[CONTRIBUTION FROM THE SLOAN-KETTERING INSTITUTE FOR CANCER RESEARCH]

The Study of Protein Binding by Partition Analysis. The Effect of Protein Charge[1,2]

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A procedure has been developed for the study of protein binding involving the distribution of the substance of interest between an aqueous phase containing the protein and an immiscible organic phase. The most important factors here are the partition coefficient and the effect of the organic solvent on the protein as, *e.g.*, surface denaturation. The optimum conditions for the system containing methyl orange and bovine albumin have been worked out. The use of the quaternary salt, benzyltrimethylammonium chloride, offers a decisive advantage. It was possible to study the effect of protein charge on the dye binding free from the complications due to the competitive binding of salt ions and the heterogeneity of the binding sites. The observed effect of altering the charge is much less than that predicted by the Debye–Hückel theory. The results also suggest a slight structural change in the protein, associated with the titration of the imidazole groups, which affects the binding properties of the protein.

Much of the recent quantitative work on the binding properties of purified proteins has been based on the method of equilibrium dialysis.[3-6] Though it is of rather wide applicability this method is subject to several significant limitations. (1) It is required, on the one hand, that the protein molecule be sufficiently large to be retained by the dialysis bag. This limits the study of the binding properties of protein fragments and polypeptides by this method. On the other hand, the substance which is bound must be small enough to be readily dialyzable. Therefore large molecules cannot be investigated. (2) Another factor of less importance, though often of considerable inconvenience, is the extended time interval required for equilibration in the dialysis method. (3) In binding studies with dyes it has been found that usually a significant correction must be made for the membrane adsorption of the dye. Under certain circumstances the variability of this correction leads to a lower precision in the determination of the amount of dye bound than the analytical method permits. (4) Whenever the protein carries a net electrostatic charge it is the usual practice to add dialyzable electrolyte to minimize the Donnan effect. In the case of serum albumins, at least, this is particularly undesirable because these proteins bind even the simplest anion, *e.g.*, chloride. As a result the protein charge is altered, usually to an unknown extent.

This binding of salt anions is especially objectionable when it is competitive with the binding of other anions. Such has been the case in previous studies with organic anions. This situation obscures the interpretation of binding results in terms of the structural characteristics of the protein because the association constants and related thermodynamic quantities are relative to the kind and concentration of competitive anion. A further complication, in this connection, arises from the heterogeneity in the binding of simple anions[7] and organic anions.[5]

The competitive effect adds an additional difficulty to the theoretical calculation of the electrostatic interaction between bound ions because of the uncertainty as to the change in protein charge. This uncertainty arises from the possibility that the binding of the ion may involve a displacement from the protein of the competitive inorganic ion.

In this paper we describe a new method of studying protein binding which avoids the difficulties cited above. This method involves the equilibration of an aqueous phase, containing protein and substance under study, with an appropriate immiscible organic solvent. The concentration of this substance in the organic phase is measured. This value together with a knowledge of the partition coefficient permits the calculation of the concentration of unbound substance in the aqueous phase, as well as the amount bound to the protein. The method depends on the assumption that the partition coefficient for the uncombined material is not affected by the protein. For the protein concentration used in this study this assumption is undoubtedly justified. A similar assumption also underlies the method of equilibrium dialysis.

The use of an organic phase in the method of partition analysis leads to several characteristic considerations associated with the proper choice of organic solvent.

(1) The selection of solvent depends, of course, on the particular small molecule or ion which is under study since the substance must be partitioned between the aqueous and organic phases. It is generally desirable that the value of the partition coefficient be in the neighborhood of one. However, under conditions where the concentration in the aqueous phase of the unbound substance is very small, a much larger value may be needed for analytical purposes. The possibility of working with a partition coefficient much larger than one may make feasible binding studies by partition analysis not possible by equilibrium dialysis. (2) The existence of a liquid–liquid interface in the partition analysis procedure leads to an increased tendency to surface denaturation of the protein. This restricts considerably the kinds of solvents which may be used and is probably the most serious limitation of the method. (3) No component of the organic

(1) Presented in part before the Division of Biological Chemistry at the Philadelphia Meeting of the American Chemical Society, April, 1950. Investigation conducted during the writer's tenure of a Fellowship in Cancer Research of the American Cancer Society, recommended by the Committee on Growth of the National Research Council; present address: Dept. of Pediatrics, University of Pennsylvania School of Medicine.

(2) A preliminary description of this method has been published; see ref. 11. W. L. Hughes, Jr. has also employed essentially the same procedure in a study of the reaction between mercaptalbumin and methylmercury iodide (*Cold Spr. Harb. Symposium Quant. Biol.*, **14**, 79 (1950)).

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

(4) J. D. Teresi and J. M. Luck, *J. Biol. Chem.*, **173**, 21 (1948).

