

1952

# Interaction between proteins and surface active ions

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INTERACTION BETWEEN PROTEINS  
AND SURFACE ACTIVE IONS

by

Jen-Tsi Yang

A Dissertation Submitted to the  
Graduate Faculty in Partial Fulfillment of  
The Requirements for the Degree of  
DOCTOR OF PHILOSOPHY

Major Subject: Biophysical Chemistry

Approved:

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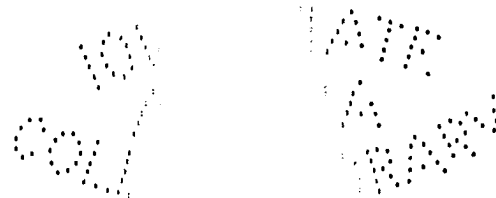
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## I. INTRODUCTION

In the study of protein structure in aqueous solution difficulties have been encountered due partly to the complexity of the structure and partly to the absence of any direct experimental method. In recent years, however, one experimental approach is the study of complex formation of proteins with ions, which offers promise of yielding information on protein configuration. Of particular interest are the surface active ions, which have exhibited markedly strong affinity for proteins as compared with other organic ions. Rarely does any simple ion produce so many diverse effects on proteins and biological systems. These include protein denaturation, dispersion and precipitation, complex formation, inhibition of activation, bactericidal and bacteriostatic action, hemolysis and splitting of natural complexes and conjugated proteins. The commercial availability of the surface active ions at the present time has further stimulated investigation in this field of chemistry. Most of the studies, however, have been qualitative in nature, chiefly because of the lack of readily available, purified surface active compounds for research, which, again is a reflection of the emphasis on their industrial application.

It is not within the scope of this dissertation to attempt an investigation of the potent biological properties of surface active ions. Rather, the main interest will be limited to the

study of binding affinity between proteins and surface active ions. Briefly, the purpose of this research was fourfold:

(1) A competitive study between proteins and surface active ions was attempted. Previous studies in the literature have indicated that the binding affinity of such ions is far superior to that of the simple organic ions. It is not yet known whether different proteins will react differently toward the same surface active ion. In this laboratory the aqueous solution of sodium dodecylbenzenesulfonate (SDBS) has been used as a dispersing agent for certain water-insoluble proteins such as zein in corn meal (28). As a first thought water-soluble proteins such as serum albumin and ovalbumin may compete with zein-SDBS complex. The study of such competitive interaction was therefore carried out. As work progressed, it was further extended to a comparable study of albumin-SDBS complexes.

(2) In the literature, adequate data are lacking for the surface active cations. It was therefore desirable to study the interaction between proteins and such cations, the results of which were expected to further confirm the nature of complex formation.

(3) During the progress of research, there appeared an entirely conflicting opinion about the nature of the complex formation between protein and surface active ions. Thus the

third objective of this dissertation was to conduct a quantitative study of the binding process and aim at clarifying such confusion.

(4) Due to the limit of scope, another important field of interest was only briefly studied, that is, denaturation of protein with surface active ions. Further study along this line will certainly shed more light on the nature of protein-ion interaction and consequently the protein structure and configuration.

## II. REVIEW OF LITERATURE

Probably the oldest surface active agent is soap; and, in recent years, the best known are the so-called detergents. It is the ionic types of the latter which will form the main subject matter of this review, due to the fact that all non-ionic compounds so far investigated lack any affinity toward proteins, a finding perhaps indicative of the nature of the interaction. The term detergent is discarded here, and the compounds are called surface active ions instead, in distinction to the ill-defined terminology currently used in the technical field.

### A. Chemistry of Surface Active Ions

Most surface active ions are characterized by a molecular structure which is essentially linear, i.e. considerably longer than it is wide. Usually the elongated nonpolar portion, "tail", of the molecule comprises a hydrocarbon radical of lyophobic nature, whereas the small polar group, "head", is of a lyophilic nature. Typical anionic types are the salts of sulfuric, sulfonic, phosphonium and arsonium derivatives of alkyl or aromatic residues. The soaps, by contrast, are the salts only of the higher fatty acids. Cationic types are chiefly the higher alkyl quaternary ammonium salts, including the alkylpyridinium halides. Of lesser importance are the ampholytic types. A typical example is cetylamino-

acetic acid.

1. Micelle formation

Physicochemical studies of solutions of surface active ions, such as colligative properties, equivalent conductivity and transference numbers, diffusion, viscosity, light scattering, and X-ray diffraction, have indicated that the ions exist both as simple ions and as colloidal aggregates known as micelles (66). It is also well recognized that the micelles are dissociable electrolytes which are in equilibrium with simple ions. Shift of the equilibrium depends on the critical micelle concentration which is characteristic of the length and nature of the hydrocarbon chain and is affected by changes in temperature, or the addition of salts.

The fact that surface active ions in aqueous solution can aggregate to form micelles may be explained in terms of the forces involved (17, 18). Work has to be done in order to bring the lyophilic "heads" together because of the Coulomb repulsion. On the other hand the lyophobic "tails" tend to segregate themselves from the water molecules and lose energy when they reach the hydrocarbon surroundings. Thus micelle structure represents an equilibrium between the repulsive long-range Coulomb forces among the "heads" and the short-range attractive van der Waals forces among the "tails". Addition of salts to the solution tends to diminish



the electrical work, because of the shielding effect of the ionic atmosphere. Thereby it will lower the critical micelle concentration. Likewise, this same effect may explain the cause of the increase in size of the micelles as was observed by Debye (17, 18, 19). It may also account for the dependence of the critical micelle concentration on the kind or valency of added gegenions, instead of ionic strength of the salts added (18). According to Debye, a calculation of the potential at the micelle indicates that the concentration of ions of equal sign around the micelle will at least be  $3 \times 10^{-4}$  times smaller than in the bulk of the solution. Thereby, it has little effect on the micelle formation.

## 2. Models of micelles

The structure of the micelles has been a subject of study for many years. Different views are held by different workers on the size and shape of the micelles. McBain (67, 68, 69) has proposed two models: The small, highly charged spherical micelles of varying sizes and the large weakly ionized lamellar micelles. Harkins (32) has supported a cylindrical model on the basis of X-ray diffraction. Hartley (53, 54), however, believes that only the spherical model of uniform size is tenable and thinks the anomalous properties arise from a change in the degree of ionization of the gegenions. Recently Corrin (16) and Brady (9) have questioned

the validity of the assumption of a quasi-crystalline arrangement of a micelle, and have concluded that the spherical model is compatible with the X-ray evidence. Nevertheless, the anomalous viscosity, flow birefringence and light scattering results do indicate the presence of elongated particles. Debye and Anacker (19) observed that the micelles formed from alkyl trimethyl ammonium bromide are elongated, rod like structures whose length increases with salt concentration. Debye and Anacker (19) has further postulated that the hydrocarbon tails project radially from the axis of the double-layer cylindrical micelle ending in the polar heads which make up the surface of the particle. The effect of salt on the size and stability of the micelle has been estimated by Hobbs (36) in the case of dilute salt solutions. More recently, Scheraga and Backus (6, 94) have supported Debye's model on the basis of flow birefringence studies.

Confusing as different views may be, all the evidence now available indicates that the surface active ions may aggregate or pack together in many different forms relating to the steric configuration of the ions themselves and their environment. Earlier in an objective review, Halston (85) stated that no single theory suffices. Likewise, Winsor (118) has attempted to interpret the X-ray measurements on the basis of an intermicellar equilibrium. From thermodynamic consideration (98, 99), the change in free energy ( $\Delta F$ ) between

spherical and rod-like or lamellar structures is comparatively small. Energy factors ( $\Delta H$ ) favor the lamellar model rather than rods or spheres. On the other hand, entropy factors ( $\Delta S$ ) tend to oppose the more ordered lamellar arrangement. Consequently the actual configuration taken up is the resulting compromise. In dilute aqueous solution, the spherical micelles would be predominant. With increasing concentration or on addition of salts, the overall effect of increase in  $\Delta H$  and decrease in  $\Delta S$  will favor the formation of rod-like or plate-like aggregates. Finally plates, a more ordered structure than rods, may be formed; that is, salting-out occurs.

### 3. Size of micelles

Since the micelles are in equilibrium with single ions, it seems rational to assume that there exists a distribution of varying sizes which, in turn, depends upon the nature and concentration of the ions, the concentration of added salts, temperature, and other factors. Indeed, Vold (114) has applied successfully the law of mass action to the aggregation of such colloidal electrolytes. Usually, an optimum size of a micelle is considered to have 50 to 100 simple ions. From molecular kinetic studies of sodium dodecyl sulphate Hakala (31) obtained a molecular weight of about 25,000 and Neurath and Putnam (73) estimated a symmetrical aggregate of

some 75 anions. Lundgren and O'Connell (63) reported a molecular weight of approximately 15,000 for sodium alkylbenzene sulfonate. Recently, however, Debye and Anacker (19) observed, by means of light-scattering measurements, the molecular weight of a cationic detergent to be of the order of a million. This value seems surprisingly large. It is possible that partial crystallization might have occurred in this case.

It should be emphasized here that the state of micelles relating to their forms and sizes need not govern their interaction in biological systems, as will be further discussed latter. The concentration of surface active ions may affect its rate of reaction, but not the final results. It is conceivable that dissociation of the micelles may occur if the single ions have greater affinity for some substrate than for each other.

#### B. Evidence of Protein-ion Interaction

In recent years, many investigations on the interactions of proteins and ions have been published. It is well recognized that many ions are bound by proteins with varying degree of affinity. Some of the evidence comes from the measurements of osmotic pressure (90), membrane equilibrium (92,93) electromotive force (92,93), pH displacement (2, 57, 91), polarography (108, 109), ultrafiltration (7), partition analysis (39), polarization of fluorescence (51, 116, 117), absorption

spectra (42, 44, 45, 48, 75), equilibrium-dialysis (40, 49, 92, 93, 111, 112) and electrophoresis (3, 4, 57, 58, 62, 84, 96). Of the methods mentioned, the last two procedures are among the most satisfactory.

### 1. Electrophoresis

Electrophoretic analysis has a great advantage over the other methods in its visual identification. Evidence for complex formation between proteins and ions consists in an increase in the mobility together with the difference in the patterns obtained from that of the native protein. The patterns also reveal the nature of the interaction. A statistical combination will result in a single spreading boundary, an "all-or-none" reaction in two well-defined boundaries (62). Furthermore, dissociation, if any, of the complex in the electric field will be detected by the dissymmetry of the patterns. Electrophoretic analysis has also been shown to be applicable to the quantitative evaluation of protein-ion interaction (57, 58, 62, 84).

Interpretation of the results, however, is frequently complicated by the poor resolution of the individual components and by the false boundary anomalies and, in some cases, artifacts. They may be minimized or even suppressed by choosing as supernatant a suitable buffer, by increasing the ionic strength of the medium or by decreasing the protein

concentration (113). Completely normal boundary behavior represents an ideal that is never realized in practice. The moving boundary method, free from all anomalies, is a contradiction in itself (104). For a proper understanding of the fundamental principles involved as well as for a critical evaluation of the results obtained, references are made to the papers by Longworth (54), Svensson (105, 106), Dole (20), Moore (70) and the monograph by Abramson and co-authors (1).

As a rule the ascending boundaries exhibit a higher mobility and a smaller area than the corresponding boundaries in the descending side. No general agreement has as yet been reached as to which of these patterns should be used for the computation of the experimental results. The somewhat better resolution of the individual components and, in some cases, the absence of false boundary anomalies on the ascending side render it easier to evaluate. For valid theoretical reasons Longworth (54) prefers to compute data from the descending pattern. Frequently the values reported for the mobility and percentage composition of the components represent the mean average derived from both sides.

## 2. Equilibrium-dialysis

Quantitative results can be conveniently obtained by the equilibrium-dialysis method. It is particularly valuable in the case where very low concentrations of protein and ions

may be employed, provided that a sufficiently sensitive analytical method can be developed. This method is, however, limited only to the ions which are dialyzable through the cellophane membrane. Thus, it is not applicable to the study of interactions of proteins with other proteins, polysaccharides, nucleic acids or other large ions. In the case of surface active ions, the single ions can easily pass through the membrane, but not the micelles due to their bulky sizes. However, the equilibrium will be shifted in favor of the single ions when some of them are removed on dialysis. The rate of diffusion is thereby somewhat slower than that for the other simple ions. Appropriate buffer concentration should be used so as to be sufficient enough to suppress Donnan correction within the range of protein concentration studies. Caution has to be taken in many cases where the blank error from the dialysis bags and the adsorption of ions on the bags may produce erroneous results.

Combination of the two methods mentioned seems most desirable for the study of protein-ion interaction. A typical example is the binding of bovine serum albumin with methyl orange. Smith and Briggs (96) have shown that the same quantitative information concerning the interaction may be obtained by the moving boundary method as by the dialysis method of Klotz and coworkers (49). It is of interest to note that boundary spreading was observed in this case, indicating the statistical character of the interaction. Dissymmetry of the patterns on both sides was accounted for by the dissociation of albumin-

methyl orange complex under the influence of the electrical field, a finding indicative of weak binding affinity.

### C. Controversy on Complex Formation

All investigators agree that long-chain alkylsulphates and alkyl aryl sulfonates are strongly bound to proteins in a manner of complex formation. Disagreement, however, arises as to the number of surface active ions bound and also the nature of the binding process. Until recently, such complex formation has been considered to be of "all-or-none" character. The other view proposed instead that a "statistical" reaction can be as well applied to surface active ions as to simple ions.

#### 1. "All-or-none" reaction

In an extensive study Lundgren, Elam and O'Connell (62) first established the formation of complexes between egg albumin and sodium dodecylbenzenesulfonate (SDBS). Electrophoretic patterns revealed that in the region of protein excess, a complex formed that possessed a constant ratio of protein to SDBS (three to one by weight) and the free protein formed a separate boundary. The well-defined complex boundary migrated with a mobility intermediate between that characteristic of the native protein and of the SDBS. The amount of uncombined albumin diminished with increase in anion-protein mixing ration (I/P). Mixtures of albumin and SDBS in about

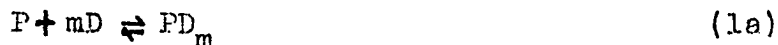


equal proportions gave a single boundary. In the region of SDBS excess, two boundaries again appeared, the one with varying mobility representing a complex whose composition depended upon the mixing ratio and the other the free SDBS. The constant composition of the complex with minimum binding ratio was also confirmed by nitrogen analysis of the salted out complex. On the other hand, heat-denatured albumin no longer possessed the "all-or-none" character and combined with SDBS in all proportions, a finding perhaps indicative of the mechanism of interaction.

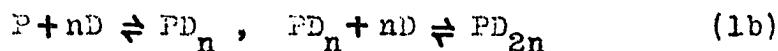
Comparable conclusions were reached by Putnam and Neurath in their study of mixtures of horse serum albumin and sodium dodecylsulphate (SDS), which has been reviewed in detail by Putnam (82). From the electrophoretic analysis, three components could be identified: (1) free albumin; (2) a complex having 0.22 gram of SDS per gram of albumin; and (3) another complex having 0.42-0.45 grams of SDS per gram of albumin. The composition of the latter approximately corresponded to the total acid binding capacity of the protein. This was also confirmed from the study of precipitation of the complex in the acid medium (see, Section D).

In the foregoing studies, it was assumed that all the surface active ions were bound to the proteins. This might be erroneous, if the complex formation was considered to be reversible in itself. Indeed, partial removal of the bound ions can be achieved by prolonged dialysis. Complete regener-

ation of the proteins can be accomplished by extraction of the bound ions with 60% acetone in the presence of electrolytes (60, 63) or by precipitation of the bound ions as the barium salt (83). Thus, the binding process may be represented by the equilibria below:



in the case of alkylbenzene sulfonate, or



in the case of alkyl sulphate, where  $m$  or  $2n$  represents the number of basic groups on the proteins. On the other hand, the disruption of the protein structure may not be fully reversible (83), as evidenced from the detectable differences between the regenerated and native proteins. Perhaps equation (1) actually involves two steps: first an irreversible complex formation together with denaturation and then, second, a reversible equilibrium between the complex and the regenerated protein.

In the range of combination studied by Lundgren and by Putnam, it is beyond any doubt that an "all-or-none" phenomenon has taken place. This, however, does not necessarily exclude the domination of statistical factors in the binding of the first few anions. Probably the bound ions are distributed randomly among the protein molecules and are also in equilibrium with the free ions. After this stage, free ions will penetrate the intact protein molecules and force the polypeptide chains to unfold. Thus, it brings about denatur-

ation, as evidenced by molecular kinetic studies. Indeed, Duggan and Luck (21) have found that about eight molecules of SDS combine with one molecule of bovine albumin to form a stable complex which prevents the normal viscosity rise of albumin in 6 M urea, but additional SDS will cause rapid increase in viscosity. Once, a few structural bonds are broken, the potential barrier is lowered and additional anions will be anchored only on these favored molecules. The resultant is then an "all-or-none" reaction. It is not necessary to postulate that statistically bound ions are denuded in this stage, for a slight increase in the mobility of the so-called uncombined protein is actually observed. In the final stage, the protein-ion complex behaves like denatured protein and again combines statistically with extra ions up to the maximum binding capacity, as has been confirmed from the electrophoretic analysis. Such is proposed hypothesis that will be put to a test in this dissertation.

## 2. Statistical reaction

Recently, Karush and Sonenberg (40) questioned the general validity of the "all-or-none" reaction. Instead, they postulated a statistical binding process for protein and alkyl sulphates, similar to that for simple ions.

a. Simple theory. The law of mass action has been applied first by von Muralto (71) and simplified by Klotz and

coworkers (41, 49) to the case of multiple combination between proteins (P) and ions (I). The successive protein-ion equilibria may be represented by a series of equations shown below:



Mathematical analysis indicates that the average number of bound ions per mole of total protein,  $r$ , can then be expressed by the equation:

$$r = \frac{\sum_{i=1}^n i \left( \prod_{j=1}^i K_j \right) (I)^i}{1 + \sum_{i=1}^n \left( \prod_{j=1}^i K_j \right) (I)^i} \qquad (3)$$

If all the binding sites on the protein have the same intrinsic association constant,  $K$ , and, aside from the statistical factor, the free energy of binding to any site is independent of binding to other sites, Equation (3) may then be reduced to a linear form.

$$r = \frac{K n (I)}{1 + K(I)} \quad \text{or} \quad \frac{1}{r} = \frac{1}{Kn} \cdot \frac{1}{(I)} + \frac{1}{n} \quad (4)$$

where  $n$  is the average maximum number of sites per protein molecule. Accordingly, a plot of  $1/r$  against  $1/(I)$  will give a straight line. From the slope of this line and its intercept with the ordinate, the values of  $n$  and  $K$  can be obtained.

A similar conclusion has been reached by Scatchard (89), and expressed in the form.

$$\frac{r}{(I)} = Kn - Kr \quad (5)$$

which is fundamentally equivalent to equation (4), but has less uncertainty in the intercept from which  $n$  is calculated, thus eliminating the disadvantage of concealing deviations from the ideal laws. Furthermore, if the experimental points do not fall on a straight line, it may be inferred that the intrinsic association constants are not equal and/or there is interaction among the bound ions. The intercept on the  $r$  axis will still give the value of  $n$ .

In some cases, the simple theory fits quite well with the experimental results (43, 110, 111, 112). This, however, is not true in many other cases, where electrostatic interaction and other factors can no longer be neglected.

It should also be pointed out that the value of  $n$  is dependent on the structure of the ion. For example the maximum number of sites available on serum albumin varied from 6 up to 40 (43). Doubt has been raised on the reliability of the methods of extrapolation (40, 89). Indeed it is still an open question to be clarified.

b. Electrostatic interaction. Since the binding of ions may cause a net change in the average charge of the protein, it is necessary to introduce an electrostatic correction, analogous to the acid-base titration curves of soluble proteins and the effect of ionic strength on these curves (11, 14, p. 444). A modified form of the simple theory has been developed by Scatchard (89).

$$\frac{r}{(I)} e^{2w'r} = nK - rK, n > 4 \quad (6)$$

Here  $w' = (1 + 1/n)w$  and  $w$  is calculated from the Debye-Huckel theory, i.e.

$$w = \frac{\epsilon^2 z^2}{2DkT} \left( \frac{1}{b} - \frac{\kappa}{1 + \kappa a} \right)$$

in which  $\epsilon$  is the electronic charge,  $z$  the valence of the ion,  $D$  the dielectric constant of the medium,  $K$  Boltamann's constant,  $T$  the absolute temperature,  $b$  the radius of the protein molecule,  $a$  the distance of closest approach, and  $\kappa$

has its usual significance in the Debye theory. It has been reported that such correction worked quite satisfactorily in some cases (89).

c. Heterogeneity theory. One of the simplest heterogeneity theories assumes that the binding can be described by only two different intrinsic association constants (92, 93). In this case, equation (4) is modified into

$$\frac{r}{(I)} = \frac{n_1 K_1}{1 + K_1(I)} + \frac{n_2 K_2}{1 + K_2(I)} \quad (7)$$

where  $n = n_1 + n_2$

This is adequately represented by the combination of human serum albumin with chloride ion and with thiocyanate ion (92, 93) and also the binding of an azo-dye with bovine serum albumin (37).

d. Gaussian distribution theory. Although the binding of simple ions with proteins is statistical in nature, the reaction between proteins and higher homologues of surface active ions has, until recently, been considered "all-or-none". Karush and Sonenberg, however, though otherwise and attempted to prove the latter as a statistical process (40). Failure to apply the previous theories in the studies of bovine albumin and alkyl sulfates led them to introduce another heterogeneity theory. It was assumed that the

number and distribution of the intrinsic binding constants are such as to be adequately described by a Gauss error function. This formulation is similar to that used by Pauling, Pressman and Grossberg to describe the heterogeneous binding of hapten by antibody in competition with antigen (81). The derivations finally led to equation (8) below.

$$\frac{n}{r} = \frac{1}{1 - f [ (I) ]} \quad (8)$$

where

$$f [ (I) ] = \frac{1}{\sqrt{\pi}} \int_{-\infty}^{\infty} \frac{e^{-\alpha^2}}{1 + K_0(I) e^{\alpha\sigma}} d\alpha$$

in which  $K_0$  is an average binding constant,  $\sigma$  measures the range of values of  $K$ , and

$$\alpha = \ln (K/K_0) / \sigma$$

The integral can be evaluated numerically for the computation of  $f(I)$  by trying various values of  $\sigma$  and using Weddle's rule (64).

By this method, it has been shown that the theoretical curves fit rather satisfactorily with the experimental data of the binding of serum albumin and alkyl sulphates. Such results are quite contrary to the earlier "all-or-none" con-



cept. It was argued that in earlier studies much higher concentrations of alkyl sulphates as micelles were employed. Thus, the interpretation of the data was complicated by micelle formation and, in many cases, denaturation. Such explanation however, seems not too convincing. Since the micelles are in equilibrium with single ions, the protein may take up the ions singly, if it has greater affinity for the single ions than the ions for themselves. To be sure, higher concentration of the ions will accelerate the rate of reaction, but not necessarily change the course of reactions. Karush and Sonenberg have also pointed out that their data covered only a rather narrow range of  $1/r$  so that the suitability of the distribution function is not put to a severe test. Especially it is very questionable that in their plot of  $1/r$  vs  $1/I$ , extrapolation to the higher concentration (i.e.  $1/I \rightarrow 0$ ) did not involve too much risk. Furthermore, their calculated thermodynamic data seems rather confusing. Recently, Goddard and Pethica (30) found it difficult to reconcile such calculations with their direct calorimetric measurements.

In the foregoing discussion, one important thing has thus far been neglected, that is, the adaptability of protein toward ions. It seems conceivable that interaction between protein and surface active ions may not be limited to one type of binding process. Statistical reaction will probably

not be ruled out at very low or very high concentrations of ions. On the other hand, within certain range of protein-surface active ion ratio, "all-or-none" reaction may predominate. In this dissertation, it is attempted to clarify part of the confusion as it exists.

#### D. Diverse Effects of Surface Active Ions on Proteins

In addition to complex formation, the action of surface active ions on proteins may result in denaturation, precipitation and dispersion. These phenomena, in general, depend on the conditions of study and perhaps follow the same mechanism.

##### 1. Denaturation

An excellent review on protein denaturation has been published by Neurath and coworkers (72). Anson (5) in 1939 first discovered that surface active ions denature proteins such as hemoglobin and egg albumin, at their isoelectric point and keep the denatured protein in solution. Significantly, these ions have much higher protein-denaturing potency than other chemical reagents. Even at very low concentrations, they have the same effects as produced by very high concentrations of such other denaturants as urea and guanidine hydrochloride (about 0.008 M versus about 8M). However, the amount of ions required is proportional to the amount of protein present, a fact indicative of complex formation.

Many investigations indicate that surface active ions produce a profound change in molecular structure of protein. Quantitative evidence of these changes comes from the measurement of the sulfhydryl groups liberated, viscosity measurements, X-ray diffraction studies and many others. More recently, the study of flow birefringence looks very promising. Such technique has already been applied to heat and urea denaturation by Foster and coworkers (25, 26, 27, 88). The interpretation of their results, however, was complicated by the formation of aggregates. Thus, confirmation of these studies by other methods seems desirable.

## 2. Precipitation

Earlier study by Matsumura (65) indicated that horse serum was precipitated with 0.01M. sodium oleate and was completely redissolved with 0.025 M. sodium oleate. The interpretation of this phenomenon was however obscured by the complication of hydrolysis of the soap. Bull and Neurath (10) were the first to report the precipitation of egg albumin by sodium dodecylsulphate (SDS) and the re-dispersion by excess SDS. Quantitative studies of surface active cations by Schmidt (95) and anions by Putnam and Neurath (82, 83) have shown that precipitation was influenced by the protein-ion weight concentration ratio, pH, temperature and ionic strength, the first two factors being most decisive.

Kuhn and Bielig (50) found that proteins were precipitated

by surface active cations only if the former were present as anions. Putnam and Neurath extended the conclusion that the surface active ions will precipitate the proteins only when the two species bear an opposite sign of charge. This, however, is only true in the absence of salt of high concentrations. The pH range of precipitation can be considerably shifted by addition of a neutral salt, whereas the protein and the ion may even possess the same sign of charge.

In the optimum pH range, three regions of interaction between serum albumin and SDS were observed (83): (a) That of protein-excess where the protein was incompletely precipitated, (b) the equivalence zone where complete precipitation occurred, and (c) that of surface active ion excess where partial or complete re-dispersion occurred. Significantly, the minimum and maximum mixing ratio requisite for complete precipitation corresponded respectively to one-half and to the total number of cationic groups in the protein, as has been confirmed from the electrophoretic analysis. More recently, comparable results were also obtained for the precipitation of lysozyme by both surface active anions and cations (29). In this research, advantage has been taken of this phenomenon for the preparation of protein-surface active anion complexes.

### 3. Dispersion

Surface active ions can be advantageously employed as dispersing solvents for many proteins which are extremely

insoluble in neutral solvents. Lundgren and coworkers (115) found that chicken feather keratin forms in the presence of a reducing agent a soluble complex in neutral aqueous sodium alkylbenzenesulfonate. An interesting application of surface active ions in the manufacture of protein fibers has been fully reviewed by Lundgren (61)

Alcohol-soluble zein can also be dispersed in aqueous solution of sodium dodecylbenzenesulfonate (SDBS) and the physical chemistry of such solutions has recently been studied by Foster (24). In this laboratory substantially quantitative extraction of the proteins of corn was attained using dilute aqueous SDBS solution buffered at pH 10 containing in addition a small amount of reducing agent such as sodium bisulphite (28). All these observations again demonstrate the extraordinarily strong affinity of surface active ions for the proteins.

#### E. Competitive Interaction Between Proteins and Ions

Ions of different affinities for the proteins may compete with each other for interaction with the same proteins. Klotz (42) found that addition of any carboxylate or sulfonate ions to a solution of protein-dye complex reversed the effect of the protein on the spectrum of the dye, due to the fact that the bound dyes were displaced from the complex by the anions of higher binding capacity. This reversing ability decreased

with increase in pH, owing to increasing electrostatic repulsion of the anions at higher pH. The importance of this repulsion in determining the relative attraction of two organic anions to a protein will depend on van der Waals forces as well as electrostatic forces. The former would contribute more to the stability of the protein-dye complex than that of the protein-acid complex. Thus, the competitive ability of the acids was lessened at high pH.

