ethylmagnesium chloride. Properties of carbinol: b. p. 140° at 25 mm.; $n^{20}D$ 1.5198.

The carbinol was dehydrated over activated alumina at 300–350°, and the olefin hydrogenated as for *o*- and *m*-cymenes.

***t*-Butylbenzene: First Method, by Alkylation.**—*t*-Butyl chloride (1.108 g.) was added to a stirred mixture of benzene (5.320 g.) and powdered anhydrous aluminum chloride over a period of two hours. Stirring was continued for a further thirty minutes after which the hydrocarbon was decanted from the catalyst and worked up in the usual manner. The residue in the flask was used as catalyst for eleven similar runs with addition of 20 g. of fresh aluminium chloride in between each batch.

A total quantity of 11 gallons of fractionated hydrocarbon was prepared.

Second Method. By Disproportionation of *p*-Di-*t*-Butylbenzene and Benzene.—The conditions used for this reaction were those described by Ipatieff and Corson,[24] approximately 2 kg. of *t*-butylbenzene being prepared by this method. The yield of final hydrocarbon was 85% based on *p*-di-*t*-butylbenzene initially taken.

Acknowledgment.—The authors wish to express their indebtedness to Miss D. Macken, Messrs. G. J. Curteis, E. A. Johnson, D. T. McAllan, W. S. Nathan, W. J. Oldham and C. L. Walters who were responsible for a considerable amount of the experimental work.

(24) Ipatieff and Corson, THIS JOURNAL, **59**, 1417 (1937).

Summary

Selected methods for the preparation of the twenty-two monocyclic C_{10} aromatic hydrocarbons are described. Selection was based upon readily available starting materials, which could be conveniently purified, and upon reactions easy to carry out on a relatively large scale. Excepting where a required hydrocarbon could be readily separated from a commercially available material by fractionation, *e.g.*, *m*-diethylbenzene, syntheses have been chosen to avoid the presence of impurities in the final hydrocarbon which would prove difficult to separate.

Physical constants have been determined on the hydrocarbons and on most of the amines, nitriles, ketones and nitro compounds which were obtained as intermediates in the syntheses.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, NEW YORK UNIVERSITY COLLEGE OF MEDICINE]

Interaction of Homologous Alkyl Sulfates with Bovine Serum Albumin

BY FRED KARUSH[1] AND MARTIN SONENBERG

The study of the structure of the protein molecule as it exists in aqueous solution meets formidable difficulties not only because of the complexity of the structure but also because of the absence of any really direct and general experimental method. It is true, of course, that important structural information has come and more will undoubtedly follow from X-ray diffraction investigations with protein crystals and that much of this knowledge can be carried over to the dissolved molecule. However, it is still an open question whether the diffraction method can disclose the fine details of protein structure, particularly those associated with the configuration of side chains. These elements of structure are undoubtedly of crucial significance in the determination of specificity. In any case, it would appear that the environment of the protein molecule in the crystal is sufficiently different from that in solution that significant limits on the extrapolation from solid to solution must be imposed. It seems very likely that distinctive structural properties would accrue to the protein molecule in the transition from the crystalline to the dissolved state.

One experimental approach which offers promise of obtaining information which can be interpreted in terms of protein configuration is the study of complex formation of protein with small organic ions or molecules of known structure. In the specialized field of immunochemistry this method has been intensively employed[2] and has led to considerable clarification with regard to the problem of serological specificity. It is particularly applicable to the study of serum albumins since these proteins are able to combine reversibly with a great variety of compounds of known structure, in particular, organic anions.[3–7]

In the investigation described here we have studied the reversible binding by bovine serum albumin of an homologous series of straight chain alkyl sulfates. These compounds offer the possibility of distinguishing between polar and nonpolar contributions to the binding. The work was conducted with a view to obtaining the maximum amount of thermodynamic information about the binding process. Earlier studies[8] of the interaction of serum albumin and alkyl sulfates, as well as other synthetic detergents, have involved much higher concentrations of detergent

(1) Investigation conducted during 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: Sloan-Kettering Institute for Cancer Research, New York.

(2) K. Landsteiner, "The Specificity of Serological Reactions," Harvard University Press, Cambridge, Mass., 1945.

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

(4) B. D. Davis, *Am. Scientist*, **34**, 611 (1946).

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

(6) I. M. Klotz and J. M. Urquhart, *J. Biol. Chem.*, **173**, 21 (1948).

(7) J. D. Teresi and J. M. Luck, *ibid.*, **174**, 653 (1948).

(8) F. W. Putnam, *Adv. Prot. Chem.*, **4**, 79 (1948).

than employed here. As a result the interpretation of the data in terms of the binding properties of the native protein has been complicated by micelle formation and, in many cases, denaturation.

Experimental

Materials.—The serum albumin used was crystallized bovine serum albumin obtained from Armour & Co. The actual protein content of the powdered material was determined by Kjeldahl analysis using a value of 16.07%[9] for nitrogen. The particular lot of albumin used in these experiments contained 94.5% protein.

