RATIO OF SICKLE-CELL ANEMIA HEMOGLOBIN TO NORMAL HEMOGLOBIN IN SICKLEMICS*

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The erythrocytes of certain human individuals undergo a reversible change in shape known as sickling if deprived of oxygen (1, 2). Taliaferro and Huck (3) postulated that this characteristic is transmitted by a single dominant gene, but failed to account genetically for the wide divergence in clinical signs and symptoms among individuals who possess sickling red cells. A small fraction, about 1 in 40, of these individuals have a severe chronic anemia called sickle-cell anemia; the others have no symptoms which can be associated with this erythrocyte characteristic, and their condition is termed sickle-cell trait or sicklemia (4). Neel (5) has postulated that there exists in the Negro population a gene which in the heterozygous condition results in sicklemia and in the homozygous condition in sickle-cell anemia. His statement is based on the finding that the trait was present in every parent of a sickle-cell anemia patient that he tested. This hypothesis is in accord with the results of recent electrophoretic studies of the hemoglobins in these conditions (6, 7). These studies showed that the erythrocytes of individuals with sickle-cell anemia contain a new kind of hemoglobin, different from that of normal individuals, and also that the hemoglobin of sicklemic persons consists of two components which behave electrophoretically as normal hemoglobin and sickle-cell anemia hemoglobin. These results have aided in the clarification of both the genetic and the clinical aspects of sickle-cell anemia and sickle-cell trait. In the attempt to obtain information about the nature of sickle-cell anemia hemoglobin, amino acid analyses of hydrolysates of normal, human, adult hemoglobin and of sickle-cell anemia hemoglobin were carried out, which showed that there are probably small differences

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in the number of residues of leucine, serine, valine, and threonine in these molecules (8).

It has been observed that all of the erythrocytes of individuals with sickle-cell trait undergo sickling and that a greater reduction in partial pressure of oxygen is required to produce complete sickling in sickle-cell trait erythrocytes than in those of sickle-cell anemics (1). These observations indicate that each sicklemia erythrocyte contains both normal hemoglobin and sickle-cell anemia hemoglobin. In a pooled sample of blood from five sicklemic individuals the ratio of the abnormal to the normal hemoglobin was found to be 39:61 (7).

In the light of this knowledge it was considered pertinent to ascertain the extent of the variation of the ratio of sickle-cell anemia hemoglobin (SCA hemoglobin) to normal hemoglobin in sicklemic individuals and the effect of various factors on this ratio. Such information not only would increase the knowledge of sickle-cell disease but also might shed some light on the general problem of the control of hemoglobin anabolism.

EXPERIMENTAL

Blood Samples—Samples of blood to which either citrate or oxalate had been added were obtained from parents of sickle-cell anemic children or from brothers or sisters of sickle-cell anemic individuals and were kept at 4° until solutions of hemoglobin could be prepared from them.[1] Other samples were obtained in examining the cell residues of blood obtained from Negro donors at a commercial blood bank.[2] For this purpose the rapid diagnostic test of Itano and Pauling (9) was employed, and samples of those cell residues which responded positively to this test were stored as noted above. As far as it was possible to determine, these persons were otherwise normal and had not received a blood transfusion within a year previous to the time the blood or cell samples were obtained.

Solutions of hemoglobin were prepared from these samples of blood and erythrocytes according to the method of Drabkin (10). The hemoglobin in each solution was converted to the carbonmonoxy derivative by saturating the solutions with carbon monoxide and each solution was then stored at 4° in tightly stoppered, brown glass bottles. The hemoglobin concentration of each solution was determined spectrophotometrically at 540 mμ. For this purpose a Coleman junior spectrophotometer was used and the standard curve employed was obtained by using a pure solution of normal

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carbonmonoxyhemoglobin, the concentration of which had been determined by iron analysis (8).

