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Studies of Sickle Cell Anemia

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Studies of Sickle Cell Anemia*

I. The Transport of Phosphorus Across the Red Cell Envelope As Measured With Radiophosphorus (P^{32})

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IN the eight years since the appearance of the pioneer study by Pauling and his associates¹ on sickle cell anemia in which it was described as "a molecular disease," a truly impressive number of researchers have reported on what we may summarize as the physico-chemical properties of the blood of sickle cell anemia patients. It cannot be concluded, however, that these studies have exhausted the possibilities for investigation. The physical and chemical information that one might deem procurable from the blood of sickle cell patients may be conveniently summarized in Table 1. Although much of this information does not exist, the table cannot claim to be exhaustive.

The data from several areas are offered at present for the most reasonable physical factors responsible for the sickling of the red blood cells. One group offers the crystallization of the hemoglobin within the cell as the causative factor. Another believes that the hemoglobin does not crystallize but undergoes gelation and forms tactoids. A third position is that the hemoglobins and lipids near the surface of the cell undergo precipitation which twists the cells into the observed crescent and sickled shapes. The bulk of evidence seems to favor the second position inasmuch as x-ray studies of sickled cells and studies of the thermal properties of specific heat and heat of compression do not favor crystallization. This evidence is in favor of the gelation process as the cause of sickling. The third position is relatively recent and has not had time for critical examination.²

In this first of three short papers we shall report

* As part of a research program with radioactive and stable isotopes in the biophysics laboratory of the department of physics at Howard University, investigations of some properties of blood in normal and sickle cell anemia patients have been undertaken. Some results on these problems are reported in this series of three papers. We are deeply grateful to Professor Roland B. Scott, Head, Department of Pediatrics, for many blood samples and for helpful discussions and to Dr. William Bullock, Head, Hematology Laboratory of Freedmen's Hospital, for blood samples and other assistance. This work has been supported by the Atomic Energy Commission, AT (30-1)-892.

some measurements made in our biophysics laboratory on the transport of phosphorus across the red blood cell membrane in normal and sickle cell anemia blood samples in vitro as measured with radioactive phosphorus (P^{32}) as PO_4 . Preliminary data reported earlier on the initial experiments in this group indicated that there was a quantitative difference in the statistical means for normal and sickle cell anemic blood.⁴

MATERIALS AND METHODS

Blood samples were obtained from the Hematology Clinic of Freedmen's Hospital. Samples

TABLE 1. PHYSICAL AND CHEMICAL PROPERTIES OF SICKLE CELL BLOOD

I. Cells
A. General
1. Shape and size
2. Electrophoretic behavior
3. Optical studies—microspectrometry, birefringency
4. Energy utilization
5. Thermal properties: specific heat and heat of compression
6. Osmotic pressure
B. The Membrane
1. Electron microscopy studies
2. Permeability to various substances
3. Attachment of hemoglobin molecules
4. Fragility—lysis
5. Enzymes and enzyme systems
6. Composition
C. The Interior
1. Hemoglobin
a. Ultracentrifuge studies
b. Electrophoretic studies
c. X-ray crystallographic studies
d. Solubility
e. Amino acid composition
f. Viscosity
g. Dissociation constants
h. Oxygen dissociation curve
i. Buffering capacity
j. Optical studies
k. State within the cell
l. Distribution of various hemoglobins
2. Other Constituents
a. Identification and amounts
b. Enzyme systems
3. Metabolism
II. Plasma
A. Composition
B. Electrophoretic studies of protein constituents
C. Viscosity
D. Enzyme systems
E. Osmotic pressure

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from hematologically normal patients or staff members were also taken. The samples were grouped according to their origin as sickle cell, sickle cell trait, or normal. Approximately twenty ml. of blood were drawn into brown bottles containing two mg. of sodium heparin. Two ten ml. samples were then transferred to Warburg vessels, without center wells, and to each solution of normal saline (0.5 ml.) containing approximately 1 microcurie of radioactive phosphate as PO_4^{3-} was added. The steps in handling the samples are outlined in the flow sheets. The flasks were placed in the Warburg thermostat at 37.5°C . They were flushed with oxygen for two minutes, closed and shaken at the rate of 60 oscillations per minute for four hours. The samples were removed and centrifuged. The plasma was drawn off and the red cells were washed carefully three times with normal saline. The steps in the handling of the red blood cells and the plasma are summarized in the flow sheets. In general the phosphorus was determined by the method of Fiske and Subbarow.³ As indicated in the flow sheets, the radioactivity of a sample was determined by counting a sample, dried under an infrared lamp, in a small metal salve box. The determinations were made on a Nuclear of Chicago, model 172, scaling unit with an end window Geiger Muller tube, $3.2\text{mg}/\text{Cm}^2$. Each sample was counted for at least three one