The alkyl sulfates ($C_nH_{2n+1}SO_4Na$) employed, sodium dodecyl, sodium decyl and sodium octyl sulfates, were specially purified samples generously supplied by the Fine Chemicals Division of E. I. du Pont de Nemours and Co. The purity of the dodecyl sulfate was checked by determining the additional acidity developed after acid hydrolysis. Accurately weighed samples of about 0.3 g. were placed in Pyrex test-tubes with 5 ml. of 0.1 *N* HCl and 2 ml. of water. The tubes were sealed and kept at 110° for forty-eight hours. After cooling and solidification of the dodecyl alcohol, the aqueous phase was quantitatively transferred and titrated. A value of 99.7% for the purity of the dodecyl sulfate was thus obtained. Other samples of these three materials from the same source have also been analyzed[10] for sodium and on this basis calculated to be practically 100% alkyl sulfate.

Dialysis Method.—The binding of alkyl sulfates by albumin was determined by equilibrium dialysis. All the experiments were conducted at *p*H 6.1 with a final buffer concentration of 0.025 *M* phosphate. This concentration was selected so as to be just large enough to render the Donnan correction negligible within the range of protein concentration employed, namely, 0.05 to 0.5%, and permit a maximum free alkyl sulfate concentration. The latter is limited by the salting-out effect of the buffer salt.

The dialysis was carried out with 20 ml. of protein solution in 0.05 *M* phosphate contained in a bag of Visking sausage casing. This bag was placed in a large test-tube with 20 ml. of a solution of alkyl sulfate free of buffer and the tube stoppered with a serum cap. All the phosphate was placed in the protein solution to allow larger initial concentrations of the alkyl sulfate to be used than would be possible if the original sulfate solution were made up in 0.025 *M* phosphate. The tubes were placed on a rocker, with a capacity of 24 tubes, in a horizontal position and gently rocked at a frequency of 1 or 2 cycles per minute. If the bag is prepared so that it is taut and an air bubble is enclosed and if an air space is provided inside the tube, very efficient mixing, both inside and outside the bag, takes place. With this arrangement at room temperature, equilibrium is attained in four hours or less and at low temperature (2°) overnight rocking is more than adequate.

To ensure maximum accuracy protein concentrations were selected so that the concentration of bound anion, wherever feasible, would be two to three times that anticipated for the free anion at equilibrium. This precaution avoids the situation in which the calculation of the amount of bound anion depends on a small difference between two large numbers. The initial outside concentrations of alkyl sulfate were chosen in such a way that the resulting equilibrium values of free anion when plotted as described below would be fairly evenly distributed over the range encompassed by the plot.

Equilibrium controls consisting of alkyl sulfate outside and buffer inside the bag were frequently included in the experiments. In addition the possibility that loss of alkyl sulfate might occur, either by adsorption or chemical reaction, was investigated. It was found that no significant adsorption took place in the range of anion concentration of interest, namely, 10^{-3} to 10^{-5} *M*. Fortunately also, chemical losses were negligible for these dilute solutions when subjected to room temperature for not more than several hours and to low temperature for not more than two days. It is worth noting, however, that longer exposure at room temperature, say, twenty-four to forty-eight hours, usually led to detectable loss of alkyl sulfate, particularly with the very dilute solutions.

In order to calculate the values of the thermodynamic functions ΔH and ΔS for the binding process, experiments were conducted at room temperature (25–28°) and at low temperature (1–2°). For each experiment fresh solutions of protein and alkyl sulfate were prepared and all determinations were done in duplicate. The concentration of free anion in equilibrium with bound anion was obtained by the determination of the concentration of alkyl sulfate anion outside the dialysis bag.

Analytical Method.—The investigation reported here required the quantitative measurement of long chain alkyl sulfate concentrations as low as 10^{-5} *M*. A search of the literature revealed that no adequate method was available. We therefore undertook the development of a suitable colorimetric method following along lines initiated by Brodie and Udenfriend.[11] The method is based upon the fact that octyl and higher alkyl sulfates form complexes with rosaniline hydrochloride which can be extracted into organic solvents. A detailed description of the method will be published elsewhere. It is sufficient to mention here that the procedure involves the addition of 4 ml. or less of alkyl sulfate solution to 1 ml. of 4×10^{-4} M rosaniline hydrochloride in 0.025 *M* phosphate, *p*H 6.1, in a total volume of 5 ml., and the extraction of the colored complex with 5 ml. of a mixed solvent consisting of 50% chloroform and 50% ethyl acetate. The two-phase system is centrifuged and the light absorption of the lower phase, which contains the complex, is measured with a Klett colorimeter using a green filter. The method is suitable for concentrations as low as 10^{-5} *M* and has an accuracy of about 2%.

Results

The protocol with attendant calculations for a typical dialysis experiment is shown in Table I. The values in the first column are simply one-half the initial outside concentrations of alkyl sulfate. The molar concentration of protein is based upon a value of 69,000[12] for the molecular weight of bovine serum albumin. The last two columns give the reciprocal of the average number of anions bound per molecule of protein ($1/r$) and the reciprocal of the free equilibrium anion concentration ($1/c$). These quantities are most convenient for graphical representation of the data. It may be noted that although the concentrations of protein and bound anion shown in columns 3 and 4 may be somewhat in error due to volume changes inside the dialysis bags, the values of $1/r$ and $1/c$ are independent of such variations.

