much greater amount of P is rendered ether soluble. Indeed, this P was shown to be due to the long chain ether of glycerol phosphate V.

P. Production of Lysolecithin and Long Chain Aldehyde by Mild Acid Hydrolysis of Lecithin XI.—The unreduced lecithin fraction XI (7.4 mg.) was dissolved in 1 ml. of glacial acetic acid, 2 drops of saturated $HgCl_2$ solution added, and the mixture heated in a boiling water-bath for 20 minutes and then let stand at room temperature for 20 hours. After the hydrolysis free aldehyde was liberated and lysolecithin was produced. However, only 40% of XI was hydrolyzed by this procedure. The products were easily detected by chromatography on silicic acid impregnated paper using diisobutyl ketone–acetic acid–water 40:20:3 as solvent.[10] Chromatography was carried out at 0–5°. The R_f values of lecithin, lysolecithin and the long chain aldehyde were 0.38, 0.22 and 0.90, respectively. The lecithin and lysolecithin were analyzed quantitatively by paper chromatography as described previously.[10] For this analysis the acetic acid solution was evaporated to dryness under nitrogen and the residue dissolved in exactly 0.74 ml. of isoamyl alcohol–benzene 1:1. Six 20-μl. aliquots were placed on silicic acid impregnated paper and the chromatograms developed in the solvent mentioned above. The lipids were detected on the chromatograms with Rhodamine 6G. The lecithin and lysolecithin spots were cut off and analyzed for total lipid P. The results showed that after hydrolysis 60% of the P occurred in the lecithin fraction and 40% in the lysolecithin fraction. Control chromatograms of the lecithin which was not hydrolyzed with acetic acid showed that only trace amounts of lysolecithin were present. Furthermore, when the reduced lecithin XIII was hydrolyzed with acetic acid as described above, no lysolecithin or free aldehyde was produced. These results are in agreement with the data given in section O. The data also show that 40% of the pig heart lecithin exists as the plasmalogen and 60% as the classical diester structure.

In order to clarify the apparent discrepancy between our findings and those of Rapport and co-workers,[11,12] we have prepared the phosphatides of beef heart and subjected these to the same reactions given in this paper. The beef heart plasmalogens, as well as those of pig heart, occur mainly in the lecithin and cephalin fractions. By our method beef heart lecithin and cephalin contain 40 and 37%, respectively, of the plasmalogen form, the remaining being the diester type. When the hydrolysis products of the glycerophosphate ethers were chromatographed on silicic acid as outlined in section G (Experimental section), we have subsequently found that the beta-glycerol ethers occur predominantly in the chloroform eluate whereas the alpha-glycerol ethers occur predominantly in the ethyl ether eluate. This procedure, coupled with chemical, infrared spectral, and paper chromatographic analysis and with the reactivity of these ethers toward periodate has now shown that the pig heart glycerol ethers are mainly alpha-derivatives (75% alpha and 25% beta) but the beef heart glycerol ethers are predominantly beta-derivatives (87% beta and 13% alpha). Hence a species difference explains the apparent discrepancy between our work and that of Rapport and collaborators.

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[CONTRIBUTION NO. 2257 FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY]

Observations on the Chromatographic Heterogeneity of Normal Adult and Fetal Human Hemoglobin: A Study of the Effects of Crystallization and Chromatography on the Heterogeneity and Isoleucine Content

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By chromatographic methods, it has been shown that both crystallized and uncrystallized normal adult human hemoglobin contain a main component that comprises about 90% of the heme proteins. Three minor heme proteins and a non-heme protein also have been detected. The several components are not artifacts of preparation or chromatography, and they have been shown to differ in their content of isoleucine. Cord blood hemoglobin also contains minor components. These results are discussed in relation to other reports of the heterogeneity of human hemoglobin. The methods described may be used for the isolation of preparative amounts of the main components.

Introduction

Although there have been numerous reports of the heterogeneity of normal adult human hemoglobin,[1] it has generally been assumed that hemoglobin carefully crystallized, for example by Drabkin's method,[2] is homogeneous. Thus, crystallized hemoglobin has been used for amino acid analysis,[3–5] for the determination of N-terminal amino acids[6] and for other measurements in this and other laboratories. Indeed, Rhinesmith, Schroeder and Pauling[6] reported that crystallized hemoglobin did not show the heterogeneity on starch block electrophoresis that Kunkel and Wallenius[7] described for uncrystallized hemoglobin. In the course of experiments that required pure ferrihemoglobin cyanide, a study of the homogeneity of hemoglobin preparations was prompted by the observation that a minor component could be detected chromatographically in ferrihemoglobin cyanide that had been prepared from crystallized oxyhemoglobin. Later experiments showed that oxyhemoglobin and carbonmonoxyhemoglobin likewise were heterogeneous and that the minor component was not an artifact of preparation or chromatography nor could it be removed by crystallization under the most exacting conditions.

Although the chromatographic techniques employed in this study differ from those of previous workers, their published descriptions of the use of the ion exchange resin IRC-50 were of great assistance in the selection of proper conditions. The

(1) H. Itano, *Ann. Rev. Biochem.*, **25**, 331 (1956).

(2) D. L. Drabkin, *Arch. Biochem.*, **21**, 224 (1949).

(3) W. A. Schroeder, L. M. Kay and I. C. Wells, *J. Biol. Chem.*, **187**, 221 (1950).

