[CONTRIBUTION FROM THE UNITED STATES PUBLIC HEALTH SERVICE, TUBERCULOSIS RESEARCH LABORATORY, CORNELL UNIVERSITY MEDICAL COLLEGE]

Competition in the Binding of Long Chain Fatty Acids and Methyl Orange to Bovine Serum Albumin[1]

BY GERALDINE E. COGIN[2,3] AND BERNARD D. DAVIS

The binding of long chain fatty acids by bovine serum albumin was investigated with the hope that the results obtained would permit inferences about the forces involved and the topography of the binding sites on the protein. The interaction between albumin and oleic, elaidic and stearic acids has been investigated at *p*H 6.6 in 0.10 *M* phosphate buffer at 6° by observing the competition, in binding to the protein, between each acid and methyl orange. In the regions of very small molar ratios of added fatty acid to albumin there is almost no competition; this has been interpreted in terms of the existence of more than one set of binding sites. As more fatty acid is added to the system the competition increases until one fatty acid displaces more than one dye anion; it has been suggested that the binding of a long chain anion at one site results in steric hindrance at other sites. As still more fatty acid is added, a saturation limit, beyond which there is no further increase in competition, is reached; it is believed that the solubility of the acid determines this limit. Thus it can be seen that no single hypothesis is sufficient to explain the entire competition curve.

In attempting to elucidate the structure of protein molecules in solution, modern studies have been increasingly directed at their interactions with other compounds. The emphasis has been placed upon the combination of proteins with small molecules of known structure in the hope that analysis of the results obtained would permit inferences as to the forces involved and the topography of the binding sites on the protein. Serum albumin has been shown to be unique among proteins in its ability to combine with an extremely varied assortment of compounds; it is therefore a convenient as well as a particularly interesting protein to use in these experiments.

The binding by serum albumin of lower fatty acid anions up to C_9 has been demonstrated in ultrafiltration studies.[4] It has also been shown that these compounds increase the anionic electrophoretic mobility of albumin,[5] and protect it against heat[6,7] and urea[8-10] denaturation as determined by cloud-point and viscosity studies, respectively. All evidence indicates that the binding to albumin is increased by increasing the length of the fatty acid chain. Early work by one of the authors,[11] using solubility and microbiological techniques, indicated that oleate ion is bound much more strongly than other anions previously studied. The investigation of the interaction between bovine serum albumin and some long chain fatty acids was therefore undertaken with the thought that the high affinity of these anions for the available loci would be useful in the study of the distribution of the reactivities of the binding sites as well as the structural configuration in the neighborhood of these sites.

Because cellophane is impermeable to long chain fatty acids the equilibrium dialysis technique, widely and successfully used in binding studies, could not be employed in these experiments. The interaction was therefore studied indirectly by observing the competition between a nondialyzable fatty acid and a dialyzable dye anion for the albumin. Competition between methyl orange and other organic anions has been studied by Klotz, Triwush and Walker[12] using spectral displacement. Karush[13] has studied the competitive interaction of an anionic azo dye and sodium dodecyl sulfate with albumin using the dialysis technique.

Experimental

Materials.—Armour crystallized bovine serum albumin was used in these experiments. The methyl orange was obtained from the National Aniline Company. We are indebted to Dr. Leo Shedlovsky of the Colgate–Palmolive–Peet Company for the sodium oleate. The stearic acid (m.p. 69.8°; neut. equiv. 285) and elaidic acid (m.p. 43.7–46.0°; neut. equiv. 283) were generously supplied by Professor Charles O. Beckmann of Columbia University. The dialyses were carried out in Visking sausage casing.

Procedure.—Sodium phosphate buffer was used in all the experiments reported here; the final salt concentration was 0.10 *M* ($\Gamma/2 = 0.176$) and the *p*H was 6.6. The final albumin concentration was approximately 1% and the temperature 6 ± 1°. Two stock solutions of albumin were prepared: 2% in 0.60 *M* phosphate, 1% in 0.30 *M* phosphate. The protein concentration of these solutions was determined by Kjeldahl analysis using 16.07%[14] as the nitrogen content. The fatty acid solutions were prepared by weight, using a minimum of KOH for solution, and were diluted with water to give the desired concentration. The methyl orange was dissolved in water because salt markedly decreases its solubility.

