Protein-Ion Binding Measurements by Radioactive Tracers. II. The Binding Constants of Iodide and Acetate Ions to Bovine Serum Albumin*

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The binding of iodide ion from solutions of sodium iodide to deionized bovine serum albumin was studied by equilibrium dialysis using radioactive ^{131}I as a tracer ion and nonbinding ^{24}Na as the Donnan-ratio ion. From these data the sum of the binding constants for all sites, $\Sigma_i n_i K^\circ_{i\mathrm{I}} = 7700$, was calculated as two-thirds of the value for iodide ion (11,700) previously reported for bovine serum mercaptalbumin. Mathematical analysis of the bovine serum albumin binding curve indicated the presence of three classes of sites with their corresponding binding constants as follows: $n_1 = 1$, $K^\circ_{1\mathrm{I}} = 4900$; $n_2 = 6$, $K^\circ_{2\mathrm{I}} = 440$; and $n_3 = 14$, $K^\circ_{3\mathrm{I}} = 14$. The binding constant of acetate ion was determined with the same experimental technique through measurement of its competitive effect on the binding constant of iodide ion to bovine serum albumin. The overall binding constant, $\Sigma_i n_i K_{i\mathrm{Ac}} = 830$, for acetate ion was subdivided for the three classes of binding sites as follows: $n_1 = 1$, $K^\circ_{1\mathrm{Ac}} = 530$; $n_2 = 6$, $K^\circ_{2\mathrm{Ac}} = 50$; and $n_3 = 14$, $K^\circ_{3\mathrm{Ac}} = 2$. The radioactive-tracer–equilibrium-dialysis technique described here should be applicable to the quantitative measurement of the binding constants of a large number of other ions including those for which no suitable radioactive tracers exist.

In a previous paper (Saifer and Steigman, 1961), a method for the measurement of the Donnan ratio by radioactive tracers and its application to protein-ion binding was described. For quantitative measurements of binding constants, it possessed a number of important advantages over the equilibrium dialysis procedure (Klotz, 1953; Hughes and Klotz, 1956) as well as the emf method (Carr, 1952, 1953a,b,c; Scatchard *et al.*, 1950). The data obtained with the radioactive tracer method were used to calculate the binding constant of iodide ion to BSM.[1] The experimental value found was in good agreement with that obtained by Scatchard *et al.* (1957).

The present paper reports the binding constant of iodide ion to BSA using the same technique. The binding constant of acetate to BSA was not measured directly as was the iodide ion. Instead it was calculated through measurement of its competitive effect on the binding constant of iodide ion to BSA. This variation in technique is capable of being extended to the quantitative measurement of the binding constants of other ions including those for which radioactive tracers are not commonly available.

Experimental

The procedure used for determining the binding constant of iodide ion to BSA was similar to that described in detail (Saifer and Steigman, 1961) for BSM including the technique for the deionization of the protein and the utilization of the equilibrium-dialysis procedure for the binding measurements. Two sets of experiments were performed in the study of acetate-ion binding to BSA. In the first set a constant concentration of 10^{-3} M acetate buffer, *p*H 5.10, was present in the 50.0 ml outside solution, while in the second the concentration of buffer acetate ion was maintained at 10^{-2} M. The outside solutions of both sets also contained a known amount of sodium iodide which was varied from 10^{-5} to 10^{-2} M for each set together with trace amounts of ^{131}I (~50,000 cpm) and ^{24}Na (~100,-000 cpm).

The deionized BSA solutions (~5 g/100 ml) used in these experiments were prepared from 1 × recrystallized material obtained from Pentex, Inc. (Kankakee, Ill.), to which a molecular weight of 69,000 was assigned. The exact protein concentration for each tube was determined from ultraviolet-absorbance measurements as previously described (Foster and Sterman, 1956). The dialysis experiments and all other measurements were carried out at a temperature of 20° ± 2°.

Results

From the radioactive-tracer measurements the Donnan ratio was found, and from Donnan ratio the number of moles of I^- bound per mole of protein ($\bar{\nu}$) and the ratio $\bar{\nu}/\alpha$ were calculated according to the relationships described in detail in the previous paper (Saifer and Steigman, 1961):

$$\bar{\nu} = \bar{Z}_p = \frac{(\mathrm{I}^-)_B}{(\mathrm{P})} \qquad (1)$$

$$\alpha = f_\pm (\mathrm{I}^-)_F e^{-2.303 w' \bar{Z}_p \bar{Z}_A} \qquad (2)$$

and

$$\frac{\bar{\nu}}{\alpha} = \frac{(\mathrm{I}^-)_B}{f_\pm (\mathrm{I}^-)_F (\mathrm{P}) e^{2.303 w' \bar{Z}_p \bar{Z}_A}} \qquad (3)$$

where $f_\pm$ is the factor for the activity coefficient corrections. The factor $f_\pm$ was obtained by using the Debye-Huckel equation, $\ln f_\pm = 0.509 \bar{Z}^2 \sqrt{\mu}$, where $\bar{Z}$ is the charge on the iodide ion and μ is the ionic strength of the solution.