(4) A. Rossi-Fanelli, D. Cavallini and C. de Marco, *Biochem. et Biophys. Acta*, **17**, 377 (1955).

(5) D. W. Allen and W. A. Schroeder, *J. Clin. Invest.* **36**, 1343 (1957).

(6) H. S. Rhinesmith, W. A. Schroeder and L. Pauling, THIS JOURNAL, **79**, 609 (1957).

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

first successful chromatograms of hemoglobin on IRC-50 were made by Boardman and Partridge,[8] who were able to separate sheep fetal and maternal hemoglobin and bovine hemoglobin and who outlined clearly the problems of chromatography on this resin. Huisman and Prins[9,10] demonstrated that human hemoglobins, F, A, E, S and C could be separated well on IRC-50 and emerged from the column in the order given. Morrison and Cook[11] were the first workers to demonstrate by chromatography on IRC-50 that oxyhemoglobin from hemolysates of normal adult red blood cells is heterogeneous. These workers found three components in normal adult oxyhemoglobin; they comprised 10, 84 and 6% of the total in the order of elution. The spectra of these components were reported to be identical with that of normal oxyhemoglobin, but the brief report[11] did not state whether the components could be rechromatographed without change in chromatographic behavior. Prins and Huisman[10] using uncrystallized carbonmonoxyhemoglobin confirmed the presence of 10% of a rapidly moving component but were unable to detect the most slowly moving component.

Cook and Morrison[12] likewise reported the presence of three components in a hemolysate of umbilical cord red cells: these components, in the order of elution, comprised the major fetal fraction (70%), a component (20%) that corresponded to the major fraction of adult hemoglobin and one (10%) that corresponded to their slowest component from adult hemoglobin. Prins and Huisman's findings[10] were different: they observed 10% of a fast moving fraction that preceded the main fetal component, but the most slowly moving component was absent.

By means of starch block electrophoresis Kunkel and Wallenius[7] isolated a component that comprised 1.8–3.5% of normal uncrystallized adult hemoglobin and that electrophoretically was more slowly moving than the main component. A faster moving component also was observed. These authors showed that the behavior of these components was unchanged on reëlectrophoresis.

Prior to our observation that crystallized hemoglobin is heterogeneous crystallized hemoglobin was reported by different groups of investigators to contain[3] isoleucine and to be free[4] of isoleucine whereas electrophoretically purified hemoglobin was shown to be free of isoleucine.[13] On the other hand, fetal hemoglobin is known to contain about 10 residues of isoleucine per molecule.[14] Therefore, it was of interest to determine the isoleucine content of the chromatographically isolated components of hemoglobin.

Although at the start of this investigation it was clear that hemolysates of normal adult and fetal red cells contain several hemoglobin components, it was not known whether this heterogeneity applied to crystallized hemoglobin; it had been assumed that it did not.[6] Moreover, the preliminary reports that described the heterogeneity did not rule out the possibility that the minor components were preparative or chromatographic artifacts.

The present investigation, therefore, presents further information about the nature and the isolation of the minor components in crystallized and uncrystallized hemoglobin. The procedures may be used on a larger scale to obtain the main component of normal adult human hemoglobin in a form that is free of several minor components normally present.

Experimental

Preparation of Hemoglobin Solutions.—Hemoglobin was prepared by the method of Drabkin[2] and crystallized either as oxyhemoglobin or as carbonmonoxyhemoglobin. In some instances, in order to rule out artifacts of preparation, blood from an investigator (D. W. A.) was used immediately to prepare hemolysates by Drabkin's method and the hemoglobin was not crystallized but was dialyzed at 2° against the developer for 12 hr. with vigorous magnetic stirring. In one experiment toluene was omitted from the hemolysis, without effect on the result. Ferrihemoglobin cyanide was prepared in the cold either from crystallized oxyhemoglobin or from fresh hemolysates by adding developer that contained excess 0.1 *M* or 0.01 *M* potassium ferricyanide. All solutions before being chromatographed were dialyzed against the developer.

Preparation of Chromatographic Columns.—Amberlite IRC-50 synthetic cation-exchange resin (Lot 761224 obtained from Fisher Scientific Company) was purified according to the method of Hirs, Moore and Stein.[15] Then, the resin in the hydrogen form was sifted wet and the portion that passed a 200-mesh sieve and was retained by a 250-mesh sieve was used for pouring all columns. After being sifted, the resin was washed again with 3 *N* hydrochloric acid and water. Before a column was poured, a portion of the resin was suspended in the appropriate buffer and the suspension was mechanically stirred. The *p*H was brought back to that of the buffer by careful addition of 40% aqueous sodium hydroxide at 15 minute intervals and stirring was continued for several hours thereafter. The resin was washed thoroughly with the buffer and then suspended in sufficient buffer so that the volume of supernatant buffer was twice that of the settled resin. The resin was allowed to stand overnight, or until ready for pouring of the column. Columns were poured in sections in the manner described by Moore and Stein[16] except that no air pressure was applied while the resin settled. Columns (1 × 35-cm. in dimension) for analytical work were poured at room temperature in jacketed tubes through which water at 5–6° later was circulated. A 2.5 × 35-cm. unjacketed column was used in a cold room at 5–6° for preparative work. After a column had been poured, the developer was passed through at 5 to 10 ml. per hr. for 4 or 5 days at room temperature or until the *p*H of the influent and effluent were equal. The temperature was then reduced to 5–6° and equilibration was continued for 2 or 3 more days. Even then the column apparently had not reached equilibrium because the relative positions of hemoglobin components were not constant until several chromatograms had been completed. A change in either the sodium ion or hydrogen ion concentration required reëquilibration of the column.

