material was observed to be present, although about 50 mg. of *meso*-inositol containing more ash was left in the mother liquor.

We wish to take this opportunity to express our thanks to Standard Brands, Inc., for a fellowship which made possible this work. Also we wish to thank Merck and Co., Inc., for the microanalyses reported in this paper.

Summary

A new method for the preparation of hexahydroxybenzene is described.

Hexahydroxybenzene has been found not to reduce with platinum catalysts as previously reported but has been reduced using Raney nickel to give two known cyclitols; scyllitol, m. p. 353–355°; *meso*-inositol, m. p. 224–225°; and one new cyclitol, m. p. 213–214° (hexaacetate 205–206°). Evidence is also given for the presence of *epi*-inositol and another new cyclitol (hexaacetate, m. p. 139–140°) in the reduction mixture.

A discussion of these results is given.

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[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF NORTHWESTERN UNIVERSITY]

The Binding of Organic Ions by Proteins. Competition Phenomena and Denaturation Effects

BY IRVING M. KLOTZ, HENRY TRIWUSH[1] AND F. MARIAN WALKER

Competition between organic anions for loci of attachment on protein molecules has been demonstrated recently by spectral methods.[2] From changes in optical absorption of a given reference dye, it has been possible to arrange a series of noncolored anions in the order of their relative abilities to displace the dye and hence to combine with the protein. These investigations have now been extended to several series of isomeric compounds to elucidate certain structural effects and have been placed on a more quantitative basis for evaluating molecular weight and substituent influences.

Since the change in absorption of the dye is also a reflection of the state of the protein molecule, the spectral method has been used also to examine the effects of various denaturants on anion-protein complexes.

Experimental

Reagents.—All experiments involving protein have utilized crystallized bovine serum albumin from Armour and Co. The azo dyes which served as reference ions have been described previously.[2]

Salicylic acid, *p*-toluenesulfonic acid, naphthalene β-sulfonic acid, urea and urethan were Eastman "highest purity" grade. The sodium dodecyl sulfate was a specially purified sample generously supplied by the du Pont Co. Acetyl-*l*-tryptophan was prepared for us by Dr. A. H. Schlesinger following the procedure of du Vigneaud and Sealock.[3] The product had a m. p. of 189–190°. Acetyl-*d,l*-leucine was also prepared by Dr. Schlesinger, in an analogous fashion to the tryptophan derivative, and exhibited a m. p. of 160.5–161.5°.

Procedure.—All buffers were made from reagent grade phosphate salts. In the measurement of displacement effects, the following order of additions was generally used. Weighed quantities of albumin and of dye, respectively, were added to separate flasks containing known quantities of the buffer solution. A weighed quantity of the competing substance was then placed in a third dry volumetric flask to which were added 10 cc. of the dye solution, measured with a pipet, and enough protein solution to bring the volume to the 50 cc. mark on the flask. In every case two controls were also prepared, one of which contained 10 cc. of dye diluted to 50 cc. with buffer, and the second of which consisted of 10 cc. of dye diluted to 50 cc. with protein-bufler solution.

In the case of urea and urethan, however, this procedure was not convenient because large quantities of these substances were required. The required quantity of urea, or urethan, was added, therefore, to the initial buffer solution, in separate portions of which the dye and protein were subsequently dissolved. In experiments in which the order of addition was to be varied, the following procedure was adopted. Methyl orange was dissolved in a sample of buffer containing no urea. Practically equal quantities of albumin were added to each of two dry volumetric flasks. In one of these was placed 10 cc. of the dye solution, whereas the other was given 10 cc. of urea-buffer solution. Each flask was permitted to stand 10 minutes. The first was then filled with urea-buffer solution, the second with 10 cc. of dye solution and additional urea-buffer solution, to the 50 cc. mark. Spectra were taken immediately after complete mixing in each case.

Spectra were obtained in 1-cm. silica cells with the Beckman spectrophotometer.

The dialysis-equilibrium experiments have been described adequately in previous publications.[4,5]

Results and Discussion

As has been demonstrated previously,[2] a dye ion bound by a protein molecule shows a spectral absorption curve distinctly different from that of the free ion (Fig. 1). In the presence of a competing ion, some of the bound dye may be displaced from the protein, and hence the spectrum will be shifted back toward that of the free ion. The extent of the reversal is a measure of the displacing ability of the competitor ion and therefore of its affinity for the protein. On this basis it has

(1) Lederle Laboratories Fellow, 1947.