Data thus obtained for iodide-ion binding to BSA are summarized in Table I. From these, a plot was made of $\bar{\nu}/\alpha$ versus $\bar{\nu}$, as illustrated in Figure 1, curve 2. In order to compare these results with our data for BSM, as reported in our previous paper (Saifer and Steigman, 1961), the latter were plotted in the same figure as curve 1 except that ordinates were divided by 1.52 so as to have the ordinate intercepts of the two curves fall together. As shown by Scatchard *et al.* (1957), the fact that their shapes are concave upward indicates that for both BSA and BSM, the iodide ion is binding to more than one class of sites.

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[1] Abbreviations used in this work: BSM, bovine serum mercaptalbumin; BSA, bovine serum albumin.

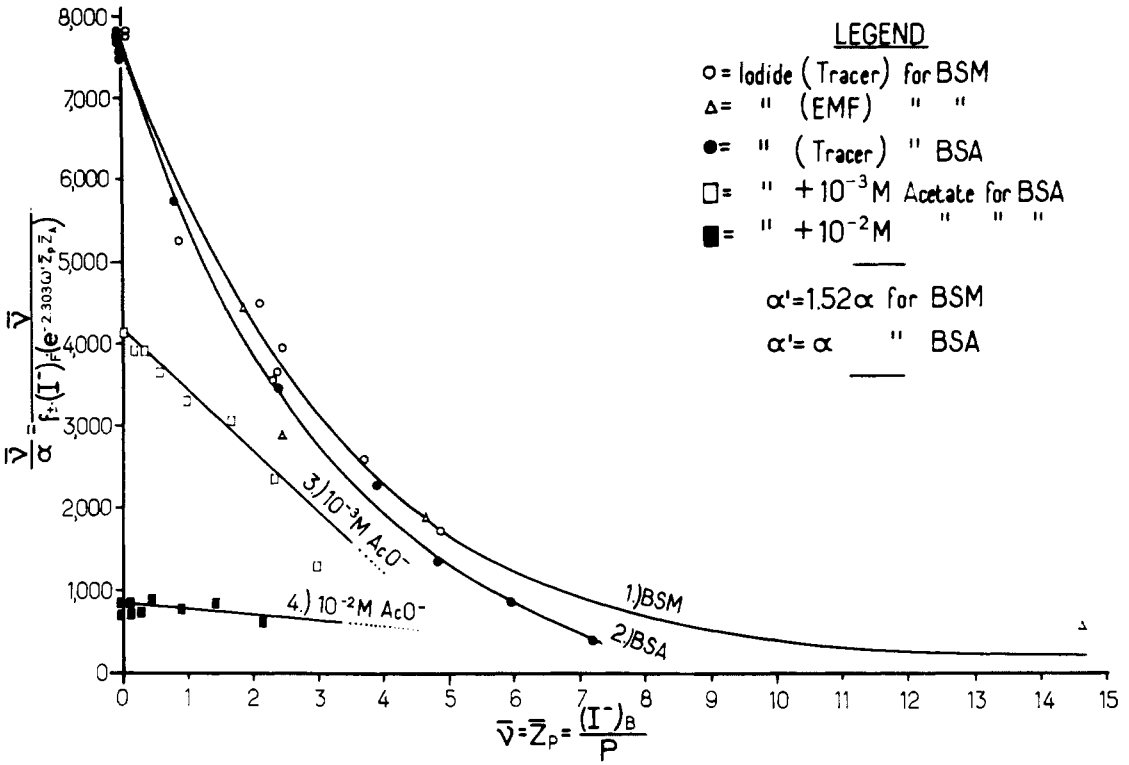

FIG. 1.—Iodide-ion binding to bovine serum albumin and bovine serum mercaptalbumin. Curve 1, iodide-ion binding to isoionic bovine serum mercaptalbumin (ordinate values should be multiplied by 1.52); curve 2, iodide-ion binding to isoionic bovine serum albumin; curve 3, apparent iodide-ion binding to isoionic bovine serum albumin in the presence of 10^{-3} M acetate buffer; curve 4, same as curve 3 except that 10^{-2} M acetate buffer was used.