Electrophoresis Experiments—The electrophoresis experiments carried out in this investigation were very similar to those previously described (7). The buffer solution used consisted of 0.08 M NaCl, 0.02 M sodium cacodylate, and 0.008 M cacodylic acid and had a pH of 6.50 and ionic strength of 0.10 (11). A portion of each hemoglobin solution was diluted to a concentration of 1 per cent with buffer and then dialyzed at 4° for 18 hours

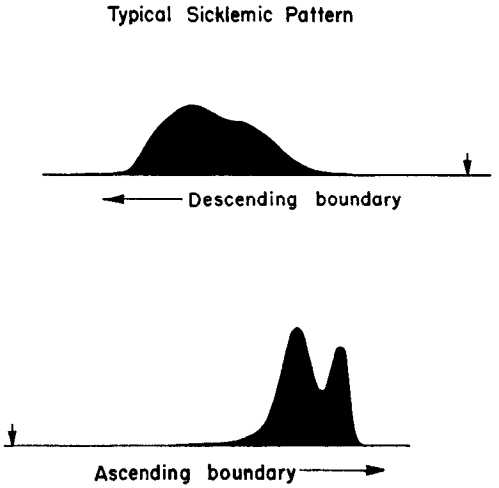

FIG. 1. The ascending and descending boundaries of an electrophoresis experiment on the carbonmonoxyhemoglobin from a typical sicklemic individual. The leading peak is due to SCA hemoglobin. In some experiments, including those on known mixtures of SCA hemoglobin and normal hemoglobin, extraneous spikes have occurred in the descending boundary. The cause of this difficulty is undetermined; however, no such effects ever occur in the ascending boundary under the conditions used for electrophoresis.

against the buffer saturated with carbon monoxide. Finally the dialyzed solutions were completely clarified by centrifugation in the cold at 36,500 $\times$ g for 20 minutes and then subjected to electrophoresis for 15 hours under a potential gradient of 3.57 volts per cm., with the equipment previously described (7).

In Fig. 1 are shown typical Longsworth scanning diagrams of an electrophoresis experiment on a hemoglobin sample from a sicklemic individual. Under the conditions used for electrophoresis, the descending boundary is much more diffuse than the ascending one; therefore, the latter boundary was chosen for measurement.

The image of the ascending boundary was projected and enlarged by

means of a photographic enlarger and traced on a large piece of paper. Routinely, six tracings were made in order to minimize the subjective errors. Since the leading and trailing edges of the boundary are not symmetrical, the two peaks were resolved by means of the method of Tiselius and Kabat (12) of dropping a perpendicular to the base-line from the minimum point between the two peaks. The total area under the curve and the area under the SCA hemoglobin peak were measured for each

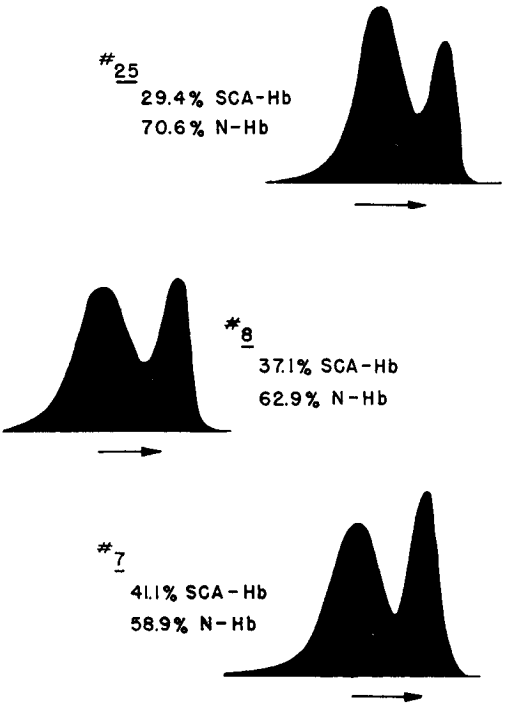

FIG. 2. Ascending boundaries of electrophoresis experiments on the carbonmonoxyhemoglobin from some individual sicklemics. The leading peak is due to SCA hemoglobin.

tracing with a planimeter. After the average value for each quantity was determined, the "measured" percentage of SCA hemoglobin in the sample was calculated. In order to convert this measured percentage to the actual percentage a calibration curve was used, which was made in the following manner. Known mixtures of normal and SCA hemoglobin, having a total protein concentration of 1 per cent, were subjected to electrophoresis under the conditions already described for sicklemia hemoglobin. The ascending boundaries were treated as above and the measured percentages of SCA hemoglobin were plotted against the known percentages

(7). The equation of the linear curve thus obtained, calculated by means of the method of least squares, is $Y = 1.17\,X - 5.5$, where Y is the actual percentage of SCA hemoglobin and X is the measured percentage. The measured percentage of SCA hemoglobin from an electrophoresis experiment of a sample of sicklemia hemoglobin, as described above, is substituted in the equation of the calibration curve and the actual percentage of SCA hemoglobin in the sample calculated.