(5) F. Karush and M. Sonenberg, THIS JOURNAL, **71**, 1369 (1949).

(6) G. Scatchard, I. H. Scheinberg and S. H. Armstrong, Jr., *ibid.*, **72**, 535 (1950).

(7) G. Scatchard, I. H. Scheinberg and S. H. Armstrong, Jr., *ibid.*, **72**, 540 (1950).

phase should be sufficiently soluble in the aqueous phase to alter significantly the solvent properties of the latter. Even a slight solubility may lead in some cases to a competitive effect on the protein binding. Such an effect must be taken into account when comparing results obtained by partition analysis with those of another method.

This method has been applied to the study of the binding of methyl orange by bovine serum albumin. The optimum conditions for this system have been worked out and are described below. Particular attention was directed to the effect of net protein charge on the binding in order to evaluate the electrostatic interaction arising from this source. This was feasible in the present instance because: (a) buffer salts were not used, (b) the competitive effect of the chloride ion was slight because of its low concentration (0.01 *M*) and (c) the complications due to the heterogeneity of the binding sites were avoided by using the appropriate extrapolation of the experimental results (see below).

Experimental

Materials.—The protein used was crystallized bovine serum albumin obtained from Armour and Company. Its moisture content was determined by drying to constant weight at 105°. Correction was also made for its ash content.

The anionic azo dye was a sample of reagent grade methyl orange which was recrystallized twice from water and dried *in vacuo*.

The method also involves the use of the quaternary ammonium salt, benzyltrimethylammonium chloride. This was contributed by the Commercial Solvents Corporation in the form of a 60% aqueous solution. Its purification was effected by the following procedure: (1) complete removal of water by evaporating to dryness under vacuum on a steam-bath and exposure to drying agent under vacuum; (2) solution of the solid in the minimum amount of boiling absolute alcohol; (3) precipitation of salt by addition of approximately equal volume of ether and cooling; (4) precipitate washed with ether and dried *in vacuo*.[8]

The organic medium finally selected was a mixed solvent consisting of 60% by volume of redistilled 1-hexanol and 40% practical "heptane." It was washed with 0.1 *N* alkali to remove organic acids. This step also served to saturate the solvent with water.

Procedure.—Binding determinations were carried out with threaded glass vials stoppered with tinfoil-lined caps, each containing 10 ml. of the aqueous phase and 10 ml. of solvent. The vials were placed on a rocker immersed in a constant temperature bath maintained at 25.0 ± 0.1°. They were rocked at a frequency of 5 cycles per minute, a rate sufficient to permit mixing in each phase without excessive disturbance of the interface. Under these conditions a rocking period of 4 hours was found sufficient for equilibration.

All solid solutes were initially present in the aqueous phase. The albumin concentration was 1.00×10^{-4} *M*, based on a molecular weight of 69,000, and that of the benzyltrimethylammonium chloride was 0.0100 *M*. Various initial dye concentrations were used, the maximum being 8.00×10^{-5} *M*. Usually, no buffer was employed. Stock solutions of 2.50×10^{-4} *M* albumin were prepared daily including an amount of 0.100 *N* sodium hydroxide required to give the protein a specified average charge. The binding determinations were always done in duplicate, the results agreeing usually to within 1%. Control tubes were included in every experiment for the determination of the partition coefficient. These contained initially in the aqueous phase 0.0100 *M* quaternary salt, 0.001 *M* phosphate buffer, *p*H 7.0 and a convenient concentration of dye, usually 2.00×10^{-5} *M*. The quaternary salt appears to be negligibly soluble in the organic phase.

(8) I am indebted to Dr. M. E. Balis of the Sloan-Kettering Institute for the purification of the quaternary salt.

For purposes of comparison a few binding determinations were made by equilibrium dialysis. Ten-ml. portions of a solution of 1.00×10^{-4} *M* protein, 0.0100 *M* quaternary salt and 0.0500 *M* phosphate buffer, *p*H 7.0, contained in dialysis bags, were equilibrated against 10-ml. portions of dye in a similar solution without protein. The solutions were rocked overnight in a constant temperature water-bath, 25.0 ± 0.1°. The electrolyte concentration was large enough to render the Donnan correction negligible. Controls were run without protein to ascertain the casing adsorption correction. In the range of free dye concentrations used, this correction varied from 5.5 to 6.7% of the free dye depending on the presence of dissolved hexanol.

Analytical Method.—The concentration of methyl orange was determined by measurement of its light absorption with a model DU Beckman spectrophotometer. In the partition analysis experiments the absorption of the dye in the organic phase was measured. For the determination of the partition coefficient the dye concentrations in both phases were ascertained. In the particular solvent used the wave length of maximum absorption, in the visible region, of the quaternary cation-methyl orange complex is 416 mμ, and the molar extinction coefficient is 3.11×10^4. For methyl orange in an aqueous medium containing 0.01 *M* of the quaternary salt and saturated with solvent, the corresponding quantities are 464 mμ and 2.695×10^4.

