The Chromatography of Hemins from Normal and Abnormal Human Erythrocytes


FISCHER determined the chemical structure of protohemin, the prosthetic group of hemoglobin, in 1929. Since that time, it has been widely assumed that protohemin is a homogeneous compound and that its chemical structure does not vary in health or in disease. This assumption has survived an era noted for the discovery of molecularly abnormal human hemoglobins and the evolution of the concept of "molecular disease." In the case of sickle hemoglobin, the first example of this group of diseases, the molecular change was localized to the protein portion of the molecule, and the hemin was found to be normal. Thus, the concept of protohemin homogeneity remained unchallenged until the preliminary report of Morrison and Cook. These investigators were able to separate erythrocyte hemins into as many as four components, using column and paper chromatography.

The potential physiologic importance of this observation cannot be ignored. Further, the possibility that molecular changes in protohemin could play a role in the pathogenesis of diseases, especially anemias associated with disordered heme synthesis, is an intriguing one.

The investigations to be described were undertaken to evaluate the reported heterogeneity of protohemin and the possible significance of this finding in health and disease. A preliminary report of some of these investigations has appeared in abstract.

Materials and Methods

Preparation of Hemins

Hemins were extracted from erythrocytes by a modification of the method of Anson and Mirsky and were precipitated and dried by modifications of the methods of Connolly et al. Three ml. of whole, washed erythrocytes were added dropwise to 50 ml. chilled acid-acetone, freshly prepared by adding 0.6 ml. concentrated HCl to 100 ml. re-distilled, reagent grade acetone. The mixture was homogenized in a Waring Blender for 60 seconds, then centrifuged and the precipitate discarded. Concentrated NH₄OH was added to the supernate until an olive-green precipitate appeared. The mixture was centrifuged and the precipitate dried under a nitrogen stream. The mixture was centrifuged and the precipitate dried under a nitrogen stream. The precipitate was washed successively with 0.6 N HCl, 0.3 N HCl and distilled water and was dried overnight in a vacuum desiccator. Specimens were stored in a freezer. The spectrum of the reduced pyridine hemochromogen prepared from such specimens was characterized by spectral maxima at 547 and 525 μμ.

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Three different procedures were used to produce preparations rich in \( \beta \)- and \( \gamma \)-hematins. These methods were modified from those of Hamsik and of Lemberg and Legge. In all three methods, the starting material was hemin prepared by the method of Fischer and recrystallized by the method of Shemin et al. 

1. Approximately 100 mg. hemin were dissolved in 5 ml. pyridine, and the solution allowed to stand at room temperature for 24 hours. The pyridine was allowed to evaporate and the resulting solid was dried in a vacuum desiccator.

2. Approximately 25 mg. hemin were dissolved in 30 ml. 0.05 N NaOH. The mixture was heated to 80°C. and concentrated HCl was added until a red-brown precipitate formed. The suspension was kept at 80°C. for 2 hours and at room temperature for an additional 24 hours, after which it was centrifuged and the precipitate dried in an oven at 105°C.

3. Approximately 25 mg. hemin were dissolved in 10 ml. absolute methanol to which several drops of saturated methanolic NaOH had been added. An excess quantity of glacial acetic acid was added. The precipitate which appeared after the mixture stood for 24 hours at room temperature was harvested and dried in an oven at 105°C.

Coprohemin, mesohemin, deuterohemin, and hematohemin were prepared from their respective porphyrins by the method of Erdman and Corwin.

**Column Chromatography**

To 6 Gm. silicic acid, prepared according to the method of Morrison and Stotz, were added 2.4 ml. 0.15 N HCl. After thorough mixing, 9.6 ml. redistilled CHCl₃ were added. The suspension was rinsed into a chromatographic column of 0.9 cm. internal diameter and allowed to settle into a column about 10 cm. in height.

