[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF NORTHWESTERN UNIVERSITY]

# The Binding of Organic Ions by Proteins. Comparison of Native and of Modified Proteins

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Extensive investigations have been made of the binding of organic and inorganic ions by serum albumin under a variety of physico-chemical conditions.[1] From these studies it has become apparent that this protein forms strong complexes with small ions even under conditions where both species have the same charge. It has been observed also that several other proteins such as β-lactoglobulin[2,3,4] egg albumin,[2,5] and gelatin[6,7,8] bind large anions, such as the detergent sodium dodecyl sulfate, even at *p*H's alkaline to the isoelectric point. Nevertheless, serum albumin is outstanding among native proteins in its very marked affinity for anions under conditions in which other proteins give no evidence of binding ability.

Whether this behavior of serum albumin is truly unique has been difficult to decide, for data on complexes between dissolved proteins and anions other than dodecyl sulfate are very sparse and usually in a form not suitable for quantitative comparison. To obtain some insight into the unusual character of serum albumin, a comparative quantitative examination has been made of the binding of a common anion by a large group of proteins, mostly of crystalline nature, under very nearly identical environmental conditions.

Within recent years, many investigators have developed methods of treating proteins, under very mild conditions, with reagents which are quite specific in the groups with which they will react, and which apparently will not injure the structural framework of the macromolecule.[9] In a consideration of the nature of the groups participating in the anion–protein bond, it has seemed appropriate, therefore, to examine also the binding properties of serum albumin chemically modified in a controlled fashion. These experiments, coupled with the comparative studies on native proteins, enable one to construct a general picture of the nature of the binding process, which is consistent with the observed behavior of these large molecules.

(1) I. M. Klotz and J. M. Urquhart, THIS JOURNAL, **71**, 847 (1949). Earlier work, and investigations by other laboratories, are cited in this reference.

(2) H. P. Lundgren, *Textile Research J.*, **15**, 335 (1945).

(3) H. B. Bull, THIS JOURNAL, **68**, 747 (1946).

(4) T. L. McMeekin, B. D. Polis, E. S. Della Monica and J. H. Custer, *Federation Proc.*, **7**, 172 (1948).

(5) H. B. Bull, THIS JOURNAL, **67**, 10 (1945).

(6) S. E. Sheppard, R. C. Houck and C. Dittmar, *J. Phys. Chem.*, **46**, 158 (1942).

(7) K. G. A. Pankhurst and R. C. M. Smith, *Trans. Faraday Soc.*, **40**, 565 (1944); **43**, 511 (1947).

(8) F. W. Putnam, *Advances in Protein Chemistry*, **4**, 79 (1948).

(9) For recent reviews of these methods see H. S. Olcott and H. Fraenkel-Conrat, *Chem. Rev.*, **41**, 151 (1947), and R. M. Herriott, *Advances in Protein Chemistry*, **3**, 169 (1947).

## Experimental

The extent of binding of the anion, methyl orange, was measured by the differential dialysis technique described in detail previously.[10] Most of the experiments were carried out, with mechanical shaking, for an eighteen-hour period in an ice-bath at 0.0 ± 0.1°, but a few were equilibrated in a cold room at 5°.

Analyses for the dye were made with the Beckman spectrophotometer.

The bovine plasma proteins, fractions II, III-1, IV-1, and crystallized albumin were obtained from Armour and Company, the first three as generous gifts. Of the native non-plasma proteins, β-lactoglobulin was supplied through the courtesy of Dr. T. L. McMeekin of the Eastern Regional Research Laboratory, and the others were obtained by gift or purchase from Armour and Company.

Of the modified proteins, the acetyl-amino bovine albumin was a sample kindly supplied by Dr. H. Fraenkel-Conrat of the Western Regional Research Laboratory. The guanidinated human serum albumin was obtained through the courtesy of Dr. W. L. Hughes, Jr., and Dr. H. A. Saroff of the Harvard Medical School.[10a] Professor E. J. Cohn supplied us with a quantity of the parent human albumin from which the guanidinated substance had been made. We are very much indebted to the sources indicated for the assistance rendered in this project.

