[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF NORTHWESTERN UNIVERSITY]

# The Binding of Organic Ions by Proteins[1]

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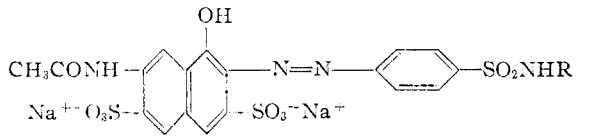
## Introduction

It has been recognized from many investigations[2-15] that organic anions interact with proteins on the basic side as well as on the acid side of their isoelectric points. Nevertheless quantitative studies from the point of view of the law of mass action are lacking. To contribute to the clarification of these interactions a quantitative investigation has been made of the binding of two azosulfonic acids by bovine serum albumin, and the data have been analyzed, in terms of the statistical and electrostatic factors which influence binding, by an approach analogous to that used so successfully in interpreting the acid-base reactions of proteins.[16]

## Experimental

**Reagents.**—Crystalline bovine serum albumin was obtained through the courtesy of Armour and Company. Corrections for water content were made by heating a small sample in an oven at 110° until constant weight was attained.

The methyl orange, $Na^{+-}O_3SC_6H_4N{=}NC_6H_4N(CH_3)_2$, was a commercial sample of reagent grade. Azosulfathiazole




where R is a thiazole ring, was kindly supplied by the Winthrop Chemical Company. A purity of 97.2% had been found from titration with methylene blue. As indicated by the manufacturer, the impurity is probably only sodium chloride, used in salting out the dye.

**Dialysis Experiments.**—Cellophane bags were prepared from commercial sausage casing and were filled with a measured amount of a protein solution usually near 0.1% concentration. The bag was immersed in a solution of the methyl orange or azosulfathiazole and placed in a cold room at approximately 5° for a period of seventy-two hours, an interval sufficient for the attainment of equilibrium. The bag was then removed and the external solution analyzed spectrophotometrically for the colored anion. For each anion concentration a control tube was also prepared which differed from the primary tube only in that the former contained buffer rather than a protein solution inside the bag. By this method it was possible to minimize any errors arising from binding of the dye by the cellophane membrane. Preliminary experiments indicated that about 8% of the free dye in solution was bound by the cellophane membrane.

A typical experiment, with attendant calculations, is summarized in Table I.

TABLE I

A TYPICAL DIALYSIS EXPERIMENT

| | Protein tube | | Control tube | |
|---|---|---|---|---|
| | Outside of bag | Inside of bag | Outside of bag | Inside of bag |
| Vol. of soln., cc. | 20.00 | 10.00 | 20.00 | 10.00 |
| Moles of albumin[a] | ..... | $1.394 \times 10^{-7}$ | ..... | ... |
| Concn. of methyl orange, $M$ | $7.46 \times 10^{-5}$ | ..... | $9.06 \times 10^{-5}$ | ... |
| Moles bound methyl orange[b] | ..... | $4.80 \times 10^{-7}$ | ..... | ... |
| Bound dye / Total protein | ..... | 3.44 | ..... | ... |
| $p$H | 5.68 | 5.68 | 5.68 | ... |

[a] Calculated on assumption of molecular weight of 70,000. [b] Under the conditions of the present experiments, particularly with such low protein concentrations, the influence of the Donnan effect on the dye distribution is negligible.

The dialysis experiments were carried out in 0.1 $M$ phosphate buffers. $p$H's were measured with a glass electrode. No difference could be detected between the $p$H inside and outside of the cellophane bag.

The spectrophotometric analyses were made with a Beckman spectrophotometer using cells of 1-cm. depth.

## Results and Discussion

The extent of binding of methyl orange and azosulfathiazole, respectively, by bovine serum albumin is illustrated in Figs. 1 and 2. The data indicate clearly that many molecules of the organic anion may be bound by a single protein molecule, and, consequently, that the equilibrium cannot, in general, be expressed by a single equilibrium constant.

Before considering the anion-protein equilibria in detail it is necessary to know whether the methyl orange or azosulfathiazole molecules form aggregates among themselves in aqueous solution. This added complication has been shown not to exist in the concentration region under investigation, by the establishment of the close adherence to Beer's law by each dye.

Thus it is possible to limit our considerations

(1) Presented in part before the Division of Physical Chemistry at the Atlantic City meeting of the American Chemical Society, April, 1946.

(2) A. Grollman, *J. Biol. Chem.*, **64**, 141 (1925).

(3) L. M. C. Rawlins and C. L. A. Schmidt, *ibid.*, **88**, 271 (1930).