Results and Discussion

The Partition Coefficient.—As noted above the choice of solvent depends, among other factors, on the nature of the constituent which is being partitioned. In general, it is preferable to use a solvent of minimum polarity in order to avoid surface denaturation. This also serves to reduce the solubility of the solvent in the aqueous phase. However, when working with substances which are ionic in the aqueous phase we have been unable to avoid the use of a substantial fraction of a polar component. For methyl orange, in particular, the higher alcohols have been especially useful, probably because of their ability to form hydrogen bonds.

In our initial attempts to partition methyl orange in the form of its sodium salt it was necessary to work with solutions containing 0.1 *M* sodium chloride. This concentration of anion, whether it be chloride or any other, is objectionable because of the binding of anions by albumin accompanied by an unknown change in protein charge.[9] Furthermore, this binding is probably competitive with respect to the binding of dye anions, including methyl orange.[10,11]

For these reasons the use of an organic quaternary ammonium salt to increase the partition coefficient offered a decisive advantage. The readily available salt benzyltrimethylammonium chloride was found particularly suitable and it has, therefore, been incorporated as an essential feature of our procedure. It is most satisfactorily employed at a concentration of 0.01 *M* when methyl orange is to be partitioned. Under these conditions it gives a partition coefficient close to 2 in favor of the organic phase with a solvent, described above, which is neither excessively soluble in the aqueous phase nor harmful to the protein.

A value of 0.01 *M* for the quaternary salt concentration is also a satisfactory compromise with respect to two further considerations. On the one

(9) G. Scatchard and E. S. Black, *J. Phys. Colloid Chem.*, **53**, 88 (1949).

(10) I. M. Klotz and J. M. Urquhart, *ibid.*, **53**, 100 (1949).

(11) F. Karush, THIS JOURNAL, **72**, 2705 (1950).

hand the concentration of chloride ion is sufficiently low so that, based on the studies of Scatchard, Batchelder and Brown,[12] we may be assured that there is only slight binding of this anion. On the other hand, this amount of electrolyte is adequate to ensure that the ionic strength of the aqueous phase will remain fairly constant within the range of dye concentration and protein charge encompassed by our experiments.

Because of the extreme difference in solvent properties of the two constituents of the organic phase the partition coefficient is extremely sensitive to their relative amounts. Although the solubility of 1-hexanol in water is only 0.59% by weight at 20°, it was observed that the change in composition due to solution of hexanol in the aqueous phase during equilibration caused a 2% reduction in the partition coefficient. This was established by comparison with the value found when the aqueous phase contained, before contact with the organic phase, an amount of 1-hexanol whose fraction of its solubility was approximately the same as its mole fraction in the organic medium. In view of this sensitivity it is highly desirable that controls be included in every experiment, and it is essential to redetermine the partition coefficient when fresh solvent is prepared.

Experiments were carried out, without protein, to ascertain the possible dependence of the partition coefficient (dye concentration in organic phase/dye concentration in aqueous phase) on the final dye concentration. It was found to increase slowly with decrease in the dye concentration. For example, at a dye concentration in the organic medium of 1.91×10^{-5} M the partition coefficient is 1.78 but at 0.65×10^{-5} M dye its value has risen to 1.86. This variation is probably due to partial dissociation of the quaternary cation–dye complex in the organic medium. To secure the most accurate binding results the preparation and use of an appropriate calibration curve is required.

Since the binding experiments to be described subsequently involved a wide range of pH and small variations in ionic strength, the effect of these factors on the partition coefficient was investigated. With the particular system used in this study the partition coefficient was found to be independent of pH in the range of 5.3 to 9.4 and to be experimentally insensitive to the addition of 0.002 M sodium chloride.

Stability of Protein.—A characteristic property of soluble globular proteins is their ability to undergo surface denaturation. This tendency is often accentuated when the interface exists between an aqueous phase and an immiscible organic phase. We have therefore made some observations designed to reveal whether any significant amount of denaturation of the albumin occurs during the rocking period.

It is to be expected that the protein would be most sensitive to surface effects in the isoelectric region. When a 1.00×10^{-4} M albumin solution, pH 5.2, containing 0.01 M quaternary salt, was rocked with solvent for 4.5 hours at 25°, a thin skin formed at the interface. An analysis of the aqueous phase showed that 1.5% of the protein had been rendered insoluble. The concentration of the soluble protein was determined by measurement of the light absorption at λ291 of a 12.5-fold dilution of the solution in 0.1 N sodium hydroxide. On the other hand when enough alkali was added to the albumin solution to give it an average charge of −8 (pH 6.4), no measurable amount of protein was precipitated. This was also true at higher pH values. Though indicative, these results do not exclude structural changes of the protein which would nevertheless leave it soluble, particularly under conditions where the protein carries a net charge different from zero.