Hemin was prepared for chromatography by adding one drop of pyridine to 0.6 mg. of the dried hemin. After several minutes, 0.5 to 1.0 ml. chloroform was added. This solution was layered onto the surface of the silica.

The chromatogram was developed with an increasing concentration gradient from 0 to 8.5 volumes per cent ethanol in chloroform. For this purpose a three-chambered system of reservoirs was used. Eighty-five ml. redistilled chloroform were poured into a mixing chamber attached to the column. After 10 ml. had flowed through the column, the mixing chamber was sealed from the atmosphere in order to form a constant volume vessel, and a supply reservoir containing 5.75 volumes per cent ethanol in chloroform was allowed to communicate with it. After an additional 40 ml. of flow, the supply reservoir was replaced with one containing 15 volumes per cent ethanol in chloroform.

The per cent transmittance of the column eluate at 405 μm was monitored by a continuous flow absorption meter.*

**Paper Chromatography**

Reverse phase paper chromatography was performed by the method of Chu and Chu, utilizing siliconized filter paper and a mixture of 5.5 ml. water, 0.1 ml. n-propanol and 0.4 ml. pyridine as the developing solvent. Hemin dissolved in acid-acetone was inserted at the origin in the form of a line, and the solvents were allowed to ascend for about 90 minutes. In order to intensify the bands thus formed, the dried chromatogram was sprayed with a solution of 50 mg. dimethoxybenzidine in 100 ml. methanol to which 3 volumes per cent of 3 per cent \( \text{H}_2\text{O}_2 \) had been added.

**Absorption Spectra**

Reduced pyridine hemochromogen spectra were determined on a Cary Recording Spectrophotometer, Model 15. The hemochromogen was prepared as described by Connelly et al.*

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*Manufactured by Gilson Medical Electronics, Middleton, Wis. The instrument was modified by replacing the Corning #7-54 filter between sample and light source with a Corning #5-58 filter.
CHROMATOGRAPHY OF ERYTHROCYTE HEMINS

Fig. 1.—Per cent transmittance at 405 mμ (— — —) and concentration of ethanol in the developing solvent (— — —) are shown relative to the volume of column effluent. The per cent transmittance curve represents data from nine normal subjects and was obtained by averaging transmittance values at selected eluate volumes. The range of volumes at which peaks appeared in the nine determinations is indicated by the limit markers above each peak. For explanation of "A," "B" and "C," see text.

RESULTS

Hemins from Normal Erythrocytes

Silicic acid chromatography. The results of column chromatography of hemins prepared from the erythrocytes of nine normal human subjects are shown in figure 1. Three fractions were noted: a small, rapidly migrating fraction, eluted at about 6–7 ml., designated "A"; a major fraction of intermediate mobility, eluted at about 40 ml., designated "B"; and a slowly moving fraction, eluted at about 70 ml., designated "C." A portion of the hemin remaining at the top of the column was not eluted, even with concentrations of ethanol as high as 15 volumes per cent. This portion was designated "D." Each of the hemin preparations from nine subjects contained all four frac-
Table 1.—Spectral Maxima for Reduced Pyridine Hemochromogens of Various Hemin Preparations

<table>
<thead>
<tr>
<th></th>
<th>I (mλ)</th>
<th>II (mλ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythrocyte hemins</td>
<td>557</td>
<td>525</td>
</tr>
<tr>
<td>Fraction “A”</td>
<td>557</td>
<td>525</td>
</tr>
<tr>
<td>Fraction “B”</td>
<td>557</td>
<td>525</td>
</tr>
<tr>
<td>Fraction “C”</td>
<td>557</td>
<td>525</td>
</tr>
<tr>
<td>Mesohemin</td>
<td>545</td>
<td>517</td>
</tr>
<tr>
<td>Coprohemin</td>
<td>546</td>
<td>517</td>
</tr>
<tr>
<td>Hemohemin</td>
<td>548</td>
<td>519</td>
</tr>
<tr>
<td>Deuterohemin</td>
<td>550.5</td>
<td>517</td>
</tr>
</tbody>
</table>

Repeated chromatography of the rapidly migrating fraction “A” enabled isolation of a chromatographically homogeneous material free of slowly migrating components. However, neither fraction “B” nor “C” could be made to be homogeneous by this technic. Each subsequent attempt at chromatography of these fractions produced the full four-component pattern.