Binding of Methyl Orange by Cellophane.—Before use, the cellophane casing was soaked overnight in distilled water. Ten ml. of 0.30 *M* phosphate buffer was placed inside a cellophane sac which was immersed in 20.0 ml. of methyl orange of known concentration. After the tubes were shaken for three days at 6° to attain equilibrium, the concentration of free dye (c) was determined with a Beckman spectrophotometer. From the volume of the system the number of moles of free dye was calculated and subtracted from the total number in the system to determine the number (B) bound by the dialysis sac. A plot of log B *vs.* log c was a straight line. The same curve was obtained when fatty acid was placed inside the bag with the buffer.

Binding of Methyl Orange by Albumin.—Most of the determinations of dye bound to protein were carried out by an indirect method. Ten ml. of 1% albumin in 0.30 *M*

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(2) Public Health Service Research Fellow of the National Institutes of Health, April, 1948–May, 1950.

(3) The Sloan-Kettering Institute for Cancer Research, New York.

(4) P. D. Boyer, G. A. Ballou and J. M. Luck, *J. Biol. Chem.*, **167**, 407 (1947).

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

(6) G. A. Ballou, P. D. Boyer, J. M. Luck and F. G. Lum, *ibid.*, **163**, 589 (1944).

(7) P. D. Boyer, F. G. Lum, G. A. Ballou, J. M. Luck and R. G. Rice, *ibid.*, **162**, 181 (1946).

(8) P. D. Boyer, *ibid.*, **158**, 715 (1945).

(9) P. D. Boyer, G. A. Ballou and J. M. Luck, *ibid.*, **162**, 199 (1946).

(10) E. L. Duggan and J. M. Luck, *ibid.*, **172**, 205 (1948).

(11) B. D. Davis and R. J. Dubos, *J. Expt. Med.*, **86**, 215 (1947).

(12) I. M. Klotz, H. Triwush and F. M. Walker, THIS JOURNAL, **70**, 2935 (1948).

(13) F. Karush, *ibid.*, **72**, 2714 (1950).

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

phosphate was placed inside a cellophane sac which was immersed in 20.0 ml. of methyl orange of known concentration. After equilibration at 6°, the *p*H of the solutions inside and outside the sac were measured with a Beckman *p*H meter; no difference could be detected. The concentration of free dye outside the sac (which equals that inside) was determined spectrophotometrically. The number of moles of free dye, calculated from the volume of the system, was subtracted from the total number present, giving the total number of moles of dye bound; by subtracting the number of moles bound by the dialysis sac itself at the equilibrium concentration of free dye, the number of moles of methyl orange bound by the albumin was computed. The molar ratio of bound dye to albumin was also calculated.

To determine whether the correction for the binding of dye by cellophane, applied in the indirect method of analysis, was valid in the presence of albumin, a direct method of analysis was also employed. In these experiments the albumin in an aliquot of the solution inside the sac at equilibrium was precipitated with 3% trichloroacetic acid. The solution was filtered and the precipitate washed with TCA until free of color. Control experiments in which known mixtures of dye and protein were used showed that the recovery of dye was complete. The filtrate was collected in volumetric flasks and the dye concentration measured spectrophotometrically. This concentration is the sum of the free and bound methyl orange. The concentration of free dye is known since it equals that of the solution outside the sac, the Donnan effect being negligible. The albumin concentration was determined by Kjeldahl analysis. With these data the concentration of bound methyl orange can be computed. The ratio of bound methyl orange to albumin calculated by this direct method agreed within the experimental error with that obtained by the indirect method.