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

(3) V. du Vigneaud and R. R. Sealock, *J. Biol. Chem.*, **96**, 514 (1932).

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

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

been possible to determine the relative displacing abilities of several organic acids.

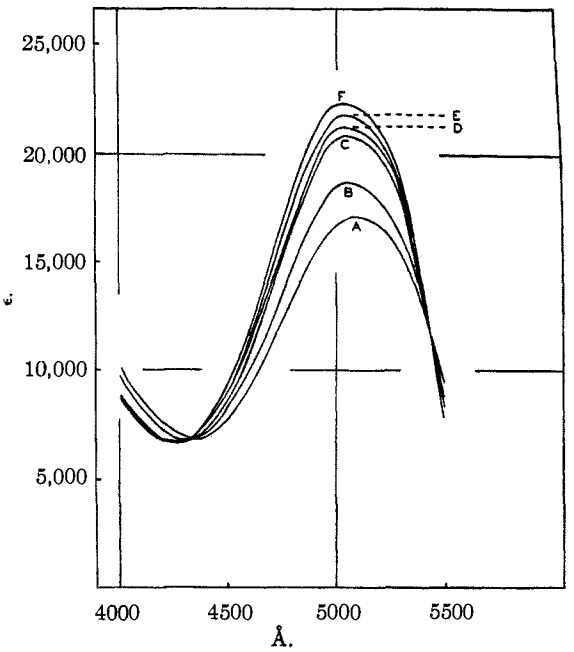

Fig. 1.—Influence of competing anions on spectrum of azosulfathiazole at *p*H 6.8: A, with **bovine** albumin; B, bovine albumin and *p*-aminobenzene **sulfonic** acid (0.200%); C, bovine albumin and *p*-hydroxybenzoic acid (0.202%); D, bovine albumin and *o*-aminobenzene sulfonic acid (0.198%); E, bovine albumin and *o*-hydroxybenzoic acid (0.202%); F, buffer only. In each case the protein concentration was 0.155% and the dye 1.546×10^{-5} *M*.

Relative Affinities of Some Ortho–para Isomers.—From preceding investigations[2] it was evident that the anion of an organic acid is a more effective displacing agent than the parent non-ionized compound. An attempt was made also to uncover structural effects by an examination of the relative affinities of several ortho–para isomers of some aromatic acids, but a consistent trend was not apparent. Extension of these studies to some additional series, however, now shows a consistently higher relative affinity for albumin in ortho compounds in which the substituent is a hydrogen donor. Some of the pertinent data are illustrated in Fig. 1, which shows the comparative displacing abilities of the ortho and para isomers of aminobenzenesulfonic acid and hydroxybenzoic acid. Data on the aminobenzoic acids have been reported previously.[2] Examination of the results for each pair of isomers shows that the ortho compound is bound more strongly than the corresponding para isomer.

With the chlorobenzoic acids, however, the ortho and para isomers are rather close in their respective affinities for albumin, as judged by previous displacement experiments.[2] The difference, if any, is in favor of the para compound, in contrast to the behavior observed with the hydroxyl- and amino-substituted aromatic acids. Evidently the "ortho-effect" is limited to substituents which can be donors in a hydrogen-bond. In the ortho position the hydrogen-donating group forms a bridge with the adjacent substituent which removes the former from the aqueous phase and hence decreases interactions with the water molecules. On the other hand in the para position, the hydrogen-donating group is free to interact with water molecules, and hence the attraction of the solvent tends to keep the molecule from being bound by the protein. A similar effect has been pointed out previously with the orange dyes.[2]

Mass-law Analysis of Competitive Binding.—In the displacement experiments which have have been described, one observes essentially the result of competition between two types of ion for the same locus on the protein molecule. It would be highly desirable to obtain more than just *relative* binding affinities from these data, which are inherently quantitative in nature. An examination has been made, therefore, of the multiple equilibria which may be involved, and of the conditions under which these may be reduced to a simple equation to correlate the binding of the competing species.

In the situation under consideration with two types of ion, represented by A and B, respectively, competing for sites of attachment on the protein molecule, numerous complexes may exist, any one of which may be represented by the notation PA_iB_j. P represents the protein and i and j may each vary from zero to n, the maximum number of sites available on the protein molecule, so long as $(i + j)$ does not exceed n. This latter restriction is obviously necessary if we are considering competition among the limit of n available sites.