Developers.—Because hemoglobin is most stable at a neutral or slightly alkaline *p*H, a search was made for developers of *p*H 7.0 to 7.2 and of such composition that hemoglobin would be adsorbed by the resin. This result can be accomplished, as Boardman and Partridge's work[8] suggests, by lowering the sodium ion concentration. The chromatographic behavior of hemoglobin is extremely sensitive to slight changes in sodium ion concentration or *p*H, as is shown by the behavior of hemoglobin with the different

(8) N. K. Boardman and S. M. Partridge, *Biochem. J.*, **59**, 543 (1955).

(9) T. H. J. Huisman and H. K. Prins, *J. Lab. Clin. Med.*, **46**, 255 (1955).

(10) H. K. Prins and T. H. J. Huisman, *Nature*, **177**, 840 (1956).

(11) M. Morrison and J. L. Cook, *Science*, **122**, 920 (1955).

(12) J. L. Cook and M. Morrison, *Federation Proc.*, **15**, 235 (1956).

(13) W. H. Stein, H. G. Kunkel, R. D. Cole, D. H. Spackman and S. Moore, *Biochim. et Biophys. Acta*, **24**, 640 (1957). These results were kindly communicated to us by Dr. Stein prior to publication.

(14) P. C. van der Schaaf and T. H. J. Huisman, *ibid.*, **17**, 81 (1955).

(15) C. H. W. Hirs, S. Moore and W. H. Stein, *J. Biol. Chem.*, **200**, 493 (1953).

(16) S. Moore and W. H. Stein, *ibid.*, **192**, 663 (1951).

developers that are listed in Table I. Potassium cyanide was originally included in the developers in order to decrease the dissociation of ferrihemoglobin cyanide during chromatography. It was not removed from the developers when oxyhemoglobin was chromatographed because ferrihemoglobin cyanide and oxyhemoglobin have identical chromatographic behavior. Thus, traces of ferrihemoglobin (methemoglobin) in solutions of oxyhemoglobin are converted to ferrihemoglobin cyanide and do not produce slow moving extraneous zones on the column.

TABLE I

COMPOSITION OF BUFFERS USED AS DEVELOPERS

No.	*p*H at 25°	Concn. Na^+, *M*	Concn. KCN, *M*	Grams required to make 4 l. of buffer $NaH_2PO_4 \cdot H_2O$	Na_2HPO_4	KCN
1	7.22 ± 0.02	0.075	0.01	13.80	14.20	2.60
2	7.18 ± .02	.0625	.01	13.80	10.65	2.60
3	7.02 ± .02	.05	.01	13.80	7.10	2.60
4	6.91 ± .02	.05	.01	16.56	5.68	2.60

Operation of the Column.—The surface of the column was made flat by stirring and then allowing the resin to settle. A hemoglobin solution that had been dialyzed against the developer was pipetted slowly onto the column with a bent-tip pipet while 1 to 2 mm. of developer remained above the surface of the column. The sample was allowed to flow in under gravity and was washed in with developer. Usually 30 to 60 mg. of hemoglobin dissolved in 0.5 to 2.0 ml. of developer were chromatographed on the 1 × 35-cm. columns and 200 to 400 mg. in 2 to 6 ml. on the 2.5 × 35-cm. column. The flow rate of developer in the smaller columns was usually 5 ml. per hr. and 1-ml. fractions were collected. After more rapidly moving zones had emerged from a column, the development of more strongly adsorbed zones was completed with doubled flow rate and fraction size.

When a zone was to be rechromatographed the effluent was collected in a vessel chilled in ice-water and then promptly refrigerated. Only the most concentrated effluent, and not the entire effluent that contained a zone, was used for rechromatography. If the zone was to be rechromatographed with a different developer, the sample was first dialyzed against this developer in the cold.

When all zones had been washed from a column, it was used again without further treatment if the same developer had been used throughout.

Spectrophotometry.—The fractions were diluted with distilled water to 3 ml. or 5 ml. or more if the fraction were very concentrated. The optical densities were determined with a Coleman junior spectrophotometer, model 6A at 415 mμ, or with a Beckman spectrophotometer, model DU at 280 and 415 mμ. Because many readings were required, the spectrophotometric readings with the Beckman spectrophotometer were simplified by using the hydrogen lamp at both wave lengths. The values at 415 mμ with the hydrogen lamp are about 3% lower than when the tungsten lamp is used at 415 mμ.

Determination of Phenylalanine, Leucine and Isoleucine.—Samples from hemoglobin preparations or from chromatographic zones were hydrolyzed in refluxing doubly distilled 6 *N* hydrochloric acid for 22 hr. and the amounts of phenylalanine, leucine and isoleucine were determined by starch chromatography.[17]

Results and Discussion

Chromatography of Ferrihemoglobin Cyanide.—Figure 1 illustrates a typical chromatogram of oxyhemoglobin with Developer No. 1; it is also typical of ferrihemoglobin cyanide that has been oxidized at *p*H 7.0 with 0.01 *M* potassium ferricyanide. In this and other figures the fractions with optical density above 1.0 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 least diluted fraction. Two zones, A_I and A_{II},[18] are obvious; they have peaks at effluent volumes of 17 and 24 ml., respectively. Like Prins and Huisman,[10] we have never observed the third component reported by Morrison and Cook[11]: our chromatographic conditions are vastly different, however, from either Prins and Huisman or Morrison and Cook. Zone A_I usually comprises about 10% of the total. When ferrihemoglobin cyanide was oxidized at alkaline *p*H and with more concentrated ferricyanide solutions zone A_{II} gave indications of heterogeneity, but this heterogeneity depended on conditions of preparation and was partially reversed when cysteine subsequently was added to the preparation.

