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The Acetylcholine–Cholinesterase System

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Evidence recently has been obtained which indicates that a fundamental phenomenon underlying the blood-depressor action produced by choline derivatives may be an exchange adsorption between the cations of the administered choline derivative and those of acetylcholine naturally fixed to tissue surfaces.[2]

It was pointed out early, from this Laboratory,[3] that all substances which exhibit an acetylcholine action on blood pressure are cations, and the suggestion was made that the action of such compounds on the blood pressure is fundamentally bound up with their ionic condition. It seemed necessary to assume that a desorption process was involved in their activities.

A number of studies have been made[4] of the properties of onium ions in connection with their physiological activities. Welch and Roepke[5] have suggested that a "permutit-like" exchange with inorganic ions may be involved in the physiological activity of acetylcholine. As the present work was being completed, Roepke[6] described a study of cholinesterase based on the Michaelis–Menten theory. He evaluated the relative affinities of various substances for the surface of cholinesterase, and discussed the relationship between affinity and physiological activity. Other aspects of this problem recently have received attention.[7]

In the work here described, the esterase hydrolysis of acetylcholine has been considered from a kinetic point of view, and the adsorption affinities of a series of choline derivatives for cholinesterase have been measured by an application of equations of the type derived by Langmuir for adsorption on solid surfaces.

(1) This is a second paper constructed from a thesis presented by Morris Ziff, May, 1937, for the degree of Doctor of Philosophy at New York University.

(2) A paper on this subject by Renshaw, Green, and Ziff will appear soon.

(3) (a) Renshaw, *Science*, **62**, 384 (1925); (b) Renshaw and Hotchkiss, THIS JOURNAL, **48**, 2698 (1926).

(4) (a) Harvery, *J. Exptl. Zool.*, **10**, 507 (1911); (b) Renshaw and Bacon, THIS JOURNAL, **48**, 1726 (1926); (c) Bencowitz and Renshaw, *ibid.*, **47**, 1904 (1925); **48**, 2146 (1926); (d) Renshaw and Hotchkiss, *ibid.*, **48**, 2698 (1926); (e) Ing and Wright, *Proc. Roy. Soc.* (London), **109B**, 337 (1932); **114**, 48 (1933).

(5) Welch and Roepke, *J. Pharmacol.*, **56**, 319 (1936).

(6) Roepke, *ibid.*, **59**, 264 (1937).

(7) (a) Kahane and Lévy, *Compt. rend.*, **202**, 781 (1936); (b) Hall and Ettinger, *J. Pharmacol.*, **59**, 29 (1937); (c) Broun and Scheiner, *Bull. soc. chim. biol.*, **17**, 1647 (1937).

Experimental Part

The rates of hydrolysis of acetylcholine bromide have been measured under different conditions using the experimental method described by Renshaw and Bacon[4b] for low, constant alkalinity. This method was used later by Willstätter and coworkers[8] in the study of hydrolysis in the presence of esterase.

The titrations were carried on at a constant *p*H of 7.4 in a bath maintained at 37°. The *p*H was controlled by a cresol red buffer mixture made up according to Clark[9] and barium hydroxide solution, restandardized at frequent intervals, was used for the titrations. The choline derivatives were prepared in this Laboratory and their purity was demonstrated. The source of the cholinesterase was normal horse serum (Lederle) which was preserved with 0.4% chloroform. It was found experimentally that it was unnecessary to use a purified cholinesterase preparation and, therefore, the serum was used directly. In these experiments, a 50-cc. volume of distilled water containing a measured amount of horse serum, usually 0.5 cc., was added to a weighed amount of substrate.

Acetylcholine bromide was used in these experiments, since it has been the experience of the authors that the bromide salt can be prepared in the most satisfactory state of purity. It was found that the bromide and iodide ions, in the concentrations used in these experiments, have no measurably different effect on the activity of the esterase from that of the chloride ion.