minute intervals and the counts averaged. The basic data in this study are the specific activities expressed as counts per mg. of phosphorus.

Three different phosphorus samples in the cell are reported in this paper: The total phosphorus, the acid soluble phosphorus, and the inorganic phosphorus. In Tables 2-4 are listed the results for these three groups of compounds. The quantity listed is the ratio of specific activity of the plasma to the specific activity of the red blood cell.

When this ratio is large it means that more of the radioactive phosphorus remains in the plasma. If this ratio were equal to one, equilibrium between the two phosphorus functions would have been achieved between the plasma and the red blood cell.

In order to discuss these data we shall need the quantities of phosphorus in the plasma and red blood cells. Our determinations are in Table 5.

DISCUSSION

The data for all fractions in Tables 2-4 reveal that the specific activity of the phosphorus in the plasma is greater than that for the red blood cell and therefore no equilibrium has been reached. The mean of the ratios for sickle cell anemic blood is without exception greater than that for the normal blood. The trait is so close to normal that statistically they may be taken as equal although there is a small difference which has persisted in

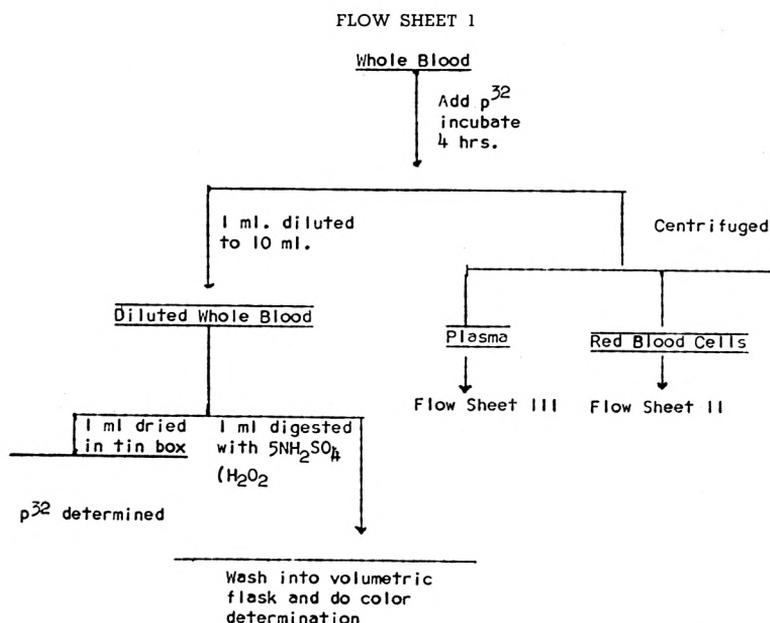


TABLE 2.—RATIOS FOR TOTAL PHOSPHORUS (EXPT. A)

	No.	Mean	Range	Standard Deviation
Normal Blood	9	1.69	1.26-2.06	0.103
Sickle cell trait	8	1.70	1.24-2.55	0.168
Sickle cell anemia	9	2.18	1.78-2.83	0.144

TABLE 3.—RATIOS FOR ACID SOLUBLE PHOSPHORUS (EXPT. A)

	No.	Mean	Range	Standard Deviation
Normal Blood	9	2.42	1.78-2.84	0.31
Sickle cell trait	8	2.80	1.92-2.93	0.66
Sickle cell Anemia	9	3.48	1.17-3.77	0.10

TABLE 4.—RATIOS FOR INORGANIC PHOSPHORUS (EXPT. B)

