PART I

CONTRIBUTIONS TO THE STUDY OF SICKLE CELL HEMOGLOBIN

PART II

A RAPID DIAGNOSTIC TEST FOR SICKLE CELL ANEMIA

Thesis by

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ABSTRACT

The red blood cells of individuals who have either sickle cell anemia or sicklemia possess the capacity to undergo reversible changes in shape in response to changes in the partial pressure of oxygen. When the oxygen pressure is lowered, these cells change their forms from the normal biconcave disk to crescent, holly wreath, and other forms. This process, known as sickling, occurs more readily in sickle cell anemia than in sicklemia. The evidence available at the time this investigation was begun indicated that the process of sickling might be intimately associated with the state and nature of the hemoglobin in the sickle cell erythrocyte. Electrophoretic investigations revealed a difference in net charge between the hemoglobins of normal individuals and sickle cell anemia individuals. The hemoglobin from the erythrocytes of sicklemic individuals was found to consist of two electrophoretic components, identifiable with sickle cell anemia hemoglobin and normal hemoglobin. The globins derived from these hemoglobins cannot be distinguished from each other on the basis of electrophoretic experiments. Titration studies on these hemoglobins failed to reveal any significant differences in number and type of ionizable groups. A rapid chemical method was developed for producing sickle cells by the use of sodium dithionite.

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PART I

CONTRIBUTIONS TO THE STUDY OF SICKLE CELL HEMOGLOBIN

INTRODUCTION

The existence of the disease now called sickle cell anemia was first brought to the attention of the medical profession by the description of a case by Herrick (1) in 1910. The most striking hematological characteristic of this disease is the presence of peculiar sickle shaped erythrocytes which are less resistant to destruction within the circulation than normal erythrocytes. A state of chronic anemia exists in the afflicted individual, and this anemia is periodically accentuated by "crises" during which the rate of destruction of red cells is greatly increased. The symptoms and tissue changes in sickle cell anemia have been attributed to the chronic anemia and to multiple thromboses in small blood vessels of masses of abnormally shaped red cells. The condition is hereditary, and is confined almost exclusively to negroes. While whole blood transfusions are employed to alleviate temporarily the severe anemia following crises, no specific treatment is known for the disease, and most victims do not survive the third decade of life. Death may occur from intercurrent infections or from the destructive effects of the anemia and thromboses on the vital organs.

A closely related hematological condition is sickle cell trait or sicklemia. Individuals in whom this condition exists have no symptoms of sickle cell disease but possess erythrocytes

which assume the sickle shape under appropriate conditions. The existence of this trait in an individual apparently bears no relation to his health and life span. Whereas in sickle cell anemia the history and symptoms of the patient frequently lead to its diagnosis even before his blood is examined, in sicklemia the presence of cells which sickle is the only criterion of diagnosis. In studies made in the United States it has been found that about seven or eight per cent of negroes have this trait. Studies in Africa have yielded an average incidence of about twenty per cent (2). The ratio of incidence of the anemia to the trait in this country is about one to forty. The ratio of occurrence at the time of birth must be considerably higher because of the high mortality among sickle cell anemia individuals in early life.

Neel (3) has recently 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.

Of primary interest to the chemist is the change in shape of the erythrocytes in sickle cell anemia and sickle cell trait in response to the presence or absence of oxygen or carbon monoxide. In the presence of either of these gases the cells

are binconcave discs indistinguishable in shape from normal erythrocytes. Removal of the gas causes the cells to undergo remarkable shape changes which lead eventually to the formation of typical sickle shaped cells. Initially the hemoglobin appears to aggregate at one or more foci within the cell. This is followed by the collapse of the cell membrane and the joining of the hemoglobin masses into a single elongated mass. The process is reversible, the sickled cells reverting to the biconcave disc shape upon the addition of oxygen or carbon monoxide. This procedure may be repeated over and over with same cells without any noticeable diminution in their capacity to sickle. The existence of "irreversibly sickled" cells (4) has been reported. This phenomenon occurs in cells which have been maintained in a sickled condition for long periods of time, and the apparent inability to resume the discoidal form may be due to a permanent damage to the cell membrane (5).

Aside from numerous clinical reports the bulk of the studies on sickle cell anemia have been concerned with the erythrocyte and the sickling process. It was first noted in 1917 (6) that sickling of the erythrocytes of a sickle cell anemia patient occurs when a drop of his blood has been sealed off from the atmosphere for varying periods of time. This test is still the one most widely used in the diagnosis of sickle cell anemia and trait, and it has been employed by numerous

investigators to study the effects of varying physical and chemical conditions on the sickling process. It was found that sickle cell formation is accelerated by increase of temperature and by the presence of white blood cells and bacteria. Serum was found to play no part in the sickling process, and sickling was observed in saline suspensions of susceptible cells. The significance of these findings was clarified by the experiments of Hahn and Gillespie (7), which established for the first time the relationship of sickling to the presence or absence of oxygen and carbon monoxide. Erythrocytes from sickle cell anemia individuals were suspended in chamber and observed through a microscope while different gases were passed through the chamber. It was found that passing carbon dioxide, nitrous oxide, or hydrogen through the chamber caused the cells to sickle and that passing oxygen or carbon monoxide through the cell caused them to revert to the discoid form. The reversibility of the process was demonstrated by alternately passing one of the inert gases and either oxygen or carbon monoxide through the chamber. The observation of these workers that acidification of the cell suspension favors the sickling process is in accord with the dependence of oxygen saturation of hemoglobin on pH.

Sherman (8) used the technique of Hahn and Gillespie to compare the resistance to sickling of sickle cell anemia and

trait cells. He found that a greater reduction of partial pressure of oxygen is required to cause trait cells to sickle than anemia cells. The marked difference in resistance to sickling is evident in his plot of per cent of cells sickled against air pressure. 65 per cent of sickle cell anemia cells are sickled at 80 millimeters air pressure; at 20 millimeters 99 per cent are sickled. None of the trait cells are sickled at 80 millimeters and 12 per cent at 20 millimeters. The air pressure must be reduced to 10 millimeters to cause 95 per cent sickling in trait blood. A corollary finding was that in the venous circulation of sickle cell anemia individuals 30 to 60 per cent of the red cells were sickled in contrast to less than 1 per cent in trait individuals. In the course of these studies Sherman noted that sickled cells were birefringent.

The erythrocytes in sickle cell anemia are more resistant to hemolysis in hypotonic saline than normal cells. When these cells are sickled, they are less resistant to destruction by mechanical trauma (9) than normally shaped cells. The latter observation is in accord with the observation that sickled cells are rigid while discoidal cells are flexible. Ghosts of sickle cells never have the sickle shape (5). Cardozo (10) in his immunological studies of cells from sickle cell anemia

patients and normal individuals could not find any difference in their agglutinating reactions. He concluded that there was no correlation between the sickling of erythrocytes and the blood agglutinogens, A, B, M, or N, or combinations of these. More recently Snyder and his co-workers (11) have presented evidence for linkage between the sickle cell trait and the MN blood group.

In spite of the apparent relationship between the state of combination of hemoglobin and the sickling process no report was found in the literature of any studies of hemoglobin from sickle cells. Heretofore the only method for distinguishing sickle cell from normal hemoglobin has been its effect on the shape of erythrocytes. It was felt that the detection of even one measurable abnormality in sickle cell hemoglobin would be a significant step in the study of the properties of hemoglobin and the solution of the problem of characterizing sickle cell hemoglobin. The unique properties of hemoglobin suggested a number of approaches to the study of sickle cell hemoglobin. The role of oxygen and carbon monoxide in reversing sickling demonstrated that the heme sites on the molecule played some role in the sickling process. Magnetic susceptibility studies were proposed to compare the susceptibilities of corresponding compounds of normal and sickle cell

hemoglobin and to determine the effect of sickle cell formation on the susceptibility of sickle cell ferrohemoglobin. At the time these experiments were proposed the amount of sickle cell anemia blood available was not sufficiently large to permit measurements on the Gouy balance, and the work was deferred pending the completion of a microsusceptimeter. The effect on sickling of molecules and ions other than oxygen and carbon monoxide was investigated. This study led to the use of sodium dithionite for the rapid production of sickling.

Since there was no obvious clue as to the nature of the abnormality in sickle cell hemoglobin, it was necessary to consider all possible methods of characterizing protein molecules. The first method which was applied was electrophoresis; the apparatus was available, and the amount of hemoglobin required for each experiment was small. The choice was a fortunate one as the electrophoretic method proved to be particularly sensitive in demonstrating the type of difference which exists between the normal and sickle cell hemoglobin molecules. This difference is in the net charge of the molecules. Titration studies were then undertaken in an attempt to elucidate the cause of the difference. Preliminary diffusion and sedimentation studies were also performed.

ELECTROPHORETIC STUDIES

A. Hemoglobin from normal and sickle cell anemic individuals.

(1) Introductory note.

Little work has been reported on the electrophoresis of human hemoglobin. Andersch, Wilson, and Menten (12) studied the electrophoretic mobilities of adult and fetal human carbonmonoxyhemoglobin. Stern, Reiner, and Silber (13) hemolyzed erythrocytes and analyzed the entire hemolysate electrophoretically. These papers did not supply a sufficient number of results to be taken as controls for similar studies on sickle cell anemia hemoglobin: furthermore there was no assurance that the experimental conditions of these workers would be duplicated. Normal hemoglobin was therefore examined in order to establish a set of experimental conditions and results with which sickle cell hemoglobin could be compared. Ferrohemoglobin and carbonmonoxyhemoglobin were selected for study because of their presence in sickled and non-sickled cells, respectively. The carbonmonoxide compound was taken in preference to the oxygen compound because of its greater stability. The amphoteric behavior of these two compounds is the same. The hemoglobins from normal, sickle cell anemic, and sicklemic individuals were examined.

(2) Experimental methods.

Three types of experiments were performed in the comparison of normal and sickle cell anemia hemoglobins: (a) with carbonmonoxyhemoglobins; (b) with uncombined ferrohemoglobins in the presence of dithionite ion which prevents oxygenation and oxidation to ferrihemoglobin; and (c) with carbonmonoxyhemoglobins in the presence of dithionite ion. The presence of dithionite does not affect the equilibrium between ferrohemoglobin and carbon monoxide. The experiments of type c were performed and compared with those of type a in order to ascertain whether the presence of dithionite ion causes any specific electrophoretic effect. Gralen (14) has already shown that if a dithionite treated carbonmonoxyhemoglobin solution is freed of the ion by dialysis, the electrophoretic behavior of the resulting protein solution is the same as that of the untreated protein.

Potassium phosphate buffers of 0.1 ionic strength and pH values of 5.7, 6.9, 7.0, and 8.0 were prepared. Since it was not feasible to manipulate the hemoglobin solutions in an oxygen free atmosphere, it was necessary to add a reducing agent, sodium dithionite, to the buffer to prevent the oxygenation of ferrohemoglobin. The amount of dithionite necessary to maintain the buffer and hemoglobin solution oxygen free during the course of the experiment was determined empirically; a large excess was avoided in order to minimize

the effects of dithionite ion and its oxidation and decomposition products on the pH and ionic strength of the buffer.

The pH and conductivity of the buffer were measured for each experiment. The conductivity measurements were made at the temperature of the electrophoresis bath; the pH values were taken on buffer samples which were at room temperature.

Normal human red blood cells were obtained from a commercial laboratory within two days after the blood was drawn. Sickle cell anemia blood was obtained from patients in the Los Angeles County Hospital and the Children's Hospital of Los Angeles. These patients were clinically diagnosed as having sickle cell anemia, and had not received any transfusions of normal blood within three months prior to the time of sampling. Stroma free concentrated solutions of human adult hemoglobin were prepared by the method used by Drabkin (15). The solutions were analyzed for nitrogen and diluted just before use with the appropriate buffer until the hemoglobin concentrations were close to 0.5 grams per 100 milliliters and were then dialyzed against large volumes of these buffers for 12 to 24 hours at 4°C.

The buffers for the experiments with ferrohemoglobin and carbonmonoxyhemoglobin in presence of dithionite were prepared by adding 300 milliliters of 0.1 ionic strength sodium

dithionite solution to 3.5 liters of 0.1 ionic strength buffer. One hundred milliliters of 0.1 molar sodium hydroxide was then added to bring the pH of the buffer to approximately its original value. Ferrohemoglobin solutions were prepared for electrophoresis by diluting the concentrated solutions with this dithionite containing buffer and dialyzing against it under a nitrogen atmosphere. The hemoglobin solutions for the experiments of type c were made up similarly except that they were saturated with carbon monoxide after dilution and were dialyzed under a carbon monoxide atmosphere. The experiments of type b were set up similarly except that dithionite was not added. To accelerate the attainment of equilibrium the dialysis bags were kept in continuous motion in the buffers by means of a stirrer. A mercury seal was employed to prevent the escape of the nitrogen and carbon monoxide and contamination with atmospheric oxygen, which would have exhausted the small amount of dithionite in the buffers.

The experiments were carried out in the modified Tiselius electrophoresis apparatus described by Swingle (16). Potential gradients of 4.8 to 8.4 volts per centimeter were employed, and the duration of the runs varied from 6 to 20 hours. The temperature at which the runs were performed was 1.5°C.

(3) Results.

The Longsworth scanning diagrams of sickle cell anemia ferrohemoglobin and carbonmonoxyhemoglobin did not differ significantly from those of the corresponding normal compounds. In each case the hemoglobin molecules migrated as a single peak although the ferrohemoglobin peaks appeared broader than the carbonmonoxyhemoglobin peaks. That this broadening was not due to the presence of dithionite ion was shown by the diagrams of carbonmonoxyhemoglobin solutions containing dithionite, which did not differ from those not containing dithionite.

Occasionally small amounts (less than 5 per cent of the total protein) of material with mobilities different from that of either kind of hemoglobin were observed in these uncrystallized hemoglobin preparations. According to the observations of Stern, Reiner, and Silber (13) a small amount of a component with a mobility smallerthan that of oxyhemoglobin at pH 8.6 is present in human erythrocyte hemolysates. This component is observable in the sickle cell anemia and mixture diagrams of Figure 3.

A significant difference was found in the electrophoretic mobilities of hemoglobin derived from erythrocytes of normal individuals and from those of sickle cell anemia

individuals. In the pH range 5.7 to 8.1 sickle cell anemia ferrohemoglobin and carbonmonoxyhemoglobin behaved as more positive ions than the corresponding normal compounds. The mobility difference between the ferrohemoglobins was approximately the same as that between the carbonmonoxyhemoglobins. The mobility data are shown in Tables I to VI and Figures 1 and 2. The two types of hemoglobin are particularly easily distinguished as the carbonmonoxy compounds in phosphate buffers of 0.1 ionic strength at pH 6.9 and 7.0. In this pH region, which is between the isoelectric points of the two compounds, sickle cell anemia carbonmonoxyhemoglobin moves as a positive ion while the normal compound moves as a negative ion, and there is no detectable amount of one type present in the other. The results on the carbonmonoxyhemoglobins show that their mobilities are not affected by the presence of dithionite ions.

It is of interest that ferrohemoglobin was found to have a lower isoelectric point in phosphate buffer than carbonmonoxyhemoglobin. Titration studies have indicated that oxyhemoglobin (similar in electrophoretic properties to the carbonmonoxy compound) has a lower isoelectric point than ferrohemoglobin in sodium chloride solutions or in the absence of added salt (17). These results might be reconciled by assuming that the ferrous iron of ferrohemoglobin forms complexes with phosphate ions which cannot be formed when the iron is combined with oxygen or carbon monoxide.

Mobility experiments in cacodylate buffer of ionic strength 0.1 and pH 6.9 yielded mobilities of 1.30×10^{-5} and 1.57×10^{-5} cm^2 sec^{-1} $volt^{-1}$ for normal and sickle cell anemia carbonmonoxyhemoglobins, respectively. A similar difference was found in cacodylate buffer of pH 6.52 (18). These buffers were 0.08 molar with respect to sodium chloride. These experiments with a buffer quite different from phosphate buffer demonstrate that the difference between the hemoglobins is essentially independent of type of buffer ions present.

Table I.

Electrophoretic Mobilities of Normal Ferrohemoglobin in Presence of Dithionite.

Exp.	Individual	pH	$\mu \times 10^5$*
22	N 2	8.08	-2.43
24	N 3	8.08	-2.52
30	N 4	8.05	-2.36
39	N 7	7.91	-2.32
42	N 7	5.79	1.52
48	N 8	5.80	1.63

Table II.

Electrophoretic Mobilities of Normal CO-Hemoglobin in Presence of Dithionite.

Exp.	Individual	pH	$\mu \times 10^5$*
23	N 2	8.12	-2.30
25	N 3	8.08	-2.02
31	N 4	8.02	-2.20
32	N 5	8.12	-2.31
34	N 6	7.81	-2.30
58	N 9	5.67	2.61
59	N 9	5.78	2.44

* Mobility (μ) is given in $cm.^2$ $sec.^{-1}$ $volt^{-1}$ in Tables I to VI.

Table III.

Electrophoretic Mobilities of Normal CO-Hemoglobin in Absence of Dithionite.

Exp.	Individual	pH	$\mu \times 10^5$
18	N 1	7.99	-2.21
40	N 7	8.01	-2.65
49	N 8	8.01	-2.36
51	N 8	7.00	-0.16
57	N 9	7.00	-0.07
90	N 11	6.90	-0.20
66	N 11	6.90	-0.08
41	N 7	5.71	2.34
50	N 8	5.70	2.31

Table IV.

Electrophoretic Mobilities of Sickle Cell Anemia Ferrohemoglobin in Presence of Dithionite.

Exp.	Individual	pH	$\mu \times 10^5$
37	A 1	7.87	-1.91
36	A 1	7.75	-1.86
44	A 2	7.75	-1.46
45	A 2	5.78	2.28
55	A 3	5.78	2.24

Table V.

Electrophoretic Mobilities of Sickle Cell Anemia CO-Hemoglobin in Presence of Dithionite.

Exp.	Individual	pH	$\mu \times 10^5$
38	A 1	7.93	-1.78
58	A 3	5.78	2.68

Table VI.

Electrophoretic Mobilities of Sickle Cell Anemia CO-Hemoglobin in Absence of Dithionite.