(4) M. I. Gregersen and J. G. Gibson, *Am. J. Physiol.*, **120**, 494 (1937).

(5) W. W. Smith and H. W. Smith, *J. Biol. Chem.*, **124**, 107 (1938).

(6) H. W. Robinson and C. S. Hogden, *ibid.*, **137**, 239 (1941).

(7) J. Steinhardt, C. H. Fugitt and M. Harris, *J. Research Nat. Bur. Standards*, **26**, 293 (1941).

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

(9) H. P. Lundgren, D. W. Blum and R. A. O'Connell, *J. Biol. Chem.*, **149**, 183 (1943).

(10) F. W. Putnam and H. Neurath, *ibid.*, **150**, 263 (1943).

(11) K. G. A. Pankhurst and R. C. M. Smith, *Trans. Faraday Soc.*, **40**, 565 (1944).

(12) P. D. Boyer, *J. Biol. Chem.*, **158**, 715 (1945).

(13) G. A. Ballou, P. D. Boyer and J. M. Luck, *ibid.*, **159**, 111 (1945).

(14) L. Michaelis and S. Granick, THIS JOURNAL, **67**, 1212 (1945).

(15) E. K. Rideal, *Endeavour*, **4**, 83 (1945).

(16) K. Linderström-Lang, *Compt. rend. trav. lab. Carlsberg*, **15**, No. 7 (1924); R. K. Cannan, *Chem. Rev.*, **30**, 395 (1942); E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides," Reinhold Publishing Corp., New York, N. Y., 1943, pp. 449-477.

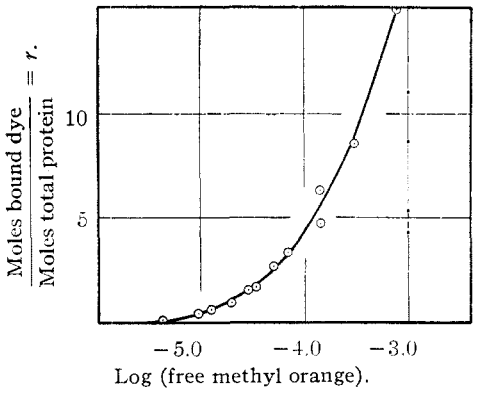



Fig. 1.—Binding of methyl orange by bovine serum albumin, *p*H 5.67.

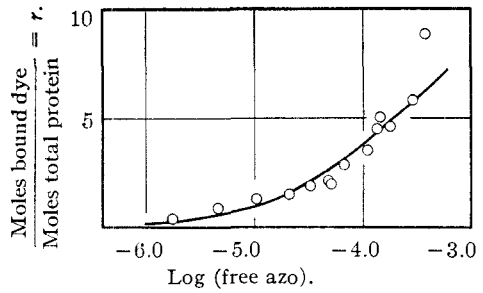



Fig. 2.—Comparison of theoretical and experimental values in binding of azosulfathiazole.

to the successive protein–anion equilibria represented by the equations

$$\begin{aligned} P + A &= PA \\ PA + A &= PA_2 \\ &\cdots\cdots\cdots\cdots \\ PA_{i-1} + A &= PA_i \\ &\cdots\cdots\cdots\cdots \\ PA_{n-1} + A &= PA_n \end{aligned} \qquad (1)$$

where P indicates a molecule of free protein, A one of the organic anion and $n$ the maximum possible number of bound anions per protein molecule. The classical equilibrium constants will be given by the relations

$$\begin{aligned} (PA)/(P)(A) &= k_1 \\ (PA_2)/(PA)(A) &= k_2 \\ &\cdots\cdots\cdots\cdots \\ (PA_i)/(PA_{i-1})(A) &= k_i \\ &\cdots\cdots\cdots\cdots \\ (PA_n)/(PA_{n-1})(A) &= k_n \end{aligned} \qquad (2)$$

It can be shown[17] that the ratio, $r$, of the moles of bound anion to the total moles of protein is given in general by the equation

$$r = \frac{k_1(A) + 2k_1k_2(A)^2 + \cdots + n(k_1k_2\cdots k_n)(A)^n}{1 + k_1(A) + k_1k_2(A)^2 + \cdots + (k_1k_2\cdots k_n)(A)^n} \qquad (3)$$

To convert this equation to a less unwieldy form it is necessary to consider in some detail the nature of the binding process.

