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Electrophoresis of Leghemoglobin

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Electrophoresis has generally been used to determine the purity and homogeneity of proteins. Up to the present time the purity of leghemoglobin has been estimated by comparing its analyses with those of blood hemoglobin. In the work to be reported we have used the electrophoretic technique for determination of the homogeneity and the isoelectric point of the purified leghemoglobin preparations.

EXPERIMENTAL

Leghemoglobin preparations

The leghemoglobin was prepared essentially in the same manner as previously described [1]. Nodules of soya bean were crushed in a mortar at 0° C and the leghemoglobin was precipitated at 58–75 per cent saturation of ammonium sulphate. The precipitate was dissolved in a small quantity of water and dialyzed against running water until free from ammonium sulphate and then diluted with distilled water to contain 0.3–0.5 % hemoglobin. The iron in this prepraation is always trivalent. Hence, all our findings concern leghemiglobin.

Other samples used in this work were prepared by extracting the uncrushed nodules with distilled water at room temperature for 48 hrs. (10 % toluene as an antiseptic.)

Apparatus

The apparatus employed by us in the mobility determinations and in the analytical work was the Tiselius electrophoresis apparatus (Perkin Elmer). The principle of this apparatus has been described by Moore and White [2].

In the preparative electrophoretic experiments a Klett electrophoresis apparatus was used. Separation was carried out in 150 ml cells.

Measurements

For the homogeneity studies 0.3 to 0.4 per cent leghemiglobin solutions were used. Before the protein solutions were placed into the electrophoretic cell they were dialyzed against 2 litres of buffer in a cellophane tube at 4° C until there was no difference in the

pH and the conductance between the dialysate and the buffer. To eliminate a possible denaturation of protein within the acid region of the pH scale we used a mechanical dialyzer according to Reiner and Fenichel [3], whereby the equilibrium between the protein solution and the buffer was established within 2—3 hrs. The outside buffer solutions were then used to fill the upper compartments of the electrophoresis apparatus.

The usual Wheatstone bridge type of circuit and a conductivity cell were employed in measuring the specific conductance of the solutions at the same temperature as the electrophoretic experiments were performed. The movement of the boundaries during electrophoresis was followed at intervals of 1 hr. The average displacement of the boundary per second represents the apparent speed of the boundary because the refractive index gradients in the cell are recorded on films at a unit magnification. The hydrogen ion concentration of the protein and the buffer solutions were determined with a glass electrode.

The spectrum of the leghemiglobin was determined with a Beckman spectrophotometer.

The buffer solutions used in all experiments were prepared by mixtures of sodium acetate-acetic acid and sodium dihydrogen phosphate-sodium monohydrogen phosphate. Sodium chloride was added to both mixtures to obtain a ionic strength of $\mu = 0.1$.

Protein-N was determined by the Kjeldahl procedure. Distillation was carried out according to Kirk [4].

Mobility calculations

The mobility is calculated by a simple equation: $u = S/F$ where S is the apparent speed of the boundary as defined above and F the potential gradient $F = i/q \cdot \varkappa$ where i is the current in ampere, q cross-section of the electrophoretic cell in sq. cm and $\varkappa$ the specific conductance of the solutions.

RESULTS

The concentration of the protein solutions used for homogeneity studies was 0.4—0.5 per cent. The studies were carried out at pH values between 4 and 7.2. At all pH values two boundaries were present (Fig. 1). Only at pH 4.0 was a single sharp ascending boundary with a very diffuse descending boundary observed. At this acidity both of the components migrated to the negative pole and obviously were denatured. By magnifying the electrophoretic patterns 5 times and measuring the areas with a planimeter we found out that the concentrations of the two components were practically equal (Table 1). These leghemiglobin solutions were then diluted to 0.2 per cent for the mobility

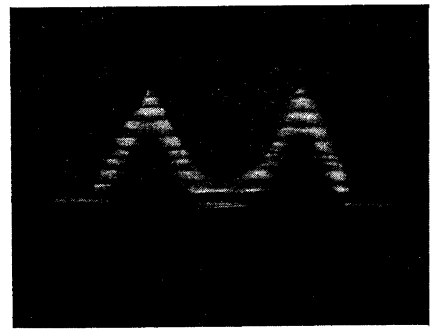

Fig. 1. Electrophoresis of leghemoglobin at pH 7.15. Ascending limb.

Table 1. Electrophoretical analyses.

No.	Faster comp.	Slower comp.
1	55.5	44.5
2	54.5	45.5
3	50.5	49.5
4	51.5	48.5
5	50.8	49.2
Average:	52.6	47.4

studies. The potential gradients were kept practically constant in all experiments (ca 8 volt/cm). The results are summarized in Fig. 2 which represents the mobilities of the sharper boundaries as a function of the pH.

The plus and minus signs refer to the charges on the protein. It will be seen from Fig. 2 that the isoelectric points of the two components are pH $\sim$ 4.4 and $\sim$ 4.7.

To test whether the leghemiglobin can be split into components by precipitation with ammonium sulphate, experiments were carried out with preparations which were obtained by extracting the uncrushed nodules with distilled water. The electrophoretic picture in this case showed the two components mentioned above, and furthermore a possible big salt component with a very small mobility.