TABLE I
IODIDE-ION BINDING TO ISOIONIC BOVINE SERUM ALBUMIN

$(I^-)_T$ (molality)	$(I^-)_F$ (molality)	$(I^-)_B$ (molality)	$1/r$ (Donnan ratio)	$\bar{\nu} = \frac{(I^-)_B}{(P)}$	$\frac{\bar{\nu}}{\alpha_\pm} = \Sigma_{KI}$	$\alpha = f_\pm \alpha_0{}^a$	$f_\pm$
3.44×10^{-12}	6.94×10^{-13}	2.81×10^{-12}	0.242	5.41×10^{-9}	7800	6.939×10^{-13}	1.000
2.75×10^{-12}	8.11×10^{-13}	1.94×10^{-12}	0.270	6.32×10^{-9}	7791	8.106×10^{-13}	1.000
3.54×10^{-12}	7.37×10^{-13}	2.80×10^{-12}	0.246	5.53×10^{-9}	7509	7.366×10^{-13}	1.000
9.92×10^{-6}	1.48×10^{-6}	8.44×10^{-6}	0.154	1.14×10^{-2}	7757	1.468×10^{-6}	0.996
1.25×10^{-6}	2.16×10^{-7}	1.03×10^{-6}	0.150	1.66×10^{-3}	7719	2.152×10^{-7}	0.999
1.11×10^{-5}	1.80×10^{-6}	9.28×10^{-6}	0.192	1.35×10^{-2}	7564	1.789×10^{-6}	0.996
6.87×10^{-4}	1.66×10^{-4}	5.22×10^{-4}	0.372	7.85×10^{-1}	5677	1.383×10^{-4}	0.970
2.68×10^{-3}	1.04×10^{-3}	1.63×10^{-3}	0.580	2.33	3457	6.754×10^{-4}	0.941
5.95×10^{-3}	3.26×10^{-3}	2.69×10^{-3}	0.707	3.89	2250	1.729×10^{-3}	0.914
1.08×10^{-2}	7.32×10^{-3}	3.49×10^{-3}	0.803	4.83	1358	3.564×10^{-3}	0.885
1.99×10^{-2}	1.61×10^{-2}	3.86×10^{-3}	0.891	5.95	829	7.186×10^{-3}	0.847
4.91×10^{-2}	4.31×10^{-2}	6.00×10^{-3}	0.910	7.24	400	1.812×10^{-2}	0.771

[a] Where $\alpha_o = (I^-)_F e^{-2.303 w' \bar{Z}_p \bar{Z}_A}$.

If the number of sites and the binding constants in every class are, respectively, n_1, n_2, n_3, and $K°_{1A}$, $K°_{2A}$, $K°_{3A}$, then the following relationships exist:

$$\bar{\nu}_A = \Sigma_i \nu_{iA} \quad (4)$$

and

$$\frac{\bar{\nu}_A}{\alpha_A} = \Sigma_i \frac{\bar{\nu}_{iA}}{\alpha_A} \quad (5)$$

The subdivision of the binding sites into classes and the determination of binding constants for each class might be performed by using two methods, i.e., a geometrical method or a logarithmic one. Both were described in detail by Scatchard *et al.* (1957) and need not be discussed here. However, it should be stressed that for both methods the intercepts of the curve on both axes are important. Thus at $\bar{\nu}_A = 0$ the intercept is $\bar{\nu}_A/\alpha_A = \Sigma_i n_i K°_{iA}$; while at $\bar{\nu}_A/\alpha_A = 0$ the intercept is $\bar{\nu}_A = \Sigma_i n_i$. Scatchard *et al.* (1957) found that the limits at $\bar{\nu}_A/\alpha_A = 0$ were usually indeterminate, but those at $\bar{\nu}_A = 0$ could be determined with moderate precision.

Our data for BSM fit best the lower limits of the number of binding sites proposed by them, i.e.,

$$n_1 = 1,\ n_2 = 6, \text{ and } n_3 = 14 \quad (6)$$

Thus the limit at $\bar{\nu}_I = 0$ will be $\bar{\nu}_I/\alpha_I = \Sigma_i n_i K_{iI} =$ 11,700 (where subscript I is for iodide ion).

As shown in the previous paper, the limit at $\bar{\nu}_I = 0$ ($<10^{-5}$ M I^-) was determined directly with the equilibrium-dialysis-tracer method and was found to be 11,800, which agrees very well with Scatchard's data. In addition, the whole curve for BSM coincided with that of Scatchard's, when they were superimposed, as is shown in the upper curve of Figure 1. We also found that the limits at $\bar{\nu}_I/\alpha_I = 0$ were indeterminate.

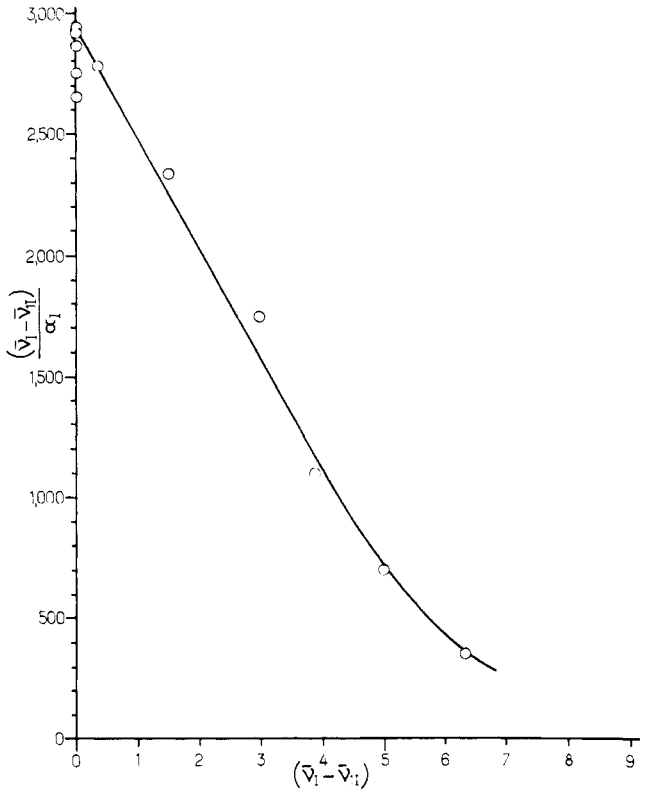

FIG. 2.—Iodide-ion binding to isoionic bovine serum albumin. Plot of $(\bar{\nu}_I - \bar{\nu}_{II})/\alpha_I$ versus $(\bar{\nu}_I - \bar{\nu}_{II})$.