Experimentally we have measured the free and bound quantities of only *one* of the ions, *e. g.*, A, but not that of the other species. We are interested, however, in the dependence of bound A on the quantity of B present in the solution.

If we let r represent the moles of bound A per mole of total protein, it becomes evident by an analysis similar to that used in the simpler case[4] that

$$r = \frac{\text{moles of bound A}}{\text{moles total protein}} \tag{1}$$

$$= \frac{\sum_{j=0}^{n-1}(PA_1B_j) + 2\sum_{j=0}^{n-2}(PA_2B_j) + \cdots + i\sum_{j=0}^{n-i}(PA_iB_j) + \cdots + n(PA_n)}{\sum_{j=0}^{n}(PB_j) + \sum_{j=0}^{n-1}(PA_1B_j) + \sum_{j=0}^{n-2}(PA_2B_j) + \cdots + \sum_{j=0}^{n-i}(PA_iB_j) + \cdots + (PA_n)} \tag{2}$$

$$= \frac{\sum_{i=1}^{n}\sum_{j=0}^{n-i} i(PA_iB_j)}{\sum_{i=0}^{n}\sum_{j=0}^{n-i}(PA_iB_j)} \qquad (i + j) \gg n \tag{3}$$

If electrostatic interactions between successively bound anions are neglected, the generalized expression (3) may be replaced readily by a very simple equation, for in such a situation each locus on the protein may be treated as a separate individual. Corresponding to the relation

$$r = \frac{nk_a(A)}{1 + k_a(A)} \quad (4)$$

when a single species, A, is being bound, we have the equation[6]

$$r = \frac{nk_a(A)}{1 + k_a(A) + k_b(B)} \quad (5)$$

in the event of competition. k_a and k_b represent the intrinsic binding constants for the respective species. It also follows, of course, that if s represents the moles of bound ion B per mole of protein, then it will be given by the relation

$$s = \frac{nk_b(B)}{1 + k_b(B) + k_a(A)} \quad (6)$$

Thus the ratio of bound A to bound B becomes

$$\frac{r}{s} = \frac{k_a(A)}{k_b(B)} \quad (7)$$

A combination of equations (5) and (7) allows one to evaluate both the binding constant for B, k_b, and the concentration of free B from a knowledge of n, k_a, (A) and r. The latter two may be determined from the spectrum of the reference dye, in the presence of the competing ion, B, and the protein. k_a and n may be obtained from a separate series of spectral or dialysis-equilibrium studies.

Since equation (5) neglects the effects of electrostatic interactions on the binding constants it is desirable to use it only where such interactions are small. Previous work has indicated that such interactions are small for monovalent ions. Hence the quantitative aspect of the work to be described has been limited to monovalent anions, and the singly-charged dye, methyl orange has been used as the reference anion.

Binding of Methyl Orange.—Earlier quantitative work on methyl orange–albumin complexes[4] was carried out at *p*H 5.7 and a temperature of 5°. The spectral studies, however, have been made at *p*H 7.6 and 24°, so that it is necessary to obtain further quantitative data to evaluate k_a and n.

The dialysis-equilibrium technique has been used for this purpose and the results obtained are illustrated in Fig. 2. Application of least square methods to these data leads to a value of 4.19×10^3 for the intrinsic constant, k_a, and to 12.8 for the (average) maximum number of methyl orange anions which may be bound by a single albumin molecule. With this information it is possible to proceed to evaluate binding constants for noncolored anions from competiton experiments.

It is of interest perhaps to note that there is a substantial difference between the value of n in the present study and that obtained in previous work[4] with a different buffer at *p*H 5.7 and 5°. Buffer anions in themselves are capable of competing with dye anions and specific effects may be introduced. On the other hand, the reality of the difference in n may be open to some question in view of the difficulty of obtaining precise values of n at high concentrations of dye, since r is calculated from the difference between two large values of (A). In view of this problem rather detailed studies of buffer effects are being carried out at present in this laboratory.

(6) E. C. Markham and A. F. Benton, THIS JOURNAL, **53**, 497 (1931).

