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*DIFFERENTIAL TITRATION BY MEANS OF PAPER ELECTROPHORESIS AND THE STRUCTURE OF HUMAN HEMOGLOBINS**

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Patients suffering from sickle-cell anemia possess hemoglobin (hemoglobin b) which differs from normal adult hemoglobin (hemoglobin a). A molecular basis for this difference was first demonstrated experimentally by Pauling, Itano, Singer, and Wells in 1949 by means of free electrophoresis in the Tiselius apparatus.[1] They showed that the hemoglobin b molecule possesses a net charge about three units more positive than hemoglobin a, within a pH range of about 1.5 units on either side of neutrality. Since that time, three additional abnormal hemoglobins, hemoglobin c,[2] with an even greater net positive charge than hemoglobin b, hemoglobin d[3], and hemoglobin e[4] have been described. The present paper describes a new experimental method of differential titration by means of which information was obtained concerning the chemical basis of the difference in charge between hemoglobins a, b, and c.

As the pH of a protein solution is increased, various groups within the protein molecule lose protons. Neutral terminal carboxyl groups and the neutral free carboxyl groups of aspartic and glutamic acid residues become negatively charged in the pH range from 2 to 5.5; imidazole groups of histidine residues lose their positive charges in the range from 5.5 to 8.5; terminal amino groups lose their positive charges from 6.5 to 9.0; epsilon amino groups of lysine residues lose their positive charges in the range from 9 to 11.5; neutral tyrosine hydroxyl groups become negatively charged in the range from 9 to 12; and guanidinium groups of arginine residues lose their positive charges above—often considerably above—pH 11.[5] In some instances groups may be tied off so that they are completely unreactive in the native protein,[6, 7] and the characteristic pH ranges within which the groups ionize may be modified by neighboring substituents.[5]

Suppose that hemoglobin b possesses three more net positive charges than hemoglobin a by virtue of possessing three *more* positively charged nitrogen atoms such as, for example, those in lysine residues. In solutions with a pH of about 11.5 or higher the difference in charge between the two hemoglobins should disappear, since all epsilon amino groups are uncharged. On the other hand, if we assume that hemoglobin b possesses its greater net positivity by possessing three *less* free carboxyl groups than hemoglobin a, then the difference in charge between the two

hemoglobins should disappear in a solution with a pH of about 3.5 or less, since most carboxyl groups are uncharged around this pH. Therefore, comparisons of the charges on hemoglobins a, b, and c at selected pH values may be expected to yield information as to the nature of the groups responsible for the differences in charge when the comparisons are considered in the light of the pK values of the various groups.

Paper electrophoresis is a simple technique for comparing relative mobilities[8] and therefore for comparing relative charges of proteins which are as similar in size and shape as hemoglobins a, b, and, presumably, c.[1, 9] If paper electrophoretic experiments are made on several paper strips, each with the three hemoglobins but each in a buffer of different pH, the differences in the distances which the hemoglobins migrate on a given strip should bear a close relation to the differences in charge between the hemoglobins at that pH. If electro-osmotic flow can be measured[8] and if, as seems reasonable, the hemoglobins do not bind different numbers of any small ions other than protons,[1] the ratio of the distances which the hemoglobins migrate should equal the ratio of the numbers of protons bound by each hemoglobin at that pH. Such experiments can be considered to be differential titrations of the three forms of hemoglobin and are similar to experiments performed by Durrum[10] in which histidine could be distinguished from arginine and lysine as well as from the neutral amino acids.

MATERIALS AND METHODS

Hemoglobins.—Blood was drawn into acid-citrate-dextrose solution from subjects who were homozygous for hemoglobins a, b, or c.[11] Hemoglobin solutions were prepared according to the method of Drabkin,[12] and were converted to carbonmonoxyhemoglobin.

Hemoglobin solutions for use in paper electrophoretic experiments were generally made isoionic by passage over columns of ion-exchange resins, according to the method of Dintzis.[13] For determination of the isoionic points, 5–7 per cent solutions of carbonmonoxyhemoglobin flowing from the columns were collected under mineral oil, and the pH was measured without added salt. All pH measurements were made in a Beckman Model G pH-meter at room temperature.

Paper Electrophoresis.—Electrophoretic experiments on paper were carried out essentially as described by Kunkel and Tiselius.[8] Two spots of hemoglobin *a* were generally used, and these, together with two spots of a 10 per cent dextran solution, provided evidence regarding the uniformity of the electric field and an estimate of the electro-osmotic flow. Runs were made, at least in duplicate, at room temperature, for 1–4 hours, with a potential of 350 volts. A current of 7–10 milliamperes flowed in most experiments. Papers were dried at 120° C. and stained with alcoholic bromphenol blue.[8]

The pH of the buffers was measured at room temperature (22–27° C.) before, and after, most runs. The change in pH of the buffer during a run was seldom more than 0.03 pH units. The ionic strength of all buffers was 0.06.