Competition between Methyl Orange and Fatty Acid for Albumin.—Five ml. each of 2% albumin in 0.60 *M* phosphate buffer and a fatty acid solution of known concentration were placed inside a dialysis sac which was immersed in 20.0 ml. of methyl orange of known concentration. After equilibration, no *p*H difference could be detected between the solution inside and outside the sac. The ratio of bound methyl orange to albumin in the presence of a known quantity of fatty acid was determined by the indirect method.

Results

The data obtained from a typical experiment on the binding of methyl orange by albumin, and the accompanying calculations, are given in Table I. The results are plotted in Fig. 1 according to the method suggested by Scatchard[15]: r/c *vs.* r, where r is the molar ratio of bound dye to albumin and c the concentration of free dye in equilibrium with the albumin–dye complex. r has been calculated on the basis of 69,000[16] as the molecular weight of albumin. The data can be fitted by the equation

$$r/c = kn - kr \qquad (1)$$

where k is the intrinsic equilibrium constant for the reaction at a single site and n is the total number of sites available to the dye. By the method of least squares values of $n = 13.8$ and $k = 4.37 \times 10^3$ (with a probable error of 4.6%) have been obtained.

In Table II are listed the results of a typical experiment on the competition between methyl orange and a fatty acid for the albumin, and the necessary calculations. In all of the competition experiments reported here the same total number of moles of methyl orange was used. A summary of the results for stearate, elaidate and oleate are

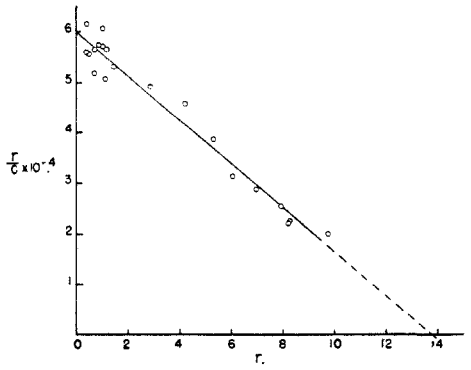

Fig. 1.—Binding of methyl orange by bovine serum albumin: 0.10 *M* phosphate buffer, *p*H 6.6, 6°.

TABLE I

BINDING OF METHYL ORANGE BY BOVINE SERUM ALBUMIN

Temperature 6°; albumin 1.45×10^{-4} *M*; buffer, 0.10 *M* phosphate ($\Gamma/2 = 0.176$); *p*H 6.6

Concn. of free dye, m./l. $\times 10^5$, c	Moles total dye in system $\times 10^5$	Moles bound dye[a] $\times 10^6$	Moles dye bound by sac $\times 10^5$	Moles dye bound per mole protein r[b]	$r/c \times 10^{-4}$
49.2	30.2	15.4	1.3	9.75	1.99
37.4	24.0	12.8	0.91	8.24	2.21
36.9	24.0	12.9	.91	8.28	2.24
31.5	21.8	12.3	.76	7.95	2.53
24.4	18.1	10.8	.56	6.99	2.87
19.2	15.0	9.2	.41	6.09	3.16
13.8	12.1	8.0	.28	5.33	3.87
9.29	9.10	6.32	.17	4.25	4.60
5.87	6.02	4.26	.076	2.89	4.83
2.82	3.04	2.20	.041	1.49	5.30
2.29	2.40	1.71	.032	1.16	5.17
2.52	2.40	1.64	.036	1.11	4.40
1.80	2.14	1.60	.023	1.09	6.05
1.40	1.49	1.07	.018	0.73	5.20
1.33	1.20	0.80	.017	.54	4.07
0.797	0.896	0.657	.009	.45	5.63

[a] Calculated from volume of system = 30.0 ml. [b] Moles of protein = 1.45×10^{-6}.

