ELECTROPHORETIC HETEROGENEITY OF NORMAL ADULT HUMAN HEMOGLOBIN AT LOW IONIC STRENGTHS AND HIGHER TEMPERATURES

by

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INTRODUCTION

In recent years there has been a growing interest in the electrophoretic analysis of human hemoglobin. Since PAULING *et al.*[1] found that the mobility of sickle-cell hemoglobin differs from that of normal hemoglobin, several additional abnormal hemoglobins have been found by the use of electrophoretic techniques[2].

However, these experiments were carried out under standard conditions, *i.e.*, at a temperature of about 1° C, at 0.1 ionic strength, and at pH 6.5. Under such conditions normal human hemoglobin was found to be electrophoretically homogeneous.

On the other hand, GEIGER[3] found that the oxyhemoglobin of various animals can be separated by cataphoresis into two different fractions at 25° C and at pH about 7, in solutions of 0.02 *M* sodium phosphate and in salt-free solutions. Under these conditions, he also found two oxyhemoglobins in some of the samples of human blood.

MOORE AND REINER[4] found that two fractions appear at pH 2.5 in the electrophoretic analysis of the red-cell components of normal human blood. Small fractions accompanying human hemoglobin were found at pH 8.6, ionic strength 0.1[5]. It has been reported[6] that in a 0.01 *M* sodium phosphate solution of erythrocyte extracts also two components besides oxyhemoglobin are found in electrophoretic analysis.

Recently, electrophoretic heterogeneity of human CO-hemoglobin at pH 6.5, ionic strength 0.1 and 2° C has been reported by DERRIEN AND RAYNAUD[7].

In the present work, moving-boundary electrophoresis was used in a systematic investigation of the role of ionic strength, temperature, and different ionic species on the electrophoretic behaviour of normal human CO-hemoglobin.

It was found that when the ionic strength of the solution was lowered from $\mu = 0.1$ to 0.005 and the temperature raised from about 1° C to 20° C, the CO-hemoglobin exhibits progressive electrophoretic heterogeneity.

EXPERIMENTAL

Preparation of stock CO-hemoglobin solution. Red blood cells were separated by centrifugation from oxalated normal human blood immediately after hypodermic withdrawal. The red cells were first washed three times, each with 0.9% saline solutions and then with 1.2% NaCl + 0.0025 *M* $AlCl_3$ solutions according to DRABKIN[8].

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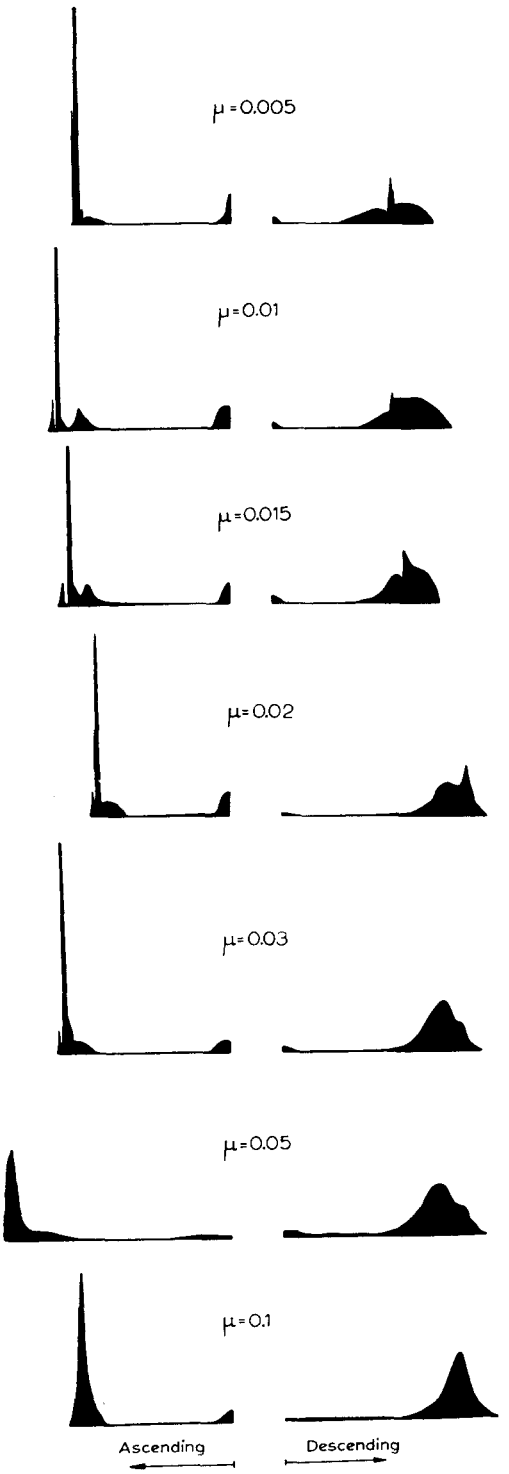