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

**Statistical Effect.**—The simplest situation in a case of multiple binding of the anions by the protein would be that in which a bound ion exerts no electrostatic influence on the succeeding bindings, and in which each anion is bound to the same kind of group on the protein. In such a case the strength of attachment would be the same for each bound anion and the *relative* values of the successive equilibrium constants would be determined solely by statistical factors. For this situation the equilibrium constant of the $i$th reaction in (1) is given by the relation

$$k_i = \frac{n - (i - 1)}{i}\frac{1}{K} \qquad (4)$$

as has been indicated frequently in analogous problems.[16,18] $K$ is a constant which depends on the nature of the anion as well as on the character of the protein and hence must be determined experimentally.

It can be shown readily[17] that where the statistical effect is predominant and the equilibrium constants are given by (4), the relatively involved equation (3) may be reduced to a very simple linear form.

$$\frac{1}{r} = \frac{K}{n}\frac{1}{(A)} + \frac{1}{n} \qquad (5)$$

Thus a simple method of determining whether the relative values of the binding constants of a given anion are fixed by statistical considerations alone is to plot the ratio of the moles of total protein to moles of bound anion *versus* the concentration of free anion. If a linear relationship is obtained, statistical factors are predominant.

Such a graph for methyl orange is shown in Fig. 3. It is evident immediately that methyl orange fits the statistical situation. Therefore one is led to the conclusion that the binding of one of these anions does not affect the binding of a second, except insofar as the first ion reduces the number of spaces available to the second. Each focus of attachment on the albumin molecule

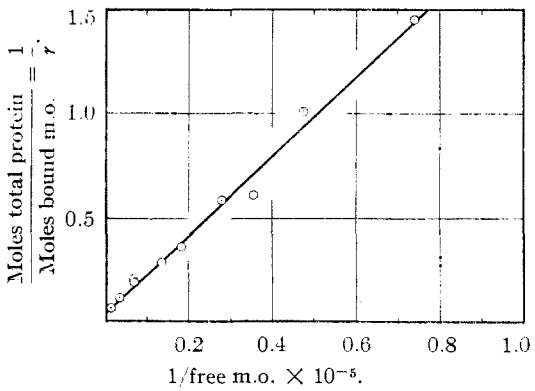



Fig. 3.—Validity of statistical interpretation of binding of methyl orange.

(18) E. Q. Adams, THIS JOURNAL, **38**, 1503 (1916); H. S. Simms, *ibid.*, **48**, 1239 (1926); A. L. von Muralt, *ibid.*, **52**, 3518 (1930).

must have the same inherent tendency for attaching an anion.

In view of the fact that the data for methyl orange can be fitted to equation (5), the constants $K$ and $n$ have been evaluated by the method of least squares, with each point weighted in proportion to the concentration of free anion present. This method of weighting has been adopted to give the higher concentrations more nearly equal influence, in comparison with the lower concentrations, in fixing the constants of (5), than they would have in an unweighted equation. The result obtained is

$$\frac{1}{r} = \frac{4.48 \times 10^{-4}}{22.4}\frac{1}{(A)} + \frac{1}{22.4} \qquad (6)$$

The fractional value of $n$ must be attributed to experimental error. Presumably 22 is the maximum number of bound methyl orange anions, under the conditions of the present experiments.

Azosulfathiazole stands in marked contrast to methyl orange, as is evident from the pronounced curvature in Fig. 4. The first few anions of

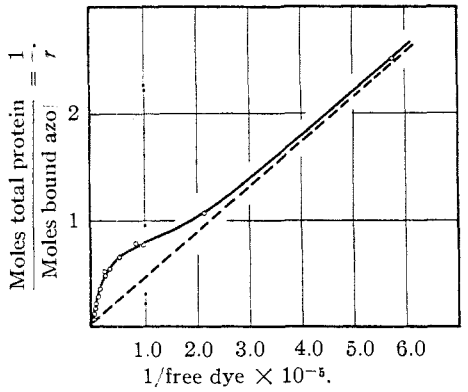



Fig. 4.—Statistical and electrostatic effects in binding of azosulfathiazole by bovine serum albumin.

azosulfathiazole are more strongly bound than are those of methyl orange, no doubt because of the additional van der Waals interaction in the larger molecule. Each added anion, however, exerts an additional electrostatic repulsion toward further oncoming anions. Consequently, the relative extent of binding decreases until it even falls below that for methyl orange. While it is difficult to extrapolate the curve for azosulfathiazole, the indications are that its maximum number of bound anions is the same as that for methyl orange. Knowing this limiting value it is possible to make a first approximation to the line which would be obtained if the azosulfathiazole binding were also determined only by statistical factors, for as the solution becomes more and more dilute, the complexes become almost exclusively the PA type in which there would be no mutual repulsion of bound anions. This first approximation to the idealized statistical behavior is indicated by the dotted line in Fig. 4.