The pyridine hemochromogen spectra (table 1) of the purified fraction “A” and the rechromatographed but non-homogeneous fractions “B” and “C” did not differ from one another nor from the spectrum of the original extract.

Paper chromatography. Normal erythrocyte hemins separated into three components on paper chromatograms (fig. 2, A). Again, the major fraction had intermediate mobility.

When fraction “A,” prepared by repeated column chromatography as described above, was subjected to paper chromatography, all material remained at the origin (fig. 2, B).

**Effect of Carbon Monoxide**

The possibility that oxidative changes in the hemin molecule resulted from

![Fig. 2.—Reverse phase paper chromatography of normal erythrocyte hemins. A. Hemins derived from normal erythrocytes. Three fractions are seen. B. Paper chromatography of fraction “A.” All material remains at origin.](image-url)
Fig. 3.—Silicic acid column chromatography of normal hemins before and after exposure to acid-methanol. The rapidly migrating fraction “A” is markedly increased after exposure.

liberation of free oxygen during cleavage of the heme-globin linkage in oxyhemoglobin was considered. Consequently, carbon monoxide gas was allowed to bubble through two blood samples until all the hemoglobin was converted to carboxyhemoglobin as measured spectrophotometrically. Hemins prepared from these specimens were subjected to column and paper chromatography. No differences were noted in the pattern produced as compared with specimens which had not been so treated.

Results with Special Hemin Preparations

Effect of acid-methanol exposure. A hemin preparation known to give the previously described pattern on column chromatography was dissolved in absolute methanol to which several drops of concentrated HCl had been added. The mixture was allowed to stand overnight at room temperature, and the hemin was precipitated and dried as outlined in Methods. The results of silicic acid chromatography before and after acid-methanol exposure appears
Fig. 4.—Radiochromatography of normal hemins after exposure to methanol-C\textsuperscript{14}. The only hemin fraction containing radioactivity is that which remains at the origin. Radioactivity at the solvent front corresponds to free methanol not attached to hemin.

in figure 3. The relative proportion of hemin migrating as fraction “A” was markedly increased by exposure to acid-methanol.

This fast-moving fraction was purified by repeated chromatography until it behaved as a homogeneous compound in both the column and paper chromatographic systems. The material so prepared was submitted for microanalysis\textsuperscript{*} and was found to contain 7.8 per cent methoxy groups. This value is intermediate between the theoretical proportion of 9.1 per cent for dimethyl protohemin and 4.7 per cent for monomethyl protohemin, suggesting either a mixture of the two esters or contamination by other substances.

Acid-methanol exposure as described above was repeated using methanol-C\textsuperscript{14}. The resulting compounds were subjected to paper chromatography and the chromatogram was scanned for radioactivity with the apparatus described by Berliner et al.\textsuperscript{15} As is illustrated in figure 4, the only hemin fraction containing radioactivity was that which remained at the origin, the site at which fraction “A” is found. Radioactivity was also detected at the solvent front, probably because of the presence of free methanol-C\textsuperscript{14} at this site. When the

\textsuperscript{*}Performed by the University of Melbourne Microanalytical Service, Melbourne, Australia.
labeled solvent was chromatographed in the absence of hemins, all radioactivity appeared at the solvent front.