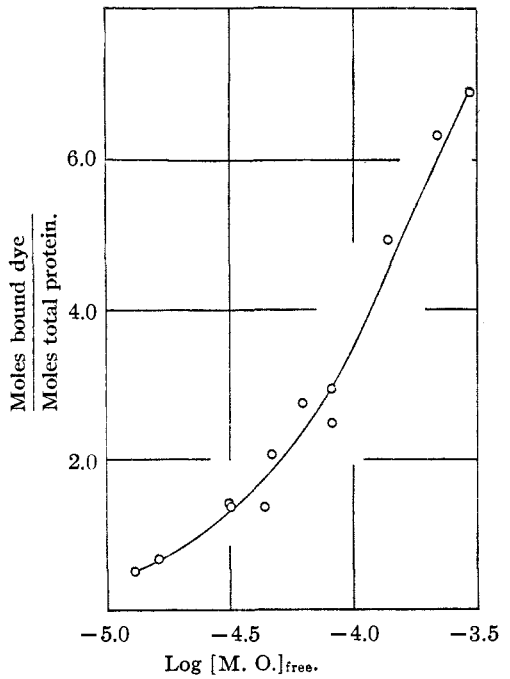

Fig. 2.—Binding of methyl orange by bovine serum albumin at *p*H 7.6 and 24°.

Binding of Salicylate Ions.—A series of spectra of methyl orange was obtained in solutions of variable concentration of salicylate but fixed concentrations of bovine serum albumin and of the reference dye. From the magnitude of the absorption in one of these solutions, as compared to that of the free dye and of the albumin-dye complex, respectively, it is possible to calculate the fraction of methyl orange, α, which is in the free unbound form, by methods previously described.[2] Values of α in methyl orange solutions containing salicylate, as well as protein, are assembled in Table I.

If we define the following terms

A_t = total reference dye in moles per liter
B_t = total competitor ion in moles per liter
P_t = total protein in moles per liter
$r' = rP_t$ = bound reference dye in moles per liter
$s' = sP_t$ = bound competitor ion in moles per liter

equations (5) and (7) may be rearranged by simple algebraic manipulation to the following forms

TABLE I

CALCULATION OF BINDING CONSTANTS WITH BOVINE SERUM ALBUMIN FROM DISPLACEMENT EXPERIMENTS WITH METHYL ORANGE: *p*H 7.6, 24°

B_t	A_t	α	P_t	$k_a\alpha$	$k_a\frac{\alpha}{1-\alpha}$	$nk_a\frac{\alpha}{1-\alpha}$	s'	k_b
Salicylate								
0.01012	1.173×10^{-5}	0.69	2.704×10^{-5}	2.90×10^{3}	9.35×10^{3}	11.9×10^{4}	0.000233	2.2×10^{2}
.00495	1.173×10^{-5}	.62	2.704×10^{-5}	2.61×10^{3}	6.85×10^{3}	8.75×10^{4}	.000192	2.8×10^{2}
.00245	1.173×10^{-5}	.54	2.704×10^{-5}	2.27×10^{3}	4.95×10^{3}	6.30×10^{4}	.000137	2.9×10^{2}
.00115	1.173×10^{-5}	.49	2.722×10^{-5}	2.04×10^{3}	4.02×10^{3}	5.15×10^{4}	.000094	3.6×10^{2}
.00043	1.173×10^{-5}	.42	2.722×10^{-5}	1.74×10^{3}	3.03×10^{3}	3.88×10^{4}	.000012	0.9×10^{2}
							Av. =	2.5×10^{2}
Dodecyl sulfate								
0.00209	1.110×10^{-5}	0.86	7.89×10^{-5}	3.59×10^{3}	25.1×10^{3}	32.5×10^{4}	0.00098	2×10^{4}
.00102	2.208×10^{-5}	.83	8.37×10^{-5}	3.47×10^{3}	20.1×10^{3}	26.0×10^{4}	.00102	.....
.000765	2.208×10^{-5}	.76	8.37×10^{-5}	3.18×10^{3}	13.2×10^{3}	17.0×10^{4}	.00099	.....
.000612	2.208×10^{-5}	.70	8.37×10^{-5}	2.95×10^{3}	9.80×10^{3}	12.5×10^{4}	.00096	.....
.000510	2.208×10^{-5}	.63	8.37×10^{-5}	2.65×10^{3}	7.18×10^{3}	9.10×10^{4}	.00092	.....
.000437	2.208×10^{-5}	.51	8.37×10^{-5}	2.13×10^{3}	4.38×10^{3}	5.60×10^{4}	.00083	.....
.000340	2.208×10^{-5}	.37	8.37×10^{-5}	1.54×10^{3}	2.45×10^{3}	3.13×10^{4}	.00065	.....
.000306	2.208×10^{-5}	.36	8.37×10^{-5}	1.50×10^{3}	2.35×10^{3}	3.01×10^{4}	.00064	.....
Acetylleucine								
0.01146	1.160×10^{-5}	0.47	3.26×10^{-5}	1.96×10^{3}	3.72×10^{3}	4.75×10^{4}	0.000141	4.6×10^{1}
Acetyltryptophan								
0.00800	1.160×10^{-5}	0.55	3.26×10^{-5}	2.31×10^{3}	5.15×10^{3}	6.55×10^{4}	0.000215	1.4×10^{2}
p-Toluenesulfonate								
0.01076	1.160×10^{-5}	0.75	3.26×10^{-5}	3.13×10^{3}	12.5×10^{3}	16.1×10^{4}	0.000337	2.8×10^{2}
Naphthalene-β-sulfonate								
0.00890	1.160×10^{-5}	1.00	3.26×10^{-5}	4.19×10^{3}	.....	.....	.....	.....
Urea								
3.0	1.160×10^{-5}	0.56	2.22×10^{-5}	2.36×10^{3}	5.35×10^{3}	6.82×10^{4}	.000097	0.17
Urethan								
1.00	1.160×10^{-5}	0.70	2.26×10^{-5}	2.95×10^{3}	9.80×10^{3}	12.5×10^{4}	.000183	1.8