## Results

**Plasma Proteins.**—The ability of plasma proteins to bind anions, and the physiological significance of such interactions, have been recognized for some time.[11] It has been demonstrated also by electrophoretic studies,[12] as well as by rough salt-fractionations,[13] that the albumin constituent is primarily responsible for this property. Direct binding studies on separate fractions, where protein-protein interactions would be minimized, have been lacking, however, largely because of the difficulty of obtaining adequate quantities of highly homogeneous fractions, until the recent development of low-temperature, alcohol-fractionation procedures.[14]

Four such fractions of bovine origin have been examined in the present study. The conditions under which the binding experiments were carried out are summarized in Table I. Previous investigations[1,15] have demonstrated already that crystallized albumin binds methyl orange, the reference anion, and that γ-globulin has practically no binding ability, but since these experiments were

(10) I. M. Klotz, F. M. Walker and R. B. Pivan, THIS JOURNAL, **68**, 1486 (1946).

(10a) The preparation and properties of the guanidinated albumin will be described in a forthcoming paper, in THIS JOURNAL, by W. L. Hughes, Jr., and H. A. Saroff.

(11) H. Bennhold, *Erg. inn. Med. Kinderh.*, **42**, 273 (1932).

(12) R. A. Rawson, *Am. J. Physiol.*, **138**, 708 (1943).

(13) B. D. Davis, *J. Clin. Invest.*, **22**, 753 (1943).

(14) E. J. Cohn, L. E. Strong, W. L. Hughes, Jr., D. J. Mulford, J. N. Ashworth, M. Melin and H. L. Taylor, THIS JOURNAL, **68**, 459 (1946).

(15) I. M. Klotz and F. M. Walker, *J. Phys. and Colloid Chem.*, **51**, 666 (1947).

TABLE I

BINDING STUDIES WITH BOVINE PLASMA PROTEINS: PHOSPHATE BUFFER

| Protein[16] | Concn. of protein, % | pH | Temp., °C. | Concn. of methyl orange—Lowest | Concn. of methyl orange—Highest |
|---|---|---|---|---|---|
| Fraction II ($\gamma$-globulin) | 0.2 | 5.7 | 5 | $0.30 \times 10^{-5}$ | $40.0 \times 10^{-5}$ |
| | .2 | 6.8 | 0.0 | $.28 \times 10^{-5}$ | $14.9 \times 10^{-5}$ |
| Fraction III-1 ($\beta_2$-globulin) | .2 | 6.8 | .0 | $.35 \times 10^{-5}$ | $9.06 \times 10^{-5}$ |
| Fraction IV-1 ($\alpha_2$-globulin) | .1 | 6.8 | .0 | $.32 \times 10^{-5}$ | $8.76 \times 10^{-5}$ |
| Crystallized albumin | .2 | 6.8 | .0 | $.18 \times 10^{-5}$ | $5.37 \times 10^{-5}$ |

TABLE II

BINDING STUDIES WITH NATIVE CRYSTALLIZED PROTEINS

| Protein | Concn. of protein, % | Buffer | pH | Temp., °C. | Concn. of methyl orange—Lowest | Concn. of methyl orange—Highest |
|---|---|---|---|---|---|---|
| $\beta$-Lactoglobulin | 0.2 | Phosphate | 6.8 | 0.0 | $0.38 \times 10^{-5}$ | $6.90 \times 10^{-5}$ |
| Egg albumin | .2 | Phosphate | 6.8 | .0 | $.39 \times 10^{-5}$ | $8.64 \times 10^{-5}$ |
| Insulin | .1 | Bicarbonate[a] | 8.2 | .0 | $.80 \times 10^{-5}$ | $9.00 \times 10^{-5}$ |
| Lysozyme | .2 | Phosphate | 7.6 | .0 | $.39 \times 10^{-5}$ | $3.94 \times 10^{-5}$ |
| Ribonuclease | .1 | Phosphate | 6.8 | .0 | $.44 \times 10^{-5}$ | $3.40 \times 10^{-5}$ |
| Pepsin | .2 | Phosphate | 6.8 | .0 | $.28 \times 10^{-5}$ | $8.16 \times 10^{-5}$ |
| Trypsin | .2 | Phosphate | 6.8 | .0 | $.71 \times 10^{-5}$ | $7.26 \times 10^{-5}$ |
| Chymotrypsin | .2 | Phosphate | 6.8 | .0 | $.73 \times 10^{-5}$ | $7.92 \times 10^{-5}$ |

[a] Sodium chloride was added also to a concentration of 0.15 *M*.

carried out under slightly different conditions, they have been repeated, for purposes of comparison with the other fractions, in a common buffer and at a common temperature.

