Some Characteristic Properties of Hemoglobin C

By T. H. J. Huisman, P. C. van der Schaaf and A. van der Sar

Thanks to the extensive use of physico-chemical methods, electrophoresis in particular, many abnormal types of hemoglobin have been discovered in the last few years. Some have been found in Negroes (Hb-S, Hb-C, Hb-D), while another abnormal hemoglobin (Hb-E) has been noted in a race in East Asia (Thailand). One of the hemoglobins occurring in Negroes (hemoglobin C) was discovered a few years ago by Itano and Neel. They described two Negro families, in whom the combinations of Hb-A and Hb-C (heterozygous Hb-C disease), and of Hb-S and Hb-C (sickle-cell hemoglobin-C disease) were present. Later other investigators also described this sickle-cell Hb-C disease. According to Motulsky et al., the occurrence of Hb-C in association with another type of hemoglobin is not at all rare and is found in 1.4–3 per cent of the American Negro population.

Homozygous hemoglobin-C disease was first described by Spaet et al., in 1953. This observation was confirmed soon afterwards. The disease was observed not only in the United States but also in a Negro family in Algiers. Homozygous hemoglobin-C disease is characterized by a mild, normochromic, normocytic anemia; a slight reticulocytosis; a great number of target cells; an increased osmotic fragility; a sometimes marked splenomegaly; a mild hyperbilirubinemia and an increased urobilinogen excretion in the feces. A definite diagnosis may be made with the aid of electrophoresis, as the electrophoretic mobility of Hb-C is greater than that of Hb-A or Hb-S. Further, it should be noted that this abnormal hemoglobin shows no increased resistance against alkali, while tests for sickle cell formation are negative. Differentiation between Hb-C and hemoglobin D, recently discovered by Itano, can also be made by electrophoresis, since the mobility of Hb-D is similar to that of Hb-S and therefore less than Hb-C. The present paper will describe two families, residing on the island of Curacao (Netherlands Antilles). In addition it will describe the results of amino acid analysis of hemoglobin taken from the blood of four patients with homozygous Hb-C disease; this is a follow-up of previous work from this laboratory concerning differences in the amino acid composition of Hb-A.

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The authors are indebted to Professor J. H. P. Jongis for his advice and criticism during these experiments and for his help in the preparation of the manuscript; to Dr. H. I. Scheinberg of New York for placing at our disposal a sample of pure Hb-C; to Miss Andree Dozy, for her capable technical assistance. One of us (P. C. v.d. S.) holds a grant from the Netherlands Organization for Pure Research (Z.W.O.).

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and Hb-F. The data obtained will be compared with the analysis of Hb-A and Hb-F and also of the hemoglobin from four patients afflicted with sickle-cell anemia.

A few differences in the amino composition of Hb-C will be pointed out. Attention will also be directed toward two other characteristic properties of this hemoglobin, namely the reduced solubility of the carbonmonoxy form in phosphate buffer and the abnormal behavior of Hb-C, while separating from various hemoglobins during ion exchange chromatography.\(^\text{13}\)

REPORT OF CASES

Family Z

A. Z., a 34 year old male, apparently white, was first treated in 1945 for gastro-intestinal complaints. Physical examination revealed an enlarged spleen, extending two fingers' breadths below the costal margin. No other abnormalities were noted. The blood picture showed a rise in hemoglobin and R.B.C. with a slightly elevated number of reticulocytes. Sternal puncture showed a marked increase in the erythrocytic series while the leukocytic series was normal. Until August 1954 the clinical course was uneventful. The spleen remained constant in size; hemolytic crises were not observed. The hematologic data ranging over these 8 years are listed in table 1. It will be noted that the hemoglobin rise was not observed again. It seemed probable that we were dealing with a hemolytic syndrome, as indicated by the increased serum bilirubin, the increased osmotic fragility, and the occurrence of a great number of target cells. Sickle cell tests were all negative. The diagnosis of homozygous Hb-C disease was confirmed by electrophoretic and chromatographic experiments.

B. Z., a 31 year old housewife, sister of A. Z., was treated in 1950 for ocular complaints. Physical examination showed an enlarged spleen also in this case. The blood picture showed the same deviations as that of her brother, A. Z. (table 2). Outstanding here, is that the patient has a marked anemia, which was refractory to all therapy. Diagnosis: homozygous—Hb-C disease.

