Gould, H. J., Arnstein, H. R. V., and Cox, R. A. (1966), *J. Mol. Biol.*, (in press).
Hamilton, M. J. (1964), *Proc. Am. Biophys. Soc.*, 107.
Huppert, J., and Pelmont, J. (1962), *Arch. Biochem. Biophys. 98*, 214.
Langridge, R. (1963), *Science 140*, 1000.
McPhie, P., Hounsell, J., and Gratzer, W. B. (1966), *Biochemistry 5*, 988.
Richards, E. G., Coll, J. A., and Gratzer, W. B. (1965), *Anal. Biochem. 12*, 452.
Sanger, F., Brownlee, G. G., and Barrell, B. G. (1965), *J. Mol. Biol. 13*, 373.
Spirin, A. S. (1964), Struktur und Funktion des genetischen Materials, Akademie Verlag Berlin, p 163.
Timasheff, S. N., Witz, J., and Luzzati, V. (1961), *Biophys. J. 1*, 525.
Witzel, H., and Barnard, E. A. (1962), *Biochem. Biophys. Res. Commun. 7*, 289.

Multiple Electrophoretic Zones Arising from Protein–Buffer Interaction*

John R. Cann

ABSTRACT: Moving-boundary experiments demonstrate that in the pH range 6.2–9.2, BSA (bovine serum albumin) interacts reversibly with phosphate–borate and borate buffers to give electrophoretic patterns showing two or more peaks. The two or three zones shown on cellulose acetate by BSA in these buffers arise from the same interaction and are not indicative of true heterogeneity.

In the many applications of zone electrophoresis to biological problems, it is imperative that cognizance be taken of the fact that multiple zones need not necessarily indicate heterogeneity.

Recently Cann and Goad, (1965a,b) have presented a theory of electrophoresis of reversibly interacting systems of the type

$$P + nHA \rightleftharpoons P(HA)_n \qquad (1)$$

where P represents a protein molecule or other macromolecular ion in solution and $P(HA)_n$ its complex formed by binding n moles of a small, uncharged constituent, HA, of the solvent medium, *e.g.*, undissociated buffer acid. It is assumed that P and $P(HA)_n$ possess different electrophoretic mobilities and that equilibrium is established instantaneously. One of the results of computations for zone electrophoresis (Cann and Goad, 1965b) is that under appropriate conditions a single macromolecule, interacting with an uncharged constituent of the solvent, can give two zones. Since zone electrophoresis is such a powerful method for separating macromolecules and has found extensive application to a variety of biological problems, it is important to verify the prediction experimentally. The experiments described herein were designed with this in mind and demonstrate that a protein can give multiple zones due to interaction with the buffer solvent.

Experimental Section

Moving-boundary electrophoresis was carried out in the standard 11-cc Tiselius cell with the Spinco Model H electrophoresis–diffusion instrument. Schlieren patterns were recorded photographically with the cylindrical lens system. Values of the mobilities, $cm^2\ sec^{-1}\ v^{-1} \times (10^{-5})$, and apparent mobilities are shown above or beside the corresponding peaks in the patterns presented in Figure 1.

Zone electrophoresis was carried out on 5 × 20-cm S and S cellulose acetate strips with a Colab Shandon apparatus. Between 10 and 15 μl of protein solution was applied to the strip with a Beckman sample applicator. On completion of electrophoresis the zone patterns were developed in the usual manner by staining with Ponceau S. In the case of fractionation experiments in phosphate–borate buffer, the zones on an unstained strip were visualized with ultraviolet light. After cutting out the desired zone, the rest of the strip was stained to make certain that there was no contamination with the other zone. The protein was eluted by floating the excised zone on 70 μl of buffer in a capped vial for 3–4 hr with occasional agitation. In the case of borate buffer, a narrow longitudinal piece was cut from one

* From the Department of Biophysics, Contribution No. 247, Florence R. Sabin Laboratories, University of Colorado Medical Center, Denver, Colorado. *Received October 4, 1965.* Supported in part by Research Grant AI-01482-14 from the National Institute of Allergy and Infectious Diseases of the National Institutes of Health, U. S. Public Health Service, and in part by the American Cancer Society and the Damon Runyon Memorial Fund for Cancer Research.