It has been found by Klotz[13] that the value of r depends on the protein concentration as well as on c, for the range 0.2 to 1%, in the binding of methyl orange by bovine serum albumin. A few observations with our systems on the effect of protein concentration in the range 0.5 to 0.05% have not revealed any significant dependence of r on protein content, though we have not investigated this question in any great detail.

In Figs. 1 and 2 we present a summary of the

(9) E. Brand, *Ann. N. Y. Acad. Sci.*, **47**, 187 (1946).

(10) F. D. Hoffner, G. A. Piccione and C. Rosenblum, *J. Phys. Chem.*, **46**, 662 (1942).

(11) B. B. Brodie and S. Udenfriend, personal communication.

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

(13) I. M. Klotz, personal communication.

binding data for sodium octyl, decyl and dodecyl sulfates at room and low temperatures. The experimental results are the points in the plot of $1/r$ *vs.* $1/c$. The reason for this mode of representation will be indicated below.

TABLE I

BINDING OF DECYL SULFATE ANION BY BOVINE ALBUMIN AT ROOM TEMPERATURE IN 0.025 *M* PHOSPHATE BUFFER, *p*H 6.1

Equil. concn. without protein m./l. × 10^5	Equil. concn. with protein m./l. × 10^5	Concn. of bound anion m./l. × 10^5	Concn. of protein m./l. × 10^5	Moles anion bound per mole protein, r	$1/r$	$1/c$ × 10^{-4}
80.0	43.0	74.0	6.84	10.8	0.0925	0.232
50.0	17.6	64.8	6.84	9.46	.1057	.568
25.00	13.2	23.6	2.74	8.61	.1161	.758
17.50	6.84	21.3	2.74	7.77	.1287	1.46
8.75	4.01	9.48	1.37	6.93	.1444	2.49
7.00	2.64	8.72	1.37	6.37	.1570	3.79
6.25	2.10	8.30	1.37	6.06	.1650	4.76
3.375	1.44	3.88	0.684	5.66	.1765	6.95
3.125	1.24	3.76	0.684	5.49	.1822	8.07
2.875	1.06	3.64	0.684	5.32	.1881	9.43

Because of the importance of a knowledge of the binding capacity of the protein, several experiments were done at room temperature using 2% protein and as high concentration of decyl and dodecyl sulfates as feasible. For the decyl sulfate salt the maximum equilibrium concentration of the free anion was 1.7×10^{-3} *M* and that for the dodecyl sulfate was 3.25×10^{-4}. Under these conditions values of r very close to 14 but not exceeding it were obtained. It must be emphasized, however, that this figure is not necessarily a maximum one since the data when plotted, for example, in the form of r against log c do not indicate 14 to be a limiting value. The significance of this fact will be explored in the following section.

Discussion

General Considerations.—In order to justify a thermodynamic analysis of our data, it is necessary to show that the binding process is a reversible one. This can be done experimentally by immersing dialysis bags containing protein which have previously been equilibrated against an alkyl sulfate solution into 20 ml. of 0.025 *M* PO_4 buffer, *p*H 6.1 and determining the new equilibrium values of r and c. The results of such an experiment with dodecyl sulfate at low temperature are shown by the two points in Fig. 2 indicated by arrows. This second equilibration, in contrast to the usual binding experiment, involves a net removal of anion from combination with the protein.

A complication which must be avoided with long-chain anions to permit a thermodynamic treatment is the formation of micelles. This has been achieved in the present study by working well below the critical concentrations[10] for micelle formation, taking into account the effect of salts on these values.[14]

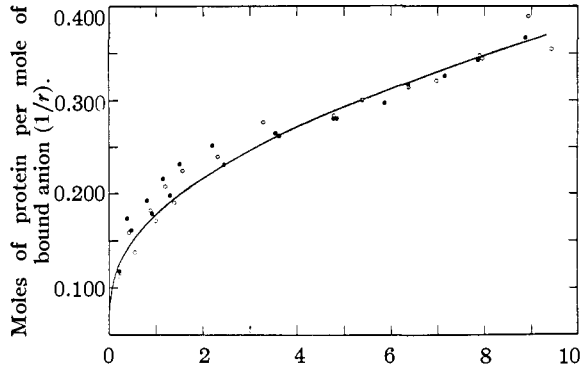

Fig. 1.—Binding data for octyl sulfate: O, low temp.; ●, room temp.

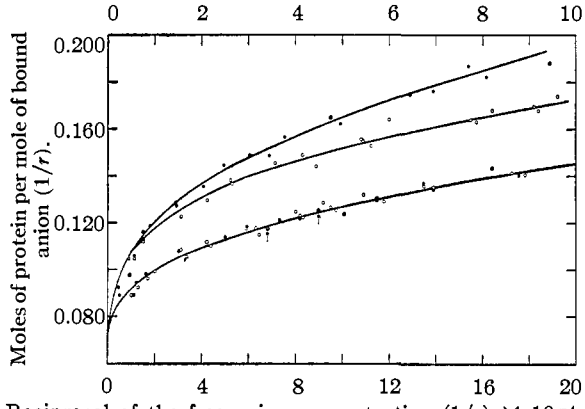

Fig. 2.—Binding data for decyl and dodecyl sulfates; upper two curves and top scale refer to decyl sulfate and lower curve and bottom scale to dodecyl sulfate; reversibility points (see text) indicated by arrows; O, low temp.; ●, room temp.