TABLE II

COMPETITION IN THE BINDING OF OLEATE AND METHYL ORANGE TO BOVINE SERUM ALBUMIN

Temperature 6°; albumin 1.37×10^{-4} *M*; buffer: 0.10 *M* phosphate ($\Gamma/2 = 0.176$); *p*H 6.6; total methyl orange in system: 5.88×10^{-6} moles

Moles oleate added per mole protein[a] f	Concn. of free dye m./l. $\times 10^5$ c	Moles bound dye[b] $\times 10^6$	Moles dye bound by sac $\times 10^6$	Moles dye bound per mole protein r
20.2	14.7	1.48	0.30	0.862
16.3	14.5	1.56	.29	.929
12.1	14.3	1.63	.28	.986
10.1	13.5	1.84	.27	1.05
8.95	12.9	2.08	.26	1.33
7.08	11.3	2.51	.21	1.68
6.05	10.5	2.76	.20	1.87
5.05	8.84	3.23	.17	2.23
2.43	6.40	3.96	.11	2.81
1.01	5.62	4.19	.093	2.99
0	5.29	4.30	.087	3.07

[a] Moles of protein = 1.37×10^{-6}. [b] Calculated from volume of system = 30.0 ml.

(15) G. Scatchard, *Ann. N. Y. Acad. Sci.*, **51**, 660 (1949).

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

plotted in Fig. 2. The ordinate is f, the molar ratio of added fatty acid to protein; the abscissa is r, the molar ratio of bound methyl orange to albumin.

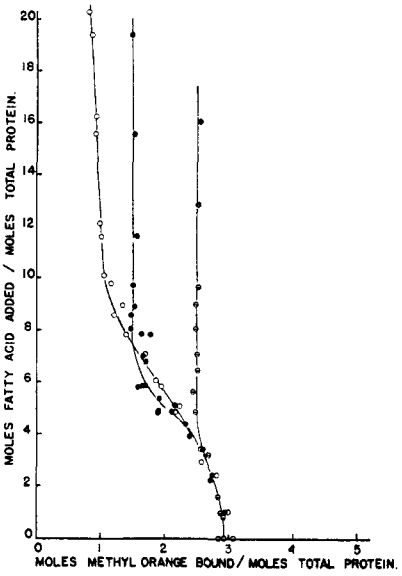

Fig. 2.—Competition between methyl orange and long chain fatty acids for serum albumin, 0.10 M phosphate buffer, pH 6.6, 6°: ○, oleate; ●, elaidate; ⊖, stearate.

Discussion

When increasing amounts of fatty acid are added to a given amount of albumin before dialysis against a fixed concentration of methyl orange, the ratio of bound dye to albumin decreases, as can be seen from Fig. 2. Until three moles of fatty acid have been added to a mole of albumin, the acids show identical competition with the dye. It can therefore be inferred that the reactive sites on the albumin which are involved are equally available to all three fatty acids. The addition of more than four moles of stearate, however, effects no further reduction of the ratio of bound methyl orange to albumin, and also produces a turbid solution, indicating saturation of the aqueous solution with fatty acid. The elaidate curve reaches a limiting value at $f = 7$, oleate at $f = 9$, each producing a turbid solution at these respective values. It should be noted here that the solutions have already become faintly turbid in regions where competition is still increasing. It appears likely that the limit of effectiveness of each acid as a competitor is determined by its solubility, though one cannot exclude the possibility that the value of f beyond which there is no further competition represents the total number of sites available to a given kind of fatty acid anion. Good solubility data in this buffer system are not available, but it is known that in water oleate is most soluble and stearate least.

In order to study the competition reaction itself in more detail, the results have been plotted in another way in Fig. 3. Here the abscissa is f, the molar ratio of added fatty acid to albumin, and the ordinate is $1 - r/r_0$, the fraction of competition; r is the molar ratio of bound dye to albumin in the presence of f moles of fatty acid and

