[CONTRIBUTION NO. 1109 FROM THE STERLING CHEMISTRY LABORATORY, YALE UNIVERSITY]

Electrophoresis-convection Applied to Substances Forming Soluble Complexes[1]

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The application of electrophoresis-convection to systems of high molecular weight substances in equilibrium is studied by means of experiments with a model system of bovine serum albumin (BSA) and fish sperm desoxypentosenucleic acid (DNA). It is found that under conditions in which the two components form complexes with one another, the DNA greatly enhances the transport of BSA. The utility of such complex-formation in electrophoresis-convection fractionations is indicated.

Electrophoresis-convection has been successfully applied to the fractionation of a number of systems of proteins. The technique and apparatus have been previously described.[2] The cell in which the fractionation occurs consists of two reservoirs connected by a narrow, vertical, semi-permeable channel formed between two sheets of sausage casing. The cell is filled with the protein solution to be fractionated and immersed in the appropriate buffer between two flat platinum electrodes which, when current is passed between them, supply a homogeneous electric field across the channel. The horizontal migration of the protein components in the electric field creates horizontal density gradients in the channel, and under the action of gravity, there results a transport of the proteins into the lower reservoir. In a system of non-interacting proteins with different electrophoretic mobilities in the particular buffer, the components are differentially transported into the bottom reservoir, and fractionation may be achieved. In particular, if one component in the mixture is at its isoelectric point, while the others are not, this component may ideally be obtained in pure condition in the top reservoir after the mobile components have been transported to the bottom reservoir. The most efficient fractionation obtains under such circumstances.

This method has achieved, among other separations, a refined fractionation of the γ-globulin fraction of animal sera into a series of fractions of different average isoelectric points[3,4] and this has permitted studies to be made of the distribution of antibodies within antisera.[5,6] In the application of the method to other systems, however, in certain cases fractionation may be made difficult by either of two factors: (a) the components of interest may have too closely similar isoelectric points; or (b) they may be similar electrophoretically and insoluble in solutions near their isoelectric *p*H's. Under these conditions, the isoelectric procedure mentioned above may prove to be inadequate or impossible. If such components, however, were differentially capable of forming soluble complexes with some suitable complexing agent, their fractionation might be facilitated. For if the complexing agent had electrophoretic properties sufficiently different from the original components, electrophoretic resolution of the components, in their complexes, would be enhanced and electrophoresis-convection separation made feasible.

Before attempting fractionations by this method, it was decided first to determine whether the transport of a typical protein could be sufficiently altered by complex-formation to warrant interest in the method. For this purpose a model system was investigated consisting of bovine serum albumin (BSA) and, as the complexing agent, fish sperm desoxypentosenucleic acid (DNA). These substances were chosen for their ready availability and because electrophoretic studies with similar systems have already been made.[7,8] The results of electrophoresis-convection transport experiments with this system are the subject of this paper.

Materials and Methods.—Bovine serum albumin was the crystalline product obtained from Armour. The fish sperm desoxypentosenucleic acid was obtained from Schwarz Laboratories. It contained a small amount of material which gave a Folin test, and which partially dialyzed out of the channel during an electrophoresis-convection experiment. Since the amount of this material was quantitatively negligible for our purposes, the DNA was not further purified.

Tiselius electrophoretic analyses were performed at 2° in a Klett–Tiselius apparatus. The electrophoresis-convection experiments were carried out in a cold room at 4–5°. One hundred-thirty ml. of solution was placed in the electrophoresis-convection cell at the start of each run, of which 50 ml. was in the top reservoir. During the run, about six 1-ml. samples were withdrawn from the top reservoir at known times and analyzed for BSA and DNA. Parallel runs were treated as similarly as possible. Where two experiments in different buffers were to be compared, the currents employed were calculated, knowing the buffer conductivities, to give the same electric field strength in both runs.