Simple Theory.—The application of the law of mass action to the case of multiple binding leads to the following simple relation between r and c under the conditions that all the binding sites have the same intrinsic association constant K and that, aside from the statistical factor, the free energy of binding to any particular site is independent of binding to other sites.[15,16] The value

$$\frac{1}{r} = \frac{1}{Kn}\,\frac{1}{c} + \frac{1}{n} \qquad (1)$$

of K depends, of course, on the particular system under consideration and n represents the average maximum number of sites per molecule, *i. e.*, the binding capacity. According to equation (1) a plot of $1/r$ *vs.* $1/c$ will give a straight line. From the slope of this line and its intercept with the $1/r$ axis the values of K and n can be obtained. Such

(14) M. L. Corrin and W. D. Harkins, THIS JOURNAL, **69**, 683 (1947).

(15) A. L. von Muralt, *ibid.*, **52**, 3518 (1930).

(16) I. M. Klotz, *Arch. Biochem.*, **9**, 109 (1946).

a procedure has been used by Klotz, Walker and Pivan[5] in their study of the binding of anionic dyes by bovine serum albumin and by Teresi and Luck[7] in an investigation of the binding of other organic anions by the same protein.

As is evident from Figs. 1 and 2 the binding of alkyl sulfates by bovine albumin does not satisfy the conditions noted above since the experimental points deviate significantly from linearity and increasingly so with decrease of $1/c$. This deviation is more striking with decyl sulfate than with dodecyl sulfate because the data for the former cover a wider range of $1/r$. From the shape of the curves it appears that, ignoring the statistical contribution, the binding of each additional anion on an albumin molecule involves a smaller decrease in free energy than the binding of the previous one. There are a number of possible factors which would lead to such an effect and we now proceed to consider them.

Electrostatic Interaction.—Studies of the acid–base titration curves of soluble proteins and the effect of ionic strength on these curves have shown that it is necessary to introduce an electrostatic correction to account adequately for the shapes of these curves.[17,18] This correction is associated with the variation of charge on the protein during the titration and is based on the Debye–Huckel theory of interionic attraction.

The contribution of the electrostatic interaction to the free energy of binding has been calculated by Klotz, *et al.*,[5] for the binding to bovine serum albumin of the monovalent anion methyl orange, and the divalent anion azosulfathiazole. More recently Scatchard[19] has developed an explicit relation between r and c containing an electrostatic interaction term which is applicable with high accuracy for values of $n > 4$. This relation can be put in a form analogous to (1) as follows

$$\frac{1}{r} = \frac{1}{Kn}\frac{e^{2w'r}}{e^{w}}\frac{1}{c} + \frac{1}{n} \qquad (2)$$

where $w' = (1 + 1/n)w$ and w is given by

$$w = \frac{\epsilon^2 z^2}{2DkT}\left(\frac{1}{b} - \frac{\kappa}{1 + \kappa a}\right) \qquad (3)$$

in which ϵ is the electron charge, Z, the charge on the small anion, D, the dielectric constant of the medium, k, Boltzmann's constant, T the absolute temperature, b, the radius of the protein molecule, a, the distance of closest approach, and

$$\kappa = \left(\frac{4\pi N\epsilon^2}{1000\,DkT}\right)^{1/2}\Gamma^{1/2} \qquad (4)$$

Here, N is Avogadro's number and Γ is twice the ionic strength of the medium. To calculate w we have assumed, following Klotz, *et al.*,[5] a value of 30 Å. for b and 31.5 Å. for a. Since Γ for 0.025 M PO_4 buffer at pH 6.1 is 0.0644, we have calculated a value of 0.0451 for w at 27°. This represents an electrostatic free energy contribution of 54 cal./mole for the binding of each additional anion to the protein.

To test the adequacy of the electrostatic interpretation of our results by the application of equation (2), we have plotted our experimental data in the form of $1/r$ *vs.* $e^{2w'r}/c$. For this purpose a value of 10 was assigned to n to obtain w'. An examination of such a plot reveals no reduction in the deviation of the data from a straight line. It appears that the electrostatic free energy correction is too small by almost an order of magnitude. Even if w' is treated as a parameter it is impossible to adjust its value so that the experimental points fall on a linear curve. It is clear, therefore, that not only is the electrostatic effect unable to explain our results but that any interaction or correction which takes the mathematical form represented in equation (2) will be unsatisfactory.

There is some question whether any electrostatic correction should be made in our case since Klotz, *et al.*,[5] found, in the presence of phosphate buffer, that the binding of the monovalent anion methyl orange obeyed a linear relation between $1/r$ and $1/c$ without such a correction. We have therefore chosen in the following discussion and calculations to omit further consideration of the electrostatic effect.

