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*A THIRD ABNORMAL HEMOGLOBIN ASSOCIATED WITH HEREDITARY HEMOLYTIC ANEMIA**

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Previous work from these Laboratories has established the existence of an abnormal hemoglobin in sickle cell trait and sickle cell anemia and has provided the basis for a theory relating the presence of the abnormal hemoglobin to the clinical pictures in sickle cell trait and sickle cell anemia.[1] It was shown in this work that the electrophoretic picture in these conditions is compatible with the usual genetic picture that both parents of an individual suffering from sickle cell anemia have the sickle cell trait.[2] There are in the literature, however, a number of reports in which one of the parents of a sickle cell anemia victim possesses non-sickling erythrocytes. In some cases of this type the parent whose cells do not sickle has been shown to have thalassemia minor (Cooley's trait), and hematologic studies on the erythrocytes of the anemic individual have revealed the presence of properties which are present in the erythrocytes both of sickle cell disease and of thalassemia.[3,4] In other cases the hemoglobin of the parent having the non-sickling cells has a greater electrophoretic mobility on the acid side of the isoelectric point than either normal hemoglobin or sickle cell hemoglobin, and the hemoglobin of the anemic child contains both this new hemoglobin and sickle cell hemoglobin.[5]

The present report deals with the identification of still another form of human hemoglobin in five members of a family in which the genetic picture is not typical of sickle cell anemia, although two of the members have in the past been diagnosed as having sickle cell anemia. An earlier study of this family disclosed that the two anemic children and the father, who was not anemic, had sickling erythrocytes while the mother, two sisters and two brothers of the anemic children had non-sickling erythrocytes and were not anemic.[6] The father, one brother and one sister were not available for the present study.

Experimental Methods.—(*a*) *Sickling tests.* The sodium dithionite,[7]

sodium metabisulfite[8] and moist seal[9] methods were employed. The tests were repeated on specimens which were negative for sickling.

(*b*) *Electrophoretic studies.* The methods described in previous reports from these Laboratories were employed.[1,10] The carbonmonoxyhemoglobins were studied.

(*c*) *Solubility studies.* The solubilities of the ferrohemoglobins prepared from the erythrocytes of the members of this family were determined in a series of concentrated phosphate buffers. The amorphous rather than the crystalline state was examined for reasons which will be discussed later. Similar studies were conducted on the ferrohemoglobins from normal, sickle cell trait, and sickle cell anemia individuals for control purposes. (1) *Materials:* Concentrated hemoglobin solutions were prepared and were dialyzed against distilled water. A potassium phosphate buffer having a total phosphate concentration of 2.8 molar and a pH of 6.8[11] was prepared from reagent grade crystalline potassium dihydrogen phosphate and dipotassium phosphate powder. (2) *Experimental method:* From 8.00 to 9.60 ml. of the concentrated phosphate buffer were delivered from a buret into 10-ml. volumetric flasks, and 100 mg. of sodium dithionite, $Na_2S_2O_4$, was added to the buffer in each flask. The flasks were then filled to the 10-ml. mark with aqueous hemoglobin solution or with the hemoglobin solution and distilled water. The contents of the flasks were mixed by manual shaking, and the precipitate was separated from the solution by centrifugation. Experiments were discarded unless a visible precipitate formed within a few seconds of the mixing. The absence of a precipitate indicates either undersaturation or supersaturation, the latter occurring in some cases when the hemoglobin is present in but small excess.[12] Following the centrifugation, which caused the excess solid hemoglobin to collect above the dense solution, the solution of hemoglobin in phosphate buffer was removed. Its concentration was determined spectrophotometrically immediately after saturation with carbon monoxide. The temperature of the solutions at the end of the centrifugations was 18° ± 2° C. The ionic strength of each solution was calculated from the amounts of 2.8 molar phosphate buffer and sodium dithionite added. The amount of hemoglobin present in each of the equilibrium mixtures was calculated from the volume and concentration of the hemoglobin solutions added.