which are more convenient for purposes of calculation

$$s'\frac{k_a\alpha}{1-\alpha} = nk_a\frac{\alpha}{1-\alpha}P_t - k_a\alpha A_t - 1 \qquad (8)$$

$$k_b = k_a\frac{\alpha}{1-\alpha}\frac{s'}{B_t - s'} \qquad (9)$$

The calculations of k_b for salicylate complexes of bovine serum albumin have been made with the aid of equations (8) and (9) and are summarized in Table I.

The results obtained give some indication of a decrease in k_b with increasing salicylate concentration. This may be a reflection of the cumulative influence of the electrostatic factors which have been neglected. However, a small error in the (extrapolated) value of the extinction coefficient of the methyl orange–albumin complex may also contribute to this trend, so that its significance is still a matter of some doubt. For the present the average value of 2.5×10^2 for k_b will be used.

It is of interest to note that the intrinsic binding constant for methyl orange is almost seventeen times greater than that for salicylate ion. In terms of energies of binding, ΔF_1^0, for the first bound ion, is −6460 calories/mole for methyl orange and −4780 calories/mole for salicylate ion. The greater affinity of the dye is undoubtedly an expression of the increased van der Waals interaction in the larger molecule as has been emphasized particularly by Steinhardt.[7]

Binding of Sodium Dodecyl Sulfate.—Several series of spectra have been obtained for methyl orange–albumin solutions with varying quantities of sodium dodecyl sulfate. It has been evident immediately that sodium dodecyl sulfate is bound very strongly by albumin, for at concentrations of less than 0.001 *M* it is capable of displacing methyl orange almost completely from its albumin complex, whereas a substance such as salicylate ion requires more than ten times that concentration to do likewise.

Attempts have been made, furthermore, to evaluate the intrinsic binding constant from the equations derived above. Since these equations assume implicitly that the competition is a reversible one, the spectra of two solutions were compared in which the concentrations of methyl orange, sodium dodecyl sulfate and albumin were

(7) J. Steinhardt, *Ann. N. Y. Acad. Sci.*, **41**, 287 (1941).

identical, but the order of addition of the former two reagents was altered. The optical absorption was found to be independent of the order of addition. Duggan and Luck's[8] work also indicates that sodium dodecyl sulfate, in low concentrations, does not denature bovine albumin.

It has seemed appropriate, therefore, to apply equations (8) and (9) to competition between sodium dodecyl sulfate and methyl orange. The calculations which have been made are assembled in Table I. At first glance they may seem puzzling in that the concentration *calculated* for bound sodium dodecyl sulfate exceeds the total concentration actually present, except for the solution above 1×10^{-3} *M*. A likely interpretation may be that practically all of the dodecyl sulfate is in the bound state and that the discrepancies between the calculated bound values and the concentrations actually present are due to the neglect of electrostatic factors in deriving equation (5). These electrostatic repulsions of successively bound ions, being cumulative, may be significant at high degrees of binding, and hence particularly serious for a strongly bound anion. In any event it is evident that most of the data cannot be used to calculate an intrinsic binding constant for sodium dodecyl sulfate. The experiment at 0.002 molar concentration indicates a k_b of about 2×10^4. However, its significance may be subject to some question, for the experiments of Corrin and Harkins[9] show that the critical concentration of sodium dodecyl sulfate is near 0.001 *M* at equivalent concentrations of added electrolyte of 0.1 or more.[10] Since micelle formation has not been considered at all in the derivation of the equations for competition, any intrinsic constants calculated therefrom may be in error.