**Electrostatic Effect.**—It is most convenient in these calculations to eliminate all but the statistical and electrostatic-interaction factors by considering the ratio of two successive equilibrium constants, $k_{i-1}/k_i$. Such a procedure is analogous to that used by Kirkwood and Westheimer[19] in treating the first and second ionization constants of a dibasic acid. This ratio of equilibrium constants corresponds to that for the reaction

$$PA_i + PA_{i-2} = 2PA_{i-1} \qquad (7)$$

in which an A ion is being transferred from one complex to another of lower degree. The free energy change for this reaction would be given by the equation

$$-\Delta F^0 = +RT \ln (k_{i-1}/k_i) = -\Delta F_{elec.} + RT \ln \left[\frac{n-(i-2)}{n-(i-1)}\frac{i}{i-1}\right] \qquad (8)$$

where $\Delta F_{e\,ec.}$ is the change in electrostatic free-energy in reaction (7). The second term in equation (8) is the statistical one and has been evaluated from equation (4).

The electrostatic free-energy change may be considered as the difference between the charging energy of two $PA_{i-1}$ ions and the sum of the charging energies of the $PA_i$ and $PA_{i-2}$ ions. For the charging energy of an ion in an electrolyte solution one may use the relation[20] developed from the Born and Debye–Hückel theories and hence obtain

$$\Delta F_{elec.} = \frac{-Nz^2e^2}{D}\left(\frac{1}{b} - \frac{\kappa}{1+\kappa a}\right) \qquad (9)$$

where $N$ is Avogadro's number; $z$, the number of charges on A; $e$, the electronic charge; $D$, the dielectric constant of the medium; $b$, the radius of the protein molecule; $a$, the "distance of closest approach" to the protein of a charged ion; and $\kappa$ is given by

$$\kappa = \left(\frac{4\pi Ne^2}{1000DkT}\right)^{1/2}\Gamma^{1/2} \qquad (10)$$

where $k$ is the Boltzmann constant, $T$, the absolute temperature and $\Gamma$, twice the ionic strength of the medium. To the approximation with which the present calculations can be carried out it is probably unnecessary to introduce the "effective" dielectric constant used by Kirkwood and Westheimer[19] in their treatment of dibasic acids, for in the relatively large protein molecule, the distance of the charged anions from the center of the protein molecule would be nearly the same as the radius of the latter. It is of interest to note also that the value of $\Delta F_{elec.}$, as given by equation (9), is a function of the valence *and* size of the organic anion A, but depends only on the radius, and not on the valence, of the protein ion.

(19) J. G. Kirkwood and F. H. Westheimer, *J. Chem. Phys.*, **6**, 506 (1938).

(20) D. A. MacInnes, "Principles of Electrochemistry," Reinhold Publishing Corp., New York, N. Y., 1939, page 146; E. J. Cohn and J. T. Edsall, ref. 16, pp. 473–475.

Making use of equations (8), (9) and (10), one can calculate readily the magnitude of the electrostatic-interaction term for azosulfathiazole. For the purpose of this calculation the radius of the protein molecule has been computed from the partial specific volume of 0.748[21] and found to be 27.5 Å. Such a procedure has been used previously by Edsall.[20] If account is taken also of the hydration of the serum albumin molecule, a radius of 30 Å. is obtained,[22] and this value has been assumed for $b$. To obtain $a$, 4 Å. has been added to $b$, since the distance between the two sulfonate ions on azosulfathiazole is approximately 8 Å. In the buffer used, the ionic strength was 0.120. Using the radii indicated and accepted values for the other constants one may readily calculate $\Delta F_{\text{elec.}}$ for the binding of azosulfathiazole. This value may be combined with the statistical term of equation (8) and the ratio $k_{i-1}/k_i$ may be determined.

To calculate each $k_i$ explicitly it is necessary to evaluate one constant empirically. A first approximation was obtained by calculating the slope of the dotted line in Fig. 4, computing $K$ from (5) and $k_1$ from (4). Knowing $k_1$, one may obtain the other equilibrium constants which may be substituted in (3) and $r$ calculated.

