sample was allowed to flow in under gravity and washed in with 3 × 1 ml. of developer. The columns equilibrated with Developer No. 1, 2 and 4 were run as described[7] at 5–6°. When using columns equilibrated with Developer No. 5 at 5–6°, the flow rate was regulated initially to deliver 6 ml. per hr. and 2-ml. fractions were collected. After 120 ml. of effluent, the flow rate was increased to 10 ml. per hr. for the next 500 ml. and 4-ml. fractions were collected. At this point, the leading edge of the main component had either reached or was very close to the bottom of the column. The column was then allowed to warm to room temperature; the flow rate automatically increased to 15 ml. per hr. Under these conditions, the remaining zones were removed from the column rapidly. After the column had been warmed, it was not used again until 600 ml. of developer had been passed through in the cold in the course of 4 days.

(Since these conditions were first devised and used, it has become apparent that a few degrees difference in room temperature during the last part of the chromatogram markedly influences the results. Therefore, chromatograms of this type are now routinely thermostated at 28° during that period when the main component and more slowly moving components are passing from the column. (Furthermore, reconditioning is now done with 1000 ml. instead of 600 ml. in 4 days.)

Before rechromatography, the pooled fractions of a zone were concentrated at 0–5° by dialyzing against a 20% solution of polyvinylpyrrolidone (PVP K-30 from Oxford Laboratories, San Francisco 4, California) in developer in order to maintain a suitable *p*H. By this method, 30 ml. could be concentrated to 3 ml. in 24 hr. by dialyzing in 300 ml. of PVP solution. The sample was then dialyzed against the proper developer before use.

Spectrophotometry.—The optical density of each fraction was read in the Beckman DU spectrophotometer at 280 and 415 mμ as described by Allen, *et al.*[7]

Results and Discussion

Chromatographic Methods.—A first comparison was made by separating the combined more rapidly moving minor components from the major component with Developer No. 2, followed by rechromatography of the minor components with Developer No. 4 for further analysis. However, the separation by Developer No. 2 was less satisfactory than previously described[7] and this method was found to be unsuitable because the quantity of zone A_{Ic}[17] was not reproducible in duplicate chromatograms with the same sample. Apparently, a variable quantity of the minor components trailed into the major zone with Developer No. 2. Furthermore, on rechromatography with Developer No. 4, a small amount of a slowly moving component which remained on the top of the column suggested the presence of still another minor component. The quantities of zones A_{Ia} and A_{Ib} were in satisfactory agreement in duplicate chromatograms and these two zones were found in a constant ratio to each other in normal and phenylketonuric individuals.

Inasmuch as the main difficulty with this procedure lay in the inability to achieve a complete initial separation of the major and minor components, a procedure with a new developer, No. 5, was devised to eliminate the problems of rechromatography by permitting whole hemoglobin to be chromatographed and the entire analysis to be completed with one chromatogram. A typical chromatogram is shown in Fig. 1. Zones A_{Ia} and A_{Ib} emerge as a single zone which is well separated from A_{Ic}. More strongly adsorbed minor components still remain on the column and are removed more rapidly by doubling the flow rate after 120 ml. of effluent; zones A_{Id} and A_{Ie} are eluted with peaks at 140 ± 10 and 300 ± 20 ml. The chromatogram is continued until the front of the main component reaches the bottom of the column (about 650 to 700 ml.[18]). At this time, the main zone is spread over the lower two-thirds of the column. A slowly moving zone is faintly visible near the top of this region. If now the column is warmed to room temperature (better 28°), the major component moves off the col-