Heterogeneity Theory.—On *a priori* grounds there is no reason to believe that the intrinsic association constants of all the binding sites are identical. We must therefore consider the possibility that a variation of these constants would account for the shape of our binding curves. Indeed, qualitatively, it is clear that if the binding constants are not the same, the slope of the curve of $1/r$ *vs.* $1/c$ should decrease with increasing $1/c$, as is experimentally the case. Before we attempt a quantitative formulation, we want to consider two other possible effects associated with the paraffin chain character of the anions.

It is well known that due to the large organic groups in these anions, they tend to interact with one another in aqueous systems as evidenced by the formation of micelles. One may ask therefore if the association of one anion on a particular site may not lead with increase in concentration of free anion to the adsorption of at least a second one in the same neighborhood of the protein due to interaction between the paraffin chains. This phenomenon would be similar to micelle formation but might be expected to occur at a somewhat lower concentration due to the immobilization of half of the dimer by the protein. However, it can be inferred from an inspection of our curves that if such were the case significant dimerization should have already occurred at concentrations of 5×10^{-6} M for dodecyl sulfate, 3×10^{-5} M for decyl sulfate and 1×10^{-4} M for octyl sulfate. These values are approximately 1000-fold less than the critical concentrations for micelle formation[9]

(17) R. K. Cannan, *Chem. Rev.*, **30**, 395 (1942).

(18) E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides," Reinhold Publishing Corp., New York, N. Y., 1943, Chap. 20.

(19) G. Scatchard, *Ann. N. Y. Acad. Sci.*, in press.

and for this reason it appears unlikely to us that such an effect is significant for the interpretation of our results.

Due to the relatively great length of the alkyl sulfate it is imaginable that steric interference may lead to a continuous reduction of binding energy with increase in r. That this is probably not important here is indicated by a comparison of the data for decyl and dodecyl sulfates. If one considers a specified range of $1/r$, say 0.100 to 0.140, and compares the curvature exhibited by the experimental points in this range for the two anions, there is no indication of a greater curvature for the dodecyl sulfate. Such a difference would be expected as a consequence of the greater length of the dodecyl anion resulting in more extensive steric interference. The probable absence of steric effects also is suggested by the fact that the value of σ (see below) is not greater for dodecyl than for decyl sulfate. The quantity σ is a measure of the spread of K values which must be assumed to account for our data in accordance with the heterogeneity theory which we will now discuss.

We take as our model a protein molecule with a large number of binding sites which combine with small ions or molecules independently of each other. We then assume that the number and distribution of the intrinsic binding constants are such as to be adequately described in terms of the free energy of binding by a Gauss error function which when normalized is

$$(1/\sqrt{\pi}\sigma)e^{-[\ln(K/K_0)]^2/\sigma^2} \qquad (5)$$

where K_0 is an average binding constant and σ measures the range of values of K. This formulation is similar to that used by Pauling, Pressman and Grossberg[20] to describe the heterogeneity of binding of hapten by antibody in competition with antigen. In their case the heterogeneity was associated with differences among the antibody molecules whereas we are concerned with differences among the sites on the same molecule. The shape of this distribution function for $\sigma = 1$ to 4 is shown in Fig. 1 of their paper. The fraction of the total number of sites on the molecule which have a particular value of K in an infinitesimal region is given by

$$\frac{dn}{n} = \frac{1}{\sqrt{\pi}} e^{-[\ln(K/K_0)]^2/\sigma^2} d\,[\ln (K/K_0)] \qquad (6)$$

With the aid of this relation and the condition that the sites act independently it is easily shown that the average fraction of sites occupied as a function of c is expressed by

$$\frac{r}{n} = 1 - \frac{1}{\sqrt{\pi}} \int_{-\infty}^{\infty} \frac{e^{-\alpha^2}}{1 + K_0 c e^{\alpha\sigma}}\, d\alpha \qquad (7)$$

in which α has been substituted for $\ln(K/K_0)/\sigma$. For purposes of calculation and plotting it is convenient to put equation (7) in the form

$$\frac{n}{r} = \frac{1}{1 - f(c)} \qquad (8)$$

where

$$f(c) = \frac{1}{\sqrt{\pi}} \int_{-\infty}^{\infty} \frac{e^{-\alpha^2}}{1 + K_0 c e^{\alpha\sigma}}\, d\alpha \qquad (9)$$

Unfortunately, the integral in (9) cannot be evaluated analytically and recourse must be had to numerical or graphical integration for the computation of $f(c)$. Prior to such integration the values of the parameters K_0 and σ must be fixed. It turns out, however, that $f(c) = {}^1/_2$ for all values of σ when $K_0 c = 1$. This means that K_0 is equal to the reciprocal of the free anion concentration at which the protein is half-saturated and its value can be obtained directly from the experimental data if the value of n has been previously ascertained. The value of σ is taken to be that which gives the best agreement between experimental and calculated binding curves. It is of interest to note that for $\sigma = 0$, $f(c) = 1/(1+K_0c)$ and equation (8) reduces to (1). That is, the simple theory is a limiting case of the more general heterogeneity theory.