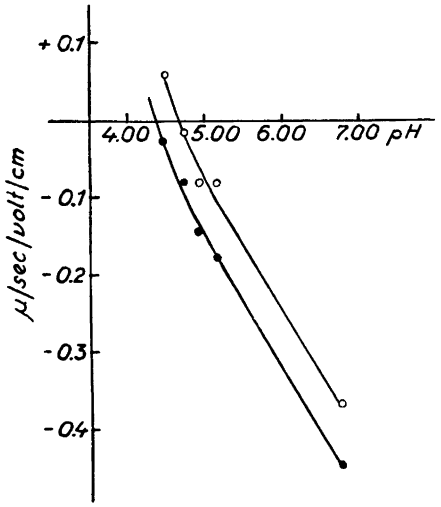

Fig. 2. Electrophoretic mobilities of the two components of leghemiglobin at 4° C in acetate buffer at varying pH and constant ionic strength (0.1).

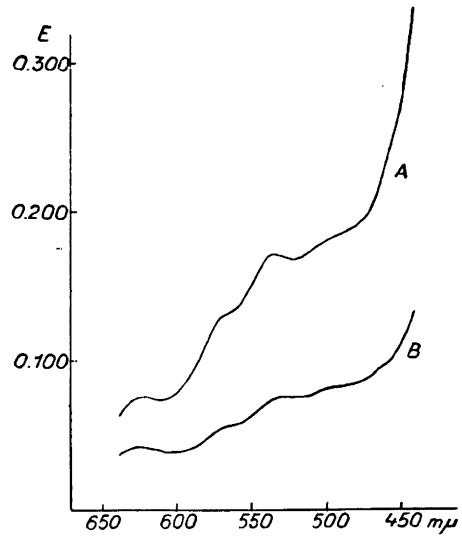

Fig. 3. The spectra of the two components of leghemiglobin calculated at the same protein concentration (~ 0.07 mg/ml) at pH 7.10.

A: spectrum of the faster component (I. P. 4.4).
B: spectrum of the slower component (I. P. 4.7).

Because the region between the two ascending boundaries will contain only the faster component and that between the two descending boundaries in the other limb only the slower component, the two components can be electrophoretically separated as pure proteins. This separation was carried out at pH 7.0 and $\mu = 0.1$ in a phosphate buffer. Both components proved to be hemin proteins.

From the spectra of these components we could also see a quantitative difference in the hemin content in respect to protein-N. The component with I. P. 4.4 has twice as high hemin content as that with I. P. 4.7 (Fig. 3).

A re-electrophoresis of the faster component at pH 3.40 and 7.25 indicated that the protein in question is electrophoretically homogeneous at these reactions (Fig. 4).

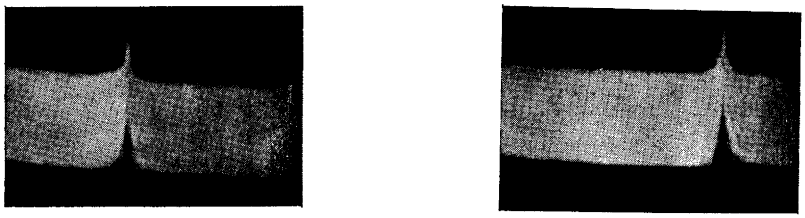

Fig. 4. Electrophoresis of the faster component.

A: ascending limb, pH 5.40, $\mu = 0.1$, $t = 210$ min.
B: ascending limb, pH 7.25, $\mu = 0.1$, $t = 180$ min.

DISCUSSION

The electrophoretic picture of the leghemiglobin indicates the existence of two components in this hemin protein. The anomality at pH 4.0 must be interpreted as a denaturation of globin. The two components of leghemiglobin are not splitting products caused by the high ammonium sulphate concentration of the preparation. The spectrum of the two components of leghemiglobin show that both of them are hemin proteins. The hemin content of the faster component seems to be about twice that of the slower one.

The leghemiglobin purified by ammonium sulphate precipitations contains usually about 0.27 % iron. In one preparation precipitated 5 times the percentage was 0.34 Fe[1], hence, its iron content was equal to that of crystalline blood hemoglobin. On the basis of this it seems that even by means of ammonium sulphate precipitations it may be possible to separate a leghemiglobin which corresponds to the faster component in the electrophoretic separation. The iron content less than 0.3 % found in most of the leghemiglobin preparations is evidently due to the fact that the preparations contained both of the electrophoretically separable components. Whether this slower fraction with a smaller hemin content represents a homogeneous substance or whether it possibly is a mixture or an adsorption compound of leghemiglobin and globin (possibly denatured) remains still to be solved. Besides, the possibility must not be overlooked that the quantitative difference in the hemin content of the two components may be due to the splitting of one hemin group from the leghemiglobin during electrophoresis. Hence, the separation method employed would cause the differences observed.

SUMMARY

Leghemiglobin isolated from the crushed nodules of soya bean and precipitated at 58—75 per cent saturation of ammonium sulphate contains two components with the isoelectric points pH 4.4 and 4.7.

Both of the components are hemin proteins.

The component with the I. P. 4.4 seems to be homogeneous between pH 5.4 and 7.20.

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