C. Z., a 30 year old female, sister of A. Z. and B. Z., had no complaints. Physical examination showed a normal spleen. Hematologic examination revealed an increased osmotic

<table>
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<tr>
<th>Date</th>
<th>Hb Gm%</th>
<th>RBC millions per cu. mm</th>
<th>WBC per cu. mm</th>
<th>Retic %</th>
<th>Osmotic fragility % saline</th>
<th>Indirect serum bilirubin mg %</th>
<th>Target cells</th>
<th>Nucleated RBC per 100 WBC</th>
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TABLE 2.—Hematologic data of Family Z (August 1954)

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<th>Age</th>
<th>Cu. mm</th>
<th>WBC per 100 cu. mm</th>
<th>Hct %</th>
<th>Hb Gm. E</th>
<th>Osmotic fragility %</th>
<th>Target cells thousand</th>
<th>Platelets</th>
<th>Hb-C %</th>
<th>Hb-A %</th>
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Hematologic data of Family M

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<th>WBC per 100 cu. mm</th>
<th>Hct %</th>
<th>Hb Gm. E</th>
<th>Osmotic fragility %</th>
<th>Target cells thousand</th>
<th>Platelets</th>
<th>Hb-C %</th>
<th>Hb-A %</th>
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<td>0.40-0.15</td>
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<td>342</td>
<td>240.000</td>
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</table>

It is striking that, in an apparent white family of German and Dutch origin, a disease was found which is normally confined to Negroes. The possibility of racial admixture and the family relationship in the third generation (fig. 1) may explain the origin of the homozygous and heterozygous Hb-C disease from a negroid (?) ancestor (i.e., A. B., see figure 1) who must have suffered from the heterozygous or more probably the homozygous Hb-C disease. On the other hand, the possibility of a second influence via another ancestor (i.e. H. C. or P. C.) remains.

Family M

R. M., a 28 year old Negro, was admitted to the hospital for gastro-intestinal complaints. Physical examination revealed an enlarged spleen (2-3 fingers' breadths below the costal margin) and a palpable liver. The blood picture, which is also included in table 2, showed no anemia, but did show a marked increase in target cells. Osmotic fragility and serum bilirubin were increased. The slightly dark colored urine contained an increased amount of porphyrine (16 µ/100 ml.), while urobilinogen excretion in the feces was also increased. Tests for sickle cell formation were negative. Sternal puncture revealed an overactive erythrocytic series. Radiologic examination gave no indication of hemolytic disease. Electrophoretic technic established the diagnosis of homozygous Hb-C disease.

G. M., a 48 year old Negro, father of R. M., was examined and found to have no abnormalities. The spleen was not enlarged. The hematologic data, included in table 2, showed an enhanced osmotic fragility while target cells were also increased to 40 per cent.

Diagnosis: heterozygous Hb-C disease. Other members of the family could not be examined by us.

For the investigations described below the blood samples were taken from the patients with the homozygous Hb-C disease (A. Z., B. Z. and R. M.), while Dr. H. J. Scheinberg of New York supplied us with a sample of pure hemoglobin C (which will be designated as sample 1).

METHODS

Hemoglobin from the blood of patients with homozygous Hb-C disease was isolated and purified in the same manner as described earlier for Hb-A and Hb-F, while sample 1 hemoglobin was also crystallized according to Drabkin's method.
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Horizontally lined: possible presence of Hb-C.
Black: Hb-C. White: Hb-A.

The method of Brinkman and Jonxis was used for alkali denaturation tests, while the electrophoresis was done in a Tiselius apparatus (Perkin Elmer Co.) according to the method indicated by Itano and Neel. The hemoglobin solutions were diluted to a concentration of about 1 gm per cent and dialyzed for 3 days at 0°C in a 0.02 M phosphate buffer at pH 6.75, to which 0.08 M NaCl was added. This buffer was also used during the electrophoresis, which lasted approximately 6 hours (at 5.5 mA and 120 V).

Chromatographic investigations were performed with the cation exchanger IRC 50 (NP 64) as described by Prins and Huisman. The columns used were flat cuvettes of 6.5 x 3.0 x 20.0 cm, filled up to 15 cm with this resin. The separation of the different hemoglobins was performed with a sodium citrate-citric acid buffer of pH 6 and a sodium ion concentration of 0.15. About 30 mg of hemoglobin dissolved in 1 ml was chromatographed.