Exp.	Individual	pH	$\mu \times 10^5$
56	A 3	8.01	-1.91
43	A 2	7.99	-1.76
53	A 3	7.00	0.47
70	A 4	6.90	0.34
87	A 5	6.90	0.37
89	A 5	6.90	0.31
95	A 6	6.90	0.34
96	A 7	6.90	0.38
97	A 8	6.90	0.30
98	A 9	6.90	0.30
99	A 10	6.90	0.33
100	A 11	6.90	0.35
46	A 2	5.71	3.15
52	A 3	5.68	2.64

Table VII.

Isoelectric Points in Phosphate Buffer, $\mu = 0.1$, at 1.5°C.

Compound	Normal	Sickle Cell Anemia	Difference
Carbonmonoxyhemoglobin	6.87	7.09	0.22
Ferrohemoglobin	6.68	6.91	0.23

Figure 1.

Mobility (μ) - pH curves for carbonmonoxyhemoglobins in phosphate buffers of 0.1 ionic strength. The black circles and black squares denote the data for experiments performed with buffers containing dithionite ion. The open square designated by the arrow represents an average value of 10 experiments on the hemoglobin of different individuals with sickle cell anemia. The mobilities recorded in this graph are averages of the mobilities in the ascending and descending limbs.

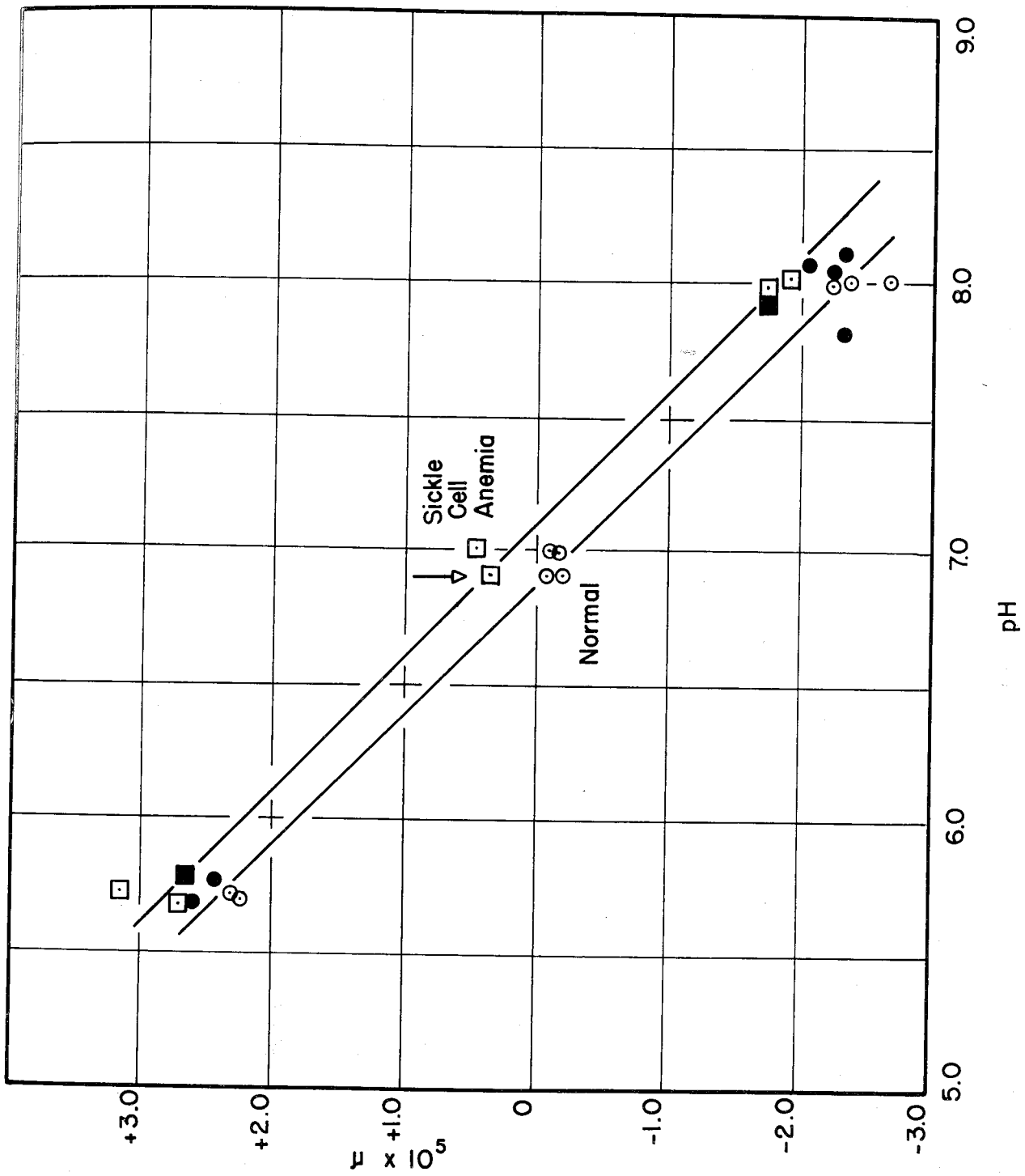
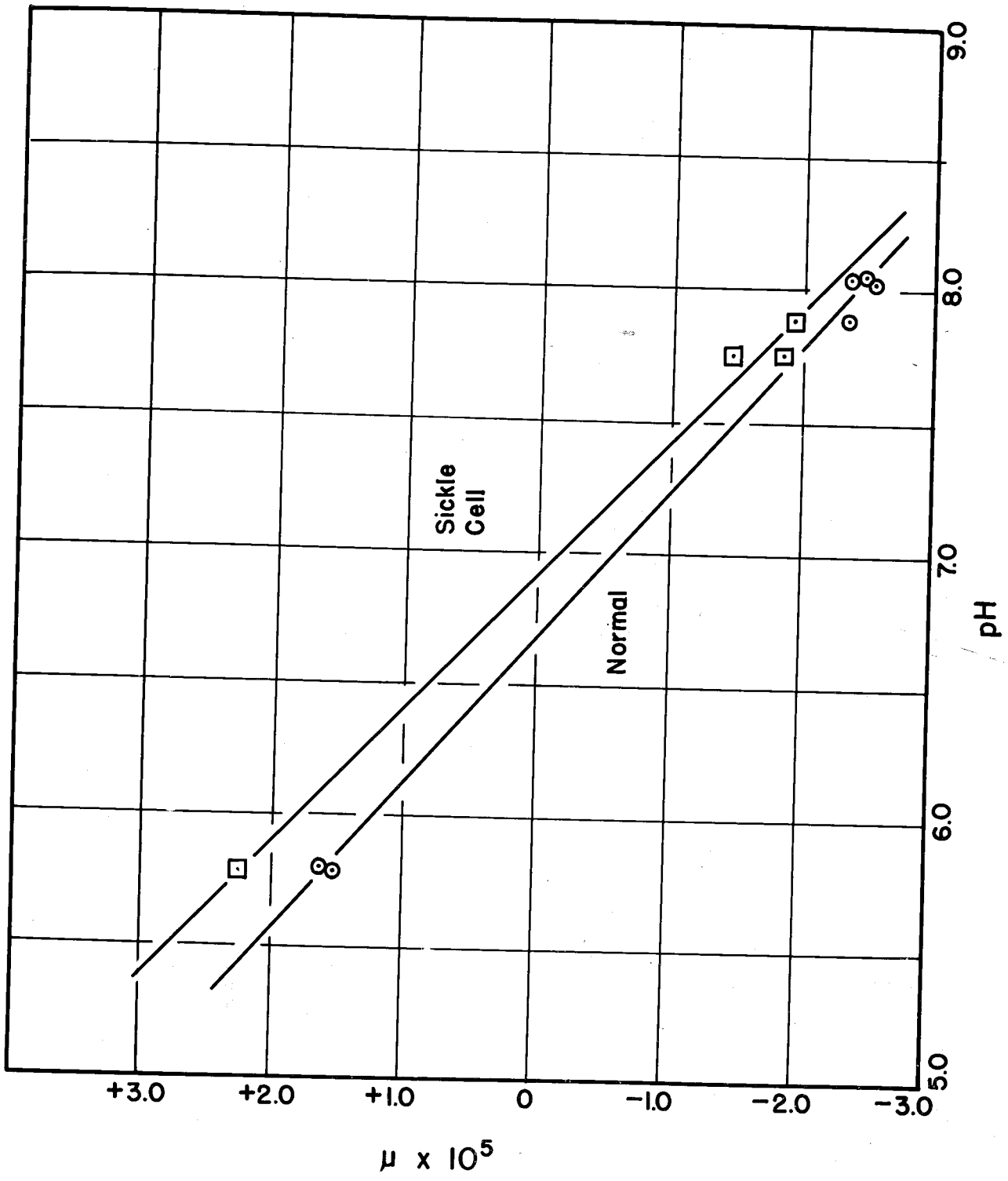

Sickle Cell Anemia
Normal
+3.0
+2.0
+1.0
0
-1.0
-2.0
-3.0
μ x 10^5
5.0
6.0
7.0
8.0
9.0
pH

Figure 2.

Mobility (μ) - pH curves for ferrohemoglobins in phosphate buffers of 0.1 ionic strength containing dithionite ion. The mobilities recorded in the graph are averages of the mobilities in the ascending and descending limbs.


9.0
8.0
7.0
6.0
5.0
pH
Sickle Cell
Normal
+3.0
+2.0
+1.0
0
-1.0
-2.0
-3.0
μ x 10⁵

B. Hemoglobin from sicklemic individuals and mixtures of hemoglobins.

(1) Introductory note.

The limitation on the amount of blood which can be obtained from sickle cell anemia patients, the majority of whom are children, has hampered the hemoglobin studies. The blood of sickle cell trait individuals was therefore investigated as a possible source of large quantities of the abnormal hemoglobin. These individuals have erythrocytes which sickle, but they do not suffer any ill effects from this trait. The removal of the usual transfusion donation volume of 500 milliliters of whole bloodfrom a healthy individual having this trait would not involve any abnormal risks. The erythrocytes which are usually discarded after the removal of plasma were obtained from a commercial laboratory and tested for sickling. The hemoglobin prepared from cells which showed sickling were then examined electrophoretically and were found to contain two hemoglobin components. In order to establish the nature of these components a series of experiments were performed on mixtures of hemoglobins from normal, sickle cell anemic, and sicklemic individuals.

(2) Experimental methods.

A specimen of red cells from each bleeding, which contained from 200 to 250 milliliters of cells, was tested for

sickling with sodium dithionite reagent. Only the erythrocytes from negro donors were tested. Out of 41 specimens tested 5 were positive for sickling. Hemoglobin solutions were prepared from each of these speciments individually for electrophoretic examination. Only the carbonmonoxy compound was examined, and pH 6.9 phosphate buffer of ionic strength 0.1, inwhich normal and sickle cell anemia carbonmonoxyhemoglobins have opposite charges, was employed. A potential gradient of 4.7 volts per centimeter was used throughout. After a preliminary run of six hours established the existence of two peaks in the trait hemoglobin scanning diagram, subsequent runs of fifteen to twenty hours were performed to resolve these peaks. The six hour run was performed on a 0.5 per cent hemoglobin solution; the other runs were made at a concentration of 1.0 per cent, which yields a better diagram of the resolving peaks. The experiments on mixtures of normal and sickle cell anemia hemoglobins and of normal and sickle cell trait hemoglobins were performed under the same conditions.

(3) Results.

The six hour Longsworth scanning diagram of 0.5 per cent hemoglobin from sickle cell trait cells showed a definite skewing and spreading of one boundary and a partial resolution into two peaks of the other. Comparable diagrams of hemoglobin from normal and sickle cell anemia cells show single

homogeneous peaks. These diagrams are shown in Figure 4.

The twenty hour scanning diagrams are shown in Figure 3. Sickle cell trait hemoglobin at 1.0 per cent concentration shows resolution into two peaks at both boundaries at this time. Normal and sickle cell anemia hemoglobin diagrams show that these compounds exist as single electrophoretic components even after twenty hours. The mobilities of the two components present in the hemoglobin of trait cells approximate those of normal and sickle cell hemoglobins. The apparent mobilities of the components in trait hemoglobin are somewhat lower than those of normal and sickle cell anemia hemoglobin. This anomolous behavior in the diagrams is due in part to the superposition of two protein concentration gradients on each other (19). The same phenomenon is evident in the analysis of a solution containing equal amounts of normal and sickle cell anemia hemoglobins at a total concentration of 1.0 per cent, the diagram of which is also shown in Figure 3. Experiments on mixtures of normal and sickle cell trait hemoglobins showed only two components, and the mobilities of these were the same as those in the trait hemoglobin and the normal-anemia hemoglobin mixture.

The mobilities of the components in the various mixtures and in trait hemoglobin are given in Table VIII. These mobilities were computed from the boundary in which the peaks were symmetrical, namely the one in which the normal component

ascends and the anemia component descends. In the region of the isoelectric points where the absolute mobilities are small the calculated mobilities are subject to large percentage errors from slight variations in the buffer pH and temperature. However, the separation of the peaks depends on the quantity of electricity passed through the solution, a factor which was closely controlled in these experiments. The difference between the mobilities of the two components is therefore of greater significance than the absolute mobilities and has been given in the last column of Table VIII. The agreement among these figures is excellent for the various types of experiments.

It is apparent from Figure 3 that the sicklemia material contains less than 50 per cent of the anemia component. In order to determine this quantity mixtures containing normal and sickle cell anemia hemoglobins at several different ratios at a total concentration of 1.0 per cent were prepared. These mixtures were analyzed electrophoretically by Dr. Ibert C. Wells in cacodylate buffer at pH 6.52 (18). Comparison of these results with an analysis of sicklemia carbonmonoxyhemoglobin in the same buffer yielded a value of 39 per cent for the amount of sickle cell anemia component in the sicklemia hemoglobin. The hemoglobins of five sicklemic individuals were pooled for this experiment. Subsequent experiments in individual

specimens have shown some variation in composition among individuals. In every case the anemia component comprises less than half of the total hemoglobin.

Table VIII.

Electrophoretic Mobilities of Normal and Anemia Components in Sickle Cell Trait CO-Hemoglobin and Mixtures at 1.0% Total Concentration in Phosphate Buffer of pH 6.9, μ 0.1.

Exp.	Individual	Conc.	Type of Experiment	Mobility x 10^5 Normal Comp.	Anemia Comp.	Difference
88	T 3	1.0%	Trait	-0.094	0.245	0.339
92	T 3	1.0%	Trait	-0.068	0.264	0.332
69	T 1 T 2	0.5% 0.5%	Mixture of two traits	-0.108	0.242	0.350
67	N 11 T 1	0.5% 0.5%	Mixture of normal and trait	-0.061	0.260	0.321
68	N 10 T 1	0.5% 0.5%	Mixture of normal and trait	-0.053	0.272	0.325
72	N 11 A 4	0.5% 0.5%	Mixture of normal and anemia	-0.103	0.212	0.315
91	N 11 A 5	0.5% 0.5%	Mixture of normal and anemia	-0.037	0.305	0.342

Figure 3.

Longsworth scanning diagrams of carbonmonoxyhemoglobin in phosphate buffer of 0.1 ionic strength and pH 6.90 taken after 20 hours electrophoresic at a potential gradient of 4.73 volts/cm.

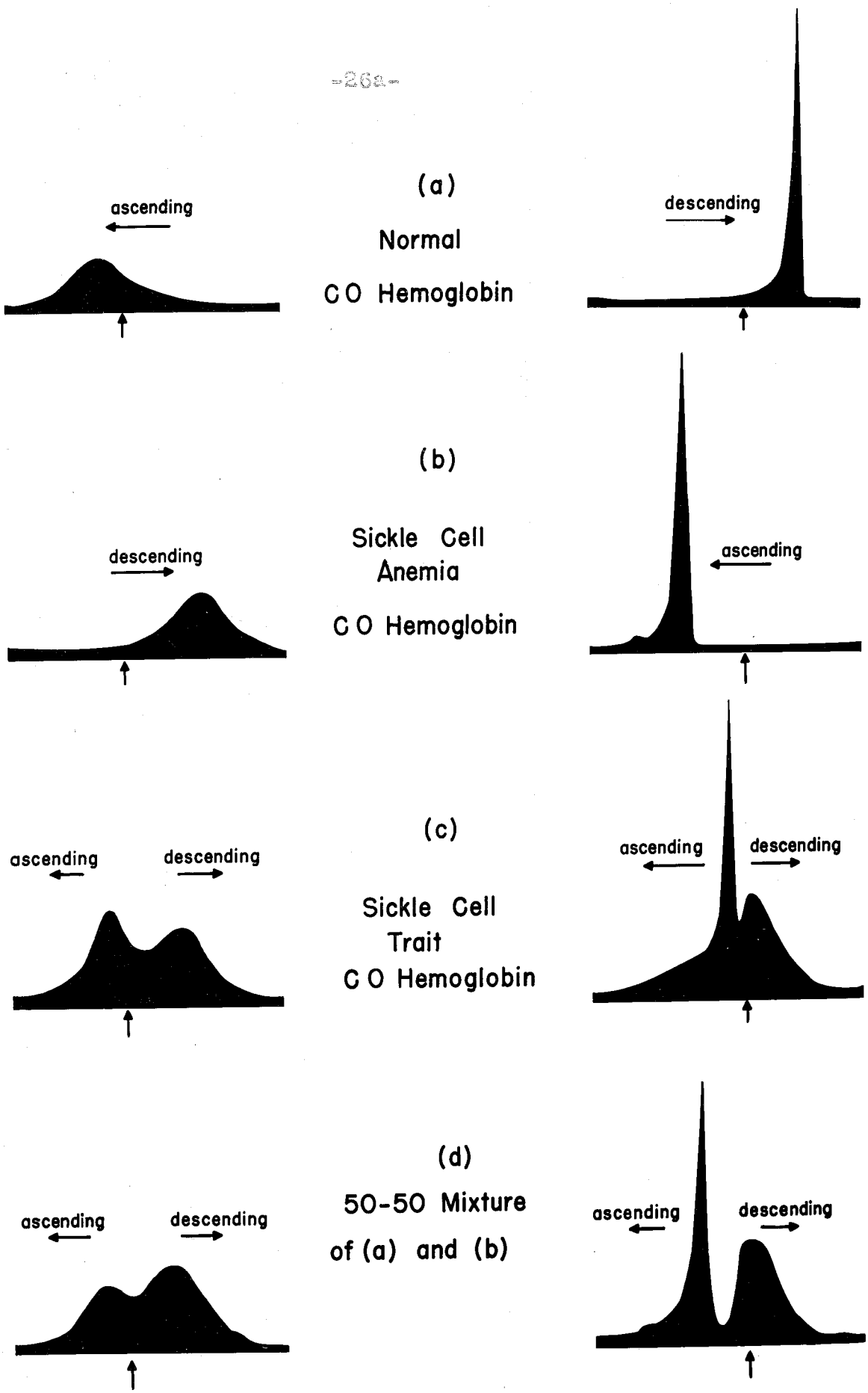

ascending
descending
(a)
Normal
CO Hemoglobin
descending
ascending
(b)
Sickle Cell
Anemia
CO Hemoglobin
ascending
descending
ascending
descending
(c)
Sickle Cell
Trait
CO Hemoglobin
ascending
descending
ascending
descending
(d)
50-50 Mixture
of (a) and (b)

Figure 4.