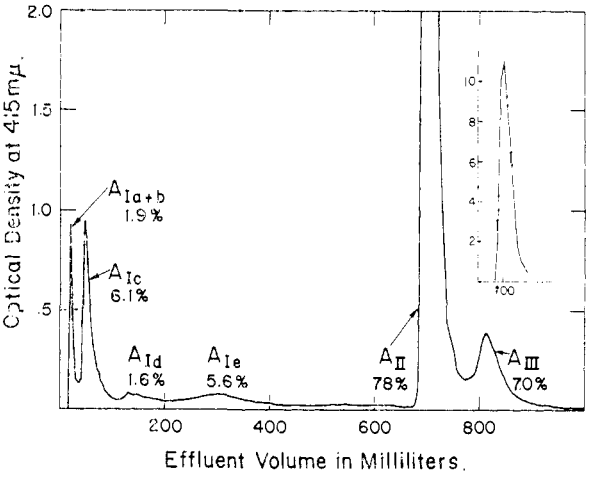

Fig. 1.—Chromatogram of whole adult oxyhemoglobin on a 1 × 35-cm. column of IRC-50 with Developer No. 5. The inset shows that portion of the chromatogram near 700 ml. of effluent volume on a reduced vertical scale. The chromatogram was warmed from 5–6° to room temperature (28°) after 675 ml. of effluent.

(17) The system of nomenclature used in this paper follows that of Allen, *et al.*[7] When the identities of the minor zones as isolated by chromatography and by starch block electrophoresis have been correlated, a definitive nomenclature can be established.

(18) This volume applies when the total hemoglobin on the column is 50 mg. It will be greater or less in reverse relation to the quantity on the chromatogram.

umn in 50 ml. of effluent. A zone, A_{III}, more strongly adsorbed than the major component shows up distinctly. If the chromatogram had been continued in the cold, zone A_{II} would, no doubt, have trailed so much as almost to mask zone A_{III} but by warming the column, the separation of zones A_{II} and A_{III} is enhanced. Zone A_{III} usually appears as a single zone, but occasionally it seems to be formed by two poorly separated zones.[19] This type of chromatogram is reproducible in the position and amount of each zone present.

Allen, *et al.*,[7] have demonstrated that zones A_{Ia}, A_{Ib} and A_{Ic} are not artifacts. Two experiments were carried out to ascertain whether or not the minor components, including the very slowly moving zone, were artifacts of this method of chromatography. In the first experiment, whole hemoglobin was chromatographed with Developer No. 1. On this type of column, there is very little separation between the rapidly moving minor components and the major fraction; the peak of the combined rapidly moving minor components (zone A_I) comes out at 17 ml. and the peak of the major fraction (zone A_{II}) at 24 ml. Approximately 75% of the major fraction emerges in the 10 ml. after the peak. The fractions that contained zone A_I as well as the first 12 ml. of the effluent that contained zone A_{II} were combined, dialyzed against Developer No. 5, and rechromatographed with Developer No. 5. Zones A_{Ia} through A_{Ie} were present in the usual amounts whereas zone A_{III} was present to the extent of about 15% of the usual amount. A decrease in the amount of zone A_{III} was to be expected if this zone was not formed on the column because the trailing portion of the main component was discarded at an arbitrary point before rechromatography. The conclusion may be drawn that chromatography does not produce the minor components or increase their amounts.

In the second experiment, about twice the usual amount of whole hemoglobin was chromatographed with Developer No. 5. As zone A_{II} emerged from the column, the fractions were immediately chilled and the five most concentrated fractions which contained about 90% of the amount usually chromatographed were pooled and rechromatographed with Developer No. 5. All of the minor zones were eliminated by this procedure. Zone A_{II} trailed less obviously as it moved down the column. About 1% of the total material was present in an ill-defined zone between zone A_{II} and the usual position of zone A_{III}. Thus, chromatography is not responsible for the minor components.

Experiments were not made to determine whether the minor components are produced during the preparation of the hemoglobin solution. However, the experiments of Kunkel, *et al.*,[10] and Allen, *et al.*,[7] suggest that they are not so produced. It seems probable, therefore, that the minor components exist in the red blood cell.