Binding of Acetylleucine, Acetyltryptophan, *p*-Toluenesulfonic Acid and Naphthalene-β-sulfonic Acid.—Preliminary experiments had indicated that these compounds are progressively better displacing agents in the order listed. It seemed of interest, therefore, to evaluate their intrinsic binding constants with bovine albumin. The results obtained are summarized in Table I. The affinity constants increase in the order of the qualitative observations, though a value could not be estimated for the naphthalene-β-sulfonate ion because the displacement of methyl orange was complete, within the error of observation. Further experiments must be carried out at lower concentrations.

Though these are only the beginnings of more extensive measurements, it is already evident that the polar nature of substituents may overbalance any tendency of increased molecular weight to increase binding, for the acetyltryptophan ion though of higher weight is not bound as strongly as the naphthalenesulfonate anion. It may also be pertinent to note that the *p*-toluenesulfonate ion has an intrinsic affinity constant substantially the same as that of the salicylate ion, a further emphasis of the reduction of polar interactions with the aqueous solvent when the hydroxyl group is ortho to the anionic group.

Binding of Urea and of Urethan.—Much attention has been paid to the interaction of urea with proteins, as measured by several physical and chemical criteria.[8,11] Nevertheless, since many aspects of the action of urea are still obscure, an investigation of its displacing ability toward methyl orange–albumin complexes has seemed in order.

Urea has been found capable of displacing methyl orange and of producing intermediate spectra similar to those illustrated in Fig. 1. The degree of displacement depends on the concentration of urea, as is illustrated in Fig. 3, no effect being observed in 1 molar solutions and complete displacement being obtained at 6 molar concentration. The shape of the concentration curve in Fig. 3 is very similar to that obtained by Neurath, Cooper and Erickson[12] using certain solubility tests as the measure of activity of the urea.

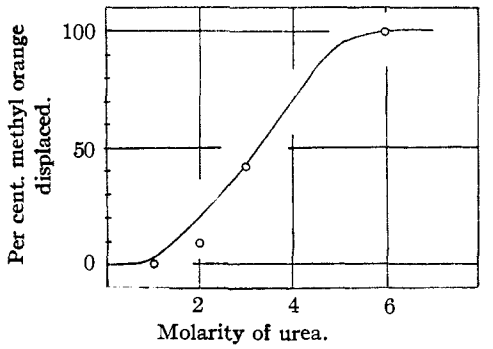

Fig. 3.—Displacing ability of urea as a function of concentration.

The 1 and 6 molar solutions, respectively, were each examined spectrally at two different times, once within ten minutes of the preparation of the solution and again after having stood at room temperature for twenty-four hours. The spectra with and without standing did not differ significantly at either concentration. Thus, insofar as displacement phenomena are concerned, the action of urea is very rapid. It is possible of course that other aspects of urea interaction with albumin are much slower in developing.

In the 3 molar solution, a comparison was made also of the effect of order of addition of urea and methyl orange to the albumin on the resultant spectra. Typical readings are illustrated in Table II. It is evident that the small differences

(8) E. L. Duggan and J. M. Luck, *J. Biol. Chem.*, **172**, 205 (1948).

(9) M. L. Corrin and W. D. Harkins, THIS JOURNAL, **69**, 1428 (1947).

(10) It is of interest to note that the minimum in Duggan and Luck's data on the viscosity of sodium dodecyl sulfate solutions containing albumin and urea occurs at the critical concentration of the detergent.

(11) H. Neurath, J. P. Greenstein, F. W. Putnam and J. O. Erickson, *Chem. Rev.*, **34**, 157 (1944).

(12) H. Neurath, G. R. Cooper and J. O. Erickson, *J. Biol. Chem.*, **142**, 249 (1942).

are within experimental error. In any event the addition of urea first, does not increase the degree of displacement. Apparently we are dealing with a true competition between the dye anion and the urea molecules for sites on the albumin.