Longsworth scanning diagrams of carbonmonoxyhemoglobins in phosphate buffer of 0.1 ionic strength and pH 6.90 taken after 5½ and 6 hours electrophoresis at a potential gradient of 4.73 volts/cm. These diagrams show that a prolonged run is not necessary to establish the type of hemoglobin present. The longer runs are of value in determining the ratio of the hemoglobins present in a mixture.

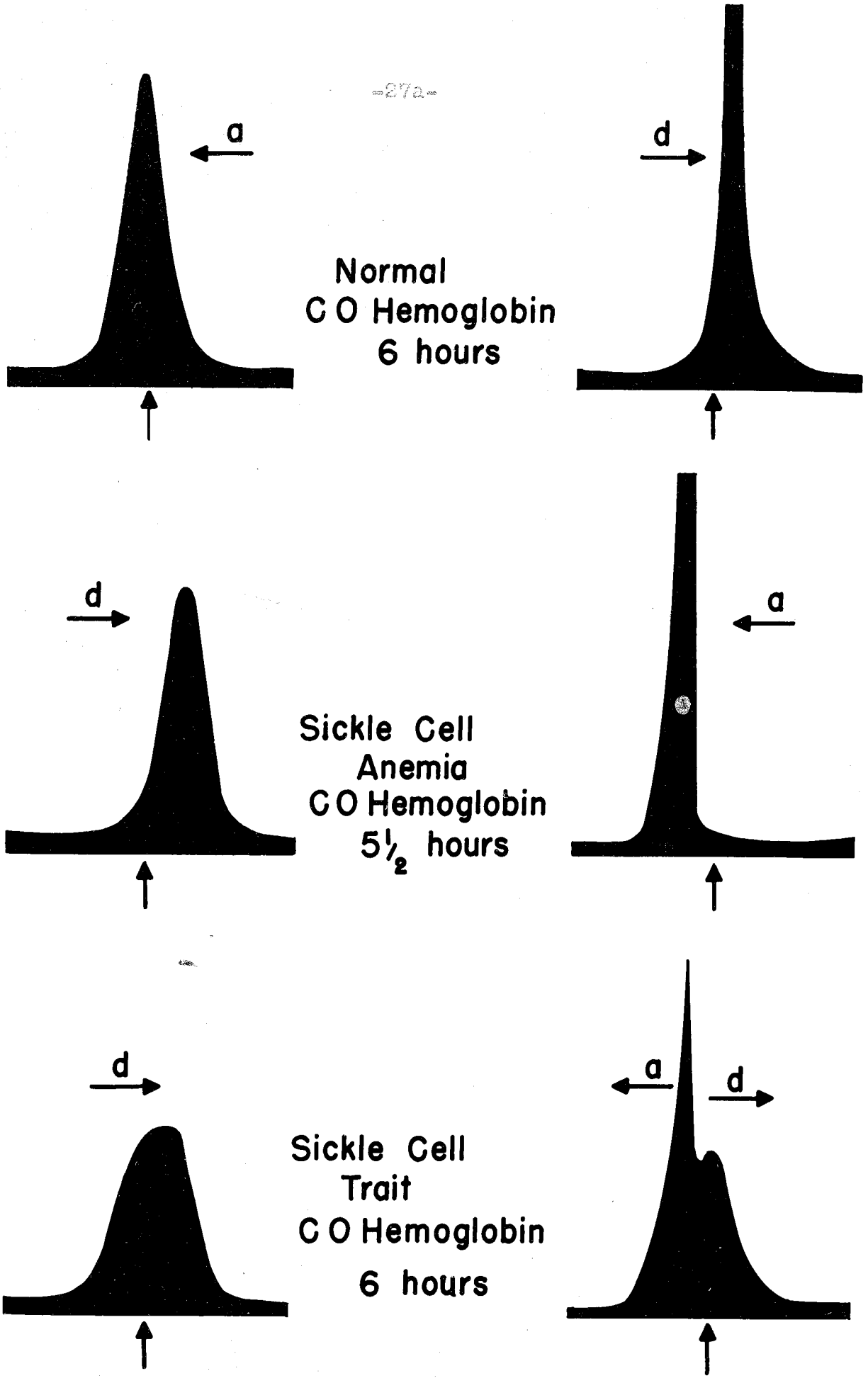
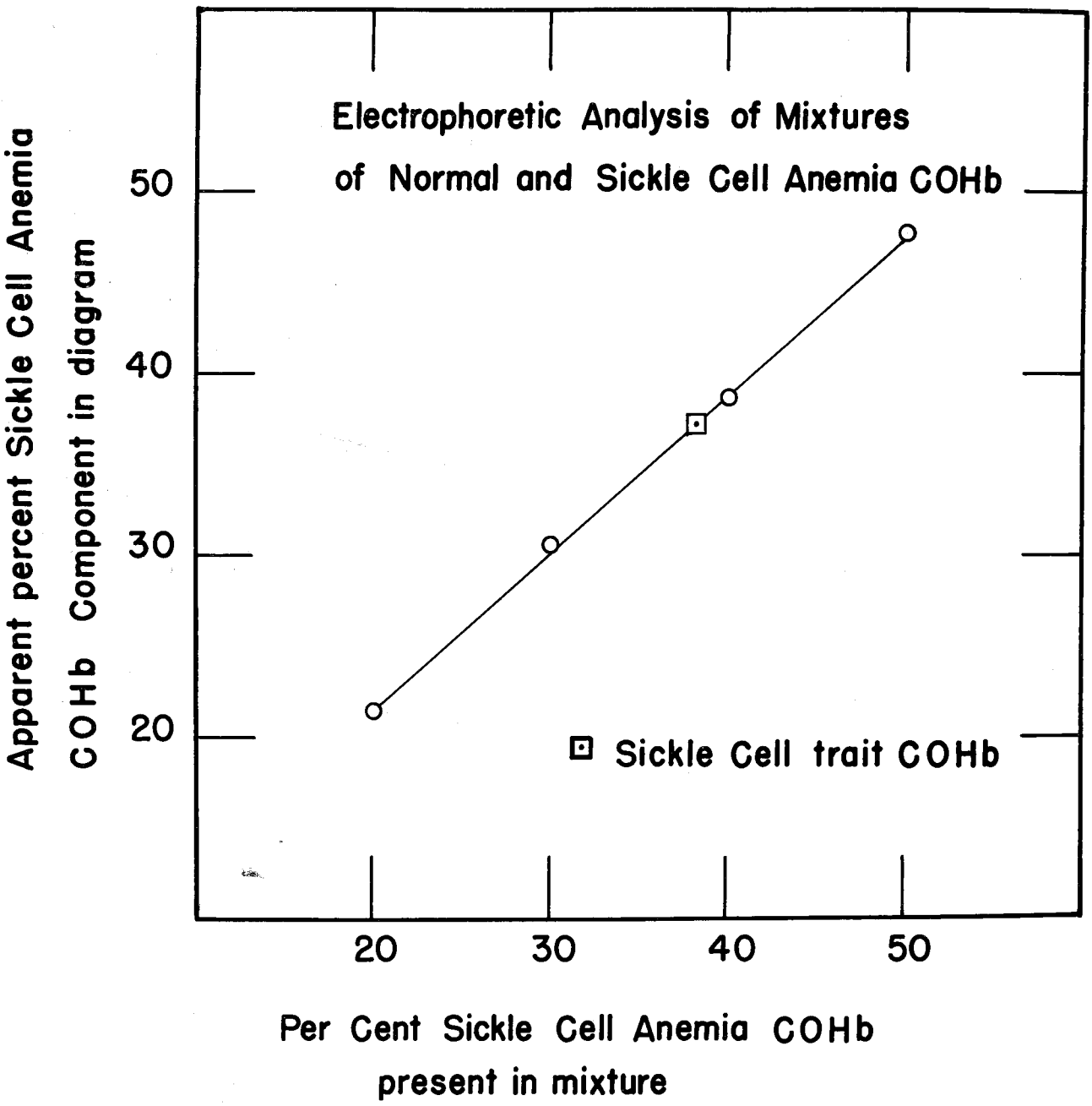

a
d
Normal
C O Hemoglobin
6 hours
d
a
Sickle Cell
Anemia
C O Hemoglobin
5½ hours
d
a
d
Sickle Cell
Trait
C O Hemoglobin
6 hours

Figure 5.

The determination of the percentage of sickle cell anemia carbonmonoxyhemoglobin in known mixtures of the protein with normal carbonmonoxyhemoglobin by means of electrophoretic analysis. The square shows the composition of a solution in which the hemoglobins of 5 sicklemic individuals were pooled.


Electrophoretic Analysis of Mixtures
of Normal and Sickle Cell Anemia COHb
Apparent percent Sickle Cell Anemia
COHb Component in diagram
50
40
30
20
Sickle Cell trait COHb
20
30
40
50
Per Cent Sickle Cell Anemia COHb
present in mixture

C. Globins from normal, sickle cell anemic, and sicklemic individuals.

(1) Introductory note.

The next step in the investigation was to determine whether the heme or the globin portion of the sickle cell anemia hemoglobin molecule was responsible for its difference from the normal hemoglobin molecule. The obvious approach to this problem was to examine the globins by electrophoresis, the method which first detected the difference between the hemoglobins. The preparation of heme and globin and the resynthesis of hemoglobin is a relatively simple procedure; however, the process of dissociating heme from globin with acid denatures the globin, and this denatured globin, which is soluble in acid, must be converted to "native" globin by a process of slow neutralization before it can be used in the resynthesis of hemoglobin (20).

Although natural and resynthesized hemoglobins have the same absorption spectrum, solubility, and crystal form, they differ electrophoretically. Gralen (14) examined three preparations of resynthesized hemoglobin electrophoretically and found that one was similar to natural hemoglobin while the other two were different, in that the isoelectric point was 0.2 pH unit more acid than that of the natural substance. The process of producing native globin from denatured globin

leaves a considerable residue of insoluble protein material which is discarded. For the comparison of the globins the entire yield of denatured globin was examined without renaturation, not only to conserve the material available but also to avoid examining just one product of a possible fractionating process. This precluded the performance of experiments near the isoelectric point where denatured globin is insoluble.

(2) Experimental methods.

The procedure of Anson and Mirsky (20) was followed in the preparation of denatured globin from normal, sickle cell trait, and sickle cell anemia hemoglobins. The globin solutions were slightly turbid, and solid material collected at the boundaries during an electrophoretic run. Centrifugation of the solutions for 30 minutes at 35,500g prior to a run removed the material and eliminated this difficulty. Two normal and two sickle cell trait globin samples of 0.5 per cent concentration were dialyzed against acetate buffer of pH 4.6 and ionic strength 0.1. These solutions were analyzed electrophoretically in pairs, each pair consisting of one specimen each of normal and trait globin. The globin solutions in each pair of experiments were dialyzed simultaneously in the same buffer. The current through the electrophoresis cells in which these solutions were analyzed was connected in series

so that the same quantity of electricity passed through each solution. The potential gradient was 4.9 volts per centimeter, and the duration of the runs five hours. One pair of runs on normal and sickle cell anemia globins was performed in the same manner in phosphate buffer of pH 5.54 and ionic strength 0.1 at 4.0 volts per centimeter for five hours. This buffer is similar to one in which normal and sickle cell anemia hemoglobins showed a significant difference in mobility.

(3) Results.

The Longsworth scanning diagrams of each of the globins showed the two components described by previous workers (21). These diagrams are illustrated in Figure 6. Comparable runs of normal, trait, and anemia globins yielded nearly identical patterns and mobilities. The mobilities are listed in Table IX.

Table IX.

Electrophoresis of Globin.

Exp.*	Individual	Buffer	pH	Mobility x 10^5 Slow Component	Mobility x 10^5 Fast Component
83	N 11	Acetate 0.1 μ	4.64	5.78	6.53
84	T 1			5.69	6.45
85	N 10	Acetate 0.1 μ	4.64	5.74	6.49
86	T 2			5.65	6.48
93	N 11	Phosphate 0.1 μ	5.54	4.01	4.10
94	A 5			4.09	4.19

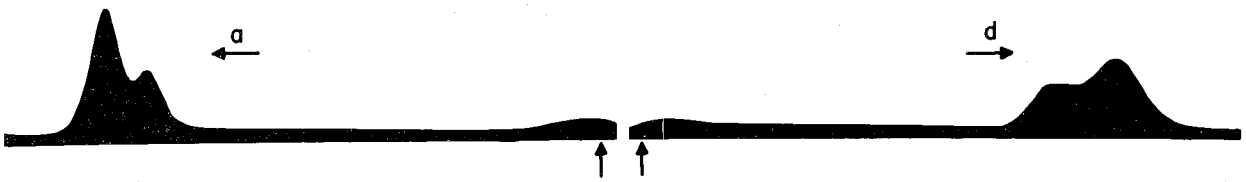
* The globin solutions in each pair of experiments were dialyzed simultaneously in the same buffer solution. The electrophoresis cells containing these solutions were connected in series so that the same current passed through each cell.

Figure 6.

Longsworth scanning diagrams of denatured globin prepared from normal, sickle cell anemia, and sicklemia hemoglobins. Potential gradients of 4.90 and 3.98 volts per centimeter were used for the acetate and phosphate experiments, respectively.

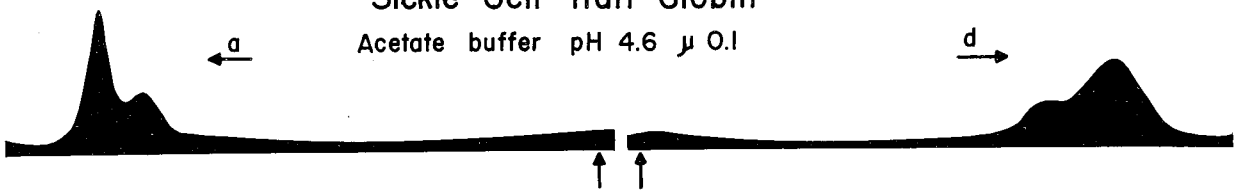
Normal Globin

Acetate buffer pH 4.6 μ 0.1



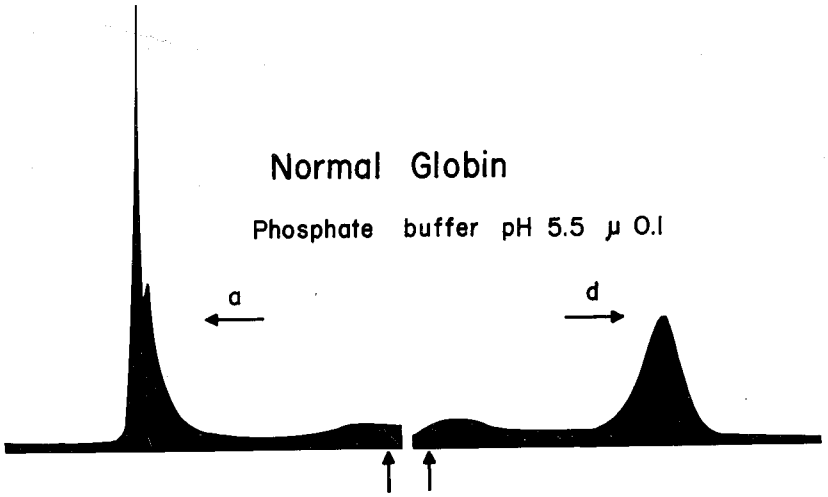
Sickle Cell Trait Globin

Acetate buffer pH 4.6 μ 0.1



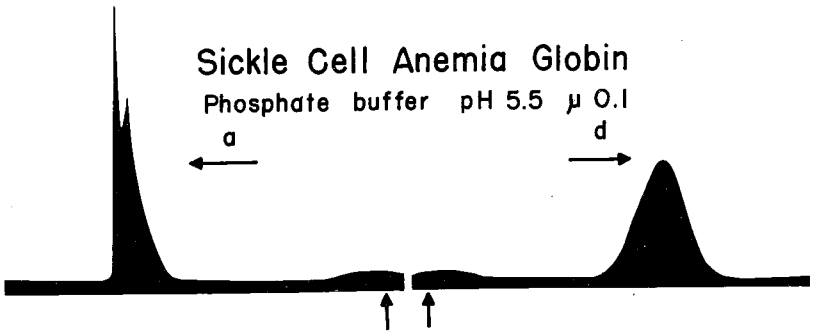
Normal Globin

Phosphate buffer pH 5.5 μ 0.1



Sickle Cell Anemia Globin

Phosphate buffer pH 5.5 μ 0.1



D. Discussion of electrophoretic results.

The factors which must be considered in discussing the difference in mobilities of normal and sickle cell anemia hemoglobins are the net charges on the molecules at a given pH and the frictional resistance of the molecules to movement in a buffer. Since the proteins were compared under identical conditions, the effects of buffer ions and of protein concentrations may be ignored except as they may reflect differences in the protein molecules. It is impossible to ascribe the difference entirely to dissimilarities in the particle weights or shapes of the two hemoglobins in solution; a purely frictional effect would cause one species to move more slowly than the other throughout the entire pH range and would not produce a shift in the isoelectric point. Moreover, preliminary velocity ultracentrifuge and free diffusion measurements indicate that the two hemoglobins have the same sedimentation and diffusion constants.