Comparison of Hemoglobin from Normal and Phenylketonuric Individuals.—Five samples of normal hemoglobin and six of phenylketonuric hemoglobin were compared chromatographically; two normal samples were repeated with fresh specimens of blood after two months had elapsed. Only one chromatogram, that of a normal hemoglobin, no. 3, differed from all the others. In addition to all the other minor components, another, the peak of which was eluted at 530 ml., preceded zone A_{II}. This slow minor component was detected again when a fresh sample of the hemoglobin was checked two months later. (In later samples, it has not been detected; indeed, zone A_{Ie} is not very apparent in most samples.)

Not only the number but also the quantity of the minor components was the same in the phenylketonurics and the normal individuals as Table I clearly shows. Comparison of the average amounts of corresponding zones in the two kinds of hemoglobin with the spread of results in each group suggests that the differences are well within the error of the method. It would therefore seem that the presence of high levels of phenylalanine in the blood does not alter the biosynthesis of hemoglobin in amount or type. The minor components are not changed by the presence of an excessive amount of a normal constituent of the blood.

TABLE I

THE AMOUNT OF EACH OF THE CHROMATOGRAPHICALLY SEPARATED COMPONENTS OF HEMOGLOBIN FROM NORMAL ADULT AND PHENYLKETONURIC INDIVIDUALS EXPRESSED AS A PERCENTAGE OF THE TOTAL OPTICAL DENSITY AT 415 mμ

	A_{Ia+b}	A_{Ic}	A_{Id}	A_{Ie}[a]	A_{II}	A_{III}
Normal						
1	1.9	5.5	1.5	4.6	81.7	4.8
2	2.1	6.3	1.8	3.9	81.1	4.8
3	2.0	5.7	1.3	3.9[b]	82.8	4.6
	1.9	5.2	1.5	3.6[b]	81.7	5.7
4	1.9	5.6	1.8	4.7	80.8	5.5
	1.7	5.4	1.7	4.3	82.0	5.0
5	2.4	6.3	1.9	5.6	79.5	4.3
Av.	2.0	5.7	1.6	4.4	81.3	5.0
Phenylketonuric						
1	1.9	6.1	1.6	5.6	77.8	7.0
2	2.0	5.7	1.4	3.8	83.0	4.7
3	1.8	4.0	1.4	3.5	84.7	4.6
4	1.9	5.2	1.4	2.8	85.1	3.7
5	2.1	5.4	1.3	3.5	83.9	3.8
6	2.2	5.2	1.3	3.7	83.4	4.2
Av.	2.0	5.3	1.4	3.8	83.0	4.7

[a] This zone is rather arbitrarily taken to be all material above the blank reading from the lowest point between zones A_{Id} and A_{Ie} and the point of emergence of zone A_{II}. [b] In this case A_{Ie} is the sum of two separate zones. See text.

Conclusions

The results of this investigation bear on three topics: (1) the chromatography of hemoglobin, (2) the detection of minor components in the hemoglobin from hemolysates of red blood cells, and (3) the effect of abnormal conditions on the biosynthesis of hemoglobin.

Continued use of the chromatographic methods for hemoglobin that were described by Allen, *et al.*,[7] has shown that very satisfactory separations can be obtained by judicious modification of the condi-

(19) Recent unpublished experiments suggest that the separation of A_{III} into two zones is a yet undetermined function of the chromatographic conditions. It is consistently obtained with Developer No. 5 on some columns but not on others.

tions of pH, sodium ion concentration and temperature. However, reproducible results require the very careful control of all conditions and the re-equilibration of the column if any condition is altered. Thus, when Developer No. 5 is used and the column is warmed, re-equilibration for 4 days at 6° with 1000 ml. of developer is required.