Effect of pH. A hemin preparation was dissolved in 0.14 M sodium bicarbonate and divided into two portions. Concentrated HCl was added to one portion to bring the final concentration to about 4 N HCl. The precipitate which formed was allowed to stand in the mother liquor for 2 hours, and was then washed with 4 N HCl and dried under vacuum. The second portion was brought to pH 7 by adding 0.5 M phosphate buffer. The precipitate which formed was allowed to stand in the mother liquor for 2 hours and was then washed with the phosphate buffer and dried under vacuum. The magnitude of fraction “A” after column chromatography of the two preparations was compared with that of the original hemin (fig. 5, A). Fraction “A” was increased by the acid exposure and disappeared on exposure to pH 7 buffer.

In order to investigate the effect of column pH, water, 0.15 N HCl and 1.0 N HCl were used in the preparation of silicic acid for chromatography. The pH of the aqueous phase of columns formed from these materials was determined with indicator dyes (table 2). When the acid-exposed hemin from the previous experiment was subjected to chromatography on these columns, the magnitude of fraction “A” was found to increase as the pH decreased (fig. 5, B).

Storage in acid-acetone. Hemin was extracted in the usual way from whole erythrocytes, and the acid-acetone extract was allowed to stand at room temperature for 48 hours before continuing the preparative process. The column chromatogram produced by this preparation is shown in figure 6. A marked increase in the slowly moving fraction “C” was noted, as was the appearance of certain new minor peaks. In addition, there was an apparent increase in the insoluble fraction “D” on the column surface, although this fraction was not
Table 2.—pH of Silicic Acid for Chromatography

<table>
<thead>
<tr>
<th>Aqueous Solvent*</th>
<th>pH</th>
<th>Indicators</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled H₂O</td>
<td>3.5-4</td>
<td>cresol red, methyl violet</td>
</tr>
<tr>
<td>0.15 N HCl</td>
<td>2</td>
<td>methyl red, congo red</td>
</tr>
<tr>
<td>1.0 N HCl</td>
<td>&lt;1.0</td>
<td>picric acid</td>
</tr>
</tbody>
</table>

*2.4 ml of the designated solvent were added to 6 Gm. silicic acid after which 9.6 ml.
of chloroform were added.

quantitated. The paper chromatogram (fig. 7, B) was also markedly altered.

Two possible explanations for the altered pattern were considered: (1) the formation of β- and γ-hematin during the storage procedure, and (2) conversion of protohemin to other hemins, particularly mesohemin, deutero-

hemin or hematohemin.

Results with β- and γ-hematin. To investigate the first possibility, prepara-

tions designed to be rich in β- and γ-hematin were made as described in

Methods and subjected to column chromatography. With all three prepara-

tions, the insoluble fraction at the column top (fraction "D") constituted the

major portion of the hemin. This phenomenon was so striking that 5 to 10

times the usual amount of hemin was required in order to obtain a pattern

in which the other fractions could be evaluated. The patterns produced by

this excess quantity are illustrated in figure 8, B, C, and D. In all cases, frac-

tion "C" was increased in relative proportion to fraction "B" as compared with

the normal pattern (fig. 8, A).

On paper chromatograms, the hemin in these preparations remained at the

origin.

Results with protohemin derivatives. In order to investigate the possibility

of mesohemin, hematohemin or deuterohemin formation during the storage

period, the chromatographic behavior of these protohemin derivatives was

studied. Column chromatographic patterns appear in figure 9, along with that

of coprohemin.

Several observations can be made from these patterns. First of all, there is

a marked similarity of the patterns produced by hematohemin and deutero-

hemin to that produced by hemins derived from erythrocytes. All these

chromatograms are composed of three peaks and, although the peaks are of

different magnitudes, their mobilities are quite similar. On the other hand,

the patterns produced by mesohemin and coprohemin differ from one another

and from the patterns of the other three hemins studied. Mesohemin ex-

hibits only two peaks, and the major fraction has a much more rapid mobility

than that of the other hemins. By contrast, coprohemin shows possible sepa-

ration into two components, and the major component has slow mobility; in

fact, its mobility corresponds with that of fraction “C” noted in normal

erthrocyte hemin preparations.