In mixtures of the two components, the BSA concentration was determined by the Folin reaction employing Cu^{++}, and the DNA by the Dische cysteine–HCl method.[9] A Fisher Electrophotometer was used for the colorimetric assays.

Tiselius Electrophoretic Analysis of the BSA–DNA System.—Preliminary experiments with mixtures of BSA and DNA showed that precipitation occurs in the system at *p*H 5.0 and below. In acetate buffer at *p*H 5.20 and ionic strength, μ 0.02, the Tiselius electrophoresis patterns indicate that interaction exists between the components, while at *p*H 5.50 and μ 0.20, the two components do not interact and migrate independently of one another. The electrophoresis diagrams of representative experiments are reproduced in Fig. 1. At *p*H 5.20 and μ 0.02, interaction is dem-

(1) Presented at the Meeting of the American Chemical Society, Buffalo, New York, March, 1952.

(2) J. R. Cann, J. G. Kirkwood, R. A. Brown and O. J. Plescia, THIS JOURNAL, **71**, 1603 (1949).

(3) J. R. Cann, R. A. Brown and J. G. Kirkwood, *ibid.*, **71**, 2687 (1949).

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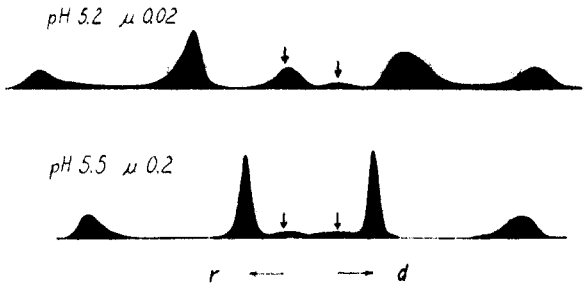

Fig. 1.—Tiselius electrophoresis patterns for a 1:1 BSA–DNA mixture, under interacting conditions (top) and non-interacting conditions (bottom). The vertical arrows indicate the starting positions.

onstrated by the fact that the slower of the two peaks in both rising and descending limbs is moving much more rapidly and is much more asymmetric than is characteristic of a free BSA peak. The apparent mobilities of the peaks in a number of experiments under a variety of conditions is given in Table I.

TABLE I

TISELIUS ELECTROPHORESIS DATA FOR BSA–DNA MIXTURES

Buffer	Mixture, g. BSA/g. DNA	Boundary	Mobility cm./sec./volt/cm. × 10^5 Ascending	Descending
Acetate,	Pure BSA	..	− 1.23	− 1.30
*p*H 5.2	Pure DNA	..	−18.2	−12.0
μ 0.02	½	Slow	− 8.4	− 5.3
		Fast	−18.3	−13.5
	1	Slow	− 8.0	− 5.0
		Fast	−18.6	−13.6
	2	Slow	− 5.9	− 4.3
		Fast	−18.1	−13.8
	5	Slow	− 5.7	− 3.7
		Fast	No peak	−12.3
Acetate,	Pure BSA	..	− 2.38	− 2.38
*p*H 5.5	Pure DNA	..	−12.3	−11.3
μ 0.2	1	Slow	− 2.39	− 2.19
		Fast	−11.8	−11.5

If the complexes formed between BSA and DNA at *p*H 5.2 were in rapidly adjusted equilibrium, then the slow peak in the descending limb would have the mobility of free BSA.[7] Furthermore, the region between the slow and fast peak in this limb would be essentially free of DNA. Neither of these criteria are met with in these experiments. The mobility of the slow peak is much larger than that of BSA under the same conditions, and direct sampling of the contents of the region between the slow and fast peaks in the descending limb demonstrated that the DNA concentration there is about 20% of the DNA concentration of the original solution. On the other hand, if BSA and DNA formed a complex which was in very slowly adjusted equilibrium with other components of the solutions, the slow peak in both limbs would have a characteristic mobility independent of the ratio of total BSA and DNA in the solution. The results in Table I demonstrate, on the contrary, that the mobility of the slow peak does depend on the over-all BSA–DNA ratio of the solution, increasing with increasing relative DNA content. We conclude that the rate of equilibration of the complexes in this system is neither very rapid nor very slow, but is intermediate between these two extremes.