	No.	Mean	Range	Standard Deviation
Normal Blood	3	1.64	1.38-2.05	0.35
Sickle cell trait	0			
Sickle cell anemia	5	2.87	2.34-3.41	0.49

two groups of experiments.⁴ In our opinion the data on the inorganic fraction is the most significant for this study since the radiophosphorus was introduced into the plasma as inorganic phosphorus. The low rate of synthesis in the red blood cell results in little production of organically bound phosphorus. Thus, the radioactivity in the acid soluble and the total is almost exclusively due to the inorganic. Determinations of the radioactivity of other organic fractions not included in the organic soluble fraction, e.g. the phospholipid gave a specific activity in one sample for the

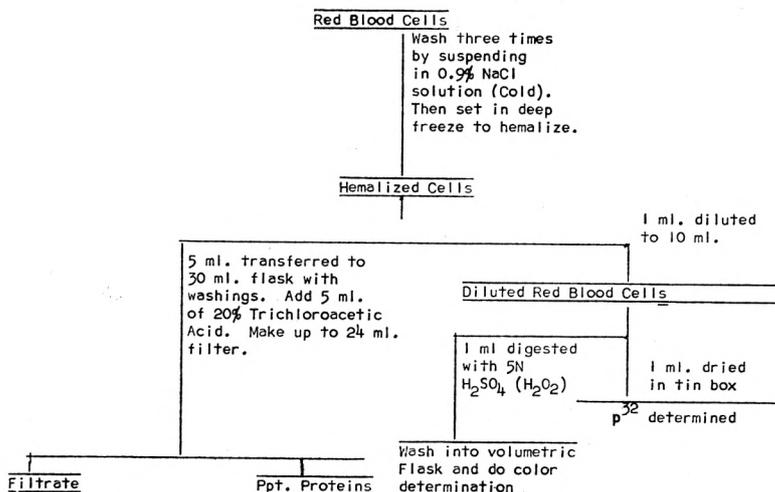
TABLE 5.—QUANTITIES OF PHOSPHORUS IN THE BLOOD IN MILLIGRAMS PER 100 MILLILITER

	Total Phosphorus	Inorganic Phosphorus
Normal		
Plasma	22.25	2.28
Red Blood Cells	102.36	3.24
Sickle cell anemic		
Plasma	20.05	2.22
Red Blood Cells	89.72	10.19

plasma of 0.047 and for the red blood cell 0.002 while the organic of the plasma read 6.12 and that of the red blood cell 4.09. A slight contamination could alter these values disastrously, hence we have not considered them.

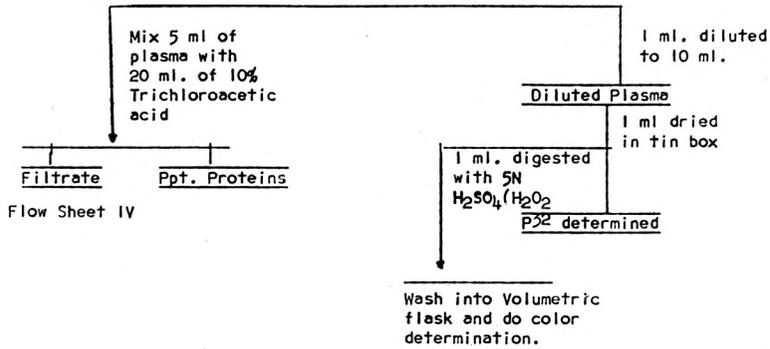
The inorganic phosphorus sample reveals that in these patients every normal value is below every sickle cell value. The ratio for the largest normal is 2.05, for the smallest sickle cell anemic it is 2.34. These results suggest that a slower equilibration rate of the inorganic phosphorus in comparison with the normal is characteristic of sickle cell anemia. This interpretation must be examined, however, in the light of the data in Table 5 which shows that there is over three times as much inorganic phosphorus in the red blood cell as there is in the plasma. This result can be believed as reporting the true value of the inorganic phosphorus following our chemical methods or that the methods employed lead to a hydrolysis of labile phosphorus compounds in the sickle cell anemic rbc, compounds that are more resistant in the normal. We have, on the other hand, additional data in the total phosphorus samples, Table 2, where the

FLOW SHEET 2



FLOW SHEET 3

Plasma



larger plasma to rbc specific activities in the sickle cell anemic case are also found.