As is shown in figure 7, paper chromatography revealed that the mobility

of mesohem in corresponded to that of the rapidly migrating fraction in eryth-

rocyte hemins. In addition, the mobility of hematohem corresponded to

that of one of the fractions appearing after storage of hemin in acid-acetone.

Spectral curves provide another means of distinguishing among the hemins,
Fig. 6.—Silicic acid column chromatography of normal erythrocyte hemins after storage in acid-acetone. Note the increase in the slowly moving fraction “C” after storage.

and the spectral maxima for the reduced pyridine hemochromogen derivatives of each of the special preparations is given in table 1. The spectral characteristics of all these protohemin derivatives are distinctly different from those of hemins derived from erythrocytes.

Results with Hemins from Abnormal Erythrocytes

Erythrocyte hemins from a variety of diseases in man as well as from certain experimental conditions in swine were studied (table 3). These diseases provided a selection of hypochromic and microcytic anemias, as well as other conditions in which disordered heme synthesis might be pathogenetically important. Hemins derived from the abnormal hemoglobin reported by Reissman et al.16 were of interest because this hemoglobin demonstrated abnormalities of spectrum, heme-heme interactions and oxygen-carrying capacity. In addition, hemins from normal and abnormal myoglobin17,18 were studied.

In all cases the columns and paper chromatographic patterns produced
Fig. 7.—Reverse phase paper chromatography of various hemin preparations. A. Hemins from normal erythrocytes. B. Normal erythrocyte hemins after acid-acetone storage. C. Mesohemin. D. Deuterohemin. E. Hematohemin. F. Coprohemin. Could not be distinguished from those produced by normal erythrocyte hemins.

Discussion

Chromatographic heterogeneity of hemins extracted from erythrocytes into acid-acetone was consistently observed, confirming the observations of others. The heterogeneity was not altered by prior treatment of the erythrocytes with carbon monoxide and therefore does not result from oxidation of the hemin by liberated free oxygen. Several conclusions may be advanced concerning the nature of the observed components.

The chromatographic behavior of fraction “A” is that of a non-polar molecule: it travels with the organic solvent front during silicic acid chromatography in which the aqueous phase is stationary, and it remains at the origin on reverse-phase paper chromatograms in which the aqueous phase is mobile. The polarity of the protohemin molecule depends primarily on the state of ionization of its propionic acid carboxyl groups. Chemical conditions preventing ionization of these groups would result in decreased polarity of the molecule. Such conditions were observed to result in increased amounts of fraction “A.” This phenomenon is illustrated by the increase in fraction “A” that occurred following exposure to acid and methanol. The results of microanalysis of frac-
tion “A” after such exposure show that it is largely composed of esters. That the esterification took place after cleavage from the globin is shown by the C\textsuperscript{14}-methanol experiment in which all labeled hemin had the mobility of fraction “A.” Esterification of the protohemin molecule is a reaction involving the carboxyl group of the propionic acid side chains, and one which would make ionization of these groups impossible.

It is possible that esterification occurs during acid-acetone extraction because of the presence of methanol contaminating the acetone. Reagent grade acetone may contain as much as 0.04 per cent methanol and yet meet American Chemical Society standards.\textsuperscript{19} The boiling points of acetone and methanol are so similar that separation by distillation is difficult. We found that acetone that had been redistilled in glass 3 times contained traces of methanol by a spot test.\textsuperscript{20}

The effect of pH on fraction “A” can also be explained by the effect produced on the propionic acid groups. At a low pH, the carboxyl groups are not ionized, and again the molecule would be non-polar. This accounts for the observed increase in fraction “A” on acid exposure as well as the inverse relationship between the magnitude of fraction “A” and column pH.