Table II summarizes the results that were obtained with ferrihemoglobin cyanide. The ferrihemoglobin cyanide that was used in chromatograms no. A76-A78 was prepared from one sample of oxyhemoglobin with different concentrations of potassium ferricyanide. The increased amount of zone A_I with increased concentration of potassium ferricyanide may be significant. When ferrihemoglobin cyanide is prepared at *p*H 9.0 (chromatogram no. A87) the increase in zone A_I very likely is significant because of the oxidation of sulfhydryl groups at *p*H 9.0.[19]

TABLE II

CHROMATOGRAPHY OF FERRIHEMOGLOBIN CYANIDE WITH DEVELOPER NO. 1

Chrom. no.	*p*H	Concn. of $K_3Fe(CN)_6$, *M*	% A_I	% A_{II}
A76	7.0	0.01	12	88
A78	7.0	.01	11	89
A77	7.0	.1	15	85
A87	9.0	.1	21	79
A90	7.0	.01	12	88
			% F	
A82	7.0	.01	92	8

Ferrihemoglobin cyanide prepared from umbilical cord blood of newborn infants was chromatographed in chromatogram no. A82. In this instance 92% of the hemoglobin moved like zone A_I; it was assumed to be fetal hemoglobin.

The sample for chromatogram no. A90 had been prepared by adding potassium ferricyanide to the oxyhemoglobin in an amount less than that required to oxidize it completely to ferrihemoglobin cyanide. The resulting sample was a mixture in which some of the heme groups of the hemoglobin molecules were combined with oxygen, and some were oxidized and combined with cyanide. This was indicated by the persistence of a spectral maximum at 580 mμ where a maximum is present in the spectrum of oxyhemoglobin but not in that of ferrihemoglobin cyanide. The ratio of the optical densities at 540 to 580 mμ was 1.18. This mixture chromatographed exactly as did ferrihemoglobin cyanide. The spectra of zones A_I and A_{II} had identical ratios of optical densities at 540 to 580 mμ not only in each zone as a whole but in the individual fractions that were collected. These re-

(17) W. H. Stein and S. Moore, *J. Biol. Chem.*, **176**, 337 (1948).

(18) The designations, A_I, A_{II}, and others, are given for convenience of reference in this discussion. It is not our purpose to propose a system of nomenclature.

(19) A. E. Mirsky and M. L. Anson, *J. Gen. Physiol.*, **18**, 307 (1934).

sults clearly demonstrate that there is no difference in the effect on chromatography of the hemoglobin molecule between an oxyferroheme group and a ferriheme cyanide group. It was evident that oxyhemoglobin would be chromatographically indistinguishable from ferrihemoglobin cyanide and, therefore, oxyhemoglobin was used in subsequent experiments to avoid the possibility of the formation of artifacts during the preparation of ferrihemoglobin cyanide.

Chromatography of Oxyhemoglobin.—Table III illustrates the results obtained with oxyhemoglobin and one sample of carbonmonoxyhemoglobin.

TABLE III

CHROMATOGRAPHY OF OXYHEMOGLOBIN WITH DEVELOPER NO. 1

Chrom. no.	Nature of preparation	% Non-heme protein	% A_I	% A_{II}
A91	Hemolysate, 18 hr. after drawing blood		10[a]	90[a]
A95	Same sample 6 days later		10	90
A97	Hemolysate[b]		11	89
A98	Two flawless crystals dissolved in developer[b]		14	86
A99	HbO_2, twice fractionally crystallized		10	90
A112	Fresh hemolysate	2.1[c]	9	91
A113	Same (different donor)	2.0	10	90
A115	HbCO, once crystallized	1.6	13	87
A94	Zone A_I from HbO_2		100	0
A96	Zone A_{II} from HbO_2		0	100
A111	Zone A_I from HbCO		100	0
A123	Zone A_{II} from HbO_2	0	0	100
A128	Zone A_I freed of non-heme protein	0.1	100	0

[a] The percentages in these columns refer only to the heme proteins. [b] A portion of the hemolysate that was used for chromatogram no. A97 was crystallized to produce the sample for chromatogram no. A98. [c] Percentage of the total protein of the sample. Not determined in all chromatograms.

Chromatograms no. A91, A95, A97, A112 and A113 demonstrate that freshly prepared hemolysates of red cells contain about 10% of a minor hemoglobin component (A_I) and that about 2% of a non-heme protein precedes and accompanies zone A_I. This non-heme protein easily is detected spectroscopically because its absorption spectrum has a maximum at 280 mμ but none at 415 mμ. It also has been characterized by its relative content of some amino acids and its sedimentation constant in the ultracentrifuge.[20] The amount of non-heme protein has been calculated roughly on the basis of the assumption that the absorption coefficients for hemoglobin and for this non-heme protein are the same at 280 mμ.

