ELECTROPHORETIC MOBILITY-IONIC STRENGTH STUDIES OF PROTEINS

II. SPECIES DIFFERENTIATION OF CROSS-REACTING ALBUMINS*

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Shortly after the Tiselius (1) moving boundary method of electrophoresis became available for the study of proteins of biological importance, it was employed by Landsteiner and coworkers (2) in an attempt to differentiate the serum albumins of fowls of closely related species. These investigators were able to show some differences in the electrophoretic mobilities of these serum albumins in acetate buffer, pH 5.2, 0.02 ionic strength, but only for fowls of different orders. Sharp et al. (3) studied the electrophoretic behavior of crystallized horse serum albumin in the pH region of the isoelectric point and in buffers of 0.1 and 0.02 ionic strength. They considered the complex electrophoretic patterns in this pH range to consist of "anomalies" because of the lack of symmetry between ascending and descending boundaries. However, they did obtain similar patterns with several crystalline horse albumin fractions as well as with the albumin of whole, untreated horse serum. They concluded from this that these "anomalies" reflected the complex nature of horse serum albumin in general and were not caused by secondary changes produced during the purification process. The fact that electrophoretic patterns in the isoelectric point region reflect properties inherent in the protein (3) which are specific enough to permit species differentiation can also be seen by comparing the pattern obtained by Longsworth and Jacobsen (4) for bovine serum albumin with that obtained by Sharp et al. (3) for horse serum albumin, both being determined in acetate buffer at 0.1 ionic strength. Although both kinds of albumins show multiple ascending boundaries, as compared to spread out descending ones, the differences in the size and shape of these rising peaks are most striking. Perhaps the most clear-cut evidence of the usefulness of the electrophoretic pattern in the isoelectric region for species differentiation of physiologically related proteins is provided by the work of Alberty (5). He found that crystallized human serum albumin was resolved into two "components," whereas crystallized bovine

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albumin gave three "components" in the isoelectric point region with 0.15 N sodium chloride solutions. In a previous publication (6) a technique was described for the electrophoretic subfractionation of human serum albumin by the Tiselius moving boundary method. As is shown in Fig. 1, 0.5 to 5 per cent solutions of this "purified" protein, which give a single moving boundary in barbiturate buffer, pH 8.6, 0.1 ionic strength, yield at least four distinct ascending boundaries at pH 4.0, acetate-NaCl buffer (7), 0.05 ionic strength.

The present study deals with the application of this subfractionation procedure to the separation of various purified and crystallized animal albumins, in addition to human serum albumin, into a number of distinct electrophoretic patterns. The differences in the number and shape of separate peaks formed and the relative per cent of their areas permit the clear-cut species differentiation of the various cross-reacting animal albumins employed in these experiments (8–10).

EXPERIMENTAL

5 per cent solutions were prepared from sterile concentrated human1 (25 per cent), bovine2 (35 per cent), and porcine2 (25 per cent) serum albumins and from egg albumin2 recrystallized five times, by dilution with barbiturate buffer (pH 8.6, 0.1 ionic strength). 10 ml. portions of each solution were dialyzed mechanically (12) at 4° overnight against 400 ml. of buffer. The samples were then analyzed in the portable Aminco-Stern electrophoresis apparatus with the standard double channel Tiselius cell by the technique previously described (6, 13). Fig. 2 shows the ascending and descending electrophoretic patterns obtained for human serum albumin at pH 8.6 and is typical of the results obtained for the other species under these conditions, except for egg albumin which revealed a second trace component only after prolonged electrophoresis. 5 per cent solutions of the various animal albumins were now prepared by dilution with acetate-NaCl buffer (7) (pH 4.0, 0.04 ionic strength). These were dialyzed and analyzed electrophoretically exactly as described above. The ascending and descending patterns obtained for human serum albumin are shown in Fig. 3 and the ascending patterns for the other species in Figs. 4 to 6. The mobilities of the leading peaks were calculated for each albumin fraction in the barbiturate buffer system, and the values are given in Table I. The values for the relative area of each peak obtained in acetate-NaCl

1 Human serum albumin was obtained from the American National Red Cross through the courtesy of Dr. J. N. Ashworth. It was prepared by the ethanol-low temperature fractionation procedure of Cohn et al. (11).