Results.—(*a*) *Sickling tests.* The erythrocytes of R. H., a male, and B. H., a female, the two individuals who were previously reported as being anemic, were positive for sickling, and virtually all of their red cells sickled. The erythrocytes of their mother, M. H., a brother, G. H., and a sister, H. H. D., did not sickle. Three different specimens of M. H.'s blood were drawn and tested over a six-month period; at no time was any sickling elicited in her cells in spite of numerous tests. These results are in agreement with those reported in the previous study of this family.[6]

(*b*) *Electrophoretic studies.* The electrophoretic behavior of the hemoglobins of the two sickling individuals in this study, R. H. and B. H., appears to be the same as that previously found in cases of sickle cell anemia. R. H.'s carbonmonoxyhemoglobin was analyzed both in phosphate buffer and in cacodylate buffer. The scanning diagram in phosphate buffer of pH 6.90 and ionic strength 0.1 is identical to that previously published,[1] and the mobility in this buffer is 0.34×10^{-5} cm.^{2} sec.$^{-1}$ volt^{-1}. The mobility determinations of seven different specimens of sickle cell anemia carbonmonoxyhemoglobin in this buffer averaged 0.33×10^{-5} cm.^{2} sec.$^{-1}$ volt^{-1}.[13] In cacodylate-sodium chloride buffer of pH 6.50 and ionic strength 0.1, the major component of the carbonmonoxyhemoglobins from both R. H. and B. H. has the mobility of sickle cell carbonmonoxyhemoglobin. About 6% of R. H.'s hemoglobin and 12% of B. H.'s hemoglobin are present in this buffer as a slow-moving component having the approximate mobility of normal carbonmonoxyhemoglobin. These patterns in cacodylate buffer are very similar to those found in some cases of sickle cell anemia.[5,10] The scanning diagrams of the carbonmonoxyhemoglobins from M. H., G. H., and H. H. D. in the cacodylate buffer are indistinguishable from those obtained from individuals having sickle cell trait.[10] Normal hemoglobin and a hemoglobin component which has the same mobility as sickle cell hemoglobin are present. The percentages of the abnormal hemoglobin present are 42, 35, and 49%, respectively, for M. H., H. H. D. and G. H.

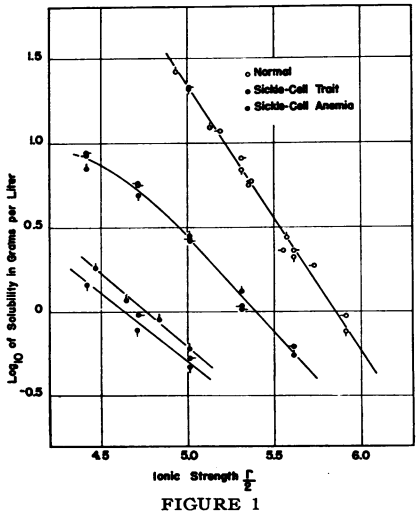

FIGURE 1

The solubilities in phosphate buffers at 18° C. of amorphous ferrohemoglobins from three normal individuals, three individuals having sickle cell trait and two individuals having sickle cell anemia. One of the normal specimens and the sickle cell anemia specimen having the lower solubility were examined twice and are represented by two series of points. Two curves are drawn for the sickle cell anemia points because the two specimens have slightly different electrophoretic patterns (see discussion section).

(*c*) *Solubility studies.* The results are indicated by figures 1 and 2. The points on the solubility curve for normal ferrohemoglobin represent experiments on three different specimens of normal hemoglobin, one of which was examined in two series of determinations. Specimens of hemoglobin from three individuals having sickle cell trait and two having sickle

cell anemia were examined. The anemia curve showing the higher solubility represents a single series of determinations on a sickle cell anemia hemoglobin specimen which shows on electrophoretic analysis approximately 16% of a slow component having the mobility of normal hemoglobin. The other anemia curve represents two series of determinations on a sickle cell anemia hemoglobin specimen having less than 5% of the slow component. The solubilities of the hemoglobins of the sickling members of the family being studied, R. H. and B. H., are between those of sickle cell trait individuals and sickle cell anemia individuals. The solubilities of the hemoglobins from the non-sickling individuals, M. H. and G. H., are nearly the same as those of specimens of normal hemoglobin. H. H. D.'s hemoglobin was not available in sufficient quantity to run the solubility experiments.

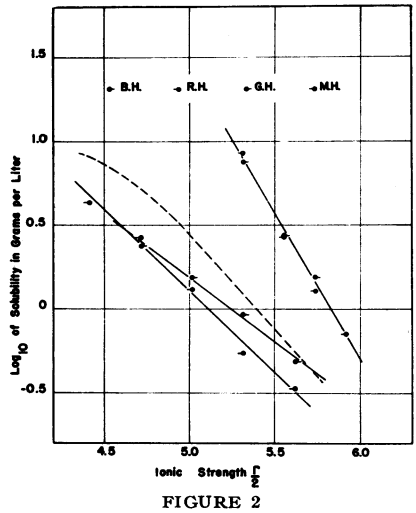

FIGURE 2

The solubilities in phosphate buffers at 18° C. of amorphous ferrohemoglobins from four members of the family under study. The dashed curve is the sickle cell trait curve from Figure 1. M. H. and G. H. have two-component electrophoretic patterns similar to those found in sickle cell trait. R. H. and B. H. have electrophoretic patterns with one major component, similar to those found in sickle cell anemia.