Clear-cut negative results were obtained in the experiments with fractions II and III-1. Thus the evidence is quite conclusive that neither $\gamma$-globulin nor $\beta_2$-globulin binds methyl orange anions. On the other hand, the experiments with fraction IV-1 were not nearly as satisfactory, perhaps because of the presence of an insoluble component in the sample used, or possibly because of the dissociation of some of the lipid from the lipoprotein. Evidence for such dissociation has been reported by Oncley, Scatchard and Brown.[15] In either event, when the insoluble material was permitted to remain in suspension in the protein chamber, definite indications of binding were obtained, to an extent of about 30% of that observed with an equivalent weight of crystallized bovine albumin. Removal of the insoluble material by centrifugation in an ordinary laboratory centrifuge, however, reduced the observed binding almost to zero. Further experiments on this plasma fraction will be carried out when better samples are available. At present it seems reasonable to conclude that the extent of binding of methyl orange by this protein fraction is quite small. Such a conclusion is also supported by the fact that clear samples of fraction IV-1 produce no shift in the spectrum of methyl orange.

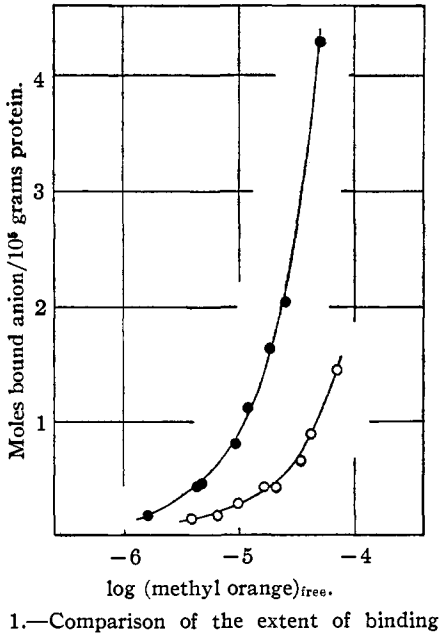


Fig. 1.—Comparison of the extent of binding of $\beta$-lactoglobulin, O, with that of (an equal weight of) serum albumin, ●.

Thus it becomes apparent from these experiments on individual fractions of the serum proteins that albumin is far superior to any other constituents of the plasma which have been examined, in its ability to form complexes with anions.

**Other Native Proteins.**—Turning to the non-plasma proteins, one finds again that the ability to bind a small anion, such as methyl orange, is an uncommon property. The crystallized proteins investigated, and the conditions used, are summarized in Table II. Of these, only $\beta$-lactoglobulin shows appreciable uptake of anions (Fig. 1).

It is perhaps pertinent to mention also that $\beta$-lactoglobulin displaces the spectrum of methyl orange in a direction closely similar to that ob-

(16) For characteristics of these fractions, see J. L. Oncley, G. Scatchard and A. Brown, *J. Phys. and Colloid Chem.*, **51**, 184 (1947).

served with bovine albumin.[17] On the other hand, among the non-binding proteins examined, lysozyme, pepsin, trypsin and chymotrypsin, none produced any significant alteration in the optical absorption of the anion.

**Modified Proteins.**—Previous studies[18] have demonstrated that the decreased binding by serum albumin with increasing *p*H occurs in the region in which the quaternary nitrogen atoms of the lysine residues are losing their charge, and hence indicated that these residues are the primary loci at which the anions are bound. A further test of this viewpoint is available in an examination of the binding properties of an albumin in which the amine groups have been selectively acetylated. The extent of binding of methyl orange by such an acetyl-amino bovine albumin (90% of amine groups acetylated) is illustrated in Fig. 2, together with some data on the unacetylated protein under substantially identical conditions. A marked drop in complex formation is apparent. Nevertheless, the decrease in binding is not proportional to the decrease in the number of available lysine cationic loci. It seems likely, therefore, that the guanidinium groups of the arginine residues also participate in anion binding.

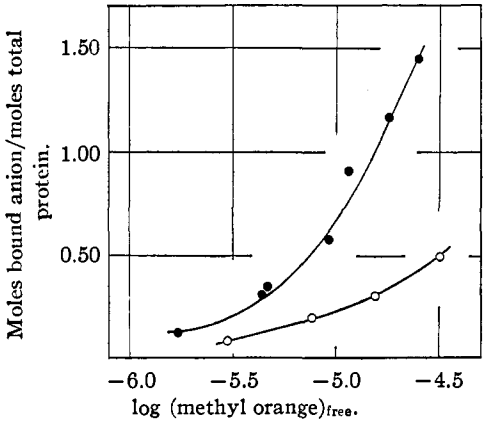


Fig. 2.—Comparison of the extent of binding of acetyl-amino bovine serum albumin, O, with that of the parent albumin, ●. Both sets of measurements were made in a phosphate buffer at *p*H 6.8 and at 0°. The protein concentration was 0.1% for the acetylated sample, 0.2% for the original material.