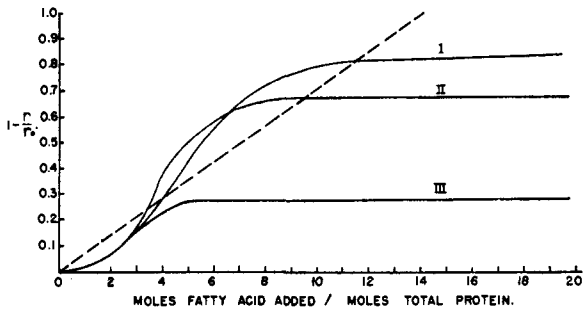

Fig. 3.—Competition between methyl orange and long chain fatty acids for bovine serum albumin: I, oleate; II, elaidate; III, stearate.

r_0 the value this ratio would have if f were 0 and the concentration of free dye were unchanged; it is calculated from the binding data obtained in the absence of fatty acid. The reason for the choice of this method of representing the competition reaction is as follows: The interaction between methyl orange and bovine serum albumin can apparently be described by the equation $r/c = kn - kr$ which implies that the initial probability of reaction between methyl orange and albumin is the same at each site and that the electrostatic interaction is negligible.[15] These results agree with those obtained by Klotz and Urquhart[17a] and by Teresi.[17b] If one assumes, therefore, that the reaction between albumin and fatty acid anions can be described by a similar equation

$$F/C = Kn - KF \qquad (2)$$

where F is the molar ratio of bound fatty acid to albumin, C the concentration of free fatty acid and K the equilibrium constant for the reaction at a single site; also, that n the total number of sites available to fatty acid is the same as that available for methyl orange binding ($n = 14$, obtained from the methyl orange–albumin reaction, has been used here) then

$$1 - r/r_0 = F/n^{18} \qquad (3)$$

If all the fatty acid added were bound by the albumin (*i.e.*, $F = f$) the curve shown as the dashed line in Fig. 3 would represent the competition reaction. Crude solubility data, obtained in this Laboratory, for these fatty acids in this buffer, indicate that at least 95% of the fatty acid is bound at saturation but of course a larger percentage is bound at lower fatty acid concentrations. Therefore it must be emphasized that a plot of $1 - r/r_0$ *vs.* f would not be linear; it would fall off at high values of f and at low values approach the dashed line in Fig. 3. Examination of Fig. 3 shows that the experimental values lie on an S shaped curve; when a small fraction of the total number of sites is occupied by fatty acid anions there

(17) (a) I. M. Klotz and J. M. Urquhart, THIS JOURNAL, **71**, 1597 (1949); (b) J. D. Teresi, *ibid.*, **72**, 3972 (1950).

(18) In the absence of fatty acid

$$r_0/c = kn - kr_0 \text{ or } r_0 = nkc/1 + kc$$

In the event of competition, the equations given by Klotz, Triwush and Walker are used

$$r = nkc/1 + kc + KC \text{ and } F = nKC/1 + KC + kc$$

and from these relationships Eq. (3) can be derived. Equation (3) can also be derived assuming that when competition occurs

$$r = k(n - F) - kr$$

is almost no competition but as f increases, the experimental curves I and II rise until the theoretical value for competition is exceeded and one fatty acid anion displaces more than one dye anion.

The crude solubility data can be used to approximate a lower limit of binding at any value of added fatty acid. If the concentration of free acid is assumed to be equal to the solubility of elaidic acid, an estimate of the fraction bound when $1 - r/r_0 = 0.025$ can be made; this value is about twice that calculated from $F/n = (1 - r/r_0)$, so that there is indeed very much less competition than would be expected. The almost complete lack of competition must be explained. The complicating presence of micelles in solutions of fatty acids most probably is not responsible for this situation. Micelle formation would of course be expected to decrease the competing ability of the fatty acid, but as more fatty acid is added to the system there should be an even greater tendency toward micelle formation with a resultant further decrease in competition. Experimentally it is observed that as increasing amounts of fatty acid are added competition increases until one fatty acid is effective in displacing more than one dye anion. If, however, there were additional sites of low reactivity available only to fatty acid, not to methyl orange, it would be possible to account for the almost complete lack of competition at low values of f. Recent work of Karush,[19] Karush and Sonenberg,[20] Scatchard, Scheinberg and Armstrong[21,22] and Teresi[18] has shown that the binding of anions by serum albumin can best be described in terms of more than a single set of reactive sites.