Competitive interaction between fatty acids and serum albumin methyl orange complex has been investigated by Cugin and Davis (13). In the region of very small molar ratios of added fatty acids to albumin there is almost no competition. This has been explained in terms of the existence of more than one set of binding sites. As more fatty acid was added to the solution of the complex, the displacing effect increased until one fatty acid molecule displaced more than one dye ion. It has been suggested that the binding of a long-chain alkyl anion at one site caused steric hindrance at other sites. As still more fatty acid was added, a saturation limit was reached beyond which there was no further increase in competition. It was thought that the solubility of the acid determined this limit. Thus, it can be seen that no single hypothesis is sufficient to explain the entire competition curve.

As can be expected, competitive action between surface active ions and other ions should be much in favor of the

former. This is true in the case of sodium dodecylsulphate (SDS). Klotz and coworkers (46) found that at concentrations of less than 0.001 M. SDS was capable of displacing methyl orange almost completely from its albumin complex, whereas other ions such as salicylate required more than ten times that concentration to do likewise. Comparable results were obtained by Karush (38) for the interaction between SDS and an azo dye with bovine albumin. Hypotheses have been postulated in terms of homogeneous sites by Klotz (46) and heterogeneous sites by Karush (38). A clear understanding seems to await further investigation.

As two ions will compete with each other for the same protein, so it seems logical that one protein may displace another protein from the protein-ion complex. No work, however, has been reported on this subject.

## F. Mechanism of Interaction

### 1. Complex formation

a. Electrostatic forces. The unusual affinity of proteins for ions has been the subject of much recent speculation. Abundant evidence now available supports the view that electrostatic forces play an essential role in the mechanism of binding. Indirect evidence comes from comparison of binding capacity of compounds of practically identical structure, one being charge and the other uncharged. It has been reported.

that certain amides and esters have much less effect on the proteins than the corresponding anions (8, 42). Likewise, all nonionic surface active compounds so far investigated lack the capacity for interaction with proteins, indicating the requisite of charged groups on the ions (60, 86). Another fact is that rise in pH beyond a certain limit usually deprives the protein almost completely of the bound ions, due to neutralization of the charged loci on the protein. Equally convincing evidence is found in the studies of modified proteins, [for methods of preparation, see (35, 74)]. Elimination of the free  $\epsilon$ - amino groups by acetylation and of the quaternary groups by formaldehyde treatment shows marked changes in the anionic binding (44, 110). Therefore, it seems reasonable to conclude that protein-anion interaction would involve, for the most part, a salt linkage between the positively charged groups on the protein molecule, i.e. histidine, lysine and arginine, and the anions.

b. Van der Waals forces and hydrogen bonding. The presence of positive groups in the protein is a necessary but not sufficient condition for anionic binding. In addition, the role of van der Waals forces cannot be neglected, especially where nonpolar groups are also involved as in the case of surface active anions. Electrophoretic analysis indicates that no combination occurs between egg albumin and either benzene sulfonate or naphthalene sulfonate as contrasted



with long-chain alkylbenzenesulfonate (60). Increase in chain length of aliphatic carboxylates increases their capacity to stabilize serum albumin against thermal or urea denaturation (7). These observations are in accord with Steinhardt's conclusions with regard to the increase in anion affinity with increase in chain length within an homologous series (102, 103). The role of van der Waals forces, however, is lessened in the case of ions with side-chain polar groups such as dyes. Perhaps this also explains why surface active ions have extraordinarily stronger affinity for proteins than most simple ions.

Since van der Waals forces play an important role in the binding of fatty acids, Luck (59) proposed the hypothesis that the binding capacity of anions with proteins is a dual function of the large number of positively charged groups and the close juxtaposition of some of these to non-polar side chains of certain amino acids, especially leucine, isoleucine, valine, and phenylalanine. Klotz objected to this viewpoint on the ground of thermodynamic considerations and steric configurations (43, 47). Instead, Klotz (43) proposed an internal hydrogen-bonding hypothesis, assuming that within the protein molecule -OH groups form bonds with  $\text{-COO}^-$  groups preferentially to bonds with  $\text{=NH}$  residues. This assumption seems warranted by the greater energy of  $\text{OH}\cdots\text{O}$  bonds as compared to  $\text{O}\cdots\text{HN}$  bonds (78). It, therefore, follows that the binding capacity of a protein is a direct function of the number of

positively charged groups and an inverse function of the number of carboxyl and hydroxyl groups and the "binding index" ( $\Sigma(\equiv\text{NH}^+)/[\Sigma(-\text{OH})-\Sigma(-\text{COO}^-)]$ ) will predict the relative affinity of different proteins for the same anion. As such, methyl orange is strongly bound by serum albumin, weakly by  $\beta$ -lactoglobulin and virtually not at all by egg albumin. Tanford (107), however, reported that egg albumin binds much more strongly with chloride ions than  $\beta$ -lactoglobulin does. In fact, the latter is incapable of binding at its isoelectric point at all. It therefore appears that the "binding index" does not have general validity.

That hydrogen-bonding plays an important part in the protein structure is without any doubt (79, 80). This is particularly clear in the case of denaturation, where the breaking of hydrogen bonds by physical or chemical means contributes to unfold the once highly condensed polypeptide chains. However, it seems rather risky to minimize the role of van der Waals forces in the binding process. Furthermore, it is inconceivable that the increase in affinity for larger ions is not attributed to such forces at all, although thermo-dynamically it can be explained by the increase in entropy change due to greater release of water molecules. To determine binding affinity, it is necessary to consider the contribution of all forces involved. The insufficient evidence now available precludes any acceptable conclusion. It therefore must await

further study of the structure of both proteins and ions involved.

Another point is raised in the binding mechanism of "extra" anions to the proteins. It is well known that proteins can bind an amount of surface active anions in excess of the number of basic groups present in the protein molecule. Different views are held with regard to the sites of adsorption of such extra ions. It has been proposed that the extra anions combine electrostatically with the weakly positive nitrogen atoms of the amide and peptide groups (76, 77, 100, 101), or attach by weak nonpolar forces to those already electrostatically bound (60). In the absence of any evidence, it is improper to regard either as an acceptable explanation of the facts.

## 2. Denaturation

The mode of denaturation has been speculated on by Neurath and coworkers (72) and also by Pauling and coworkers (79, 80). A clue is revealed by the high affinity of surface active ions for the proteins. The penetration of the tightly folded protein molecule by surface active ions will force the polypeptide chain to unfold, thus reducing the potential barrier to the entrance of more ions and consequently breaking all internal linkages. In this process, an "all-or-none" reaction will be favored. Once it is unfolded, the binding

of extra ions will contribute to further disorientation of the protein molecule. On the other hand, when at very low concentrations only a few surface active ions are bound, probably statistically, to the protein molecules, there is no reason to expect the unfolding of the protein molecules. Much of these speculations, however, is still an open question and needs further studies.

### 3. Precipitation and dispersion

The precipitating action of surface active ions on proteins seems to follow the same mechanism as other protein-precipitants. It can be attributed to the elimination of the free charge of highly ionized protein through interaction with counter-ions. Accordingly, these reactions are carried out by altering the net charge through pH adjustment, and at the same time, by adding counter-ions which possess strong affinity for the protein. Thus, heavy metal salts precipitate proteins only in alkaline solution, and complex acids, acid dyes, ferrocyanides and picrates in acid medium. The same is true for surface active anions or cations. That the stability of the proteins is not at a minimum at their isoelectric point, but rather in the ionized form can also be understood on the basis of zwitter-ion theory. At the isoelectric point, the proteins retain a sufficient amount of both positive and negative charges to effect hydration. Lowering in pH suppresses the

ionization of the carboxylic groups and thereby increases the net positive charges. The latter, however, are reduced by anionic binding and the residual hydration of the proteins is eliminated. Consequently, the stability of the protein solution is destroyed and precipitation occurs. The effect of neutral salt on the precipitation can also be explained as the salting-out of the protein-ion complex. Probably the ionization of the complex is depressed at the high concentration of the electrolytes in the solution. The dispersing effect of excess surface active ions, likewise, can be understood by considering the electric charge of the protein. When excess ions are absorbed, the protein acquires increasing electric charge of the same sign as the ions, its hydration is increased and therefore the precipitate is redispersed. This dispersing effect is characteristic for the interaction of the proteins with surface active ions, due probably to their high affinity for each other as well as for the proteins.

In conclusion, surface active ions possess remarkably strong intrinsic affinity for proteins and therefore readily form complexes with them. This combination causes disturbance of the intermolecular and intramolecular structure of proteins by upsetting the balance of the electrostatic forces, van der Waal's forces and hydrogen bondings in the molecule. Denaturation, precipitation, dispersion and many other biochemical effects are the manifest results of this binding process.

### III. MATERIALS AND METHODS

#### A. Materials

##### 1. Proteins

Bovine serum albumin was obtained through courtesy of Armour and Company (Lot No. 69, substandard), and used without further crystallization. Usually a 1-2% solution was prepared and kept in the cold room (1-3°C.). For spectrophotometric analysis, the protein solution was first dialyzed against repeatedly-changed buffer for several days.

Ovalbumin was prepared in the cold room (1-3°C.) from fresh egg white according to Sørensen's procedure (97). The fresh eggs were obtained from the College Poultry Farm. Crude ovalbumin was recrystallized three times from ammonium sulphate at its isoelectric point ( $\text{pH} = 4.8$ ), dialyzed free of salt against distilled water, lyophilized to dryness and finally kept in the cold room.

Zein was supplied by the Corn Products Refining Corporation and used without further purification.

All protein concentrations were determined by micro-Kjeldahl method, using the data listed in Table I.

##### 2. Surface active ions

Santomerse #3, principally sodium dodecylbenzenesulfonate, was supplied by the Monsanto Chemical Company and further

purified with 95% ethanol. The alcoholic solution was filtered free of inorganic salts, vacuum-distilled to remove ethanol, diluted with distilled water, lyophilized to dryness and kept in an air-tight bottle. Its apparent molecular weight was 354, as determined by Paar bomb sulfur analysis.

Table 1. Molecular weight and nitrogen contents of some proteins

Proteins	Molecular Weight	Nitrogen Content
Bovine serum albumin	69,000 (15)	15.95% (22)
Ovalbumin	44,000 (23)	15.8% (12,52)
Zein	40,000 (14 pp. 390, 428)	16.0%

Zephiran chloride solution was purchased from Winthrop Chemical Company. The principle constituent was n-dodecyl dimethyl benzyl ammonium chloride. Its molar concentration was determined by micro-Kjeldahl analysis, on the basis of one equivalent nitrogen per mole zephiran chloride.

### 3. Buffers

All buffers were made up with reagent-grade chemicals. The corresponding pH values were measured at room temperature with a Leeds and Northrup Universal pH assembly, equipped with a glass electrode.

Table 2. The compositions of some buffer solutions

	Composition	pH measured	Ionic Strength
Glycine	0.030 M	10.0	0.10
NaOH	0.020 M		
NaCl	0.080 M		
Sodium barbiturate (1) (veronal)	0.024 M (2)	8.5	0.10
HCl	0.004 M		
NaCl	0.076 M	8.9	0.10
	0.018 M (1)		
	0.002 M		
	0.080 M (2)		
Glycine	0.09 M	3.3	0.10
HCl	0.01 M		
NaCl	0.09 M		
K <sub>2</sub> HPO <sub>4</sub>	0.0321 M	7.6	0.20
KH <sub>2</sub> PO <sub>4</sub>	0.0036 M		
NaCl	0.100 M		
Sodium acetate	0.20 M	4.5	0.20
Acetic acid	0.30 M		

Other buffers used will be mentioned in the sections under experimental results.

## B. Methods

### 1. Electrophoresis

Electrophoretic analyses were carried out at 2.0°C. in the Tiselius-type cell, purchased from the Klett Manufacturing Company, with a modified Philpot-Svensson optical system. The construction of the equipment has been adequately described by Longworth (53, 55, 56).



The protein concentrations were adjusted to about 0.4% and clarified, if necessary, in the Sorvall SS-1 high-speed angle centrifuge. All electrophoretic runs were performed at a constant field strength of 4.0 to 4.5 volts  $\text{cm}^{-1}$ , and stopped just before the fastest boundary migrated out of the visual field. In order to facilitate comparison of the experiments, pictures were also taken at definite intervals during the run.

All calculations of mobilities and percentage compositions were made on enlarged tracings of the patterns in the customary manner. In cases of incomplete resolution of protein components, arbitrary separation was made by drawing a vertical line from the minimum between the peaks. No correction was attempted for the dilution effect at the  $\delta$ -boundary, nor for the conductivity of the solution on the descending side.

## 2. Equilibrium-dialysis

Visking cellophane casings, 20/32 inches in diameter, were used for routine analysis. The bags were first dialyzed against a large volume of phosphate buffer for ten days, fresh buffer being used every two or three days. Portions (20ml) of buffered protein-SDBS solution contained in the air-tight bags were then equilibrated against equal volumes of the buffer in test tubes. Controls containing buffer only inside the bag were prepared in the same manner. The rubber stoppers were covered

with tin foil. The tubes were shaken gently in the cold room for two days. The dialyzates were than diluted, if necessary, and analyzed spectrophotometrically.

### 3. Spectrophotometric analysis

SDBS concentrations were determined by measurement of the light absorption with the model DU Beckman spectrophotometer at  $\lambda_{\max} 223m\mu$ . Both control buffer and SDBS solution were first centrifuged at 13,000 r.p.m. in the Sorvall centrifuge for 30 minutes.

### 4. Nitrogen determination

Nitrogen analysis was made by the micro-Kjeldahl method, using selenium oxychloride as catalyst. The ammonia was distilled into 4% boric acid and titrated with 0.01N HCl, using as a mixed indicator methylene blue and methyl red in 95% ethanol.

#### IV. EXPERIMENTAL RESULTS AND DISCUSSION

##### Part I. Competitive Interaction Between Proteins and Surface Active Anions

Electrophoretic analysis has proved to be one of the few useful methods for the investigation of protein-ion interaction. Evidence comes from changes in the number of boundaries, the relative areas of the boundaries and their corresponding mobilities. In Part I competitive interaction between three proteins and a surface active anion, sodium dodecylbenzenesulfonate (SDBS) was studied electrophoretically, the results of which studies would reveal the relative binding affinity of the anion by the proteins.

##### A. Competitive Interaction Between Zein-Sodium dodecylbenzenesulfonate (SDBS) Complex and Albumins

###### 1. Preparation of zein-SDBS complex

Zein is insoluble in water but can be easily dispersed in an aqueous solution of a surface active anion such as SDBS. A stable complex could be prepared by either of two methods:

(1) Dialysis method. A mixture of about 3 gm. zein and 100 ml 1% SDBS solution was shaken gently until the protein was completely dispersed. The clear solution was then dialyzed against repeatedly-changed distilled water until free of

loosely bound excess SDBS. (2) Solubility method. A mixture of 6 or 7 gm. zein and 100 ml 1% SDBS was shaken gently for a day or two and then centrifuged to remove the excess (undispersed) zein.

By electrophoretic analysis, the mobility of the complex prepared by both methods was found to be about  $-8.5 \times 10^{-5}$   $\text{cm}^2 \text{ volt}^{-1} \text{ sec}^{-1}$  in veronal-NaCl buffer ( $\text{pH} = 8.9$ ,  $\Gamma/2 = 0.10$ ), indicating that a well-defined complex was formed. The dialysis method, however, had two disadvantages. First, it was tedious and time-consuming. Secondly, the amount of SDBS removed was not easily controlled. Indeed, it was not known whether the complex would loose some bound anion through prolonged dialysis in addition to those loosely adsorbed. Thus, the solubility method was preferred in the subsequent experiments.

## 2. Choice of electrophoretic buffer

In order to study interaction of proteins with SDBS it was necessary to find a suitable buffer for electrophoretic analysis. For this purpose, a mixture of zein-SDBS and ovalbumin-SDBS of known concentration was prepared by the dialysis method. In a preliminary study, it was found that both proteins formed stable complexes with SDBS. Among the buffers studied veronal-NaCl ( $\text{pH} = 8.5$ ,  $\Gamma/2 = 0.10$ ) and glycine-NaCl ( $\text{pH} = 10.0$ ,  $\Gamma/2 = 0.10$ ) were the most satisfactory, yielding

quantitative resolution. For the sake of comparison, both buffers were employed for subsequent analyses. In general, the results were in good agreement with each other, a fact perhaps indicative of their reliability.

### 3. Interaction between zein-SDBS and ovalbumin

Mixtures of zein-SDBS (solubility method) and ovalbumin of various proportions were stored in the cold room (1-3°C.) for two days and then equilibrated against either veronal-NaCl or glycine-NaCl buffer for electrophoretic analysis. In Figure 1 are shown some representative runs against veronal-NaCl buffer. The corresponding electrophoretic data are listed in Table 3.

Since combination of a protein with anions would increase its net negative charge, the protein-SDBS complex would move much faster toward the anode in an electric field than the protein itself. Preliminary studies showed their mobilities to increase in the order of ovalbumin, zein-SDBS and ovalbumin-SDBS.

Definite interaction was clearly revealed in the electrophoretic patterns. Even in the low ovalbumin/zein-SDBS region, there appeared a small fast boundary marked "OI", the mobility of which corresponded closely to that of ovalbumin-SDBS complex, but which was somewhat slower than the latter. Thus, it was suggested that part of the anion was taken away from the zein-SDBS complex and an ovalbumin-SDBS complex formed.

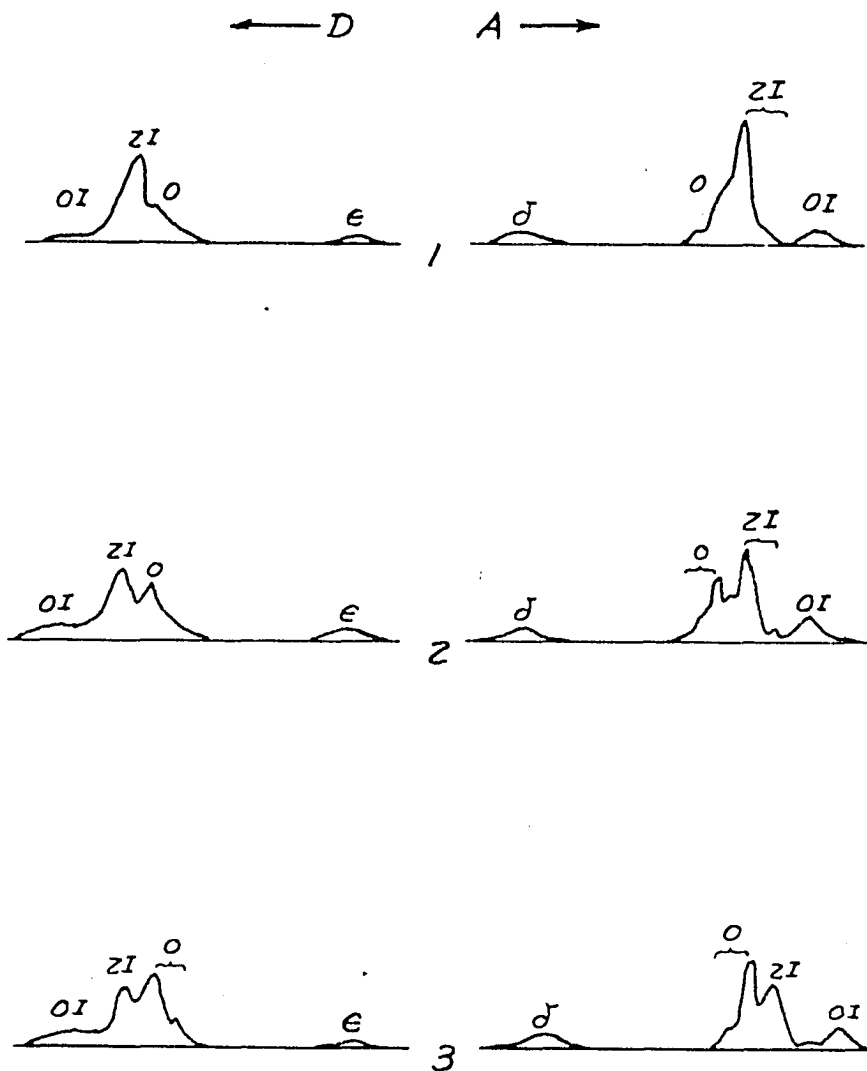


Fig. 1. Electrophoretic analyses of mixtures of zein-SDBS (ZI) and ovalbumin (O) in veronal-NaCl buffer (pH = 8.9,  $\Gamma/2 = 0.10$ ).

1. Z/O = 67/33. 2. Z/O = 51/49.  
3. Z/O = 34/66.

Table 3. Electrophoretic analyses of mixtures of zein-SDBS and ovalbumin at 2°C in veronal-NaCl buffer (pH = 8.9,  $\Gamma/2 = 0.10$ )(1)

Relative Compn.		Bound-ary	Mobilities				Relative Area(3)		
ZI(2)	O		O	ZI	OI	O	ZI	OI	
%	%		$-\mu \times 10^5 \text{ cm}^2 \text{ volt}^{-1} \text{ sec}^{-1}$						
100		D		8.5					
		A		8.5	9.4		99	11	
	100(4)	D				11.7	(14.8)(5)		
		A				12.8			100
	100	D	6.3	7.2					
		A	6.7	7.5			16	84	
67	33	D	6.3	7.1	8.0	10.4			
		A	6.6	7.4	8.2	9.1	4	18	64
		D		7.2	8.3	10.2			
51	49	A		7.4	8.5	9.4	30	59	3
		D	6.4	7.2	8.2	9.8			
34	66	A	6.7	7.4	8.2	9.5	9	45	37
							1		8

- (1) O = ovalbumin, OI = ovalbumin-SDBS, ZI = zein-SDBS.  
 (2) Based on the protein concentrations only.  
 (3) Based on the ascending patterns.  
 (4) Ovalbumin-SDBS complex, instead of native ovalbumin.  
 (5) A fast boundary anomaly.

This displacement reaction seemed to reach a certain limit. Thus, as the relative concentration of ovalbumin increased a separate boundary, presumably uncombined ovalbumin, began to increase in proportion.

Nitrogen analysis of the mixture did not show any loss of insoluble zein. That zein lost some of its bound anion to native ovalbumin but still remained in solution was rather unexpected. Presumably, the zein-SDBS complex retained enough anion to be kept in solution. Since the number of anions bound was much in excess of the basic groups in the protein, it is suspected that some weakly bound anions were displaced by native ovalbumin.

With veronal-NaCl buffer (pH=8.9) as electrolyte fast boundary anomaly usually appeared on the descending pattern. Increase of the ionic strength to 0.20 by addition of salt destroyed the anomaly. However, at this concentration of salt, part of the zein was salted out. In the case of glycine-NaCl buffer (pH=10) no anomaly has been observed, but the resolution between zein-SDBS and ovalbumin boundaries was not as good as in veronal-NaCl buffer, due to the fact that the mobility of ovalbumin was close to that of zein-SDBS complex and the boundaries were thereby overlapped with each other.

#### 4. Interaction between zein-SDBS and bovine albumin

Mixtures of zein-SDBS (solubility method) and bovine albumin were studied electrophoretically (Table 4) in a manner



similar to section 3. In Figure 2. are shown some representative patterns using glycine-NaCl buffer. There appeared virtually only two boundaries, the mobilities of which corresponded to zein-SDBS and bovine albumin (Table 4). No faster albumin-SDBS boundary was detected in any patterns. Furthermore, the relative areas were nearly equal to the original composition. To be sure, the actual percentage of zein should be smaller than its relative area owing to the presence of SDBS. Such correction however was not very large. From these facts, it might be inferred that no competitive interaction between zein-SDBS and bovine albumin occurred.

It is interesting to note that bovine albumin migrated even more rapidly than zein-SDBS at pH 10. The reverse was true with veronal-NaCl buffer at pH 8.5. Comparable results were also obtained in the latter case. However, the boundaries were not resolved too well, probably due to their close mobilities.

Comparison of the foregoing results seems to indicate that ovalbumin has a higher affinity for SDBS than zein, whereas bovine albumin does not compete with zein-SDBS complex. If this is true, it would be of interest to compare the binding affinities to the two albumins. In section B, it will be shown that ovalbumin does bind SDBS more strongly than bovine albumin.

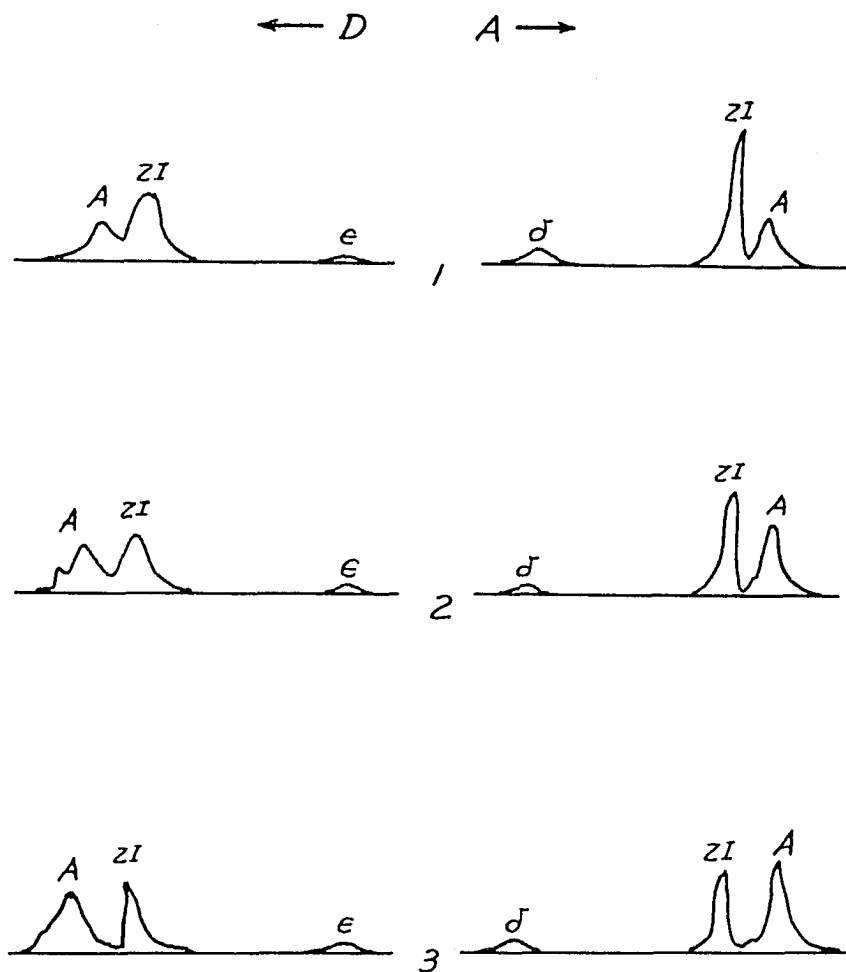


Fig. 2 . Electrophoretic analyses of mixture of zein-SDBS (ZI) and bovine albumin (A) in glycine-NaCl buffer (pH = 10.0,  $\Gamma/2 = 0.10$ ).

1. Z/A = 69/31.    2 . Z/A = 53/47.  
3. Z/A = 36/64.

Table 4. Electrophoretic analysis of mixtures of zein-SDBS and bovine albumin at 2°C. in glycine-NaCl buffer (pH = 10.0,  $\Gamma/2 = 0.10$ ) (1)

Relative Composition		Bound-ary	Mobilities		Relative area	
ZI (2)	A		ZI	A	ZI	A
%	%		$-\mu \times 10^5 \text{ cm}^2 \text{ volt}^{-1} \text{ sec}^{-1}$			
100		D	8.6			
		A	8.6		100	
	100	D		8.7		
		A		9.0		100
69	31	D	7.8	9.4		
		A	8.0	9.1	69	31
53	47	D	7.7	9.3		
		A	7.7	9.0	49	51
36	64	D	7.4	9.4		
		A	7.7	9.6	36	64

(1) A = bovine albumin, ZI = zein-SDBS.

(2) Based on protein concentration only.

## B. Competitive Interaction Between Ovalbumin and Bovine

### Albumin with SDBS

#### 1. Choice of electrophoretic buffer

Mixtures of ovalbumin and bovine albumin of known composition were studied electrophoretically against several buffers. Among them, phosphate-NaCl ( $\text{pH} = 7.6, \Gamma/2 = 0.20$ ) and acetate-NaCl ( $\text{pH} = 5.6, \Gamma/2 = 0.20$ ) were found unsuitable for routine analysis, yielding extremely diffuse and unsymmetric patterns on both descending and ascending sides. On the other hand, with glycine-NaCl and veronal-NaCl buffers, as used previously, the mixture was quantitatively well-resolved. Variation of ionic strength from 0.10 to 0.20 by addition of salt also did not affect the resolution in either buffer system. Thus 0.10 ionic strength was used for subsequent analyses.