A similar conclusion is reached from a comparison (Fig. 3) of the binding of human serum albumin with a sample in which the ε-amine groups of lysine have been converted to guanidinium groups. No significant difference is observed in the affinity of each protein for the organic anion. Evidently the amine group of lysine is not essential as such but merely because it forms a positively-charged locus. It is also pertinent to point out, in anticipation of further discussion, that the spatial displacement of the cationic center on substitution of the guanidinium group for the ε-ammonium does not seem to affect the extent of binding.

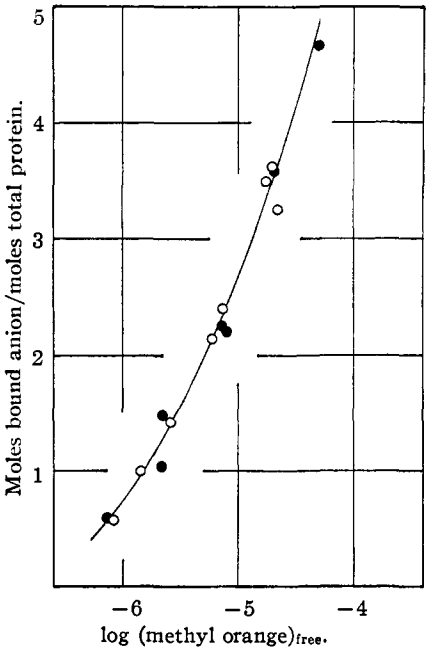


Fig. 3.—Comparison of the extent of binding of guanidinated human serum albumin, O, with that of the parent albumin, ●. Both sets of measurements were made in a phosphate buffer at *p*H 7.6 and at 5°. The protein concentration was 0.1% in both cases.

## Discussion

**Comparative Binding Abilities of Various Proteins.**—A survey of the results obtained with various native proteins indicates that the ability to bind methyl orange at *p*H's near 7 is limited, within this group, to two examples—serum albumin and β-lactoglobulin—with the former being far superior to the latter. It should be mentioned also, however, that studies on the plasma of *Limulus polyphemus*[19] under conditions not too different from those used in the present paper, indicate that hemocyanin also is capable of forming complexes with methyl orange, but to a degree even less than β-lactoglobulin.

Further attention should be drawn also to the behavior of egg albumin. While it is true that significant binding, as measured by the dialysis technique, does not occur at *p*H 6.8, it is apparent from the shift in indicator equilibria observed by Thiel and Schulz[20] and by Danielli,[21] as well as from the titration curves described by Steinhardt, Fugitt and Harris,[22] that egg albumin does form complexes with methyl orange, and other small

(17) I. M. Klotz, THIS JOURNAL, **68**, 2299 (1946).

(18) I. M. Klotz and F. M. Walker, *ibid.*, **69**, 1609 (1947).

(19) I. M. Klotz, A. H. Schlesinger and F. Tietze, *Biol. Bull.*, **94**, 40 (1948).

(20) A. Thiel and G. Schulz, *Z. anorg. allgem. Chem.*, **220**, 225 (1934).

(21) J. F. Danielli, *Biochem. J.*, **35**, 470 (1941).

(22) J. Steinhardt, *Ann. N. Y. Acad. Sci.*, **41**, 287 (1941); J. Steinhardt, C. H. Fugitt and M. Harris, *J. Research Nat. Bur. Stds.*, **26**, 293 (1941).

anions, in solutions acidic to the isoelectric point of this protein. That shifts in indicator equilibria parallel binding affinity is indicated by the absence of any anionic indicator shift in egg albumin solutions at *p*H 7.[23]

From the experiments of Thiel and Schulz,[20] it is evident also that casein binds methyl orange anions, at a *p*H near 3, with an affinity almost equal to that of egg albumin.

Consideration of all of these data leads one to the conclusion that proteins may be arranged in a linear array with respect to their binding affinities toward a common anion, such as methyl orange. When this anion is one which is not bound too strongly, the order of the proteins can be discerned quite clearly, as in the present case with methyl orange, and could even be expressed in a quantitative fashion in terms of the free energy of binding. On the other hand, when an anion is used which is bound very strongly, such as the detergent dodecyl sulfate,[24,25] even proteins low in the scale of binding affinities will form complexes. Thus electrophoretic investigations[2] and ultracentrifugal studies[26] have revealed complexes of dodecyl sulfate with egg albumin, and with insulin. Similarly there are indications that insulin binds thiocyanate ion,[27] an anion which is known to be bound very extensively by serum albumin,[28] in solutions of high (0.15 *M*) anion concentration.