The solubility behavior of a single specimen of hemoglobin which contains 65% of normal hemoglobin and 35% of the new inherited abnormality reported by Itano and Neel[5] and of a single specimen of hemoglobin from an individual suffering from thalassemia major (Cooley's anemia) were investigated. The plot of the logarithm of the solubility against ionic strength for both these specimens fell within 0.1 logarithm unit of the plot for normal hemoglobin.

(*d*) *Summary of results.* The hemoglobins from M. H., G. H., and H. H. D. resemble the hemoglobins from individuals having sickle cell trait in their electrophoretic behavior but differ in having a greater solubility and in failing to cause sickling. Likewise, the hemoglobins from R. H. and B. H. resemble hemoglobins from individuals having sickle cell anemia in their electrophoretic behavior but differ in having a greater solubility.

Nomenclature.—In order to facilitate the discussion in the present paper and to avoid confusion in future works, it seems desirable at this time to establish a system of symbols for identifying the various forms of

adult human hemoglobin. Table 1 lists the characteristics of the four forms of adult human hemoglobin which have been identified to date. While the names normal hemoglobin and sickle cell hemoglobin adequately describe the first two forms and will be used in this paper, names of equally descriptive nature have not yet been proposed for the two newly recognized forms. Therefore for purposes of identification and discussion normal hemoglobin, sickle cell hemoglobin,[1] the abnormal hemoglobin reported by Itano and Neel,[5] and the abnormal hemoglobin reported in the present paper will be designated adult human hemoglobins *a*, *b*, *c* and *d*, respectively, more briefly as hemoglobins *a*, *b*, *c* and *d*. Mixtures of these hemo-

TABLE I

CLASSIFICATION OF ADULT HUMAN HEMOGLOBINS[a]

NAME OF HEMOGLOBIN	IDENTIFICATION LETTER	CAUSES SICKLING	ELECTROPHORETIC MOBILITY[b]	SOLUBILITY[c]
Normal	*a*	No	Slow	High
Sickle cell	*b*	Yes	Intermediate	Low
...	*c*	No	Fast	High
...	*d*	No	Intermediate	High

[a] The hemoglobin present in individuals having thalassemia major is not included in this tabulation; some investigators are of the opinion that the abnormal properties of this hemoglobin are due to the presence of fetal hemoglobin.[29, 30] The possibility that the low-mobility component in the hemoglobins of R. H., B. H. and certain sickle cell anemics may be fetal hemoglobin is being investigated.

[b] As carbonmonoxyhemoglobin in cacodylate-sodium chloride buffer of pH 6.50 and ionic strength 0.1. In this buffer each of these carbonmonoxyhemoglobins migrates as a positive ion.

[c] As amorphous ferrohemoglobin in concentrated phosphate buffers of pH 6.8 at 18°C.

globins which occur naturally may then be called hemoglobins *ab*, *ad*, *bc*, etc. Hemoglobins *c* and *d* have not been found free of other hemoglobins, and their behavior with respect to sickling and solubility are inferred from the behavior of mixtures of *c* and *d* with *a* and *b*. The solubilities of *c* and *d* are therefore not precisely known at this time, and they have been grouped together with that of normal hemoglobin as "high" with respect to that of sickle cell hemoglobin.

Discussion.—The results of the sickling tests conducted on the individuals who were available for this study are in complete agreement with those found by the previous investigators of this family.[6] These workers noted in addition that the sickling was of the slow type in the sickling individuals, an observation which implies that the constitution of their erythrocytes is not identical with that of the fast sickling cells typically found in sickle cell anemia.

The observation that the non-sickling erythrocytes of M. H., G. H. and H. H. D. contain hemoglobin components[14] which migrate like those

found in sickle cell trait suggests two possible explanations. One explanation, that the membrane and internal structure of these cells are abnormally rigid and are able to withstand the tendency of the sickle cell hemoglobin to aggregate, is a difficult one to test unambiguously. The other explanation, that the abnormal component in these cells represents a hemoglobin which, while migrating with the same mobility as sickle cell hemoglobin, is actually a different molecular species, can be tested if an independent criterion for differentiating hemoglobins can be employed. Solubility differences exist among the hemoglobins of different species,[15] between adult and fetal hemoglobins[16] and between normal and sickle cell hemoglobins.[17, 18] The possibility that the abnormal hemoglobin in this family might have a characteristic solubility was therefore investigated.