This investigation has added three minor heme-containing components to the three that were detected by Allen, *et al.*,[7] in normal adult hemoglobin. Although Allen, *et al.*, failed to observe a component that moved more slowly than the main zone, such a component is apparent under the conditions employed here. Presumably, it is the same as the more slowly moving component observed by Morrison and Cook,[5] Huisman, *et al.*,[8] and Gutter, *et al.*[9] It may well be that undetected minor components are hidden in the main component or that the zones of some minor components may be heterogeneous. Zones A_{Ia} and A_{Ib} move through the column so rapidly even with Developer No. 4 that heterogeneity could well be hidden. The ratio of the heme to protein as derived from the ratio of the optical densities at 415 to 280 mμ is constant in all zones except zone A_{Ia+b}. A non-heme protein has been found to be eluted immediately ahead of A_{Ia}, and there are indications that either another non-heme protein is present in the region of A_{Ib} or that there are fewer hemes in this hemoglobin component. The point has not been pursued further.

The biosynthesis of hemoglobin does not seem to be influenced by the mass action effect of an excessively large amount of one of the constituents that goes to make it up. In the phenylketonuric individual, the presence of 30 to 40 times the normal amount of phenylalanine in the blood does not alter the nature of the hemoglobin components.

Although the present procedures are time consuming, they offer a sensitive method for the study of the hemoglobin components in hematological disorders.

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PASADENA, CALIFORNIA

[CONTRIBUTION FROM THE PLANT, SOIL AND NUTRITION LABORATORY]

Isolation of a New Acidic Aromatic Amino Acid (*m*-Carboxy-α-phenylglycine) from Iris Bulb

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A new acidic aromatic amino acid, *m*-carboxy-α-phenylglycine was isolated from iris bulbs (*Iris tingitana var. Wedgewood*) by the use of ion-exchange resins and column chromatography. Its structure was determined from oxidation studies, elemental analysis, melting point and infrared absorption spectra. Synthetic *m*-carboxy-α-phenylglycine was prepared and found to be identical with the isolated material. *m*-Phthalic acid and *m*-phthalaldehydic acid were identified after oxidation of the amino acid, respectively, with alkaline permanganate and chloramine-T. The amount of this amino acid in the iris bulb was determined by quantitative paper chromatography with isolated material as the standard.

Introduction

Plant materials contain many amino acids which do not occur in proteins. In recent years, many new amino acids have been isolated from plants and characterized.[2] Storage organs have been particularly rich sources.

In an investigation of the non-protein nitrogen fraction of the iris bulb (*Iris tingitana var Wedgewood*), β-aminoisobutyric acid was isolated and identified.[3] In the isolation procedure, the acidic amino acids were removed by absorption on the salt form of an anion-exchange resin. Paper chromatography of this acidic fraction revealed several unknown compounds. One of them occupied the spot marked unknown No. 18 in Fig. 1 of our previous paper. This compound has been isolated and identified as *m*-carboxy-α-phenylglycine, an amino acid hitherto undescribed in the literature.

(1) United States Plant, Soil and Nutrition Laboratory, Ithaca, New York.

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(3) S. Asen, J. F. Thompson, C. J. Morris and F. J. Irreverre, *J. Biol. Chem.*, **234**, 343 (1959).

Discussion

The acidic fraction from our previous isolation[2] was chromatographed on the acetate form of Dowex 1 and eluted with acetic acid. This process purified an unknown amino acid which was uncontaminated by any other ninhydrin active substance. After several recrystallizations from hot water, 400 mg. of a pure white crystalline material was obtained. Elemental analysis established an empirical formula of $C_9H_9O_4N$. The compound remained unchanged when heated with 6 N HCl for 24 hr. at 120°. Reaction with ninhydrin on paper produced a yellow color which gradually changed to the normal blue violet complex. When the ninhydrin reaction was performed in the test-tube under quantitative conditions, the color yield was 90% of theory.[4]

Migration of the isolated substance toward the anode in paper electrophoresis at pH 7 further confirmed the acidic nature of this compound. The rate of migration indicated no strongly acidic groups. With four oxygen atoms in the empirical formula, a dicarboxylic acid was likely.

(4) W. Troll and R. K. Cannan, *ibid.*, **200**, 803 (1953).