Qualitatively, the electrophoretic behavior of this system is similar to that found with analogous systems,[7,8] for which it has been suggested that ionic interactions are responsible for the formation of complexes between the components.

Electrophoresis-convection of the BSA–DNA System.—As a control measure, it was decided to compare the transport of the two components in a mixture under given conditions, with the transport of each of the components in the absence of the other. Three individual cells, in which runs 1, 2 and 3 were performed, were filled with a BSA–DNA mixture, BSA alone, and DNA alone, respectively, all in acetate buffer, *p*H 5.2, μ 0.02. The cells were then connected in series, and the circulating buffer was cascaded through them. More detailed data for these experiments are given in Table II, and the results are presented in Fig. 2.

TABLE II

TRANSPORT DATA FOR BSA–DNA SYSTEM IN ELECTROPHORESIS-CONVECTION

Run	Acetate buffer *p*H	Acetate buffer μ	Initial concn., g./l. BSA	Initial concn., g./l. DNA	*E*, v./cm.	Composition of top after 26 hr. g./l. BSA	Composition of top after 26 hr. g./l. DNA
1	5.2	0.02	7.6	6.0	0.5	1.3	0.33
2	5.2	.02	6.4	0.0	.5	4.6	..
3	5.2	.02	0.0	5.7	.5	..	.11
4	5.5	.20	7.3	5.0	.4	4.4	.24
5	5.5	.20	7.3	0.0	.4	4.5	..
6	5.5	.20	0.0	4.6	.4	...	.50
7	5.2	.02	9.1	3.4	.5	2.1	.32
8	5.2	.02	4.3	6.8	.5	0.4	.31

Under these interacting conditions, after 26 hours about 85% of the BSA in the BSA–DNA mixture had been transported out of the top reservoir, whereas only 25% of the BSA by itself was transported under the same conditions.

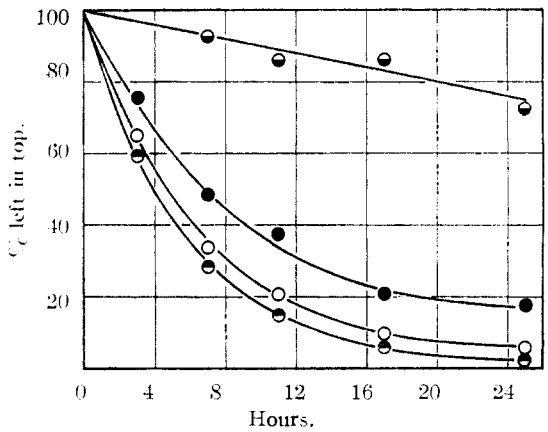

Fig. 2.—Electrophoresis-convection experiments 1, 2 and 3, under interacting conditions: ◒, BSA alone; ●, BSA in mixture; ○, DNA in mixture; ◒, DNA alone.

Another set of experiments under non-interacting conditions was carried out. Runs 4, 5 and 6 were performed in series upon a BSA–DNA mixture, BSA alone, and DNA alone, respectively, all in acetate buffer, *p*H 5.5, μ 0.2. The results, given in Fig. 3, indicate that under these non-inter-