Although in the majority of protein solubility studies crystalline protein preparations are used, this procedure was undesirable in the present work because the process of preparing crystals is likely to cause partial fractionation of the components.[19] Other undesirable features of solubility work on crystalline proteins are the length of time required for equilibration and the danger of supersaturation.[12] The low solubility of ferrohemoglobin in comparison with other compounds of hemoglobin[16] and the large difference in the solubilities of normal and sickle cell ferrohemoglobins suggested the use of this compound throughout the solubility experiments. Another advantage of using this compound is the very small dependence of its solubility on temperature.[16] The use of sodium dithionite served a double purpose; any ferrihemoglobin which forms during the preparation of the hemoglobin solutions is reduced to ferrohemoglobin by this compound, and no oxyhemoglobin can form in the presence of an excess of sodium dithionite because of the high rate of reaction of oxygen with this reducing agent. Both ferrihemoglobin and oxyhemoglobin are considerably more soluble than ferrohemoglobin, and their temporary presence might result in supersaturation. The amount of dithionite, while sufficient to maintain the hemoglobin[20] in the reduced, unoxygenated state, was small in relation to the total phosphate concentration of the buffer, so that its decomposition did not alter significantly the pH or the total ionic strength of the solutions. It was found that the equilibrium concentration of hemoglobin was attained within the time required to precipitate and separate by centrifugation the excess hemoglobin as amorphous solid. In the case of normal hemoglobin, presumably a one-component protein, the solubility of the amorphous phase was independent of the amount of the solid present. The solubility of sickle cell trait hemoglobin increased with the amount of the solid phase present, approaching a limiting value in the presence of a large excess of the two-component hemoglobin. Repeated centrifugation and resuspension of an amorphous precipitate of normal hemoglobin resulted in the formation of crystalline ferrohemo-

globin, which has a lower solubility than the amorphous form. The foregoing results, plus the reproducibility of the results as indicated by figure 1, indicate that amorphous hemoglobin possesses a metastable equilibrium solubility which is characteristic of the type of hemoglobin and of the concentration of the solvent buffer, and which is greater than that of the crystalline form. These results are in accord with those obtained by other workers[16,21] on the crystalline and amorphous hemoglobins of several species.

The actual solubility in moles per liter[22] of sickle cell trait hemoglobin falls much closer to that of sickle cell anemia hemoglobin than to that of normal hemoglobin although the percentage of sickle cell hemoglobin in sickle cell trait is always smaller than that of normal hemoglobin.[10] This indicates that, unlike the system treated by Northrop and Kunitz,[23] the amorphous solid solution of normal and sickle cell hemoglobin does not show a Raoult's Law type of behavior in its solubility but has a solubility much lower than that predicted from Raoult's Law. This finding precludes the determination of the composition of sickle cell trait hemoglobin in its two components from solubility data alone. The deviation of the sickle cell trait solubility data from a straight line in the high solubility region is undoubtedly due to the fact that in the experiments in this region the excess of hemoglobin present was small, and the composition of the solid phase was significantly altered by the solution of a large fraction of the added hemoglobin.

The observations that the solubilities of the two-component hemoglobins of M. H. and G. H. are nearly the same as those of normal hemoglobin and five to seven times as great as that of sickle cell trait hemoglobin, which has the same electrophoretic pattern, provide conclusive proof that the abnormal component in the hemoglobins of these individuals is not sickle cell hemoglobin. These results also suggest that, barring large interaction effects, the solubility of this abnormal hemoglobin is of the same order of magnitude as that of normal hemoglobin, which is about 14 times as soluble as sickle cell hemoglobin in the amorphous state. Thus the presence of a hitherto unreported hemoglobin component in the erythrocytes of M. H., the mother, G. H., and probably H. H. D. has been established without consideration of the data presented by the studies on R. H. and B. H. Probably the major electrophoretic component of the hemoglobins of the latter individuals contains two abnormal hemoglobins migrating with the same mobility. The presence of sickle cell hemoglobin is indicated by (1) the positive sickling test and relatively low solubility of their hemoglobins, (2) the identity of the electrophoretic mobility of the major component of their hemoglobins with that of sickle cell hemoglobin and (3) the presence of sickle cell trait in their father. The presence of the other hemoglobin, which henceforth will be called hemoglobin *d*, is indicated by (1) the greater solubility of their hemoglobins in com-

parison with sickle cell anemia hemoglobins having the same or very similar electrophoretic patterns and mobilities and (2) the presence of this hemoglobin in their mother and two siblings.