RESULTS AND DISCUSSION

The isoionic points[14] of the three hemoglobins, measured, as described above, in the absence of salt, were as follows: hemoglobin a, 7.11; hemoglobin b, 7.21;

hemoglobin c, 7.42. If the hemoglobins bind no other small ions present in the solution except hydrogen ions, each isoionic point should be equal to the isoelectric point as measured electrophoretically, except for the electrostatic change of isoionic point due to the ionic strength of the buffers used in electrophoretic experiments.[14] In a solution somewhat more acid than the lowest pH of the three isoionic points, all three hemoglobins will move as cations in an electrophoretic experiment. Hemoglobin c, with the greatest positive charge, will move faster than hemoglobin b, while hemoglobin a, with the smallest positive charge, will move as the slowest of the three. In a solution somewhat more alkaline than the highest pH of the three isoionic points, the relative mobilities of the hemoglobins, now all anions, will be reversed, and hemoglobin c, with the greatest positive charge, or smallest negative charge, will move as the slowest.

Table 1 gives the relative mobilities of the three hemoglobins in buffers of differing pH. The pH values given for the isoelectric points (e.g., pH 7.0 for a) are probably no more accurate than 0.1 pH unit, since electro-osmotic flow was only estimated with dextran. Photographs of some of the filter papers of these runs are shown in Figures 1–6.

TABLE 1

RELATIVE MOBILITIES OF HEMOGLOBINS a, b, AND c AS MEASURED BY PAPER ELECTROPHORESIS IN VARIOUS BUFFERS (IONIC STRENGTH = 0.06)

Buffer	pH	Relative Mobilities*
Glycine–glycine HCl	3.0	+a = +b = +c
Glycine–glycine HCl	3.4	+a = +b = +c
Sodium acetate–acetic acid	4.1	+a = +b = +c
Sodium acetate–acetic acid	4.8	+a < + b < + c
Sodium acetate–acetic acid	5.8	+a < + b < + c
Na_2HPO_4–NaH_2PO_4	7.0	a = 0 + b < +c
Na_2HPO_4–NaH_2PO_4	7.2	−a b = 0 + c
Na_2HPO_4–NaH_2PO_4	7.4	−a > −b c = 0
Sodium diethyl barbiturate–diethyl barbituric acid	8.6	−a > −b > − c
Sodium glycinate–glycine	9.8	−a > −b > − c
Sodium glycinate–glycine	10.8	−a > −b > − c
Na_2HPO_4–Na_3PO_4	11.7	−a > −b > − c
Na_2HPO_4–Na_3PO_4	12.0	−a > −b > − c

* + denotes mobility as cation toward cathode; − denotes mobility as anion toward anode; > and < denote "mobility greater than" and "mobility less than," respectively.

It can be seen from Table 1 and Figures 1 and 2 that at all alkaline pH values investigated hemoglobin a moves faster than hemoglobin b, and hemoglobin b moves faster than hemoglobin c. This shows that the net charge of hemoglobin c is more positive than that of hemoglobin b, and the net charge of the latter is more positive than that of hemoglobin a, even at a pH of 12.0. Since most lysine and some arginine groups in proteins have lost their positive charges at a pH of 12.0,[5] these experiments indicate that the differences in charge between the three hemoglobins are not due to differences in their content of lysine or arginine residues.

The paper electrophoretic experiments performed at acid pH values show that the three hemoglobins continue to differ in net positive charge down to a pH of 4.8. At a pH of 4.1, 3.4, or 3.0, however, the mobilities of the three hemoglobins are essentially equal (Table 1 and Figs. 3 and 4). These experiments provide evidence that the differences in charge between hemoglobins a, b, and c are due to differences in their content of free carboxyl groups. For, in a pH range where only these

groups are losing their charges, the differences in charge between the three proteins disappear.