It has been pointed out to us by Professor Scatchard[23] that the calculation of $k_1$ from the dotted line in Fig. 4 gives undue weight to that portion of our data which is poorest in experimental precision. To improve the agreement between calculated and observed values of $r$, other values of $k_1$ have been assumed, and curves for $r$ *versus* log (A) computed. By means of successive approximations, a "best" value of $1.25 \times 10^5$ was obtained. The calculated curve for this value of $k_1$ is illustrated as the solid line in Fig. 2. The agreement with the experimental observations is probably better than one might anticipate in view of the assumptions implicit in the calculations.

It is also of interest to consider the magnitude of the electrostatic effect in the binding of methyl orange by bovine serum albumin. The calculation is analogous to that for azosulfathiazole, except that a new value must be obtained for $a$. It has been assumed, at Professor Scatchard's suggestion,[23] that only the $SO_3^-$ group need be considered in the interaction, and hence a value of 31.5 Å. has been taken for $a$. With this radius, $\Delta F_{\text{elec.}}$ becomes 33 calories/mole. For purposes of comparison, the statistical term in equation (8) has also been converted into calories for various values of $i$, and the results are listed in Table II. The electrostatic term is about an order of magnitude smaller than the statistical one. Furthermore the percentage effect on $\varphi$(i) in Table II is much less than on $RT$ ln $\varphi(i)$. Nevertheless an electrostatic effect of 33 calories/mole is not in complete agreement with the experimental data illustrated in Fig. 1. A theoretical curve for $r$, calculated in the manner described for azosulfathiazole, lies slightly below the experimental curve. Thus at a concentration of $10^{-4}$ $M$ the theoretical value of $r$ is 3.39 and at $10^{-3}$ $M$, 11.7. In view of the oversimplified model of a spherical protein molecule with a symmetrical charge distribution, these relatively small discrepancies are perhaps to be expected.

TABLE II

EVALUATION OF THE STATISTICAL CONTRIBUTION TO THE FREE-ENERGY CHANGE

| | $\frac{22-i+2}{22-i+1}\frac{i}{i-1} = \varphi(i)$ | $RT \ln \varphi(i)$[a] in calories |
|---|---|---|
| 2 | 2.10 | 410 |
| 3 | 1.575 | 251 |
| 4 | 1.40 | 186 |
| 5 | 1.32 | 153 |
| 6 | 1.27 | 133 |
| 7 | 1.24 | 120 |
| 8 | 1.22 | 110 |
| 9 | 1.205 | 103 |
| 10 | 1.20 | 101 |
| 11 | 1.19 | 96 |
| 12 | 1.19 | 96 |
| 13 | 1.19 | 96 |
| 14 | 1.20 | 101 |
| 15 | 1.205 | 103 |
| 16 | 1.22 | 110 |
| 17 | 1.24 | 120 |
| 18 | 1.27 | 133 |
| 19 | 1.32 | 153 |
| 20 | 1.40 | 186 |
| 21 | 1.575 | 251 |
| 22 | 2.10 | 410 |

[a] Calculated for a temperature of 5°.

**Nature of the Anion–Protein Bond.**—In the interaction of detergent anions with proteins it has been pointed out by Putnam and Neurath[10] that the points of attachment are probably cationic groups on the protein. A similar mechanism seems likely with the sulfonate groups described in this paper. In this connection it is of interest to note that the maximum number of bound anions, 22, corresponds roughly to the number of arginine residues, 25, in the bovine albumin molecule.[24]

In comparing the affinity of a protein for various anions, it is appropriate to consider the relative values of the first binding constant, $k_1$, since after the addition of one or more anions, the complexes with different added groups are no longer strictly analogous. It would be suitable also to compare the intrinsic constants, $K$ or $1/K$, and thereby eliminate the statistical factor. However, in the present situation the maximum number of bound anions is the same for both com-

(21) E. J. Cohn and J. T. Edsall, ref. 16, p. 428.

(22) G. Scatchard, paper presented in the Symposium on Interactions in Protein Solutions at the Atlantic City meeting of the American Chemical Society, April, 1946.

(23) G. Scatchard, private communication.

(24) E. Brand, B. Kassel and L. Saidel, *J. Clin. Investigation*, **23**, 437 (1944).

pounds, and hence the use of the intrinsic constant will not change the *relative* affinities of the two substances.