The solubility experiments were performed as described by Derrien et al. with a 3.5 M potassium phosphate buffer at pH 6.5 (designated as 100 per cent). The solubility of COHb-C was compared in various dilutions of this buffer (64-95 per cent phosphate buffer) with those of COHb-A, COHb-S and COHb prepared from umbilical cord blood. The phosphate buffer was diluted in all cases in graduated steps of 2 per cent. Twenty-five mg of hemoglobin were used in a total volume of 10 ml. The amount of dissolved hemoglobin was determined by measuring the extinction at 5420 Å. In addition to these salting-out experiments solubility curves were also plotted for hemoglobin. These curves were obtained in the same manner (salting out at 50-90 per cent phosphate buffer). The reduced form of hemoglobin was prepared by mixing 25 mgm HbO2, dissolved in 1 ml, with 9 ml phosphate buffer of various strengths to which a measured quantity (100 mgm Na2S2O4) was added. Contact with air was avoided. In these experiments also the amount of dissolved Hb was determined by measuring the extinction at 5420 Å.

The amino acid analysis was performed according to a slightly altered column chromatographic method, as developed recently by Moore and Stein. The proteins were hydrolysed by refluxing about 150 mg of COHb with excess hydrochloric acid (20 ml. 6 N HCl). After removing the hydrochloric acid in vacuo and redissolving the residue in a smaller volume (10-20 ml), 12-16 mg of the original hemoglobin were analyzed. Technical details of the procedure are given in a previous paper (13).
Fig. 2.—The diagrams of the hemoglobin of the individuals under study compared to the diagrams of known hemoglobins and of various mixtures.

RESULTS AND DISCUSSION

This study deals with two families in which the occurrence of the homozygous form of hemoglobin C disease gave rise to the following signs and symptoms: (a) a somewhat sickly general condition with various vague complaints, (b) practically normal hemoglobin with the exception of patient B. Z., whose anemia was possibly due to other causes, (c) an elevated serum bilirubin, caused by an
increased hemoglobin destruction, (d) splenomegaly, (e) a large number of target
cells and a few nucleated erythrocytes, (f) an increased osmotic fragility. It is
striking that the symptoms are the same in a Negro family, and in an apparently
white family; from this it seems probable, that hemoglobin C disease runs a like
course in colored and white. The clinical symptoms described here are about the
same as reported by others.10

1. Identification of hemoglobin C

The hemoglobin of the six patients described was first analyzed electrophoreti-
cally. One of these analyses (A. Z.) is shown in figure 2. It is observed that the
hemoglobin of this patient is electrophoretically pure and moves much faster
than adult hemoglobin. Addition of Hb-A shows two well separated boundaries.
Addition of a sample hemoglobin derived from cord blood to a mixture of Hb-A
and the hemoglobin to be analyzed gives rise to a third band, while a fourth is
obtained when sickle-cell hemoglobin (Hb-S) is added. A separation of the un-
known hemoglobin, and Hb-S alone and combined with normal adult hemoglobin
was likewise obtained. In patients B. Z. and R. M. similar pictures were found,
while in the other three cases examined the presence of the same abnormal hemo-
globin next to Hb-A could be established. Patient F. D. showed 67.5 per cent
abnormal hemoglobin, C. Z. 41.5 per cent, and G. M. 44 per cent (table 2).

These experiments show that the abnormal hemoglobin derived from the
blood of the patients examined was electrophoretically not identical with Hb-A,
Hb-S or Hb-F. Hemoglobin D can be excluded, as its electrophoretic mobility
is the same as that of Hb-S.12 The absence of Hb-S is further proved by negative
sickle cell tests. As shown in figure 3, the alkali denaturation curves of the hemo-

![Graph](image-url)
globin of the three patients with the homozygous form of the disease are compared with that of normal adult hemoglobin. It may be that the abnormal Hb has a slightly increased resistance against alkali, but the differences as compared with normal hemoglobin is so small, that no conclusion can be drawn. It is clear, however, that significant amounts of fetal hemoglobin are not present. The clinical picture and the results described here indicate that the abnormal hemoglobin is identical to hemoglobin C, described by Itano and Neel.¹

2. Chromatographic investigations

Figure 4 shows the chromatographic behavior of hemoglobin taken from the blood of a patient with homozygous Hb-C disease (A. Z.), of a patient with heterozygous Hb-C disease (F. D.), and of some other kinds of human hemoglobin. It will be clear that Hb-C moves markedly slower than the other hemoglobins. If the rate of displacement of COHb-A is set at 1.00, then the rate for COHb-C is 0.33 (0.30–0.40), for COHb-F 1.33 (1.23–1.43), and for COHb-S 0.72 (0.67–0.75). As mentioned elsewhere this method is well suited to show the presence of abnormal hemoglobins. All the blood samples of the patients described were examined in the same manner, and the results showed a good correlation with those obtained by using electrophoretic technics. The relatively slow rate of displacement of COHb-C in this chromatographic experiment should probably be regarded as a specific property of this hemoglobin.