A more crucial observation is the effect of continued rocking on the binding of the dye by the protein. Here it was found that the dye concentration in the organic phase after 8 hours was the same as after 4.5 hours of rocking. In this experiment the protein had a charge of about −16. This result demonstrates that there is not occurring a slow process during the rocking period which significantly alters the measurable binding properties of the protein.

Finally, the comparison of results obtained by partition analysis and equilibrium dialysis discussed below indicates that the binding properties of the albumin are not irreversibly affected by the rocking. We may conclude, therefore, that the albumin probably does not undergo any gross structural alterations in the partition analysis procedure except that associated with the insolubilization of a slight amount of protein in the isoelectric region.

Comparison of Partition Analysis and Equilibrium Dialysis.—Binding experiments have been carried out by these two methods under experimental conditions as closely similar as possible. Since 0.05 M phosphate buffer was used in the dialysis procedure, this was also included in the comparable partition analysis experiment. The results of this partition experiment are shown near the end of Table I. Here, r is the average number of dye anions bound per albumin molecule at the free aqueous equilibrium dye concentration c.

The comparison of the results of the two methods has led to the disclosure of two characteristic factors in the partition method which influence the observed binding behavior of the protein. The initial failure to obtain agreement gave rise to experiments which suggested that, on the one hand, the hexanol dissolved in the aqueous phase was exerting an inhibitory effect and, on the other hand, the exposure of the protein to the organic phase produced an enhancement of its binding of the dye.

In Table II are given the results of dialysis experiments designed to demonstrate the significance of these factors. For purposes of this demonstration the values of r/c can be directly compared. There is also included for comparison, in the last line of the table, one of the partition binding determinations described above. As shown by (4) and (5) of Table II, under similar conditions partition analysis yields a higher value of r/c, indicating greater binding, than does the dialysis

(12) G. Scatchard, A. C. Batchelder and A. Brown, THIS JOURNAL, 68, 2320 (1946).

TABLE I

PARTITION ANALYSIS RESULTS FOR BINDING OF METHYL ORANGE BY BOVINE SERUM ALBUMIN AT 25°, 0.0100 *M* QUATERNARY SALT, 1.00 × 10^{-4} *M* ALBUMIN

Initial dye concn. in aqueous phase, m./l. × 10^5	Dye concn. in organic phase, m./l. × 10^5	Free dye concn. in aqueous phase, c m./l. × 10^5		r/c × 10^{-4}
h = 0, pH = 5.3, albumin = 0.985 × 10^{-4} *M*				
2.40	0.280	0.147	0.200	13.6
4.00	.455	.241	.336	13.9
5.60	.645	.345	.468	13.6
8.00	.940	.508	.665	13.1
h = −8, pH = 6.4				
2.40	0.304	0.160	0.194	12.10
4.00	.510	.271	.322	11.87
5.60	.726	.391	.448	11.47
8.00	1.069	.582	.635	10.92
h = −8, pH = 6.4				
3.20	0.407	0.216	0.258	11.96
4.80	.625	.334	.384	11.50
6.40	.850	.460	.509	11.06
h = −16, pH = 7.6				
2.40	0.292	0.158	0.195	12.25
4.00	.498	.274	.323	11.79
5.60	.712	.396	.449	11.33
8.00	1.091	.613	.630	10.30
h = −16, pH = 7.6				
3.20	0.396	0.214	0.259	12.10
4.80	.614	.335	.385	11.50
6.40	.838	.462	.510	11.04
8.00	1.095	.609	.630	10.34
h = −16, pH = 7.6				
2.40	0.303	0.158	0.194	12.28
4.00	.522	.276	.320	11.60
5.60	.755	.403	.444	11.02
8.00	1.125	.609	.627	10.30
h = −20, pH = 7.9				
2.40	0.332	0.176	0.189	10.75
4.00	.570	.307	.312	10.18
5.60	.824	.448	.433	9.66
8.00	1.229	.675	.610	9.04
h = −24, pH = 8.5				
2.40	0.394	0.212	0.179	8.46
4.00	.674	.368	.296	8.04
5.60	.980	.542	.408	7.53
8.00	1.460	.820	.572	6.97
h = −32, pH = 9.0				
2.40	0.565	0.307	0.153	4.98
4.00	0.957	.530	.251	4.74
5.60	1.342	.750	.351	4.68
8.00	2.046	1.169	.478	4.10
0.0500 *M* phosphate, pH 7.0				
2.40	0.386	0.213	0.180	8.46
4.00	.658	.370	.297	8.04
5.60	.941	.535	.412	7.71
8.00	1.390	.800	.581	7.26
h = −16 + x, pH = 7.6, 0.0100 *M* NaSCN				
2.40	0.572	0.356	0.147	4.14
4.00	0.975	.617	.241	3.90
5.60	1.384	.887	.333	3.76
8.00	2.027	1.325	.465	3.51