The application of equation (8) to experimental binding data is most readily and generally done by preparing theoretical curves of n/r *vs.* $1/K_0c$ for various values of σ. For this purpose numerical integration was carried out using Weddle's rule[21] with integration limits -2.4 to 1.2 or -2.4 to 2.4 depending largely on the value of σ. The theoretical curves are shown in Figs. 3 and 4 for two different ranges of $1/K_0c$. It is important to note that beyond $1/K_0c = 1$, the curves do not deviate much from linearity. In fact, if binding data of not high accuracy were to fall on such a curve, the temptation would be to draw a straight line through the points and conclude that the binding obeys the simple theory. This situation has important implications for the interpretation of data in the literature which we shall discuss below.

On the basis of the curves in Figs. 3 and 4 we have calculated the theoretical curves shown as solid lines in Figs. 1 and 2 using $n = 14$. The values of K_0 and σ employed are listed in Table II. It is clear that a satisfactory fit of the curves with the data is obtained except possibly for very

TABLE II

CONSTANTS AND THERMODYNAMIC DATA FOR BINDING OF ALKYL SULFATES BY BOVINE SERUM ALBUMIN. THE DATA REFER TO THE BINDING OF ONE MOLE OF ALKYL SULFATE

Compound	Temperature	σ	$K_0 \times 10^{-4}$	$-\Delta F^0$, kcal.	ΔS^0, E. U.
Octyl sulfate	Room and low	4.5	0.4	5.01[a]	16.7
Decyl sulfate[c]	Room	5	2.5	6.03[a]	13.3
Decyl sulfate[c]	Low	6	3.4	5.70[b]	13.3
Dodecyl sulfate	Room and low	5	18.2	7.22[a]	24.0

[a] Calculated for 27°. [b] Calculated for 2°. [c] $\Delta H^\circ = -2.0$ kcal.

(20) L. Pauling, D. Pressman and A. L. Grossberg, THIS JOURNAL, **66**, 784 (1944).

(21) H. Margenau and G. M. Murphy, "The Mathematics of Physics and Chemistry," D. Van Nostrand Co., New York, N. Y., 1943. Chap. 13.

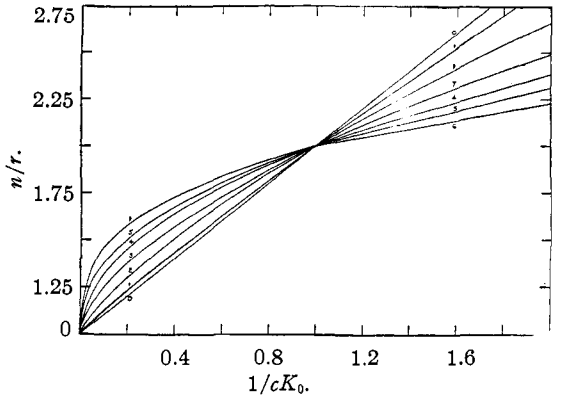

Fig. 3.—Theoretical binding curves for $\sigma = 0$ to 6 for range of high binding.

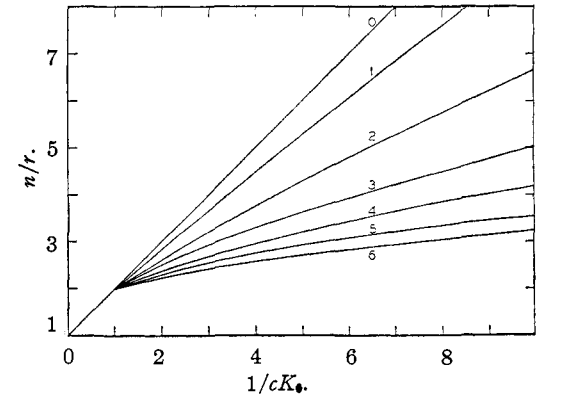

Fig. 4.—Theoretical binding curves for $\sigma = 0$ to 6 for range of low binding.

low values of $1/c$ where the curves tend to be slightly higher than the points. It may be noted that our procedure involves the approximation of substituting a continuous spectrum of K values for what must be a discontinuous set for any particular molecular species of albumin. There is, however, the possibility that crystallized albumin is not molecularly homogeneous with respect to its binding properties. Our analysis would not reveal such a condition because it does not really distinguish between variation of binding energy among the sites on a single molecule as compared to differences among different molecules. However, because of the large range of values of K indicated by values of σ of 4 or 5, which in turn would imply considerable differences among the albumin molecules, we are inclined to attribute most of the effect to variation among the sites within the individual molecules. A rough calculation shows that for $\sigma = 5$ and a value of K_0 corresponding to $-\Delta F^\circ = 6$ kcal, the range of K values would correspond to a range of $-\Delta F^\circ$ from approximately 2 kcal. to 10 kcal.

It will be noted in Table II that there is some variation in the values of σ. It would be premature to attempt at this point to assign any physical significance to this variation both because of the relatively small differences involved and because of the limited range of r for which σ is employed in the theoretical calculation.

The question may be raised as to whether our choice of distribution function is uniquely suitable to account for our data. It appears to us that it is very likely not so and that other distribution functions may be equally adequate. This view is based on the fact that our data cover a rather narrow range of $1/r$ so that the suitability of the distribution function is not put to a severe test. Further, this distribution does not fit in with the kind of physical picture of the interaction which we favor though, of course, no definite judgment on this point can be made at the present time.