The difference in mobilities must be ascribed to a difference in net charge on the molecules. These experiments indicate that the net number of positive charges is greater for sickle cell anemia hemoglobin than for normal hemoglobin in the pH region near their isoelectric points. The most plausible explanation for this difference in net charge is

that there is a difference in the number or kind of ionizable groups in the two hemoglobins. The groups capable of forming ions in the pH range of these experiments are the carboxyl groups in the heme and the carboxyl, imidazole, amino, and guanidino groups in the globin. The charge on a protein molecule depends also on the number of adsorbed cations and anions, but the difference in the mobilities of the proteins in cacodylate buffer was the same as that in phosphate buffer. This lack of a specific buffer effect indicates that a difference exists which is probably independent of buffer ion adsorption; however, it does not eliminate the possibility that the difference in ion adsorption may be the same in both buffers; the sickle cell anemia hemoglobin molecule may adsorb more cations or fewer anions than the normal hemoglobin molecule idependently of the ions involved.

It has been well established that the electrophoretic mobility of a protein molecule is directly proportional to its net charge (22). If the assumption is made that the change in net charge of the hemoglobin molecule between pH 5.7 and 8.0 is due entirely to the dissociation of hydrogen ions, the difference in net charge of normal and sickle cell anemia hemoglobins can be calculated from consideration of titration data. The acid base titration curve of normal human carbonmonoxyhemoglobin (Figure 8) is nearly linear in the neighborhood

of the isoelectric point of the protein, and a change of one pH unit in the hemoglobin solution in this region is associated with a dissociation by the hemoglobin molecule of about 13 hydrogen ions per molecule. The same value was obtained by German and Wyman (17) with horse oxyhemoglobin. The difference in isoelectric points of the two hemoglobins under the conditions of these experiments is 0.23 for ferrohemoglobin and 0.22 for carbonmonoxyhemoglobin. This difference corresponds to about three charges per molecule. With consideration of the experimental error, sickle cell anemia hemoglobin therefore has between two and four greater net positive charges per molecule than normal hemoglobin in the pH region 5.7 to 8.0.

The isoelectric points of the ferrohemoglobins are about 0.2 unit lower than those of the corresponding carbonmonxy-hemoglobins. This is a reversal of the relative isoelectric points obtained from the differential titration of horse oxyhemoglobin and ferrohemoglobin (17). This anomalous effect may be due to the coordination of phosphate ion with the uncombined site on the iron of ferrohemoglobin.

Up to this stage it has been assumed that one of the two components of sicklemia hemoglobin is identical with sickle cell anemia hemoglobin and the other is identical with the normal compound. Aside from the genetic evidence (3) which

makes this assumption very probable electrophoresis experiments afford direct evidence that the assumption is valid. The experiments on the pooled sicklemia carbonmonoxyhemoglobin and the mixture containing 40 per cent sickle cell anemia carbonmonoxyhemoglobin and 60 per cent normal carbonmonoxyhemoglobin in cacodylate-sodium chloride buffer were compared, and it was found that the mobilities of the respective components were essentially identical (18). Experiments on sicklemia carbonmonoxyhemoglobin and on several mixtures of normal, sickle cell anemia, and sicklemia carbonmonoxyhemoglobins in phosphate buffer at pH 6.9 give added support to this assumption. The mobility calculations on these experiments, which are summarized in Table VIII, show that the difference in mobilities of the two components was the same in sicklemia carbonmonoxyhemoglobin and in the mixtures. There was no indication of a new component upon the addition of normal carbonmonoxyhemoglobin to sicklemia carbonmonoxyhemoglobin. The area under the peak corresponding to the normal component had increased by the amount expected. These sensitive tests reveal that at least electrophoretically the two components in sicklemia hemoglobin are identifiable with sickle cell anemia hemoglobin and normal hemoglobin.

The Longsworth scanning diagrams of globins derived from normal, sickle cell anemia, and sicklemia hemoglobins show the

two components previously described by other workers in normal globins (21). The relative amounts and mobilities of the components are nearly the same for normal and sickle cell trait globins in acetate buffer and for normal and sickle cell anemia globins in phosphate buffer. While the number of experiments was small, they were performed under the carefully controlled conditions previously described, and the results in acetate buffer show good reproducibility. It is therefore of interest that the mobilities do not show the same differences which are present in the hemoglobins. This loss of specificity in mobility is probably due to the denaturing process which accompanies the acid dissociation of heme from hemoglobin. The changes in isoelectric points and mobilities following denaturation have been reported for several proteins including horse carbonmonoxyhemoglobin (14).

TITRATION STUDIES

(1) Introductory note.

Titration studies have been employed to estimate the amino acid composition of several proteins, and differential studies of heme proteins have been extremely valuable in the investigation of heme linked acid groups. The comparison of normal and sickle cell anemia hemoglobins by titration methods was therefore proposed as a possible method of determining which of the ionizable groups was responsible for the difference in net charge between the molecules. By carrying out the titrations under identical conditions it was hoped that small differences in the nature of the ionizable groups would be detected.

(2) Experimental methods.

Materials. Normal carbonmonoxyhemoglobin solution was prepared from the pooled blood of several individuals whose erythrocytes did not sickle upon testing with sodium dithionite reagent. The sickle cell anemia carbonmonoxyhemoglobin solution was prepared from the erythrocytes of six individuals diagnosed clinically and electrophoretically (Table VI, individuals A6 to A11) as having sickle cell anemia. In order to conserve material no attempt was made to crystallize the hemoglobins. The solutions were dialyzed at 4°C. against redistilled water

for two weeks with frequent changes of water. The concentrations of these stock solutions were determined by iron analyses to be 0.619 and 0.652 millimolar in normal and sickle cell anemia hemoglobins, respectively.

Carbonate free sodium hydroxide solution was prepared and standardized against potassium acid phthalate after dilution with redistilled water to approximately 0.1 N. Three determinations yielded for the hydroxide concentration the value 0.1020 N with an error of less than one part in 1000. The hydrochloric acid solution was prepared by dilution of C. P. grade concentrated acid with redistilled water. This solution was titrated with the previously standardized sodium hydroxide solution and was 0.1233 N in acid. Titration of the acid solution with the standard hydroxide solution six weeks later, two weeks before the titration studies were completed, revealed no change in the ratio of the normalities within the precision of the titration.

Reagent grade "neutral" formaldehyde (36%-38%) was allowed to stand over activated alumina (23) overnight before its use in the formol titrations. Suspended particles of aluminum oxide were removed by use of a sintered glass filter.

The Beckman Model G pH meter with the Type E glass electrode was used in all the titrations. This electrode is suitable for use in strongly alkaline solutions. The instrument

used in these studies was calibrated at 25 degrees C. with Beckman standard pH 7.00 buffer. With this calibration pH determinations of Beckman standard buffers at pH 4.00 and 10.00 checked to with 0.01 unit of the given values. Secondary pH standard solutions were carefully prepared according to the directions of Clark (24) at pH 4.00, 7.00, and 10.00. On comparison against the Beckman buffers these prepared buffers had pH values at 25 degrees of 4.01, 7.00, and 9.36, respectively, to ±0.01 unit. The cause of the discrepancy in the last case was not determined.

Titration procedure. 10.0 milliliters of 2.5 molar KCl solution were delivered into a 25 milliliter volumetric flask from a buret. To this was added 1.00 milliliter of stock carbonmonoxyhemoglobin solution. A measured aliquot of standard acid or base was then added from a syringe microburet. The contents were diluted to the 25 milliliter mark with redistilled water and shaken manually. The flask was then immersed in a constant temperature water bath and the contents allowed to come to temperature equilibrium. Water baths at temperatures of 1.5, 25, and 50 degrees were used. The temperatures did not vary more than ±0.3 degree from these values.

pH measurements were made in a 30 milliliter beaker partially immersed in the constant temperature bath. The contents of

the volumetric flask were emptied into the beaker, the electrodes lowered into the titration mixture, and the reading taken when the pH reached a constant value. A gasket made of heavy sheet rubber with holes for the electrodes was used to cover the beaker in order to minimize temperature variation and carbon dioxide absorption. Control runs were made at each temperature on 1 molar KCl solutions containing no hemoglobin.

For the formol titrations 5.00 milliliters of neutral formaldehyde were added to the titration mixture before the addition of standard base. Otherwise the procedure was identical with that previously described. The control, normal carbonmonoxyhemoglobin, and sickle cell anemia carbonmonoxyhemoglobin titrations were performed on the same day using the same preparation of formaldehyde. The temperature of the titrations was 25°C.

Calculations. The equivalents of acidic or basic groups titrated is the difference between the equivalents of acid or base added and the equivalents remaining in solution. The latter figure can be calculated from comparison of the pH values obtained in the protein titration and the control titration, assuming that the presence of protein molecules does not affect the activity of the hydrogen and hydroxide ions in solution. Consider two titration mixtures at the same pH, one containing hemoglobin and inorganic ions and the

other, the control, containing only the inorganic ions. Because of the buffering capacity of the protein molecules more acid or base must be added to the protein solution than to the control solution to reach the same pH. The additional acid or base added is equal to the amount of acid or base bound by the protein, assuming equal activity coefficients in the two solutions. This assumption is generally made in the acid base titration of proteins (25).

The pH values obtained from the control titration of 1.0 molar KCl solutions with standard acid and base were plotted against the equivalents of acid or base added. A smooth curve was drawn through these points. This was done for each of the three temperatures at which the titrations were performed. The alkaline portions of these curves are shown in Figure 7. The points from the carbonmonoxyhemoglobin titrations were then plotted on the same scale. The horizontal distance, in other words the distance at constant pH, from the control curve to the hemoglobin titration point was taken as the number of equivalents of acid or base bound by the hemoglobin at that pH.

The same method was employed to compute the formol titration data. At a given pH the effect of the dissociation or formaldehyde and of formic acid is assumed to be the same in the absence and in the presence of hemoglobin. Levy's

empirical formula (26) was not applicable because it was derived for a different temperature and for titration mixtures which contained no added salt.

The average values of the pH of the stock solutions in 1 molar KCl were 7.03 and 6.59, respectively, for normal and sickle cell anemia carbonmonoxyhemoglobins. In calculating the equivalents of acid or base bound the amount required to take the stock solution to the electrophoretic isoelectric points was included. These figures, determined in phosphate buffers, are undoubtedly not the same as the isoionic points in potassium chloride solution, but their use was necessitated by the lack of any good determinations of the isoionic point of human hemoglobin. Moreover, the experiments in cacodylate buffer indicated that the difference in isoelectric points was independent of the inorganic ions present. A series of isoionic point determinations on human hemoglobin by simple dialysis against water to constant pH were reported by Levy, Mignon, and Netter (27). The values obtained from the oxyhemoglobins of 13 adults varied from 6.93 to 7.24. The electrophoretic results in phosphate buffer at pH 6.9 and 7.0 show much better reproducibility and justify the taking of the electrophoretic rather than the dialysis results as indicative of the real difference in charge in the region of the isoelectric point.

(3) Results.

The titration curves are shown in Figures 8 to 10, and the data are tabulated in Tables X to XXI. These results are discussed in the following section.

Table X.

Control Titrations of 1.0 Molar Potassium Chloride Solutions at 1.5°C.

meq. H^+ or OH^- added	pH	meq. H^+ or OH^- added	pH
.0278	2.92	-.0192	11.42
.0185	3.10	-.0192	11.43
.0093	3.43	-.0230	11.52
--	5.49	-.0307	11.68
--	5.61	-.0384	11.78
-.0038	9.08	-.0461	11.86
-.0077	10.19	-.0614	12.00
-.0115	11.15	-.0768	12.11
-.0154	11.41	-.0998	12.23

Table XI.

Control Titrations of 1.0 Molar Potassium Chloride Solutions at 25°C.

meq. H^+ or OH^- added	pH	meq. H^+ or OH^- added	pH
.1854	2.13	--	5.32
.1483	2.23	--	5.77
.1205	2.32	-.0008	7.20
.0927	2.44	-.0015	8.69
.0742	2.53	-.0023	9.18
.0556	2.66	-.0038	9.46
.0371	2.83	-.0077	10.01
.0278	2.97	-.0154	10.48
.0185	3.14	-.0307	10.82
.0093	3.44	-.0461	11.03
.0046	3.78	-.0615	11.16
.0019	4.14	-.0768	11.27
.0009	4.51	-.0998	11.39

Table XII.

Control Titrations of 1.0 Molar Potassium Chloride Solutions at 50°C.

meq. H^+ or OH^- added	pH
.0185	3.22
.0093	3.52
--	5.55
-.0038	8.95
-.0077	9.32
-.0115	9.60
-.0154	9.69
-.0192	9.85
-.0230	9.92
-.0307	10.05
-.0461	10.31
-.0614	10.43
-.0768	10.52
-.0998	10.63

Table XIII.

Titration of Normal Human Carbonmonoxyhemoglobin in 1.0 Molar Potassium Chloride at 25°C.[1]

pH	meq. H^+ or OH^- added	Control	meq. H^+ or OH^- bound	h	h corrected
2.34	.1854	.1160	.0694	112.4	109.2
2.37	.1761	.1090	.0671	108.6	105.4
2.41	.1669	.0997	.0672	108.8	105.6
2.44	.1576	.0927	.0649	105.0	101.8
2.49	.1483	.0820	.0663	107.3	104.1
2.53	.1390	.0742	.0648	104.9	101.7
2.59	.1298	.0641	.0657	106.3	103.1
2.64	.1205	.0579	.0626	101.3	98.1
2.71	.1112	.0500	.0612	99.1	95.9
2.80	.1020	.0403	.0617	99.8	96.6
2.88	.0927	.0335	.0592	95.8	92.6
2.90	.0835	.0322	.0513	83.0	79.8
3.19	.0742	.0164	.0578	93.5	90.3
3.37	.0649	.0109	.0540	87.4	84.2
3.65	.0556	.0060	.0496	80.2	77.0
4.01	.0464	.0027	.0437	70.7	67.5
4.25	.0371	.0016	.0355	57.5	54.3
4.51	.0278	.0009	.0269	43.5	40.3
4.92	.0185	.0003	.0182	28.5	25.3
5.90	.0093	-.0002	.0095	15.4	12.2
6.99	--	-.0007	.0007	1.1	-2.1
7.00	--	-.0007	.0007	1.1	-2.1
7.08	--	-.0008	.0008	1.2	-2.0
7.30	-.0015	-.0009	-.0006	-1.0	-4.2
7.44	-.0031	-.0009	-.0022	-3.6	-6.8
7.51	-.0038	-.0010	-.0028	-4.5	-7.9
7.58	-.0046	-.0010	-.0038	-5.8	-9.0
7.78	-.0061	-.0011	-.0050	-8.1	-11.3
8.02	-.0077	-.0012	-.0065	-10.5	-13.7
8.07	-.0077	-.0012	-.0065	-10.5	-13.7
8.42	-.0092	-.0014	-.0078	-12.6	-15.8
8.59	-.0108	-.0015	-.0093	-15.1	-18.3
8.90	-.0115	-.0018	-.0097	-15.7	-18.9

[1] 6.18×10^{-4} millimoles of carbonmonoxyhemoglobin were added to each titration mixture. h values were obtained by dividing this figure into the figures in the fourth column.

Table XIII (Continued)

pH	meq. H^+ or OH^- added	Control	meq. H^+ or OH^- bound	h	h corrected
9.02	-.0123	-.0020	-.0103	-16.7	-19.9
9.28	-.0138	-.0028	-.0110	-17.8	-21.0
9.42	-.0154	-.0034	-.0121	-19.6	-22.8
9.43	-.0154	-.0035	-.0120	-19.4	-22.6
9.81	-.0192	-.0059	-.0133	-21.5	-24.7
10.01	-.0230	-.0077	-.0153	-24.8	-28.0
10.17	-.0269	-.0096	-.0173	-28.0	-31.2
10.27	-.0307	-.0111	-.0196	-31.7	-34.9
10.31	-.0346	-.0119	-.0227	-36.7	-39.9
10.36	-.0346	-.0128	-.0218	-35.3	-38.5
10.43	-.0384	-.0142	-.0242	--39.2	-42.4
10.60	-.0461	-.0195	-.0266	-43.1	-46.3
10.72	-.0538	-.0252	-.0286	-45.3	-48.5
10.82	-.0615	-.0307	-.0308	-49.9	-53.1
10.99	-.0768	-.0422	-.0346	-56.0	-59.2
11.08	-.0845	-.0516	-.0329	-53.2	-56.4
11.13	-.0922	-.0576	-.0346	-56.0	-59.2
11.18	-.0998	-.0642	-.0356	-57.6	-60.8

Table XIV.

Titration of Normal Human Carbonmonoxyhemoglobin in 1.0 Molar Potassium Chloride at 1.5°C.[1]

pH	meq. H^+ or OH^- added	Control	meq. H^+ or OH^- bound	h	h corrected
4.68	.0278	.0006	.0272	44.0	34.9
5.40	.0185	.0001	.0184	29.8	20.7
6.38	.0093	-.0003	.0090	15.5	6.4
7.47	--	-.0006	.0006	0.9	-8.2
7.58	--	-.0007	.0007	1.0	-8.1
8.00	-.0038	-.0009	-.0029	-4.7	-13.8
8.69	-.0077	-.0011	-.0066	-10.7	-19.8
9.44	-.0115	-.0015	-.0100	-16.2	-25.3
9.84	-.0154	-.0019	-.0135	-21.8	-30.9
10.09	-.0192	-.0024	-.0168	-24.1	-33.2
10.40	-.0230	-.0035	-.0195	-31.5	-40.6
10.82	-.0307	-.0065	-.0242	-39.2	-48.3
11.08	-.0384	-.0096	-.0288	-46.6	-55.7
11.32	-.0461	-.0137	-.0324	-52.4	-61.5
11.60	-.0614	-.0250	-.0354	-58.9	-68.0

[1] See Table XIII.

Table XV.

Titration of Normal Human Carbonmonoxyhemoglobin in 1.0 Molar Potassium Chloride at 1.5°C.[1]

pH	meq. H^+ or OH^- added	Control	meq. H^+ or OH^- bound	h	h corrected
4.85	.0278	.0006	.0272	44.0	41.5
5.40	.0185	.0002	.0183	29.2	26.7
6.29	.0093	-.0003	.0096	15.5	13.0
6.83	--	-.0006	.0006	1.0	-1.5
6.88	--	-.0008	.0008	1.3	-1.2
6.92	--	-.0008	.0008	1.3	-1.2
6.95	--	-.0008	.0008	1.3	-1.2
7.34	-.0038	-.0010	-.0028	-4.5	-7.0
7.63	-.0077	-.0011	-.0066	-9.5	-12.0
8.28	-.0115	-.0016	-.0099	-16.0	-18.5
8.58	-.0154	-.0022	-.0132	-21.4	-23.9
8.60	-.0154	-.0023	-.0131	-21.2	-23.7
8.83	-.0192	-.0030	-.0162	-26.2	-28.7
9.01	-.0230	-.0040	-.0190	-30.7	-33.2
9.33	-.0307	-.0071	-.0236	-38.2	-40.7
9.66	-.0461	-.0136	-.0325	-52.6	-55.1
10.00	-.0614	-.0267	-.0347	-56.2	-58.7

[1] See Table XIII.