An important ratio calculable from our data may be introduced here. It is the fraction of total phosphorus found within the cell at the end of the four hour period. This ratio is for the total phosphorus

$$\frac{(P \text{ of cells}) \times (S.A. \text{ cells})}{(P \text{ of plasma}) (S.A. \text{ plasma}) + (P \text{ of cells}) (S.A. \text{ cells})}$$

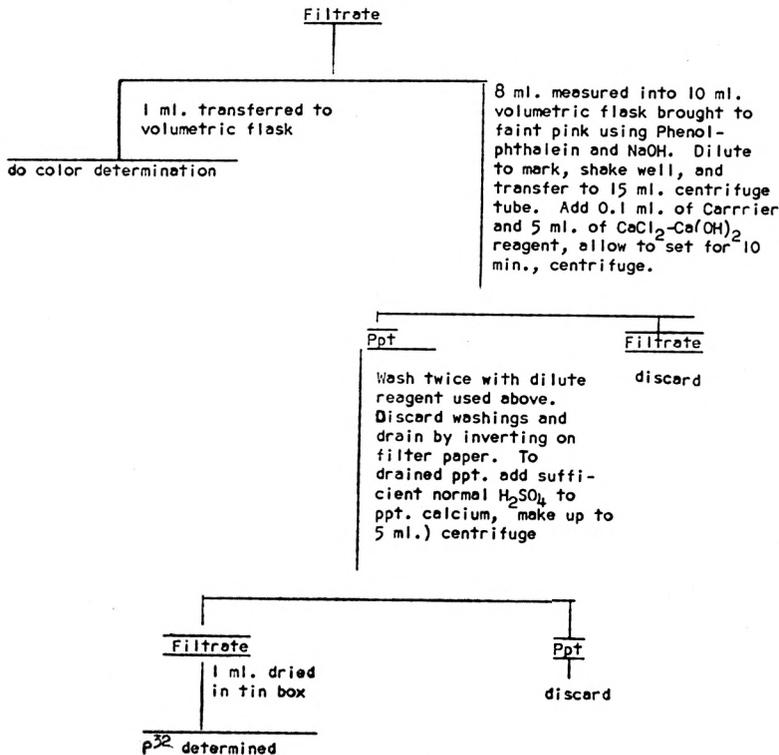
where P stands for phosphorus and S.A. is the specific activity. Dividing numerator and denominator by (S.A. cells), which is the specific activity in Table 2, we have the data for the normal in Tables 2 and 5,

$$\frac{102.34}{22.75 \times 1.69 + 102.36} = 0.73,$$

while for the sickle cell anemic

$$\frac{89.72}{20.04 \times 2.18 + 89.72} = 0.65.$$

FLOW SHEET 4



That is the normal takes up 73 per cent of the total radioactivity; the sickle cell anemic only 65 per cent. This ratio shows that independent of the conclusion with respect to the inorganic phosphorus determinations, the normal cell takes up slightly more of the radioactivity from the solution than the sickle cell anemic although the difference is not as impressive as the inorganic fraction ratios. The value are sufficiently close, however, 73 versus 65, to account for some of the references in the literature which state that the sickle cell and normal rbc take up the same amount of radioactivity from a phosphate solution containing P^{32} . This study reveals that although such a conclusion is technically correct, it exhibits a most insensitive experimental technique.

These data are consistent with the results reported by Taylor, et al,⁷ that approximately $\frac{1}{3}$ of the P^{32} remains in the plasma after four hours of incubation at 37.5°C . We do not find, however, that the specific activity of the inorganic phosphorus within the cell ever equals that of the plasma which they report.

Our data support a mechanism for the transport of the PO_4 across the red cell membrane which is relatively independent of the concentration of the inorganic phosphorus within the cell inasmuch as the higher concentration in the sickle cell anemia rbc does not lead to a more rapid interchange of the PO_4 .⁸

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Studies of Sickle Cell Anemia

II. Phosphatase Activities in Normal and Sickle Cell Anemic Blood

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SINCE it has been demonstrated experimentally through the use of radioactive P^{32} that the erythrocyte of the individual with sickle cell anemia exhibits a prolonged turnover time for phosphorus thereby utilizing inorganic phosphorus less rapidly than the erythrocyte of the normal individual,¹ it has become of interest to investigate the enzymes in the plasma which render inorganic phosphorus available to the erythrocyte through the process of splitting phosphoric esters present in the plasma. The point of question in this undertaking centered about the probability of decreased enzymatic activity in the sickle cell plasma making less inorganic phosphorus available to the sickle cell erythrocyte and accounting for a diminution in uptake and increased turnover time.