**Configurational Basis of Binding Affinity.**—At some point in these considerations, the question naturally arises whether it is possible to account for the binding characteristics of the different proteins in terms of their fundamental molecular structure. When initial quantitative data with methyl orange were limited to only two serum proteins—albumin and γ-globulin—it seemed possible that the difference in behavior of the two proteins might be due to their pronounced differences in shape, the γ-globulin being much more asymmetric.[15] With the accumulation of further data, however, such an hypothesis becomes untenable, for several molecules with smaller axial ratios[15,29] than serum albumin, such as egg albumin or insulin, show decreased, rather than increased, binding affinities.

Fundamentally, of course, the properties of a protein must be an expression of its constituent amino acids, both with respect to content and arrangement. In regards to composition, serum albumin is not unique in the possession of any particular amino acid residue not present in the nonbinding proteins. On the other hand, an examination of the *distribution* in content of amino acids does suggest a correlation with binding affinity, which, in turn, can be interpreted in terms of the configurational pattern within the protein molecule.

The particular amino acids to which one would pay most attention in comparing distributions depends on the point of view which is adopted with respect to the molecular nature of the binding process. As has been mentioned earlier, the drop in binding ability at *p*H's near 11, as well as on acetylation of the amino groups suggests that cationic residues play a dominant role. On the other hand mere possession of such groups does not seem to be adequate, for several of the nonbinding proteins, γ-globulin, for example, contain very substantial quantities of arginine, lysine and histidine residues and yet do not exhibit significant binding properties. In fact, lysozyme,[30] which does not bind methyl orange under the conditions described in this paper, contains as large a percentage of basic amino acids as does serum albumin.[31]

Since the presence of cationic groups in a protein is not a sufficient condition for binding, it is suggested frequently that one or more other residues must be in proper juxtaposition to the quaternary nitrogen to supply the additional specific attraction to insure binding. It seems unlikely, however, that a particular secondary residue need be at a specific distance from the cationic locus, since anions of all sizes and shapes, organic and inorganic, form associations with serum albumin. Furthermore, the replacement of the ε-amino group of lysine by the more extended guanidinium group does not produce a decrease in binding affinity of albumin.

Since the presence of a *specific* secondary amino acid to reinforce the attraction by the cationic group does not seem to be a rigid requirement for binding ability by a protein, the alternative possibility was explored, that the *absence* of a particular pattern might be conducive toward the attraction of anions. In particular the possibility suggested itself that functional groups within the protein might act as competitive agents for the cationic groups, just as buffer ions have been observed to compete with methyl orange anions.[32] To examine this proposal, available amino acid determinations on the proteins of present interest have been converted to a common unit weight ($10^5$ grams). The total number of cationic nitrogen residues, $\Sigma(\equiv NH^+)$, and of free carboxyl groups (*i. e.*, corrected for amide nitrogen), $\Sigma(COO^-)$, could then be calculated on a comparable basis for each protein, and the sums obtained have been placed in Table III.

It is apparent from the data that competition by carboxylate groups alone could not account for

(23) E. H. Lepper and C. J. Martin, *Biochem. J.*, **21**, 356 (1927).
(24) I. M. Klotz, H. Triwush and F. M. Walker, THIS JOURNAL, **70**, 2935 (1948).
(25) F. Karush and M. Sonenberg, *ibid.*, **71**, 1369 (1949).
(26) G. L. Miller and K. J. I. Andersson, *J. Biol. Chem.*, **144**, 475 (1942).
(27) E. Volkin, *ibid.*, **175**, 675 (1948).
(28) G. Scatchard and E. S. Black, *J. Phys. and Colloid Chem.*, **53**, 88 (1949).
(29) H. Neurath, THIS JOURNAL, **61**, 1841 (1939).
(30) E. P. Abraham, *Biochem. J.*, **33**, 622 (1939).
(31) E. Brand, *Ann. N. Y. Acad. Sci.*, **47**, 187 (1946).
(32) I. M. Klotz and J. M. Urquhart, *J. Phys. and Colloid Chem.* **53**, 100 (1949).