In preparing the sample for chromatogram no. A98, two flawless crystals about 2 or 3 mm. in length were selected from the mother liquor of an oxyhemoglobin solution that had been crystallized to the extent of only 5 to 10% by dialysis against 2.8 *M* phosphate buffer according to Drabkin's procedure.[2] These crystals were washed with 2.8 *M* phosphate buffer and dissolved in developer, and the solution was chromatographed.

(20) U. Hasserodt and J. Vinograd, private communication.

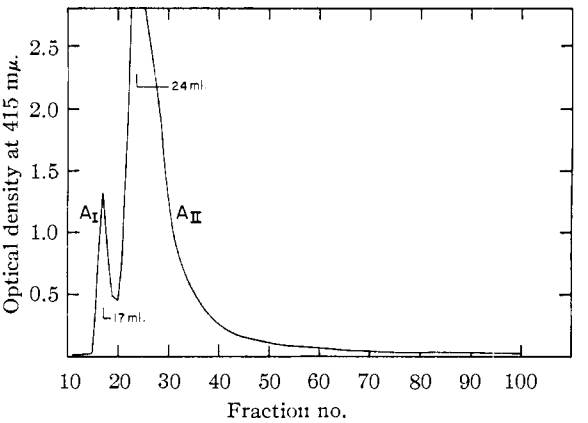

Fig. 1.—Chromatogram of adult oxyhemoglobin on a 1 × 35-cm. column of IRC-50 with developer no. 1.

Zone A_I was still present as indeed it is in twice fractionally crystallized oxyhemoglobin (chromatogram no. A99) and in crystallized carbonmonoxyhemoglobin (chromatogram no. A115). The latter also was contaminated with the non-heme protein.

Because zone A_I and non-heme protein are present in fresh hemoglobin solutions from red cells that have been hemolyzed with distilled water in the presence or absence of toluene, it is unlikely that they are artifacts of preparation. It was possible, however, that they were artifacts of chromatography and, therefore, these subfractions were rechromatographed. In chromatograms no. A94, A111 and A128 Zone A_I was rechromatographed without evidence of the formation of Zone A_{II}, and in chromatograms no. A96 and A123 zone A_{II} was rechromatographed without evidence of zone A_I. Another possibility that arises is that zone A_I is simply zone A_{II} in equilibrium with the non-heme protein. This possibility was excluded in chromatogram no. A128: zone A_I that had been almost completely separated from the non-heme protein by chromatography behaved as zone A_I and not as zone A_{II} on rechromatographing. Chromatograms no. A128 and A123 illustrate that the non-heme protein can be separated from zones A_I and A_{II} and that it is not formed by the removal of hemes from hemoglobin by the column.

Chromatography with Other Developers and the Fractionation of Zone A_I.—In order to obtain a better separation of zone A_I from A_{II} and of fetal hemoglobin from zone A_{II}, developer no. 2 was prepared. The results from a fresh hemolysate of adult red cells are presented in Fig. 2 and from a hemolysate of cord blood cells in Fig. 3. This developer is very satisfactory for preparative work on larger columns because the separation of the components is easy and complete. Although zone A_{II} is appreciably retarded when developer no. 2 is used, the peaks of zone A_I and zone F still emerge from the 1 × 35-cm. columns in 17 ml. of effluent. Therefore, both zone A_I and zone F are virtually unabsorbed by the resin in the presence of developer no. 1 or 2 because 15 ml. is approximately the hold-up volume of the 1 × 35-cm. columns. The fact that neither zone A_I nor zone F is adsorbed by the column does not constitute proof that they are chromatographically identical, and, consequently,

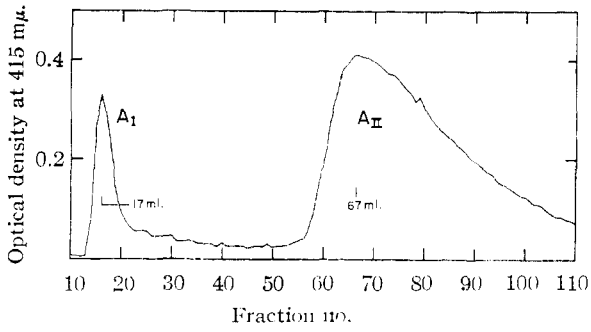

Fig. 2.—Chromatogram of adult oxyhemoglobin on a 1 × 35-cm. column of IRC-50 with developer no. 2.

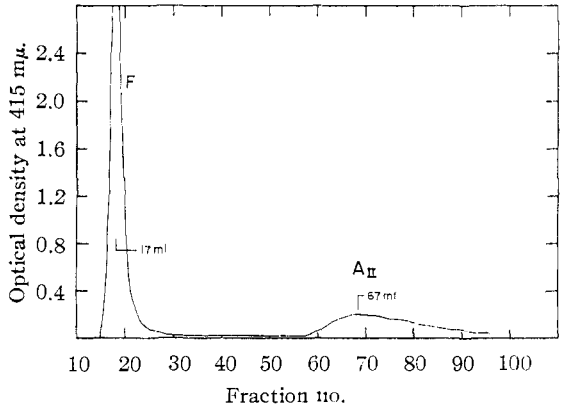

Fig. 3.—Chromatogram of cord blood oxyhemoglobin on a 1 × 35-cm. column of IRC-50 with developer no. 2.

conditions were sought whereby they might be adsorbed by the resin and effectively chromatographed. Developers no. 3 and 4 were somewhat successful in achieving this end and developer no. 4 is the more generally useful.