The washed, packed cells were hemolyzed by shaking with 1.4 volumes of a 1:0.4 mixture of distilled water and toluene and were left overnight at a temperature of about 4° C. The hemoglobin solution was separated by centrifugation at high speed in the cold and carbon monoxide was bubbled through it for about 30 minutes. The stock HbCO solution was stored at about −5° C and was used for electrophoresis within the following three weeks.

The concentration of the HbCO was determined by measuring the optical density at $\lambda = 5374$ Å according to SCHÖNBERGER[9].

Buffer. Cacodylate buffer solutions of pH 6.5 were prepared at the required ionic strength and then saturated with carbon monoxide.

Electrophoretic measurements. Measured volumes of the stock HbCO were dialyzed against the buffer in cellophane tubes for about two days at 2° C and were then diluted with the buffer to yield 0.5% HbCO solutions. The pH of the solutions was measured by a Model G Beckmann pH-meter.

A Klett Electrophoresis Apparatus was used, with 11 ml standard cells; the temperature was kept constant to within 0.1° C or less. Light source was a single-filament tungsten lamp, and Ilford F.P.3, fine-grain panchromatic sheet films were employed for the scanning photography. At equal intervals during each experiment, three photographs were taken of both the ascending and descending boundaries. The scanning photographs were enlarged about five-fold and the curves were traced on millimetric paper.

The areas of the different peaks were measured by a planimeter according to SVEDBERG[10], and the first-moment axis of each peak was determined with an Amsler-Integrator No. 1. The mobility of each boundary was calculated from the velocity of the first moment and from the conductivity of the CO-hemoglobin solution as measured with an Industrial Instruments conductivity bridge, Type RC-1.

RESULTS

Ionic strength series

All the experiments were carried out at 1.4° C, using Na-cacodylate-cacodylic-acid buffer of pH about 6.5, at different ionic strengths. The electrophoretic patterns obtained in the range of $\mu = 0.1$ to $\mu = 0.005$ are represented in Fig. 1. At pH 6.5, all the boundaries giving Schlieren peaks moved towards the cathode. Only a turbidity (not registered by the scanning photographs) moved towards the anode. The turbidity also made the descending leg less transparent than the ascending leg.

Fig. 1. Electrophoretic patterns of 0.5% normal adult human CO-hemoglobin at 1.4° C in Na-cacodylate-cacodylic acid solutions of pH 6.5 at various ionic strengths.

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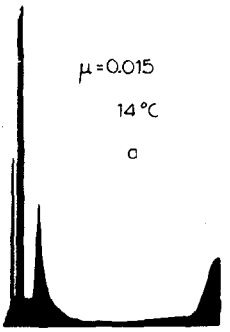
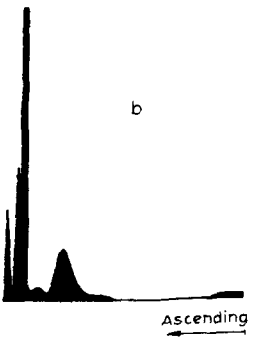
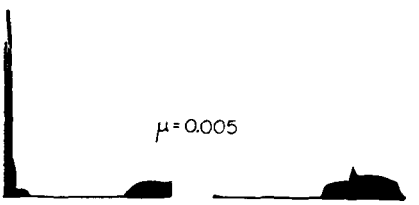
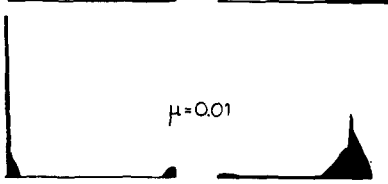
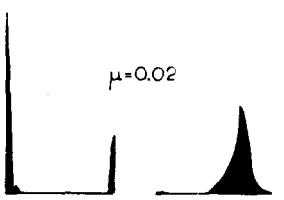


Fig. 2. CO-hemoglobin in cacodylate buffer of pH 6.5, ionic strength 0.015. (Electrophoresis at 1.4° C and 12.2 volts/cm). Scanning photographs taken: (a) after 8350 sec, and (b) after 19522 sec, using a compensator.