side of the undeveloped strip and stained. The stained piece was used as a guide in excising the slow zone. The remainder of the strip was stained. The excised strip was cut into small square pieces and packed onto the bottom of the upper section of a cylindrical centrifuge tube with a capillary attached to the bottom. The protein solution was then spun from the cellulose acetate into the capillary using a clinical centrifuge. Usually about 20 μl of solution was obtained; in the few cases in which only about 10 μl was obtained, an additional volume of fresh buffer was spun through the cellulose. The fractions were aged about 0.5 hr before electrophoretic analysis.

The bovine serum albumin (BSA)[1] used for moving-boundary electrophoresis was Armour's Lot No. W69102 except in the experiments shown in Figures 1A and B and accompanying fractionations for which Lot No. W69312 was used. Lot No. A69908 was used for zone electrophoresis. Samples of BSA dissolved in buffer were dialyzed *vs.* buffer in the cold for about 24 hr with one change of dialyzate prior to electrophoresis. The symbol Γ designates ionic strength.

Results

Moving-Boundary Experiments. A survey of the moving-boundary electrophoretic behavior of BSA in several different buffers was made in order to provide necessary background information for understanding subsequent zone electrophoresis on supporting media. The results of this survey (Figure 1) establish that BSA interacts with borate buffer. Compare, *e.g.*, the reasonably normal patterns (Figure 1A) obtained in 0.043 Γ phosphate buffer, pH 6.1, with those (Figure 1B) in a phosphate–borate buffer of about the same ionic strength and pH. The latter are typical of interacting systems, the ascending (but not descending) pattern showing a rapidly moving major peak and a considerable slower, minor one. That the two peaks actually constitute a single reaction boundary and do not indicate true heterogeneity[2] was demonstrated by fractionation experiments. The protein disappearing across the rapidly moving peak was withdrawn from the ascending limb of the electrophoresis cell, dialyzed against fresh buffer, and then analyzed electrophoretically. Like the unfractionated protein, the ascending (but not descending) patterns of the fractions showed two peaks. In fact, the patterns of the fractions were, within experimental error, indistinguishable from those of unfractionated controls.

Comparisons of electrophoretic patterns were also made under other sets of conditions; whereas 1% BSA migrates as a single, reasonably symmetrical boundary in 0.043 Γ or 0.01 Γ sodium barbital buffer at pH 8.9

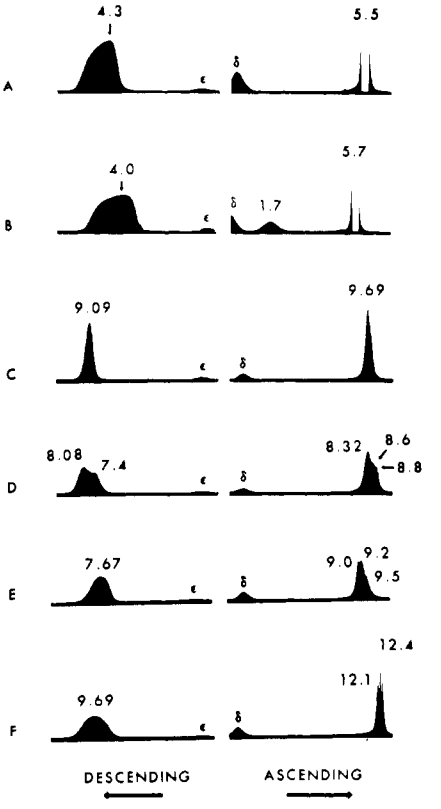

FIGURE 1: Comparison of moving-boundary electrophoretic patterns of BSA in phosphate and barbital buffers with those in phosphate–borate and borate buffers: A, 0.0043 M Na_2HPO_4–0.030 M NaH_2PO_4, pH 6.07, 3% protein; B, 0.012 M Na_2HPO_4–0.0081 M NaH_2PO_4–0.4 M H_3BO_3, pH 6.15, 3% (1% protein gave very similar patterns); C, 0.043 Γ sodium barbital, pH 8.90, 1% (essentially same patterns were obtained in 0.01 M buffer); D, 0.043 Γ sodium borate (0.043 M NaOH–0.108 M H_3BO_3, originally added to prepare buffer), pH 8.90, 1%; E, 0.011 Γ sodium borate, pH 7.57, 1%; F, 0.011 Γ sodium borate, pH 8.90, 1%.