Table XVI.

Titration of Sickle Cell Anemia Carbonmonoxyhemoglobin in 1.0 Molar Potassium Chloride at 25°C.[2]

pH	meq. H^+ or OH^- added	Control	meq. H^+ or OH^- bound	h	h corrected
2.32	.1854	.1205	.0649	99.7	105.7
2.39	.1669	.1044	.0625	96.0	102.0
2.58	.1298	.0655	.0643	98.8	104.8
2.88	.0927	.0269	.0658	101.1	107.8
3.00	.0835	.0256	.0579	88.9	94.9
3.16	.0742	.0176	.0566	86.9	92.9
3.34	.0649	.0117	.0532	81.7	87.7
3.61	.0556	.0065	.0491	75.4	81.4
3.88	.0464	.0036	.0428	65.8	71.8
3.97	.0464	.0030	.0434	58.8	64.8
4.23	.0371	.0017	.0354	54.1	60.1
4.40	.0278	.0011	.0267	41.0	47.0
4.54	.0232	.0008	.0224	38.8	44.8
4.76	.0185	.0005	.0180	27.6	33.6
5.02	.0139	.0002	.0137	21.1	27.4
5.42	.0093	--	.0093	14.3	20.7
5.96	.0046	-.0002	.0048	7.4	13.4
6.57	--	-.0005	.0005	0.8	6.9
6.60	--	-.0005	.0005	0.8	6.8
6.61	--	-.0005	.0005	0.8	6.8
6.77	-.0015	-.0006	-.0009	-1.4	5.6
6.93	-.0031	-.0007	-.0024	-3.7	2.3
7.07	-.0046	-.0008	-.0038	-5.7	0.3
7.22	-.0061	-.0008	-.0053	-8.1	-2.1
7.39	-.0077	-.0009	-.0068	-10.4	-4.4
7.57	-.0092	-.0010	-.0082	-12.6	-6.6
7.72	-.0108	-.0011	-.0097	-14.9	-8.9
7.89	-.0123	-.0012	-.0111	-17.1	-11.1
8.04	-.0138	-.0012	-.0127	-19.5	-13.5
8.40	-.0154	-.0014	-.0140	-21.5	-15.5
8.99	-.0184	-.0020	-.0164	-25.2	-19.2
9.20	-.0200	-.0025	-.0175	-26.9	-20.9

[2] 6.51×10^{-4} millimoles of carbonmonoxyhemoglobin were added to each titration mixture. h values were obtained by dividing this figure into the figures in the fourth column.

Table XVI (Continued).

pH	meq. H^+ or OH^- added	Control	meq. H^+ or OH^- bound	h	h corrected
9.82	-.0230	-.0045	-.0185	-28.4	-22.4
9.88	-.0269	-.0065	-.0204	-31.4	-25.4
10.04	-.0307	-.0080	-.0227	-34.9	-28.9
10.30	-.0384	-.0116	-.0268	-41.2	-35.2
10.48	-.0461	-.0154	-.0307	-47.2	-41.2
10.74	-.0615	-.0262	-.0353	-54.2	-48.2
10.92	-.0768	-.0368	-.0400	-61.5	-55.5
11.07	-.0922	-.0504	-.0418	-64.2	-58.2

Table XVII.

Titration of Sickle Cell Anemia Carbonmonoxyhemoglobin in 1.0 Molar Potassium Chloride at 1.5°C.[2]

pH	meq. H^+ or OH^- added	Control	meq. H^+ or OH^- bound	h	h corrected
4.53	.0278	.0008	.0270	41.5	39.2
5.02	.0185	.0002	.0183	28.1	25.8
5.97	.0093	-.0001	.0094	14.4	12.1
7.08	--	-.0006	.0006	0.9	-1.4
7.36	--	-.0007	.0007	1.1	-1.2
7.84	-.0038	-.0008	-.0030	-4.6	-6.9
8.17	-.0077	-.0010	-.0067	-10.3	-12.6
9.07	-.0115	-.0013	-.0102	-15.7	-18.0
9.77	-.0154	-.0018	-.0136	-20.9	-23.2
10.11	-.0192	-.0024	-.0168	-25.8	-28.1
10.38	-.0230	-.0034	-.0196	-30.1	-32.4
10.76	-.0307	-.0059	-.0248	-38.1	-40.4
11.06	-.0384	-.0093	-.0291	-44.7	-47.0
11.23	-.0461	-.0110	-.0351	-53.9	-56.2
11.52	-.0614	-.0204	-.0410	-63.0	-65.3

[2] See Table XVI.

Table XVIII.

Titration of Sickle Cell Anemia Carbonmonoxyhemoglobin in 1.0 Molar Potassium Chloride at 50°C.[2]

pH	meq. H^+ or OH^- added	Control	meq. H^+ or OH^- bound	h	h corrected
4.72	.0278	.0008	.0271	41.6	49.3
4.76	.0278	.0007	.0270	41.5	49.2
5.19	.0185	.0003	.0182	28.0	35.7
5.90	.0093	-.0001	.0094	14.4	22.1
6.48	--	-.0005	.0005	0.8	8.5
6.48	--	-.0005	.0005	0.8	8.5
6.49	--	-.0005	.0005	0.8	8.5
7.00	-.0038	-.0008	-.0030	-4.6	3.1
7.18	-.0077	-.0009	-.0068	-10.4	-2.7
7.67	-.0115	-.0011	-.0104	-16.0	-8.3
8.02	-.0154	-.0013	-.0141	-21.7	-14.0
8.31	-.0154	-.0016	-.0138	-21.2	-13.5
8.60	-.0192	-.0023	-.0169	-26.0	-18.3
8.82	-.0230	-.0030	-.0200	-30.7	-23.0
9.22	-.0307	-.0058	-.0249	-38.2	-30.5
9.62	-.0461	-.0110	-.0351	-53.9	-46.2
9.98	-.0614	-.0254	-.0350	-53.8	-46.1

[2] See Table XVI.

Table XIX.

Control Titration of Formaldehyde
in 1.0 Molar Potassium Chloride at 25°C.

pH	meq. OH^- added	pH	meq. OH^- added
5.35	--	8.97	.0154
5.78	.0008	9.20	.0230
6.18	.0015	9.32	.0307
7.34	.0023	9.44	.0384
7.90	.0031	9.52	.0461
8.22	.0046	9.58	.0538
8.42	.0061	9.66	.0614
8.50	.0077	9.71	.0692
8.79	.0100	9.76	.0768
8.91	.0123		

Table XX.

Formal Titration of Normal
Human Carbonmonoxyhemoglobin at 25°C.[1]

pH	meq. OH^- added	Control	meq. OH^- bound	h	h corrected
6.57	.0230	.0018	.0212	-34.3	-37.5
6.90	.0307	.0020	.0287	-46.5	-49.7
7.34	.0384	.0023	.0381	-58.4	-61.6
7.92	.0461	.0032	.0429	-69.4	-72.6
8.58	.0538	.0075	.0463	-74.9	-78.1
8.94	.0614	.0130	.0484	-78.3	-81.5
9.13	.0692	.0189	.0583	-81.4	-84.6
9.31	.0768	.0285	.0483	-78.2	-81.4

[1] See Table XIII.

Table XXI.

Formol Titration of Sickle Cell

Anemia Carbonmonoxyhemoglobin at 25°C.[2]

pH	meq. OH^- added	Control	meq. OH^- bound	h	h corrected
6.52	.0230	.0018	.0212	32.6	26.6
6.86	.0307	.0020	.0287	44.2	38.2
7.30	.0384	.0022	.0362	55.6	49.6
7.32	.0384	.0023	.0361	55.4	49.4
7.81	.0461	.0029	.0432	66.4	60.4
8.12	.0461	.0040	.0421	64.7	58.7
8.31	.0538	.0052	.0486	74.6	68.6
8.58	.0538	.0075	.0463	71.1	65.1
8.84	.0614	.0110	.0504	77.4	71.4
9.10	.0692	.0176	.0516	79.3	73.3
9.27	.0768	.0259	.0509	78.2	72.2

[2] See Table XVI.

Figure 7.

Control titration curves at three temperatures. The pH values of 1.0 molar KCl solutions were measured after the addition of aliquots of standard acid or base. The alkaline portions of the control curves constructed from these measurements are shown here.

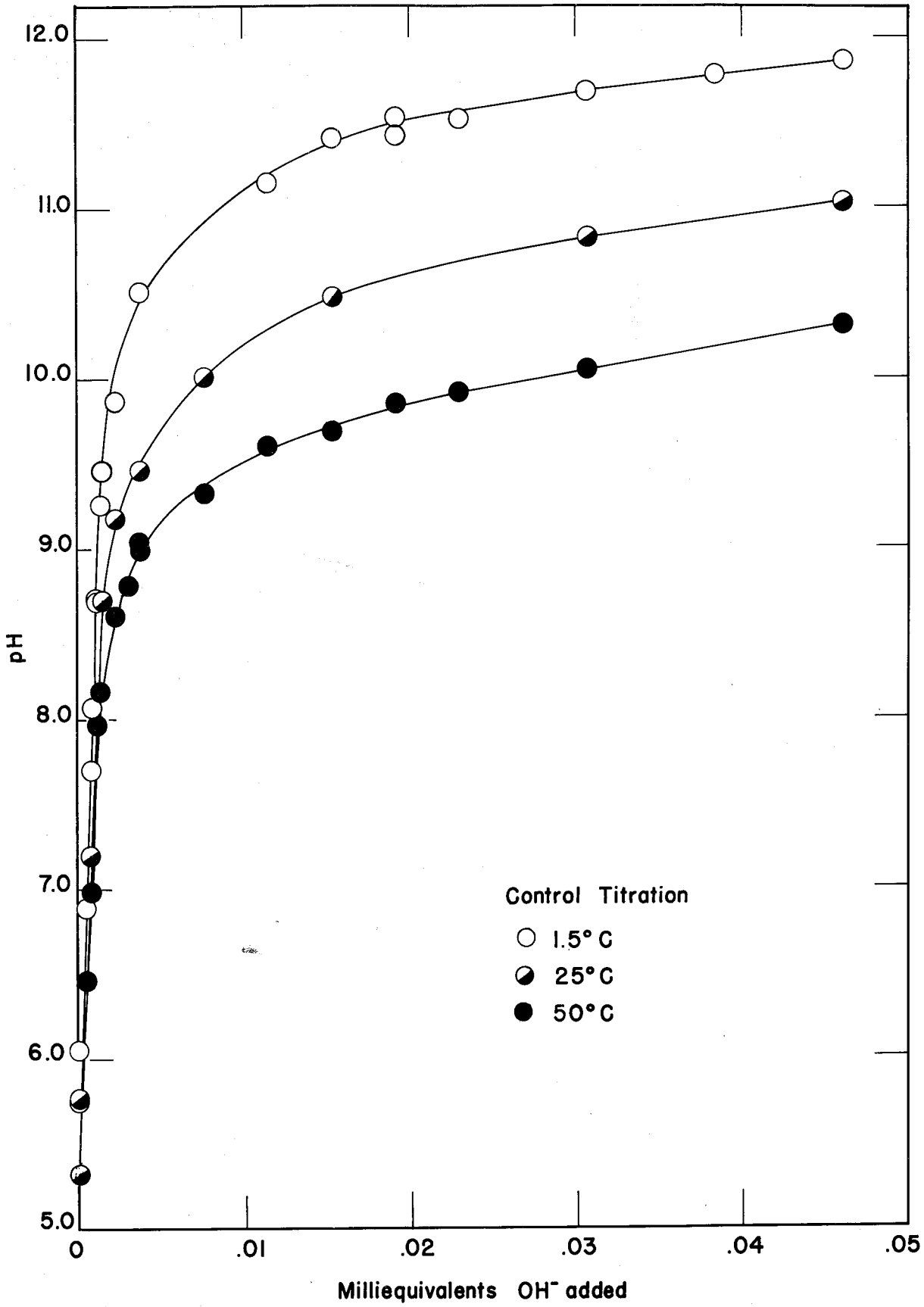

12.0
11.0
10.0
9.0
8.0
7.0
6.0
5.0
pH
0
.01
.02
.03
.04
.05
Milliequivalents OH⁻ added
Control Titration
1.5° C
25° C
50° C

Figure 8.

Titration curves of normal and sickle cell anemia carbonmonoxyhemoglobins at 25°C.

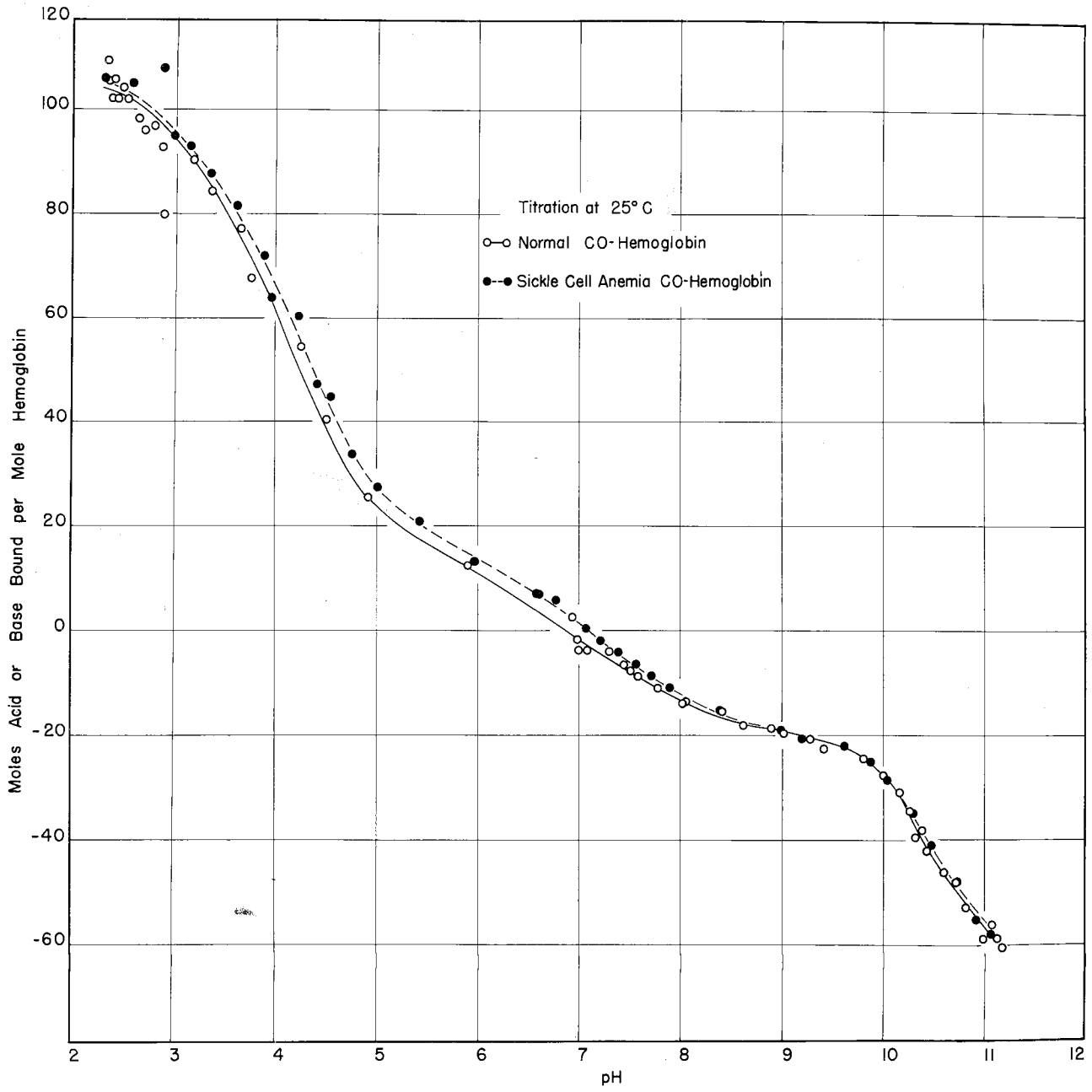
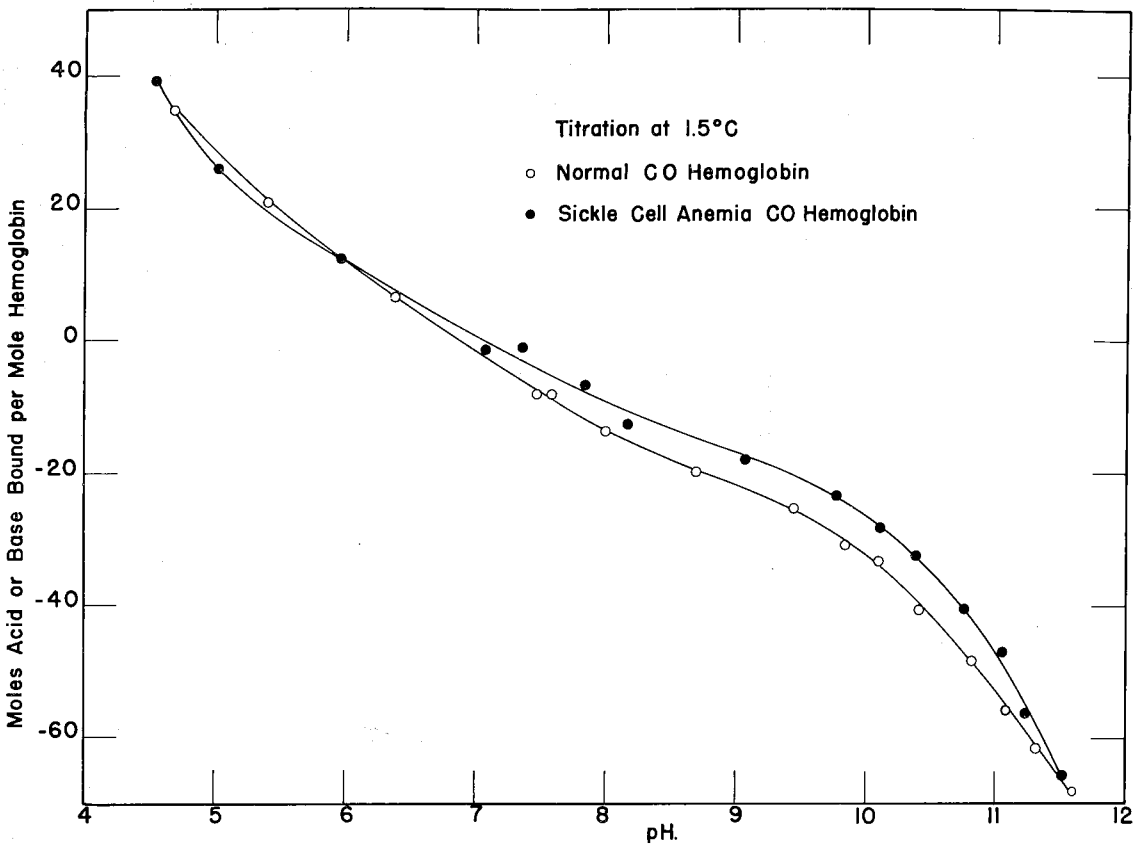

Titration at 25° C
Normal CO-Hemoglobin
Sickle Cell Anemia CO-Hemoglobin
Moles Acid or Base Bound per Mole Hemoglobin
120
100
80
60
40
20
0
-20
-40
-60
2
3
4
5
6
7
8
9
10
11
12
pH

Figure 9.