Thermodynamic Considerations.—Although the values of K_0 listed in Table II are average values, their comparison with one another is nevertheless of significance. It is clear that the binding becomes greater with increase in the length of the paraffin chain. This is, of course, obvious from the data of Figs. 1 and 2. Its importance follows from the fact that it shows that probably the whole alkyl group is involved in the interaction with the protein in addition to the electrostatic interaction between charged groups.

The study of the temperature dependence of the binding of alkyl sulfates has also led to interesting conclusions. Although the data for octyl sulfate are not as good as that for the other homologs, it appears that the binding of the octyl anion is practically independent of temperature as with dodecyl sulfate. On the other hand, decyl sulfate binding is dependent on temperature and an average value for ΔH° of -2.0 kcal. is obtained. This is a rather unexpected difference among the homologs and, though it may have important implications for the structure of albumin, confirmation and extension of this kind of data is required before theoretical interpretation is justified.

The binding of the alkyl sulfates is largely or wholly an entropy effect. The standard entropy changes for the process are listed in the last column of Table II. To what extent these changes are associated with the release of water molecules bound to protein and anion and/or structural changes of the protein, it is not possible to decide at this time. The predominance of the entropy contribution to the free energy of complex formation between copper (cupric) and bovine albumin has been demonstrated by Klotz and Curme.[22]

Another important point which emerges from the thermodynamic data is that there is danger in the tendency, which is found in the literature, to interpret an increased binding as due to larger van der Waals forces because of increase in size of the non-polar portion of the bound molecule.

(22) I. M. Klotz and H. G. Curme, THIS JOURNAL, **70**, 939 (1948).

The comparison of dodecyl with decyl sulfate shows that the binding of the smaller anion yields a larger reduction in the energy of the system and serves to emphasize the desirability of measuring the temperature dependence of binding whenever possible.

Significance of n.—Previous work[5,7] on the binding of organic anions by albumin has proceeded on the assumption that it is possible to define a value of n for the anions studied by the extrapolation of the $1/r$ *vs.* $1/c$ plot, corrected where necessary for electrostatic effects. We have already indicated that though we have taken $n = 14$ for the alkyl sulfates, we do not regard this value as necessarily the binding capacity of albumin. In the binding of methyl orange Klotz, *et al.*,[5] obtained a value of 22 for n by extrapolation. However, the maximum value of r obtained experimentally was apparently about 15 and the curve of r *vs.* log c gave no indication of tending toward a limiting value. It is therefore quite possible that the correct value of n is larger than 22 and that all the binding sites do not have the same value of K as the apparent linearity of the $1/r$ *vs.* $1/c$ plot would suggest.

The interpretation of Teresi and Luck's data[7] according to the simple theory is even more questionable because the range of $1/r$ and especially $1/c$ is more restricted.[23] Furthermore the data are not of high accuracy due to the fact that the amount of bound anion was only about one tenth of free anion. According to their results *o*-nitrophenolate is bound more strongly than *p*-nitrophenolate though $n = 6$ for the former and 25 for the latter compound. This appears rather unlikely to us on physical grounds and is especially open to question because the calculation of the intrinsic constants depends on the prior determination of n. In fact, if n is assumed to be the same for the two compounds the fact that the binding curve of *p*-nitrophenolate lies below that of *o*-nitrophenolate would indicate that contrary to the conclusion of Teresi and Luck, the former compound is bound more strongly.

Such a reversal could arise if the portions of the binding curves of the two anions being compared corresponded to different ranges of $1/K_0c$ as shown in Figs. 3 and 4. The possible danger of being misled because of such a situation is readily seen by a comparison of our octyl and decyl sulfate data. If one considers the octyl sulfate data for the range of $1/c = 2 \times 10^4$ to 10×10^4, one could draw the best straight line through the points and conclude that $n = 5$ and that the intrinsic association constant K is 10×10^4. On the other hand, if the data for decyl sulfate were limited to the range $1/c = 1 \times 10^4$ to 3×10^4, the conclusion would be reached that $n = 10$ and $K = 5 \times 10^4$. Thus a comparison of the two limited sets of data would lead to the inference that the binding capacity of the protein for octyl sulfate is less than for decyl but that the former is more strongly bound, though both conclusions are contrary to fact.

It appears, therefore, that the interpretation of the extrapolated value of $1/r$ as measuring the binding capacity of the protein for a particular anion is of dubious validity. Such a value may be quite adventitious depending on the concentration range studied and the magnitude of σ or any other parameter which reflects the variation of K values. A possibility that deserves serious consideration is that the binding capacity is the same for all organic anions, within a certain size range, and is equal to the number of positively charged sites on the surface of the protein molecule. If this were actually the case it would mean that for any particular organic anion the values of K for the various sites would cover a very wide range.

Structural Implications.—The binding of organic anions to serum albumin very probably involves the electrostatic interaction between a positively charged group on the protein and the negative group of the anion.[24,25] However, not all of the positive groups of the protein are equally available for binding. This is clear from the analytical data for bovine serum albumin[8] which shows that there are 84 such groups at *p*H 6.1 from the arginine and lysine residues alone. Yet according to our results only 14 groups are involved in binding.

