[CONTRIBUTION FROM THE BUREAU OF BIOLOGICAL RESEARCH, RUTGERS UNIVERSITY]

Binding of Sodium Deoxycholate by Cytochrome *c*[1]

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The binding of sodium deoxycholate by the basic protein cytochrome *c* has been studied by several methods. It was demonstrated by electrophoresis that an equilibrium governs the amount of anion bound by the protein. The molar ratio of protein to total anion at which the former was converted at *p*H 8.55 from a cation to a non-migrating molecule was 1:17. Equilibrium dialysis indicates that the binding can be represented by the equation $1/R = 1/n - (K/n)(1/A)$ where $1/R$ and $1/A$ have the usual significance. n equals the maximum number of bound anions and was found to be 12 per protein molecule. This value is in close agreement with the number of excess positive charges (+13) calculated to be present on the protein molecule at *p*H 8.55. Binding at *p*H 11.9 was negligible. A 315 mμ maximum was observed in the absorption spectrum of ferrocytochrome *c* in the absence of sodium dithionite.

The purpose of this study was the attempt to determine the nature of the interaction between cytochrome *c* and sodium deoxycholate. Bile salts, especially sodium deoxycholate, have proved effective as agents promoting the separation of the enzyme cytochrome oxidase from extraneous cellular material. Wainio and his associates[3,4] were able to separate cytochrome oxidase from the other cytochromes by the serial addition of sodium deoxycholate. Smith and Stotz,[5] as well as other investigators before them, have employed sodium cholate. An understanding of the binding of a surface active agent by a pure cytochrome would be useful in the further purification of cytochrome oxidase.

The only recorded studies of anion binding by cytochrome *c* are those of Boeri, Ehrenberg, Paul and Theorell[6] and Colvin.[7] The former found that when increasing amounts of chloride ions (or Br^- or $SO_4^=$) were added to an acid solution of ferricytochrome *c*, present mainly as Cyt-$2H^+$, 2 chloride ions were taken up simultaneously to form Cyt-$2H^+$–$2Cl^-$. The absorption spectrum and the magnetic properties of the protein were affected. Colvin found that the binding of Orange II was enhanced by lowering the *p*H and that the binding of individual anions was affected by previously bound anions. The binding curve is sigmoid in shape.

Methods

All studies were conducted with cytochrome *c* prepared from beef heart by the method of Keilin and Hartree[8] as further purified by the method of Margoliash.[9] The purity of the resulting cytochrome *c* was measured by its iron content (0.42–0.47%), nitrogen content (14.4–15.1%) and electrophoretic homogeneity (one peak at *p*H 8.55 and $\Gamma/2$ of 0.2). Difco Laboratories sodium deoxycholate which assayed at 99.00% was used. Iron determinations were made by the α,α′-dipyridyl method of Klein[10] and nitrogen determinations by the semi-micro Kjeldahl method.

Electrophoresis.—Determinations were made at 0.0–0.5° with a field current of 9.5 milliamp. using the Perkin-Elmer apparatus, Model 38. The cytochrome *c* was dissolved in the buffers of *p*H 8.55 and 11.90 to give a final concentration of 5 mg. per ml. The sodium deoxycholate was added to the buffer to give final concentrations of from 0.0 to 0.089 *M*. The solutions were dialyzed overnight at 4° against the buffer unless the protein solutions only contained sodium deoxycholate. No loss of the protein was ever detected; probably the concentration of salts used here prevented penetration of the membrane by cytochrome *c*.[11] Because of the intense color of cytochrome *c*, base lines were difficult to obtain with the scanning mechanism. Area measurements were limited, therefore, to three determinations for a study of the separation of the protein–anion complex. The mobility values were consistently reproducible on successive trials even though the time of dialysis varied from 16 to 48 hours. This is good evidence that equilibrium had been achieved.