The curve obtained for BSA was somewhat different than that for BSM. For BSA the intercept at $\bar{\nu}_I = 0$ ($<10^{-5}$ M I^-), as determined directly with the equilibrium-dialysis-tracer method, was found to be $\Sigma_i n_i K_{iI} = 7700$. This figure represents the average of the first six values in the column for $\bar{\nu}/\alpha$ (Table I). The values reported by Scatchard for the number of each class of sites are respectively

$$n_1 = 1;\ n_2 = 7 \pm 1;\ \text{and}\ n_3 = 18 \pm 4 \qquad (7)$$

In order to confirm these results the subdivision of the binding sites into classes was performed using both the geometrical and logarithmic methods. The results of the subdivision for the first two classes of sites are:

$$n_1 = 1, \quad n_2 = 6 \qquad (8)$$

$$K^\circ_{1I} = 11K^\circ_{2I} \qquad (9)$$

Since the intercept at $\bar{\nu}_I = 0$ is

$$\bar{\nu}_I/\alpha_I = \Sigma_i n_i K^\circ_{iI} = 7700 \qquad (10)$$

This gives:

$$K^\circ_{1I} = 4900 \text{ and } K^\circ_{2I} = 440 \qquad (11)$$

Once K°_{1I} is determined, the values of $\bar{\nu}_{1I}$ for every experimental point, as well as the differences $(\bar{\nu}_I - \bar{\nu}_{1I}$ and the ratios $(\bar{\nu}_I - \bar{\nu}_{1I})/\alpha_I$ can be calculated. Figure 2 shows the plot of $(\bar{\nu}_I - \bar{\nu}_{1I}/\alpha_{1I}$ versus $(\bar{\nu}_I - \bar{\nu}_{1I})$. The asymptotic slope of the curve intersects the x axis at $(\bar{\nu}_I - \bar{\nu}_{1I}) = 6$ indicating that for the second class of sites, $n_2 = 6$.

The results, obtained by the logarithmic method, for n_2 and K°_{2I} are shown in Figure 3. The curve, without circles, represents the plot of $\log y = \log[x/(1 + x)]$ versus $\log x$ and the values correspond to the left vertical scale and lower horizontal scale. The circles represent the points obtained from the experimental data and are related to the right vertical scale and upper horizontal scale.

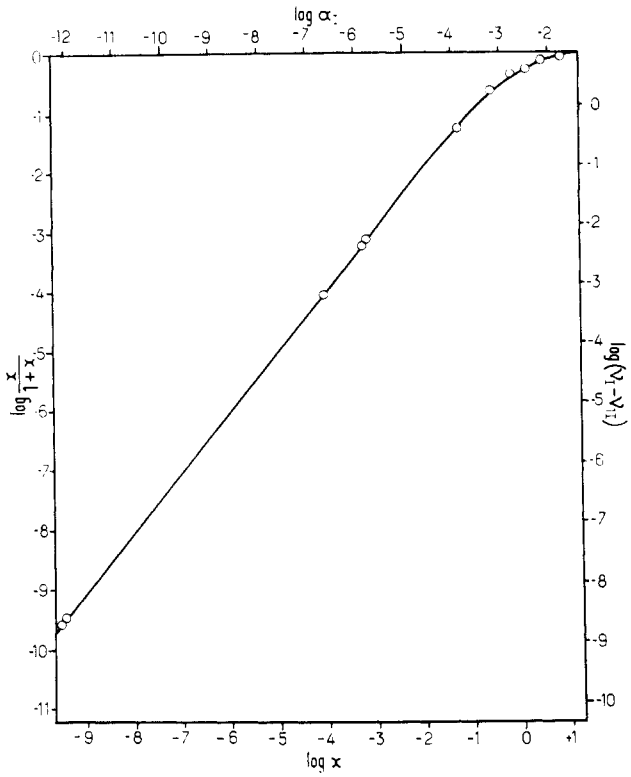

FIG. 3.—Iodide-ion binding to isoionic bovine serum albumin. The curve itself represents the theoretical plot of $\log y = \log[x/(1 + x)]$ versus $\log x$ (left ordinate and lower abscissa, respectively). The circles represent the experimental data $\log(\bar{\nu}_I - \bar{\nu}_{1I})$ versus $\log \alpha_I$ (right ordinate and upper abscissa, respectively.)