It is necessary to prove that the equality of the mobilities of the three hemoglobins at a pH of about 4.1 and lower is not due to irreversible denaturation of the proteins. Solutions of the three hemoglobins, brought to a pH of 4.1 with hydrochloric acid, were dialyzed at room temperature against an acetate buffer of pH 4.1 for 197 minutes. The dialysis sacs were then placed in a veronal buffer of pH 8.6 and dialyzed overnight. This procedure causes a portion of each hemoglobin to precipitate, and the precipitates were removed by centrifugation. A paper electrophoretic experiment was performed with the supernatant solutions in veronal buffer of pH 8.6. The result is pictured in Figure 5 and shows that the same differences in mobility were observed as had been seen at this pH in experiments in which the three hemoglobins had not been exposed to acid (Fig. 1). In a similar experiment the precipitated hemoglobins were dissolved in a small amount of sodium hydroxide and electrophoresis was performed at a pH of 10.8. The result, seen in Figure 6, shows that some of the denatured hemoglobin remained at the starting point but that the portions that migrated did so with the usual differences in mobility between hemoglobins a, b, and c, which are observed at alkaline pH. The exposure to a pH of 4.1, therefore, has not irreversibly denatured the hemoglobins with respect to the differences in structure which are responsible for the differences in charge at higher pH values. Exposure of the hemoglobins for 2–3 hours to a buffer of pH 3.4, 3.0, or 11.8, with subsequent dialysis against a veronal buffer of pH 8.6, also yielded supernatant hemoglobins still possessing the characteristic differences in mobility at pH 8.6.

Havinga and Itano have cited experiments in which electrophoretic analyses in acidic buffers failed to disclose any significant differences in mobility between denatured globins prepared from hemoglobins a and b.[15] They also report experiments in which the usual differences in mobility characteristic of the two hemoglobins were observed in the native globins of these proteins at a pH of 7.0 and in a 0.01 molar solution of Na_2HPO_4. They do not report experiments on the denatured globins in alkaline buffers.

On the basis of these results it would appear unlikely that ionizable groups other than carboxyl groups could account for the differences in charge between the proteins. Since the imidazole nitrogen of histidine ionizes in a pH range of around 5.5–8.5,[5] such a nitrogen atom would be expected to be charged at pH 4.0 and uncharged at pH 11. Therefore, a difference in the histidine content of the hemoglobins would result in differences in mobility at acid pH values and in no differences in mobility in alkaline buffers. This is the opposite of what was observed. Differences in tyrosine content should not contribute to differences in charge between the hemoglobins, except at pH values near and above the pK value of the tyrosine hydroxyl group, which is about 10.8.[5] The phosphorus content of hemoglobin a is not large enough for differences in the content of phosphate groups between hemoglobins a, b, and c to account for their differences in charge.[16] Differences in specific binding of buffer ions by the hemoglobins cannot account for the

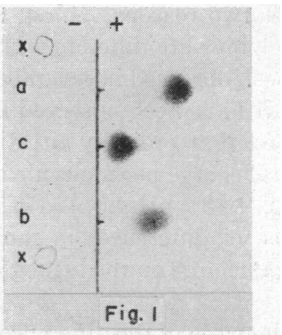

Fig. 1

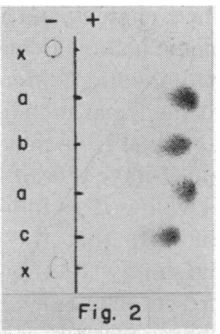

Fig. 2

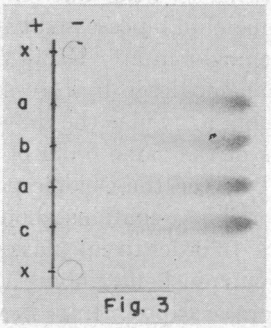

Fig. 3

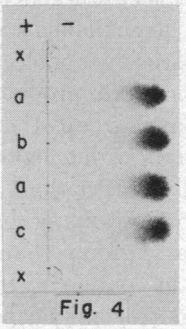

Fig. 4

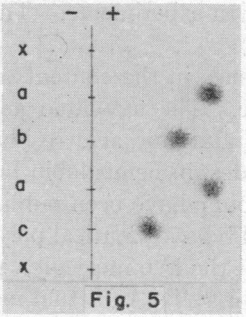

Fig. 5

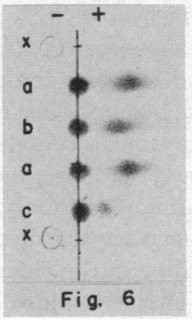

Fig. 6

FIGS. 1–6.—Paper electrophoresis of hemoglobins a, b, and c and dextran, x. The line is the locus of the starting points for hemoglobins and dextran. The outlined circles are locations of dextran spots which stained too faintly to be photographed. The + and − signs indicate on which side of the starting line the anode and cathode, respectively, are located.

Fig. 1, veronal buffer, pH = 8.6, $\Gamma/2 = 0.06$; run for 240 minutes at 400 volts. Fig. 2, phosphate buffer, pH = 11.7, $\Gamma/2 = 0.06$; run for 126 minutes at 350 volts. Fig. 3, acetate buffer, pH = 4.08, $\Gamma/2 = 0.06$; run for 57 minutes at 400 volts and 140 minutes at 200 volts. Fig. 4, glycine buffer, pH = 3.35, $\Gamma/2 = 0.06$; run for 75 minutes at 350 volts. Fig. 5, veronal buffer, pH = 8.65, $\Gamma/2 = 0.06$; run for 225 minutes at 350 volts. Hemoglobin solutions, adjusted to pH 4.1, were dialyzed against pH 4.08 acetate buffer for 197 minutes, then against pH 8.6 veronal buffer overnight. Supernatant hemoglobin solutions from veronal dialysis were run in this experiment.