#### 2. Preparation of albumin-SDBS complexes

Albumin-SDBS complexes of minimum binding ratio could be prepared by several methods: (1) extraction with 60% acetone, (2) salting out, (3) prolonged dialysis and (4) acid precipitation. The last two procedures were employed in this research. In the dialysis method, a mixture of albumin and excess SDBS was stored for two days in the cold room (1-3°C.) Removal of excess anion was achieved through prolonged dialysis against a large volume of repeatedly-changed distilled water for several days. For ovalbumin, a stable complex was formed with

an electrophoretic mobility of about  $-11$  to  $-12 \times 10^{-5}$   $\text{cm}^2$  volt $^{-1}$  sec $^{-1}$  in glycine-NaCl or veronal-NaCl buffer. In the case of bovine albumin, the electrophoretic boundaries tended to split into two of nearly equal size after prolonged dialysis. The effect of dialysis time on the removal of SDBS is shown in Table 5.

Table 5. Effect of dialysis time on bovine albumin-SDBS complex from electrophoretic measurements

Dialysis time	Boundary	Mobilities (1)2		Relative (2) area	
		1	2	1	2
Days		$-\mu \times 10^5 \text{cm}^2 \text{volt}^{-1} \text{sec}^{-1}$			
4	D	11.0	(13.9)		
	A	11.2	12.2	trace	100
7	D	10.3	(13.8)		
	A	10.7	11.5	54	46
15	D	10.1	(13.8)		
	A	10.5	11.6	53	47

(1) A fast boundary anomaly in veronal-NaCl buffer (pH= 8.5,  $\Gamma/2 = 0.10$ ) as indicated by brackets ( ).

(2) Based on ascending patterns.

It might be assumed that part of the bound anion could be taken away through dialysis.

In the acid precipitation method, ovalbumin and SDBS solutions were mixed in a protein/ion (P/I) ratio of 3 to 1, and bovine albumin and SDBS solution in a P/I ratio of 2.5 to 1. The complexes were precipitated by adding an equal volume of acetate buffer (pH=4.5,  $\Gamma/2=0.20$ ). The precipitate was centrifuged down and re-dispersed in an alkaline buffer (veronal-NaCl or glycine-NaCl in this research). In this manner, stable complexes could be prepared as evidenced from electrophoretic mobilities in Table 6, which corresponded closely to those prepared by the dialysis method.

Table 6. Electrophoretic mobilities of albumin-SDBS complexes by acid precipitation method at 20°C.

Solution	Buffer	Boundary	Mobilities <sup>(1)</sup>
			$-\mu \times 10^5 \text{cm}^2 \text{volt}^{-1} \text{sec}^{-1}$
Ovalbumin-SDBS	Veronal-NaCl pH= 8.9, $\Gamma/2 = 0.10$	D	11.7 (14.8)
		A	12.8
	Glycine-NaCl pH= 10.0, $\Gamma/2 = 0.10$	D	11.5
		A	12.0
Bovine albumin-SDBS )	Veronal-NaCl pH= 8.5, $\Gamma/2 = 0.10$	D	11.3 (14.5)
		A	11.9
	Glycine-NaCl pH= 10.0, $\Gamma/2 = 0.10$	D	11.6
		A	10.9 (trace) 11.9

(1) The figures in brackets ( ) indicated fast boundary anomalies.

In a preliminary study, excess protein or excess SDBS failed to precipitate the complex. This is in good agreement with the observation by Putnam (83). Thus, the complex precipitated only in a narrow P/I range. This method was preferred to the dialysis method in that it is easier to control the amount of bound SDBS and is much less time consuming. It is also interesting to note that the electrophoretic pattern of bovine albumin-SDBS corresponded closely to that from 4-day dialyzed complex. Presumably, dissociation would take place during prolonged dialysis.

### 3. "All-or-none" reaction

Electrophoretic analyses were performed for mixtures of albumin and SDBS. The results were in good agreement with the findings of Lundgren (62) and others. In the P/I range studied, an "all-or-none" reaction has taken place. This will be discussed in detail in Part III.

### 4. Interaction between bovine albumin-SDBS and ovalbumin

Mixtures of bovine albumin-SDBS (acid precipitation method) and ovalbumin were studied electrophoretically in the same manner as described previously. Definite interaction between native ovalbumin and bovine albumin-SDBS was clearly revealed in electrophoretic analysis (Table 7). Some of the representative patterns are shown in Figure 3. The relative composition

of the observed ovalbumin boundary was much smaller than that present in the original mixture, whereas a new boundary marked "A" appeared with a mobility corresponding to bovine albumin. It is therefore assumed that some of the bovine albumin was displaced from its SDBS complex, whereas part of the ovalbumin formed a complex with the SDBS. It seems also that there existed a limit for such displacement reaction. For reasons unexplained, the albumin-SDBS boundaries were split into two, or even three in one case. It is suspected that the boundaries of ovalbumin-SDBS and bovine albumin-SDBS could be somewhat resolved although their mobilities were very close to each other. Quite possibly the split was simply due to a false boundary, as sometimes occurred.

##### 5. Interaction between ovalbumin-SDBS and bovine albumin

Similar experiments were performed for mixtures of ovalbumin-SDBS (acid precipitated) and bovine albumin. In Table 8 are listed electrophoretic analyses. Some representative patterns are shown in Figure 4. With glycine-NaCl buffer, only two boundaries were observed, with mobilities close to those of bovine albumin and ovalbumin-SDBS complex. Furthermore, the relative area was close to the relative composition in the original mixture. These results therefore clearly indicated that there was no interaction between bovine-albumin and ovalbumin-SDBS. In other words, bovine albumin could



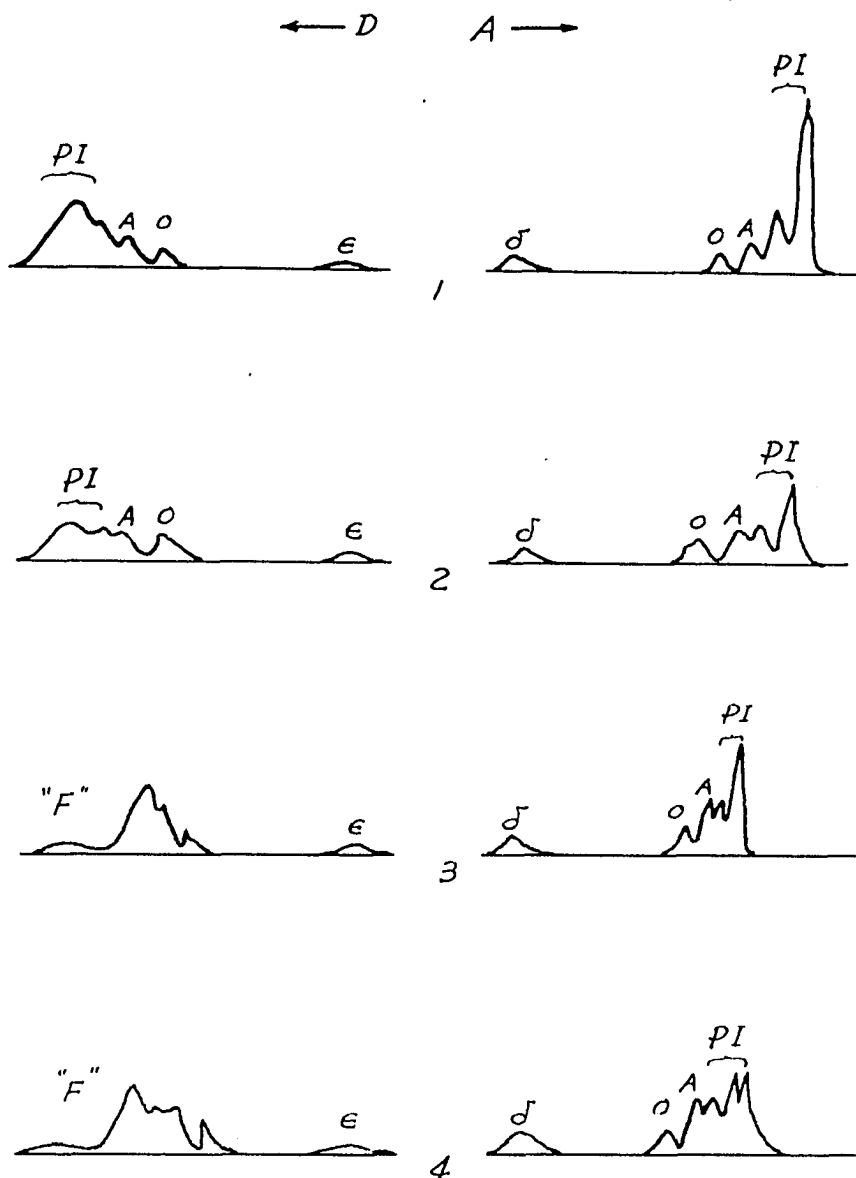


Fig. 3. Electrophoretic analyses of mixtures of bovine albumin-SDBS (AI) and obalbumin (O). 1 and 2 in glycine-NaCl buffer (pH = 10.0,  $\Gamma/2 = 0.10$ ). 3 and 4 in veronal-NaCl buffer (pH = 8.5,  $\Gamma/2 = 0.10$ ). 1 and 3. A/O = 66/34. 2 and 4. A/O = 49/51.

Table 7. Electrophoretic analyses of mixtures of bovine albumin-SDBS<sup>(1)</sup> and ovalbumin at 20°C.<sup>(2)</sup>

Relative Compn. Bound-ary			Mobilities				Relative Area			
O	AI(3)		O	A	PI		O	A	PI	
%	%		- $\mu \times 10^5$ cm <sup>2</sup> volt <sup>-1</sup> sec <sup>-1</sup>				%	%	%	
Glycine-NaCl buffer pH = 10.0, $\Gamma/2 = 0.10$										
34	66	D	7.7	9.0	10.0	11.1	7	11	18	64
		A	8.3	9.6	10.5	11.5	4	9	18	69
51	49	D	7.4	8.8	9.6	10.7	15	22	20	43
		A	7.6	9.2	9.8	10.9	17	20	18	45
65	35	D	7.9	9.3	10.3	11.3	31	11	20	38
		A	8.3	9.6	10.4	11.2	31	8	10	51
Veronal-NaCl buffer <sup>(4)</sup> pH = 8.5, $\Gamma/2 = 0.10$										
34	66	D	8.4	9.6	10.4	(14.4)	--	--		--
		A	8.9	10.0	11.3		10	23		66
51	49	D	8.6	10.1	11.1	12.4 (16.6)	--	--		--
		A	9.1	10.5	11.4	12.7 13.3	11	25	21 23	20
67	33	D	7.9	9.5	11.0	(13.9)	--	--		--
		A	8.2	9.6	11.0	11.5	32	31	22	25

(1) Prepared by acid precipitation and re-dispersion in alkaline buffer.

(2) O = ovalbumin, OI = ovalbumin-SDBS, AI = bovine albumin-SDBS, PI = protein-SDBS.

(3) Based on protein concentration only.

(4) A fast boundary anomaly appeared on the descending side as indicated by brackets( ).

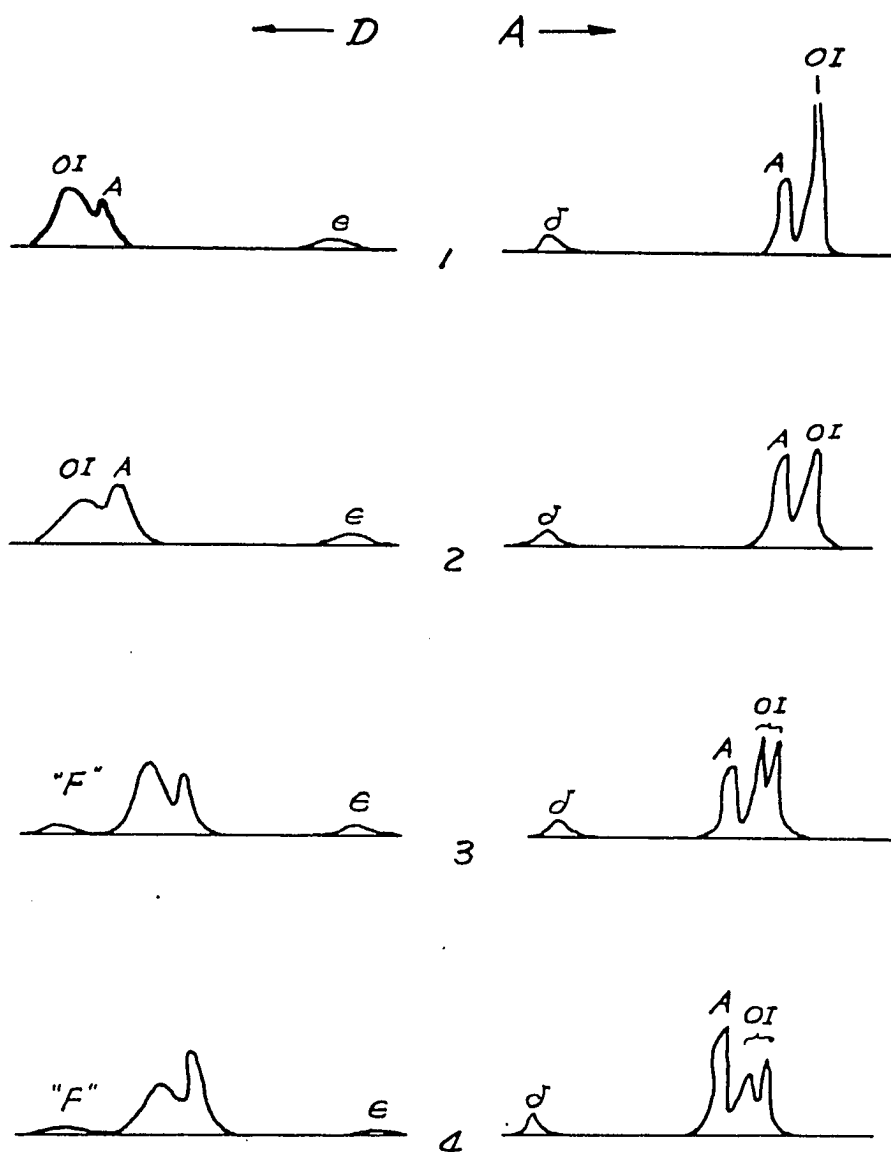


Fig. 4. Electrophoretic analyses of mixtures of ovalbumin-SDBS (OI) and bovine albumin (A). 1 and 2 in glycine-NaCl buffer (pH = 10.0,  $\Gamma/2 = 0.10$ ). 3 and 4 in veronal-NaCl buffer (pH = 8.5,  $\Gamma/2 = 0.10$ ). 1 and 3. O/A = 66/34. 2 and 4. O/A = 48/52. "F" = false boundary anomaly.

Table 8. Electrophoretic analysis of mixtures of ovalbumin-SDBS<sup>(1)</sup> and bovine albumin at 20°C.<sup>(2)</sup>

Relative Composition		Boundary	Mobilities		Relative area	
A	OI <sup>(3)</sup>		A	OI	A	OI
%	%		$-\mu \times 10^5 \text{ cm}^2 \text{ volt}^{-1} \text{ sec}^{-1}$		%	%
glycine-NaCl buffer pH = 10.0, $r/2 = 0.10$						
34	66	D	10.2	11.6	33	67
		A	10.5	11.7	29	71
52	48	D	9.8	11.0	48	52
		A	9.7	10.9	45	55
68	32	D	9.3	--	--	--
		A	9.7	10.4	69	31
Veronal-NaCl buffer <sup>(4)</sup> pH = 8.5, $r/2 = 0.10$						
34	66	D	8.7	10.4 (14.2)	--	--
		A	9.1	10.5 11.2	30	36 34
52	48	D	8.4	9.9 (13.8)	--	--
		A	8.7	9.8 10.5	46	32 22
68	32	D	9.3	10.5 (14.9)	--	--
		A	9.6	9.6 10.3	67	17 16

(1) Prepared by acid precipitation and re-dispersion in alkaline buffer.

(2) A = bovine albumin, OI = ovalbumin-SDBS.

(3) Based on protein concentration only.

(4) A fast boundary anomaly appeared on the descending side as indicated by brackets ( ).

not compete for the anion and displace ovalbumin from its SDBS complex. Analogous results were obtained with veronal-NaCl buffer, where, however, the ovalbumin-SDBS boundary was again split into two. This might also be a false boundary, similar to that observed in section 4.

Comparison of the foregoing results led to the following conclusion. The binding affinity of ovalbumin with SDBS is much stronger than that of bovine albumin with SDBS. This would also be in agreement with the previous results that ovalbumin could displace the anion from zein-SDBS complex but bovine albumin could not.

## Part II. Interaction Between Proteins and Surface Active Cations

Quantitative studies of the interaction between proteins and surface active cations are so far lacking in the literature. In Part II it was therefore attempted to conduct an electrophoretic analysis of protein surface active cation mixtures. An alkyl dimethylbenzyl ammonium chloride, known commercially as Zephiran, was used for this purpose.

### A. Choice of Electrophoretic Buffer

Since surface active cations would precipitate proteins on the alkaline side of the isoelectric point, it was necessary to study the complex formation only when the proteins were in the anionic form. Thus, electrophoretic buffers were so selected that their pH values were lower than 4.6 to 4.8. Among the buffers studied, only glycine-NaCl yielded good quantitative resolution for a mixture of bovine serum albumin and ovalbumin. Hence it was used for all routine analyses.

### B. Electrophoretic Analysis of Complex Formation

#### 1. Ovalbumin-zephiran complex

Mixtures of constant protein concentration (about 0.40%) and varying zephiran concentration were prepared by mixing the stock solution and diluting with glycine-NaCl buffer to a known volume. To insure complete interaction, the solutions

were allowed to stand two days in the cold room (1-3°C.). Dialysis of the mixtures was omitted in order to prevent loss of zephiran through diffusion. Some of the representative electrophoretic patterns are shown in Figure 5. The corresponding data on the cationic mobility and relative composition are given in Table 9.

The "all-or-none" character of complex formation was clearly indicated by the migration of excess native ovalbumin as a separate component at the boundary marked "O". At constant protein concentration, the amount of uncombined ovalbumin (O) diminished with increasing zephiran (I) concentration. Finally all ovalbumin was changed into the complex (OI) form. Mixtures of ovalbumin and zephiran at high I/O ratio usually became very turbid on standing, thus making electrophoretic analysis impossible.

Analysis of the relative areas was somewhat complicated by the lack of well-defined boundaries on both the descending and ascending patterns. As the mixing ratio (I/O) increased, the ascending boundaries became too sharp to resolve and usually appeared as an open peak. On the other hand, the descending boundaries were frequently rather diffuse, indicating the presence of several poorly resolved components. As a first approximation, assuming that (1) all zephiran was bound to the protein in the complex, and (2) the two components of ovalbumin combined with zephiran identically, and neglecting the correction for refractive-index increments, a minimum

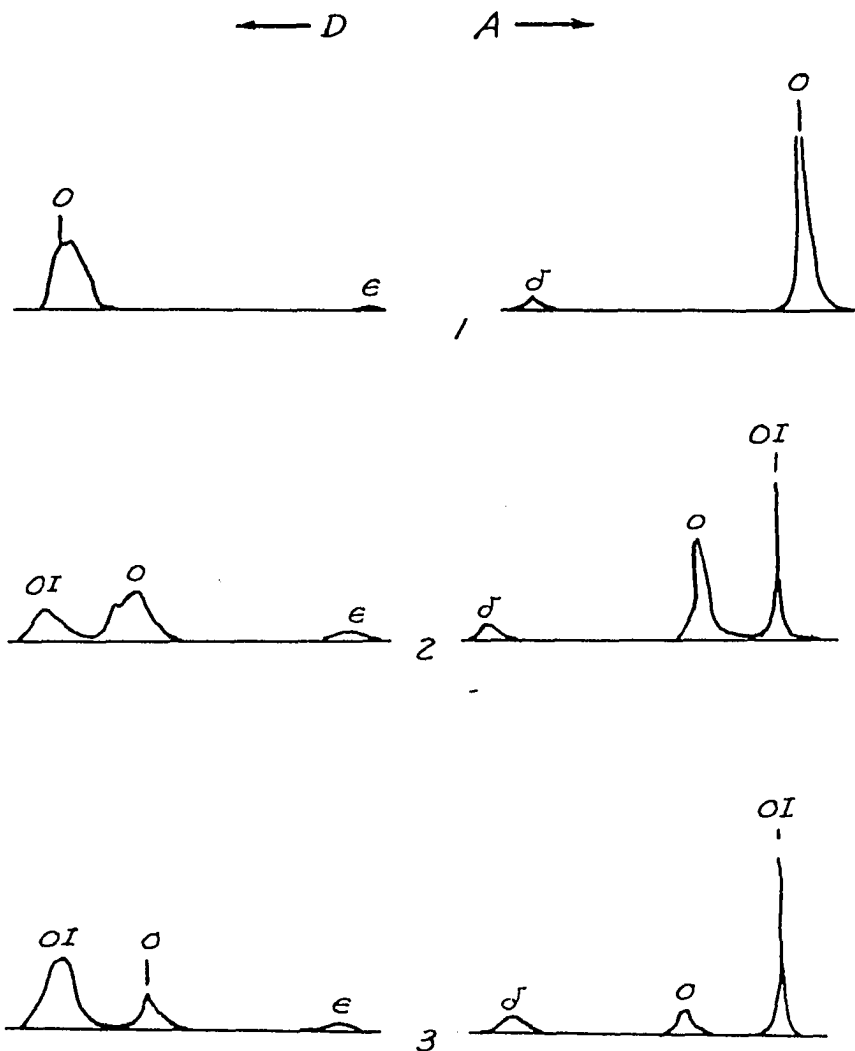


Fig. 5. Electrophoretic analyses of mixtures of ovalbumin (O) and zephiran (I) in glycine-NaCl buffer (pH = 3.3,  $\Gamma/2 = 0.10$ ). Protein concentration = 0.40%. Zephiran concentrations: 1. 0%, 2. 0.054% and 3. 0.108%.



Table 9. Electrophoretic analyses of ovalbumin-zephiran mixtures at 2°C. in glycine-NaCl buffer. (pH = 3.3,  $\Gamma/2 = 0.10$ ) (1)

Solution		Bound-ary	Mobilities		Relative Area	
O	I		O	OI	O	OI
%	%		$+ \mu \times 10^5 \text{cm}^2 \text{volt}^{-1} \text{sec}^{-1}$			
0.40		D	5.6			100
		A	5.8			100
0.40	0.054	D	5.1	5.8	7.6	63
		A	5.2		7.3	77
0.40	0.108	D	4.8		7.5	16
		A	4.5		7.2	--

(1) O = ovalbumin, I = zephiran, OI = ovalbumin-zephiran.

Table 10. Electrophoretic analyses of bovine albumin-zephiran mixtures at 2°C. in glycine-NaCl buffer (pH = 3.3,  $\Gamma/2 = 0.10$ ) (1)

Solution		Bound-ary	Mobilities		Relative Area	
A	I		A	AI	A	AI
%	%		$+ \mu \times 10^5 \text{cm}^2 \text{volt}^{-1} \text{sec}^{-1}$		%	%
0.42		D	7.5			100
		A	7.8			100
0.42	0.054	D	8.2			100
		A	8.1			100
0.42	0.108	D	8.4			100
		A	8.4			100
0.42	0.162	D	9.1			100
		A	9.4			100

(1) A = bovine albumin, I = zephiran, AI = bovine albumin-zephiran.

binding ratio (I/P) in the complex was estimated to be 40-60 moles zephiran per mole of ovalbumin. Such estimation might involve appreciable errors for the following reasons.

(1) The weight percentage of the zephiran solution was determined by nitrogen analysis, assuming one equivalent nitrogen per mole zephiran. The presence of nitrogenous substance other than zephiran would certainly yield a too high concentration by weight. The purchased zephiran solution was supposed to contain 12.8% zephiran, but the nitrogen determination gave a value of 16.9%. Direct determination of dry weight failed because of decomposition on warming. (2) The alkyl group of zephiran is principally a dodecyl carbon chain. It was therefore assumed that one equivalent nitrogen corresponded to an apparent molecular weight of 340. There was no way to determine accurately the amount of zephiran bound by weight. Even so, the estimated minimum binding ratio seemed to correspond with the acidic groups of the protein. The titration curve of ovalbumin indicated the presence of about 51 titratable acid groups and the chemical analysis yielded a total free carboxyl content of about 45 groups per molecule ovalbumin (23). Thus, it seemed to agree reasonably well with the hypothesis that stoichiometric combination exists between the acidic groups of the protein and surface active cation, similar to that between the basic groups and surface active anions (see, Part III).

## 2. Bovine albumin-zephiran mixtures

Analogous experiments were conducted for mixtures of bovine albumin and zephiran. In Figure 6 the absence of an "all-or-none" reaction is clearly shown in the electrophoretic patterns, where only a single boundary is observed at various mixing ratios. As the amount of zephiran increased, the mobility of the boundary also increased slightly (Table 10). It might be inferred that the surface active cations were loosely adsorbed on the protein molecules and thereby both migrated together in the electric field. In the presence of a large amount of zephiran the boundaries were completely blurred, probably due to the fact that a negative density gradient was developed in the cell.

### C. Preparation of Protein-Zephiran Complexes

#### 1. Ovalbumin-zephiran complex

Both dialysis and precipitation methods were tried without success. Removal of excess zephiran in an ovalbumin-zephiran mixture by prolonged dialysis against distilled water was interrupted by the formation of a gel after about two days. Use of glycine-NaCl buffers (pH = 3.3 or 2.3) instead of water gave the same results. However, the gel appeared more slowly at pH (2.3) than at higher pH (3.3). After centrifuging off the precipitate, only a small fraction of the protein was left in the supernatant solution. From its electrophoretic

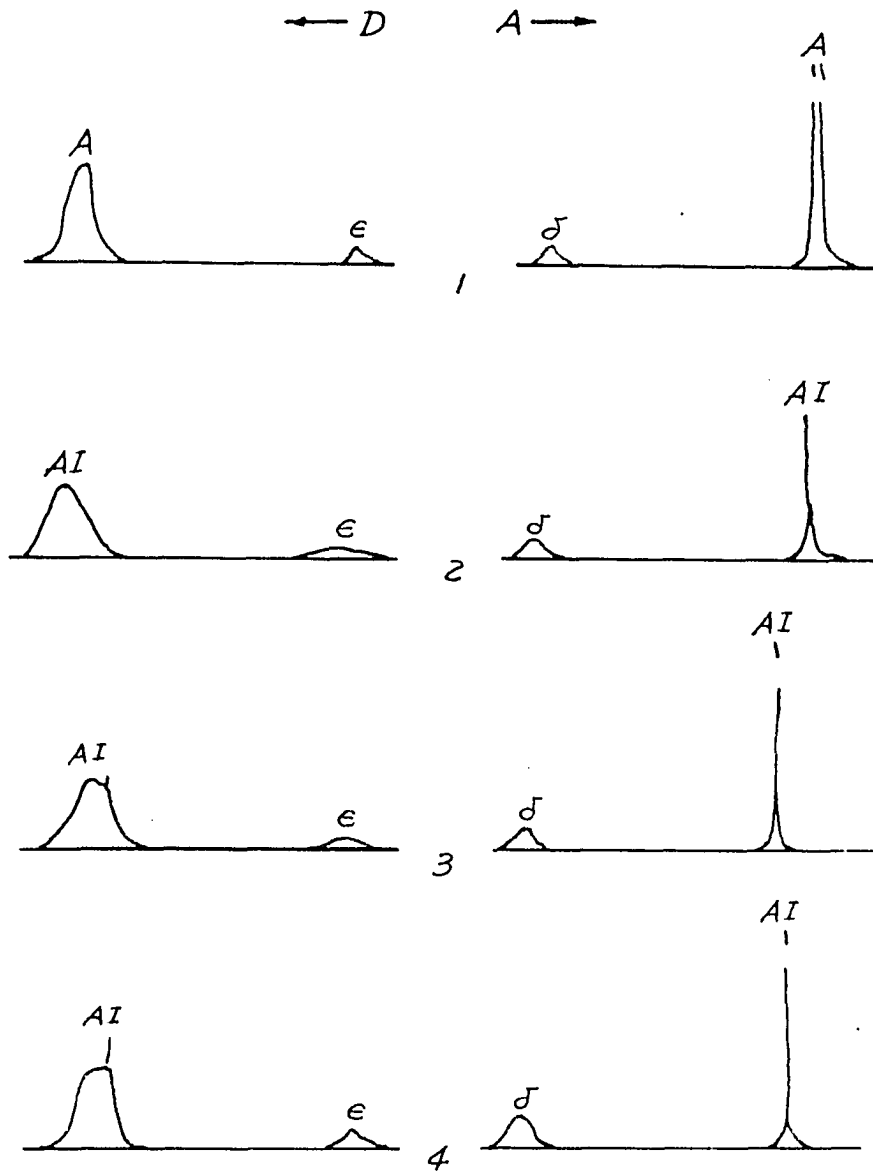


Fig. 6. Electrophoretic analyses of mixtures of bovine albumin (A) and zephiran (I) in glycine-NaCl buffer (pH = 3.3,  $\Gamma/2 = 0.10$ ). Protein concentration = 0.42%. Zephiran concentrations: 1. 0%, 2. 0.054%, 3. 0.108% and 4. 0.162%.

mobility, the remaining protein was identified as regenerated ovalbumin rather than a protein-zephiren complex.