TABLE III[34]

AMINO ACID COMPOSITION AND BINDING ABILITY OF SOME PROTEINS

| Protein | $\Sigma(COO^-)$ | $\Sigma(OH)$ | $\Sigma(\equiv NH^+)$ | $\frac{\Sigma(\equiv NH^+)}{\Sigma(COO^-) + \Sigma(OH)}$ | $\frac{\Sigma(\equiv NH^+)}{\lvert\Sigma(COO^-) - \Sigma(OH)\rvert}$ |
|---|---|---|---|---|---|
| Bovine serum albumin[a] | 133 | 128 | 145 | 0.55 | 29 |
| β-Lactoglobulin[a] | 141 | 118 | 105 | .40 | 4.6 |
| Egg albumin[b] | 98 | 134 | 90 | .39 | 2.5 |
| Casein[b] | 99 | 146 | 100 | .41 | 2.1 |
| Insulin[a] | 63 | 150 | 72 | .34 | 0.83 |
| Bovine γ-globulin[c] | 67 | 216 | 99 | .35 | 0.66 |
| Pepsin[d] | 109 | 248 | 18 | .05 | 0.13 |

Data on amino acid composition was obtained from the following sources: [a] E. Brand, *Ann. N. Y. Acad. Sci.*, **47**, 187 (1946). [b] A. C. Chibnall, *J. Int. Soc. Leather Trades Chem.*, **30**, 1 (1946). [c] Assumed to be the same as that for human γ-globulin given by E. Brand, *loc. cit.* [d] E. Brand and J. T. Edsall, *Ann. Rev. Biochem.*, 16, 223 (1947).

the differences between the proteins, for γ-globulin has a higher ratio of $\Sigma(\equiv NH^+)/\Sigma(COO^-)$ than does albumin, yet a lower binding ability. If the hydroxyamino acids are also introduced into the ratio, since these too could combine with an $\equiv NH^+$ group through a hydrogen bridge, the ratio $\Sigma(\equiv NH^+)/\{\Sigma(COO^-) + \Sigma(OH)\}$ does show some correlation with binding ability. Thus bovine albumin has a high value for this ratio whereas the other proteins have a low index (Table III). On the other hand, the ratio does not vary significantly between β-lactoglobulin and egg albumin, whereas binding ability does. Furthermore the difference in value of the ratio between serum albumin and β-lactoglobulin, or between serum albumin and γ-globulin, is much smaller than the relative binding abilities of the respective proteins. Evidently some pertinent factor has been overlooked.

Further reflection indicates that this factor may be internal interactions between the OH and $COO^-$ groups themselves. There is no compelling reason why the hydroxyl (or carboxyl) groups should always be bound to cationic loci. In fact, a very strong case might be made for a preferential bond of the type $OH\ldots{}^-OOC$ rather than $O\ldots{}^{+}HN\equiv$, since an O–H...O bond is stronger than an O...H–N bond.[33] The net result of this interaction would be to decrease the number of $COO^-$ and OH groups which can combine with the cationic $\equiv NH^+$ loci, and hence to increase the number of free cationic nitrogens, and, consequently, the possibility of anion binding by the protein.

In this hydroxyl-carboxyl interaction, it is evident that if the number of either one of these functional groups exceeds that of the other, the excess number of residues, $|\Sigma(COO^-) - \Sigma(OH)|$, would be available to combine with the quaternary nitrogens, and thereby to decrease the ability of the proteins to bind anions. Thus a measure of the net effect of this type of interaction on binding ability might be the ratio $\Sigma(\equiv N^+H)/|\Sigma(COO^-) - \Sigma(OH)|$, for the more quaternary nitrogens, the greater the possibility of binding, whereas the greater the difference between total carboxyl and total hydroxyl groups (regardless of the sign of this difference), the greater the interference with binding.

The correlation between this second type of ratio and binding affinity is very satisfactory, as can be observed in Table III. Thus serum albumin stands out among the proteins. β-Lactoglobulin, in turn, is clearly superior to its successors in the table, though far below serum albumin. Egg albumin and casein fall next into line. With lower values of the ratio $\Sigma(\equiv NH^+)/|\Sigma(COO^-) - \Sigma(OH)|$, these two proteins evidently have such weak binding properties that an observable effect cannot be obtained with methyl orange in solutions basic to the isoelectric point. In solutions acid to the isoelectric point, however, opposite charges on protein and anion tend to increase the concentration of anion at the surface of the protein, and hence supply the additional free energy needed to produce an observable degree of binding. With a protein such as γ-globulin, finally, the residue ratio, and hence the binding affinity, is so low, that even the additional contribution of electrostatic attraction at *p*H's acid to the isoelectric point is inadequate to produce binding.