MATERIALS AND METHODS

For this investigation, a comparative study was made in 20 individuals, 12 whom were normal, and 8 of whom had been diagnosed as having sickle cell anemia. The age range was 8-14 years.

The enzymes in human plasma are largely monoesterases acting upon monophosphate esters also present in plasma. The capacities of the phosphatases to attack β -glycerophosphate, inorganic pyrophosphate, 1-glucosephosphate, and 6-glucosephosphate are relatively the same.² All of the enzymes which attack phosphoric esters have been designated phosphatases and have been divided roughly into two large groups on the basis of pH optimums at which they perform. One of these groups exhibiting a maximum activity at a pH level of 9.2-9.6 has been labelled alkaline phosphatase and conversely another large group exhibiting a peak activity at a pH level of 5.0-6.0 bears the designation acid phosphatase. It must be borne in mind, however, that the pH of blood is 7.35-7.45 normally and all of these enzymes while in the plasma perform at this pH level and may show activity levels different from *in vitro* studies. β -1 glycerophosphate was chosen for the substrate and prepared after the method of Shinowara,

Jones and Reinhart.⁴ The substrates were adjusted to pH optimums of 9.3 for the alkaline phosphate and pH of 5.0 for the acid phosphatase. For the determination after the method of Bodansky,⁵ 5 ml of whole blood were collected in a centrifuge tube and allowed to clot at room temperature. The blood was then centrifuged twice and the serum taken off for determination. Nine ml. of substrate were placed in a Warburg flask and placed in the Warburg apparatus and allowed to equilibrate at 37.5°C. To this was added 1 ml. of serum and then incubated for one hour. Near the end of incubation period, a control sample was run by the same procedure save for the incubation. At the end of one hour both samples and control were cooled in ice water and 2cc of 30 per cent trichloroacetic acid were added and allowed to stand for five minutes. Through a low ash filter the precipitated proteins were removed and 8 ml. of filtrate were used for inorganic phosphorus determination. Using the technique of Fiske and Subbarrow⁶, inorganic phosphorus in the whole 8 ml. diluted with distilled water was used to minimize errors.⁷ The phosphatase activity is expressed in units of Bodansky, which is simply the expression of the number of milligrams of inorganic phosphorus split from β -glycerophosphate and obtained by taking the difference between inorganic phosphorus in the incubated sample and control sample. Utilizing the aforementioned procedure the following data were obtained:

TABLE I.—ALKALINE PHOSPHATASE ACTIVITY
IN BODANSKIES

	No.	Range	Average
Normal	16	1.46- 9.80	5.44
Sickle cell anemia	8	3.87-22.44	11.20

TABLE II.—ACID PHOSPHATASE ACTIVITY
IN BODANSKIES

	No.	Range	Average
Normal	12	0-2.22	1.20
Sickle cell anemia	5	0-2.46	0.97

DISCUSSION

Alkaline phosphatase is formed in the bone marrow and disposed of in the liver. An elevation of alkaline phosphatase is seen in obstructive and hepatogenous disease of the liver as well as in certain neoplastic diseases of the bone marrow. The bone age of the individual and rate of calcification influence alkaline phosphatase activity, the average for adults being 2.7 Bodanskies (1.5-4.0 Bodanskies), and for growing children 8.0 Bodanskies (5-12 Bodanskies).

SUMMARY

1. These studies give an average value of 11.20 Bodanskies for sickle cell alkaline phosphatase activity against 5.44 Bodanskies average for normal patients. These data suggest that in the usual sickle cell case there is serious involvement of the areas producing alkaline phosphatase. Elevated alkaline phosphatase is not a specific characteristic in sickle cell anemia, however, inasmuch as some of the normal samples have higher values than

some sickle cell samples.

2. In spite of the marked fluctuation in the sickle cell acid phosphatase determinations, they support the conclusion that there is no significant difference between the normal samples and the sickle cell anemic samples.