(Figure 1C), the patterns in sodium borate buffers of the same concentration and pH show bi- or trimodal boundaries (Figures 1D and F). Two lines of experimentation indicate that the patterns in borate buffer are reaction boundaries. First, prolonged electrophoresis in the 0.043 Γ borate buffer with back compensation to increase the effective length of the electrophoresis column did not improve the resolution of the descending pattern. Although the fast-moving peak retained its sharpness, the slow one spread excessively to form a high and long plateau. The ascending protein did give two major peaks, but they were separated by a high plateau. The second piece of evidence is afforded by the effect of field strength upon the patterns; whereas resolution occurred at 6.7 v cm^{-1} (Figure 1D), quite symmetrical unimodal boundaries were obtained at 0.58 v cm^{-1}. Nor did the boundaries spread excessively at the lower field strength. The same results were ob-

[1] Abbreviations used: BSA, bovine serum albumin.

[2] The term, true heterogeneity, is used to indicate a mixture of stable, noninteracting and nontautomeric macromolecules possessing distinctly different electrophoretic mobilities.

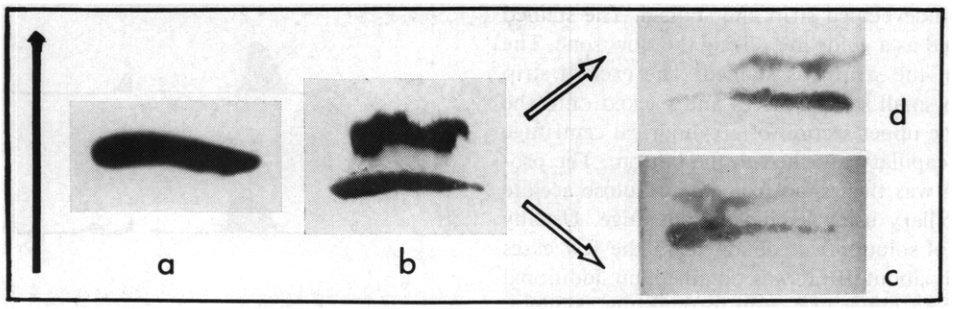

FIGURE 2: Demonstration that the two zones shown by BSA in phosphate–borate buffer on cellulose acetate arise from reversible interaction of the protein with boric acid or borate anion and are not due to true heterogeneity. (a) Zone patterns of BSA in 0.0043 M Na_2HPO_4–0.030 M NaH_2PO_4, pH 6.07, 5% protein; (b) pattern in 0.012 M Na_2HPO_4–0.0081 M $Na\ H_2PO_4$–0.4 M H_3BO_3, pH 6.15, 5% (each of the two zones obtained in the phosphate–borate buffer was cut from unstained strips and the protein eluted with buffer. The resulting fractions were then analyzed in the same phosphate–borate buffer); (c) zone pattern of material eluted from slower-migrating zone; (d) pattern of material from faster zone. Solid vertical arrow indicates direction of migration.