It appears useful to make a division of the cationic groups into two sets, one which is practically completely excluded from the association reaction in the native protein possibly because of inaccessibility, and the second set the members of which can bind organic anions but may do so with varying affinity. Some justification for such a distinction is to be found in the results of a study of the interaction between sodium dodecyl sulfate and horse serum albumin by Neurath and Putnam.[26] They observed that this protein could form a stoichiometric complex with about 55 detergent anions per albumin molecule without detectable changes in molecular shape. At higher detergent–protein ratios a second complex was formed involving the combination of about 110 anions per protein molecule. As shown by diffusion and viscosity measurements the formation of the second complex was accompanied by an increase in the molecular asymmetry of the protein. It may be remarked that the study with horse serum albumin was carried out with much higher concentrations of detergent, above the critical concentration for micelle formation, than was the case in our experiments.

(23) The values of K and ΔF listed in their paper require correction due to an oversight in calculation (see *J. Biol. Chem.*, **177**, 383 (1949)).

(24) I. M. Klotz and F. M. Walker, THIS JOURNAL, **69**, 1609 (1947).

(25) F. W. Putnam and H. Neurath, *J. Biol. Chem.*, **159**, 195 (1945).

(26) H. Neurath and F. W. Putnam, *ibid.*, **160**, 397 (1945).

Perhaps the most interesting question of structural significance raised by our results has to do with the variation of K values among the 14 sites which bind alkyl sulfate. Though, obviously no detailed answer is possible now, it does appear that the configuration of the protein in the interacting area around each positive charge is not the same for all the sites. It would be of considerable interest to determine the degree of such heterogeneity for the binding of organic anions of different sizes and shapes.

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Summary

The reversible binding of homologous alkyl sulfates by bovine serum albumin has been studied at two temperatures by the method of equilibrium dialysis. This has required the development of a colorimetric method for determining low concentrations (10^{-5} M) of these compounds. Deviations of the binding curves from the simple theory based on the mass action law have been shown to be inexplicable on the basis of electrostatic effects. A new heterogeneity theory based on a particular distribution of the intrinsic binding constants has been proposed and has been found to account quantitatively for our results. The values of the thermodynamic functions $\Delta F°$, $\Delta H°$ and $\Delta S°$, for the binding process have been calculated and the importance of the entropy contribution emphasized. The validity of the determination of binding capacity (n) by linear extrapolation has been subject to question and interpretations of binding data in the literature reconsidered. Finally, some structural implications of our results have been noted.

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Biophysical Studies of Blood Plasma Proteins. IX. Separation and Properties of the Immune Globulins of the Sera of Hyperimmunized Cows[1]

BY E. L. HESS[2,3] AND H. F. DEUTSCH

Introduction

The antibody fraction of normal bovine serum has been previously separated in high yield by means of ethanol fractionation.[4] Our attention has now been directed to the application of these procedures for the recovery of the antibody enriched fractions of the sera of cows immunized to *Brucella abortus* organisms and Newcastle virus. The various globulin fractions separated have been subjected to solubility, molecular kinetic and immunological study.

Experimental

The hyperimmune sera were prepared by inoculating two Holstein cows with Newcastle virus in whole egg embryo and with the viable *Brucella abortus* organisms at four-day intervals over a three-month period. In addition, a low titer serum pool of two calves immunized to diphtheria and tetanus toxin was studied briefly. The increase of the serum γ-globulins was followed by electrophoretic analysis and the extent of antibody production was determined by immunological assays.

Brucella abortus antibodies of two types, ag glutinins and bactericidins, were assayed accord ing to the methods published by Huddleson[5] and Irwin and Beach,[6] respectively. Newcastle virus hemagglutination inhibiting and neutralizing antibodies were assayed according to the method of Brandly, Jungherr, Moses and Jones.[7] The titers reported are based upon the assay of fractions reconstituted in phosphate buffer to their original serum concentration.

Except in certain subfractionations as indicated below, the fractionation steps follow the procedure outlined in Fig. 5 of the prior article.[4] The course of the fractionation of the hyperimmune bovine serum is shown by the electrophoretic diagrams in Fig. 1. The designation of the fractions is consistent with that adopted in the earlier report. Precipitates A from various sera were pooled for subsequent subfractionation or rework operations.

Standard electrophoretic and sedimentation procedures were followed in the physical chemical characterizations of the fractions. Solubility studies utilized the customary phase rule solubility procedures as reviewed by Herriott[8] and the successive extraction of the solid phase with

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(2) du Pont Fellow in Chemistry in 1947.

(3) Post-doctorate Fellow in Chemistry 1948.

(4) Hess and Deutsch, THIS JOURNAL, **70**, 84 (1948).

(5) Huddleson, "Brucellosis in Man and Animals," Oxford Press, New York, N. Y., 1943.

(6) Irwin and Beach, *J. of Agr. Res.*, **72**, 83 (1946).

(7) Brandly, Jungherr, Moses and Jones, *Am. Jour. Vet. Research*, **7**, 289 (1946).

(8) Herriott, *Chem. Rev.*, **30**, 413 (1942).