Figure 4 illustrates the rechromatography of zone A_I with developer no. 4. In addition to the non-heme protein, three heme-proteins are present; they emerge at the indicated effluent volumes. Zones A_{Ia}, A_{Ib} and A_{Ic} are present in the approximate ratio of 1:1:3. That the heterogeneity of zone A_I is not a result of rechromatographing is indicated by the fact that the same subfractions in zone A_I are observed when fresh oxyhemoglobin is chromatographed directly with developer no. 3. Figure 5 illustrates the rechromatography of zone F with developer no. 4 under the same conditions as shown in Fig. 4. Zone F_{II} constitutes about 80% of the fetal hemoglobin. These data alone permit the conclusion only that both zones A_I and F are chromatographically heterogeneous. The identity of any subfraction of zone A_I with a subfraction of zone F has not been established. The subfractions of zone A_I have not been rechromatographed.[20a]

The spectra of zones A_I and A_{II} are identical with that of fresh oxyhemoglobin except for increased absorption at 280 mμ in zone A_I as a result of contamination with non-heme protein. The spectra of zones A_{Ia}, A_{Ib} and A_{Ic} uniformly show a slight shift in the position of the Soret band (from

(20a) NOTE ADDED IN PROOF.—Zone F_I may be identical with a second type of fetal hemoglobin reported by A. C. Allison (*Science*, **122**, 640 (1955)); I. Halbrecht and C. Klibanski (*Nature*, **178**, 794 (1956)) and W. Künzer (*ibid.*, **179**, 477 (1957)).

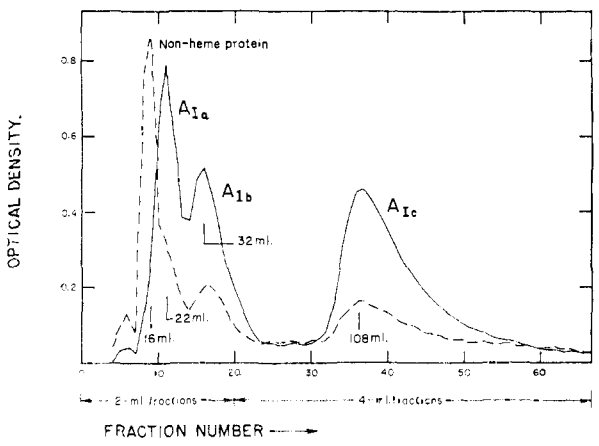

Fig. 4.—Rechromatography of zone A_I on a 1 × 35-cm. column of IRC-50 with developer no. 4: ----, optical density at 280 mμ; ———, optical density at 415 mμ.

415 to 418 mμ) and an increase in the ratio of optical density at 540 mμ to that at 580 mμ (from 0.99 to 1.1 compared to oxyhemoglobin). These changes indicate that some ferrihemoglobin cyanide has formed during the process of rechromatography.

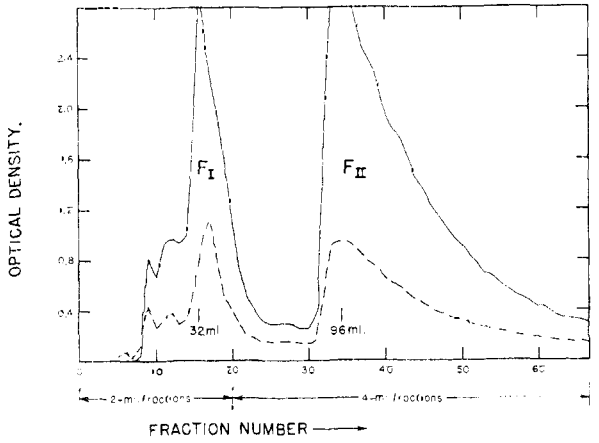

Fig. 5.—Rechromatography of zone F on a 1 × 35-cm. column of IRC-50 with developer no. 4: ----, optical density at 280 mμ; ———, optical density at 415 mμ.

Isoleucine Content of Various Hemoglobin Preparations and Fractions.—The most convincing evidence of the heterogeneity of hemoglobin is the demonstration of a difference in the amino acid content of the subfractions. Because amino acids are linked in proteins by the peptide bond, it is impossible for such a difference to be a result of denaturation. Of course, contamination by unrecognized impurities can lead to erroneous conclusions. In the present study, isoleucine determinations have been performed on uncrystallized hemoglobin, on hemoglobin crystallized in various ways, on electrophoresed hemoglobin[21] and its trailing edge, on electrophoresed zone A_I and its trailing edge, on the non-heme protein, on zone

(21) The electrophoreses were carried out by Dr. K. Bruce Jacobson in 0.05 *M* tris(hydroxymethyl)-aminomethane buffer at *p*H 8.6 by means of the column electrophoretic methods of P. Flodin and D. W. Kupke (*Biochim. et Biophys. Acta*, **21**, 368 (1956)) and J. Porath (*ibid.*, **22**, 151 (1956)).

A_{Ic} and on zone F_{II}. The results are summarized in Table IV. Because the sample of protein for hydrolysis frequently was small, it was impractical to measure the amount that was hydrolyzed. The most significant comparison, therefore, lies in the relative amounts of the three amino acids in the various preparations.