tained with 2% protein, which is particularly interesting since the ascending pattern at this concentration was very well resolved into two peaks at the higher field. Yet the unimodal boundary obtained at low field had no discernible shoulders and showed a small knob at its apex, an optical phenomenon attesting to the symmetry of the boundary. Such an effect of field strength is characteristic of at least three different types of interaction: (a) reversible isomerization reactions with a half-time of the order of the time of electrophoresis at the higher field (Cann and Bailey, 1961; Scholten, 1961); (b) proteins undergoing reaction 1 with instantaneous establishment of equilibrium; and (c) dimerization reactions of the type $2P + nHA \rightleftarrows P_2(HA)_n$ with instantaneous establishment of equilibrium. For rapidly equilibrating macromolecular systems of the type $nP \rightleftarrows P_n$, where $n > 2$, and $A + B \rightleftarrows C$, resolution is theoretically independent of field strength (Gilbert, 1955, 1959; Gilbert and Jenkins, 1960). In the case of (b) and (c), resolution at fields of the order of 10 v cm^{-1} has been shown theoretically to result from the production of gradients of HA in the electrophoresis column (Cann and Goad, 1965a; Cann and Goad, unpublished). At sufficiently lower fields and correspondingly longer times of electrophoresis, strong gradients of the small molecule cannot be maintained against diffusion so that resolution cannot occur (Cann and Goad, unpublished). Of the three possible types of interaction cited above, dimerization or higher order aggregation reactions have been eliminated by velocity–sedimentation analyses. Experiments were made at BSA concentrations of about 1 and 5.7%. Within experimental error, the sedimentation behavior in 0.011 Γ sodium borate buffer, pH 7.1, was the same as in 0.011 M (0.024 Γ) phosphate buffer of the same pH. The patterns in either buffer showed only a single boundary, which was somewhat asymmetric at the higher protein concentration; $s_{20,w}$ = 3.6–3.8 at 1% and 2.2 at 5.7%.

The results of other moving-boundary electrophoresis experiments can be summarized as follows. Interaction with borate buffer decreases somewhat the average mobility of BSA. The nature of the patterns depends in a reversible fashion upon buffer concentration; increasing the concentration at constant ionic strength and pH causes an increase in the proportion of slower moving peaks. Increasing the ionic strength by addition of NaCl also increases the proportion of slow peaks. Finally, the nature of the patterns is strongly dependent upon pH (compare Figures 1E and F) in the range studied, pH 7.6–9.2, resolution into peaks virtually disappearing at the higher pH values.

Zone Electrophoresis. Zone electrophoresis of BSA on cellulose acetate was carried out under two different sets of conditions: PHOSPHATE–BORATE BUFFER, pH 6.2. In these experiments electrophoresis was performed in the same phosphate or phosphate–borate buffer used for the moving-boundary experiments of Figures 1A and B, respectively. The zone patterns are presented in Figures 2a and b; whereas the protein gave a single zone in phosphate buffer, two zones were obtained in phosphate–borate, the faster moving zone being the major one. In order to determine whether the two zones reflect interaction or heterogeneity, the protein was eluted from each of the unstained zones with phosphate–borate buffer and the resulting fractions were analyzed by zone electrophoresis in the same buffer. Like unfractionated BSA, the pattern of each fraction showed two zones (Figures 2c and d) which demonstrates that the duplicity of zones arises from reversible interaction of BSA with boric acid or borate anion and is not due to true heterogeneity.

In the case of the fractions, which were analyzed at rather low protein concentration, the slower of the two zones was the major one. It is particularly interesting that such a large proportion of the fast fraction migrated in the slower zone when reanalyzed (Figure 2d). This result underscores the conclusion that the two zones arise from interaction.