The explanation for the greater solubility of B. H.'s hemoglobin as compared to R. H.'s may lie in the electrophoretic result that B. H.'s hemoglobin has about 12% of a component having the mobility of normal hemoglobin while R. H.'s hemoglobin has only about 6% of this component. Another possible explanation is that R. H.'s hemoglobin may contain a greater percentage of sickle cell hemoglobin than B. H.'s. The difference in the slopes of the solubility curves of the hemoglobins from these two individuals is in large part due to low values of solubilities of B. H.'s hemoglobins at ionic strengths 4.7 and 5.0. Hemoglobin was present in but small excess in these experiments, and the same effect which diminishes the solubility of sickle cell trait hemoglobin when it is present in small excess accounts for these results.

Abnormally shaped erythrocytes are more susceptible to intravascular destruction than are normal erythrocytes.[24, 25] The great difference in the pathological consequences of sickle cell anemia and sickle cell trait as well as the gradations in the severity of different cases of sickle cell anemia can be related to the fraction of the total hemoglobin which is present as sickle cell hemoglobin.[1, 4, 10] When this fraction is sufficiently high as to cause sickling at the partial pressure of oxygen in venous blood, intravascular hemolysis and anemia result. The question may well arise as to why the combination of sickle cell hemoglobin and hemoglobin *d* causes anemia when hemoglobin *d* appears to be just as soluble as normal hemoglobin. One possible answer is that the percentage of hemoglobin *d* in the erythrocytes is low; another is suggested by the solubility data on sickle cell trait hemoglobin. As was previously pointed out, these data indicate the presence of an interaction between normal and sickle cell hemoglobin which stabilizes the solid phase and diminishes the solubility of a mixture of these two hemoglobins to less than a theoretically predicted value. A similar interaction may stabilize the aggregates in sickling erythrocytes; and if the stabilizing interaction between sickle cell hemoglobin and hemoglobin *d* were greater than that between sickle cell hemoglobin and normal hemoglobin, the presence of a high percentage of sickle cell hemoglobin need not be postulated in order to explain an increased susceptibility to sickling. The same possibilities may be considered with regard to the origin of the anemia which accompanies the presence of the combination of sickle cell hemoglobin and hemoglobin *c* in an individual; in this case it has been determined electrophoretically that the erythrocytes in the known cases contain about 50% of hemoglobin *c*.[5]

The genetic picture in this family appears to be analogous to the pictures presented by the families in which the sickle cell-hemoglobin *c* combination[5] and the sickle cell-thalassemia combination[3, 4] are present. In each

of these cases an erythrocyte abnormality has been detected in the non-sickling parent of an individual having a syndrome resembling sickle cell anemia. In discussing one of these cases Neel[3] has presented three possible genetic mechanisms for the transmission of the sickle cell and thalassemia characters. The decision as to the actual mechanism involved must await the accumulation of more data involving similar families and the examination of the descendants of individuals possessing the same hemoglobin constitution as R. H. and B. H. If the marriage of R. H. or B. H. to an individual having only normal hemoglobin results in a child having only normal hemoglobin, non-allelism of the genes responsible for the formation of sickle cell hemoglobin and hemoglobin *d* would be indicated. Although neither hemoglobin *c* nor hemoglobin *d* has been found free of other hemoglobins, this work does not provide any evidence that such states would necessarily be lethal.

The existence of other genetic forms of human hemoglobin is quite probable, but the problem of detecting and characterizing forms which do not produce hematologic and clinical disorders may prove difficult. Sickle cells have been observed in deer,[26] and an abnormal form of adult sheep hemoglobin has been detected in a lamb.[12] A systematic search would probably disclose the existence of chemically and genetically different forms of hemoglobin within other species. The search need not be confined to hemoglobins; the electrophoretically distinct components of crystalline serum albumin[27] and ovalbumin[28] may be of genetic significance.

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*SPONTANEOUS AND ULTRA-VIOLET-INDUCED MUTATIONS TO PHAGE RESISTANCE IN ESCHERICHIA COLI**

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One of the fundamental problems of genetics is the nature of differences between spontaneous and induced mutation. In higher organisms, mutations induced by ionizing radiations are known to include a relatively large number of chromosomal aberrations. Yet there is no doubt that spontaneously occurring chromosomal abnormalities have been important phylogenetically, and may themselves be the secondary consequences of spontaneous genetic changes.

Bonnier and Luning[1] observe that the regression line expressing a linear relationship between intermediate doses of x-ray and mutation frequency in Drosophila fails to intercept the point derived from untreated and slightly irradiated controls. Similar findings have been obtained by Spencer and Stern[2] with departures from linearity established as significant by Boag[3] using a probit diagram. It is at least possible that the deficit