The circles coincide with the logarithmic curve when the following relationships hold:

$$\log n_2 = \log(\bar{\nu}_I - \bar{\nu}_{1I}) - \log y \qquad (12)$$

$$\log K^\circ_{2I} = \log x - \log \alpha_I \qquad (13)$$

Since in Figure 3 the circles are superimposed on the logarithmic curve, we derive from the two scales the following values:

$$\log y = -0.80\ldots \qquad [\log(\nu_I - \nu_{1I}) = 0] \qquad (14)$$

$$\log \alpha_I = -2.65\ldots \qquad (x = 0) \qquad (15)$$

Therefore, from equations (12) and (13) we get:

$$\log n_2 = 0.80 \quad \text{or} \quad n_2 = 6$$

$$\log K^\circ_{2I} = 2.65 \quad \text{or} \quad K^\circ_{2I} = 440$$

It follows from the foregoing calculations that both BSA and BSM have the same number of binding sites for the first two classes. If the assumption is made

TABLE II
COMPARATIVE IODIDE-ION-BINDING DATA FOR BSM AND BSA PROTEINS

	BSM	BSA
n_1	1	1
n_2	7 ± 1	6
n_3	18 ± 4	14
K°_{1I}	9200 (for $n_2 = 6$, $n_3 = 14$)	4900
K°_{2I}	383 (for $n_2 = 6$, $n_3 = 14$)	440
K°_{3I}	12 (for $n_2 = 6$, $n_3 = 14$)	14
$\Sigma_i k_i K^\circ_{iI}$	11,700	700
Ratio of K°_{iI} values	$K^\circ_{1I} = 24K^\circ_{2I} = 720K^\circ_{3I}$	$K^\circ_{1I} = 11K^\circ_{2I} = 350K^\circ_{3I}$

TABLE III
APPARENT IODIDE-ION BINDING TO BSA IN PRESENCE OF 10^{-2} M ACETATE

$(I^-)_T$ (molality)	$(I^-)_F$ (molality)	$(I^-)_B$ (molality)	$1/r$ (Donnan ratio)	$\bar{\nu} = \frac{(I^-)_B}{(P)}$	$\frac{\bar{\nu}}{\alpha} = \Sigma K_I^{app}$	$\alpha = f_\pm \alpha_o{}^a$	$f_\pm$
2.04×10^{-5}	1.35×10^{-5}	6.91×10^{-6}	1.651	9.45×10^{-3}	786	1.201×10^{-5}	0.889
4.25×10^{-5}	2.72×10^{-5}	1.53×10^{-5}	1.696	2.09×10^{-2}	869	2.409×10^{-5}	0.889
1.11×10^{-4}	7.11×10^{-5}	3.96×10^{-5}	1.678	7.35×10^{-2}	757	6.254×10^{-5}	0.889
2.07×10^{-4}	1.34×10^{-4}	7.29×10^{-5}	1.626	1.00×10^{-1}	860	1.163×10^{-4}	0.888
4.28×10^{-4}	2.92×10^{-4}	1.36×10^{-4}	1.592	1.81×10^{-1}	726	2.498×10^{-4}	0.887
9.76×10^{-4}	6.46×10^{-4}	3.30×10^{-4}	1.526	4.69×10^{-1}	893	5.246×10^{-4}	0.884
1.78×10^{-3}	1.23×10^{-3}	5.55×10^{-4}	1.438	8.34×10^{-1}	784	1.064×10^{-3}	0.881
3.35×10^{-3}	2.37×10^{-3}	9.87×10^{-4}	1.370	1.36	809	1.677×10^{-3}	0.873
7.11×10^{-3}	5.48×10^{-3}	1.63×10^{-3}	1.229	2.14	608	3.527×10^{-3}	0.858

[a] Where $\alpha_o = (I^-)_F e^{-2.303 w' \bar{Z}_P \bar{Z}_A}$.

TABLE IV
APPARENT IODIDE-ION BINDING TO BSA IN PRESENCE OF 10^{-3} M ACETATE

$(I^-)_T$ (molality)	$(I^-)_F$ (molality)	$(I^-)_B$ (molality)	$1/r$ (Donnan ratio)	$\bar{\nu} = \frac{(I^-)_B}{(P)}$	$\frac{\bar{\nu}}{\alpha} = \Sigma K_I^{app}$	$\alpha = f_\pm \alpha$	$f_\pm$
5.49×10^{-5}	1.47×10^{-5}	4.02×10^{-5}	0.974	5.86×10^{-2}	4185	1.401×10^{-5}	0.963
1.37×10^{-4}	3.74×10^{-5}	9.99×10^{-5}	0.979	1.38×10^{-1}	3947	3.488×10^{-5}	0.961
2.69×10^{-4}	7.69×10^{-5}	1.92×10^{-4}	0.984	2.73×10^{-1}	3917	6.97×10^{-5}	0.959
4.99×10^{-4}	1.58×10^{-4}	3.41×10^{-4}	0.958	5.02×10^{-1}	3662	1.370×10^{-5}	0.956
1.12×10^{-3}	3.73×10^{-4}	7.46×10^{-4}	0.896	9.74×10^{-1}	3228	2.963×10^{-4}	0.947
1.85×10^{-3}	7.35×10^{-4}	1.11×10^{-3}	0.856	1.63	3117	5.241×10^{-4}	0.939
3.22×10^{-3}	1.48×10^{-3}	1.74×10^{-3}	0.855	2.28	2362	9.636×10^{-4}	0.927
6.28×10^{-3}	3.74×10^{-3}	2.54×10^{-3}	0.885	2.95	1308	2.257×10^{-3}	0.905