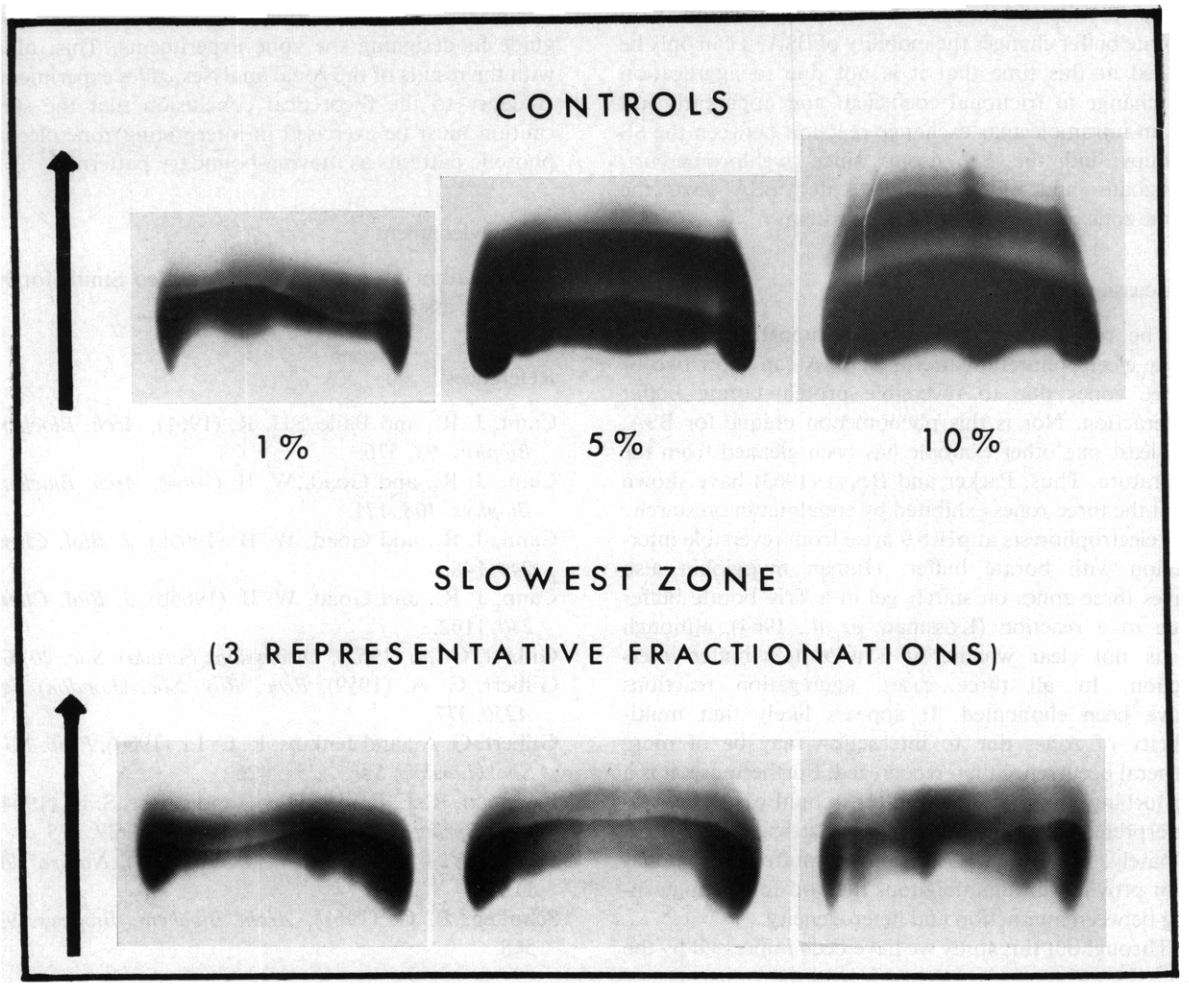

FIGURE 3: Demonstration that the two or three zones shown by BSA in borate buffer (0.0108 M NaOH–0.260 M H_3BO_3 originally added to prepare buffer, pH 7.15) on cellulose acetate arise from interaction of the protein with the buffer and are not due to true heterogeneity. Zone patterns of unfractionated controls at three different protein concentrations (0.5% protein showed only a single zone; the fastest zone shown by 10% protein shaded off in the direction of migration, but the shaded portion could not be captured photographically), and of three representative fractions obtained from 10% protein by cutting the slowest zone from unstained strips and removing the protein as described in the text. Vertical arrow indicates direction of migration.

BORATE BUFFER, pH 7.2. The zone patterns of 5% BSA in 0.011 I' sodium borate buffers showed multiple zones over the pH range 7–8.9, but the zones were most distinct below about pH 8. Patterns obtained at pH 7.2 are presented in Figure 3 (5% BSA in 0.011 M phosphate buffer, pH 7.1, gave a single zone with only a suggestion of a slower moving one). The borate system exhibited the interesting property that, although resolution into two or three zones was excellent at protein concentrations in the range 1–10%, patterns obtained at lower concentrations showed only a single zone. Accordingly, fractionation experiments to decide between interaction and heterogeneity were carried out at 10% protein. This was done in order to obtain fractions of sufficiently high concentration to ensure against possible misinterpretation in the event that electrophoresis of the fractions should give a single zone. (This illustrates one of the precautions pointed out by Cann and Goad (1964) for the validity of the fractionation test. That of dialyzing the fraction prior to analysis, while an absolute requirement for the moving-boundary method, evidently was not critical for the zone experiments described herein.) The electrophoretic patterns of fractions obtained by eluting the protein from the slowest moving (major) zone consistently showed not one but two or three zones (Figure 3). It is apparent, therefore, that the multiple zones arise from interaction of BSA with borate buffer[3] and are not due to true heterogeneity.