Typical Longsworth scanning diagrams of the ascending boundaries of electrophoresis experiments of samples of hemoglobin from individual sicklemics are reproduced in Fig. 2. It is obvious that there are significant differences in the percentages of SCA hemoglobin among these samples.

It was of importance to ascertain how reproducible were the results of any one experiment. To achieve this end the electrophoretic analyses of two samples were repeated. The percentages of SCA hemoglobin from the separate runs for each sample taken from sicklemic individuals were as follows: Case 25, 29.7 per cent, 28.9 per cent; Case 33, 34.1 per cent, 32.7 per cent. It is felt that the results of any one determination are probably reproducible to about ± 1.5 per cent.

Results

In Table I there are presented the results obtained from forty-two sicklemic individuals. These persons were chosen at random, as is indicated in the section above in which the procurement of samples is discussed, the only restriction being that the individuals should not be genetically related. Even though the names of the twelve donors obtained from the commercial blood bank were different, these samples offer some uncertainty in this latter respect.

In these forty-two sicklemic individuals the per cent of SCA hemoglobin ranged from about 24 to about 45 per cent, with an arithmetic mean of 37.5 per cent. It is to be noted that one-third of the samples contained between 40 and 42 per cent of SCA hemoglobin. An increased frequency appears to be present at about 34 per cent of SCA hemoglobin; however, the small number of samples makes this trend of doubtful significance.

Because of the wide range of percentages found, as many factors as possible have been studied in an attempt to establish their influence on the ratio of SCA hemoglobin to normal hemoglobin in sicklemic individuals.

Effect of Time—Fortunately it was possible to obtain, after variable periods of time had elapsed, new samples of blood from sicklemic individuals whose percentage of SCA hemoglobin had been previously determined. The results of these determinations are shown in Table II. It is seen that some of the final percentages are greater and some are less than the initial percentages. Undoubtedly some part of these differences

is due to inherent errors of the method used for measurement and some is due to physiological variation. Presumably the normal and SCA hemoglobins in a healthy sicklemic individual are in a state of dynamic equilib-

TABLE I

Hemoglobin in Sickle-Cell Trait

Case No.	Sex	Age	SCA hemoglobin	Case No.	Sex	Age	SCA hemoglobin
			per cent				*per cent*
11			24.3	111	F.		38.9
67	F.		27.7	37	M.		39.9
64	"		27.9	30	"	34	40.0
25	"	38	29.4	10			40.4
12			32.3	29	F.	23	40.5
80	F.		33.1	93	"		40.5
4	"	32	33.3	36			40.9
33	M.	44	33.4	101	F.		40.9
35	"	41	33.7	26	"	10	41.0
66	F.		33.9	75	M.		41.0
83	"		34.0	B. M. S.	"		41.0
46	M.		34.3	7	F.	38	41.1
71	"		34.4	39			41.3
56	F.		35.1	47	F.		41.3
28	M.	24	35.2	91	"		41.5
49	F.	35	35.6	110	M.		41.6
M. H.	"		36.6	31	F.		42.4
8	M.	65	37.1	15	"		42.6
5	F.	30	37.5	98	M.	12	43.9
34	"	25	37.5	57	"		44.5
65	M.		38.2	63	"		45.2

TABLE II

Constancy of Per Cent SCA Hemoglobin with Time

Case No.	SCA hemoglobin	Difference
	per cent	*per cent*
15	42.6, after 2 mths. 41.3	−1.3
25	29.3, " 2½ " 31.7	+2.4
7	42.0, " 3 " 39.8	−2.2
4	33.3, " 4 " 32.7	−0.6
5	37.5, " 4 " 40.1	+2.6

rium with their precursors and catabolic products (13) and small fluctuations of the ratio of the two hemoglobins around a mean value are to be expected. With these considerations in mind it is concluded that the above data support the idea that the ratio of SCA hemoglobin to normal

hemoglobin is relatively constant in a sicklemic individual, at least for periods as long as 4 months.

Effect of Age and Sex—In Table I there are given the age and sex of some of the sicklemic individuals from whom samples of blood were obtained. It is immediately apparent that both sexes have percentages occurring throughout the range of variation demonstrated above and that there is no grouping with respect to age. Consequently, the conclusion has been drawn that these two variables have no effect on the ratio of the two hemoglobins. It is of interest that the total hemoglobin level, which normally is different in the two sexes, apparently has no influence on the ratio of the two hemoglobins.