Crystallization of hemoglobin is without effect on the content of isoleucine just as it is without effect on the content of zone A_I or non-heme protein. Likewise, the procedures that must be done to prepare DNP-globin or globin do not rid the preparation of its isoleucine. Isoleucine-containing components can be removed only by electrophoresis with exclusion of trailing edge of the hemoglobin or by chromatography. No isoleucine is present in the main peak of the electrophoresed hemoglobin, and the isoleucine is concentrated in the hemoglobin and/or non-heme components of the trailing edge. Zone A_{II} contains no isoleucine and it is concentrated in zone A_I. At first glance, zone A_I might be thought to be fetal hemoglobin by comparison with zone F_{II} in which the ratio of leucine

TABLE IV

CONTENT OF SOME AMINO ACIDS IN VARIOUS HEMOGLOBIN PREPARATIONS AND FRACTIONS

Nature of preparation	μg. of amino acid phe	leu	ileu	Ratios leu/ phe	leu/ ileu
Uncrystallized hemoglobin[a]	664	1254	18	1.9	70
Crystallized oxyhemoglobin[a]	1041	1948	26	1.9	75
Crystallized carbonmonoxy-hemoglobin[a]	660	1255	19	1.9	66
Twice fractionally crystallized oxyhemoglobin[a]	1064	1988	33	1.9	60
DNP-globin[a,b]	..	1050	26	..	40
Globin[a,c]	633	1155	15	1.8	77
Main peak of electrophoresed hemoglobin (excluding trailing hemoglobin)[a]	1017	1914	0	1.9	
Trailing edge of electrophoresed hemoglobin (including hemoglobin and non-heme protein)[a]	164	307	34	1.9	9
Zone A_{II}	950	1830	0	1.9	..
Zone A_{II} (another prepn.)	565	1076	0	1.9	..
Zone A_I	524	939	93	1.8	10
Electrophoresed Zone A_I	340	622	23	1.8	27
Non-heme trailing edge electrophoresed Zone A_I	41	58	20	1.4	3
Non-heme protein	71	112	52	1.6	2
Zone F_{II}	713	1312	132	1.8	10
Zone A_{Ic}	136	270	12	2.0	23

[a] The amino acid analyses of these preparations were made by Miss Lois M. Kay. [b] A portion of the preparation used for run no. 10, Table I of ref. 6. [c] Prepared by oxalic acid method of M. L. Anson and A. E. Mirsky (*J. Gen. Physiol.*, **13**, 469 (1930)). The precipitated globin was washed well with acetone and then dissolved in water prior to lyophilizing the solution.

to isoleucine is 10. However, purification of zone A_I by electrophoresis or chromatography (zone A_{Ic}) reduces the isoleucine content to a ratio of leucine to isoleucine of about 25. Zone A_{Ic} is probably not identical with zone F_{II}, although its chromatographic behavior on IRC-50 is similar. The non-heme protein whether separated chromatographically or electrophoretically has a surprisingly high content of isoleucine relative to leucine and thus cannot be globin; its molecular weight also is greater than that of hemoglobin.[20]

Because both electrophoresis and chromatography remove minor components from hemoglobin as prepared directly by hemolysis of red blood cells, it is not unexpected that the main component should be free of isoleucine; the absence of isoleucine in the main component of electrophoresed hemoglobin is in agreement with the analysis of Stein, *et al.*[13] It is obvious from the present investigation why Schroeder, Kay and Wells[3] detected isoleucine in crystallized hemoglobin and why van der Schaaf and Huisman[14] detected it in uncrystallized hemoglobin, but it is puzzling that Rossi-Fanelli, Cavallini and de Marco[4] detected no isoleucine in hemoglobin that had been crystallized by Drabkin's procedure.

Conclusions

When uncrystallized or crystallized normal adult human hemoglobin is chromatographed under the conditions of this study, the main component that can be isolated comprises 90% of the heme proteins. It is apparent that crystallization is without avail in producing a homogeneous hemoglobin. By chromatography, though, the main component may be obtained in a purer form than by any procedure other than zone electrophoresis, but whether it consists of a single molecular species cannot be stated. It may be that the main component is "pure" for such studies as amino acid sequence in which partial denaturation or conversion to ferrihemoglobin would be of little consequence. On the other hand, it may be highly "impure," say, for oxygen dissociation studies.

In addition to the main component, both crystallized and uncrystallized adult hemoglobin contain at least three heme proteins and one non-heme protein. None of the heme proteins corresponds to the most slowly moving component described by Morrison and Cook[11]; zone A_I may be the same as their fastest moving component. Although the characterization of the three minor heme proteins of the present investigation is incomplete, none of them can be identified definitely with fetal hemoglobin, which on the basis of alkali denaturation[22] and immunochemical techniques[23] is reported to be present in adult blood.

Cord blood hemoglobin also contains minor components. Our results are in better agreement with those of Prins and Huisman[10] than with those of Cook and Morrison.[12]

The methods of the present investigation permit the ready isolation of the main components of adult and cord blood hemoglobin on a preparative scale and as such should be useful for obtaining purer hemoglobins for chemical investigation than can be obtained even by crystallization. The methods also should be capable of extension to permit the isolation of sufficient amounts of the minor components for more complete characterization. If

(22) K. Singer, A. I. Chernoff and L. Singer, *Blood*, **6**, 413 (1951).
(23) A. I. Chernoff, *ibid.*, **8**, 399 (1953).

the methods were applied to the hemoglobin mixtures that are known to occur in certain pathological conditions, it probably would be possible to determine whether entirely different components are present or whether components normally present in minor amount have simply been increased proportionately.