[3] We have carried out starch-gel electrophoresis on BSA in sodium borate buffers, pH 8.95. A whole spectrum of zone patterns (narrow zone; bearded zone; broad, diffuse zone; comet; and two zones) was obtained simply by systematic variation of buffer and protein concentration. These results might also be interpreted in terms of protein–buffer interaction, but fractionation experiments were not done.

As to the mechanism whereby interaction with borate buffer changes the mobility of BSA, it can only be stated at this time that it is not due to aggregation or change in frictional coefficient and apparently not to an intramolecular exchange reaction between the SS groups and the SH group since *p*-chloromercuribenzoate- and iodoacetamide-treated BSA gave the same zone patterns as unreacted protein.

Discussion

The results described above demonstrate that the zone electrophoretic patterns of BSA can show two or three zones due to reversible protein–borate buffer interaction. Nor is this phenomenon unique for BSA. At least one other example has been gleaned from the literature. Thus, Parker and Bearn (1963) have shown that the three zones exhibited by conalbumin on starch-gel electrophoresis at pH 8.9 arise from reversible interaction with borate buffer. Human myoglobin also gives three zones on starch-gel in a Tris–borate buffer due to a reaction (Kossman, *et al.*, 1964), although it is not clear whether it is a protein–buffer interaction. In all three cases, aggregation reactions have been eliminated. It appears likely that multiplicity of zones due to interaction may be of more general occurrence than recognized. Furthermore, it is a disturbing fact that such patterns could easily be misinterpreted as indicating true heterogeneity. Fortunately, however, with certain precautions, fractionation provides an unambiguous method for distinguishing between interaction and heterogeneity.

Throughout this study we have been impressed by the reliability of moving-boundary electrophoresis as a guide in designing the zone experiments. This, along with the results of the zonal analyses, gives experimental credence to the theoretical conclusion that the same caution must be exercised in interpreting zone electrophoretic patterns as moving-boundary patterns.

Acknowledgment

The author wishes to thank Mr. Leo Smith for his technical assistance.

References

Cann, J. R., and Bailey, H. R. (1961), *Arch. Biochem. Biophys. 93*, 576.

Cann, J. R., and Goad, W. B. (1964), *Arch. Biochem. Biophys. 108*, 171.

Cann, J. R., and Goad, W. B. (1965a), *J. Biol. Chem. 240*, 148.

Cann, J. R., and Goad, W. B. (1965b), *J. Biol. Chem. 240*, 1162.

Gilbert, G. A. (1955), *Discussions Faraday Soc. 20*, 68.

Gilbert, G. A. (1959), *Proc. Roy. Soc. (London) Ser. A250*, 377.

Gilbert, G. A., and Jenkins, R. C. Ll. (1960), *Proc. Roy. Soc. (London) Ser. A253*, 426.

Kossman, R. J., Fainer, D. C., and Boyer, S. H. (1964), *Cold Spring Harbor Symp. Quant. Biol. 29*, 375.

Parker, W. C., and Bearn, A. G. (1963), *Nature 199*, 1184.

Scholten, P. C. (1961), *Arch. Biochem. Biophys. 93*, 568.

CORRECTION

In the paper entitled Biosynthesis of Uridine Diphosphate D-Xylose. II. Uridine Diphosphate D-Glucuronate Carboxy-lyase of *Cryptococcus laurentii*, by Helmut Ankel and David Sidney Feingold, in Volume 5, No. 1, p 182, January 1966, the following correction should be made: p 184, left column, line 10 should read "efficiently stirred and 0.9 ml of 0.5 M $MnCl_2$ was added."