Titration curves of normal and sickle cell anemia hemoglobins at 1.5°C. and 50°C.


Titration at 1.5°C
Normal CO Hemoglobin
Sickle Cell Anemia CO Hemoglobin
Moles Acid or Base Bound per Mole Hemoglobin
40
20
0
-20
-40
-60
4
5
6
7
8
9
10
11
12
pH.

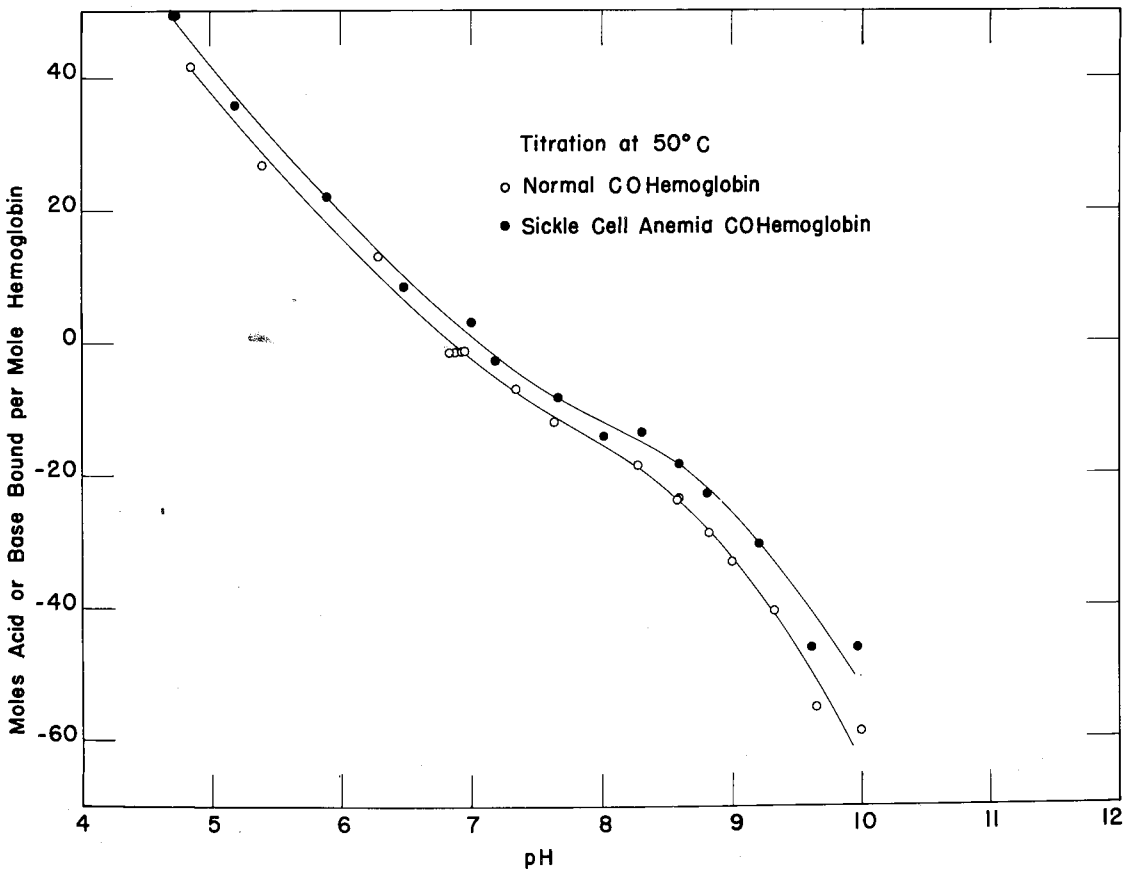
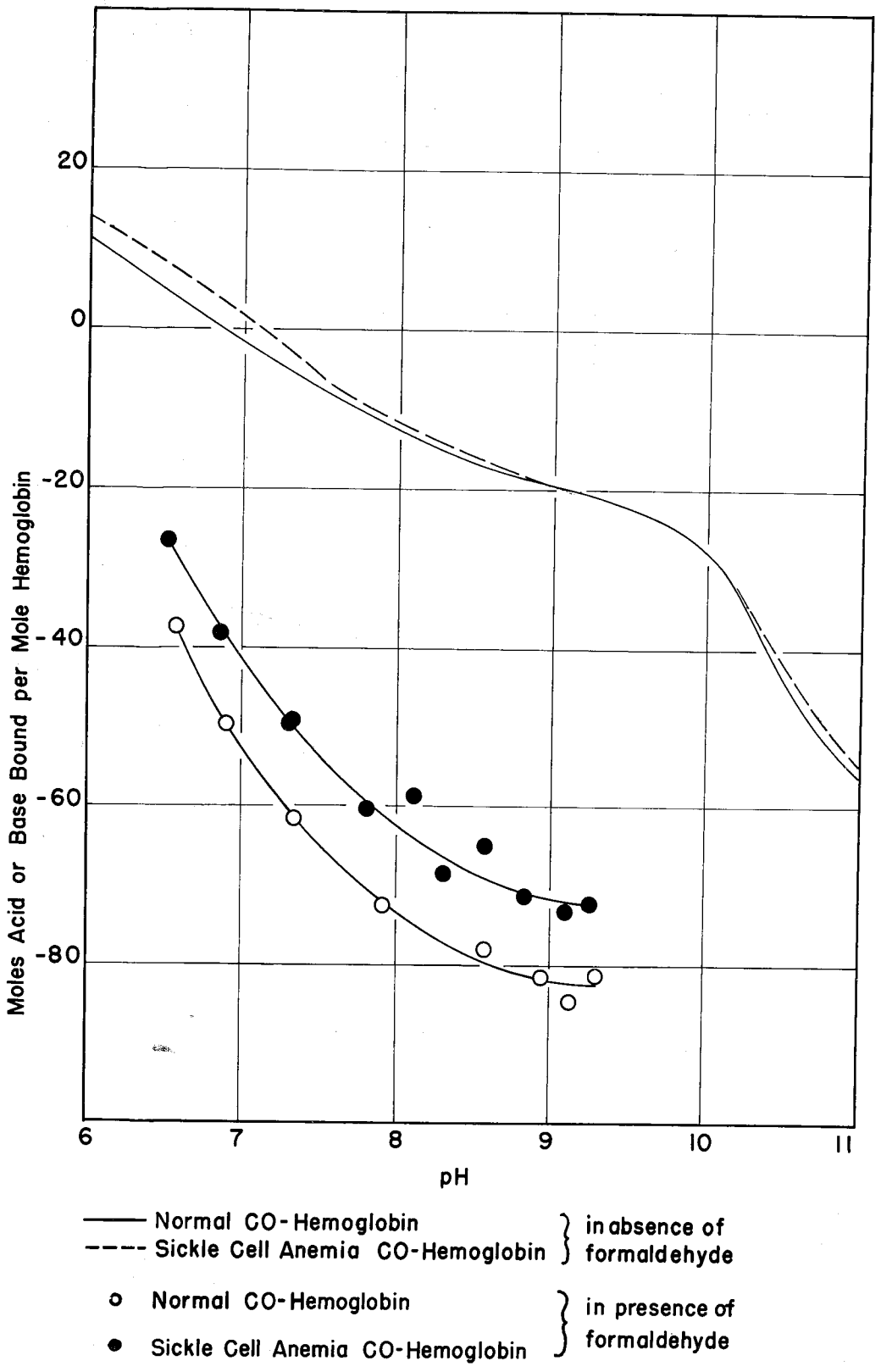

Titration at 50° C
Normal CO Hemoglobin
Sickle Cell Anemia CO Hemoglobin
Moles Acid or Base Bound per Mole Hemoglobin
40
20
0
-20
-40
-60
4
5
6
7
8
9
10
11
12
pH

Figure 10.

Formol titration curves of normal and sickle cell anemia carbonmonoxyhemoglobins at 25°C.


20
0
-20
-40
-60
-80
Moles Acid or Base Bound per Mole Hemoglobin
6
7
8
9
10
11
pH
Normal CO-Hemoglobin
Sickle Cell Anemia CO-Hemoglobin
in absence of formaldehyde
Normal CO-Hemoglobin
Sickle Cell Anemia CO-Hemoglobin
in presence of formaldehyde

(4) Discussion of titration results.

In evaluating the titration results it is desirable to recognize the limitations of this method as a means of determining the number of each type of dissociating group present in a protein molecule. The number of free amino groups as determined by the formol titration is consistently higher by about 50 per cent than the number found by amino acid analysis (25). The estimation of the number of imidazole groups depends on the determination of the apparent heat of dissociation of the protein as a function of pH, and Wyman's (28) analysis of horse oxyhemoglobin revealed about 31 groups dissociating with the heat of dissociation of the imidazole group as compared to the 33 histidine residues per molecule found by amino acid analysis. Heat of dissociation studies on other proteins have not yielded definite results on the number of imidazole groups present. The titration figure for the total number of cations (29), which is equal to the acid combining capacity of the protein, is considerably higher than the total number of basic amino acid residues found by analysis. The titration figure for the number of guanidinium groups is in good agreement with the analytical figures (29), but this agreement loses its significance in view of the fact that it is the difference of two figures which are high by the same amount.

Cohn, Green, and Blanchard (30) were able to fit their titration data for horse carbonmonoxyhemoglobin between pH 1.5 and 13 on the basis of seven apparent dissociation constants. The number of residues with titration constants 5.7 and 7.5 was found to be 33, a figure which agrees with the analytical value for histidine. These 33 groups dissociated in the pH range 5 to 9 in contrast to the 5.6 to 8.5 range assigned to the imidazole groups of horse carbonmonoxyhemoglobin by Wyman. The total number of groups with titration constants of 3.7, 4.0, and 4.8, was 87, a figure which is in marked disagreement with the analytical figure of 26 free carboxyl groups. The constants 10.5 and 11.6 were assigned to the amino, guanidino, and phenolic groups, but the number of each group present was indeterminate.

It is evident that the quantitative interpretation of an ordinary acid-base titration curve of a protein is subject to large uncertainties. On the other hand, differential titrations have been performed which have detected small differences between two compounds of the same protein. An example of this technique is the differential titration of ferrohemoglobin and oxyhemoglobin (17), which provided data for the calculation of the pK's of the heme-linked acid groups in the two compounds. The success of this technique depends on the fact that the pH of the same hemoglobin solution is measured

both before and after combination with oxygen. The protein concentration remains the same, and the effects of salt and protein on the activity coefficient of hydrogen and hydroxyl ions cancel when the difference between the curves is taken.

Since normal and sickle cell anemia hemoglobins behave as two different proteins, a differential titration comparable to the oxyhemoglobin-ferrohemoglobin titration is not feasible. A comparision can, however, be made by performing separate titrations under identical experimental conditions. As has been pointed out, attempts at quantitative interpretations of these curves probably would be unproductive. On the other hand, the proteins are presumably very similar so that comparison of corresponding portions of their respective titration curves may yield some information as to differences in their ionizable groups. Small differences would fall within the limits of experimental error, but if the net charge difference of about three charges were due to large differences in both the acidic and the basic groups, these differences would be detectable. It was with this possibility in mind that these experiments were undertaken. If such large differences were found, the analyses of these proteins could be concentrated on the particular amino acids involved.

The formol titrations indicate that sickle cell anemia hemoglobin contains fewer amino groups than normal hemoglobin.

The actual number of groups cannot be specified because of the previously mentioned uncertainty of formol titration results with proteins. Furthermore, this result is contradicted by the acid-base titration curves at 25°. The two curves are nearly identical in the pH region in which ammonium groups dissociate, indicating little or no difference in the number of free amino groups.

According to the titrations, the hemoglobins have approximately the same acid binding capacity, 105 equivalents of acid per mole of hemoglobin. The imidazole portions of the curves disclose an inconsistency in the results, sickle cell anemia hemoglobin showing a greater number of dissociating groups in the pH region 5.6 to 8.5 at 25 degrees but a smaller number at 1.5 and 50 degrees. It is necessary to go back to the mobility curves, which are roughly parallel in this region, and conclude that the number of imidazole groups must be approximately the same. The acid portions of the curves do not disclose any large differences in the number of dissociating groups.

Thus these titration studies have failed to reveal which of the amino acids are responsible for the charge difference between the hemoglobins. The conclusion which can be drawn is that there is no large difference in their basic and acidic amino acid compositions.

SUMMARY

Examination in the Tiselius apparatus of the hemoglobin from the erythrocytes of sickle cell anemic individuals has shown that it differs electrophoretically from normal hemoglobin. This finding supports the conclusion (18), based on the correlation of the observations on intact erythrocytes by previous workers that the hemoglobin was responsible for the sickling process. The presence of two electrophoretic components in the hemoglobin from the erythrocytes of sicklemic individuals is in accord with the evidence recently presented by Neel (3) on the genetic transmission of sickle cell anemia and sicklemia.

Further investigation of sickle cell anemia hemoglobin has been undertaken in order to elucidate the molecular basis for the abnormality in electrophoretic behavior. The electrophoretic mobilities and patterns of denatured globins prepared from the hemoglobins of normal, sickle cell anemic, and sicklemic indivisuals were nearly identical. Acid-base titration studies failed to show significant differences in the ionizable groups of normal and sickle cell anemia carbonmonoxyhemoglobins. The studies of Dr. Ibert Wells on the dimethyl esters of the porphyrins derived from the hemoglobins indicated that the hemes were identical (18). The amino acid analyses of normal and sickle

cell anemia hemoglobin by Dr. Walter Schroeder (31) show that very little difference exists between the proteins with respect to amino acid composition. The situation is analogous to that which exists in the comparative studies of various mammalian hemoglobins. Hemoglobins which have different crystalline forms, solubilities, and isoelectric points have very similar amino acid compositions.

Diffusion and sedimentation experiments were mentioned earlier, but it has been reported that these methods cannot distinguish renatured proteins from the corresponding native proteins (32). On the other hand, electrophoretic experiments show marked differences in mobilities and isoelectric points between these same native and reversibly denatured protein preparations. Gralen's experiments (14) also show that the reversal of denaturation of hemoglobin is not a reproducible procedure. Of three preparations one had the same isoelectric point as the original substance, and two had isoelectric points 0.2 pH units lower than that of the original substance. Thus it appears that the electrophoretic method is a particularly sensitive method of distinguishing two very similar proteins.

The ability of the sickle cell anemia hemoglobin molecule to form aggregates and cause sickling remains as an important clue to its nature. A possible mechanism for the sickling process has been proposed (18); the proposed mechanism

implies a surface configuration of the sickle cell anemia hemoglobin molecule which differs from that of the normal molecule. Immunological studies have revealed differences among the hemoglobins of various species as antigens (33) and would be useful in checking the postulate of an abnormal surface configuration of the sickle cell anemia hemoglobin molecule. If a difference of this type exists, the possibility of differences in ion adsorption cannot be overlooked. In phosphate buffer horse carbonmonoxyhemoglobin shows a dependence of isoelectric point on buffer concentration (34). This dependence has been ascribed to buffer ion adsorption. If ion adsorption is involved in the electrophoretic difference between normal and sickle cell anemia hemoglobins, this phenomenon may be manifested by a difference in the dependence of the isoelectric points on buffer concentration. Electrophoretic experiments in phosphate buffers of ionic strengths other than 0.1 should be performed in order to check this possibility.

Other differences in the properties of the hemoglobins will undoubtedly be found, but with such large molecules the probability of locating precisely the cause of their difference is small. Differences in amino acid composition, in arrangement of amino acids in the polypeptide chains, in folding of the polypeptide chains, and in adsorption of ions must all be

considered as possible causes. A better understanding of the factors involved requires the accumulation and interpretation of a large variety of chemical, physical, and immunological data.

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PART II

A RAPID DIAGNOSTIC TEST FOR SICKLE CELL ANEMIA

INTRODUCTION

Although the relationship of ferrohemoglobin, oxyhemoglobin, and carbonmonoxyhemoglobin to sickling has long been known, the possible role which other hemoglobin compounds might play has never been studied. Two approaches to this problem are suggested by the known differences between ferrohemoglobin on the one hand and oxy and carbonmonoxyhemoglobin on the other. One of the six bonding sites of the iron atom in the heme of ferrohemoglobin is unoccupied. All six are occupied in the other compounds. Magnetic susceptibility measurements have shown that ferrohemoglobin is paramagnetic while oxy- and carbonmonoxyhemoglobin are diamagnetic. Ferrihemoglobin in acid or neutral solution is the only undenatured hemoglobin compound other than ferrohemoglobin which possibly has an uncombined site. The effect on cell form of transforming its hemoglobin to ferrihemoglobin was therefore studied. Since both ferro- and ferrihemoglobin are known to form compounds with several molecules and ions, an attempt was made to produce these compounds within sickle cells. While ferrihemoglobin itself is paramagnetic, it forms both paramagnetic and diamagnetic compounds (1), and it was hoped that the study of these compounds within the cells of sickle cell anemia individuals would shed some light on the sickling process. Susceptibility

measurements on the Gouy balance were not possible because the amount of sickle cell anemia blood available at the time of these experiments was small.