As expected, mixtures of ovalbumin and zephiren were insoluble in the glycine-NaCl buffer (pH = 10.0) alkaline to the isoelectric point of the protein where the net charges of the protein and zephiren were opposite to each other. The precipitate, however, could not be redispersed in acidic glycine-NaCl buffer (pH = 2.3 or 3.3). This might be due to the fact that the pH of the buffer was too close to the isoelectric point. No lower pH ( $< 2$ ) was tried since it would be complicated by further denaturation or even decomposition of the protein. Another possibility was that drastic denaturation, followed by aggregation might make the precipitate insoluble. In view of the gel formation mentioned previously, structural change within the protein molecules should have taken place to a great extent. In this laboratory, flow birefringence study by Mr. G. F. Hanna seemed also to support this viewpoint. Mixtures of ovalbumin and zephiren gave strong double refraction, a fact perhaps indicative of protein denaturation.

## 2. Bovine albumin-zephiren mixture

Mixtures of bovine albumin and zephiren remained perfectly clear after prolonged dialysis against distilled water. Electrophoretic patterns again exhibited only a single boundary,

the mobility of which was almost identical with that of native albumin. Recalling that no "all-or-none" reaction was observed for the mixture, it is postulated that bovine albumin binds loosely with zephiran in a statistical process. It is suspected that the cation is adsorbed on the surface of the protein molecule without any significant change of the protein structure. This seems to agree with the results of flow birefringence study. Mixture of bovine albumin and zephiran did not exhibit anisotropy of flow, as contrasted with mixture of ovalbumin and zephiran. This seems to indicate that the adsorption of the cation did not disrupt the protein structure to any significant extent, thus causing denaturation.

Bovine albumin was also precipitated by zephiran in the alkaline buffer (pH = 10). The precipitate could not be re-dispersed in acidic buffers (pH = 2.3 or 3.3). Probably, the protein was denatured during drastic precipitation.

### 3. Zein-zephiran mixture

Preparation of zein-zephiran complex was also unsuccessful by the following two methods. In the first method, excess zein was suspended in zephiran so that the latter would be used up, leaving only the complex in the solution and the excess zein undispersed. In the second method, excess zephiran was used in an effort to disperse the protein into the solution. In both cases, a layer of gum, probably denatured zein, was found on the wall of the container. On dialysis of the super-

notent solution against distilled water or acidic buffer, a white precipitate immediately appeared, thus making electrophoretic analysis impossible.

Failure to prepare protein-zephiran complexes precludes the possibility of study of competitive interaction.

### Part III. Quantitative Study of Interaction Between Protein and Surface Active Anion

Controversy on the binding process of proteins with surface active ions prompted the detailed re-investigation of the nature of complex formation. In Part III, a quantitative study of the interaction between bovine serum albumin and sodium dodecylbenzenesulfonate (SDBS) was conducted at 1-3°C. in an effort to clarify some of the confusion. Also included were a few comparable studies of ovalbumin and SDBS for the sake of comparison. Both electrophoretic analysis and equilibrium-dialysis techniques were used as experimental tools.

#### A. Spectrophotometric Analysis

##### 1. Calibration curve

In order to study the mass relation between the protein, protein-SDBS complex and free SDBS, it is necessary to analyze quantitatively the concentrations of the reactants. The amount of protein in solution can be determined by the conventional micro-Kjeldahl method, the SDBS by the Parr bomb method on the basis of its sulphur content. The latter however proves not only tedious and time-consuming for routine analysis, but also insensitive at very low concentrations. Thus, a spectrophotometric method of analysis was developed. In Figure 7 is shown the ultraviolet absorption spectrum of aqueous SDBS solution of a selected concentration in phosphate-NaCl buffer



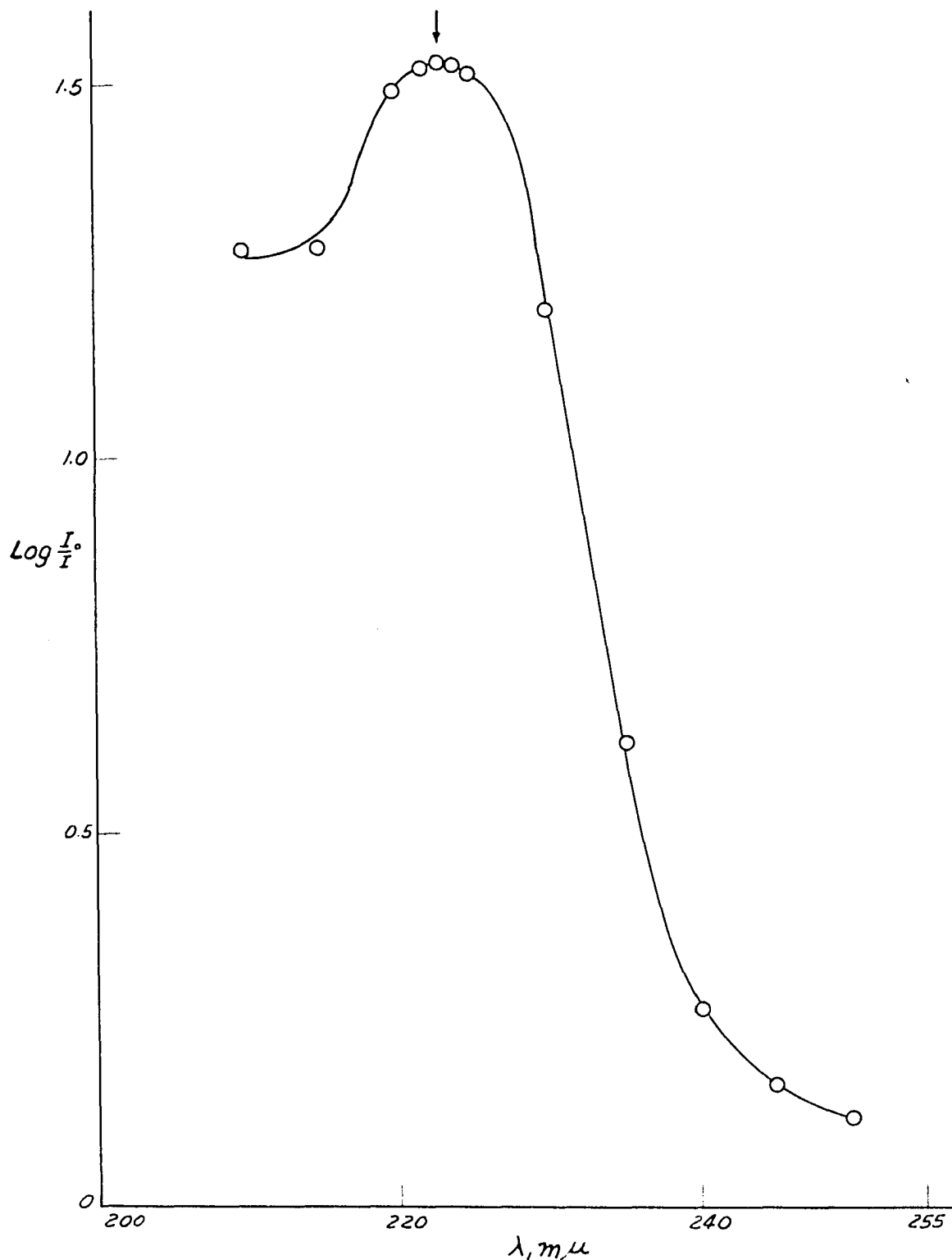


Fig. 7. Ultraviolet absorption spectrum of SDBS in phosphate-NaCl buffer (pH = 7.6,  $\Gamma/2 = 0.20$ ). Anion concentration =  $1.38 \times 10^{-4}M$ .

(pH = 7.6,  $f/2 = 0.20$ ). A maximum absorption was observed at a wave length of  $223\text{ m}\mu$ . To test the applicability of Beer's law, the optical densities at max. of a series of dilute SDBS solutions are plotted against their concentrations in Figure 8. Within the ranges studied (up to  $1.8 \times 10^{-4}\text{ M.}$ ), a straight line passing through the origin showed the law to be obeyed. The corresponding molar extinction coefficient,  $\epsilon$ , was about 11,000. Due to the uncertainty of the readings at optical density below 0.1, SDBS concentrations lower than  $10^{-5}\text{ M.}$  could not be read accurately from this calibration curve. On the other hand, a preliminary study indicated that the ionic strength of the solutions did not effect the readings within experimental error.

## 2. Difficulties in analysis and their remedies

Random errors were observed in the second decimal of the optical readings. Frequently, the densities would vary from 0.01 to 0.02 units, and sometimes even much higher. This was not too serious for the high SDBS concentrations, but it would introduce appreciably large percentage error for the low concentrations of SDBS. Thus, at least duplicate samples were used for all analyses.

Use of plastic centrifuge tubes would introduce interfering substances, probably the plasticizers, thus yielding erroneous results. Hence, stainless steel tubes were used for centrifugation.

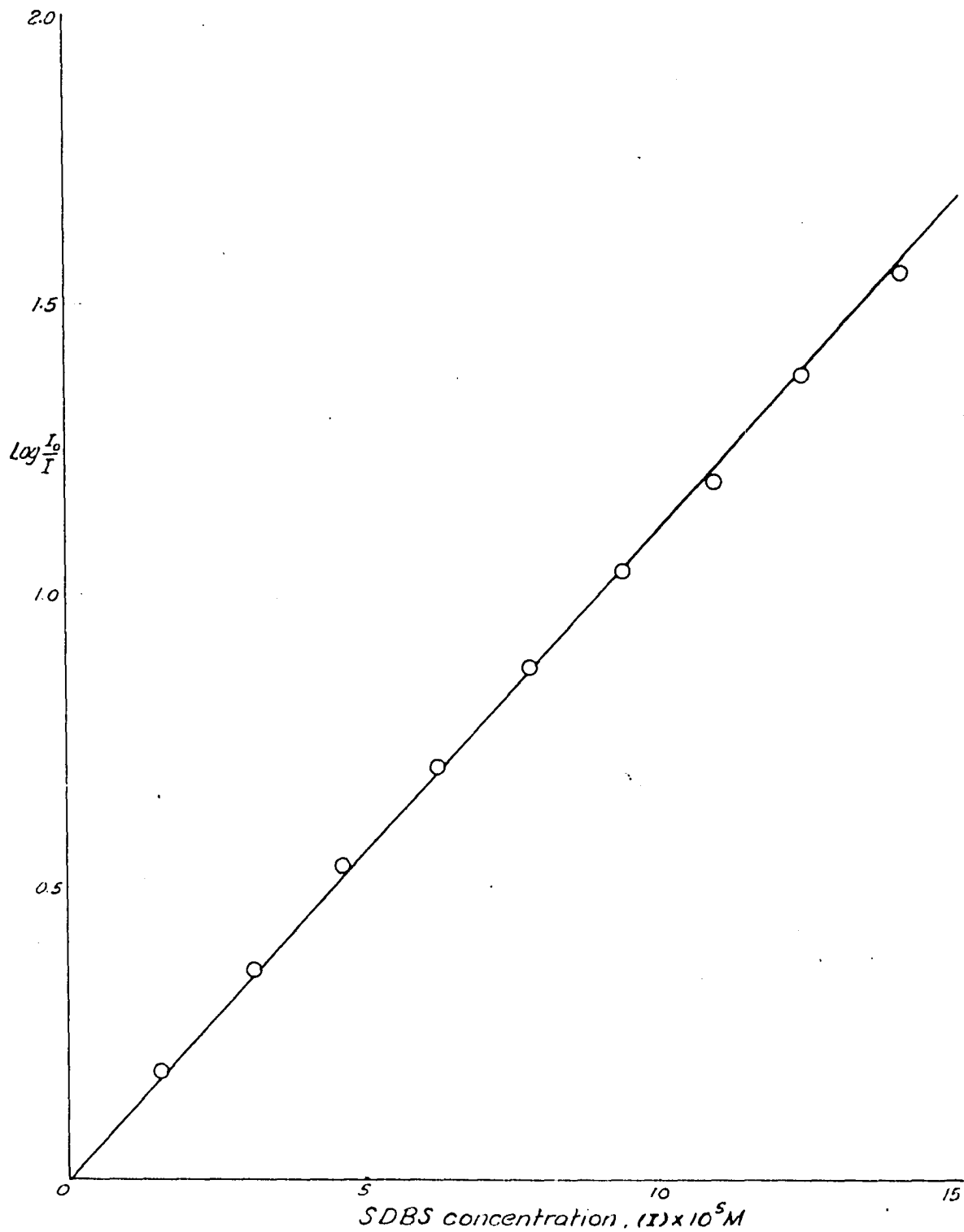


Fig. 8. Calibration curve of SDBS in phosphate-NaCl buffer at  $\lambda_{\max} = 223\mu$ .

Rubber stoppers could not be used in direct contact with the solutions. They were covered with tin foil or substituted with glass stoppers.

The most serious error came from the dialysis bags. During equilibrium dialysis of 20 ml solution against an equal volume of buffer, the blank error could be as high as 0.2 to 0.3 in optical density. Indeed, it was so large that the results were very unreliable. Two methods were developed to minimize this error. In the first method, which was used in this research, the cellophane bags were first dialyzed against a large volume of phosphate buffer for about ten days, fresh buffer being changed every two or three days. Another method was to boil the bags three times for  $1\frac{1}{2}$  hours and rinse thoroughly with distilled water after each boiling. Even with such pretreatment, a blank error of about 0.05 in optical density was frequently obtained. Thus, control runs were often made to minimize such error.

Adsorption of SDBS on the wall of the cellophane tubing was of little significance. Hence no correction was made for such error.

#### B. Equilibrium-Dialysis

Among the methods now developed, the equilibrium dialysis technique has been most satisfactorily applied for the separation of free ions from their protein complexes, thus enabling

the former to be analyzed quantitatively. In the literature this was frequently done by equilibrating the protein solution inside the cellophane bag against the outside ions studied. However, in the case of SDBS, the amount necessary for interaction was limited by its low solubility at low temperature in the presence of phosphate buffer (pH = 7.6,  $\Gamma/2 = 0.20$ ). Consequently a large volume of the dialyzate at low molar concentration was required to cover the whole range of interaction. At such low concentration, the reaction rate was so low that an equilibrium could not be reached even after one month. A typical example is shown in Figure 9, where the SDBS was taken up by bovine albumin very slowly. In Figure 10, a few representative electrophoretic patterns clearly revealed the change of relative composition between albumin and its SDBS complex. Thus, a somewhat modified procedure was employed here. To portions of albumin solution in phosphate buffer, various amounts of SDBS were added; the mixtures were stored in the cold room (1-3°C.) for at least two days, and then dialyzed against equal volumes of the same buffer. The amount of free SDBS was finally determined in the dialyzate. The Donnan correction was made negligible by the presence of high salt concentration. This modification had two advantages over the old method in that, (1) it covered a much wider range of SDBS concentration due to the fact that most of the anion was already bound to the protein

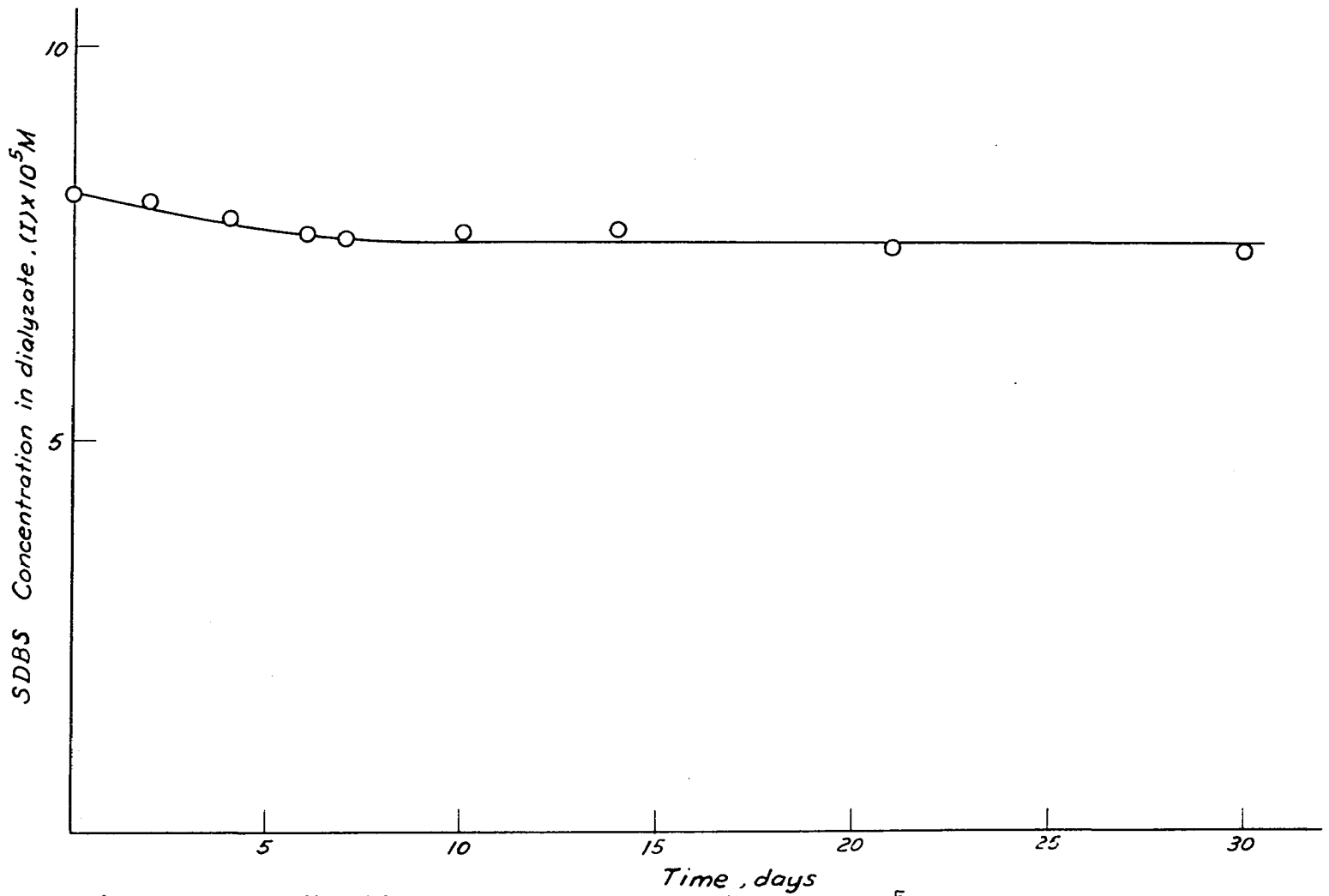


Fig. 9. Equilibration of bovine albumin ( $4.80 \times 10^{-5}M.$ ) against SDBS ( $8.10 \times 10^{-5}M.$ ) in phosphate-NaCl buffer.

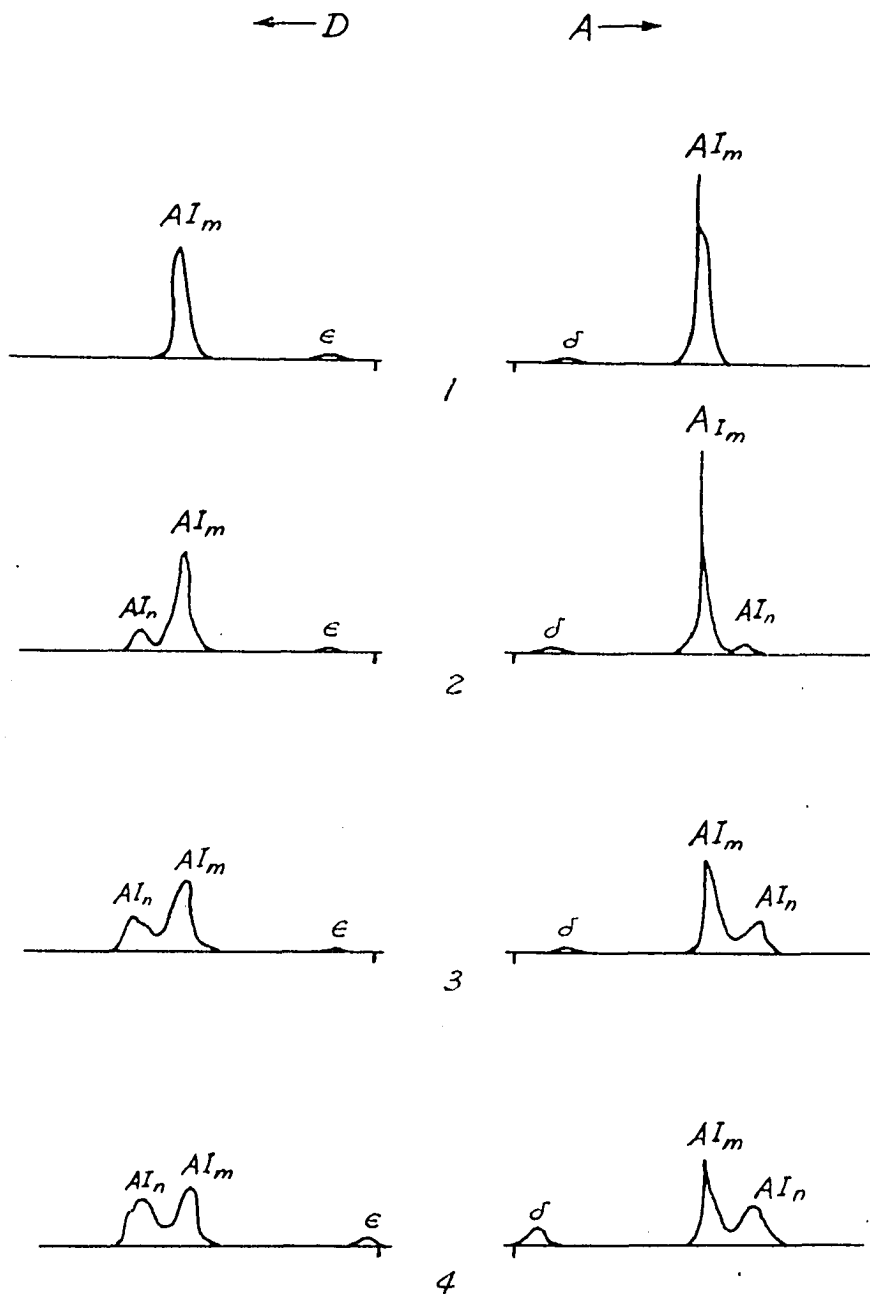


Fig. 10. Electrophoretic analyses of some bovine albumin-SDBS mixtures as represented in Fig. 9.  
1. 4 days, 2. 10 days, 3. 20 days and 4. 30 days.

Table 11. Equilibrium dialysis of bovine albumin-SDBS at 1-3°C. in phosphate-NaCl buffer

Solution	Dialysis time	Concentration of free SDBS
Albumin $6.56 \times 10^{-5} M$	Day 1	$4.58 \times 10^{-5} M$
	4	4.60
SDBS $5.57 \times 10^{-3} M$	7	4.70
	10	4.60

and (2), and equilibrium was reached after one-day dialysis, probably due to the initial high SDBS concentration. A representative experiment is shown in Table 11.

### C. Binding Curve

The effect of SDBS/albumin mixing ratio on the binding capacity is illustrated in Table 12. In Figure 11, the average number of moles SDBS bound per mole albumin,  $r$ , is plotted against the concentration of free SDBS. The initial anion concentration covered a wide range, varying from  $1 \times 10^{-4} M$ . to  $5 \times 10^{-2} M$ ., whereas the albumin concentration was kept constant at  $5.87 \times 10^{-5} M$ . The upper limit of the curve was determined in the following way. Portions of protein solution were mixed with various amounts of SDBS concentration until precipitate of excess free SDBS occurred in the cold room (1-3°C.). The supernatant solution was equilibrated against an equal volume

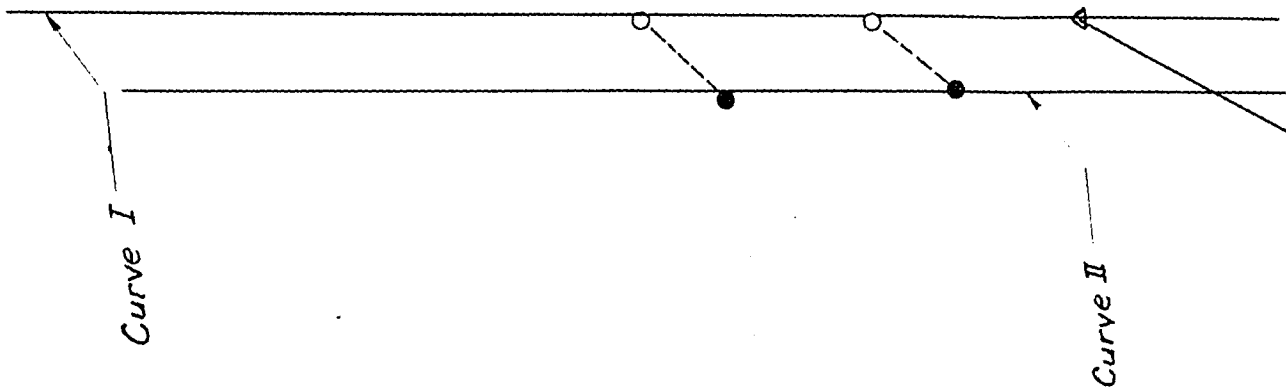


of phosphate-NaCl buffer. The free SDBS concentration in the dialyzate was taken as the limit of the curve. Hence, the amount of free SDBS present in the original solution would be expected to be much higher than that indicated in the curve.