3. Salting-out curves of COHb-C and Hb-C in concentrated phosphate solutions

First, the solubility of COHb-C in the potassium phosphate buffer at pH 6.5 in various concentrations, according to Derrien et al.,⁵ is compared with those of

![Figure 4](image-url)
COHb-A, COHb-S and COHb prepared from umbilical cord blood. As shown in figure 5, it was found that the solubility of COHb-C is significantly less than that of the other hemoglobins. Almost all COHb-C is precipitated at a phosphate concentration, which holds COHb-S and COHb-A still in solution (72 per cent and 73 per cent phosphate buffer, respectively). The absence of significant amounts of other kinds of human hemoglobin, already evident in the electrophoretic and chromatographic experiments, was confirmed. The salting-out curves determined for COHb-S, COHb-A and COHb from cord blood, are in agreement with the results already recorded by Roche and Derrien. Due to the fairly great serial increase of the phosphate concentration (two per cent), no derived curves are given. It can be seen, however, from figure 5, that COHb-C, like the other hemoglobins, does not behave as a homogeneous component in these salting-out experiments, so probably consists of more than one fraction. In contrast to the monocarboxylic form, the reduced Hb-C is salted out at phosphate concentrations which are nearly the same as those found for the corresponding adult hemoglobin. The extremely decreased solubility of reduced Hb-S is in agreement with expectations. The decreased solubility of COHb-C in phosphate buffer is a second characteristic property of this abnormal hemoglobin.

4. The amino acid composition of hemoglobin C

In connection with our studies on possible differences in the amino acid compositions of various human hemoglobins, we also analyzed COHb-C and compared the results obtained with those of COHb-A, COHb-S and purified COHb-F. The
Table 3.—The Amino Acid Composition of 48-Hour Hydrolysates of Various Kinds of Human Carbonmonoxyhemoglobin (the values are given as Gm./100 Gm. protein)

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<th>Amino Acid</th>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Sample 3</th>
<th>Sample 4</th>
<th>Mean of 4 samples</th>
<th>Mean of 4 samples</th>
<th>Mean of 4 samples</th>
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<td>3.34</td>
<td>3.36</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>108.48</td>
<td>112.27</td>
<td>109.60</td>
<td>109.57</td>
<td>109.89</td>
<td>109.71</td>
<td>109.83</td>
<td>109.19</td>
</tr>
<tr>
<td>% N recovered*</td>
<td>92.5</td>
<td>94.5</td>
<td>92.6</td>
<td>92.7</td>
<td>93.1</td>
<td>90.2</td>
<td>91.4</td>
<td>91.8</td>
</tr>
</tbody>
</table>

* Without heme, tryptophane and amide N.
in this protein is significantly higher than that present in COHb-A. Moreover it will be noted that the results of amino acid analysis of sample I, which is derived from an entirely different source, show a good correlation with the analysis of the hemoglobin samples obtained from the patients studied. This correlation also supports the diagnosis of homozygous Hb-C disease for some of the cases described here. The increased amount of lysine, therefore must be regarded as a third specific property of COHb-C.

Expressed as the number of lysine residues per Mol. of hemoglobin (MG 68000) an increase was found of 49 residues⁴ for COHb-A to 53 for COHb-C. The possible rise of histidine content is limited to one residue. This rise in number of basic amino acids with the same number of aspartic and glutamic acid residues agrees quite well with the abnormal electrophoretic behavior found for Hb-C. Itano and Neel⁵ for instance found that Hb-C had a significantly greater electrophoretic mobility in cacodylate-sodium chloride buffer solution \( \mu \) 0.1 and pH 6.50, than other human hemoglobins. The mobility for Hb-C was found to be \( 3.2 \times 10^{-5} \) cm./sec. per volt/cm, while for Hb-A and Hb-S values of \( 2.4 \times 10^{-5} \) and \( 2.9 \times 10^{-5} \) cm./sec. volt/cm were obtained, respectively. The more basic character of Hb-C may also explain its abnormal behavior in cation exchange chromatography. It will be noted that the fetal hemoglobin, whose amino acid composition shows a smaller number of basic amino acid residues, is displaced faster than normal adult hemoglobin. The more basic Hb-C displaces itself, on the contrary, at a very much lower speed than that of other hemoglobins. It should be noted, however, that the number of amide groups has not been determined; difference in this number can likewise influence the electrophoretic and chromatographic behavior of proteins.