EXPERIMENTAL METHODS AND RESULTS

The oxidation of hemoglobin to ferrihemoglobin occurs very slowly in the intact red cell, and oxidation with ferricyanide is not feasible because the cell membrane is impermeable to this ion (2). The oxidizing agent employed was sodium nitrite, which rapidly enters the cell and oxidizes ferrohemoglobin to ferrihemoglobin. Sodium nitrite is not ideally suited to use in protein solutions since it also reacts with free amino groups in acid solutions, but no other rapid oxidizing agent was found. Cells treated with this compound immediately acquired the characteristic color and spectrum of ferrihemoglobin. Microscopic examination of these cells revealed no sickling. Reduction of the ferrihemoglobin to ferrohemoglobin caused all of these cells to sickle, so the nitrite treatment had not abolished the ability of their hemoglobin to cause sickling. Addition of azide or fluoride to the ferrihemoglobin-containing cells did not alter their form, and the addition of cyanide caused lysis.

Ferrohemoglobin forms a covalent compound with ethyl isocyanide (3), but the addition of this compound to a suspension

of sickled cells did not reverse the sickling. The cells became spherical after a few minutes and lysed. It is possible that the passage of the isocyanide through the cell membrane was retarded and that the impurities in the preparation caused lysis before combination of hemoglobin and ethyl isocyanide could occur.

A buffered sodium dithionite solution which rapidly reduces the oxyhemoglobin in erythrocytes without causing hemolysis was perfected in the course of this work. This solution has proven to be effective in the rapid and accurate diagnosis of sickle cell anemia and sickle cell trait. The preparation and use of the reagent were reported in Blood, The Journal of Hematology, 4, 66 (1949).

DISCUSSION

The failure of the various compounds of ferro- and ferrihemoglobin to cause sickling is in accord with the theory that compound formation by hemoglobin diminishes the complementariness between molecules which is necessary for sickling to occur (4). The failure of ferrihemoglobin to cause sickling may be due to the binding of a water molecule by each iron atom of a heme or by repulsion at the complementary sites due to the positive charge on the iron atom.

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INTRODUCTORY NOTE

The following paper appeared in Blood, the Journal of Hematology, Vol. IV, No. 1, January, 1949.

A RAPID DIAGNOSTIC TEST FOR SICKLE CELL ANEMIA*♣

by Harvey A. Itano** and Linus Pauling

Sickle cell anemia is a congential chronic hemolytic type of anemia characterized hematologically by the development of oat-shaped and sickle-shaped erythrocytes. Other cellular abnormalities which are due to excessive blood destruction and active blood formation are also seen in blood smears. Six to ten per cent of Negroes possess the sickle trait (2,3); their blood cells have the capacity to sickle, but most of these individuals do not develop anemia.

The course of the sickling process as observed under the microscope has been described in detail by several investigators (4,6,10), but little is known about the physical processes involved in sickling. It has been established, however, that the erythrocytes of individuals with sickle cell anemia and sickle cell trait become sickled when the hemoglobin is reduced (8,14). When the hemoglobin is combined with oxygen or carbon monoxide, the cells are indistinguishable in form from normal

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* Contribution No. 1186 from the Gates and Crellin Laboratories of Chemistry, California Institute of Technology.

♣ This work was briefly reported by Dr. Dan H. Campbell at the Hematology Symposium of the U. S. Public Health Service held at St. Louis, Mo., on February 2, 1948.

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erythrocytes. The term promeniscocyte has been applied to the latter form and meniscocyte to the former (11). Hahn and Gillespie (8) and Sherman (14) obtained sickling physically by reducing the partial pressure of oxygen over suspensions of promeniscocytes. They were able to reverse the process by passing oxygen or carbon monoxide over meniscocytes. When oxygen is removed from promeniscocytes, their hemoglobin aggregates in one or more foci within the cells, and the cell membrane collapses. When oxygen is added to these cells, they resume their normal contour, and hemoglobin appears to be distributed uniformly throughout their interior. Meniscocytes are strongly birefringent under the polarizing microscope (14) while promeniscocytes are not.

When a drop of blood is sealed between a cover slip and a slide, the decline in oxygen tension due to oxidative processes in the blood cells leads to sickling (7). This is the common diagnostic test for sickle cell anemia and sickle cell trait used in clinical laboratories. Sherman found that increase in temperature, high leucocyte count, and bacterial contamination, all of which increase the rate of oxygen consumption, accelerated the sickling process. In another method a saline citrate suspension of blood is allowed to stand in a test tube under a layer of paraffin oil until sickling takes place (1). In employing any of the common diagnostic tests for sickling it is

desirable to obtain blood which has a low fraction of oxyhemoglobin. Thus the moist stasis method (13), in which blood is obtained from a patient's finger after its circulation has been occluded for five minutes, gives the most rapid and consistent results. Even with this method it is sometimes necessary to observe the preparation for several hours before the result is conclusive (5).

In order to find a more convenient and rapid method of producing meniscocytes we turned to chemical reducing agents. Sodium dithionite, $Na_2S_2O_4$, rapidly reduces oxyhemoglobin to reduced hemoglobin, and this property suggested its use in testing erythrocytes for sickling. When a solution of sodium dithionite was added to promeniscocytes, nearly all of the cells showed sickling or the early changes in the sickling process within a few seconds. Dithionite ion tends to decompose to thiosulfate and sulfite with formation of hydrogen ion so that solutions made up from commercial preparations of sodium dithionite are often strongly acid in reaction; but by adding Na_2HPO_4 to the solutions it is possible to increase the pH and at the same time provide a buffering medium. Hahn and Gillespie found that sickling was obtained most consistently if cell suspensions were buffered at a slightly acid pH. We have prepared a satisfactory reagent by adding 0.114 M aqueous Na_2HPO_4

to 0.114 M aqueous $Na_2S_2O_4$ until the final pH was 6.8. The ratio of the volumes of Na_2HPO_4 and $Na_2S_2O_4$ necessary to obtain this pH was about three to two.

The blood used in the following experiments was obtained from six different cases of sickle cell anemia, three of whom were being treated for exacerbations and three of whom were in remission. An excess of the dithionite reagent was added to promeniscocytes on a microscope slide; almost immediately changes were evident in the erythrocytes. Typical crescentic forms did not appear in large numbers, presumably because of the time required for the reduced hemoglobin molecules to become oriented in what Ponder calls the paracrystalline state (11). However, nearly all of the cells underwent changes in contour, and other changes described by earlier observers took place at an accelerated rate. The forms of many of these cells corresponded to the "holly wreath" cells of Sherman and cells classified as "abnormal" by Reinhard and his co-workers (12). After about fifteen to thirty minutes the aggregates of hemoglobin in many of the cells became birefringent. The presence of so many holly wreath cells is in accord with Sherman's observation that this form appears in large numbers when the rate of removal of oxygen is rapid. Since dithionite does not react with carbon monoxide, promeniscocytes saturated with carbon monoxide would not be expected to undergo changes in contour upon addition

of this reducing agent. This is indeed the case. Although no sickle cell trait blood was available to us for study, there is good reason to believe that such blood would behave in the same manner as sickle cell anemia blood.**

The rapidity and simplicity of this test suggests that it would be useful as a clinical laboratory procedure for diagnosing sickle cell anemia and sickle cell trait. No special precautions are necessary in collecting the blood for this test; oxygenated cells may be used since an excess of reducing agent can always be added. The test works equally well with oxalated blood or fingertip puncture specimens and may be applied in several ways. (1) About 0.05 ml. of reagent may be added to a very small drop (about 0.01 ml.) of blood on a slide. A cover slip is then laid over the mixture and cells observed under a microscope. (2) An excess of reagent may be added to a small volume of blood in a test tube and a drop of the mixture observed. (3) A convenient method for studying the entire process of sickling in a short period of time involves the use of a hemocytometer counting chamber. The chamber is half filled with a dilute saline suspension of promeniscocytes; the reagent

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** A brief note by da Silva (Science 107, 221 (1948)) which appeared since the preparation of this paper indicates that he has successfully identified sicklemia (sickle cell trait) by a procedure similar to method (1) below.

is then added to fill the rest of the chamber. The erythrocytes may be observed as the reducing agent diffuses into the part of the chamber which they occupy.

Since the dithionite reagent is unstable as mentioned above, its reducing power should be tested frequently by the addition of a test portion to a dilute suspension of oxygenated erythrocytes. If the reagent is satisfactory a change from the color of oxyhemoglobin to that of reduced hemoglobin should be observed. A large volume of stock Na_2HPO_4 solution may be prepared, but it is desirable to make up the $Na_2S_2O_4$ solution daily.

We are indebted to Dr. Edward R. Evans and Dr. Travis Winsor for their aid in obtaining the blood used in these experiments.

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APPENDIX

INTRODUCTORY NOTE

The paper which follows summarizes the combined work of the authors on the hemoglobin of sickle cell anemia up to the fall of 1949.

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Sickle Cell Anemia, a Molecular Disease[1]

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THE ERYTHROCYTES of certain individuals possess the capacity to undergo reversible changes in shape in response to changes in the partial pressure of oxygen. When the oxygen pressure is lowered, these cells change their forms from the normal biconcave disk to crescent, holly wreath, and other forms. This process is known as sickling. About 8 percent of American Negroes possess this characteristic; usually they exhibit no pathological consequences ascribable to it. These people are said to have sicklemia, or sickle cell trait. However, about 1 in 40 (*4*) of these individuals whose cells are capable of sickling suffer from a severe chronic anemia resulting from excessive destruction of their erythrocytes; the term sickle cell anemia is applied to their condition.

The main observable difference between the erythrocytes of sickle cell trait and sickle cell anemia has been that a considerably greater reduction in the partial pressure of oxygen is required for a major fraction of the trait cells to sickle than for the anemia cells (*11*). Tests *in vivo* have demonstrated that between 30 and 60 percent of the erythrocytes in the venous circulation of sickle cell anemic individuals, but less than 1 percent of those in the venous circulation of sicklemic individuals, are normally sickled. Experiments *in vitro* indicate that under sufficiently low oxygen pressure, however, all the cells of both types assume the sickled form.

The evidence available at the time that our investigation was begun indicated that the process of sickling might be intimately associated with the state and the nature of the hemoglobin within the erythrocyte. Sickle cell erythrocytes in which the hemoglobin is combined with oxygen or carbon monoxide have the biconcave disk contour and are indistinguishable in that form from normal erythrocytes. In this condition they are termed promeniscocytes. The hemoglobin appears to be uniformly distributed and randomly oriented within normal cells and promeniscocytes, and no birefringence is observed. Both types of cells are very flexible. If the oxygen or carbon monoxide is removed, however, transforming the hemoglobin to the uncombined state, the promeniscocytes undergo sickling. The hemoglobin within the sickled cells appears to aggregate into one or more foci, and the cell membranes collapse. The cells become birefringent (*11*) and quite rigid. The addition of oxygen or carbon monoxide to these cells reverses these phenomena. Thus the physical effects just described depend on the state of combination of the hemoglobin, and only secondarily, if at all, on the cell membrane. This conclusion is supported by the observation that sickled cells when lysed with water produce discoidal, rather than sickle-shaped, ghosts (*10*).

It was decided, therefore, to examine the physical and chemical properties of the hemoglobins of individuals with sicklemia and sickle cell anemia, and to compare them with the hemoglobin of normal individuals to determine whether any significant differences might be observed.

Experimental Methods

The experimental work reported in this paper deals largely with an electrophoretic study of these hemoglobins. In the first phase of the investigation, which concerned the comparison of normal and sickle cell anemia hemoglobins, three types of experiments were performed: 1) with carbonmonoxyhemoglobins; 2) with uncombined ferrohemoglobins in the presence of dithionite ion, to prevent oxidation to methemoglobins; and 3) with carbonmonoxyhemoglobins in the presence of dithionite ion. The experiments of type 3 were performed and compared with those of type 1 in order to ascertain whether the dithionite ion itself causes any specific electrophoretic effect.

Samples of blood were obtained from sickle cell anemic individuals who had not been transfused within three months prior to the time of sampling. Stroma-free concentrated solutions of human adult hemoglobin were prepared by the method used by Drabkin (*3*). These solutions were diluted just before use with the

[1] This research was carried out with the aid of a grant from the United States Public Health Service. The authors are grateful to Professor Ray D. Owen, of the Biology Division of this Institute, for his helpful suggestions. We are indebted to Dr. Edward R. Evans, of Pasadena, Dr. Travis Winsor, of Los Angeles, and Dr. G. E. Burch, of the Tulane University School of Medicine, New Orleans, for their aid in obtaining the blood used in these experiments.

[2] U. S. Public Health Service postdoctoral fellow of the National Institutes of Health.

[3] Postdoctoral fellow of the Division of Medical Sciences of the National Research Council.

[4] Contribution No. 1333.

appropriate buffer until the hemoglobin concentrations were close to 0.5 grams per 100 milliliters, and then were dialyzed against large volumes of these buffers for 12 to 24 hours at 4° C. The buffers for the experiments of types 2 and 3 were prepared by adding 300 ml of 0.1 ionic strength sodium dithionite solution to 3.5 liters of 0.1 ionic strength buffer. About 100 ml of 0.1 molar NaOH was then added to bring the pH of the buffer back to its original value. Ferrohemoglobin solutions were prepared by diluting the concentrated solutions with this dithionite-containing buffer and dialyzing against it under a nitrogen atmosphere. The hemoglobin solutions for the experiments of type 3 were made up similarly, except that they were saturated with carbon monoxide after dilution and were dialyzed under a carbon monoxide atmosphere. The dialysis bags were kept in continuous motion in the buffers by means of a stirrer with a mercury seal to prevent the escape of the nitrogen and carbon monoxide gases.

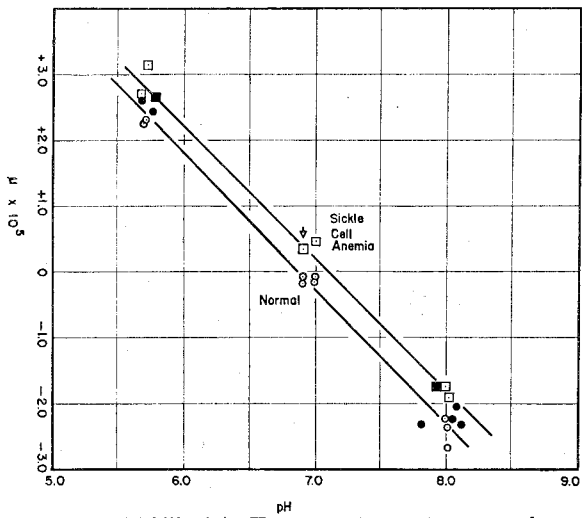

FIG. 1. Mobility(μ)-pH curves for carbonmonoxyhemoglobins in phosphate buffers of 0.1 ionic strength. The black circles and black squares denote the data for experiments performed with buffers containing dithionite ion. The open square designated by the arrow represents an average value of 10 experiments on the hemoglobin of different individuals with sickle cell anemia. The mobilities recorded in this graph are averages of the mobilities in the ascending and descending limbs.

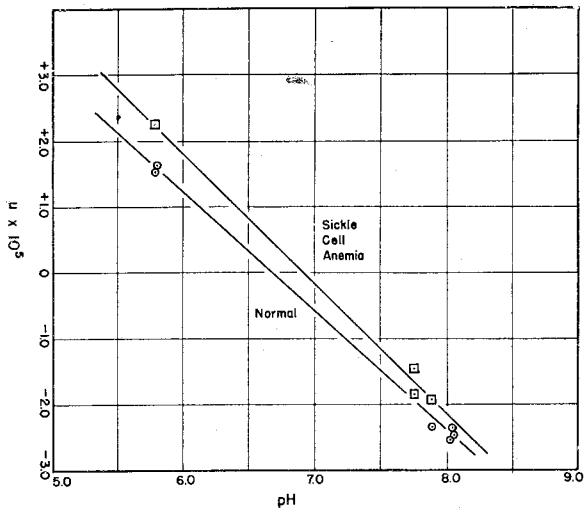

FIG. 2. Mobility(μ)-pH curves for ferrohemoglobins in phosphate buffers of 0.1 ionic strength containing dithionite ion. The mobilities recorded in the graph are averages of the mobilities in the ascending and descending limbs.

The experiments were carried out in the modified Tiselius electrophoresis apparatus described by Swingle (*14*). Potential gradients of 4.8 to 8.4 volts per centimeter were employed, and the duration of the runs varied from 6 to 20 hours. The pH values of the buffers were measured after dialysis on samples which had come to room temperature.

RESULTS

The results indicate that a significant difference exists between the electrophoretic mobilities of hemoglobin derived from erythrocytes of normal individuals and from those of sickle cell anemic individuals. The two types of hemoglobin are particularly easily distinguished as the carbonmonoxy compounds at pH 6.9 in phosphate buffer of 0.1 ionic strength. In this buffer the sickle cell anemia carbonmonoxyhemoglobin moves as a positive ion, while the normal compound moves as a negative ion, and there is no detectable amount of one type present in the other.[4] The hemoglobin derived from erythrocytes of individuals with sicklemia, however, appears to be a mixture of the normal hemoglobin and sickle cell anemia hemoglobin in roughly equal proportions. Up to the present time the hemoglobins of 15 persons with sickle cell anemia, 8 persons with sicklemia, and 7 normal adults have been examined. The hemoglobins of normal adult white and negro individuals were found to be indistinguishable.

The mobility data obtained in phosphate buffers of 0.1 ionic strength and various values of pH are summarized in Figs. 1 and 2.[5]

[4] Occasionally small amounts (less than 5 percent of the total protein) of material with mobilities different from that of either kind of hemoglobin were observed in these uncrystallized hemoglobin preparations. According to the observations of Stern, Reiner, and Silber (*12*) a small amount of a component with a mobility smaller than that of oxyhemoglobin is present in human erythrocyte hemolyzates.