Fig. 6, phosphate buffer, pH = 10.8, $\Gamma/2 = 0.06$; run for 95 minutes at 350 volts. Hemoglobin solutions, adjusted to pH 4.6, were dialyzed against pH 4.01 acetate buffer for 88 minutes, with a final pH of 4.05 of the hemoglobin solutions. Solutions were then dialyzed against veronal buffer of pH 8.6 overnight. The precipitated hemoglobins were centrifuged, and the precipitates, dissolved in a minimum amount of 0.1 N NaOH, were run in this experiment.

differences in their charges and mobilities, for two reasons. First, and more important, the isoionic points of the three hemoglobins are different in the absence of any salt. Second, mobility differences have been observed in several different alkaline buffers, and the equal mobilities at low pH have been observed in glycine and acetate buffers. Finally, it may be mentioned that ordinary titration studies of these hemoglobins[17] have revealed that the total charge per molecule of hemoglobin a at various pH values is as follows: pH 3.0, +88; pH 4.0, +47; pH 12, −67. These figures indicate that the disappearance of differences in mobility in acid buffers is not due simply to a much greater total charge on the hemoglobin molecule in acid than is present in alkali.

Pauling and his co-workers calculated, on the basis of the difference in isoelectric points between hemoglobins a and b, that hemoglobin b possessed two to four more net positive charges per molecule than did hemoglobin a.[1] Using this figure, our experiments, therefore, provide evidence that hemoglobin b possesses two to four fewer free carboxyl groups than does hemoglobin a. Since the difference between the isoionic points of hemoglobins b and c is of the same order of magnitude as, though somewhat larger than, the difference between the isoionic points of hemoglobins a and b, it seems probable that hemoglobin c contains about five to eight fewer free carboxyl groups than hemoglobin a. In order to calculate quantitatively the differences in content of free carboxyl groups in hemoglobins a, b, and c, we have attempted to combine paper electrophoretic experiments, in which electroosmotic flow is measured, with acid-base titration experiments. These results will be reported in a subsequent paper.

The structural basis for the apparent difference in the content of free carboxyl groups of these hemoglobins remains obscure. The available analytical data[18] do not indicate a smaller content of aspartic or glutamic acid residues, or a larger number of carboxyl groups which exist as amides, in hemoglobin b than in hemoglobin a. No amino acid analyses of hemoglobin c have been published.

It has been suggested that hemoglobins a and b have identical polypeptide chains but that the folding of the chains is different in the two molecules, presumably resulting in some charged groups being unreactive.[15, 18] An actual masking of acid-binding groups, perhaps due to folding of the molecule, has recently been experimentally demonstrated in native horse hemoglobin by Steinhardt and Zaiser.[6, 7] These workers have shown that thirty-six acid-binding groups are not available for titration until the protein has been exposed briefly to a pH of about 4.0 or less. When these thirty-six groups are broken out, the hemoglobin is simultaneously denatured, as judged by spectroscopic changes and loss of solubility at the isoelectric pH. Renaturation, and presumably remasking of the thirty-six groups, is effected by returning the protein to neutrality. Steinhardt and Zaiser consider these thirty-six groups to be epsilon amino or guanidinium groups, since their pK values are above 5, presumably too high for carboxyl groups, and since all the histidine residues of horse hemoglobin are titrated in the native protein.[7] It is tempting to consider whether the excess positive charge on hemoglobins b and c could be due to an analogous masking of differing numbers of charged groups in the three hemoglobins. The fact that the variations in charge persist at pH 12.0 indicates that these differences are not due to epsilon amino groups and are not very likely to be due to guanidinium groups. There is the possibility that carboxyl groups may

also be masked in hemoglobins with more of them blocked in the abnormal hemoglobins. Other more complicated masking possibilities may be imagined to account for the differences in charge, but the evidence provided by the experiments reported above makes it very probable that the three hemoglobins differ in their content of free carboxyl groups.

The method of differential titration described in this paper should be applicable to the study of other sets of closely related proteins.

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ON THE REPLICATION OF DESOXYRIBONUCLEIC ACID (DNA)

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The discoveries of Hershey and Chase[1] concerning the role of DNA in transmitting genetic information in phage and of Watson and Crick[2] concerning the structure of DNA have brought the problem of the replication of DNA into focus. The structure proposed by Watson and Crick consists of two polynucleotide chains wound helically around a common axis, tied together by hydrogen bonds between the