[a] Where $\alpha_o = (I^-)_F e^{-2.303 w' \bar{Z}_P \bar{Z}_A}$.

that this is also true for the third class of sites, the calculated value for BSA will be:

$$n_3 = 14 \quad \text{and} \quad K^\circ_{3I} = 14$$

The data obtained from the binding studies of both BSM and BSA are summarized in Table II.

Apparent Iodide Ion Binding to BSA in Presence of Acetate.—In this series of experiments it was first attempted to measure the binding of acetate ion directly, by using ^{14}C-labeled acetate ion. However, no suitable facilities were available for counting weak beta emitters with the requisite accuracy needed to obtain the binding data. Therefore an indirect experimental approach was employed, namely, that of determining the binding constants of ions such as acetate by means of competition between different ions for the same binding sites.

In these experiments crystalling BSA was used and its binding affinity for iodide ion was measured exactly as described in the absence of acetate ions. The experiments were then repeated first in presence of 10^{-3} M (*p*H values of inside solutions were 5.72–6.00 after equilibrium) acetate ion and then in the presence of 10^{-2} M (*p*H 5.20–5.40) acetate ion. The data thus obtained are presented in Tables III and IV, and plotted in Figure 1, curves 3 and 4. The equations used to determine the binding constants of two different ions competing for the same binding sites of a macromolecule, are as follows:

$$\Sigma_i n_i K_{iB} = \frac{\Sigma_i n_i K_{iA}}{\Sigma_i n_i K_{iA}{}^{app}} - 1 \qquad (16)$$

and

$$\Sigma_i n_i K_{iA}{}^{app} = \frac{\Sigma_i n_i K_{iA}}{1 + (B)\ \Sigma_i n_i K_{iB}} \qquad (17)$$

These equations are essentially the same as that derived by Langmuir (1918) to describe certain adsorption isotherms. They were used by Scatchard (1949; Scatchard *et al.*, 1954, 1957) to develop his theory on the interaction of proteins with small molecules. Klotz and his co-workers were among the first to make extensive use of the equilibrium dialysis method and the theory of multiple equilibria for determining the binding constants of small ions to proteins (Klotz, 1946, 1953; Klotz *et al.*, 1946). They show that in the case of competition of two species A and B for the same binding sites, $\Sigma_i n_i K_A{}^{app}$ depends only on the concentration of the species (B), since $\Sigma_i n_i K_{iA}$ and $\Sigma_i n_i K_{iB}$ are constants. This allows the simultaneous calculation of the sums $\Sigma_i n_i K_{iA}$ and $\Sigma_i n_i K_{iB}$, by determining experimentally two values for $\Sigma_i n_i K_{iA}{}^{app}$, namely, $\Sigma_i n_i K_{iA1}{}^{app}$ and $\Sigma_i n_i K_{iA2}{}^{app}$ at two different concentrations of B, namely, $(B)_1$ and $(B)_2$.

In the present case, the two competitive species for the same binding sites are iodide (I) and acetate (Ac). Therefore we obtain from equation (16) the two simultaneous equations:

$$\Sigma_i n_i K_{iAc} = \frac{1}{(Ac)_1} \cdot \frac{\Sigma_i n_i K_{iI}}{\Sigma_i n_i K_{iI1}{}^{app}} - 1 \qquad (18)$$

and

$$\Sigma_i n_i K_{iAc} = \frac{1}{(Ac)_2} \cdot \frac{\Sigma_i n_i K_{iI}}{\Sigma_i n_i K_{iI2}{}^{app}} - 1 \qquad (19)$$

The experimental data are:

$(Ac)_1 = 10^{-3}$ moles/liter and $\Sigma_i n_i K_{iI1}{}^{app} = 4200$

$(Ac)_2 = 10^{-2}$ moles/liter and $\Sigma_i n_i K_{iI2}{}^{app} = 828$

The last two values were found by the method of least squares, and represent the intercept at $\bar{\nu}_I = 0$ of the two apparent straight lines obtained for a 10^{-3} M and 10^{-2} M acetate concentrations, respectively, as shown in Figure 1 by curves 3 and 4.