TABLE II

EFFECT OF ORDER OF ADDITION ON DISPLACING ABILITY OF UREA

Wave length, Å.	Optical density	
	Urea added first	Dye added first
4000	0.194	0.194
4200	.253	.254
4400	.278	.283
4600	.282	.286
4700	.269	.271
4800	.241	.244
5000	.155	.158
5200	.072	.072

TABLE III

TIME INDEPENDENCE OF DISPLACING EFFECT OF URETHAN

Wave length, Å.	Optical density	
	10 minutes	24 hours
4100	0.209	0.209
4300	.264	.263
4500	.290	.288
4700	.291	.286
4900	.234	.230
5100	.139	.136

In view of this behavior it has seemed worthwhile to calculate the binding constant for urea from the displacement data in the 3 molar solution. The calculation is outlined in Table I. The constant obtained, 0.17, is the smallest yet observed, as would have been predicted from purely qualitative considerations.

A similar calculation has been made for urethan from an experiment on its displacing ability. The greater effectiveness of urethan as compared to urea is evident from the calculated binding constant, 1.8, as well as from the fact that lower concentrations produce equivalent displacements of methyl orange from the albumin.

A comparison has been made also of the displacing abilities of 1 molar urethan immediately after addition to the albumin and twenty-four hours after mixing, respectively. The optical densities of the methyl orange–albumin solutions (Table III) are substantially the same in both cases. Once again it is evident that the displacing action is very rapid, equilibrium results being obtained within an interval of minutes.

Effect of Sodium Hydroxide.—Previous experiments[5] have demonstrated that exposure of bovine serum albumin to 0.01 molar sodium hydroxide for periods up to two hours, and subsequent return to *p*H 7.6 does not alter significantly the ability of the protein to combine with methyl orange. Extension of this work to longer time intervals, however, has shown that prolonged exposure of the albumin to the alkali does slowly destroy its ability to bind dyes. As is illustrated in Fig. 4, bound methyl orange is slowly decreased, but even after forty-eight hours at 26° the protein has not been denatured entirely, as judged by the spectral criterion.

A single experiment was carried out also at 55°. After forty-eight hours exposure to 0.01 *M* sodium hydroxide, the protein produced no alteration of the spectrum of methyl orange. Denaturation was thus complete.

Effect of Heat.—Maintenance of the protein in neutral solution (*p*H 7.6) at 55° will slowly decrease its ability to change the spectrum of an anion, such as methyl orange (Fig. 5). It is evident, however, that this temperature is not a very drastic treatment as is exposure to dilute alkali. A few experiments were carried out also with the divalent anion, azosulfathiazole, at *p*H 6.8, and similar behavior was observed.

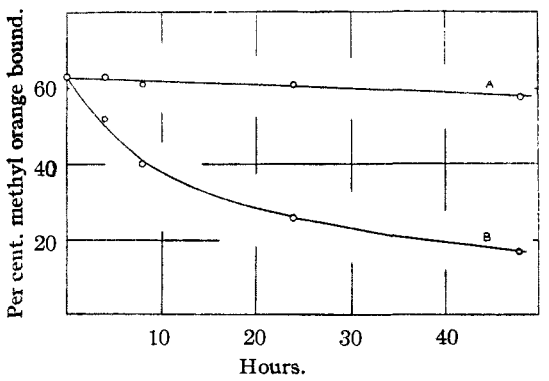

Fig. 4.—Effect of exposure to 0.01 molar sodium hydroxide at 26° on the binding ability of bovine serum albumin: A, albumin maintained at *p*H 7.6; B, albumin maintained in sodium hydroxide, neutralized to *p*H 7.6 and spectral effect on methyl orange measured. In both cases the concentration of albumin was 0.15%.

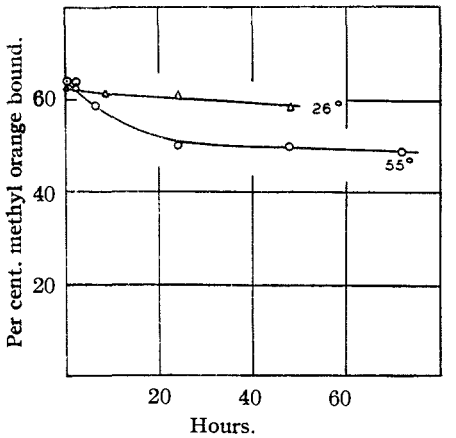

Fig. 5.—Decrease in binding ability of bovine serum albumin in phosphate buffer at *p*H 7.6.