**SUMMARY AND CONCLUSIONS**

1. The present paper describes an apparently white family in which a few members suffered from homozygous and heterozygous hemoglobin C disease. Since the paternal great grandfather and the maternal grandfather of the patients examined were in a family relation of the first order, it is surmised that the hemoglobin C was derived from the same homozygous negroid (?) ancestor. Besides its occurrence in this family Hb-C is also found in a colored family from the same island (Curaçao).

2. Hemoglobin C shows, in addition to abnormal electrophoretic mobility, three other more or less specific properties:
   a. During chromatographic examination carried out with the aid of the cation exchanger Amberlite IRC 50 (XE 64), a citrate citric acid buffer at pH 6 and sodium ion concentration of 0.15, COHb-C displaces itself noticeably slower than the three other forms of hemoglobins (S, A and F).
   b. The solubility of the COHb-C in a phosphate buffer pH 6.5 of various concentrations is significantly less than that of COHb-S, COHb-A and COHb-F. The reduced form of hemoglobin C does not show this property; the solubility of this is practically the same as that of reduced adult hemoglobin.
   c. A comparison of the amino acid composition of hemoglobin C and hemo-
globin A, S and F, shows that COHb-C contains a higher number of lysine and perhaps histidine residues. This increase amounts to four residues per mol. for lysine and one residue per mol. for histidine. The possible connection between the more basic character of the proteins and the abnormal behavior in the electrophoretic and chromatographic experiments is discussed.

3. The results obtained with five experimental methods (electrophoresis, alkali denaturation, chromatography, salting-out experiments, and amino acid composition determination) all seem to indicate that in the homozygous form of this disease only the abnormal Hb-C is present. None of the other three hemoglobins (A, S and F) were shown to be present in determinable amounts.

4. In addition to the electrophoretic abnormalities, the above three properties may be of importance in the characterization of this abnormal hemoglobin.

**SUMMARIO IN INTERLINGUA**

1. Le presente reporto describe un familia, apparentemente blanc, in que alieun membros suffeva de homozygotie e heterozygotie morbo a hemoglobin A. Proque le patern-o granpatre secunde e le materne granpatre prime del patientes examinate esseva connectite per un relation familial del prime ordine, le supposition es justificabile que le hemoglobin C esseva derivate ab le mesme homozygotie ancestrale negroide (?). Ultra su occurrentia in le familia hic discutite, hemoglobin C se trova etiam in un familia colorata del mesme insula (Curaçao).

2. A parte su anormal mobilitate electrophoretic, hemoglobin C exhibi tres altere plus o minus specific caracteristicas:

   (a) In le examine chromatographic—execute per medio del excambiatorem a cationes Amberlite IRC 50 (XE 64), con citrato de acido citric a pH 6 como tampon, e un concentration de ions de natrium de 0,15—carbomonoxyhemoglobina-C se displacia significativamente plus lentemente que le correspondent compositos de hemoglobin S, A, e F.

   (b) Le solubilitate de carbomonoxyhemoglobina-C in un tampon de phosphato a pH 6,5 de varie concentrationes es significativamente inferior al correspondent solubilitate de carbomonoxyhemoglobina-S, carbomonoxyhemoglobina-A, e carbomonoxyhemoglobina-F. Le forma reduce de hemoglobin C non exhibi iste caracteristicen; su solubilitate es practicamente identic con illo de reducete hemoglobin adulta.

   (c) Un comparation del compositos a amino-acido in le caso de hemoglobina C e in le caso del hemoglobinias A, S, e F monstra que carbomonoxyhemoglobina-C contine un plus grande numero de residuos de lysina e forsau de histidina. Iste augmento es quatro residuos per mole pro lysina e un residuo per mole pro histidina. Le possibile connexion inter le character fundamental del proteinas e le manifestationes anormal in le experimentos electrophoretic e chromatographic es discutite.

3. Le resultatos obtenite per medio de cinque methodos experimental (electrophorese, denaturacion a alkali, chromatographia, saturation salin, e determination del composition a amino-acido) pare indicar que in le forma homozygotie del morbo solmente le anormal hemoglobin C es presente. Nulle del altere tres hemoglobinias (A, S, e F) eseva presente in verificabile quantitates.
SOME CHARACTERISTIC PROPERTIES OF HEMOGLOBIN C

4. Ultra le anormalitates electrophoretic, le supra-mentionate tres caracteristicas es possibilemente de importancia in le identification de iste anormal hemoglobina.

ADDENDUM

After closing this investigation we were enabled to examine two other uiieunbers of the family M., namely I. M. (mother of the patient R. M.) and M. M. (daughter of R. M.). In both cases a heterozygous Hb-C disease was found by chromatographic analysis.

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Some Characteristic Properties of Hemoglobin C

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