Substituting these data in equations (18) and (19) we get:

$$\Sigma_i n_i K_{iI} = 7675 \quad \text{and} \quad \Sigma_i n_i K_{iAc} = 827$$

The first value agrees very well with that of 7700 obtained directly for iodide ion without acetate (Table I). However, when comparing Tables I, III, and IV, we find that there are more measurements without acetate than with and this is especially true for the limits at $\bar{\nu}_I = 0$. Therefore it is preferable to use equation (19) as the working equation, which with the proper notation then becomes:

$$\Sigma_i n_i K_{i\mathrm{Ac}} = \frac{1}{(\mathrm{Ac})} \cdot \frac{\Sigma_i n_i K_{i\mathrm{I}}}{\Sigma_i n_i K_{i\mathrm{I}}^{\mathrm{app}}} - 1 \qquad (20)$$

Using for $\Sigma_i n_i K_{i\mathrm{I}}$ the average value 7700 determined from measurements without acetate and our experimental data, we obtain an average value of $\Sigma_i n_i K_{i\mathrm{Ac}} = 830$.

As can be seen from Tables I, III, and IV, the correction factor for activity, $f\pm$, is smaller for the experiments without acetate than for those with acetate. This is especially true for a 10^{-2} M acetate concentration. The reason is that the ionic strength is considerably increased by the presence of acetate ion. If this increase in ionic strength is not taken into account one finds for $\Sigma_i n_i K_{i\mathrm{Ac}}$ a value of 930, i.e., about 100 units greater.

According to Scatchard *et al.* (1957), the ratios $K_1:K_2:K_3$ are the same for various anions which bind to BSM. A simple way of subdividing the binding constants for acetate ion is to assume that this anion binds to the same classes of sites of BSA as does the iodide anion Thus:

$$\Sigma_i n_i K_{i\mathrm{Ac}} = 830 = n_1 K^\circ_{i\mathrm{Ac}} + n_2 K^\circ_{2\mathrm{Ac}} + n_3 K^\circ_{3\mathrm{Ac}} \qquad (21)$$

$$n_1 = 1, \quad n_2 = 6, \quad n_3 = 14$$

and

$$K^\circ_{1\mathrm{Ac}} = 11 K^\circ_{2\mathrm{Ac}} = 350 K^\circ_{3\mathrm{Ac}}$$

Therefore, from (21) we get:

$$K^\circ_{1\mathrm{Ac}} = 530, \quad K^\circ_{2\mathrm{Ac}} = 50 \quad K^\circ_{3\mathrm{Ac}} = 2$$

Discussion

The comparative data for iodide-ion binding to BSM and BSA listed in Table II show that (a) the numbers of the binding sites of all three classes are the same, and (b) the value of $K^\circ_{1\mathrm{I}}$ for BSM is almost twice that for BSA while the $K^\circ_{2\mathrm{I}}$ and $K^\circ_{3\mathrm{I}}$ values are about the same for both proteins.

The main reported structural difference between BSM and BSA is that the former possesses a single free-SH group per mole which is highly reactive while BSA has only 0.66-SH group per molecule of albumin (Hughes, 1954). BSM is generally prepared from purified BSA with the procedure developed by Hughes (1947) which utilizes the interaction of the –SH group with mercury salts to separate that fraction of the serum albumin which contains such reactive groups from that portion in which the –SH groups are either absent, complexed with other ions, or buried within the folds of the molecule. It is quite probable that this purification process also results in the removal of some bound ions, e.g., fatty acids, from the BSA so as to increase the binding affinity of BSM for small ions. The markedly increased binding constant obtained for iodide ion with BSM as compared to BSA for sites of the first class may also be owing to either conformational changes in the protein produced during the preparation BSM or to the removal of a blocking group from the proximity of the primary binding site. A similar difference in the binding affinities of the same two proteins for the binding of thyroxine has been reported by Sterling *et al.* (1962). These authors found an apparent binding constant of 150,000 for thyroxine to deionized, crystalline BSM, while the apparent binding constant of the same substance to BSA gave a value of only 100,000. Our values for iodide-ion binding ($\Sigma_i n_i K^\circ_{i\mathrm{I}}$) to BSM and BSA are 11,800 and 7700, respectively. For both iodide ion and thyroxine the overall binding constant to BSA is exactly two-thirds that for BSM.

Sterling *et al.* (1962) have also reported that removal of fatty acids, or other bound lipids, from BSA by a low-temperature delipidation procedure (Goodman, 1957) results in an increased value for the binding constant of thyroxine similar to that obtained with BSM. However, one cannot be certain that the increased binding constant for BSA treated thus is not due to some denaturation of the protein resulting from the delipidation process. It would be of interest to see whether this difference also occurs for other ions such as bromide, chloride, thiocyanate, and the like, a study which is presently in progress in this laboratory. This experimental finding also raises the question as to whether some of the BSA molecules may not be present as disulfide dimers (Straessle, 1954) in which the stronger binding sites are sterically blocked. This hypothesis could be readily confirmed by a further study of the binding of I^- ion to the non-SH-containing fraction of BSA which is not precipitated by mercury ions.