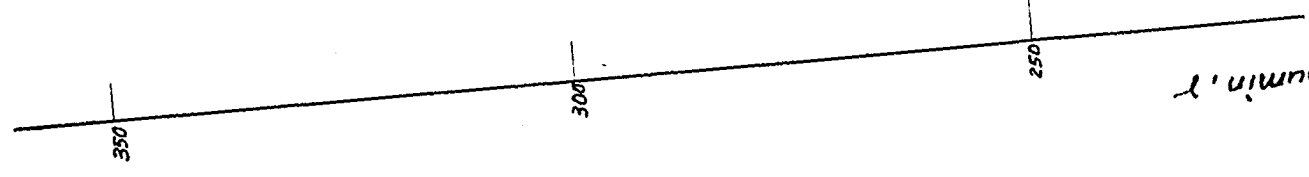
In order to determine the nature of the binding process, electrophoretic analyses were also carried out for the various equilibrium-dialyzed protein-SDBS solutions as shown in the binding curve. Some of the representative electrophoretic patterns are given in Figure 12. The corresponding data covering the whole range of the binding curve are listed in Table 13. Inspection of the patterns clearly revealed that the binding process followed three different stages, which were arbitrarily labelled as regions A, B and C and separated by the two indicated arrows in Figure 11. In region A, only a single boundary marked " $AI_m$ " was observed, the mobility of which was close to that of native albumin. It, therefore, might be suggested that only a few moles of SDBS have been bound statistically to the protein molecule. In region B, a faster albumin-SDBS complex migrated as a separate component at the boundary marked " $AI_n$ " in addition to the  $AI_m$  boundary. This second complex was presumed to contain more anions than  $AI_m$  complex as it had a higher anodic mobility, representing an increase in the net negative charge. At constant protein concentration, the amount of " $AI_m$ " diminished, with increasing SDBS concentration, whereas that of " $AI_n$ " increased, as was

Table 12. Combination of bovine albumin with sodium dodecyl benzene sulfonate at 1-3°C. in phosphate-NaCl buffer  
 (pH = 7.6,  $\Gamma/2 = 0.20$ )  
 Protein concentration =  $5.87 \times 10^{-5}M$

Total concn. of anion	Concn. of free anion	Concn. of bound anion	Av. moles anion bound per mole protein
$\times 10^5 M$	$\times 10^5 M$	$\times 10^5 M$	r
16.7	0.18	16.3	2.78
20.9	- -	- -	- -
30.3	0.44	29.4	5.01
41.8	0.97	39.9	6.80
55.8	3.00	49.9	8.51
83.6	5.23	73.1	12.5
104.4	7.90	82.6	15.1
125.4	10.5	104	17.8
209	17.1	175	29.9
293	26.1	241	41.2
418	34.6	349	59.5
502	45.3	411	70.1
649	58.0	533	91.0
735	68.3	598	102
882	83.7	715	122
1044	95.5	853	145
1233	108.5	1006	171
1457	127.4	1202	204
1871	158.5	1554	264
2050	176.0	1690	289



Protein Concentration  $5.87 \times 10^{-5} M$



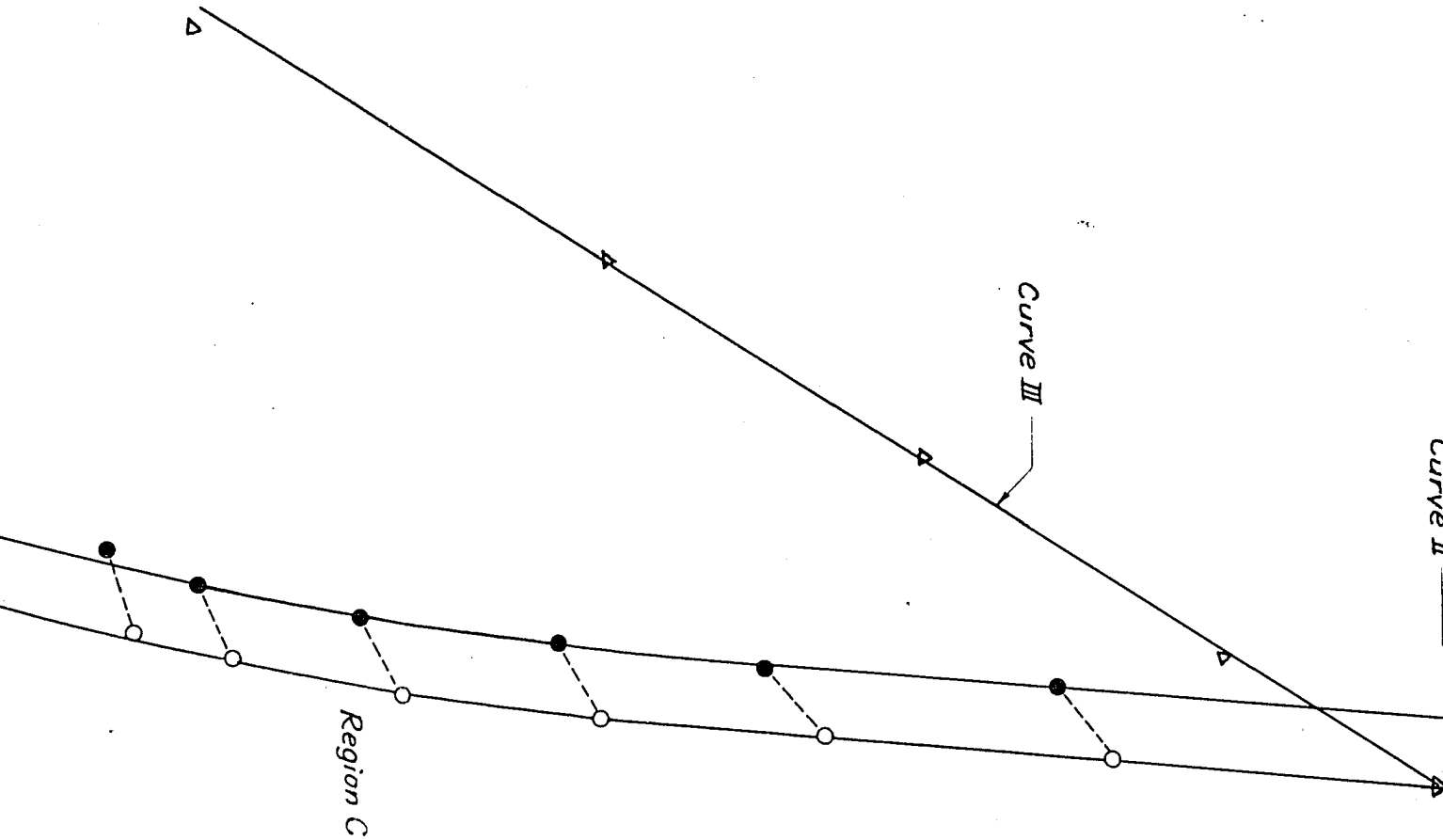


Average moles SDBS bound per mole bovine albumin,  $\bar{\nu}$

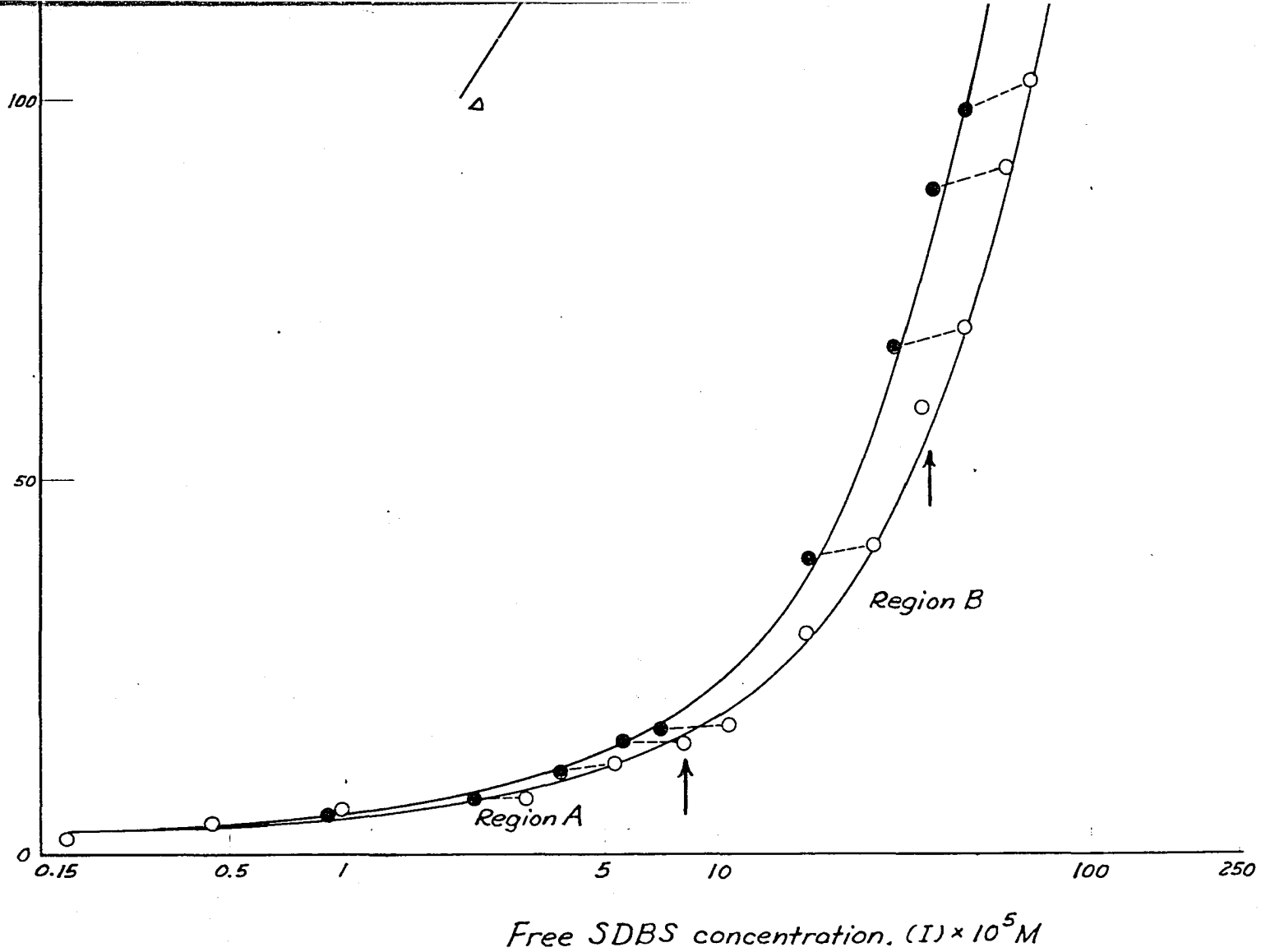
100

150

200







**Fig. 11.** Combination of bovine albumin with sodium dodecylbenzenesulfonate (SDBS) in phosphate-NaCl buffer at 1-3°C. For a discussion of the curves see text.





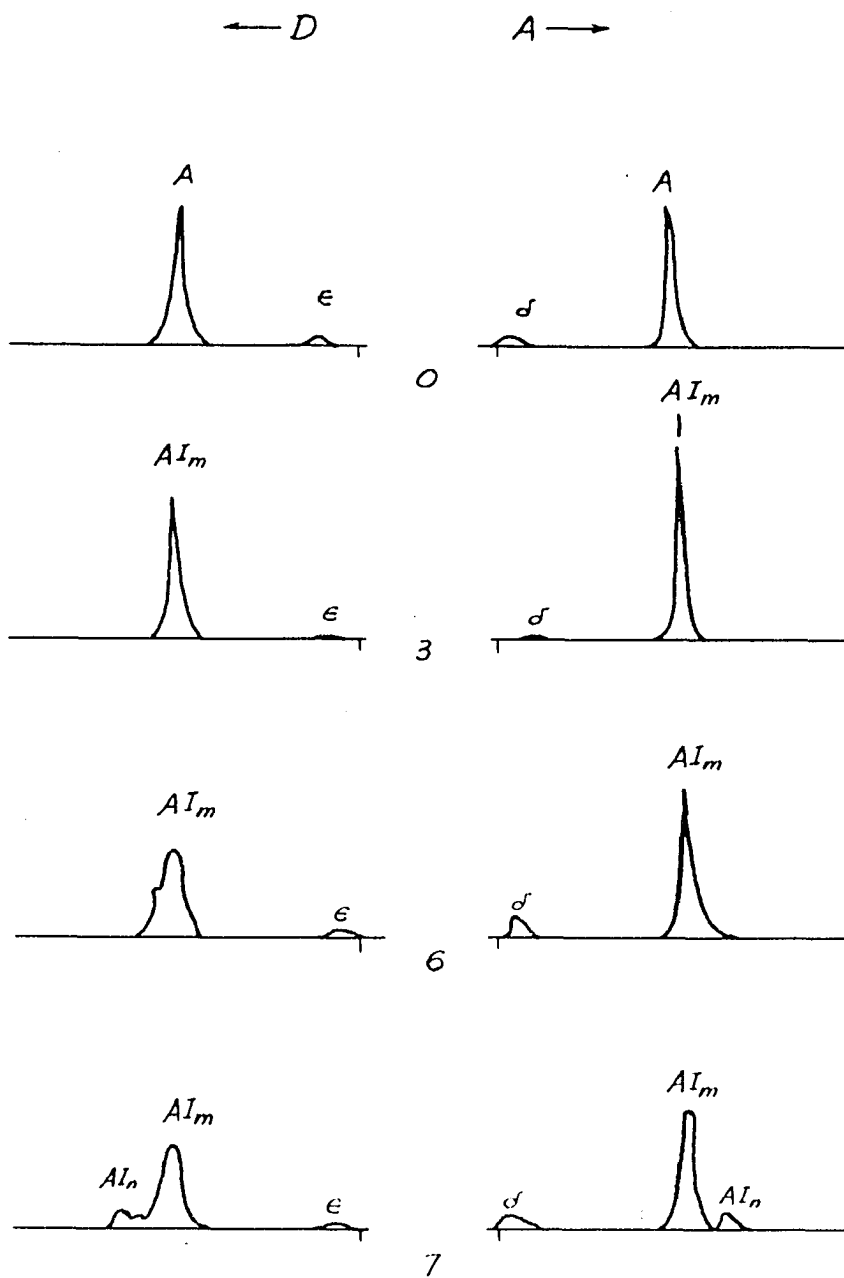


Fig. 12. Electrophoretic analyses of some bovine albumin-SDBS mixtures in phosphate-NaCl buffer. Figures between patterns represent experiment numbers in Table 13. Time of electrophoresis, 150 min. at 4-45 volts  $\text{cm}^{-1}$ , except No. 15, 140 min., and No. 20, 120 min.

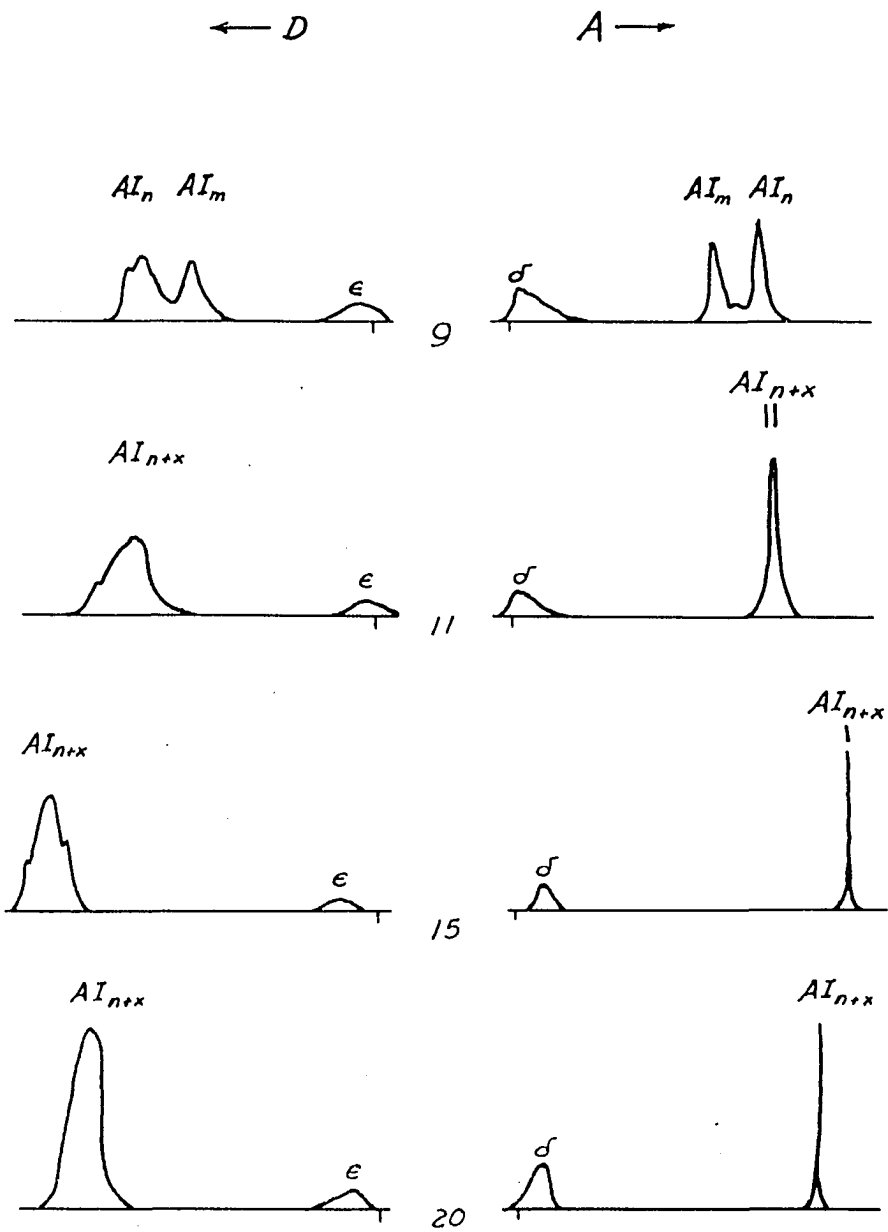


Fig. 12. (Continued).

Table 13. Electrophoretic analyses of some bovine albumin-SDBS mixtures at 2°C in phosphate-NaCl buffer (pH = 7.6,  $\bar{\Gamma}/2 = 0.20$ ). Protein concentration =  $5.87 \times 10^{-5}M$ .

Expt. No.	Av. moles anion bound per mole protein	Bound-ary	Mobilities (1)			Relative Area (2)			
			A	AI <sub>m</sub>	AI <sub>n</sub>	AI <sub>n+x</sub>	A	AI <sub>m</sub>	AI <sub>n</sub>
	r		$-\mu \times 10^5 \text{ cm}^2 \text{ volt}^{-1} \text{ sec}^{-1}$			%	%	%	%
0	0	D	6.7			100			
		A	6.4						
1	2.78	D		6.7		100			
		A		6.9					
3	5.01	D		7.1		100			
		A		7.1					
4	6.80	D		7.2		100			
		A		7.2					
5	8.51	D		7.2		100			
		A		7.5					

(1) A = bovine albumin, AI<sub>m</sub> = bovine albumin-SDBS (first statistical complex), AI<sub>n</sub> = bovine albumin-SDBS ("all-or-none" complex), AI<sub>n+x</sub> = bovine albumin-SDBS (second statistical complex).

(2) Based on the descending patterns.

Table 13. (Continued)

Expt. No.	Av. moles bound per mole protein	anion mole	Bound-ary	Mobilities				Relative Area			
				A	AI <sub>m</sub>	AI <sub>n</sub>	AI <sub>n+x</sub>	A	AI <sub>m</sub>	AI <sub>n</sub>	AI <sub>n+x</sub>
		r	$\mu \times 10^5 \text{ cm}^2 \text{ volt}^{-1} \text{ sec}^{-1}$				%				
6	12.5		D	7.2				100			
			A	7.3	8.67						
7	15.1		D	7.3	9.3		87.9	12.1			
			A	7.5	9.0						
8	17.8		D	7.2	9.2		71.6	28.4			
			A	7.5	9.2						
9	29.9		D	7.1	9.0		44.7	55.3			
			A	7.8 8.9	9.7						
10	41.2		D	7.8	9.9		15.8	84.2			
			A	8.2	10.1						
11	59.5		D			9.4			100		
			A			10.2					
12	70.1		D			11.2			100		
			A			10.9					
13	91.0		D			11.4			100		
			A			11.8					

Table 13. (Continued)

Expt. No.	Av. moles bound per mole protein	anion Bound-ary	Mobilities				Relative Area			
			A	AI <sub>m</sub>	AI <sub>n</sub>	AI <sub>n+x</sub>	A	AI <sub>m</sub>	AI <sub>n</sub>	AI <sub>n+x</sub>
	r		$-\mu \times 10^5 \text{cm}^2 \text{volt}^{-1} \text{sec}^{-1}$				%	%	%	%
14	102	D				11.7				100
		A				11.7				
15	122	D				12.5				100
		A				12.5				
16	145	D				12.3				100
		A				12.5				
17	171	D				12.7				100
		A				12.5				
18	204	D				12.6				100
		A				13.5				
19	264	D				13.1				100
		A				13.6				
20	289	D				13.3				100
		A				14.1				

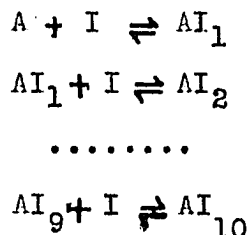
seen in the electrophoretic patterns numbered 6 to 9. From these facts it might be inferred that the reaction in this region was of the "all-or-none" character. In region C, again, there appeared only a single boundary, the composition of which varied with the SDBS/albumin mixing ratio, as evidenced by the increasing mobilities. Thus, it indicated the existence of a statistical distribution.

1. Region A

To test the applicability of the statistical theory in region A, the values  $1/r$  were plotted against  $1/(I)$  in Figure 13. According to the equations:

$$\frac{1}{r} = \frac{1}{Kn} \cdot \frac{1}{(I)} + \frac{1}{n}$$

this would represent a straight line, as indeed illustrated in Figure 13. The reciprocal of the intercept on the axis  $1/r$  gave a value of about 10, which represented a maximum number of sites,  $n$ , in region A. Thus the reactions in region A might be postulated as

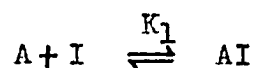


It should be emphasized that the value 10 might be subject to some errors, due to the fact that at very low free SDBS

concentration, the spectrophotometric analysis reached its limit of accuracy.

It is interesting to note that a similar conclusion was obtained by Duggan and Luck (21) from their viscosity measurements of mixtures of bovine albumin and sodium dodecyl sulphate (SDS). About eight or nine molecules of SDS combined with one molecule of bovine albumin to form a stable complex which prevented the normal viscosity rise of albumin in 6 M urea. Further addition of SDS resulted in a rapid increase in viscosity. Therefore, it might be suggested that in region A where statistical binding was predominant no denaturation has taken place.

Assuming that a statistical combination existed in region A, the intrinsic equilibrium constant  $K$  could be estimated from the slope in Figure 13, which is equal to  $1/Kn$ . For  $n = 10$ ,  $Kn$  was in the order of  $2.2 \times 10^6$ . Accordingly, the equilibrium association constant  $K_1$  of the equation:



is equal to  $nK$  (41), thus giving a value of  $2.2 \times 10^5$ .

It should be cautioned that the estimated values might involve appreciable errors for the following reasons. First, at very low SDS concentrations, the percentage experimental error became alarmingly large owing to the limit of spectrophotometric analysis. Secondly, the blank error from the

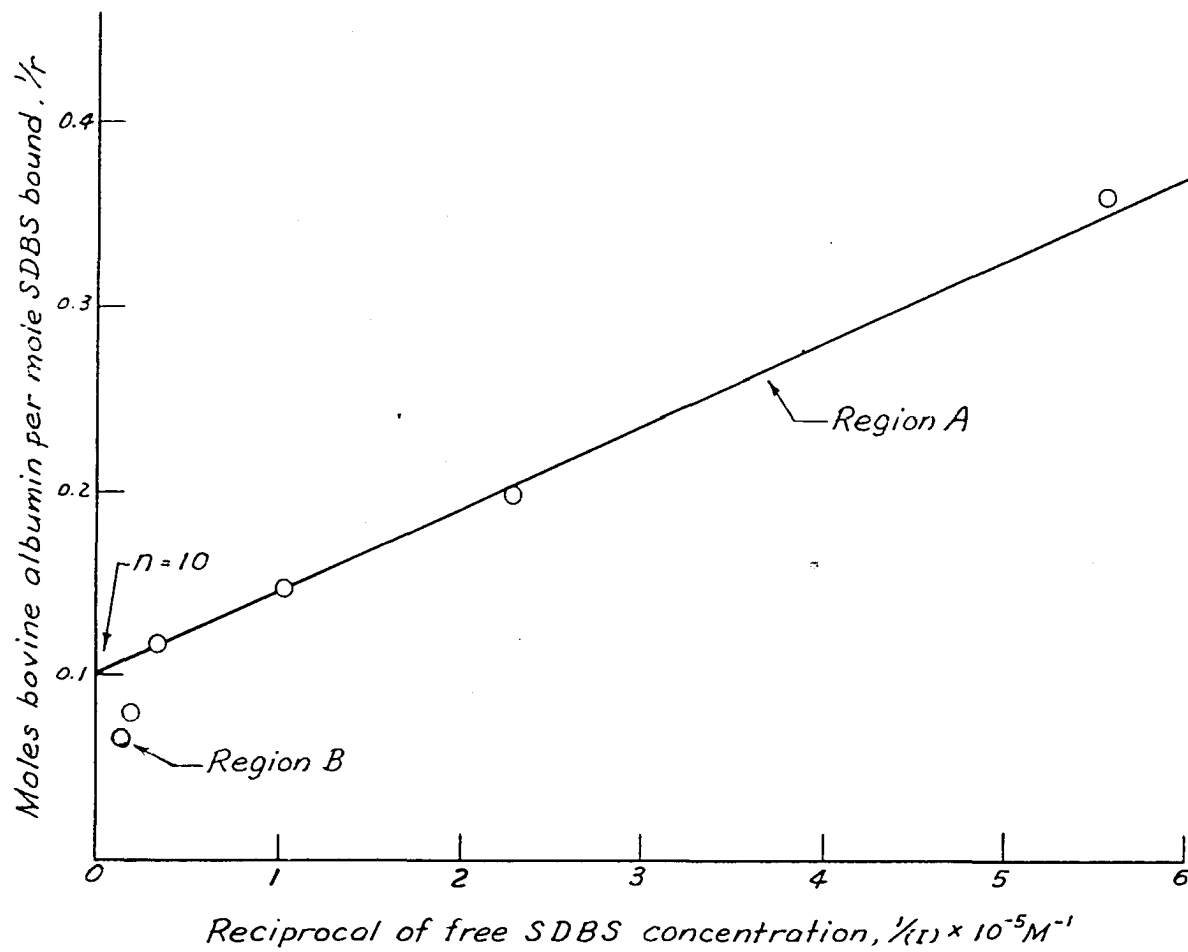


Fig. 13. The extent of binding of SDBS by bovine albumin in region A of the binding curve (see Fig. 11).



dialysis bags was quite uncontrollable, often varying from bag to bag. This became a serious matter at low SDBS concentrations. As would be seen from Figure 13, a slight variation in the amount of free SDBS would not change the  $l/r$  very much but the  $l/(I)$  would shift appreciably. Indeed, in some runs, the optical density of the dialyzate was even lower than that of the blank. An over-correction of blank error would decrease the slope appreciably and consequently increase the equilibrium constant  $K$ .

The last point in region A deviated from the linear function (Figure 13). It could not be explained on the basis of electrostatic repulsion, since it deviated below the straight line. Electrophoretically, this point still exhibited only a single boundary. At first it was thought that this point might belong to region B. Due to the limit of resolution it might not be separated into two complex boundaries. However, a quick estimation of the supposed second complex would give a new boundary of about 6 or 7 % of the relative area. Thus it seemed unlikely this was the explanation. It is also interesting to note that in Figure 13 (see also Figure 15) there seemed to exist another straight line in between region A and B. Possibly there would be a second stage of statistical combination. This question needs further investigation. More experimental data have to be collected.

2. Region B

Analysis of the relative areas under the well-defined boundaries indicated that the composition of the complexes  $AI_m$  and  $AI_n$  was nearly constant and that the distribution between the components depended on the mixing ratio (Table 14). Assuming the first complex  $AI_m$  retained ten SDBS molecules bound per molecule bovine albumin and also, as a first approximation, neglecting the slight difference in refractive increment between the protein and SDBS, the average number of binding sites for the  $AI_n$  complex was estimated to be about forty-eight to fifty.

Table 14. Minimum binding ratio of the second bovine albumin-SDBS complex from electrophoretic measurements

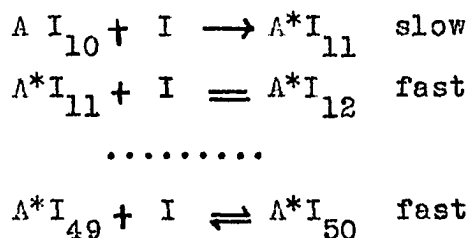
Av. moles anion bound per mole protein(1)	Relative area		Moles anion bound per mole protein
	$AI_m$	$AI_n$	
r	%	%	n
15.1	87.9	12.1	63
17.8	71.6	28.4	41
29.9	44.7	55.7	46
41.2	15.8	84.2	50
			Average 50

(1) The figures referred to those in the binding curve (Figure 11).