The slowly moving fraction “C” and the immobile fraction “D” are probably \(\beta\)- and \(\gamma\)-hematins. These compounds are polymers of half-anhydrides of the hydroxyhemin molecule and are poorly soluble in many solvent systems, both polar and non-polar.\textsuperscript{8} Preparations rich in these polymers indeed demonstrated increased proportions of fractions “C” and “D.” It is possible that conversion of polymers to the monomolecular form occurs on the column surface, accounting for the characteristic broad shape of the fraction “C” peak. This phenomenon might also explain the difficulty in purifying fraction “C” by
rechromatography. The insoluble character of these compounds explains their failure to migrate in the paper chromatographic system.

It is noteworthy that conditions used to prepare β- and γ-hematins involve a period of storage in a number of solvent systems (see Methods). Thus, the increase in fractions “C” and “D” following acid-acetone storage of normal erythrocyte hemins may be ascribed to the increased time available for polymerization. The presence of these fractions in preparations that had not been stored suggests that polymerization occurs to some extent, even without storage.

No direct evidence is given for the nature of fraction “B.” However, it constitutes the major fraction of erythrocyte hemins, is more polar than protohemin esters and is more soluble than polymerized hemins. It is postulated, therefore, that it consists of monomolecular hemin with one or both of the propionic acid carboxyl groups ionized.

Only tentative conclusions can be drawn regarding the presence of certain hemin derivatives in erythrocyte extracts. One fraction observed on paper chromatography of erythrocyte hemins is similar to that of mesohemin. However, one would expect to be able to detect the presence of mesohemin by means of column chromatography because its mobility in that system is unique. It is concluded, therefore, that mesohemin is not present in normal erythrocyte hemin preparations.

The mobility of hematohemin corresponds with that of one of the hemin fractions appearing after acid-acetone storage on paper chromatography. Column chromatography is not useful in the detection of hematohemin because the pattern produced by this compound does not differ significantly from that produced by protohemin. The same is true for deuterohemin. Furthermore, it would be difficult to detect these hemins by changes in spectra unless large quantities were present. Thus, one cannot exclude the possibility
that hematohemin and deuterohemin were formed during acid-acetone storage.

The mobility of coprohemin on column chromatography corresponds with that of fraction “C.” However, it is doubtful that the presence of fraction “C” in extracts of normal erythrocytes can be explained by the presence of coprohemin, because no difference in the pyridine hemochromogen spectrum of this fraction was detected. In addition, the presence of a very rapidly moving fraction on paper chromatography would be expected in the presence of coprohemin.

The finding of heterogeneity of hemin in various chromatographic systems, then, is seen to be a product of a number of factors unrelated to the chemical structure of hemin when attached to globin. These factors include esterification of the propionic acid carboxyl groups by traces of methanol in the organic solvents used, effects of pH on the ionization of these groups, and polymerization of the hydroxyhemin molecule. Also possible, but not proven, is the conversion of protohemin to other hemins on acid-acetone storage.

We failed to find an abnormal chromatographic pattern among hemins from examples of hematologic disorders. This, of course, does not exclude the possibility of finding such an abnormality in the future. One might cite the case of Garby et al. as an example of the types of disorders in which abnormal hemins should be looked for.

Some of the factors reported here should be applied to previous reports involving chromatography of hemins. Certain of the components noted by Connelly et al. in the hemins of beef heart muscle might be methyl esters, especially since solution in methanol preceded chromatography. Further, the “protohemin esters” found in extracts of kidney, liver, intestine, and spleen might be similarly explained.

A paper chromatographic system used for analysis of hemin extracted by acid-acetone from human and dog erythrocytes has been reported to resolve three components, the chemical natures of which were not defined. Variation in the relative proportions of these fractions was noted during the course

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Table 3.—Hemins in Human Disease and in Experimental Conditions