The first binding constant can be evaluated readily for methyl orange by substituting the known value of $K$, $4.48 \times 10^{-4}$, into (4). A value of $4.9 \times 10^{4}$ is obtained for $k_1$, $-5960$ calories/mole for $\Delta F_1$. For azosulfathiazole, a $k_1$ of $1.25 \times 10^{5}$ has been determined by methods described above. $\Delta F_1$ thus becomes $-6480$ calories/mole. The additional affinity of 520 calories which the protein has for azosulfathiazole must be attributed to the additional groups present in this molecule. Since there are many added substituents it is impossible to consider any one as most important although it seems likely that the van der Waals interaction of the added aromatic ring contributes the major portion of this extra stabilization energy. Additional investigations are in progress in which the binding of closely related analogs is being studied so that the contributions of various substituents to the binding energy may be evaluated.

**Conclusions.**—From the data and calculations presented it is obviously possible to correlate the binding of organic anions by the established principles of the law of mass action. Deviations from statistical behavior can be explained adequately in terms of electrostatic contributions and it is unnecessary to assume that the associated cations are bound simultaneously with the organic anions. The equations presented also permit the ready evaluation of equilibrium constants and free energies of binding of the first anion by the protein and consequently afford a simple quantitative method of evaluating the contributions of various structural groups to the strength of the bond.

**Acknowledgments.**—These investigations were supported in part by grants-in-aid from the Abbott Fund of Northwestern University and from the Sigma Xi Research Fund.

## Summary

Quantitative data are presented on the binding of two sulfonate anions by bovine serum albumin. The data are analyzed in terms of statistical and electrostatic factors which contribute to the strength of binding. A method of evaluating the strength of protein–anion bonds is presented.

EVANSTON, ILLINOIS RECEIVED MARCH 29, 1946

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[CONTRIBUTION FROM THE CHEMISTRY DEPARTMENT OF THE UNIVERSITY OF WASHINGTON]

# Studies of Sulfonates. VIII. Some Surface and Interfacial Tension Measurements with Aqueous Solutions of Certain Alkanesulfonates

BY E. C. LINGAFELTER, O. L. WHEELER[1] AND H. V. TARTAR

The surface properties of aqueous solutions of paraffin-chain colloidal electrolytes are of both theoretical and practical interest. The change of surface tension with time including final equilibrium values at 40° has been determined in this Laboratory[1a] by the sessile bubble method for a series of solutions of varying concentrations of sodium decane, dodecane and tetradecane sulfonate, sodium laurate buffered with 0.002 $M$ sodium carbonate, and of monoethanolammonium laurate, myristate and oleate buffered with excess monoethanolamine. Long and Nutting[2] have made a similar study at 25° of sodium laurate solutions over a $p$H range of 7 to 11. Previously it has been shown[3,4] that the surface tension of solutions of sodium soaps is markedly affected by change of $p$H. Powney and Addison[4] have determined by the drop weight method the interfacial tension of solutions of alkyl sulfates (carbon chain, $C_{12}$, $C_{14}$, $C_{16}$, $C_{18}$) and of the corresponding sodium soaps at varying $p$H values against xylene. While they may have obtained reproducible results, they evidently did not obtain equilibrium values. The drop weight method does not permit the interface to age for more than a brief time, not long enough for equilibrium conditions to become established. Furthermore, these workers did not presaturate the xylene and solutions with each other; as will be shown later, this has an influence of considerable magnitude on the values obtained.

**Surface Tensions of Solutions of Dodecane Sulfonic Acid and of Magnesium Octane Sulfonate.**—The acid was prepared by the method of Zuffanti.[5]

The magnesium octane sulfonate was taken from a stock of this compound prepared by Dr. R. D. Cadle[6] in this Laboratory by precipitation with magnesium chloride from a hot solution of sodium octane sulfonate. The salt was then purified by two crystallizations from water and finally analyzed by ignition and subsequent conversion to magnesium pyrophosphate; the results were very close to the theoretical requirement.

The surface tensions of the dodecane sulfonic acid solutions were measured by the sessile

(1) Standard Oil Company of California Fellow, 1941–1942.

(1a) Tartar, Sivertz and Reitmeier, THIS JOURNAL, **62**, 2375 (1940).

(2) Long and Nutting, *ibid.*, **63**, 84 (1941).

(3) Long, Nutting and Harkins, *ibid.*, **59**, 2197 (1937).

(4) Powney and Addison, *Trans. Faraday Soc.*, **33**, 1243 (1937); **34**, 372 (1938); **34**, 635 (1938).

(5) Zuffanti, THIS JOURNAL, **62**, 1044 (1940).

(6) Cadle, "A Study of the Properties of Salts of Certain Higher Sulfonic Acids," Doctorate Thesis, University of Washington, Seattle, 1940.