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Studies of Sickle Cell Anemia

III. Plasma Proteins in Normal and Sickle Cell Anemia Blood

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AS the final study of this group we report our preliminary work on an electrophoretic analysis of the plasma proteins. According to Krebs¹ 90 substances have been distinguished in the human plasma. In a severe hemolytic condition such as sickle cell anemia it would not be astute, then, to inquire if any of these differ significantly in amounts or characteristics from the normal. We chose for our initial investigation the relative concentrations and mobilities of the protein fractions, for close interrelations are often imputed between the plasma proteins and the proteins of the red blood cell.

MATERIALS AND METHODS

For this study an Aminco-Stern portable electrophoresis apparatus was used. Samples of normal and sickle cell blood were obtained from the Pediatrics Ward at Freedmen's Hospital.

The plasma obtained was diluted one part to two parts of buffer and then dialysed against the

same buffer for 24 hours at 2°C. The buffer used was sodium veronal (NaV-HV) of 0.1 ionic strength and a pH of 8.6. Electrophoresis was carried out in a standard clinical cell of 11 cc. capacity with a current of 10 ma passing through for a period of from one to two hours. The difference of potential across the cell is about 1000 volts. A Shedlovsky cell was used to determine the conductivity of the protein solution for the calculation of the mobilities. The cell was standardized with KCl.

The Aminco-Stern apparatus translates through its optical system the differences in the densities of the protein solution into an interpretable light pattern which is recorded on a photographic film. At pH 8.6, the proteins of the plasma are all negatively charged. Under the action of the electric field supplied by the apparatus the column of protein molecules moves towards the anode. The fastest moving component will move ahead and the slowest will bring up the rear. Thus, for

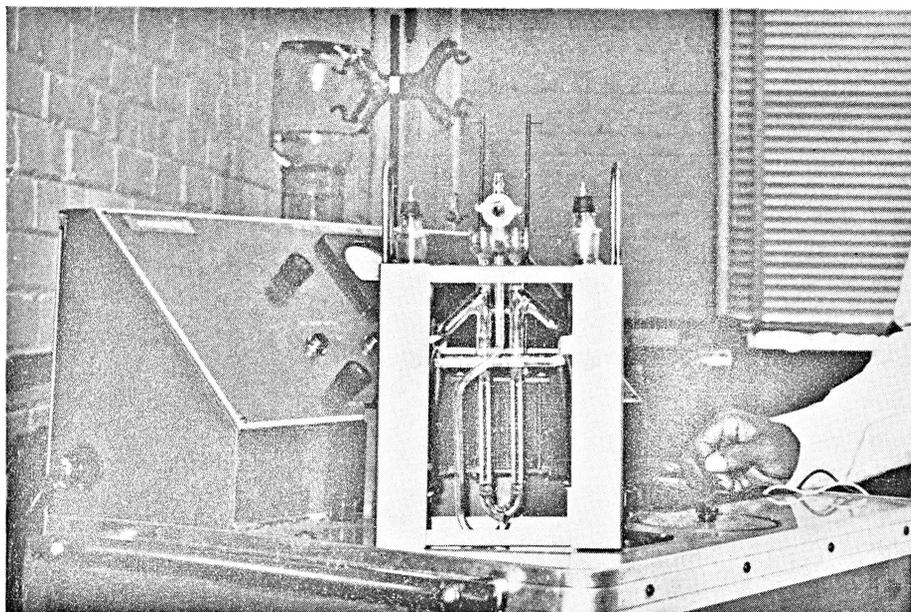


Fig. 1. The electrophoresis apparatus in use. The clinical cell is standing on the cover to the left of the thermostated bath.

plasma the leading component is albumin, the final is gamma-globulin. We may measure, therefore, how far the component moves in a given time. Moreover, the optical system gives a pattern in which each peak represents a component and, most significant, the area under each peak is proportional to the percentage of that component in the protein mixture.

Figure 1 is a picture of the apparatus with the clinical cell used for the pattern. In Figure 2 is the pattern of a normal plasma sample. The area under each of these curves has been measured with a planimeter. By noting the starting point the speed with which the component moves is easily calculated. The speed is dependent upon the molecular size and the number of surface charges. Albumin moves fastest both because of its relatively low molecular weight (70,000) with respect to the globulins (molecular weight 180,000) and the fact that albumin has more charges per molecule.