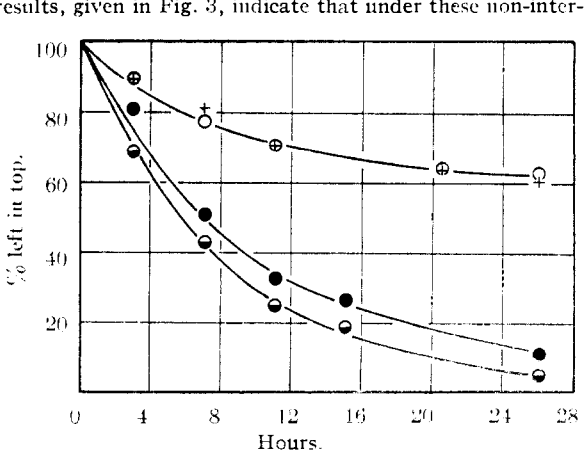

Fig. 3.—Electrophoresis-convection experiments 4, 5 and 6, under non-interacting conditions: ○, BSA alone; +, BSA in mixture; ●, DNA alone; ◒, DNA in mixture.

acting conditions, the rate of transport of BSA is not appreciably affected by the presence of DNA.

Further experiments were performed, under interacting conditions, upon BSA–DNA mixtures containing various proportions of the two components. The results, shown in Fig. 4, suggest that an increase in the proportion of the faster moving component (DNA) accelerates the transport of both components.

Discussion

It is evident that there is a very marked increase in the rate of transport of BSA in electrophoresis-convection due to the formation of complexes of BSA with DNA.[10] These results suggest, therefore, that under appropriate conditions, complex formation may indeed facilitate electrophoresis-convection fractionations.

Despite the fact that, at *p*H 5.2, μ 0.02, BSA and DNA form an interacting system, it appears possible to treat the transport phenomena in this system quantitatively, utilizing the theory of electrophoresis-convection developed for non-interacting components.[11] This is achieved as follows: wherever, in the theory for non-interacting systems, the mobilities of the individual components are called for, in applying the theory to the interacting system the constituent mobilities of the components,[12] as determined from the Tiselius patterns, are employed.

According to the theory, for a two component system the separation factor, f_2, in the top reservoir is defined by

$$f_2 = (c_2/c_2^0)/(c_1/c_1^0) \qquad (1)$$

where c_1 and c_2 are the concentrations of the two components in the top reservoir at some time during the run, and c_1^0 and c_2^0 are the initial concentrations. The separation factor can be shown to satisfy

$$[1 + X_2^0(f_2 - 1)]^\beta/f_2 = \gamma^\beta \qquad (2)$$

where $\gamma = (c_1 + c_2)/(c_1^0 + c_2^0)$, $X_2^0 = c_2^0/(c_1^0 + c_2^0)$, and $\beta = 1 - \mu_2/\mu_1$, where μ_1 and μ_2 are the electrophoretic mobilities of the two components. f_2 and γ can be determined experimentally at different times during a run, from the data presented in this paper. The observed values of f_2 can then be compared with the theoretical values calculated from equation (2), from known values of X_2^0, γ and β.

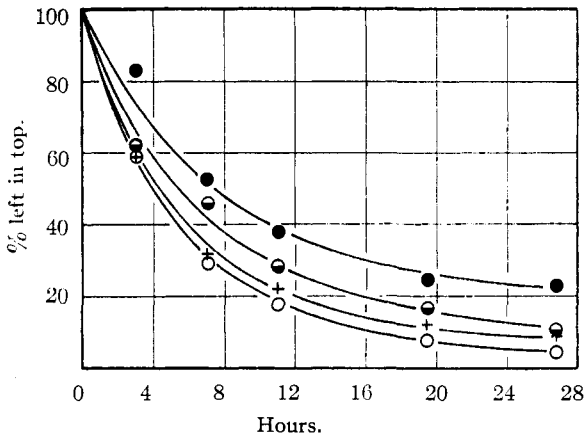

Fig. 4.—Electrophoresis-convection experiments 7 and 8, under interacting conditions: ●, BSA in 2BSA:1DNA mixture; ◒, BSA in 1BSA:2DNA mixture; +, DNA in 2BSA:1DNA mixture; O, DNA in 1BSA:2DNA mixture.