In view of the excellent agreement between binding ability and the value of the ratio $\Sigma(\equiv NH)^+)/|\Sigma(COO^-) - \Sigma(OH)|$, it seems worthwhile to use this ratio as a "binding index" from which to predict whether a given protein will show a measurable affinity for anions. The index, at present, is purely a relative one, but, nevertheless, offers a criterion on the basis of which one can place a new protein in its proper position among the list in Table III. With an ion which is bound only weakly, such as methyl orange, only proteins with a high binding index will exhibit observable effects. On the other hand, with an anion, such as dodecyl sulfate, which is bound much more strongly, even proteins low in the value of the binding index may form complexes. This anion is such a strong competing agent,[34] that it is capable of breaking up the internal interactions between cationic nitrogens and the other functional groups on the protein. Thus

(33) L. Pauling, "The Nature of the Chemical Bond," 2nd edition, Cornell University Press, Ithaca, N. Y., 1945, pp. 333–334.

(34) We are indebted to Miss Lucy Fuller for her assistance in these calculations.

dodecyl sulfate forms complexes with egg albumin[2,5] even at *p*H's alkaline to the isoelectric point. This situation thus seems to be analogous to that described by Porter[35] who found that about one-third of the $\epsilon$-$NH_2$ groups of native $\beta$-lactoglobulin would not react with 1,2,4-fluorodinitrobenzene but could be acetylated by ketene or acetic anhydride.

**Effect of Net Charge of Protein on its Binding Ability.**—The importance of the net surface charge of the protein in re-inforcing the intrinsic anion affinity of the protein at *p*H's acid to the isoelectric point can be seen also in serum albumin. Binding experiments in acetate buffer at *p*H 5 and in lactate buffer at *p*H 3.7 are compared in Fig. 4. A substantially increased binding is observed at the lower *p*H, where the protein has a net positive charge. A small part of this effect may be due to differences in the competing ability of the two types of buffer anion.[32] However, this buffer-competition effect is undoubtedly a minor one, since the increase in competing ability on adding a $-CH_3$ group, in going from acetate to lactate buffer, is probably counterbalanced by a decrease due to the simultaneous introduction of the polar -OH group.[24]

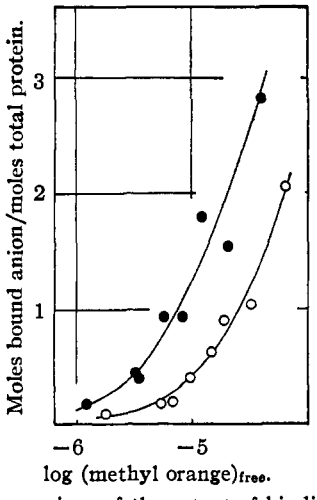


Fig. 4.—Comparison of the extent of binding of methyl orange by bovine serum albumin at *p*H 5.0, O, and at *p*H 3.7, ●.

The increase in binding should be even more magnified in the lactate buffer in a stricter comparison, for the concentration of methyl orange in anionic form at *p*H 3.7 is only about half the total indicator concentration, whereas at *p*H 5, practically all of the dye is the negatively-charged form. Thus a plot of extent of binding *versus anion concentration* at *p*H 3.7 would have the effect of shifting the curve to the left compared to that illustrated in Fig. 4, that is, to increase the observed degree of binding. Thus, there can be little doubt of the positive contribution of net protein charge to its binding ability. The effect may even be amenable to quantitative expression by an analysis similar to that used by Hartley and Roe[36] in their investigations of ionic concentrations at the interfaces of charged micelles.

**Effect of Denaturation.**—The configurational model which has been postulated also offers a basis for describing some of the changes in binding properties associated with denaturation of the proteins. In the case of serum albumin, it has been observed[24] that exposure to sodium hydroxide over prolonged periods destroys the affinity of the soluble protein for anions. A possible mechanism for this process would be the hydrolysis of the glutamine and asparagine groups with consequent liberation of carboxylate ions to join up with the free cationic nitrogens. The net result of this breakdown of amide groups would be to increase the degree of internal competition in the protein and hence to decrease the extent of binding.