The mobility measured here is defined as the velocity of an average protein molecule in unit electric field in the body of the protein solution. If Δx is the distance moved in gamma seconds and the electric field is E , then mobility = $\frac{\Delta x}{t}$.

$$E$$

From the conductivity of the solution and the current passing through the cell, we get $E = i/gk$, where K is the conductivity and g the cross section of the cell. Substituting in the equation gives $u = \frac{\Delta x}{t} \frac{gk}{i}$. In this instrument the distance is magnified

two times on the film so the final equation used was $u = \frac{(\Delta x)gk}{2it}$ cm² volts⁻¹ sec⁻¹.

The percentage concentrations were calculated from the areas under the curves obtained. The area under each peak is proportional to the concentration of the component forming the peak, that is $C = KA$ where C is concentration A is area and K is some constant, which is approximately the same for each component. In this calculation it is assumed that each protein component has the same refractive power. Then if A is the total area under the curve and A_i the area under the i th peak the per cent concentration of A_i in solution is $\frac{A_i}{A} \times 100$.

$$A$$

The electrophoretic mobilities and per cent con-

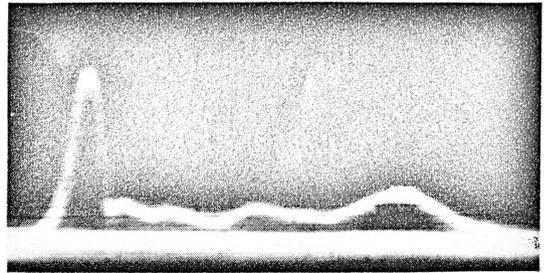


Fig. 2. The electrophoretic pattern for fresh pooled human blood run at pH 7.3 ionic strength 0.01. The mobility of the albumin is 5.08×10^{-5} . At this pH the gamma globulin moves so slowly that it is not separated from the delta peak. This pattern cannot be used then for determining relative concentrations.

RESULTS

Sickle cell Anemia MOBILITIES $\times 10^5$

Sample	1	2	3	4	Mean	Stand. Dev.
Albumin	8.30	6.9	7.5	8.09	7.69	0.63
Alpha Globulin	5.98	5.05	5.68	5.57	5.57	0.39
Beta Globulin	4.08	3.44	3.82	3.65	3.75	0.28
Gamma Globulin	0.77	0.61	0.91	0.96	0.81	0.16
Fibrinogen	2.58	2.18	2.64	2.22	2.41	0.24

centrations are shown below along with samples of the patterns obtained.

An experiment to determine the variation of mobility with pH was performed on a sample of normal and a sample of sickle cell plasma. Michaelis' universal buffer⁷ was used. The data

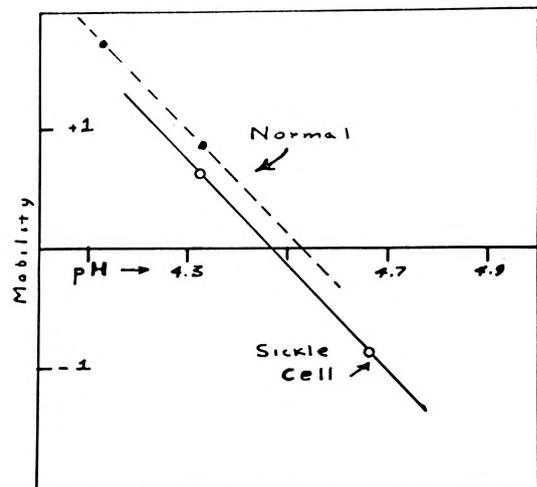


Fig. 3. The variation of mobility with pH in Michaelis' universal buffer (7). These results give the isoelectric point for normal human serum albumin as pH 4.51 while that for sickle cell serum albumin is pH 4.47 in this buffer.