Conclusions.—In comparing the quantitative and qualitative data on binding affinity for

various compounds, it is evident that there is a general correlation with molecular weight[7] so long as rather large variations are considered. Within a small range of molecular weight, however, interactions of polar substituents with the aqueous solvent play a dominant role. This is apparent not only from the striking results which have been cited for the ortho–para isomers but also in comparisons between compounds such as acetyltryptophan and naphthalenesulfonic acid. Though the former has a higher molecular weight it shows a smaller affinity for albumin, probably because of its polar amine and carbonyl substituents.

In comparing the "denaturing" agents, heat, alkali, urea and urethan, it is apparent that the action of the latter two is to be distinguished from that of the former. Whereas heat and alkali produce a gradual alteration in combining ability of the protein, urea and urethan act very rapidly in displacing the methyl orange anion from its albumin complex. The rate of formation of the urea–protein complex can thus be isolated from the subsequent denaturation processes, as measured by other criteria[12,13,14,15] and is obviously quite rapid. The slow modifications in the properties of the protein are presumably due to a subsequent slow reaction or reactions with some component of the solvent.

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(13) F. G. Hopkins, *Nature*, **126**, 328, 383 (1930).
(14) M. A. Lauffer, THIS JOURNAL, **65**, 1793 (1943).
(15) G. G. Wright and V. Schomaker, *ibid.*, **70**, 356 (1948).

Summary

A mass law analysis has been made for competitive binding of ions by a single protein. In the absence of electrostatic interactions the equations may be reduced to a convenient form for the evaluation of binding constants.

Comparison of the affinities of bovine serum albumin for a series of ortho–para isomers shows that aromatic anions with hydrogen-donating, ortho substituents are bound more strongly than corresponding para compounds. The difference is attributed to stronger interactions with the aqueous solvent in the latter substances. The importance of such interactions with polar substituents is emphasized also in comparisons of dissimilar molecules of approximately equal molecular weights.

The binding ability of bovine albumin is destroyed slowly by heat or by exposure to dilute sodium hydroxide. Urea and urethan are capable of displacing anions from their protein complexes at relatively high concentrations. Both of these substances act within a period of minutes, insofar as displacement effects are involved.

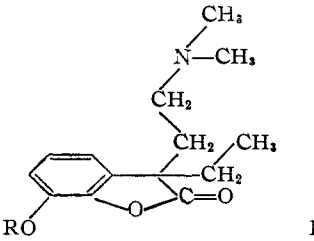
EVANSTON, ILLINOIS RECEIVED MARCH 19, 1948

[CONTRIBUTION FROM THE JOHN HARRISON LABORATORY OF THE UNIVERSITY OF PENNSYLVANIA]

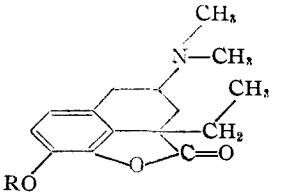
Morphine Studies. 2-Keto-7-methoxy-2,3-dihydrobenzofuran Derivatives[1]

BY E. C. HORNING AND R. U. SCHOCK, JR.[2,3]

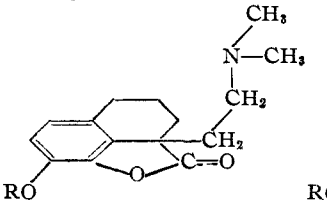
From an examination of the structure of synthetic compounds (Demerol and Amidone series) which possess physiological activity similar to that of morphine (I), it is apparent that some of the functional groups and rings of morphine do not play an important role in providing the type of activity which morphine displays. All have in common a quaternary carbon adjacent to an aromatic nucleus and a tertiary nitrogen in a β-relationship to this carbon, but a phenanthrene system is evidently not necessary. In investigating further the problem of the relation between structure and physiological activity in the morphine series, the synthesis of four model compounds has been planned (II, III, IV, V). Each of these derivatives



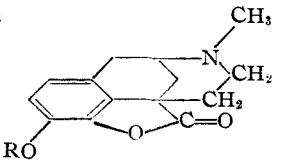
II



III



IV R = H, CH_3 or $COCH_3$



V

(1) Presented before the Division of Organic Chemistry, Second Meeting in Miniature of the Philadelphia Section of the American Chemical Society, Philadelphia, Pa., January 22, 1948.

(2) Abstracted from the thesis of R. U. Schock, Jr., presented to the Faculty of the Graduate School of the University of Pennsylvania in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

(3) Bristol Laboratories Fellow, 1946–1947; National Institute of Health Fellow, 1947–1948.