<table>
<thead>
<tr>
<th>Source of Specimen</th>
<th>Number of Cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iron deficiency anemia</td>
<td>3</td>
</tr>
<tr>
<td>Thalassemia major</td>
<td>1</td>
</tr>
<tr>
<td>Thalassemia minor</td>
<td>2</td>
</tr>
<tr>
<td>Hereditary spherocytosis</td>
<td>1</td>
</tr>
<tr>
<td>Anemia of infection</td>
<td>1</td>
</tr>
<tr>
<td>Congenital non-spherocytic hemolytic anemia</td>
<td>2</td>
</tr>
<tr>
<td>Acquired hemolytic anemia</td>
<td>3</td>
</tr>
<tr>
<td>Hemoglobin SC disease</td>
<td>1</td>
</tr>
<tr>
<td>Abnormal hemoglobin</td>
<td>1</td>
</tr>
<tr>
<td>Myoglobin—normal muscle</td>
<td>1</td>
</tr>
<tr>
<td>Myoglobin—hereditary myoglobinuria</td>
<td>1</td>
</tr>
<tr>
<td>Normal pig</td>
<td>2</td>
</tr>
<tr>
<td>Copper-deficient pig</td>
<td>2</td>
</tr>
<tr>
<td>Pyridoxine-deficient pig</td>
<td>2</td>
</tr>
</tbody>
</table>

*We are indebted to Dr. V. E. Ruth, V. A. Hospital, Kansas City, Mo., for this specimen.

†We are indebted to Dr. Gerald Perkoff for these specimens.
of hemorrhagic anemia induced in dogs, and the authors suggested that each fraction might have a different life span. Many of the factors affecting hemin heterogeneity in our studies are applicable to other chromatographic systems as well. Before attributing physiologic or pathogenetic significance to heterogeneity reported with a chromatographic system, one must be certain that the findings did not result from the factors reported here.

**Summary**

1. Erythrocyte hemins, extracted with acid-acetone, exhibited multiple components on two chromatographic systems. However, this heterogeneity can be explained by various physicochemical properties of hemin in solution and does not imply that heme, when attached to globin in the native hemoglobin molecule, is heterogeneous.

2. The presence of a non-polar fraction may be accounted for by esterification of protohemin as a result of reaction with small amounts of methanol contaminating the acetone and/or the presence of some un-ionized protohemin molecule in solutions of low pH.

3. The presence of poorly soluble fractions is probably the result of polymerization of the hydroxyhemin molecule into so-called $\beta$- and $\gamma$-hematins.

4. The formation of mesohemin is considered unlikely, but the formation of deuterohemin or hematohemin cannot be excluded.

5. No changes in hemin chromatographic patterns were noted in disease.

6. It is necessary to evaluate reports concerning chromatographic hemin heterogeneity in the light of the artifacts described above before attributing physiologic or pathogenetic significance to the findings.

**Messaggio in Interlingua**

1. Heminas erythrocytic, extrahite con acido-acetona, exhibiva multiple componentes in duo systemas chromatographic. Tamen, iste heterogeneitate pote esser explicite a base de varie proprietates physicochimic del parte de hemina in solution e non require le supposition que hem, quando illo es attachate a globina in le native molecula de hemoglobina, es un substantia heterogenee.

2. Le presentia de un non-polar fraction pote esser attribuite a esterification de protohemin como resultato de reaction con micre quantitates de methanol que contamina le acetona e/o al presentia de alicun non-ionisate molecules de protohemin in solutiones de basse pH.

3. Le presentia de mal solubile fractiones es probablemente le resultato de polymerisation del molecula de hydroxyhemina in le si-appellate hematinas $\beta$ e $\gamma$.

4. Le formation de mesohemina es reguardate como improbabile, sed le formation de deuterohemina o hematohemina non pote esser excludite.

5. Nulle alterationes in le configuration chromatographic de hemina esseva notate in le presentia de morbo.

6. Es necessari evalutar reportos concernente un heterogeneitate chromato-
graphic de hemina in le lumine del artifactos supra-describite ante que on attribue un signification physiologic o pathogenetic al constatationes.

REFERENCES

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The Chromatography of Hemins from Normal and Abnormal Human Erythrocytes

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