This theory has been verified by this procedure for model two-component non-interacting systems.[13] For the BSA–DNA system under non-interacting conditions (run 4), the results presented in Table III show that throughout the run there is good agreement between calculated and experimental values of f_2.

TABLE III

TRANSPORT OF BSA AND DNA IN NON-INTERACTING MIXTURE

Run 4, β = 0.81

Time, min.	γ	f_2, obsd.	f_2, calcd.
190	0.80	1.3	1.5
405	.67	1.9	2.1
650	.52	2.9	3.8
910	.49	3.8	4.2
1230	.40	8.9	7.9
1560	.38	12.4	10.8

For the BSA–DNA mixture under interacting conditions (run 1), two sets of theoretical f_2 values were calculated, those in column 4 of Table IV employing the constituent mobilities of the two components, while those in column 5 using the mobilities of each of the two components in the absence of the other. The latter values, therefore, ignore the effects of the interaction of the BSA and DNA. It is evident that good agreement is obtained between observed and calculated values of

TABLE IV

TRANSPORT OF BSA AND DNA IN INTERACTING MIXTURE

Run 1, β = 0.405 (interacting), β = 0.892 (non-interacting)

Time, min.	γ	f_2, obsd.	f_2, calcd. Inter.	f_2, calcd. Non-inter.
185	0.71	1.17	1.20	2.07
430	.42	1.44	1.60	17
670	.30	1.78	1.93	170
1025	.16	2.17	2.72	2.5×10^4
1500	.12	3.11	3.28	1.5×10^5

(10) It may be noted from Figs. 2 and 3 that under interacting conditions, DNA in the BSA–DNA mixture is transported somewhat less rapidly than in the absence of BSA, whereas under non-interacting conditions, DNA in the mixture is transported somewhat more rapidly than it is without BSA. These facts may be understood qualitatively, as follows. In the former case, the constituent mobility of DNA is made somewhat smaller than the mobility of free DNA due to complex formation with BSA. This results in a decreased rate of transport of DNA. In the latter case, the concurrent transport of BSA along with the DNA in the mixture enhances the density gradients in the channel, and hence increases the rate of DNA transport compared to that in the absence of BSA. On the other hand, since the DNA is largely removed early in the experiment, this effect is not reciprocal, and the BSA transport is not noticeably increased by the DNA.

(11) J. G. Kirkwood, J. R. Cann and R. A. Brown, *Biochim. et Biophys. Acta*, **5**, 301 (1950); **6**, 606 (1951).

(12) The mobilities of the slow peak in the ascending limb and of the fast peak in the descending limb are taken as the constituent mobilities of BSA and DNA, respectively. Actually, the latter is not the true constituent mobility of DNA, since, as mentioned above, the concentration of DNA behind this boundary is about 20% of the initial DNA concentration. However, for this particular mixture, in which a considerable amount of DNA remains unbound at equilibrium, the mobility of this boundary, which is very similar to that of free DNA, may be used in these calculations as a good first approximation to the true DNA constituent mobility.

(13) R. A. Brown, J. B. Shumaker, S. N. Timasheff and J. G. Kirkwood, THIS JOURNAL, **74**, 460 (1952).

f_2 utilizing the constituent mobilities of the components, but wide discrepancies exist when the effects of interaction are not considered.

It seems possible, therefore, to predict rates of transport in systems of interacting components from the theory developed for non-interacting components.

Possible applications of this technique for the fractionation of certain systems of proteins are under investigation. One system to which it might be applied is a mixture of complete and incomplete antibodies, such as appears to be present in rabbit antisera to *p*-azophenylarsonic acid-conjugated proteins.[6] A suitable conjugated protein antigen could be used as the complexing agent for this system. In another direction, Morawetz and Hughes[14] have recently investigated the interaction of proteins with certain synthetic polyelectrolytes. These polyelectrolytes might find application as complexing agents in electrophoresis-convection fractionations.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY OF COLUMBIA UNIVERSITY]