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Metabolites of Nicotine and a Synthesis of Nornicotine

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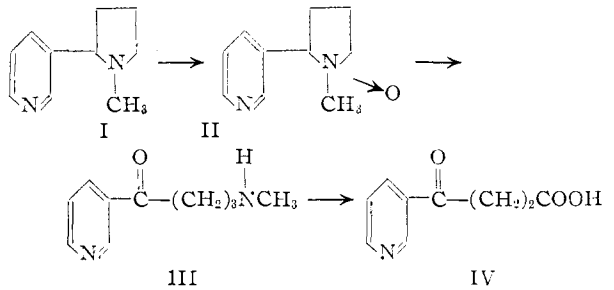
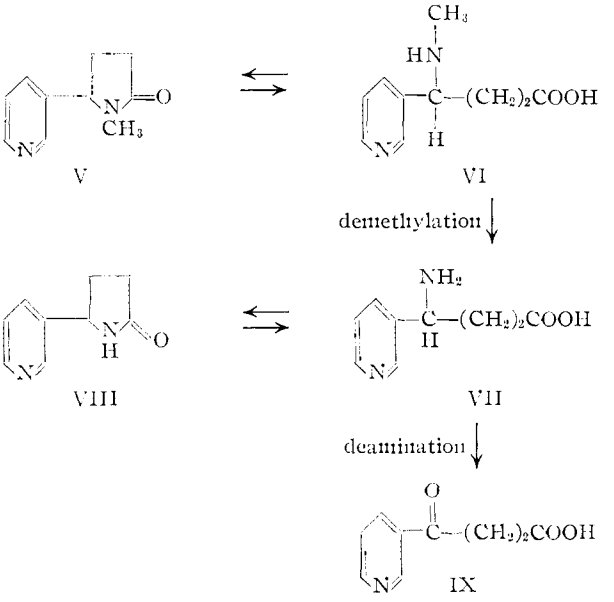
Cotinine is hydrolyzed by the action of strong barium hydroxide to γ-(3-pyridyl)-γ-methylaminobutyric acid, a possible intermediate in the biological degradation of nicotine. Zinc dust reduction of γ-(3-pyridyl)-γ-oximinobutyric acid gives a mixture from which γ-(3-pyridyl)-γ-aminobutyric acid and desmethylcotinine were obtained. The latter yields the corresponding amino acid upon hydrolysis. Nornicotine is obtained by reduction of desmethylcotinine with lithium aluminum hydride.

The demonstration by Wada and Yamasaki[2] that soil bacteria can degrade nicotine to γ-(3-pyridyl)-γ-oxobutyric acid has given rise to a number of concepts concerning the mechanism of metabolic degradation of the pyrrolidine ring of nicotine. Wada and Yamasaki[2] believed that nicotine (I) was first oxidized to oxynicotine (II) by the microbial organism, "or merely by the action of aeration." Subsequent steps, according to these authors, involved the formation of pseudo oxynicotine (III) and γ-(3-pyridyl)-γ-oxobutyric acid (IV).



As a result of the isolation of cotinine[3-5] from fermented leaves of tobacco, Frankenburg, Gottscho and Vaitekunas[3,5] have suggested γ-3-pyridyl-γ-methylaminobutyrylaldehyde as a precursor of cotinine, and Werle, *et al.*,[6] considered γ-3-pyridyl-γ-methylaminobutyric acid a possible precursor of the keto acid IV. Since cotinine itself has been obtained[4] from samples of autoxidized nicotine as well as from fermented tobacco, cotinine may play a key role in the metabolism of nicotine according to the scheme



This study was designed to provide synthetic compounds which would facilitate the acceptance or rejection of such an hypothesis. In addition the suggestion of Larson and Haag[7,8] that a compound such as γ-(3-pyridyl)-γ-methylaminobutyric acid might occur in urine of dogs following administration of nicotine gave further impetus to our investigation.

Using nicotine as a starting material, Pinner[9,10] many years ago obtained cotinine from the zinc dust reduction of dibromocotinine. γ-(3-Pyridyl)-

(1) Presented in part at the 131st meeting of the American Chemical Society, Miami, Florida, April 11, 1957. Aided by a grant from the Tobacco Industry Research Committee.

(2) E. Wada and K. Yamasaki, THIS JOURNAL, **76**, 155 (1954).

(3) W. G. Frankenburg, A. M. Gottscho and A. A. Vaitekunas, *Proc. First Internat. Sci. Cong. on Tobacco*, **2**, 419 (1955).

(4) W. G. Frankenburg and A. A. Vaitekunas, THIS JOURNAL, **79**, 149 (1957).

(5) W. G. Frankenburg, A. M. Gottscho and A. A. Vaitekunas, Abstract of Papers, Tobacco Chemists Research Conference, Oct. 6–7, 1955, Raleigh, N. C.

(6) K. Werle, H. Schievelbein and D. Spieth, *Arzneimittel-Forsch.*, **6**, 322 (1956).

(7) P. S. Larson and H. B. Haag, *J. Pharmacol. Exptl. Therap.*, **76**, 240 (1942).

(8) P. S. Larson, H. B. Haag and J. K. Finnegan, *ibid.*, **86**, 239 (1946).

(9) A. Pinner, *Ber.*, **26**, 292 (1893).

(10) A. Pinner, *Arch. Pharm.*, **231**, 378 (1893).