The effect of heat[24] and particularly of boiling[37] on the binding ability of albumin is a little more difficult to visualize. The net result, a decrease in binding, indicates a reduction in the number of free cationic groups. It is of interest to note in this connection that heating converts lysine groups in other proteins into a biologically inactive form, that is, into a state resistant to enzymic action, though not to acid hydrolysis.[38,39] It has been postulated[38] that this inactivation reaction is due to the formation of a peptide link between the $\epsilon$-amino group of lysine and a carboxyl group of glutamic or aspartic acid, and a similar process could account for the loss in binding ability of serum albumin.[40] It is also possible, however, that the disruption of the protein framework caused by the heat releases some hydrogen-acceptor groups from internal bonds and makes them available for coupling with the cationic nitrogens.

In the case of egg albumin, heat denaturation, with accompanying coagulation, converts the protein into a form capable of binding methyl orange anions[41] as well as dodecyl sulfate.[2] If the coagulation process following denaturation is due to cross-linking between disoriented polypeptide chains, it seems likely that in the stretched-out chains the side-chain functional groups, which can interact with the cationic nitrogens, will no longer be sufficiently close to do so, as they were in the compact native protein. As a result more cationic residues will be free and hence capable of binding anions.

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## Conclusions

All of the work reported so far is consistent with a picture of the binding process, for organic anions, which lays primary emphasis on the cationic groups in the protein. Thus the modified proteins described in this paper retain their affinity for anions so long as the change has not decreased the number of cationic loci. Acetylation, on the other hand, decreases the affinity of albumin for anions, just as the more drastic de-amination of gelatin reduces the number of bound dodecyl sulfates.[42]

In consideration of variations in binding ability of different proteins, a similar point of view, extended to include internal, competitive interactions, between functional groups of the protein, seems to be promising. As a minimum, it suggests a quantitative criterion, based on amino acid composition, for predicting whether a given protein will show significant binding properties. Applied in a general manner, it offers a model on the basis of which many phases of anion-protein interactions can be interpreted satisfactorily.

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## Summary

The binding abilities of four fractions of bovine plasma, $\gamma$-globulin, $\beta_2$-globulin, $\alpha_2$-globulin and crystallized albumin, have been compared. Only the latter showed significant binding properties. Among the crystallized native proteins which have been examined, including serum albumin, $\beta$-lactoglobulin, egg albumin, insulin, lysozyme, ribonuclease, pepsin, trypsin and chymotrypsin, only the first two formed complexes with the reference anion, methyl orange.

Conversion of the $\epsilon$-ammonium groups of lysine to guanidinium groups did not alter the binding ability of serum albumin. On the other hand, acetylation of the $\epsilon$-ammonium groups did reduce the degree of binding substantially.

The relative binding abilities of various native proteins can be correlated by a "binding index" based on the distribution in content of amino acids with the molecule. The index can be justified on a molecular basis in terms of internal interactions between the functional groups of the constituent amino acids of the protein.

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# The Fractionation of Proteins by Electrophoresis-Convection. An Improved Apparatus and its Use in Fractionating Diphtheria Antitoxin

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## Introduction

A method of fractionation of proteins in solution utilizing a combination of electrophoretic and convective transport of the components was suggested by Kirkwood[2] in 1941 and investigated experimentally by Nielsen and Kirkwood[3] several years later. The method is based upon the same principles as that of the Clusius column, except that horizontal electrophoretic transport instead of transport by thermal diffusion is superimposed on convective transport in a vertical convection channel. The fractionation scheme may be briefly described as follows. Two reservoirs connected by a vertical channel, of width sufficiently small to ensure laminar flow, contain a solution of the proteins to be fractionated. Upon application of a horizontal electric field, differential transport of the mobile components across the channel takes place, producing a horizontal density gradient depending upon the composition gradients. Under the action of gravity, the density gradient induces convective circulation in the channel with a velocity distribution qualitatively similar to that of the Clusius column. The result of the superposition of the horizontal electrophoretic transport and vertical convective transport is movement of the mobile components from the top reservoir to the bottom reservoir at rates depending on their mobilities, with a relative enrichment of the top reservoir with respect to the slow components and of the bottom reservoir with respect to the fast components. The mathematical theory of the transport has been worked out for representative operating conditions and will be presented in a future article. It has been used in the design of the improved fractionation cell to be described here.

In order to avoid contamination of the solution by electrolysis products, the walls of the convection channel are constructed of semi-permeable membranes, separated from the electrodes by buffer solution. The electric field across the channel is maintained by the electric current carried by the ions of the buffer electrolyte, to which the membranes are permeable. The exterior buffer solution is replenished by a cir-

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