2 Animal albumins were obtained from Pentex, Inc., Kankakee, Illinois. However, porcine albumin required additional electrophoretic purification in an analytical cell. Animal albumins were prepared by Cohn’s method (11).
Fig. 1. Effect of varying the concentration of serum albumin solutions on the resolution of their electrophoretic subfractions. Acetate-NaCl buffer, pH 4.0, ionic strength 0.05, at (a) 0.5 per cent, (b) 1 per cent, (c) 2 per cent, and (d) 5 per cent albumin. The vertical arrows indicate starting positions and the horizontal arrows the direction of migration of the ascending boundaries. \( E = \) volts per cm.; \( t = \) seconds; \( A = \) human serum albumin.

Fig. 2. Ascending (a) and descending (b) electrophoretic patterns of 5 per cent human serum albumin solution in barbiturate buffer, pH 8.6, 0.1 ionic strength. The ascending rear boundary (a) is the \( \delta \)-boundary.

Fig. 3. Ascending (a) and descending (b) electrophoretic patterns of 5 per cent human serum albumin solution in acetate-NaCl buffer, pH 4.0, 0.04 ionic strength. The ascending rear boundary (a) is probably not a \( \delta \)-boundary.
buffer and their respective mobilities are reported in Table II for duplicate determinations. Since the mobilities for all the samples in both buffer systems were calculated from ascending patterns by means of the conductance of the buffer after dialysis at 1°, the values represent "relative" rather than "true" mobilities.

![Fig. 4](image1.png)  ![Fig. 5](image2.png)

**Fig. 4.** Ascending electrophoretic pattern of 5 per cent bovine serum albumin solution in acetate-NaCl buffer, pH 4.0, 0.04 ionic strength.

**Fig. 5.** Ascending electrophoretic pattern of 5 per cent porcine serum albumin solution in acetate-NaCl buffer, pH 4.0, 0.04 ionic strength.

![Fig. 6](image3.png)

**Fig. 6.** Ascending electrophoretic pattern of 5 per cent egg albumin solution in acetate-NaCl buffer, pH 4.0, 0.04 ionic strength.

**Results**

As can be seen in Fig. 2 and Table I, the electrophoretic mobilities in barbiturate buffer at pH 8.6, 0.1 ionic strength, of the various serum albumins used in these experiments cannot in themselves be used for purposes of species differentiation. However, the low ionic strength subfractionation technique adds three additional experimental parameters for taxonomic identification. As seen in Fig. 3 and Table II, these are (a) the number and (b) shape of the ascending peaks formed and (c) the relative per cent areas under these boundaries. The significance of these parameters lies in the fact that definite dissimilarities are shown for the serum albumins of related species which cross-react immunochromically. There are similarities in the electrophoretic patterns obtained in acetate buffer as evidenced by the almost identical mobilities and shapes of the various
peaks formed. Species differences are indicated by the disappearance of the second fastest moving peak from some albumins as well as by changes in the relative areas under the various boundaries with similar mobilities. Until further investigation of the composition of these boundaries has been carried out, no explanation for their occurrence is being offered.

Some investigators (3) have referred to the type of ascending patterns shown here as "anomalies" because they are not enantiographic with the descending patterns (see Fig. 3). However, similarity of ascending or descending patterns is seldom achieved in practice, even under the most favorable circumstances; i.e., low protein concentration and high ionic strength buffers (3-5). The marked differences in the ascending and descending boundaries obtained under our experimental conditions must in some manner be related to the fact that the protein ions of the ascending boundary migrate into the buffer, whereas the protein ions of the descending boundary migrate into a solution containing ions of their own kind (3).

As demonstrated in Fig. 1, the type of pattern obtained is relatively independent of protein concentration over a wide range of values (0.5 to

### Table I

**"Relative" Ascending Mobilities of 5 Per Cent Solutions of Various Animal Albumins in Barbiturate Buffer, pH 8.6, 0.1 Ionic Strength**

<table>
<thead>
<tr>
<th>Species</th>
<th>Mobility $\times 10^4$ sq. cm. per volt per sec.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>$-6.3$</td>
</tr>
<tr>
<td>Bovine</td>
<td>$-6.8$</td>
</tr>
<tr>
<td>Egg</td>
<td>$-6.7$</td>
</tr>
<tr>
<td>Porcine</td>
<td>$-6.9$</td>
</tr>
</tbody>
</table>

### Table II

**"Relative" Ascending Mobilities and Areas of Boundaries of Animal Albumins in Acetate-NaCl Buffer (7), pH 4.0, 0.04 Ionic Strength**