[5] The results obtained with carbonmonoxyhemoglobins with and without dithionite ion in the buffers indicate that the dithionite ion plays no significant role in the electrophoretic properties of the proteins. It is therefore of interest that ferrohemoglobin was found to have a lower isoelectric point in phosphate buffer than carbonmonoxyhemoglobin. Titration studies have indicated (*5*, *6*) that oxyhemoglobin (similar in electrophoretic properties to the carbonmonoxy compound) has a lower isoelectric point than ferrohemoglobin in

The isoelectric points are listed in Table 1. These results prove that the electrophoretic difference between normal hemoglobin and sickle cell anemia hemoglobin

TABLE 1

ISOELECTRIC POINTS IN PHOSPHATE BUFFER, $\mu = 0.1$

Compound	Normal	Sickle cell anemia	Difference
Carbonmonoxyhemoglobin	6.87	7.09	0.22
Ferrohemoglobin	6.88 6.68	7.09 6.91	0.22 0.23

exists in both ferrohemoglobin and carbonmonoxyhemoglobin. We have also performed several experiments in a buffer of 0.1 ionic strength and pH 6.52 containing 0.08 M NaCl, 0.02 M sodium cacodylate, and 0.0083 M cacodylic acid. In this buffer the average mobility of sickle cell anemia carbonmonoxyhemoglobin is 2.63×10^{-5}, and that of normal carbonmonoxyhemoglobin is 2.23×10^{-5} cm/sec per volt/cm.[6]

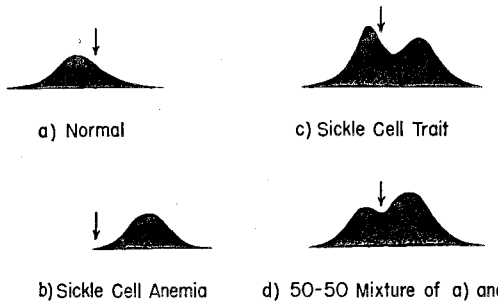

FIG. 3. Longsworth scanning diagrams of carbonmonoxyhemoglobins in phosphate buffer of 0.1 ionic strength and pH 6.90 taken after 20 hours' electrophoresis at a potential gradient of 4.73 volts/cm.

These experiments with a buffer quite different from phosphate buffer demonstrate that the difference between the hemoglobins is essentially independent of the buffer ions.

Typical Longsworth scanning diagrams of experiments with normal, sickle cell anemia, and sicklemia carbonmonoxyhemoglobins, and with a mixture of the first two compounds, all in phosphate buffer of pH 6.90 and ionic strength 0.1, are reproduced in Fig. 3. It is apparent from this figure that the sicklemia material contains less than 50 percent of the anemia component. In order to determine this quantity accurately some experiments at a total protein concentration of 1 percent were performed with known mixtures of sickle cell anemia and normal carbonmonoxyhemoglobins in the cacodylate-sodium chloride buffer of 0.1 ionic strength and pH 6.52 described above. This buffer was chosen in order to minimize the anomalous electrophoretic effects observed in phosphate buffers (*7*). Since the two hemoglobins were incompletely resolved after 15 hours of electrophoresis under a potential gradient of 2.79 volts/cm, the method of Tiselius and Kabat (*16*) was employed to allocate the

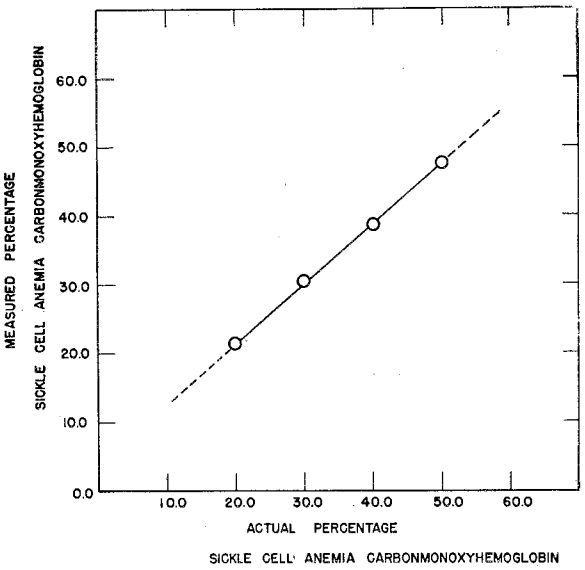

FIG. 4. The determination of the percent of sickle cell anemia carbonmonoxyhemoglobin in known mixtures of the protein with normal carbonmonoxyhemoglobin by means of electrophoretic analysis. The experiments were performed in a cacodylate sodium chloride buffer described in the text.

areas under the peaks in the electrophoresis diagrams to the two components. In Fig. 4 there is plotted the percent of the anemia component calculated from the areas so obtained against the percent of that component in the known mixtures. Similar experiments were performed with a solution in which the hemoglobins of 5 sicklemic individuals were pooled. The relative concentrations of the two hemoglobins were calculated from the electrophoresis diagrams, and the actual proportions were then determined from the plot of Fig. 4. A value of 39 percent for the amount of the sickle cell anemia component in the sicklemia hemoglobin was arrived at in this manner. From the experiments we have performed thus far it appears that this value does not vary greatly from one sicklemic individual to another, but a more extensive study of this point is required.

Up to this stage we have assumed that one of the two components of sicklemia hemoglobin is identical with sickle cell anemia hemoglobin and the other is identical with the normal compound. Aside from the

the absence of other ions. These results might be reconciled by assuming that the ferrous iron of ferrohemoglobin forms complexes with phosphate ions which cannot be formed when the iron is combined with oxygen or carbon monoxide. We propose to continue the study of this phenomenon.

[6] The mobility data show that in 0.1 ionic strength cacodylate buffers the isoelectric points of the hemoglobins are increased about 0.5 pH unit over their values in 0.1 ionic strength phosphate buffers. This effect is similar to that observed by Longsworth in his study of ovalbumin (7).

genetic evidence which makes this assumption very probable (see the discussion section), electrophoresis experiments afford direct evidence that the assumption is valid. The experiments on the pooled sicklemia carbonmonoxyhemoglobin and the mixture containing 40 percent sickle cell anemia carbonmonoxyhemoglobin and 60 percent normal carbonmonoxyhemoglobin in the cacodylate-sodium chloride buffer described above were compared, and it was found that the mobilities of the respective components were essentially identical.[7] Furthermore, we have performed experiments in which normal hemoglobin was added to a sicklemia preparation and the mixture was then subjected to electrophoretic analysis. Upon examining the Longsworth scanning diagrams we found that the area under the peak corresponding to the normal component had increased by the amount expected, and that no indication of a new component could be discerned. Similar experiments on mixtures of sickle cell anemia hemoglobin and sicklemia preparations yielded similar results. These sensitive tests reveal that, at least electrophoretically, the two components in sicklemia hemoglobin are identifiable with sickle cell anemia hemoglobin and normal hemoglobin.

Discussion

1) *On the Nature of the Difference between Sickle Cell Anemia Hemoglobin and Normal Hemoglobin*: Having found that the electrophoretic mobilities of sickle cell anemia hemoglobin and normal hemoglobin differ, we are left with the considerable problem of locating the cause of the difference. It is impossible to ascribe the difference to dissimilarities in the particle weights or shapes of the two hemoglobins in solution: a purely frictional effect would cause one species to move more slowly than the other throughout the entire pH range and would not produce a shift in the isoelectric point. Moreover, preliminary velocity ultracentrifuge[8] and free diffusion measurements indicate that the two hemoglobins have the same sedimentation and diffusion constants.

The most plausible hypothesis is that there is a difference in the number or kind of ionizable groups in the two hemoglobins. Let us assume that the only groups capable of forming ions which are present in carbonmonoxyhemoglobin are the carboxyl groups in the heme, and the carboxyl, imidazole, amino, phenolic hydroxyl, and guanidino groups in the globin. The number of ions nonspecifically adsorbed on the two proteins should be the same for the two hemoglobins under comparable conditions, and they may be neglected for our purposes. Our experiments indicate that the net number of positive charges (the total number of cationic groups minus the number of anionic groups) is greater for sickle cell anemia hemoglobin than for normal hemoglobin in the pH region near their isoelectric points.

According to titration data obtained by us, the acid-base titration curve of normal human carbonmonoxyhemoglobin is nearly linear in the neighborhood of the isoelectric point of the protein, and a change of one pH unit in the hemoglobin solution in this region is associated with a change in net charge on the hemoglobin molecule of about 13 charges per molecule. The same value was obtained by German and Wyman (*5*) with horse oxyhemoglobin. The difference in isoelectric points of the two hemoglobins under the conditions of our experiments is 0.23 for ferrohemoglobin and 0.22 for the carbonmonoxy compound. This difference corresponds to about 3 charges per molecule. With consideration of our experimental error, sickle cell anemia hemoglobin therefore has 2–4 more net positive charges per molecule than normal hemoglobin.

Studies have been initiated to elucidate the nature of this charge difference more precisely. Samples of porphyrin dimethyl esters have been prepared from normal hemoglobin and sickle cell anemia hemoglobin. These samples were shown to be identical by their x-ray powder photographs and by identity of their melting points and mixed melting point. A sample made from sicklemia hemoglobin was also found to have the same melting point. It is accordingly probable that normal and sickle cell anemia hemoglobin have different globins. Titration studies and amino acid analyses on the hemoglobins are also in progress.

2) *On the Nature of the Sickling Process*: In the introductory paragraphs we outlined the evidence which suggested that the hemoglobins in sickle cell anemia and sicklemia erythrocytes might be responsible for the sickling process. The fact that the hemoglobins in these cells have now been found to be different from that present in normal red blood cells makes it appear very probable that this is indeed so.

We can picture the mechanism of the sickling process in the following way. It is likely that it is the globins rather than the hemes of the two hemoglobins that are different. Let us propose that there is a surface region on the globin of the sickle cell anemia, hemoglobin molecule which is absent in the normal molecule and which has a configuration complementary to a different region of the surface of the hemoglobin molecule. This situation would be somewhat analogous to that which very probably exists in antigen-antibody reactions (*9*). The fact that sick-

[7] The patterns were very slightly different in that the known mixture contained 1 percent more of the sickle cell anemia component than did the sickle cell trait material.

[8] We are indebted to Dr. M. Moskowitz, of the Chemistry Department, University of California at Berkeley, for performing the ultracentrifuge experiments for us.

ling occurs only when the partial pressures of oxygen and carbon monoxide are low suggests that one of these sites is very near to the iron atom of one or more of the hemes, and that when the iron atom is combined with either one of these gases, the complementariness of the two structures is considerably diminished. Under the appropriate conditions, then, the sickle cell anemia hemoglobin molecules might be capable of interacting with one another at these sites sufficiently to cause at least a partial alignment of the molecules within the cell, resulting in the erythrocyte's becoming birefringent, and the cell membrane's being distorted to accommodate the now relatively rigid structures within its confines. The addition of oxygen or carbon monoxide to the cell might reverse these effects by disrupting some of the weak bonds between the hemoglobin molecules in favor of the bonds formed between gas molecules and iron atoms of the hemes.

Since all sicklemia erythrocytes behave more or less similarly, and all sickle at a sufficiently low oxygen pressure (*11*), it appears quite certain that normal hemoglobin and sickle cell anemia hemoglobin coexist within each sicklemia cell; otherwise there would be a mixture of normal and sickle cell anemia erythrocytes in sicklemia blood. We might expect that the normal hemoglobin molecules, lacking at least one type of complementary site present on the sickle cell anemia molecules, and so being incapable of entering into the chains or three-dimensional frameworks formed by the latter, would interfere with the alignment of these molecules within the sicklemia erythrocyte. Lower oxygen pressures, freeing more of the complementary sites near the hemes, might be required before sufficiently large aggregates of sickle cell anemia hemoglobin molecules could form to cause sickling of the erythrocytes.

This is in accord with the observations of Sherman (*11*), which were mentioned in the introduction, that a large proportion of erythrocytes in the venous circulation of persons with sickle cell anemia are sickled, but that very few have assumed the sickle forms in the venous circulation of individuals with sicklemia. Presumably, then, the sickled cells in the blood of persons with sickle cell anemia cause thromboses, and their increased fragility exposes them to the action of reticulo-endothelial cells which break them down, resulting in the anemia (*1*).

It appears, therefore, that while some of the details of this picture of the sickling process are as yet conjectural, the proposed mechanism is consistent with experimental observations at hand and offers a chemical and physical basis for many of them. Furthermore, if it is correct, it supplies a direct link between the existence of "defective" hemoglobin molecules and the pathological consequences of sickle cell disease.

3) *On the Genetics of Sickle Cell Disease*: A genetic basis for the capacity of erythrocytes to sickle was recognized early in the study of this disease (*4*). Taliaferro and Huck (*15*) suggested that a single dominant gene was involved, but the distinction between sicklemia and sickle cell anemia was not clearly understood at the time. The literature contains conflicting statements concerning the nature of the genetic mechanisms involved, but recently Neel (*8*) has reported an investigation which strongly indicates that the gene responsible for the sickling characteristic is in heterozygous condition in individuals with sicklemia, and homozygous in those with sickle cell anemia.

Our results had caused us to draw this inference before Neel's paper was published. The existence of normal hemoglobin and sickle cell anemia hemoglobin in roughly equal proportions in sicklemia hemoglobin preparations is obviously in complete accord with this hypothesis. In fact, if the mechanism proposed above to account for the sickling process is correct, we can identify the gene responsible for the sickling process with one of an alternative pair of alleles capable through some series of reactions of introducing the modification into the hemoglobin molecule that distinguishes sickle cell anemia hemoglobin from the normal protein.

The results of our investigation are compatible with a direct quantitative effect of this gene pair; in the chromosomes of a single nucleus of a normal adult somatic cell there is a complete absence of the sickle cell gene, while two doses of its allele are present; in the sicklemia somatic cell there exists one dose of each allele; and in the sickle cell anemia somatic cell there are two doses of the sickle cell gene, and a complete absence of its normal allele. Correspondingly, the erythrocytes of these individuals contain 100 percent normal hemoglobin, 40 percent sickle cell anemia hemoglobin and 60 percent normal hemoglobin, and 100 percent sickle cell anemia hemoglobin, respectively. This investigation reveals, therefore, a clear case of a change produced in a protein molecule by an allelic change in a single gene involved in synthesis.

The fact that sicklemia erythrocytes contain the two hemoglobins in the ratio 40 : 60 rather than 50 : 50 might be accounted for by a number of hypothetical schemes. For example, the two genes might compete for a common substrate in the synthesis of two different enzymes essential to the production of the two different hemoglobins. In this reaction, the sickle cell gene would be less efficient than its normal allele. Or, competition for a common substrate might occur at some later stage in the series of reactions leading to the synthesis of the two hemoglobins. Mechanisms of this sort are discussed in more elaborate detail by Stern (*13*).

The results obtained in the present study suggest that the erythrocytes of other hereditary hemolytic anemias be examined for the presence of abnormal hemoglobins. This we propose to do.

Based on a paper presented at the meeting of the National Academy of Sciences in Washington, D. C., in April, 1949, and at the meeting of the American Society of Biological Chemists in Detroit in April, 1949.

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PROPOSITIONS

1. The detection of intermediate compounds in hemoglobin reactions requires the use of compounds which are stable under the experimental conditions being employed. Furthermore, the equilibrium among the intermediates must be attained in a reversible manner. A possible method of achieving these objectives is to allow ferro- and ferrihemoglobin to come to equilibrium in the presence of a mediator (1), remove the mediator by dialysis, and saturate the ferrohemoglobin portion with carbon monoxide.

2. Differences in the chemical and physical properties of the two electrophoretic components of globin have been reported (2). A large scale separation of the components in the electrophoresis convection apparatus would permit investigations to determine whether each component is a necessary constituent of the reconstituted hemoglobin molecule.

3. The absorption spectrum of methemoglobin from an individual having congenital methemoglobinemia is abnormal (3). The oxidation potential of this hemoglobin should be determined.

4. The effect on the oxygen combining curve of hemoglobin of partial conversion to ferrihemoglobin is less than that of an equal conversion to carbonmonoxyhemoglobin (4).

PROPOSITIONS (Continued).

The in vivo production of ferrihemoglobin by nitrite is rapid and reversible. This suggests that a maintenance dose of sodium nitrite might reduce the amount of in vivo sickling in a sickle cell anemia patient.

5. Blocking Rh antibodies may be complexes of antibodies with Rh hapten from previously destroyed Rh positive red blood cells.

6. Covalent structures may be written for complexes of the ferroheme group with alkyl and aryl azides. The possible existence of ferrohemoglobin alkyl and aryl azides may be checked magnetometrically.

7. The ionic complex of catalase is probably of the form $FeOH \cdot H_2O_2$ and the covalent complex of the form $Fe \cdot H_2O_2$ or FeOOH.

8. Chance's investigations on the decomposition of H_2O_2 by catalase support the following mechanism (5,6).

$$\text{Catalase} + H_2O_2 \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{Catalase-}H_2O_2 \text{ complex}$$

$$\text{Catalase-}H_2O_2 \text{ complex} + H_2O_2 \xrightarrow{k_3} \text{Catalase} + 2\,H_2O + O_2$$

$k_1 \approx 3.0 \times 10^7\ \underline{M}^{-1}\ \text{sec.}^{-1}$ (5)

$k_2 \approx 0.02\ \text{sec.}^{-1}$ (5)

k_3 has not been measured.

PROPOSITIONS (Continued)

An independent calculation of k_1 and k_3 is possible by use of the rate constant for the catalytic decomposition of H_2O_2 by catalase and the fraction of catalase-H_2O_2 complex present at the steady state. According to this calculation,

$$k_1 = 2.5 \times 10^7 \ \underline{M}^{-1} \text{ sec.}^{-1}$$
$$k_3 = 5.8 \times 10^7 \ \underline{M}^{-1} \text{ sec.}^{-1}$$

9. In his calculation of the amount of catalase cyanide formed in the presence of H_2O_2 Chance (7) makes the incorrect assumption that the composition of the catalase-H_2O_2 complex does not depend upon the amount of cyanide added.

10. Methods are available for introducing the benzoyl group into porphyrins (8). The use of substituted benzoyl compounds and a protein containing one heme per molecule may permit the synthesis of protein antigens which are univalent with respect to known haptenic groups.

11. (a) Unless the neutrino which is emitted in the β-decay of the neutron has an absolute entropy value, the entropy change in this process is very nearly the same as that in the ionization of gaseous monatomic hydrogen.

(b) In order to have a positive absolute entropy at 25°C an electron gas could have to be at a pressure of less than 0.006 atmosphere; if the mass of a neutrino is taken as 0.15 that of an electron, a "neutrino gas" would have to be at a pressure of less than 3.5×10^{-7} atmosphere at 25°C.

PROPOSITIONS (Continued)

12. (a) Because of its low hydration and lack of complexing power, perchlorate ion should be used wherever it can replace chloride ion in kinetic and equilibrium studies.

(b) The transference numbers of perchloric acid and alkali perchlorates in dilute solutions should be measured. The concentration dependence will be small.

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