Equilibrium Dialysis.—These determinations employed the techniques of Klotz, Walker and Pivan,[12] and involved the determination of the distribution of anions between a buffered solution of the anion on one side of a differentially permeable membrane and the buffered protein–anion solution on the other.

The cytochrome *c* solutions (5 mg. per ml.) were made up in buffer containing the desired concentration of sodium deoxycholate which ranged from 0.002 to 0.01 *M*. Equilibration at *p*H 8.55 and *p*H 11.90 was carried out in $^3/_4$″ Visking cellophane tubing for from 72 to 96 hours at 4°. Controls were run at each concentration to measure the binding of the anion by the cellophane. Again there was no detectable loss of protein through the membrane.

Sodium deoxycholate concentrations were determined by the method of Szalkowski and Mader[13] as modified to meet our needs. The amount of anion bound by the protein was taken as the difference between the total concentration of sodium deoxycholate on the outside of the membrane and the total concentration on the inside. The close agreement for bound anion concentrations with successive trials varying in dialysis time from 72 to 96 hours and longer indicated that equilibrium had been substantially attained. Volume changes on the inside and consequently on the outside of the membranes were corrected for by spectrophotometric determination of the concentration changes of cytochrome *c*, *i.e.*, $D_{red} - D_{ox} \div 0.174 \times 10^3$ = moles cytochrome *c* per ml., where *D* equals optical density.

Spectral Analysis.—The absorption spectra of ferrocytochrome *c*–sodium deoxycholate mixtures were determined over a *p*H range from 5.5 to 10.5. In the first series, sodium hydrosulfite was used as the reductant and the absorption was measured from 320 to 625 mμ. In the second series, hydrogen gas and platinum black were used for reduction, and the range studied extended from 220 to 600 mμ. In each series, the cytochrome *c* and sodium deoxycholate concentrations were kept constant. The spectra of three solutions were determined simultaneously: (1) ferrocytochrome *c* in buffer of the desired *p*H (plus NaCl when necessary); (2) ferrocytochrome *c* in the same buffer plus anion; (3) ferrocytochrome *c* in 0.1 *M* Na_2HPO_4–NaH_2PO_4 buffer of *p*H 7.4.

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(2) From a thesis submitted in partial fulfillment of the Ph.D. degree. Department Biochemistry and Physiology, Rutgers University.

(3) W. W. Wainio, S. J. Cooperstein, S. Kollen and B. Eichel, *Science*, **106**, 471 (1947).

(4) B. Eichel, W. W. Wainio, P. Person and S. J. Cooperstein, *J. Biol. Chem.*, **183**, 89 (1950).

(5) L. Smith and E. Stotz, *ibid.*, **209**, 819 (1954).

(6) E. Boeri, A. Ehrenberg, K. G. Paul and H. Theorell, *Biochem. Biophys. Acta*, **12**, 273 (1953).

(7) J. R. Colvin, *Can. J. Biochem. Biophys.*, **32**, 184 (1954).

(8) D. Keilin and E. F. Hartree, *Proc. Roy. Soc. (London)*, **B122**, 298 (1937).

(9) E. Margoliash, *Nature*, **170**, 1014 (1952).

(10) J. R. Klein, personal communication.

(11) D. Keilin and E. F. Hartree, *Biochem. J.*, **39**, 289 (1945).

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

(13) C. R. Szalkowski and W. J. Mader, *Anal. Chem.*, **24**, 1602 (1952).

The cytochrome *c* concentration was 0.05 and 0.33 mg. per ml. and the sodium deoxycholate concentration 0.01 and 0.005 *M* in series 1 and 2, respectively. The ionic strength was kept constant by adding equivalent amounts of sodium chloride to cuvette #1. At wave lengths below 350 mμ, ultraviolet light was used. Readings were taken at 5 mμ intervals except where absorption maxima were observed and the intervals were reduced to 1 or 2 mμ.