Errors involved in such estimation might be briefly stated as follows: (1) A positive error in the value  $r$  would result in a higher number of  $n$ , and a negative error a lower number of  $n$ . (2) The refractive increment,  $\Delta n / \Delta c$ , of the anion (0.00181 at  $\lambda = 5780 \text{ \AA}$ ) was slightly smaller than that of the protein (0.00185 at  $\lambda = 5780 \text{ \AA}$ ). Hence the assumption made above would give a higher value of  $n$  than it should have been. (3) The error due to the boundary anomaly was minimized by using the descending patterns. (4) Theoretically, the faster boundary would tend to appear larger, the slower one smaller. Thus, it would give a negative deviation of  $n$ . (5) Personal error would be involved in the enlarging and tracing of the electrophoretic patterns. This was more serious for a small  $AI_n$  boundary, where a slight error in measurement of area would change the value of  $n$  appreciably. Thus, in Table 14, the first two values were much less reliable than the other two. (6) The assumption that  $n$  was equal to ten in the first complex  $AI_m$  would introduce some error. This again was more serious in the case where only a small second complex  $AI_n$  occurred. (7) The heterogeneity of the anions (see, section D) would affect the binding ratio ( $I/A$ ) by weight. If the higher homologues bind more strongly with the protein, the calculated value of  $n$  would be somewhat smaller than it appeared here. (8) The protein might not be homogeneous. Indeed, some small humps were observed

in the electrophoretic patterns. At present this complication was too involved to consider. For practical purpose, a value of 50 will therefore be employed for subsequent calculations.

The mechanism of interaction in region B is probably related to protein denaturation. The penetration of  $AI_{10}$  molecule by additional SDBS molecules would force the polypeptide chain to unfold. Their combination might be strengthened by a stabilization energy due to van der Waals interaction, thus reducing the potential barrier to the entrance of more SDBS ions. Consequently, in region B protein denaturation would be involved in the "all-or-none" reaction. The reaction mechanism might be postulated as:



where the asterisk \* represents denatured protein. If this is true, it would be of interest to determine the rate of denaturation in the first step. Under section F, a kinetic study of denaturation is to be described.

### 3. Region C

In Figure 14 is plotted the values  $1/r^*$  against  $1/I$ , where  $r^*$  is the number of SDBS moles bound in excess of 50 groups

per mole of protein. Evidently the simple statistical theory could not be applied in this region. The negative extrapolated intercept on the axis  $1/r^*$ , as indicated by the dotted line, is certainly meaningless. On the other hand, the curvature fits qualitatively with the hypothesis that the electrostatic interaction between bound SDBS molecules became predominant and thereby less ions were bound than should have been, due to Coulomb repulsion. The reciprocal of the intercept on the  $1/r^*$  axis gave as the maximum number of sites,  $n$ , more than 500. Although this value is not reliable, it nevertheless indicates that the protein molecule could bind appreciable amount of SDBS in excess of its basic groups. Thus, in region C, van der Waals forces and other factors would become more important than in region A and B.

In Figure 15 is plotted the values  $r/(I)$  against  $r$ , covering the whole range of the binding curve. According to Scatchard's equation for statistical combination,

$$\frac{r}{(I)} = nK - rK$$

The intercept on the  $r$  axis for region A again gave a value of ten as obtained in Figure 13. It is interesting to note that there appeared another straight line in between region A and B and all of region C, the value of  $r/(I)$  was almost independent of the value of  $r$ . In other words, the number of moles SDBS bound per mole bovine albumin was virtually a linear

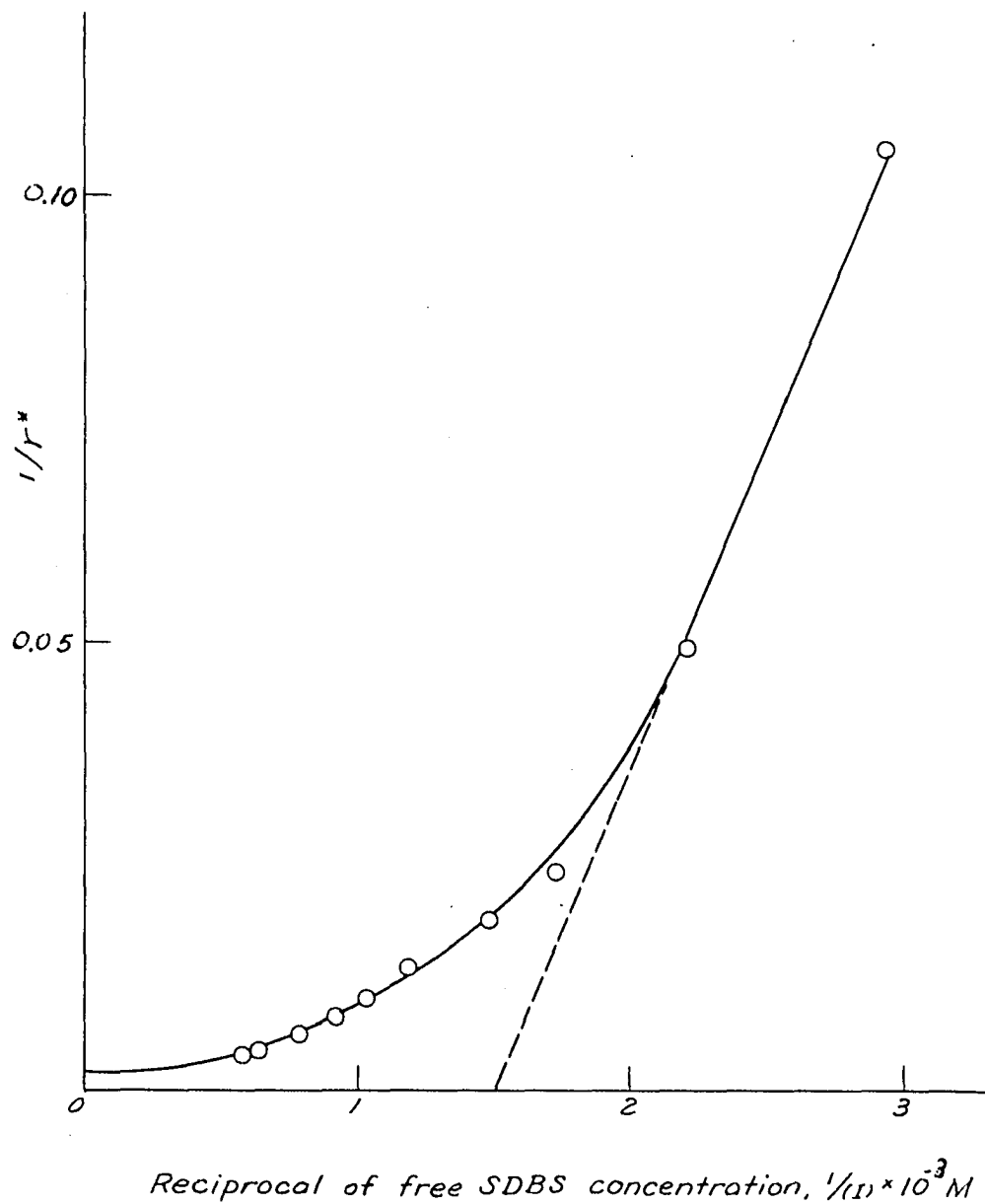


Fig. 14. The extent of binding of SDBS by bovine albumin in region C of the binding curve (see Fig. 11).

function of the free SDBS concentration. It is not yet known whether this has certain significant explanation.

Questions might be raised with regard to the validity of the binding curve for the following reasons. First, the binding curve was plotted against the total concentration of free SDBS. This is probably not justifiable in regions B and C where micelles are expected to appear. Thus, if it were possible to plot as a function of concentration of single ions the curve might be shifted to the left and the value of  $r$  would increase much more sharply than it appeared here. Secondly, the non-homogeneity of the anions (see next section) would also favor the leftward shift. The lower homologues of the anions were expected to have less affinity for the protein than the higher ones, thus leaving more free anions in the solutions. This would become more significant at higher SDBS concentrations. Consequently, it would reduce the sharpness of the rising curve. In region B where the "all-or-none" reaction was postulated to be irreversible, the curve was expected to rise vertically in a manner similar to an ideal titration curve. Quite possibly the presence of inhomogeneous anions caused such deviation from the theoretical deduction.

Despite these objections, it seems safe to postulate that the binding process between albumin and SDBS proceeds through three stages. It is also expected that the general shape of the curve would not be too different from the one

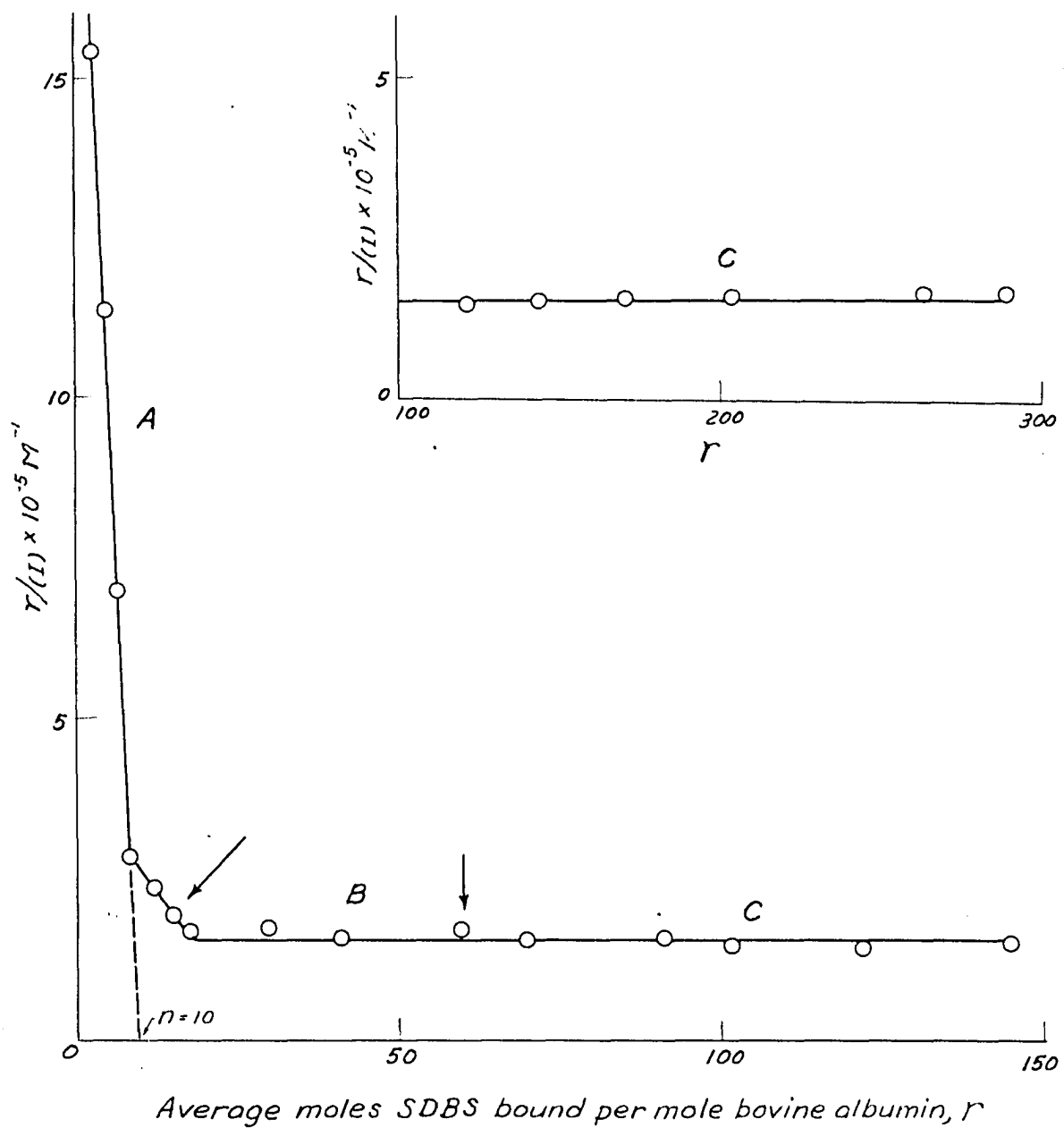


Fig. 15. The extent of binding of SDBS by bovine albumin (see Fig. 11).



described above.

D. Solubility of Sodium Dodecylbenzenesulfonate (SDBS) and  
its Heterogeneity

The solubility of SDBS at low temperatures is limited by the amount of salt present (salting-out-effect). This could be determined experimentally in the following manner. A 0.2% SDBS solution in phosphate buffer was cooled down to 1-3°C. The saturated suspension was then equilibrated against the buffer. Spectrophotometric analysis of the dialyzate gave a saturated concentration of  $7.5 \times 10^{-4}M$ . Direct determination of the supernatant solution was avoided for the reason that rise in temperature during centrifugation would increase the solubility of some SDBS. This was evidenced from the turbid appearance of the supernatant solution on standing.

Quantitative study of interaction between proteins and SDBS was complicated by the non-homogeneity of the latter. In this research, no attempt was made to separate the homologous alkyl compounds. Consequently, some inconsistent results were obtained. This was well illustrated from the binding curve and the solubility data. In Figure 11, the amount of free anions present could be well above  $7.5 \times 10^{-4}M$  and yet not precipitate was found in the dialyzate. Owing to their greater affinity, the higher homologues of the anions would be bound to the proteins in preference to the lower ones. Consequently, the latter were left in the solution in greater

proportion and the solubility of the anions was thereby increased on the molar basis. In this research it was assumed that the molar extinction coefficients were the same for the homologues. This is expected to be not too far from the fact.

#### E. Concentration Dependence of Binding

In Figure 16 are plotted two binding curves: (1) the dotted line reproduces part of Figure 11 at constant protein concentration of  $5.87 \times 10^{-5}M$  and (2) the solid line represents the binding ratios at constant protein concentration of  $3.83 \times 10^{-5}M$ . Comparison of the two curves clearly revealed the concentration dependence of the binding affinity. At the same free SDBS concentration the value of  $r$  was greater at lower protein concentration than at higher protein concentration. When the protein solution becomes more concentrated it is not unlikely that the extent of binding might be somewhat affected by change in properties of the medium. To be sure, the difference was not too great. Part of this discrepancy might also be due to the heterogeneity of the anions. For the same  $r$  value the total amount of the anions bound would be greater at higher protein concentration. Accordingly the amount of unbound lower homologues would also be greater and the binding curve tend to shift to the right. This discrepancy would become larger at higher concentration of free SDBS where the deviation due to the inhomogeneity of the anions would be much more significant, as indeed illustrated in the two curves in Figure 16.

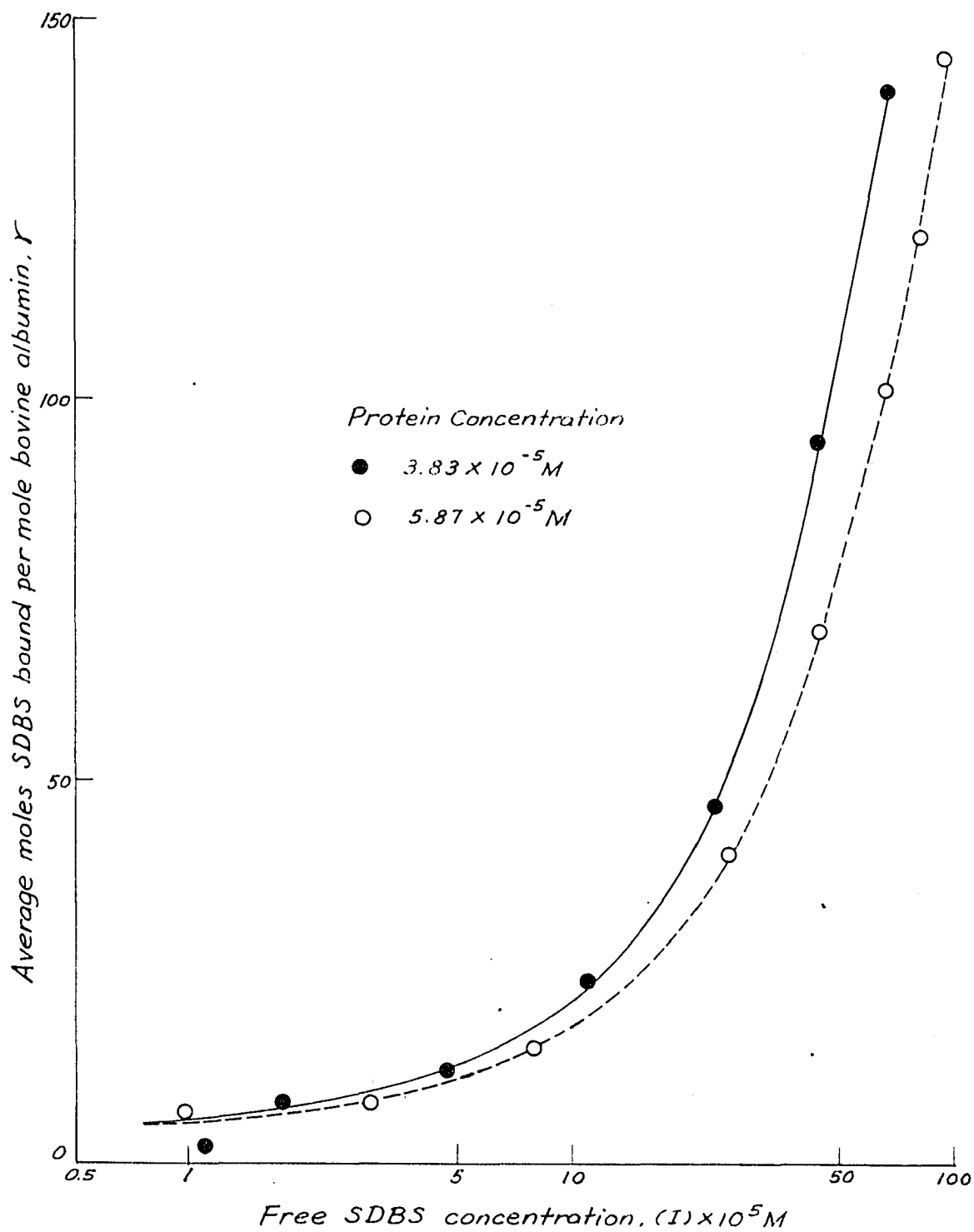
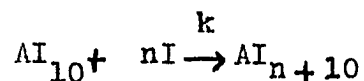


Fig. 16. Concentration dependence of the extent of binding of SDBS by bovine albumin.

## F. Kinetic Study of Interaction

As mentioned earlier, the rate of interaction between proteins and SDBS was slow when they were separated by cellophane bags and dialyzed against each other. It was therefore desirable to conduct a kinetic study of interaction, the results of which might reveal the mode of denaturation which has been postulated for region B. During equilibrium dialysis it was impossible to keep constant the total amount of SDBS inside the bags, thus complicating the calculations. The other alternative was to maintain a constant activity of free SDBS and thereby follow the rate of change of the protein. Advantage was thus taken of the low solubility of SDBS in buffer solution at low temperature. Portions of bovine albumin (0.4%) were dialyzed against an equal volume of SDBS suspension (0.2%) at 1-3°C. Electrophoretic analyses were made at definite intervals (Table 15). Since it took some time for SDBS ions to reach an initial equilibrium through diffusion and also for the formation of statistical complex  $AI_{10}$ , the amount of albumin as  $AI_{10}$  after one day dialysis was taken as the initial concentration. In Figure 17, was plotted the percentage of unchanged albumin as  $AI_{10}$  against the time. A straight line indicated a first order of reaction with respect to the albumin at constant activity of SDBS. According to the equations



$$\frac{-dAI_{10}}{dt} = k(AI_{10}) (I)^n = k'(AI_{10})$$

the reaction constant  $k$  was expected to be very large, since the concentration of SDBS was in order of  $10^{-4}M$ .

It should also be pointed out that the concentration of free SDBS in the above experiment was within the range of region C in the binding curve. Thus it was evident that the first step in region C was also an irreversible reaction, followed by a statistical combination when enough SDBS was present to form more than the  $AI_{50}$  complex.

Another experiment was carried out at lower SDBS concentration, where a large volume of dialyzate was used so that the activity of SDBS was kept virtually unchanged. The results are also shown in Table 15 and Figure 17. Inspection of the straight line indicated that the rate of reaction was nearly independent of the SDBS concentration. At the first thought, this strange phenomenon was difficult to explain. However, since the single ions were in equilibrium with their micelles, that is,

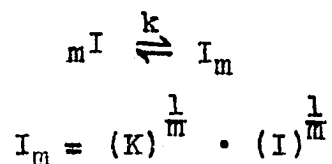


Table 15. Kinetic study of bovine albumin-SDBS complex formation at constant anion concentration at 1-3°C. from electrophoretic measurements

Concn. of anions	Time	Relative Area <sup>(1)</sup>		Calcd. compn <sup>(2)</sup>		A/A <sub>0</sub> as AI <sub>m</sub>
		AI <sub>m</sub>	AI <sub>n</sub>	AI <sub>m</sub>	AI <sub>n</sub>	
X10 <sup>5</sup> M	days	%	%	%	%	%
75.3	1	86.0	14.0	88.0	12.0	100
	4	55.6	44.4	59.8	40.2	68.2
	7	39.5	60.5	43.8	56.2	50.2
	10	23.7	76.3	27.1	72.9	30.7
	14	17.9	82.1	21.0	79.0	23.9
	21	9.0	91.0	10.7	89.3	12.2
	30	4.0	96.0	95.1	5.6	5.6
	2	100	0	100	0	100
20.8	5	73.1	26.9	76.5	23.5	76.5
	9	46.7	53.3	51.3	48.7	51.3
	14	22.2	77.8	25.7	24.3	25.7
	20 <sup>(3)</sup>	0	100	0	100	0

(1)Based on the descending patterns.

(2)Assuming M = 10 and n = 50.

(3)Appreciable amount of the protein precipitated.

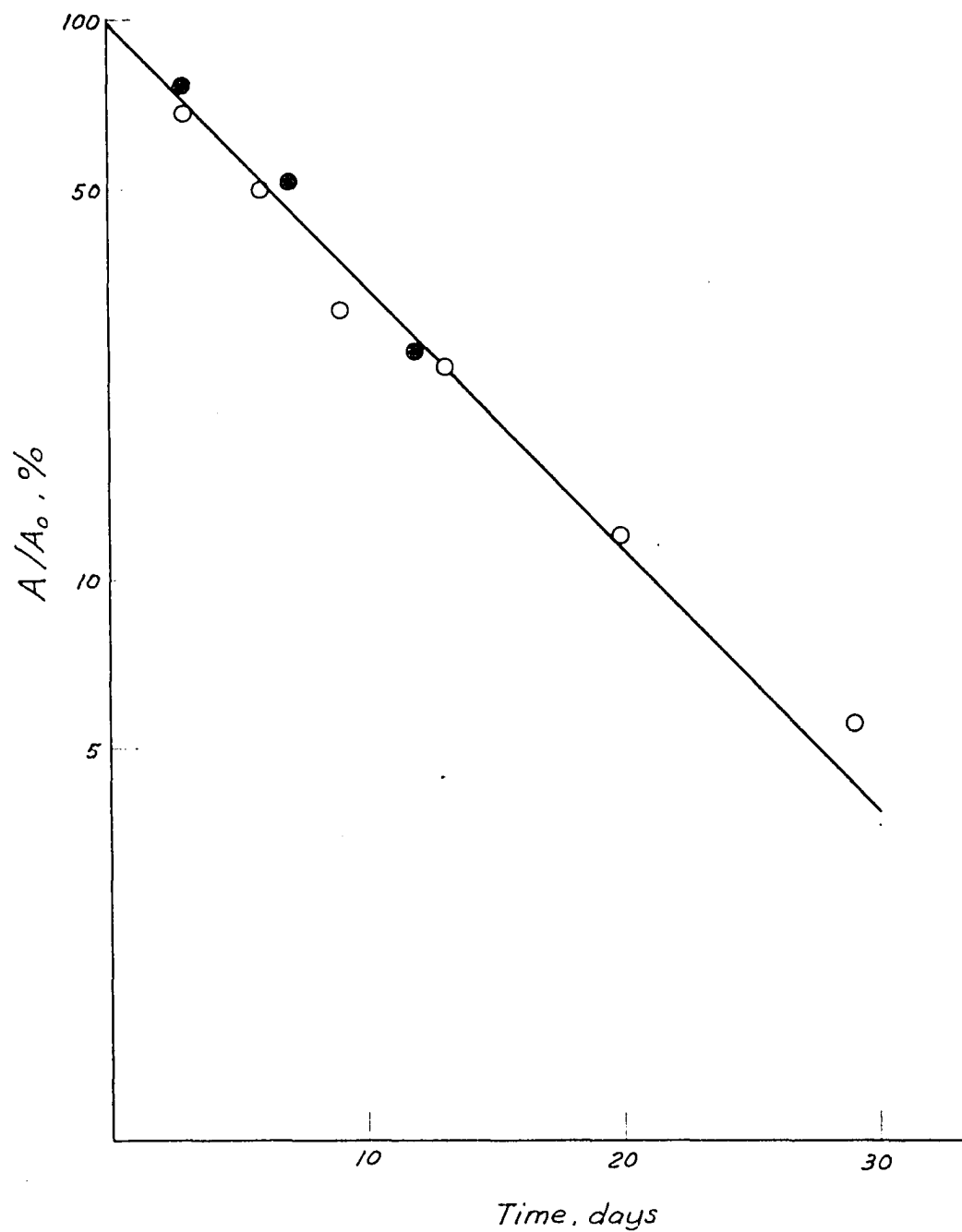


Fig. 17. Kinetic study of combination of bovine albumin with SDBS at constant anion concentration at 1-3°C.

a several-fold variation in SDBS concentration would hardly affect the amount of free single ions owing to the large value of  $m$ . Therefore, in the foregoing results, the order of reaction,  $n$ , with respect to free SDBS could not be determined.

In the second experiment the electrophoretic pattern still exhibited only a single boundary after two-days dialysis. Since the SDBS concentration was much lower than that in the first experiment, it would take a longer time to reach the second stage of the "all-or-none" reaction. Thus some error might be introduced in the calculation in Table 15, where the ratio  $A/A_0$  was taken as 100% at two-days dialysis. It was not known exactly whether the second reaction began at that time or later. Accordingly, the solid points in Figure 17 might be expected to shift slightly to the left. Such changes, however, would be rather small as could be shown in Figure 17.

After prolonged dialysis the protein solution inside the cellophane bag began to turn turbid, probably due to surface denaturation on exposure to the air during dialysis. Consequently, almost no native protein was left at the end of twenty-five days dialysis. Thus, the ratio  $A/A_0$  could no longer be determined at this point.

The dialyzate became slightly turbid on standing at 1-3°C., even though the SDBS concentration was well below its average solubility. This was again a reflection of heterogeneity of



the anions.

Two series of experiments were also carried out at different SDBS concentrations of constant activity. This was done by varying the amount of sodium chloride in the phosphate-NaCl buffer to give an ionic strength of 0.18 and 0.22 instead of 0.20. Similar calculations were made from the electrophoretic analyses. The results however turned out somewhat inconsistent with those mentioned above. Instead of a straight line, the plot of  $\log (A/A_0)$  against time was slightly curved. Thus a definite conclusion cannot be given at the present moment.

Mixtures of bovine albumin and SDBS were also studied kinetically in region A. The solution containing  $5.87 \times 10^{-5}M$  protein and  $37.5 \times 10^{-5}M$  anions was stored in the cold room. Portions were taken at 1, 4, 7 and 10 days and equilibrium-dialyzed against the phosphate buffer. Virtually no change in the optical density of the dialyzate was observed. To be sure, some errors might have been introduced as the analytic method reached the limit of accuracy. However, it is interesting to note that the initial concentration of the free anions was in the range of region B. In a short time, most of them were bound statistically to the protein, thus leaving the free anions in region A. Therefore, it might be postulated that the statistical equilibrium is a fast reaction.

### G. Reversibility of Binding Process

In order to justify the correlation between the binding affinity and denaturation of the albumin it was necessary to ascertain whether the binding process was a reversible one. This was done experimentally by immersing dialysis bags containing protein-SDBS mixture which had previously been equilibrated against phosphate buffer (Curve I, Figure 11) into an equal volume of fresh buffer and determining the new equilibrium values of  $r$  and  $I$ . The results obtained are shown by curve II in Figure 11. The corresponding points between the two curves are connected with broken lines. This second curve involved a further removal of anion from the albumin-SDBS complex. It clearly indicated that the binding process was not completely reversible; otherwise the two curves would be expected to coincide with each other.

In region A, there seemed to exist complete equilibrium. This was in good agreement with the statistical theory. However, it should be emphasized that appreciable errors might be introduced at such low concentration of the anions. Since the value  $r$  was very small in region A, slight deviation from the curve might not be detected. The discrepancy due to the heterogeneity of the anions however was not serious in this region for the fact that the amount of total free SDBS was very low itself.