TABLE II

EQUILIBRIUM DIALYSIS RESULTS FOR BINDING OF METHYL ORANGE BY BOVINE SERUM ALBUMIN AT 25°, 0.0100 *M* QUATERNARY SALT, 0.0500 *M* PHOSPHATE, pH 7.0, 1.00 × 10^{-4} *M* ALBUMIN, 8.00 × 10^{-5} *M* INITIAL OUTSIDE DYE

	Conditions	Free dye concn., c × 10^5	r	r/c × 10^{-4}
1	Inside solution previously rocked with solvent 4.5 hr., at 25°	0.752	0.640	8.51
2	Inside solution as above; outside solution 80% saturated with hexanol	.904	.609	6.74
3	5 ml. of solvent included in bag	.798	.563	7.05
4	No exposure to solvent	.945	.598	6.34
5	Comparative partition analysis results	.800	.581	7.26

method. However, when the albumin solution is rocked *versus* solvent before it is placed in the dialysis bag, an even larger value of r/c is obtained, as is evident from (1). If, in addition to a preliminary rocking, the outside solution is 80% saturated with hexanol, as in (2), r/c is substantially reduced. Finally, if the dialysis experiment is conducted without prior treatment of the albumin but with the inclusion of 5 ml. of solvent in the bag, see (3), the value of r/c found is only slightly less than that observed by partition analysis. In this experiment the dye concentrations were determined both in the outside solution and in the organic phase. In view of the large difference in (3) and (5) of the ratios of aqueous volume to solvent volume, the values of r/c can be considered to be in fairly good agreement.

The enhancement effect arising from prior rocking with solvent is in all probability due to removal of lipid from the albumin. The presence of fatty acid in albumin prepared by the alcohol fractionation procedure has been reported.[13] In addition, the use of long-chain alcohols to promote crystallization leads to their binding and, undoubtedly, to their presence in the final product. These lipid constituents probably compete with dye anions for common binding sites on the albumin molecule and thereby reduce the binding of the dye. The inhibitory effect of dissolved hexanol shown above also probably arises from its competitive binding to the protein. Thus, the greater binding shown by the partition method as compared to the dialysis method, (5) *vs.* (4), can be attributed to the net effect of lipid removal and hexanol inhibition.

The results of Table II, as was mentioned in the last section, indicate that rocking with solvent does not cause irreversible structural changes in the albumin molecule. This is suggested by the fact that a prior rocking results in an increase in the value of r/c, (1) *vs.* (4) and (5), whereas denaturation would be expected to reduce the binding.[14]

The possible effect on the dye binding of the relatively large benzyltrimethylammonium ion was also investigated by the dialysis method. By comparison with the binding observed when sodium ion was substituted for the quaternary ion, a small but definite inhibition due to the latter ion was found.

(13) E. J. Cohn, W. L. Hughes, Jr., and J. H. Weare, THIS JOURNAL, **69**, 1753 (1947).

(14) I. M. Klotz, H. Triwush and F. M. Walker, *ibid.*, **70**, 2935 (1948).

At equal values of r the larger ion causes an 8% decrease in r/c relative to the smaller ion. This small effect is consistent with the observation of Scatchard and Black[9] that trimethylphenylammonium iodide alters the isoionic point of human serum albumin about the same extent as sodium iodide. The inhibition is opposite in direction to the electrostatic effect which would arise from the binding of quaternary ion. Evidence for complex formation in the aqueous phase between the cation and methyl orange was sought by light absorption measurements. However, the quaternary ion caused less than a 1% change in the extinction coefficient of the dye.

The Effect of Protein Charge.—The multiple binding capacity of serum albumins for ions poses the problem of the effect of the electrostatic interaction among the bound ions on the observed binding curve. If, for a particular strongly bound ion, the intrinsic association constants of the binding sites of the protein were identical, then it would be possible to evaluate this effect by the deviation from linearity of the binding results when plotted in an appropriate manner. This procedure is not valid, however, because of the demonstration that the association constants for the several sites exhibit a large variation.[5,7,11]

The quantitative assessment of this variation offers a clue to the structural differences among the binding sites. However, in order to evaluate the inherent differences of the binding constants, it is necessary to consider the electrostatic interaction among the bound ions. The effect of this interaction on the binding curve is similar to that which results from the inequality of these constants. It is, therefore, very difficult to distinguish experimentally between the two effects. As a consequence it has become the practice to calculate the electrostatic correction theoretically on the basis of the Debye–Hückel formulation of the interionic attraction theory. This procedure has been most consistently followed in the work of Scatchard and his associates.[6,7]

The availability of the method of partition analysis has made possible a relatively simple experimental approach to this problem. Instead of measuring the electrostatic interaction of the bound ions, we have studied the effect of the net protein charge on the binding. This was done under conditions, as pointed out above, where the complications due to competitive binding and heterogeneity of the sites were avoided. It should be emphasized, however, that though our procedure entails a minimum theoretical ambiguity, it is not entirely without shortcoming. The limitation here arises from the possibility that the addition or removal of protons, aside from altering the charge of the protein, may cause slight structural alterations in it, resulting in a change in its binding properties. If this were the case then the experimental results could not be interpreted simply in terms of an electrostatic effect. As will be seen shortly, our results are suggestive of such a possibility.