Although the preceding discussion has dealt with differences in the binding affinities between BSM and BSA for small ions, it should be stressed that both proteins possess a single strong binding region together with twenty somewhat weaker binding sites. These similarities would serve to explain the unique properties of both these proteins for binding many diverse kinds of ions including buffer ions such as acetate. Acetate ion was chosen for this initial study of the quantitative measurement of binding constants through their competitive effect for the same binding sites, because it is a good buffer ion in the isoionic region of the serum albumins. The use of this anion minimized *p*H changes which arose in the equilibrium-dialysis system owing to variation in salt concentration. The average value of $\Sigma_i n_i K_{i\mathrm{Ac}} = 830$ for acetate ion, which is about one-tenth that of iodide ion to BSA, is in the range of results expected for the binding of this kind of ion (Teresi and Luck, 1952). In the only published study of the binding of acetate ions to crystalline BSA, these authors found $\Sigma n_i K^\circ_{i\mathrm{Ac}}$ to be 670 using an equilibrium-dialysis technique and radioactive counting of acetate labeled with ^{14}C in the carboxyl group. However, their experiments were performed at *p*H 9.1 in 0.1 M glycinate buffer. Both the higher *p*H used, as compared to the isoionic *p*H of 5.10, and the competitive effect of the high concentration of glycinate ions employed to eliminate the Donnan effect, would tend to decrease acetate-ion binding to BSA (Klotz, 1950). The value of 670 obtained by Teresi and Luck (1952) for the sum of the acetate-binding constants is therefore in reasonable agreement with our value of 830 if these different experimental conditions are taken into consideration.

It is believed that the indirect competitive ion-binding method yields results which may be somewhat less precise than those which would be obtained by direct measurements of the binding constant as was done for iodide ion. Since Scatchard *et al.* (1957) have determined the binding constants for a number of other ions to BSM with an emf procedure, it would be desirable to determine their values with the competitive ion-equilibrium-dialysis–radioactive-tracer procedure described in this paper. Such experimental work is presently in progress in our laboratory.

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The Reaction of β-Lactoglobulin Sulfenyl Iodide with Several Antithyroid Agents*

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Although β-lactoglobulin sulfenyl iodide is relatively stable toward reaction with cysteine or mercaptoethanol, it reacts very rapidly with other reagents such as thiourea, thiouracil, and related compounds which are known to interfere with the *in vivo* iodination of tyrosine in thyroglobulin. Similarly, I^- and SCN^-, which are known to exert characteristic effects upon thyroid metabolism, also react with the protein sulfenyl iodide, but these reactions are characterized by less specificity as evidenced by the approximate equilibrium constants. The reaction of thiouracil or thiourea with the sulfenyl iodide leads to the formation of the respective mixed disulfide with lactoglobulin The similarity of this product to the intermediate proposed by Maloof and Soodak (*Pharmacol. Rev. 15*, 43, 1963) in the thyroid-catalyzed desulfuration of thiourea lends support to the hypothesis that a sulfenyl iodide may function as a reactive intermediate in thyroidal-iodine metabolism.

The reactions of iodine with proteins may be grouped into essentially two classes; substitution reactions involving tyrosine and histidine residues in the peptide chain, and oxidative reactions involving cysteine and, to some extent, tryptophan (Putnam, 1953; Anson, 1941). The substitution reactions are favored in solutions of alkaline *p*H, but occur to a negligible extent at *p*H values below 7, particularly at temperatures near 0° (Putnam, 1953; Cunningham and Nuenke, 1959, 1960a). Under these conditions the predominant reaction of iodine with most proteins appears to involve oxidation of free-sulfhydryl groups of cysteine residues to the level of disulfide. However, in the case of several proteins, notably ovalbumin and β-lactoglobulin, we have been able to show (Cunningham and Nuenke, 1960a, 1961) that reaction with the sulfhydryl groups proceeds only to the level of sulfenyl iodide

$$P\text{—}SH + 2I^- \rightleftarrows P\text{—}SI + H^+ + I^- \quad (1)$$

These protein sulfenyl iodides may be quite stable depending upon the conditions of formation, but react readily with a variety of reagents including simple mercaptans to give the corresponding mixed disulfides.

$$P\text{—}SI + RSH \rightleftarrows P\text{—}SS\text{—}R + H^+ + I^- \quad (2)$$

In previous communications (Cunningham and Nuenke, 1959, 1960a,b) we have described the preparation of protein sulfenyl iodides and reported on some application of these reactions to the study of protein structure. Several aspects of the chemistry of the protein sulfenyl iodide group were suggestive of the hypothesis that it might play a role in iodide metabolism *in vivo*. Of greatest significance, perhaps, was the observation, which has been reported briefly previously (Cunningham and Nuenke, 1960b), that thiourea and thiouracil were the most reactive substances known to participate in reaction (2) with protein sulfenyl iodide.

$$P\text{—}SI + H_2N\text{—}\overset{\overset{S}{\|}}{C}\text{—}NH_2 \rightleftarrows P\text{—}S\text{—}S\text{—}\overset{\overset{NH}{\|}}{C}\text{—}NH_2 + H^+ + I^- \quad (3)$$

The report by Maloof and Soodak (1960, 1963) that a disulfide bond was formed between cellular protein and thiourea and thouracil in the course of metabolism of these substances by the thyroid further strengthened our interest in a possible physiological role for sulfenyl iodide. We have now undertaken a more comprehensive examination of the reactions of the sulfenyl iodide

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