Effect of Environment—As previously indicated, the persons from whom samples of blood were obtained for use in this study live in widely separated regions, *i.e.*, Los Angeles, California, New Orleans, Louisiana, and

TABLE III

Rôle of Heredity in SCA Hemoglobin

Case No.	Family relationship	SCA hemoglobin
		per cent
38	Mother	41.3
71	Father	34.4
42	Daughter	40.1
43	Son	40.2

Ann Arbor, Michigan. As is to be expected, persons from any one of these regions do not have a characteristic ratio of the two hemoglobins, but rather their ratios fall throughout the range of variation. An even closer scrutiny of the effect of this complex variable is afforded by the occurrence of six cases of husband and wife among the forty-two sicklemic cases mentioned above. The percentages of SCA hemoglobin found in these cases are as follows: 34.4, 42.6; 34.3, 41.3; 44.5, 35.1; 38.2, 27.9; 41.0, 40.5; 41.6, 38.9. Thus, with these sicklemic individuals, who are affected largely by the same environment, the percentages of SCA hemoglobin may be either the same or different.

Owing to the nature of this study it has not been possible to assay the effect of diet on the ratio of the two hemoglobins in sicklemics. However, since there is no known dietary therapy for sickle-cell anemia, it is considered to be unlikely that the variation of the ratio of the two hemoglobins could be traced to a dietary cause.

Influence of Heredity—Since sickle-cell anemia has been shown to be an inherited condition (5), it is not unimportant to assess the rôle heredity

may play in determining the percentage of SCA hemoglobin a sicklemic person may have.

The data in Table III indicate that heredity may be the major factor which determines the percentage of SCA hemoglobin occurring in a sicklemic person. In this family, the percentages of SCA hemoglobin which the parents have are quite different, and the percentages of the two sicklemic children (another child was a sickle-cell anemic) are the same and correspond to that of the mother instead of being intermediate between the percentage of the father and that of the mother or not corresponding with the percentage of either parent. If these results are considered with respect to the single gene hypothesis (5), they suggest that the children may have inherited the mother's allele for sickling and that this particular allele results in a characteristic proportion of SCA hemoglobin.

DISCUSSION

In view of the results of previous investigations (5–7), it is perhaps to be expected that heredity may be the dominant factor determining the ratio of SCA hemoglobin to normal hemoglobin in sicklemic individuals. While both the electrophoretic and genetic evidence is in agreement as to the qualitative aspects of the transmission of sickle-cell anemia and sickle-cell trait, the variation of the data exhibited in Table I and the clinical observation that gradations occur in the severity of the disease in sickle-cell anemia (14) imply that the simple hypothesis of Neel (5) must be extended and amplified in order to explain the quantitative aspects of sickle-cell disease. One might consider the possibility that the rates of production of both normal and SCA hemoglobins are subject to independent modification by other factors. The rate of production of hemoglobin in a sickle-cell anemic and the ratio of the two hemoglobins in an individual having a sickle-cell trait may be dependent on the modifying factors present in the individual, on the particular allelic form of the gene he possesses, or on undisclosed elements of his environment.

Anomalous Sickle-Cell Anemics

During the course of this work it became necessary to examine electrophoretically samples of hemoglobin obtained from a series of sickle-cell anemics. Among the members of this series there were some who had been able, without apparent difficulty, to exist without the need of blood transfusions for well over a year. A possible explanation for this behavior in three of these cases presented itself when the electrophoretic patterns of the hemoglobin from these persons revealed the presence of varying amounts of normal hemoglobin. Fig. 3 contains reproductions of Longsworth scanning diagrams of the ascending boundaries of electrophoretic

experiments on the hemoglobin of these three persons. Case 22 had not received a transfusion with normal blood for over a year before the first blood sample was obtained; a second blood sample obtained 6 months after the first revealed that normal hemoglobin was still present, even though no normal blood had yet been transfused. These patterns are similar to those obtained from sickle-cell anemics at varying lengths of time after transfusion with normal blood.