The theoretical basis of our experiments will be clear from the following considerations: If there are n binding sites per protein molecule with intrinsic association constants K_i, under specified conditions of pH, temperature, etc., then it follows from the law of mass action that

$$\lim_{c \to 0} \frac{r}{c} = \sum_{i=1}^{n} K_i \qquad (1)$$

If we assume that the electrostatic contribution to the free energy of binding of an ion, due to the net protein charge, is proportional to the average charge of the protein and, furthermore, that all sites are equally affected by this charge, then we may write

$$K_i = K_i^0 e^{-2wzh} \qquad (2)$$

Here h is the charge of the protein, z is the charge of the small ion and K^0_i is a characteristic association constant of the ith site, assumed to be independent of pH. The quantity w can be regarded as an empirical constant or, if certain assumptions are made, it may be calculated from the Debye–Hückel equation.

From (1) and (2) it follows that

$$\lim_{c \to 0} \frac{r}{c} \equiv \left(\frac{r}{c}\right)_0 = e^{-2wzh} \sum_i K_i^0 \qquad (3)$$

Thus we may calculate values of w corresponding to increments of h from the determination of $(r/c)_0$ for various specified values of h. This can be done by measurement of dye binding at low dye concentration and, consequently, for small values of r.

In Table I are summarized the results of binding experiments designed to yield values of $(r/c)_0$ for selected values of the protein charge. These results are also given in Fig. 1 in the form of a plot of r/c *vs.* r. The values of $(r/c)_0$, shown in Table III, are the intercepts on the ordinate and are found by linear extrapolation of the experimental results. To secure the maximum accuracy in this extrapolation the binding determinations were conducted with as low values of r (and c) as was experimentally feasible. The pH values of the various albumin solutions were determined and are included in Table I. Measurement of the pH of the aqueous phases after equilibration with solvent were also carried out. No significant pH effect due to exposure to solvent was noted.

TABLE III

EFFECT OF PROTEIN CHARGE ON THE BINDING OF METHYL ORANGE BY BOVINE SERUM ALBUMIN AT 25°

	pH	$(r/c)_0$	Interval of h	$w_{exp.}$ ($w_{theor.} = 0.0619$)
0	5.3	14.7	.....	.....
− 8	6.4	12.8	0 to −8	0.00862
−16	7.6	13.2	.....	.....
−20	7.9	11.5	−16 to −20	.0172
−24	8.5	9.1	−20 to −24	.0293
−32	9.0	5.4	−24 to −32	.0324
0.05 *M* phosphate	7.0	9.0	.....	.....
−16 + *x*, 0.01 *M* NaSCN	7.6	4.43	.....	.....

The possibility that an uncontrolled variable, such as might be associated with different batches of solvent, was affecting the results was subjected to test. Two binding experiments with $h = -16$ were carried out. One was done at the beginning

of the experiments shown in Table I and the other at their completion. The results of these two experiments are in satisfactory agreement and are included in Table I. They demonstrate that all the relevant variables were under adequate control during our experiments.

We have already noted that the quantity w can be calculated theoretically from the Debye–Hückel theory. It is easily shown from the equation for the electrical free energy of a uniformly charged sphere[15] that the effect of this charge on the binding of a small ion is given by equation (2). If the protein is taken to be a sphere of radius 30 Å. with a radius of exclusion for small ions of 32.5 Å. then w is found to be 0.0619 at 25° and ionic strength 0.01. It may be noted that w is rather insensitive to the exact difference between these radii.

In Table III are presented the experimentally derived values of w for various indicated intervals of h. These values exhibit several striking features. In the first place they are all considerably smaller than the theoretical value of w. Secondly, they are not constant but increase with larger negative charge on the protein. Thirdly, and perhaps most intriguing, is the anomalous effect which occurs between pH 6.4 ($h = -8$) and pH 7.6 ($h = -16$). For the most part, as would be qualitatively anticipated, the binding diminishes in going from pH 5.3 to 9.0. However, in the transition from 6.4 to 7.6 there appears to be a slight increase in binding in spite of the increased negative charge. Since this interval is included in the range in which the imidazole groups are titrated,[16] the possibility appears that the removal of protons from these groups gives rise to slight structural alterations of the protein which are reflected in a change of its binding properties.