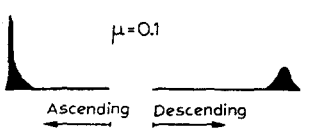
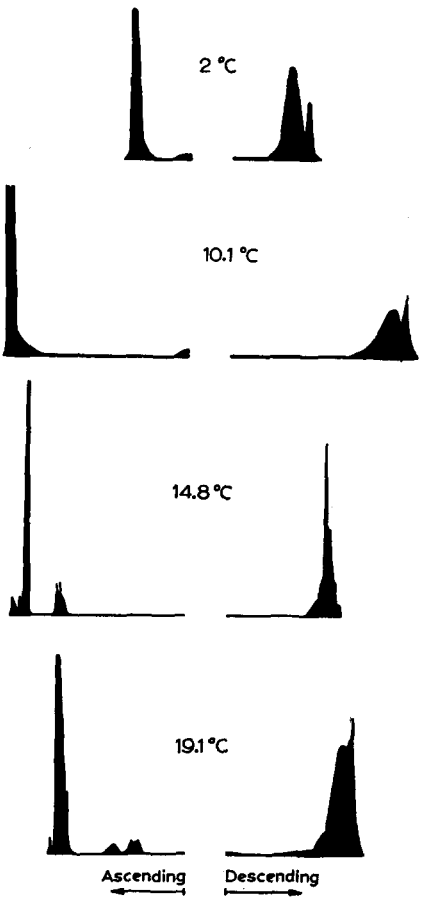


Fig. 3. Electrophoretic analyses at different temperatures of 0.5% CO-hemoglobin in cacodylate buffer of pH 6.5, ionic strength 0.1. →



At ionic strength of 0.1, one moving boundary was recorded in both the descending and ascending sides. At $\mu = 0.05$, however, the electrophoretic pattern shows heterogeneity, the ascending and descending sides each showing two peaks. At an ionic strength of 0.03 the ascending side already shows three distinct peaks, although in the descending pattern the three peaks are somewhat overlapping.

As the ionic strength is lowered to 0.02, 0.015 and 0.01, the three boundaries in each side become more distinct.

At $\mu = 0.005$, four peaks become apparent in both patterns. After prolonged electrophoresis,

Fig. 4. Electrophoresis of 0.5% CO-hemoglobin in Li-cacodylate-cacodylic acid buffer of pH 6.5, at different ionic strengths.

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the four components separated at ionic strengths of 0.015 and 0.01 also. This is illustrated in Fig. 2.

Effects of temperature

0.5% HbCO in Na-cacodylate cacodylic-acid of 0.1 ionic strength, was analyzed electrophoretically at temperatures ranging from 1.4–19.1° C.

The electrophoretic patterns become more complex as the temperature is raised. (see Fig. 3).

Replacement of Na by Li-ions

Electrophoretic experiments were carried out with Li-cacodylate, cacodylic-acid buffer at pH 6.5 and at ionic strength 0.005–0.1 (see Fig. 4). The results parallel those obtained with Na-cacodylate buffer, as shown in Fig. 1. Though here again there is an increase in the number of electrophoretic components as the ionic strength is lowered, there are significant differences:

1. the mobilities of the CO-hemoglobin components are smaller in the presence of Li-ions.

2. the progressive separation into two, three, and four peaks is achieved at somewhat lower ionic strengths than with Na.

DISCUSSION

The appearance of several peaks in the electrophoretic patterns at the lower ionic strengths of 0.03 to 0.005 and at the higher temperatures of 2° C to 19.1° C indicates that normal CO-hemoglobin is heterogeneous under these conditions.

All the experiments were carried out at currents which produced well below 0.1 watts of heat per ml in the electrophoretic cell. Thus no disturbances from thermal convection were likely to occur. Nevertheless, to check possible slight heat disturbances, several experiments were carried out at extremely low currents (giving less than 0.002 watts per ml) and these yielded patterns identical with those reported above.

Changing the field intensity several fold did not affect the number of peaks obtained, but only their sharpness. The possibility of false moving-boundaries is excluded since the system consisted of only two ionic species besides the protein.

It may be assumed that the separation of the electrophoretic components at low ionic strengths and high temperatures is due to the dissociation of the hemoglobin complex into several units.

Thus, the effect observed when temperatures were raised could result from the dissociation of certain loose bonds (*e.g.*, hydrogen bonds) which link the various components in the hemoglobin complex.