Individuals such as these may be classified as border line sickle-cell anemics; their ability to produce significant amounts of normal hemoglobin

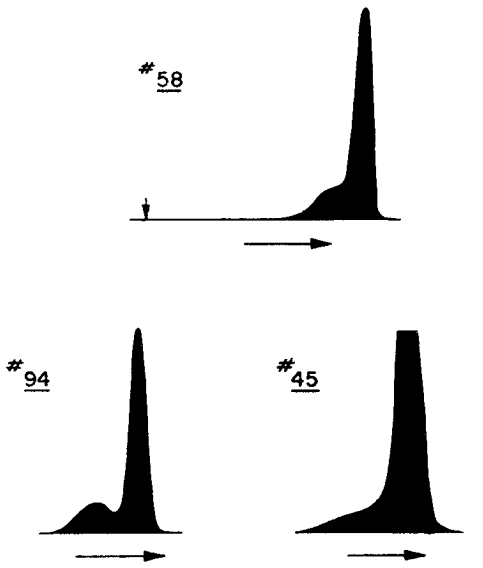

FIG. 3. Ascending boundaries of electrophoresis experiments on the carbonmonoxyhemoglobin from some anomalous sickle-cell anemics. The conditions used for electrophoresis were the same as those employed for the sicklemic samples. The leading peak is due to SCA hemoglobin. In these cases the normal hemoglobin constitutes 5 to 20 per cent of the total.

probably accounts for their improved level of existence, since the characteristics of their erythrocytes would be intermediate between those of sicklemia and sickle-cell anemia erythrocytes. The former are known to have a survival time both in normal and sickle-cell anemic individuals equal to that of normal erythrocytes (15–17).

It is felt that here is fundamental evidence to support the long held clinical feeling that "... all shades of variation between the trait and severe anemia may be encountered though no adequate evidence has been offered" (14).

The rôle of heredity of such cases is unclear, since the investigation of the parents of two of these persons revealed that they were typical sicklemics: Case 22, father 41.0, mother 40.5; Case 45, father 44.5, mother 35.1 per cent of SCA hemoglobin. Further investigation is necessary to explain these cases.

SUMMARY

The ratio of sickle-cell anemia hemoglobin to normal hemoglobin has been determined in forty-two sicklemic individuals who were otherwise normal and genetically unrelated. In this series of individuals the SCA hemoglobin varied from about 24 to 45 per cent.

Of the several factors tested, *i.e.*, time, age, sex, some environmental factors, and heredity, the latter is the only one not excluded as having an appreciable influence on the ratio of the two hemoglobins which occurs in a sicklemic person.

The discovery of some anomalous cases of sickle-cell anemia is reported. In contrast to the true sickle-cell anemic, these individuals are able to produce small amounts of normal hemoglobin.

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BIBLIOGRAPHY

1. Sherman, I. J., *Bull. Johns Hopkins Hosp.*, **67**, 309 (1940).
2. Hahn, E. V., and Gillespie, E. B., *Arch. Int. Med.*, **39**, 233 (1927).
3. Taliaferro, W. H., and Huck, J. B., *Genetics*, **8**, 594 (1923).
4. Diggs, L. W., Ahmann, C. F., and Bibb, J., *Ann. Int. Med.*, **7**, 769 (1933).
5. Neel, J. V., *Science*, **110**, 64 (1949).
6. Itano, H. A., and Pauling, L., *Federation Proc.*, **8**, 209 (1949).
7. Pauling, L., Itano, H. A., Singer, S. J., and Wells, I. C., *Science*, **110**, 543 (1949).
8. Schroeder, W. A., Kay, L. M., and Wells, I. C., *J. Biol. Chem.*, **187**, 221 (1950).
9. Itano, H. A., and Pauling, L., *Blood*, **4**, 66 (1949).
10. Drabkin, D. L., *J. Biol. Chem.*, **164**, 703 (1946).
11. Longsworth, L. G., *Ann. New York Acad. Sc.*, **41**, 267 (1941).
12. Tiselius, A., and Kabat, E., *J. Exp. Med.*, **69**, 119 (1939).
13. Schoenheimer, R., The dynamic state of body constituents, Cambridge, 25–65 (1942).
14. Wintrobe, M. M., Clinical hematology, Philadelphia, 2nd edition, 515 (1946).
15. Callender, S. T. E., and Nickel, J. F., *J. Lab. and Clin. Med.*, **32**, 1397 (1947).
16. Singer, K., Robin, S., King, J. C., and Jefferson, R. N., *J. Lab. and Clin. Med.*, **33**, 975 (1948).
17. Callender, S. T. E., Nickel, J. F., Moore, C. V., and Powell, E. O., *J. Lab. and Clin. Med.*, **34**, 90 (1949).