Results and Discussion

Figure 1 shows that cytochrome *c* which initially migrated as a cation (at *p*H 8.55) decreased its mobility with increasing concentration of sodium deoxycholate until at 0.0069 *M* sodium deoxycholate

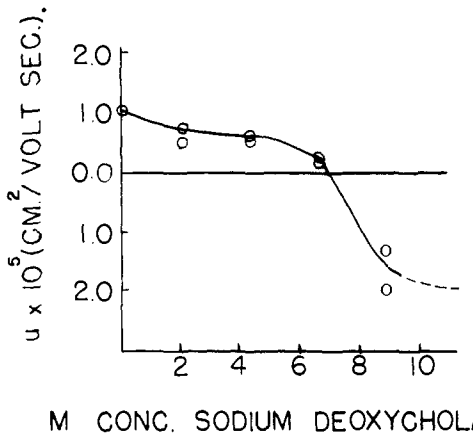

Fig. 1.—Electrophoretic mobility (μ) of cytochrome *c* at several concentrations of sodium deoxycholate in the descending limb of the cell.

it had a zero mobility. With a further increase in sodium deoxycholate, the mobility again increased but was opposite in sign, *i.e.*, the cytochrome *c* migrated as an anion. This behavior demonstrated the formation of a protein–anion complex. At zero mobility the molar ratio of sodium deoxycholate to protein was 17:1. At *p*H 11.9 the mobility of cytochrome *c* decreased with increasihg concentrations of sodium deoxycholate even though both molecules were present as anions. The mobility of the cytochrome *c*–anion mixture was less than the mobility of either cytochrome *c* or sodium deoxycholate at this *p*H. Some type of interaction was thus indicated even above the isoelectric point of cytochrome *c*. This may be ascribed to binding with residual charged basic groups on the protein molecules, *i.e.*, to NH_2 groups of lysine or to guanido groups of arginine. The number of these charged groups at this *p*H would not be numerous, however, since the *pK* values are 10.5[14] and 11.6–12.6,[15] respectively. The data are not sufficient to permit the calculation of the amount of deoxycholate bound at *p*H 11.9.

When the sodium deoxycholate was present in the protein solution only, and not in the buffer, the complex dissociated during electrophoresis as was evident from the number of migrating boundaries. Initially, only one peak was observed. Later, however, two maxima could be observed: one boundary remained stationary while the boundary corresponding to anionic material migrated with a mobility substantially the same as sodium deoxycholate. The two boundaries did not separate completely; the material between was assumed to be composed of dissociating protein–anion complex.

This behavior is believed to be caused by the rapid migration of unbound anion in the electric field away from the area of interaction; consequently, the complex dissociates to maintain an equilibrium. When sodium deoxycholate was present in the buffering medium as well as in the protein solution, only one peak was observed. This was true whether the sodium deoxycholate concentration was below or above the amount necessary to reverse the direction of migration.

The stoichiometry of the interaction is shown more clearly by equilibrium dialysis. In Fig. 2 the binding curve has been plotted as moles of total protein per mole of bound anion ($1/R$) *versus* the reciprocal of the moles of free anion ($1/A$). The points fall along a straight line indicating that the binding can be expressed according to the equation derived by Klotz, *et al.*[12]: $1/R = 1/n + (K/n)(1/A)$, where n = maximum number of anions bound per protein molecule, and K = a constant, determined empirically. The value of n was calculated from a plot of the least squares and was found to be 12. The value of K was 3.26×10^{-6}. The value of n agrees well with the number of excess positively charged groups, +13, which is calculated from the data of Velick[14] to be present at *p*H 8.55. The difference between this value of n and the molar ratio determined by electrophoresis may be explained in terms of an equilibrium. At zero mobility, there would still be free anion and protein according to the equilibrium expression $K = (AB)/(A)(B)$. The ionizable amino groups of arginine and lysine are the source of positive charge at *p*H 8.55; the binding of the anion below the isoelectric point of cytochrome *c* may therefore be assumed to be due to the ionizable guanido and ϵ-NH_2 groups.