In regions B and C, deviation from reversibility became significant as revealed from the two binding curves, I and II. If the reaction was completely irreversible, the value  $r$  was expected to be unchanged during further dialysis. The binding curve I should also be nearly vertical and independent of the free anion. This was certainly not true as evidenced from the results obtained. Indeed, the amount of free anion in the second equilibrium-dialysis was more than one half of that in the first one, indicating that some, though very little, of the bound anion was taken away from the complex. From these findings, it might be inferred that the interaction was somewhat reversible, but much in favor of the association due to mutual strong affinity between the protein and the anions. Heterogeneity of the anions might again enhance the discrepancy for the following reason: The lower homologues having less affinity for the protein remained mostly as free anion. Consequently, the amount of free anions was much greater in the first equilibrium than the second one. This was also in agreement with the experimental results where difference between the two curves was more significant at higher SDBS concentrations.

To further test the irreversibility of the binding process, portions of a mixture of albumin and SDBS in region C were equilibrated for one week against different volumes of phosphate buffer, varying from 20 ml. to 10 l. The results are

plotted as curve III in Figure 11. Evidently not all SDBS bound was removed even with 10 l. dialyzate. The lowest point in curve III still retained more than 100 moles of the anion per mole of protein. This agreed with the results described in part I, where protein-SDBS complex could be prepared through prolonged dialysis. Electrophoretic analysis of the corresponding solutions in curve III also exhibited only a single boundary. In the literature, the amount of anions in excess of that accounted for by the basic groups of the protein was termed as extra, loosely bound, anions. This seemed also true in curve III.

Complete reversal of the binding process was also attempted by other methods. Use of anion exchange resin in the dialyzate did not improve the removal of bound anions. Addition of an excess of barium chloride to the mixture of albumin precipitated not only most of the bound anion, but also an appreciable amount of the regenerated protein. The remaining protein in the solution, however, still retained the anions as a complex as shown by the electrophoretic patterns. Another method involving extraction with 60% acetone might remove the bound anions. It would, however, not be possible to distinguish whether the regenerated protein had been denaturated by the acetone or by the anion. Even though the bound anions could be removed by using barium salts or 60% acetone, the question whether the disruption

of the protein structure initiated by combination with surface active ions is fully reversible is entirely another matter. As has been postulated, region A of the binding curve involved no protein denaturation, and region B an "all-or-none" reaction, causing the unfolding of the protein molecule. For regenerated (denatured) protein these two regions might be expected to be absent. The binding process would probably begin directly with region C in a statistical manner. Indeed, Lundgren and coworkers (68) confirmed from the electrophoretic analysis that heat-denatured ovalbumin combined statistically with SDBS in all proportions. The loosely-folded regenerated protein molecule would also be expected to be penetrated by the anions more easily than the native protein molecule. Thus, the binding curve in Figure 11 might be shifted upward. In other words, at the same free SDBS concentration the average number of anions bound per mole protein,  $r$ , would be higher for the regenerated protein than for the native protein.

#### H. Binding Capacity of Ovalbumin with SDBS

For the sake of comparison of binding affinity between protein and surface active ions, the binding affinity of SDBS by ovalbumin is listed in Table 16 and plotted in Figure 18, similar to that of bovine serum albumin and SDBS. Comparable

Table 16. Combination of ovalbumin with sodium dodecyl benzene sulfonate at 1-3°C. in phosphate-NaCl buffer (pH = 7.6,  $f/2 = 0.20$ ) Protein concentration =  $9.25 \times 10^{-5}M$ .

Total concn. of anion	Concn. of free anion	Concn. of bound anion	Average moles anion bound per mole protein
$10^5M$	$10^5M$	$10^5M$	r
10.55*	1.38	7.7	0.83
20.9*	2.25	16.4	1.77
41.8*	6.75	28.3	3.06
83.6	10.8	62.0	6.70
125.4	14.4	96.6	10.4
167	17.6	132	14.3
209	20.8	167	18.1
251	25.8	199	21.6
334	32.6	269	29.1
418	39.0	340	36.8
627	57.5	512	55.3
836	75.5	685	74.0
1044	94.8	854	91.3
1276	112	1052	110
1580	141	1298	137
2210	194	1822	197

\*In these runs, part of the ovalbumin precipitated during equilibrium-dialysis.

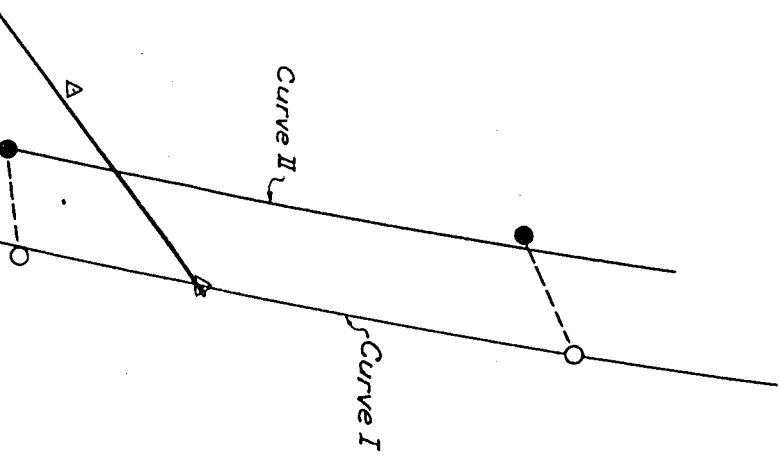
S bound per mole ovalbumin,  $r$

150

200

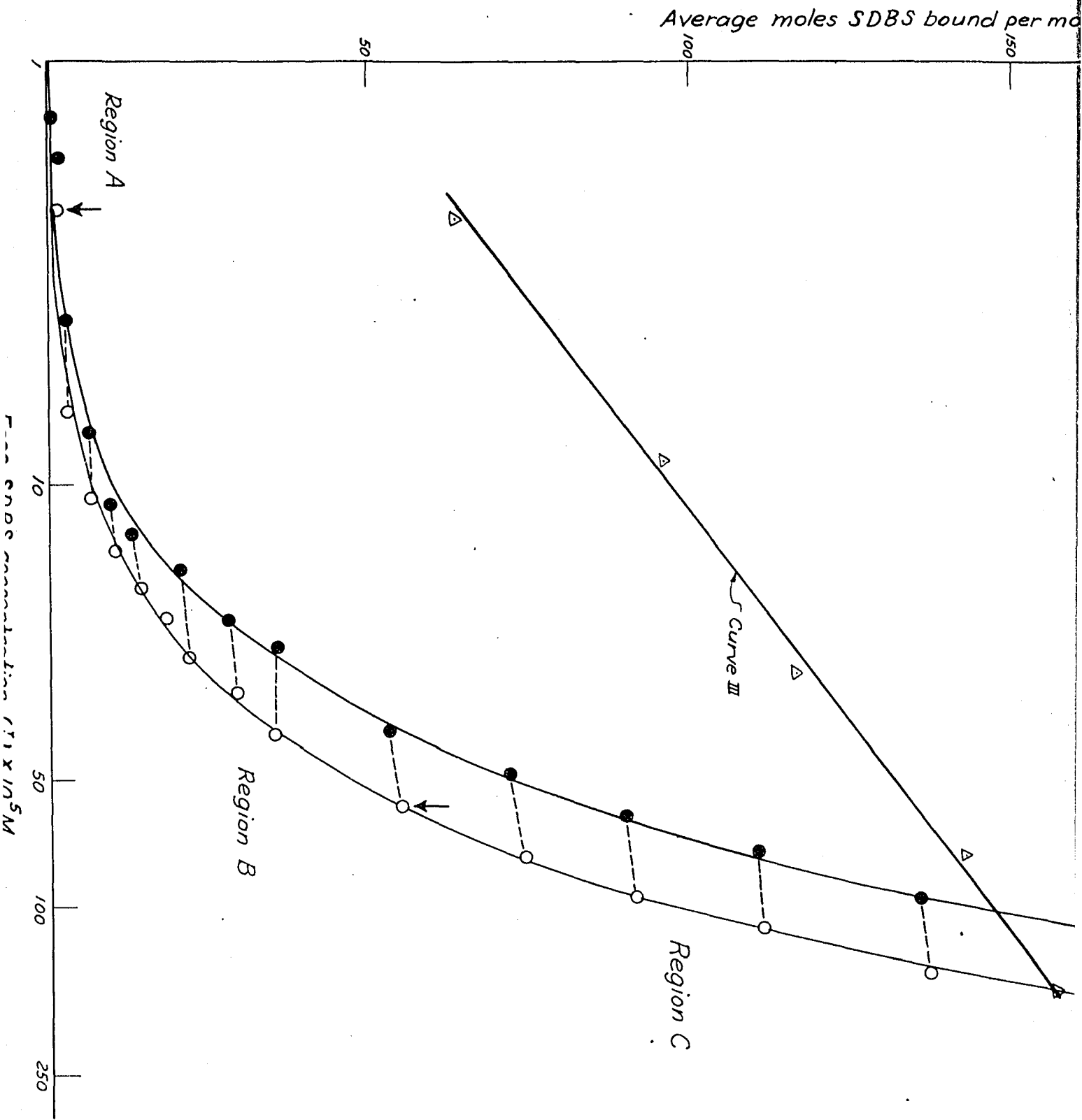
250

Protein Concentration  $9.25 \times 10^{-5} M$











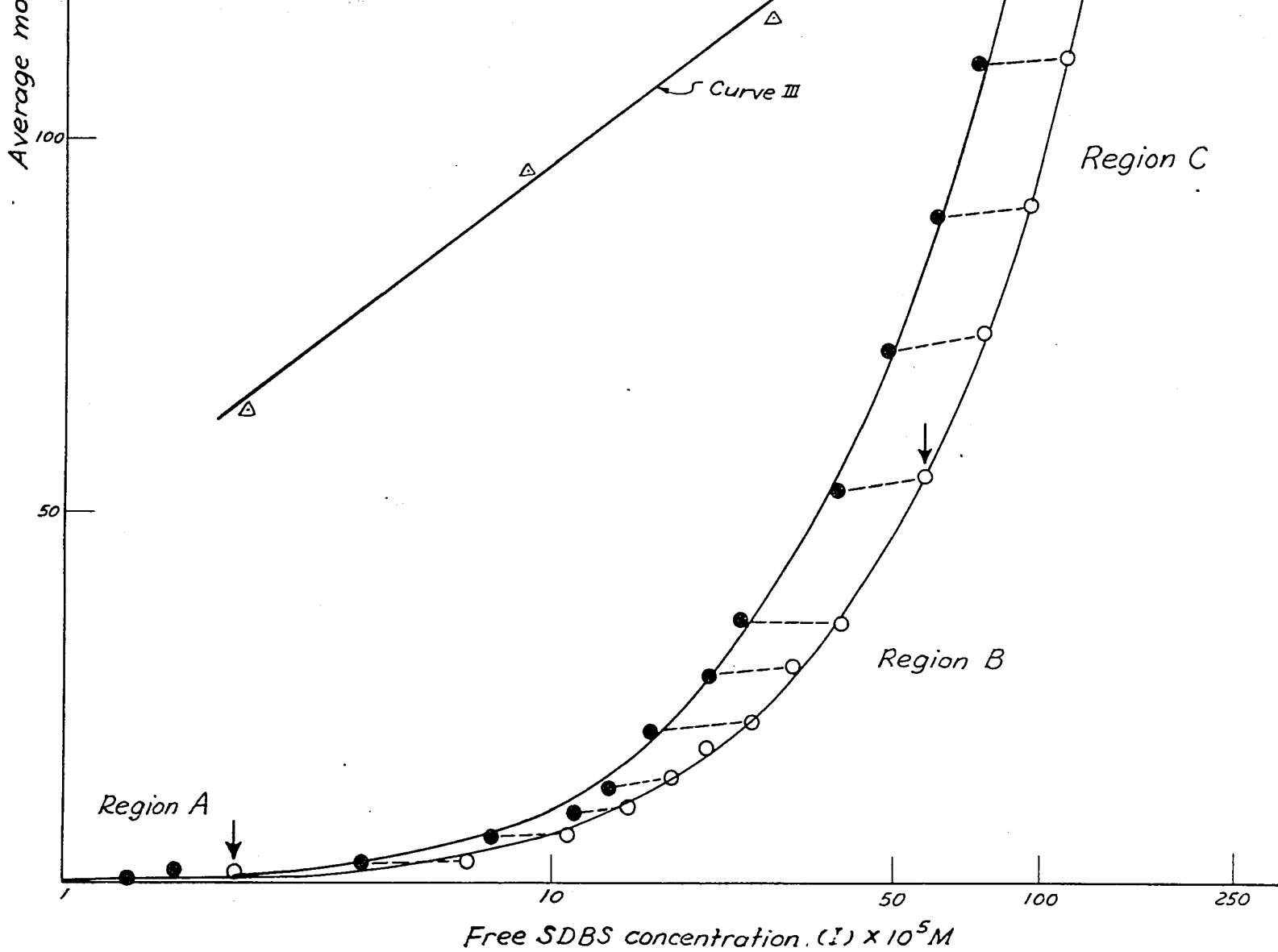


Fig. 18. Combination of ovalbumin with sodium dodecylbenzenesulfonate (SDBS) in phosphate-NaCl buffer at 1-3°C. For a discussion of the curves see text.



results were again obtained; that is, the number of SDBS moles per mole of ovalbumin,  $r$ , at first increased slowly and then rapidly bent upward, as the amount of free ions increased. However, some striking facts were revealed in the electrophoretic analysis (Figure 19 and Table 17).

### 1. Region A

There was virtually no region A present where statistical reaction was supposed to be predominant. Even at  $r$  less than two, the "all-or-none" reaction appeared and thereby began the region B. At very low SDBS concentrations, the calculated value  $r$  was further complicated by the precipitation of ovalbumin during equilibration. This again indicated that small amounts of SDBS could not stabilize the denatured ovalbumin (probably due to surface denaturation). In other words, the number of SDBS molecules bound was not sufficient enough to keep the protein in solution. The absence of region A seemed to be in good agreement with the susceptibility of ovalbumin to denaturation. Since ovalbumin is more easily denatured than bovine serum albumin, the region B was expected to occur much sooner in this case.

### 2. Region B

In Figure 19 the "all-or-none" character of complex formation was electrophoretically indicated by the migration of

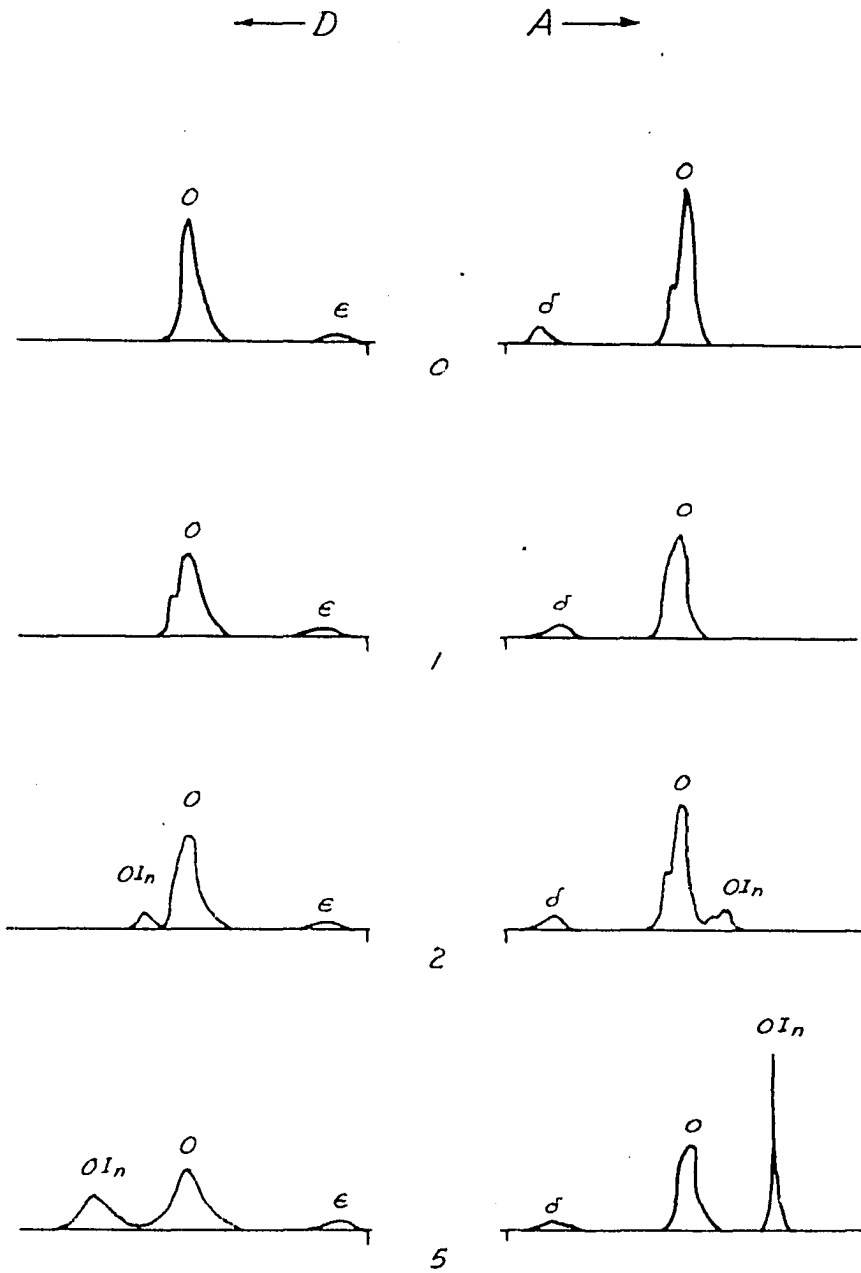


Fig. 19. Electrophoretic analyses of some ovalbumin-SDBS mixtures in phosphate-NaCl buffer. Figures between patterns represent experiment numbers in Table 17. Time of electrophoresis, 150 min. at 4-4.5 volt  $\text{cm}^{-1}$ , except No. 14, 133 min., and No. 16, 131 min.

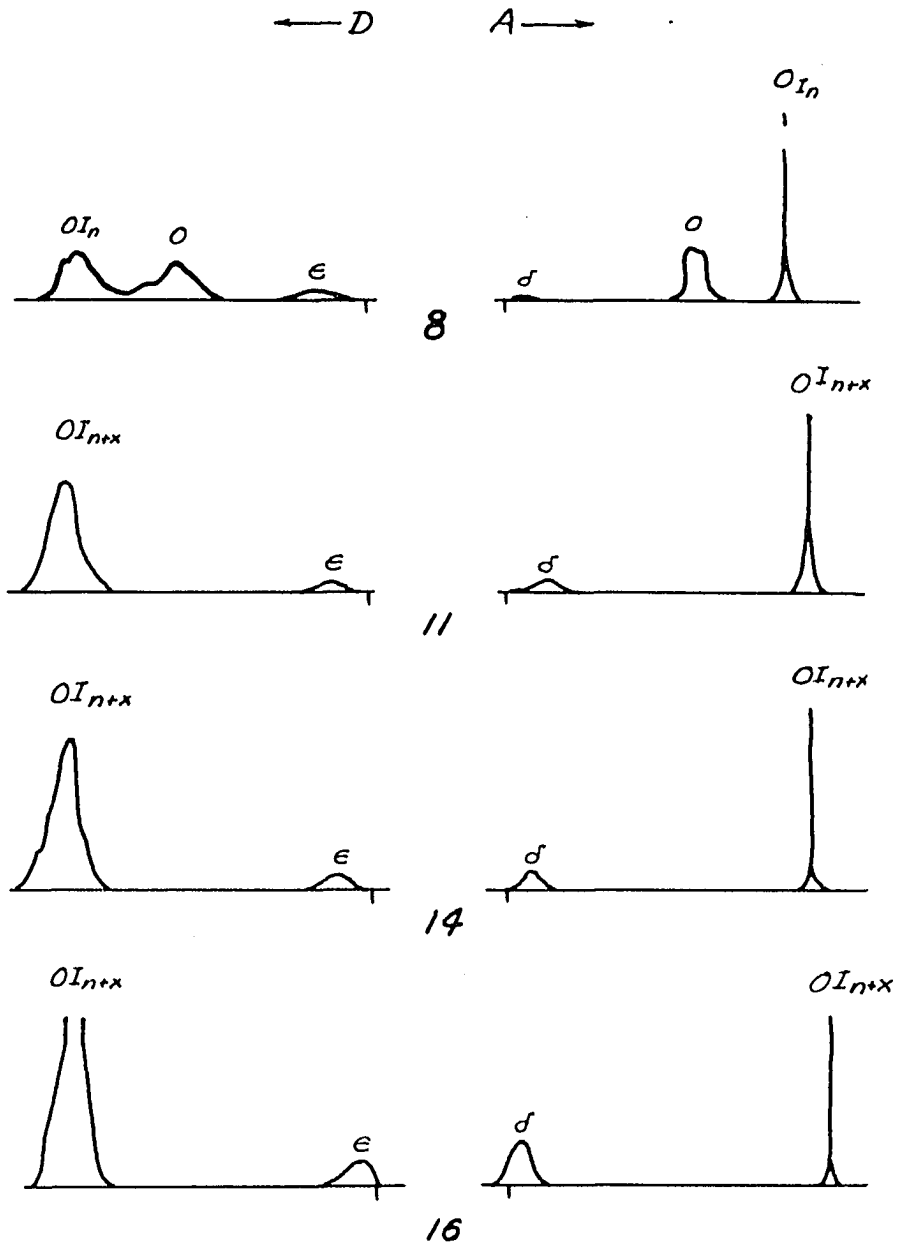


Fig. 19. (Continued).

Table 17. Electrophoretic analyses of some ovalbumin-SDBS mixtures at 20°C in phosphate-NaCl buffer (pH = 7.6,  $\Gamma/2 = 0.20$ ). Protein concentration =  $9.25 \times 10^{-5}M$ .

Expt. No.	Av. moles bound per mole protein	anion Bound-ary	Mobilities(1)			Relative Area(2)		
			0	$OI_n$	$OI_{n+x}$	0	$OI_n$	$OI_{n+x}$
	r		$-u \times 10^5 \text{ cm}^2 \text{ volt}^{-1} \text{ sec}^{-1}$			%	%	%
0	0	D A	6.6 6.4			100		
1	0.83	D A	6.8 6.9			100		
2	1.77	D A	6.5 6.5	8.1 8.1		94.0	6.0	
3	3.06	D A	6.6 6.6	9.7 9.9		90.0	10.0	
4	6.70	D A	6.8 6.8	7.8 9.9	9.9 10.4	82.2	17.8	
5	10.4	D A	6.9 6.9	10.5 10.1		67.1	32.9	

(1) 0 = ovalbumin,  $OI_n$  = ovalbumin-SDBS ("all-or-none" complex),  $OI_{n+x}$  = ovalbumin-SDBS (statistical complex).

(2) Based on the descending patterns.



Table 17. (Continued)

Expt. No.	Av. moles anion bound per mole protein	Bound-ary	Mobilities			Relative Area		
			0	$OI_n$	$OI_{n+x}$	0	$OI_n$	$OI_{n+x}$
	r		$\mu \times 10^5 \text{cm}^2 \text{volt}^{-1} \text{sec}^{-1}$			%	%	%
6	14.3	D	6.3	7.5	10.1	59.3	40.7	
		A	6.4		9.8			
7	18.1	D	6.3	7.4	10.5	52.6	47.4	
		A	6.6		10.3			
8	21.6	D	7.0	8.1	10.8	43.5	56.5	
		A	7.1		10.6			
9	29.1	D	6.7	7.6	10.7	28.8	71.2	
		A	7.2		10.5			
10	36.8	D	6.5		10.6	17.3	82.7	
		A	7.2		10.5			
11	55.3	D						100
		A			11.3 11.5			
12	74.3	D						100
		A			12.2 12.2			

Table 17. (Continued)

Expt. No.	Av. moles bound per mole protein	anion Bound-ary	Mobilities			Relative Area		
			0	$OI_n$	$OI_{n+x}$	0	$OI_n$	$OI_{n+x}$
	r		$-\mu \times 10^5 \text{cm}^2 \text{volt}^{-1} \text{sec}^{-1}$			%	%	%
13	91.3	D			12.5			100
		A			12.6			
14	111	D			12.7			100
		A			12.8			
15	137	D			12.9			100
		A			13.3			
16	197	D			13.0			100
		A			14.0			

excess native ovalbumin as a separate component at the boundary marked "0". In Table 18, analysis of the relative area under the stable boundaries were made on the following assumptions: (1) no SDBS ions were bound to the excess ovalbumin and (2), the slight difference in refractive increment of ovalbumin and SDBS was neglected. Within experimental errors the components was a linear function of the amount of SDBS bound. It is interesting to note that an average value,  $r$ , of 43 was obtained for ovalbumin which corresponded closely to the total number of basic groups in the protein (40 to 41). Considering the assumptions mentioned and the limits of accuracy in electrophoretic analysis, such an agreement seems very striking. It also indicates the importance of the role which the electrostatic forces play in the mechanism of interaction. If this is true questions might be raised with regard to bovine serum albumin, where the value of  $r$  in region B corresponded to only about one half of the basic groups in the protein. The difference in behavior between the two proteins might be explained on the basis of the difference in accessibility of foreign ions either as a result of steric arrangement or of side chain bonding. This is also compatible with the fact that bovine albumin is less easily denatured and thus less penetrated by SDBS ions than ovalbumin.

Table 18. Minimum binding ratio of ovalbumin-SDBS complex from electrophoretic measurements

Av. moles anion bound per mole protein*	Relative Area		Moles anion bound per mole protein
	O	OI <sub>n</sub>	
r	%	%	n
0.83	- -	- -	- -
1.77	94.0	6.0	37.1
3.06	90.0	10.0	39.4
6.70	82.2	17.8	47.0
10.4	67.1	32.9	38.4
14.3	59.3	40.7	42.3
18.1	52.6	47.4	45.3
21.6	43.5	56.5	44.2
29.1	28.8	71.2	45.3
36.8	17.3	82.7	47.5

Average 43  
Basic group 40-41  
of ovalbumin

\*The figures referred to those in the binding curve (Figure 18).

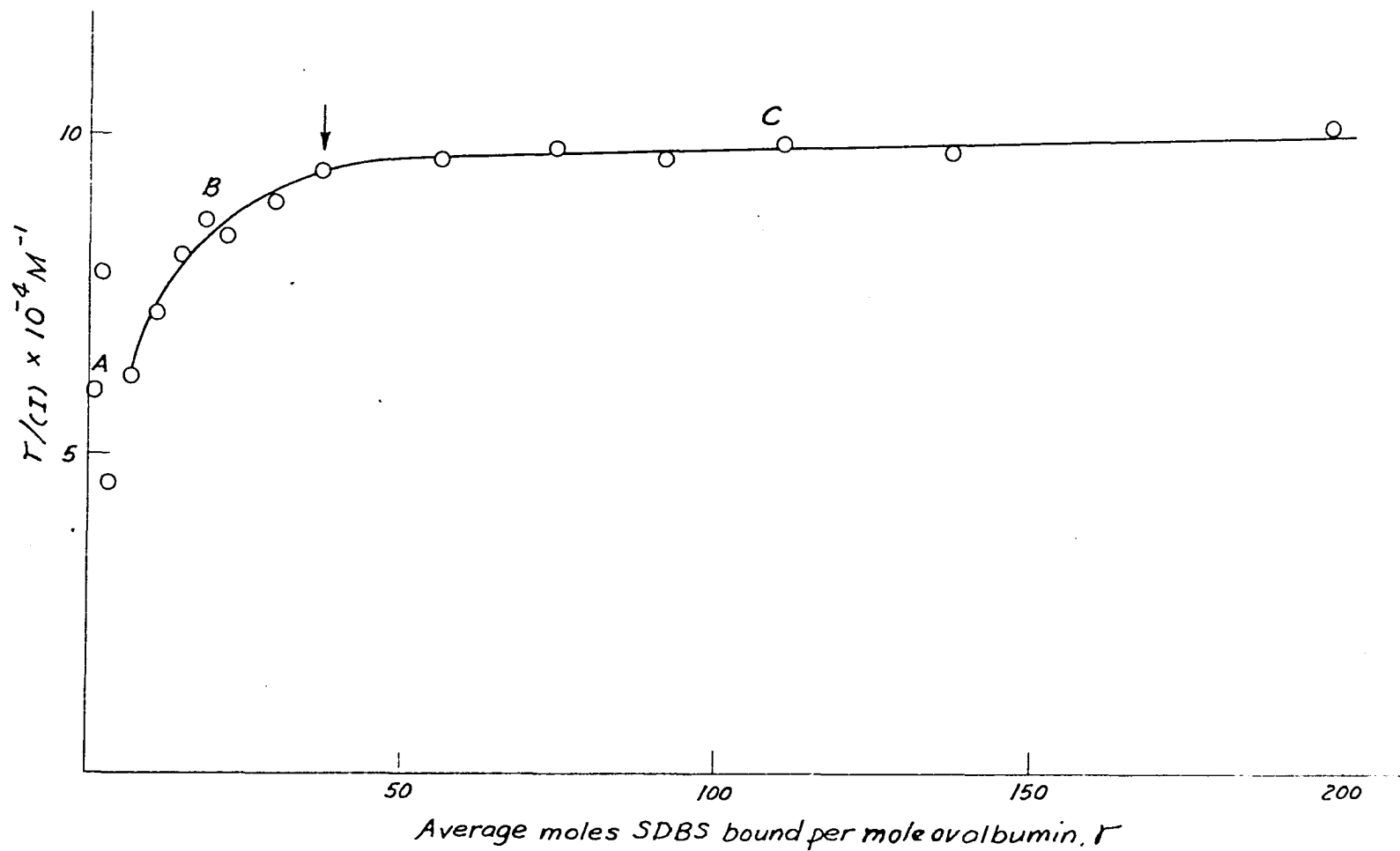


Fig. 20. The extent of binding of SDBS by ovalbumin (see Fig. 18).