It is possible that the components are held together by different types of bonds. Thus the dissociation may be progressive, different components being freed at different temperatures. This speculation is supported by the finding that the solubility-dependence of HbCO on ionic strength differs at low and high temperatures: JOPE AND O'BRIEN[11] have reported that the variation in solubility with ionic strength for HbCO in phosphate buffer at pH 6.7 is linear (at 0° C) and is represented by Cohn's equation. This suggests that at 0° C only one component is present in HbCO from human adult blood. At 24° C, however, it has been found[12] that the solubility *vs.* ionic-strength curve shows four

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discontinuities, indicating four different hemoglobins. The present work shows that these different components can be separated in electrophoresis carried out at high temperatures.

To check if this dissociation is reversible, electrophoretic experiments were carried out at 20° C, with a hemoglobin solution which had previously been kept at about 1° C for a few days. In another experiment at 1.4° C a hemoglobin solution which had been kept for about 12 hours at 20° C was used.

The electrophoretic patterns obtained were characteristic of the temperature at which the electrophoresis was carried out, and no influence of the prior temperature-conditioning of the hemoglobin could be detected. This shows that the temperature effect is reversible and that equilibrium is achieved within a few hours or less.

The ionic-strength effect may be explained as follows. Since all the components observed electrophoretically have the same sign of charge at pH 6.5, lowering the ionic strength decreases the ionic screening of each component. Thus, the Coulomb repulsive forces between them increase and the dissociation of the hemoglobin-complex into its more elementary parts is facilitated.

This hypothesis may well explain GEIGER's finding[3] that the cataphoretic separation of oxy-hemoglobin into two fractions was increased when salt was removed by electrodialysis.

LAMM AND POLSON[13] found that dissociation of HbCO is also indicated by changes in the diffusion constant obtained by changing the salt concentration or by diluting the hemoglobin at constant ionic strength.

Electrophoretic analysis of hemoglobin at concentrations of 0.5% to 2.5%, carried out at an ionic strength of 0.01, did not show any clear cut difference. The separation of the peaks, however, is not sufficient to provide accurate analysis of small changes in the relative concentrations of the various components.

The fact that there is less separation in solutions containing Li than in those containing Na, and that the mobility of hemoglobin is less in the former, may be explained by the assumption that Li ions are less adsorbable than Na-ions. The effective charge of the hemoglobin would be smaller, its mobility lower, and the repulsive forces effective in hemoglobin dissociation would become less.

The hypothesis that hemoglobin dissociates when the ionic strength of the solution is lowered agrees with the hypothesis on the oxygen equilibrium of hemoglobin, suggested by BARCROFT and others[14].

Further work on the electrophoretic separation and isolation of the different hemoglobin components is in progress.

ACKNOWLEDGEMENT

The authors are grateful to Prof. A. KATCHALSKY for his helpful discussions and interest in this research.

SUMMARY

Normal adult human CO-hemoglobin was investigated by moving-boundary electrophoresis at pH 6.5. It was found that upon lowering of the ionic strength from 0.1 to 0.005, the electrophoretic patterns exhibit an increasing number of hemoglobin components. Raising the temperature from 1.5° C to 20° C also shows increasing heterogeneity. These observations are explained by the possible dissociation of the hemoglobin complex into several different elementary units.

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RÉSUMÉ

La CO-hémoglobine adulte humaine normale a été soumise à l'électrophorèse de frontière mobile à pH 6.5. En abaissant la force ionique de 0.1 à 0.005, les diagrammes d'électrophorèse présentent un nombre croissant de constituants de l'hémoglobine. Une élévation de la température de 1.5 à 20° C met également en évidence une hétérogénéité croissante. Ces observations s'expliquent peut-être par la dissociation d'un complexe de l'hémoglobine en plusieurs unités élémentaires différentes.

ZUSAMMENFASSUNG

Normales CO-Hemoglobin des erwachsenen Menschen wurde durch Elektrophorese mit beweglichen Grenzflächen bei einem pH-Wert von 6.5 untersucht. Es wurde festgestellt, dass die elektrophoretischen Diagramme bei Herabsetzung der Ionenstärke von 0.1 bis 0.005 eine wachsende Anzahl von Hemoglobinkomponenten offenbaren. Durch Steigerungen der Temperatur von 1.5° C bis 20° C wurde gleichfalls eine wachsende Heterogenität verursacht. Diese Beobachtungen können durch die Möglichkeit der Dissoziation des Hemoglobinkomplexes in mehrere verschiedene Elementareinheiten erklärt werden.

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Received March 21st, 1955