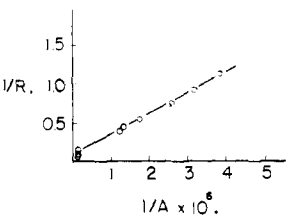

Fig. 2.—Binding of sodium deoxycholate by cytochrome *c* during equilibrium dialysis. Moles of total protein per mole of bound anion ($1/R$) are plotted against the reciprocal of the moles of free anion ($1/A$).

The previous equation was derived from an equation of the form[12] $K_i = [n - (i - 1)]/i\ (1/K)$ which permits the calculation of the binding constant for the ith anion. The value of the first binding constant was found to be 3.68×10^6 and the free energy change, calculated in the usual manner, was −8300 cal. per mole. Because a plot of the binding obeyed the equation developed by Klotz, successive binding should be independent of previous binding of the same anions, and the relative values of the successive equilibrium constants should be determined solely by statistical factors.

(14) S. F. Velick, *J. Phys. Colloid Chem.*, **53**, 135 (1949).

(15) E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides," Reinhold Publ. Corp., New York, N. Y., 1943, p. 445.

Inspection of the absorption spectra of the cytochrome *c*–deoxycholate mixtures did not reveal any significant shifts. This was not completely unexpected, however, since the binding of the iron to the protein in cytochrome *c* is very stable from *p*H 3.0 to 11.0. The absence of any effect is in contrast to the action of some other surface active agents on the prosthetic group of cytochrome *c* and of other conjugated proteins.[16,17] Our studies did, however, demonstrate the existence of a 315 mμ peak which was evident after reduction with H_2 and platinum black, *i.e.*, in the absence of hydrosulfite. This peak had been observed by Theorell[18] and Schales and Behrnts–Jensen[19] who concluded that it indicated the presence of a sulfoxide group in cytochrome *c*. Lavin, *et al.*,[20] did not observe the peak, and Lemberg and Legge[21] consider it as being due to the presence of sodium hydrosulfite used as a reducing agent. Nevertheless, it is present after reduction with hydrogen and platinum black and in the absence of any sodium hydrosulfite.

The concentration of sodium deoxycholate used in these experiments is well above the "critical" concentrations for the formation of micelles; Ekwall[22] reported it to be 0.014 *M* and McBain[23] reported it to be 0.005 *M*. Because the experimental evidence indicates that the binding is electrostatic, the micellar structure is probably not involved in the primary binding found here. The apparent binding of sodium deoxycholate by cytochrome *c* above its isoelectric point may, however, indicate a secondary interaction involving the micelles of sodium deoxycholate.

(16) M. Rabinovitz and P. D. Boyer, *J. Biol. Chem.*, **183**, 111 (1950).
(17) D. Keilin and E. F. Hartree, *Nature*, **145**, 934 (1940).
(18) H. Theorell, *Biochem. Z.*, **285**, 207 (1936).
(19) O. Schales and H. Behrnts-Jensen, *Hoppe-Seylers Z. physiol. Chem.*, **257**, 106 (1939).
(20) G. I. Lavin, C. L. Hoagland and S. M. Ward, *Proc. Soc. Expll. Biol. Med.*, **43**, 757 (1940).
(21) R. Lemberg and J. W. Legge, "Hematin Compounds and Bile Pigments, Their Constitution, Metabolism and Function," Interscience Publishing Co., New York, N. Y., 1949, p. 349.
(22) P. Ekwall, "Koninkl. Vlaam. Acad. Vetenschap., en Schone Kunsten Belgie, Kl. Vetenschap., Intern. Colloquim Biochem., Problem Lipiden."
(23) J. W. McBain, R. C. Merrill, Jr., and J. R. Vinograd, THIS JOURNAL, **63**, 670 (1941).