Normal Plasma MOBILITIES $\times 10^5$

Sample	1	2	3	4	5	6	7	Mean	Stan. Dev.
Albumin	6.65	7.15	6.53	6.39	6.45	6.30	6.10	6.51	0.33
Alpha Globulin	4.82	5.02	4.58	4.66	4.67	4.50	4.39	4.66	0.21
Beta Globulin	3.31	3.46	3.26	3.46	3.26	3.15	3.20	3.30	0.29
Gamma Globulin	0.64	0.81	0.91	0.91	0.89	0.90	0.80	0.84	0.24
Fibrinogen	2.35	2.28	2.44	2.29	2.34	2.25	2.24	2.31	0.07

The mobilities of the albumins differ significantly at about the 0.5% level, and of the alpha globulins well below this level.

Sickle Cell Relative Concentrations

Sample	1	2	3	4	Mean	Stand. Dev.
Albumin	63.7	61.3	63	68	64	2.9
Alpha Globulin	9.1	6.45	10.3	8.16	8.5	1.6
Beta Globulin	9.1	9.67	12.9	8.16	9.96	2.1
Gamma Globulin	12.49	19.35	13.8	10.88	14.13	3.7
Fibrinogen	5.68	3.23	—	4.9	4.6	—

Normal Plasma Relative Concentrations

Sample	1	2	3	4	5	6	7	Mean	Stan. Dev.
Albumin	62.75	57	53.25	52	56	57.8	60	56.97	3.7
Alpha Globulin	10.9	11.9	9.5	7.4	12.45	7.7	8.8	9.8	2.0
Beta Globulin	10.9	10.35	13.2	9.2	7.8	11.5	8.8	10.25	1.8
Gamma Globulin	10.9	15.5	24.1	24.7	18.67	19.4	19.1	18.91	4.8
Fibrinogen	4.55	5.18	—	6.18	5.17	3.85	3.7	4.77	—

Students "t" test indicates the albumin concentrations to be significantly different at about the 1% level. The concentrations of the other components are not significantly different even at the 5% level.

A/G Ratios

	Sickle cell	Normal
Range	1.59 — 2.10	1.08 — 1.70
Average	1.78	1.325

are inadequate in number but they reveal that at the same pH below the isoelectric point in this buffer, the sickle cell albumin has a greater negative mobility than the normal, (Fig. 3). This difference is considerably less than the average difference found at pH 8.6, but it is in the same direction. Alberty⁸ reported that in 0.01 M NaCl the isoelectric point of human serum albumin is pH 4.8.

SUMMARY OF DATA

These data indicate that the plasma of sickle cell patients in this study differs from that of normal subjects in the following ways at pH 8.6:

1. The albumin of sickle cell plasma has a mobility of 7.69 while that of normal plasma has a mobility of 6.51 $\text{cm}^2 \cdot \text{volt}^{-1} \cdot \text{sec}^{-1}$.
2. Alpha globulin of sickle cell plasma has a mobility of 5.57 and normal alpha globulin 4.65 $\text{cm}^2 \cdot \text{volt}^{-1} \cdot \text{sec}^{-1}$.
3. The relative concentration of albumin in sickle cell plasma is 64 per cent and that of normal plasma is 56.97 per cent.
4. The relative concentration of gamma globulin of sickle cell plasma is 14.13 per cent and in normal plasma is 17.48 per cent.
5. The ratio of albumin to globulin in sickle cell is 1.78 and in normal plasma is 1.325.

DISCUSSION

The greater mobility of the albumin of sickle cell anemia plasma could be attributed to a larger number of negative charges on the albumin molecule or to the binding by the albumin of small negative ions which are not dialyzed off during the 24 hour period of dialysis. According to Pauling et al, the hemoglobin of sickle cell anemia has two to four more net positive charges per molecule than normal hemoglobin.¹

Albumin-globulin ratio for normal blood is less than that reported by Krebs.³ It is consistent with one segment of the data of Rawnsley et al,⁴ who, although employing techniques different from ours, report finding consistently higher mean concentration of gamma globulin in Negroes, in that their results also lead to a lower A/G ratio. Our mean value on gamma globulin, however, does not differ significantly from the data on caucasoids for they report a mean 18.04 and we find 18.08 for our samples from Negroes. The smaller A/g ratio in our samples is a result of a larger value

for the other globulin fractions. Calculations from Dole's data at 25°C as reported by Antweiler⁵ give A/g 1.73 with a value of 60.3 per cent for the albumin and 11.0 ± 2.5 per cent for gamma globulin.

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