If such a structural effect did obtain the titration of the imidazole groups might extend over a wide enough pH range to account for the low and inconstant values of w found between pH 5.3 and 7.9. Nevertheless, it is apparent from the other values of w that the theory gives a value of w which is too large by at least a factor of 2.

It is clear that the Debye–Hückel approximation of the interionic attraction theory is inadequate to describe the results found in this investigation. Even if a possible structural effect is taken into account there is still a serious discrepancy between theory and experiment. One cause of this may lie in the grossly simplified structural assumptions of the theory. This pictures the net protein charge as symmetrically distributed on the surface of the sphere and assumes that the added charge is likewise distributed. These may be particularly inappropriate assumptions for the conditions of our investigation compared to the conditions associated with titration studies. In the latter case one measures the binding of a large number of protons over an extremely wide range of concentration of hydrogen ion. In any event our procedure is probably more sensitive than the titration method to changes in association constants.

In view of our results the use of the Debye–Hückel equation to calculate the electrostatic interaction of bound ions is open to serious question. In recent studies by Scatchard, *et al.*, on the combination of chloride ion and thiocyanate ion with human serum albumin,[6,7] the binding results were corrected by a factor based on this equation. In the light of the above, the quantitative validity of this procedure is questionable. The justification for it cannot be established by the experimental results both because of their unavoidable inaccuracy and, particularly, because of the ambiguity arising from the heterogeneity of the binding sites.

(15) E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides," Reinhold Publishing Corp., New York, N. Y., 1943, p. 473.

(16) R. K. Cannan, *Chem. Revs.*, **30**, 395 (1942).

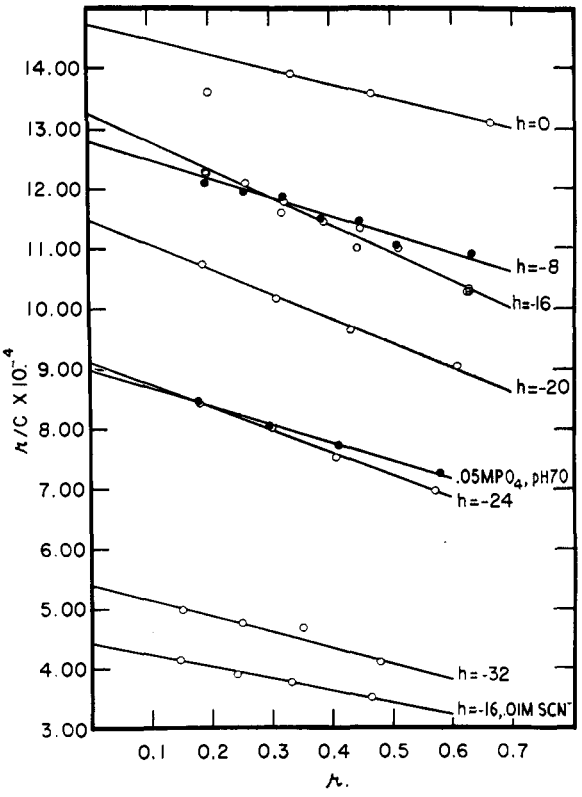

Fig. 1.—The effect of protein charge and inorganic ions on the binding of methyl orange by bovine serum albumin at 25°.

Competitive Effects.—Since many binding studies have been conducted with phosphate buffered systems, we have measured the effect of phosphate ions on the combination of methyl orange with albumin. These results are given in Tables I and III which also include measurements with 0.01 M thiocyanate. The protein charge for the latter case is shown as $-16 + x$ to indicate an unknown change in charge due to binding of thiocyanate ions. The partition coefficients required for the calculations were determined, of course, in the presence of these ions.

It is clear that both phosphate and thiocyanate ions cause a considerable reduction in the binding of methyl orange. Undoubtedly, this effect is not restricted to this dye but is generally true for organic anions. It is noteworthy that thiocyanate is much more effective than phosphate, a result which is consistent with the relatively strong binding of thiocyanate by human serum albumin as demonstrated by Scatchard, Scheinberg and Armstrong.[7]

Though the available evidence is not adequate to permit an unequivocal decision, it is probable that much of the effect of these inorganic ions is a competitive one, *i.e.*, they compete with methyl orange for common binding sites on the protein. This conclusion is clearer in the case of thiocyanate because of its greater effectiveness and because of the studies of its binding in the reference cited above. A similar conclusion was reached in a study of the effect of bound dodecyl sulfate on the combination of an anionic azo dye with bovine albumin.[17]

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(17) F. Karush, THIS JOURNAL, **72**, 2714 (1950).