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[CONTRIBUTION FROM THE DANIEL SIEFF RESEARCH INSTITUTE, THE WEIZMANN INSTITUTE OF SCIENCE]

The Wittig Reaction with Fluorenone. Formation of Cyclopropane Derivatives

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The Wittig reaction between fluorenone (I) and excess of triphenylphosphine-*n*-butylidene (IIa) has been found to yield spiro-(2,3-dipropylcyclopropane-1,9′-fluorene) (IXa). With triphenylphosphinemethylene (IIb), fluorenone gives the previously described spiro-(cyclopropane-1,9′-fluorene) (IXb). The reactions presumably proceed *via* the 9-alkylidenefluorenes (V), since treatment of 9-*n*-butylidenefluorene (Va) with triphenylphosphine-*n*-butylidene (IIa) leads to the same spiro compound IXa as obtained from fluorenone.

In connection with another investigation,[1] we were interested in determining how well the Wittig reaction[2] proceeds between ketones and the triphenylphosphine-alkylidene derived from a saturated primary bromide containing several carbon atoms. With this aim, we studied the reaction between the highly crystalline ketone fluorenone (I) and triphenylphosphine-*n*-butylidene (IIa). The results of this and related experiments are described in this paper.

Treatment of *n*-butyl bromide with triphenylphosphine yielded *n*-butyltriphenylphosphonium bromide,[3] m.p. 243°, which was dehydrobrominated with ethereal butyllithium. The resulting solution of triphenylphosphine-*n*-butylidene (IIa) in ether then was allowed to react with fluorenone (I) first at room temperature and then in refluxing tetrahydrofuran, the reagent IIa being in threefold excess. Chromatographic separation produced successively three different substances, the last two of which proved to be, respectively, triphenylphosphine and triphenylphosphine oxide. The first substance, m.p. 45°, obtained in 50% yield, was found to differ from the expected known[4] 9-*n*-butylidenefluorene (Va) of m.p. 55°. It proved to be a hydrocarbon, $C_{21}H_{24}$, *i.e.*, it was derived from the reaction of fluorenone with *two* molar equivalents of triphenylphosphine-*n*-butylidene. The fluorene carbon skeleton has been preserved, since oxidation with chromium trioxide regenerated fluorenone. The ultraviolet spectrum (two highest wave length maxima at 292 and 303 mμ) was very similar to that of a 9,9-dialkylfluorene (*cf.* 9,9-diethylfluorene, which has the corresponding maxima at 292 and 303 mμ).[5] It was significantly different from that of fluorene (λ_{max} 288.5 and 300 mμ)[6] and of a 9-monoalkylfluorene (9-methylfluorene, λ_{max} 290 and 301 mμ)[6] and quite different from that of a 9-alkylidenefluorene (9-*n*-butylidenefluorene, λ_{max} 301 and 311 mμ).[7] That the 9-position was disubstituted was confirmed by the fact that no red color was produced with Triton B in pyridine solution.[8] These facts lead us to propose

(1) *Cf.* F. Sondheimer and R. Mechoulam, THIS JOURNAL, **79**, 5029 (1957); **80**, 3087 (1958).
(2) *Cf.* G. Wittig, *Experientia*, **12**, 41 (1956); *Angew. Chem.*, **68**, 505 (1956), and earlier references cited there.
(3) Badische Anilin und Soda-Fabrik, German Patent 824,047 (*C. A.* **49**, 6998 (1955)).
(4) R. F. Schultz and C. F. Smullin, THIS JOURNAL, **62**, 2904 (1940); Y. Sprinzak, *ibid.*, **74**, 2116 (1952).
(5) E. J. Greenhow and D. McNeil, *J. Chem. Soc.*, 3204 (1956).
(6) E. J. Greenhow, D. McNeil and E. N. White, *ibid.*, 986 (1952).
(7) S. Wawzonek and E. Dufek, THIS JOURNAL, **78**, 3530 (1956).
(8) Fluorene derivatives containing one or two hydrogen atoms at C-9 give a red color under these conditions (Y. Sprinzak, Congress