<table>
<thead>
<tr>
<th>Species</th>
<th>Peak 1</th>
<th>Peak 2</th>
<th>Peak 3</th>
<th>Peak 4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Per cent of total area</td>
<td>Mobility $\times 10^4$</td>
<td>Per cent of total area</td>
<td>Mobility $\times 10^4$</td>
</tr>
<tr>
<td>Human</td>
<td>20.3</td>
<td>+6.3</td>
<td>3.8</td>
<td>+5.6</td>
</tr>
<tr>
<td>Bovine</td>
<td>19.9</td>
<td>22.1</td>
<td>+5.7</td>
<td>3.2</td>
</tr>
<tr>
<td>Porcine</td>
<td>19.7</td>
<td>33.4</td>
<td>+6.3</td>
<td>2.9</td>
</tr>
<tr>
<td>Egg</td>
<td>31.7</td>
<td>24.0</td>
<td>+5.7</td>
<td>36.9</td>
</tr>
<tr>
<td></td>
<td>22.4</td>
<td></td>
<td></td>
<td>41.0</td>
</tr>
</tbody>
</table>
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5.0 gm. per cent) and depends on ionic strength (6) as well as on pH and specific ion effects (4). Subsequent preliminary experiments with an analytical electrophoresis cell showed each boundary to contain considerable amounts of protein; i.e., 0.9 gm. per cent or greater. This would appear to rule out the possibility that one or more of these boundaries are of the salt concentration kind (ε-peaks), but not the contingency of protein-buffer salt boundaries, i.e. δ-peaks. This problem is now under investigation, as are measurements of pH and conductivity changes across each boundary.

As can be noted in Table II, the results obtained for the same protein sample are highly reproducible under our experimental conditions. Reproducible patterns were also obtained for different albumin preparations of the same species; e.g., egg, human, porcine, etc. These ascending boundaries, therefore, have utility in the detection of small structural differences among physiologically related proteins.

DISCUSSION

Serum albumins and γ-globulins of different mammalian species have been reported by some investigators (8-10) to cross-react between 3 and 25 per cent, depending upon the many factors which can affect an immune response (14). Such experimental results have led Maurer (10) to state “that one should be very cautious in interpreting the results of immunological cross reactions between various species.” It would, therefore, appear desirable to develop methods other than immunological ones for the determination of species differences of physiologically related proteins. Such published procedures include the crystallographic analysis of hemoglobins (15), ultracentrifugal analysis of respiratory proteins (16), electrophoretic analysis of various animal proteins (16-22), and the analysis of the amino acid composition of purified proteins (23, 24).

Immunological specificity depends on the particular arrangement of different polar groups on the surface of the protein molecule (25). Such techniques have been widely used for differentiation between corresponding proteins from related species. However, Colvin et al. (26) in their excellent review article on the microheterogeneity of proteins state “that immunochemistry, although sometimes useful, is not as powerful a tool for differentiating minor differences between related proteins [italics ours] as it is for detecting small amounts of distinct impurities. Hence, when immunological heterogeneity is found, the differences between protein molecules of the preparation are not necessarily minor.”

Colvin's review paper (26) discusses in some detail the usefulness of electrophoretic techniques, such as the reversible boundary-spreading technique of Alberty (27) and the electrophoresis-convection technique of
Cann and Kirkwood (28) for detecting very subtle differences between similar protein molecules, e.g. γ-globulins. These authors do not mention the interesting observation of Longsworth and Jacobsen (4) that the electrophoretic mobility near the isoelectric point is very sensitive to differences in the number of basic and acidic amino acids per molecule which determine protein specificity; this sensitivity increases with decreasing ionic strength (26). On the basis of the material presented in this paper, as well as of unpublished data with γ-globulin and other proteins, the authors believe this experimental approach to be a general one for determining the microheterogeneity of physiologically related (or similar) proteins, which possesses many practical advantages over other published procedures (26–28).

**SUMMARY**

A method previously published for low ionic strength subfractionation of pure human serum albumin into four distinct boundaries by free electrophoresis was applied to purified bovine, porcine, and egg albumin solutions. Although these albumins are known to cross-react immunologically and can only be distinguished by quantitative techniques, they give distinct electrophoretic patterns when analyzed in acetate buffer at pH 4.0, 0.04 ionic strength. These differences relate both to the number and shapes of boundaries formed and the area under each peak for the various species. This report furnishes experimental proof that, compared to immunological procedures, electrophoretic properties of physiologically related proteins as determined near their isoelectric point in low ionic strength buffers furnish a sensitive means of detecting subtle differences between similar protein molecules.

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**BIBLIOGRAPHY**

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