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Biochemical Studies on Vitamin A. IX. Biopotency of Neovitamin A in the Rat

BY PHILIP L. HARRIS, STANLEY R. AMES AND JOHN H. BRINKMAN

The physiological potencies of neovitamin A and of neovitamin A acetate have been compared with all-*trans* vitamin A and all-*trans* vitamin A acetate using standard vitamin A bioassay procedures. By rat-growth bioassay, neovitamin A has 85.3% the biopotency of all-*trans* vitamin A on an E-value basis and 80.7% on a molar basis. By liver-storage type bioassay, neovitamin A shows 75.6% the biopotency of the all-*trans* isomer on an E-value basis and 71.5% on a molar basis. Rats have the ability to convert ingested all-*trans* to neovitamin A and *vice versa*, and they tend to deposit in their liver a mixture of the two isomers containing approximately 12% of the neo form.

Pure crystalline neovitamin A (*cis–trans*) has been characterized chemically and physically[1,2] and has had assigned to it tentatively[2] a high biological potency, "substantially the same" as vitamin A (all-*trans*). Subsequent repeated bioassays of the two vitamin A isomers in pure form, both free and esterified, have confirmed their high activities, but have indicated a significant difference in their biological potencies. The present report summarizes the pertinent data obtained in this Laboratory during the past seven years.

Experimental

The pure compounds used in the study were prepared by the Organic Chemistry Department of Distillation Products Industries and were supplied as pure crystals in sealed ampoules or as solutions of the crystalline compounds in refined cottonseed oil at approximately 1% concentration. The all-*trans* vitamin A acetate used was in most instances crystalline material dissolved in cottonseed oil and distributed as the U.S.P. Reference Standard.

Spectrometric determinations of specific absorbancy ($E^{1\%}_{1\ cm.}$) of the vitamin A compounds were made using a Beckman spectrophotometer and employing isopropyl alcohol as the solvent. The estimated potencies of the neovitamin A compounds were obtained by multiplying their $E^{1\%}_{1\ cm.}$ 328 mμ by 1894. This conversion factor was that currently used to transpose *E*-value ($E^{1\%}_{1\ cm.}$ 325 mμ) of all-*trans* vitamin A to biological potency in terms of U.S.P. units per gram. Consequently, most comparisons in this report are made on a "per unit *E*-value" basis.

Bioassays from which the data were obtained were of two types, rat growth and liver storage. The rat-growth bioassays were routine tests in which two levels of a variety of substances were compared with two similar levels of vitamin A acetate, a modified U.S.P. XIII procedure. In many of the early bioassays, the design of the test was such that no estimate of the limits of uncertainty could be made. However, later bioassays were set up according to the suggestions of Bliss[3] and for these, standard errors have been calculated. The liver-storage bioassays, also used to compare the physiological activities of all-trans and of neovitamin A, were conducted by a modification[4] of previously described methods.[5,6] The results obtained were examined statistically to determine relative potency and standard error.

Results

The comparison of biopotencies, by rat-growth tests, of the two types of vitamin A alcohol are shown in the first portion of Table I. The evidence indicates that neovitamin A possesses less vitamin A potency per unit *E*-value than all-*trans* vitamin A. This indication is borne out by the results obtained by comparing the biopotency of neovitamin A acetate and all-*trans* vitamin A acetate as also shown in Table I. The over-all mean, 85.3% with a standard error of 2.58%, indicates that neovitamin A compared with all-*trans* vitamin A, per unit *E*-value, is less potent by about 15%.

Comparisons using the liver-storage type of bioassay yielded the results shown in Table II. The average of 75.6 ± 3.52% (S.E.) for the potency of neovitamin A compared with all-*trans* vitamin A determined by liver storage is somewhat lower than the 85.3 ± 2.58% relationship established for the rat-growth method. However, the difference between the two means is not statistically meaningful (t = 1.94, P = 0.05–0.1).[7] Thus, neovitamin A is probably as well utilized by the rat for storage as for growth.

A mixture of the two isomers, simulating naturally-occurring fish oils which contain vitamin A in the proportion, one-third neovitamin A and two-thirds *trans*-vitamin A, was bioassayed both by growth and by liver-storage methods. In this experiment (Table III), neovitamin A acetate

(1) C. D. Robeson and J. G. Baxter, *Nature*, **155**, 300 (1945).

(2) C. D. Robeson and J. G. Baxter, THIS JOURNAL, **69**, 136 (1947).

(3) Suggested Revision of the U. S. P. Biological Assays for Vitamins A and D Submitted to the U. S. P. by the Animal Nutrition Research Council through Dr. C. I. Bliss, November 15, 1948.

(4) S. R. Ames, P. L. Harris and H. A. Risley, to be published.

(5) K. Guggenheim and W. Koch, *Biochem. J.*, **38**, 256 (1944).

(6) J. R. Foy and K. Morgareidge, *Anal. Chem.*, **20**, 304 (1948).

(7) t = diff. of means $\sqrt{\frac{n_1\sigma_1^2 + n_2\sigma_2^2}{n_1 n_2}\left(\frac{n_1 + n_2}{n_1 + n_2 - 2}\right)}$