The Kinetics of the Formation of the Carbinol of Crystal Violet[1]

BY J. C. TURGEON[2] AND V. K. LAMER

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The fading of crystal violet in the presence of hydroxyl ion follows quantitatively the Brønsted–Debye law for primary kinetic salt effects. Crystal violet obeys Beer's law at the wave length of maximum absorption (590 mμ) in concentrations at and below 5×10^{-6} *M*. The activation energy for the reaction in water at 25° is 15.1 kcal/mole; the entropy of activation under these conditions is −12.3 cal./deg. mole. There is a strong specific solvent effect: the addition of acetone or dioxane to an aqueous solution of the dye produces a shift in the absorption peak of the crystal violet ion toward longer wave lengths and, at low concentrations of organic solvents, produces an effect on the specific reaction rate which is opposite to that expected on the basis of the decrease in dielectric constant. The use of normalized rate constants does not improve the agreement with simple electrostatic theory. The energy of activation in 40% acetone–water mixtures ($D = 59$) at $\mu = 0.005$ is 0.9 kcal./mole greater than in the case of water alone, at this ionic strength, contrary to the expected decrease due to the lowering of the coulombic activation energy in this reaction between oppositely charged ions produced by lowering the dielectric constant. The activation energy for the carbinol formation of malachite green is 0.7 kcal./mole less than that for crystal violet. The higher rate constant of the former (2.29 as against 0.28 liter/mole sec.) is due to reduction in activation energy, also to a less negative entropy of activation. The gas kinetic collision theory agrees with the observed rate for a probability factor $P = 0.098$ for $(r_a + r_b) = a_i = 2.49$ Å. *P* for the Debye theory (frequency of encounter, based on diffusion theory) is 0.84 for the reaction in water and 3.0 for the reaction in 40% acetone–water. An interaction between the crystal violet ion and the carbinol occurs which leads to spurious rate constants if the carbinol formation is slow ($t_{1/2} > 1$ hr.). Sufficient alkali was employed in these experiments to give reactions faster than this.

Introduction

Both rate and equilibrium studes[3-10] have been reported for the carbinol formation of the triphenylmethane dyes, as, for example, crystal violet (C.V., hexamethylpararosaniline), malachite green (M.G., 4,4′-bisdimethylaminotriphenylmethyl chloride), and brilliant green (B.G., the diethyl analog of malachite green). The absorption spectrum of the C.V. ion has been interpreted with regard to its structure by Lewis.[11] Figures 1 and 2 show the absorption spectrum of C.V. in water and various solvents. Biddle and Porter[7] worked with a solution of C.V. in a 6% alcohol–water mixture; they obtained a negative kinetic salt effect which they could not, of course, in 1915, interpret on the basis of modern electrolyte theory. Hochberg and LaMer,[3] using a conductimetric method, reported second-order constants (which were 25% higher than those of Biddle and Porter) for what they believed to be the homogeneous reaction

$$(\text{C.V.})^+ + {}^-\text{OH} \longrightarrow \text{carbinol}$$

Their data, however, indicate that half the initial amount of dye (5×10^{-3} mole/l.) had reacted with the NaOH before they obtained quantitative conformity with a second order kinetic law.

Shedlovsky, in a private communication to this Laboratory, reported[9] that he had been unable to obtain second-order constants for the fading reaction in water, using a spectrophotometer. He attributed his difficulty to the low solubility of the carbinol. Preliminary studies in the present investigation on C.V. showed that this dye indeed gave perceptible carbinol precipitation when the original dye concentration was of the order of 5×10^{-6} mole/l. in aqueous media.

Very recently, Laangvad[12] studied the alkaline fading of C.V.; his study of salt effects was over a limited range (< 0.02 *M*) and the precision of his measurements did not enable him to test unambiguously the limiting forms of the Brønsted–Debye equations for the velocity constant in the limit of zero ionic strength in terms of the energy and entropy of activation[13] given by Amis and La-

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