### 3. Region C

In region C, statistical reaction was predominant again, as evidenced from the electrophoretic patterns. Here, the number of SDBS molecules bound was far in excess of the basic groups of the ovalbumin. Thus, van der Waals forces and other factors must be important.

In Figure 20 are plotted the value  $r/(I)$  against  $r$ . The first three points were quite scattered, probably due to the complication of precipitation during equilibrium dialysis. In region B the curve increased slightly with increase in the value  $r$ , as contrasted with that for bovine albumin. In region C the value  $r/(I)$  was again virtually independent of  $r$  as has been found for bovine albumin.

Comparable results were also obtained with regard to the reversibility of the binding process similar to that for mixtures of bovine albumin and SDBS. This is shown by another two curves in Figure 18. In curve II the mixture of ovalbumin and SDBS was dialyzed against varying portions of the buffer (20 ml. to 10 l.). It is evident that the binding process was not completely reversible. Arguments similar to that for bovine albumin-SDBS complexes could also be applied here.

Part IV. Some Flow Birefringence Studies of Protein-Surface  
Active Anion Complexes

Flow birefringence study affords one of the most useful techniques for the investigation of protein-denaturation. Some preliminary results on protein-surface ion mixtures are described in Part IV, in an effort to correlate the nature of complex formation with the protein denaturation by the ions.

A. Bovine Albumin - Sodium Dodecylbenzenesulfonate (SDBS)  
Complex

A protein-SDBS solution of desired concentration was prepared by the precipitation method as has been described in Part I, i.e. precipitation in acetate buffer (pH = 4.5), followed by redispersion in glycine-NaCl buffer (pH = 10.0). The mixture was clarified through fine sintered-glass filter under pressure. A 10ml. portion of the filtrate was then mixed with 53.3gm. 95% glycerol. The final concentration of the albumin was about 0.6%. The viscosity of the solvent was assumed to be the same as that of 80% glycerol, i.e. 45.9 centipose at 25°C.

In Figure 21 are plotted the calculated lengths against  $G\eta/T$ . The methods of calculation as well as the operation of the equipment has already been discussed by Dr. Samsa (87), formerly of this laboratory. Three runs were made under

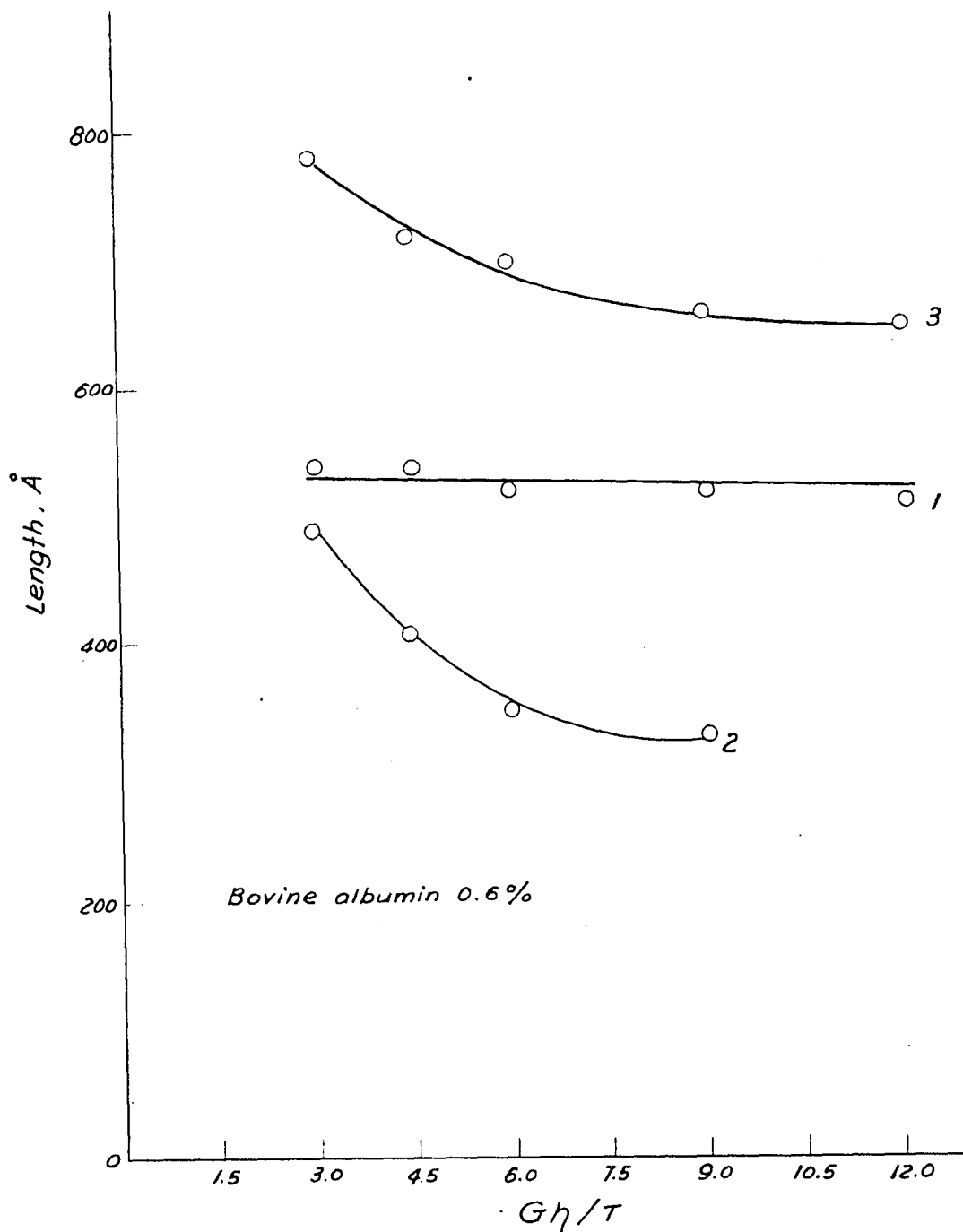


Fig. 21. Flow birefringence studies of bovine albumin-SDBS mixtures.



almost identical conditions. In run 1, the length varied from  $540\overset{\circ}{\text{Å}}$  at  $C/T = 3.02$  to  $510\overset{\circ}{\text{Å}}$  at 12.14. On the other hand, in Run 2, it was  $490\overset{\circ}{\text{Å}}$  at 3.02 and only  $330\overset{\circ}{\text{Å}}$  at 9.06. The solution foamed badly at higher speed. A third solution (Run 3) was then prepared. Even after filtration, the solution was very turbid, as observed under direct flash light. Consequently, the calculated lengths were much longer, varying from  $780\overset{\circ}{\text{Å}}$  at 3.02 to  $650\overset{\circ}{\text{Å}}$  at 12.14. That these results were not reproducible was rather disappointing. Probably the slow rate of dispersion of the precipitated complex might be responsible for such inconsistent data. For instance, to prepare a 1% solution, it took about two days to dissolve the precipitate, but to make a 3% solution, ten or more days. During such a long period, aggregation or other structural changes would be expected to occur. Among the three runs, the first one (Run 1) gave a fairly constant length at varying speeds, a fact indicative of less polydispersity than in the other two. Therefore, the value of  $540\overset{\circ}{\text{Å}}$  was probably most reliable. This was also in good agreement with the results obtained from heat or urea denaturation.

To test the state of aggregation of the denatured protein, a dilution series of the albumin-SDBS complex (Run 1) was studied by light-scattering measurements. An apparent weight-average molecular weight of about 119,000 was obtained on the basis of protein only. Thus, it seems that no

serious aggregation has taken place in Run 1.

Contrary to the acid-precipitated albumin-SDBS complex, aqueous solution of albumin and SDBS at stoichiometric mixing ratio gave no double refraction ( $\Delta$ ) at all. Even in twice concentrated solution (about 1% protein in glycerol), the  $\Delta$  value was too weak to be reliable. Another experiment was conducted under the same conditions as that used for the preparation of acid-precipitated complex, except the omission of precipitation. Use of glycine-NaCl buffer (pH  $\approx$  10) together with 11-days standing also did not improve the birefringence.

From these facts it might be deduced that in the range of mixing ratio (I/P) studied, the protein molecules were not unfolded to a great extent, except under drastic condition such as acid precipitation. It was, however, not known whether the unfolding occurred during precipitation or the low pH ( $\approx$  4.5) acidic to the isoelectric point was responsible for such structural change. It was also possible that the surface active anion in the acid medium caused such changes.

#### B. Ovalbumin-SDBS Complex

Analogous experiments were conducted for the acid-precipitated ovalbumin-SDBS complex. The filtrate through sintered glass was still too turbid to give reliable results. This indicated that denaturation, followed by aggregation had taken place to a great extent. Further filtration through a filter

pad with the aid of celite, however, caused too much loss of the protein, thus making it impossible to make flow birefringence studies. Results must await further investigation so that an optimum condition can be found where the complication of aggregation might be prevented.

### C. Zein-SDBS Complex

The solution of zein-SDBS complex was conveniently prepared by shaking excess zein with 1% SDBS in the cold room (1-3°C.). A 1% solution in 80% glycerol was used for the flow birefringence study, as shown in Figure 22, Run 1. It gave strong double refraction and a length of 950<sup>o</sup>Å at 1.82 to 940<sup>o</sup>Å at 9.06. This was in good agreement with the results obtained from other surface active ions such as sodium dodecylsulphate. In another experiment (Run 2), the protein concentration was reduced to one half by using 0.5% SDBS. It turned out that the filtrate was very turbid and, consequently, the calculated length was much longer, varying from 1610<sup>o</sup>Å at 180 RPM to 1400<sup>o</sup>Å at 900 RPM. Here again, the result was complicated by the state of aggregation. For the sake of comparison, a solution of zein in propylene glycol was prepared (Run 3). It gave length of about 400<sup>o</sup>Å, which was in good agreement with the literature value (24).

A comparison of the above results revealed some interesting facts. First, stoichiometric mixtures of either bovine

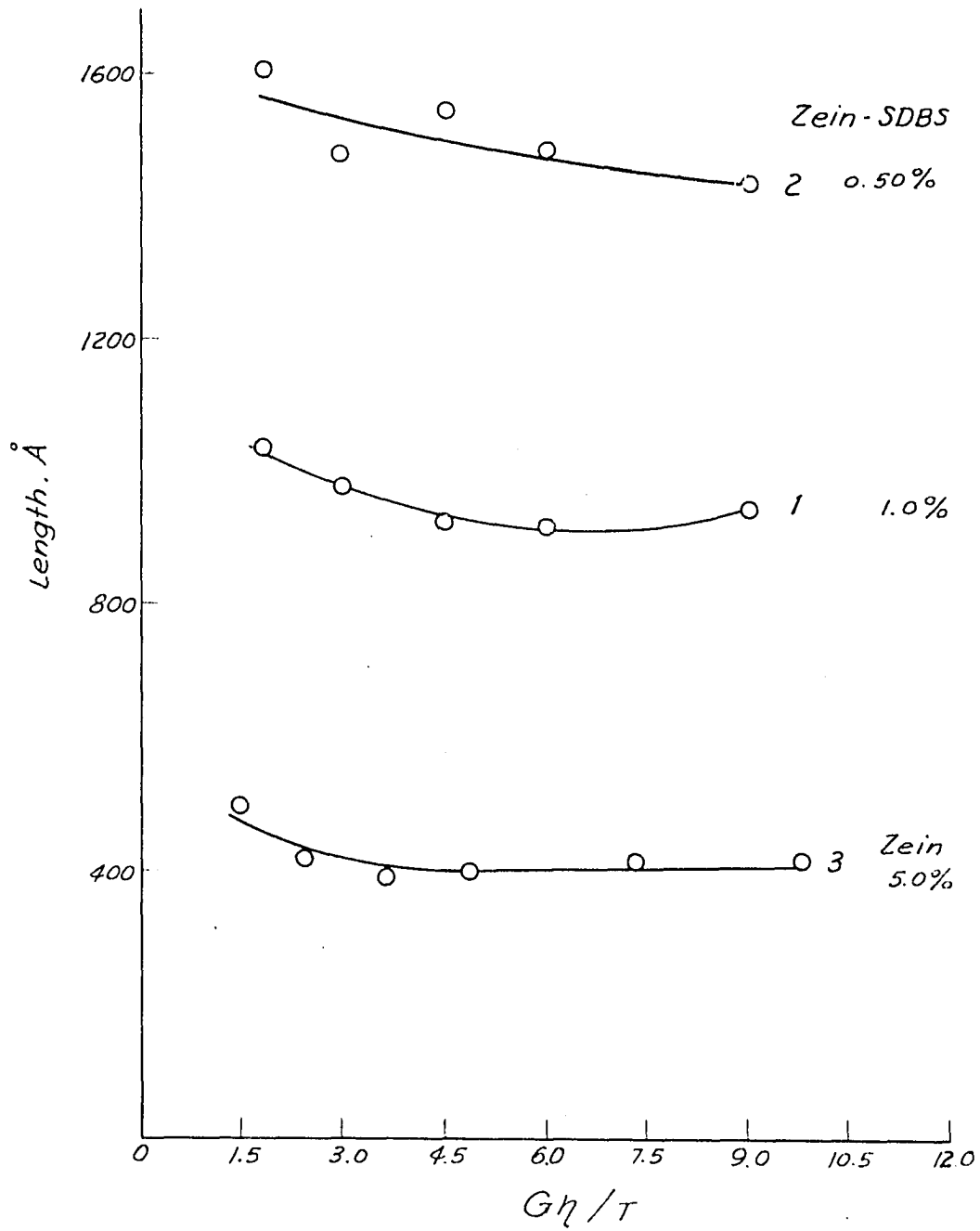


Fig. 22. Flow birefringence studies of zein and of zein-SDBS mixtures.

albumin or ovalbumin and surface active ions did not exhibit anisotropy of flow unless they were exposed to drastic treatment such as precipitation. On the other hand, zein-surface active ion complex gave strong double refraction. A clue to the mode of denaturation might be revealed by the fact that the amount of ions bound to zein was in excess of stoichiometric combination (about three times the basic groups of the protein). Owing to its insolubilization in water, zein could be dispersed only when it bound with a large amount of ions and thereby the complex was polar enough to dissolve. It might be inferred that the penetration of the protein molecule by the excess bound ions would force the polypeptide chains further apart, thus causing the unfolding of the protein.

Secondly, the albumin molecules were not completely unfolded even when they were subjected to physical or chemical denaturants. Theoretically bovine serum albumin would have a length of over  $2000\text{\AA}$  and ovalbumin about  $1400\text{\AA}$ , when completely stretched. All results now available were however in the range of 500 to  $600\text{\AA}$ . It is also interesting to note that ovalbumin was more easily denatured than serum albumin, a fact perhaps in agreement with the competitive study as described in Part I.

## V. GENERAL DISCUSSION

Controversy on the binding process of proteins with surface active anions is at least partly clarified in this research. Three stages of binding between bovine albumin and sodium dodecylbenzenesulfonate (SDBS) have been described in Part III of the experimental results. Neither of the so-called "all-or-none" and statistical reactions can cover the whole range of the binding process. Rather, it depends upon the relative concentrations of the protein to the anions. At very low anion/protein (I/P) ratios a statistical combination is predominant. This is in agreement with Karush's work (40). In region A of the binding curve, the plot of  $1/r$  against  $1/(I)$  closely resembles Karush's experimental data. At the end of this region the curve deviates from its linearity as predicated by the statistical theory, due to the fact that the second stage of binding starts. It seems rational to explain this on the basis of structural adaptation of the protein molecule rather than on the hypothesis of heterogeneity as advocated by Karush. To be sure, the binding sites on the protein molecule are by no means all equivalent. This might explain why only about ten sites bind ions statistically in the first stage. Karush's data also extrapolated to a value of about ten. It seems logical that owing to their strong binding affinity for the proteins the SDBS ions would penetrate the tightly folded protein molecule and strive for

the less accessible binding sites after the first ten or so are occupied. Accordingly, structural changes would be assumed to take place, thus causing the second stage of the "all-or-none" reaction. Definite evidence comes from the electrophoretic analysis where in region B two boundaries appear instead of one statistical boundary. It seems meaningless to apply a Gaussian distribution of binding sites to this region as Karush did. Indeed, it is very doubtful that such a distribution function would fit our data covering all three regions. Unfortunately his solutions were not subjected to electrophoretic analysis. It is suspected that his points which deviated from his binding curve would also show an "all-or-none" character, as has been found in our work (region B, Figure 11).

In regions B and C our results are in good agreement with those of Lundgren and coworkers (62) and Putnam and Neurath (84). It is beyond any doubt that an "all-or-none" reaction exists in the region B (Figure 11), as evidenced from the electrophoretic analyses. Stoichiometric complex formation corresponding to the total number of the basic groups of the protein was observed for the mixture of ovalbumin and SDBS, as has been reported by Lundgren. According to Putnam, horse serum albumin binds with sodium dodecylsulphate to form two complexes, the so-called  $AD_n$  and  $AD_{2n}$ , in which the number of the anions bound corresponds respectively

to one half and to the total number of the basic groups of the protein. Our work on bovine albumin and SDBS did not conform with such a stoichiometric relation. Instead only about fifty moles of SDBS were bound per mole of the protein, corresponding to about one half of the acid-binding capacity. Lundgren and Putnam's data were calculated on the assumption that all anions were bound to the protein. Since there also exist some free anions, their results might involve some, though very small, errors.

Objection was raised by Karush in that the "all-or-none" reaction might be attributed to the formation of anionic micelles and, in many cases, denaturation. The first point seems not very convincing. It seems unlikely that the micelle formation will change the nature of binding. Rather, it is the rate of reaction that will be affected by the presence of micelles. Since the micelles are in equilibrium with single ions, it is simply a matter of affinity between protein and simple ions and ions themselves. Quite conceivably, the equilibrium of the anions would be shifted in favor of single ions, provided the latter have greater affinity for the protein. Further evidence comes from the kinetic study as described in Part III. On equilibrating the protein solution against constant SDBS concentration ( $2.08 \times 10^{-4}M.$ ), the reaction was first statistical in nature, then followed by an "all-or-none" reaction. At such low concentration, presumably



below critical micelle concentration, still a Gaussian distribution could not be applied.

The second point raised by Karush is protein denaturation. This is just the hypothesis we tried to prove in this research. The nature of the binding process is a function of the relative ion/protein (I/P) ratio. At low I/P ratios a statistical combination involving no structural changes is predominant. After about ten or so binding sites are occupied, further combination with the anions would force the protein molecule to unfold. Thus begins an irreversible step of denaturation. Once the folded peptide chains are loosened, the protein molecule will easily bind more anions than the native protein. The result is an "all-or-none" reaction. The number of anions bound in this stage, however, is limited by the accessibility of different proteins. After the second stage a statistical reaction follows by further adsorption of the anions which are comparatively loosely bound to the protein and can be removed through prolonged dialysis or other chemical methods. An exception to the above postulation is ovalbumin, for which the first statistical binding is absent probably due to its strong binding affinity with the surface active anions.

In order to justify the hypothesis mentioned above, many questions still have to be clarified. Some of the shortcomings in this work and suggestions for future investigation can be listed as follows:

(1) It is necessary to synthesize pure SDBS free of its homologues for quantitative studies. This will certainly minimize inconsistent results and yield more accurate data than those described in this work.

(2) Concentration dependence needs further investigations. According to Klotz (43), binding of methyl orange with albumins depends to some extent upon the concentration of the protein solution used, but Karush (40) found no difference for mixtures of albumin and alkyl sulphates. With pure SDBS it may be possible to ascertain whether it is concentration-dependent as reported in this research. Another point that can be clarified from such study is the complication of micelle formation. With varying protein concentrations but constant protein/ion mixing ratios, the comparable binding curves may test the effect of micelles on the binding process.

(3) Influence of buffer and effects of pH and ionic strength on the binding capacity have not been studied in this work. A study of binding affinity with the above variants as parameters may determine the importance of such effects, which probably are not very significant because of the strong affinity between protein and surface active ions.

(4) Quantitative studies of protein-ion interaction at temperatures other than 1-3°C. will permit the determination of the effect of temperature on the extent of binding, thus enabling one to cover a much wider range than at low temperature.

(5) Some inconsistent results have been observed from the kinetic studies. Therefore it is necessary to repeat such experiments. With pure SDBS it should be possible to determine accurately the reaction rate constant. A method of determining the anion activity should be developed so as to minimize the complication of micelle formation and thereby determine the order of reaction.

(6) To test the reversibility of complex formation, it is necessary to regenerate the protein from its complex with SDBS. Comparison of the binding of SDBS with regenerated and native proteins will certainly shed more light on the nature of the binding process. Together with kinetic study it will give a better understanding of protein denaturation.

Since competitive interaction between proteins and SDBS was studied before the development of spectrophotometric analysis of SDBS, the experimental results as described in Part I have shown some serious shortcomings. Electrophoretic analysis alone has its limitations. In many cases poor resolution of boundaries or appearance of boundary anomalies made quantitative calculations very unreliable. Furthermore, it was not definitely known how much SDBS was bound to the protein and how much was left as free anions during the preparation of protein-SDBS complexes by dialysis or acid-precipitation method. It seems highly desirable to apply the equilibrium-dialysis technique and spectrophotometric analysis to the study of such interactions. This will certainly yield

more accurate quantitative data. Another serious objection is that direct evidence of competitive interaction is still lacking. Although the electrophoretic analyses in this research seemed very convincing, there is no way to identify directly and unmistakably the various boundaries in the patterns. Owing to their peculiar properties the protein-SDBS complexes could not be separated from the other proteins by methods such as precipitation and pH adjustment. Presence of other proteins or adjustment of pH to the interisoelectric zone would render only partial separation of the various components. Preparative electrophoresis could not achieve the objective in our cases either. Probably one promising approach will be the use of radioactive technique together with electrophoretic separation in identifying the different components.

In the literature little has been reported on the interaction between proteins and surface active cations. The experimental results described in Part II are far from complete. Quantitative analysis was further complicated by the inhomogeneity of the cationic zephiran. Nevertheless, the strikingly different behavior of bovine albumin and ovalbumin for the cation deserves further attention. It seems desirable to conduct quantitative studies of the binding process similar to those described in Part III. Pure surface active cation should be synthesized. A spectrophotometric method of analysis can be developed for the determination of the cation concentrations.

Denaturation of proteins by surface active ions has only been briefly studied in this work. Flow birefringence proves to be a useful technique in determining the extent of structural change. However, the results were so far complicated by the state of aggregation. It is therefore necessary to develop optimum conditions so that little or no aggregation will interfere with the experimental data. By double refraction alone it may not be possible to detect small structural change. It seems also desirable to conduct other physico-chemical studies such as viscosity measurements.

Among the three proteins studied in this work, zein is an atypical one which may have already undergone certain structural changes during its commercial preparation. Accordingly, conclusions drawn therefrom may not be applicable to the other proteins. Among the two albumins studied, some striking differences in behavior toward surface active ions are worth mentioning. (1) Evidence from their competitive interaction in Part I seems to indicate that ovalbumin has much stronger affinity for SDBS than bovine albumin. (2) From the binding curves in Part III, combination of bovine albumin with SDBS proceeds through three stages, whereas with ovalbumin the first statistical binding is absent. This seems to infer that ovalbumin is more susceptible to structural change than bovine albumin. (3) In the "all-or-none" reaction ovalbumin binds stoichiometrically with SDBS, the number of ions bound corres-

ponding to the total number of basic groups of the protein, whereas with bovine albumin this relation does not exist, a finding perhaps indicative of the difference in their accessibility to foreign ions. (4) In the case of surface active cations, again ovalbumin binds strongly with zephiran, whereas bovine albumin only adsorbs the cations very loosely, as evidenced from the results described in Part II. (5) Available evidence in this laboratory seems to point to the conclusion that ovalbumin is more easily denatured than bovine albumin. From these facts it may be inferred that there exists a close relation between structural changes of the proteins and their binding affinities for surface active ions. In other words ovalbumin is more susceptible to the penetration of surface active ions than bovine albumin. Accordingly, the former has much stronger binding affinity for the ions than the latter. Correlation of protein denaturation with the nature of binding thus offers one of the most promising sources of information on the protein structure. It is therefore suggested that future approaches on this subject should follow these two lines. From the theoretical point of view it is also pertinent to determine thermodynamic quantities for the interaction between proteins and surface active ions.

## VI. SUMMARY AND CONCLUSIONS

1. Preparation of stable complexes of ovalbumin-sodium-dodecylbenzenesulfonate (SDBS) and bovine albumin-SDBS was accomplished by two methods: (1) prolonged dialysis of mixtures of protein and excess SDBS against distilled water, or (2) acid precipitation of protein-SDBS mixture, followed by redispersion in alkaline solution. Preparation of zein-SDBS complex was made by extracting the protein in excess with SDBS solution.

2. Competitive interaction of proteins with SDBS was studied electrophoretically. Definite interaction between zein-SDBS and ovalbumin was observed with the appearance of an ovalbumin-SDBS boundary. No displacement reaction occurred with mixtures of zein-SDBS and bovine albumin. Likewise, ovalbumin removed the anion from bovine albumin-SDBS complex but no interaction was detected for mixtures of ovalbumin-SDBS and bovine albumin. Correlation between binding affinity and competitive interaction was postulated. Limitations of electrophoretic analysis were discussed.

3. Electrophoretic studies of mixtures of albumins and zephiran, a surface active cation, were performed in a manner similar to those of proteins and surface active anions. Mixtures of ovalbumin and zephiran exhibited an all-or-none character, whereas bovine albumin adsorbed the cation very loosely. Preparation of stable complexes of zephiran with

albumins or zein failed. An explanation was given on the basis of binding affinity. Correlation between interaction and denaturation was discussed.

4. A spectrophotometric method of analysis was developed for the determination of SDBS concentrations. Difficulties in analysis and their remedies were described.

5. The nature of binding between bovine albumin and SDBS was studied quantitatively, using electrophoretic analysis and equilibrium dialysis technique as experimental tools. The binding process proceeded through three stages: (1) statistical combination up to about ten moles anion bound per mole protein, (2) all-or-none reaction yielding a stable complex of about one mole protein to fifty moles anion, and (3) statistical binding up to the solubility limit of free anion at 1-3°C. Controversy in the literature was thus partly clarified. Different views held by the two schools were criticized.

6. A hypothesis was suggested for the binding process. It was postulated that the first stage involved no structural change of the protein molecule, but the second and third stages were closely related to protein denaturation.

7. A kinetic study of the all-or-none reaction was attempted. Complete reversal of the second and third stages was not achieved.

8. Quantitative study of interaction was complicated by the inhomogeneity of the surface active compounds. Future possible improvements were pointed out.



9. Comparable results of interaction between ovalbumin and SDBS were included. Electrophoretic analysis revealed the absence of statistical combination in the first stage. The minimum binding ratio in the stable complex corresponded to the total acid-binding capacity of the protein. The different behavior of bovine albumin and ovalbumin was discussed on the basis of accessibility of the proteins. Correlation of denaturation and binding affinity with regard to competitive interaction between proteins and anions was also suggested.

10. Denaturation of protein-SDBS complexes was briefly investigated with flow birefringence measurements. Future methods of approach were outlined.

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