The fact that a single fatty acid anion is effective in displacing more than one dye anion requires explanation. Similar results have been obtained in other laboratories[12,13] in studies of the methyl orange–dodecyl sulfate competition system. When Klotz, Triwush and Walker attempted to apply simultaneous equations of the type of Eq. (1) and (2) to methyl orange and dodecyl sulfate, their calculations yielded the result that more dodecyl sulfate was bound than has been added. They attributed this discrepancy to omission of electrostatic interaction terms from the binding equations. Their results for the methyl orange–salicylate system, on the other hand, did not show this inconsistency. Karush[13] treated the methyl orange–dodecyl system in another way and found that in the r range of 3 to 7 seven bound detergent anions are equivalent to eleven bound dye anions; he has explained this in terms of the heterogeneity of the binding sites of serum albumin.

While it is true that the results of these investigations cannot be explained simply in terms of a single set of binding loci of identical reactivity toward a given anion for which the two species compete without interaction they also cannot be explained completely by the theory of the inhomogeneity of the binding sites of serum albumin. At low values of f this theory is quite adequate; at high values of f additional explanation is needed. It seems quite probable that the topography of the albumin molecule is such that the addition of a long chain molecule at one binding site causes steric hindrance at another, thus permitting one fatty acid anion to displace more than one dye anion.

New York 21, N. Y. Received June 9, 1950

(19) F. Karush, This Journal, **72**, 2705 (1950).
(20) F. Karush and M. Sonenberg, *ibid.*, **71**, 1369 (1949).
(21) G. Scatchard, I. H. Scheinberg and S. H. Armstrong, Jr., *ibid.*, **72**, 535 (1950).
(22) G. Scatchard, I. H. Scheinberg and S. H. Armstrong, Jr., *ibid.*, **72**, 540 (1950).

[Contribution from the Research Laboratory of the General Electric Company]

The Pyrolysis of Diborane and the Synthesis of Pentaborane[1]

By L. V. McCarty and P. A. Di Giorgio

It has been shown that pentaborane containing only small amounts of dihydropentaborane can be synthesized from diborane in reasonable yields. Reaction time and temperature can be traded against each other which results in a relatively wide range of operating conditions. Hydrogen definitely helps in the more efficient conversion of diborane to pentaborane.

Introduction

Even since Alfred Stock[2] first demonstrated methods of making tetraborane in small amounts, methods of synthesizing one boron hydride from another have been of great interest to inorganic chemists. Recent work by Professor H. I. Schlesinger[3] and his co-workers at the University of Chicago has made diborane the easiest boron hydride to obtain in quantity. It is in the natural course of events, then, that improved syntheses for other boron hydrides from diborane should be sought.

Much work has already been done on the preparation of pentaborane by thermal decomposition of diborane in circulatory and single pass systems[2-7] particularly since the diborane synthesis of Schlesinger and Burg[4] in 1931. This paper deals with a study of the best conditions for carrying out this reaction. Some additional information pertinent to the synthesis of other boron hydrides can also be found in the data.

Experimental Part

Materials.—The diborane used in these experiments was prepared by the reaction of boron fluoride etherate with

(1) This work was done on Army Ordnance Contract T U 1-2000.
(2) Alfred Stock, "Hydrides of Boron and Silicon," Cornell University Press, Ithaca, N. Y., 1933.
(3) Unpublished work of H. I. Schlesinger and H. C. Brown and collaborators, at present contained in reports to various government agencies and in University of Chicago doctoral theses.
(4) Burg and Schlesinger, This Journal, **53**, 4321 (1931).
(5) Burg and Schlesinger, *ibid.*, **55**, 4019 (1933).
(6) Stock and Mathing, *Ber.*, **69**, 1456 (1936).
(7) Schlesinger and Burg, *Chem. Rev.*, **31**, 13 (1942).