In Table I, data are presented for the hydrolysis of acetylcholine bromide in a run in which the substrate concentration decreased from an initial concentration of 0.01 M (approx.) to a concentration of 0.0025 M. The velocity constant was calculated by substitution of cc. of barium hydroxide for the concentration terms in the unimolecular formula; t was expressed in minutes.

$$K = \frac{2.3}{t} \log \frac{a}{a - x}$$

The velocity constants in Table I have been

(8) Willstätter, Kuhn, Lind and Memmen, *Z. physiol. Chem.*, **167**, 303 (1927).

(9) Clark, "Determination of Hydrogen Ions," Williams and Wilkins Co., Baltimore, Maryland, 1925.

corrected for simultaneous alkaline hydrolysis and for dilution of the esterase concentration by alkali solution added in the course of titration.[10]

TABLE I

SERUM HYDROLYSIS OF ACETYLCHOLINE BROMIDE

Run 43: 0.1046 g. acetylcholine bromide: 0.5 cc. serum: $Ba(OH)_2$, 0.0267 N

Min.	Molar concn. $\times 10^2$	Cc. $Ba(OH)_2$ equiv.	$K \times 10^2$ min.$^{-1}$
0	0.94	17.63	..
20	.87	16.20	0.35
40	.79	14.81	.38
60	.73	13.54	.38
80	.66	12.38	.41
100	.60	11.22	.45
120	.55	10.20	.45
140	.50	9.31	.42
160	.45	8.48	.46
180	.41	7.68	.49
200	.37	6.88	.55
220	.33	6.20	.54
240	.30	5.59	.52
260	.26	5.01	.56
280	.25	4.51	.52

In this run, in which the substrate was reduced to one-fourth of its initial concentration, the unimolecular constant showed a slow rise, increasing 50% above its initial value. An interpretation of this trend will be discussed on the basis of data to be presented below.

The rate of hydrolysis of acetylcholine at various initial concentrations was measured. The serum concentration in these experiments was the same as in the previous one (0.5 cc. in 50 cc. of water). The data so obtained are presented in Table II. Column 1 gives the initial molar concentration of acetylcholine, column 2 the velocity of hydrolysis in terms of the number of cc. of 0.0267 N barium hydroxide solution required for continuous titration at constant pH for twenty minutes, and column 3 gives the value of the unimolecular velocity constant for the twenty-minute interval.

TABLE II

HYDROLYSIS AT VARYING INITIAL CONCENTRATION

1 Mol. concn. acetylcholine (C_a)	2 Cc. of $Ba(OH)_2$, corr. for alk. hydrolysis	3 $K \times 10^2$ min.$^{-1}$
0.0176	1.26	0.19
.0088	1.20	.37
.0044	1.25	.84
.0022	1.22	1.70

(10) The rate of alkaline hydrolysis was determined previously, and the velocity constant was found to be 0.7 $\times 10^{-3}$ min.$^{-1}$. The correction for dilution was made on the assumption that the velocity of reaction was proportional to the concentration of the esterase.

A comparison of the values of K in Tables I and II indicates that, within a given run, K rises slowly as the substrate concentration falls, but when the initial acetylcholine concentration is decreased in the same concentration range, the hydrolytic rate remains constant, independent of the decrease in substrate concentration, and K rises sharply.

It was thought that the formation of choline by the hydrolysis of acetylcholine might account for the fact that the velocity fell during runs (and at such a rate as to cause a relatively slow rise in the value of K), whereas, the initial velocity remained unchanged when the initial concentration of acetylcholine was decreased. Choline bromide, when added to the reaction mixture, did, in fact, decrease the velocity of hydrolysis. It was thus possible to postulate a mechanism for the hydrolysis involving inhibition of the esterase by choline. This mechanism describes quantitatively the dependance of the velocity constant on both the initial substrate concentration and the concentration of the choline produced in the hydrolysis. Roepke, whose experiments undoubtedly antedated ours, has also observed the inhibitory action of choline.

Concentrations of choline bromide relative to the acetylcholine concentration, as shown in Table III, were added to solutions of 0.0044 M acetylcholine bromide (50 mg. in 50 cc.), containing serum in 1% concentration. The unimolecular constant K was determined for the hydrolysis of acetylcholine during twenty-minute intervals, and the values of $1/K$, given in Table III, were plotted against C_b, the initial choline concentration in each run. The result is given in Curve I, Fig. 1.

TABLE III

INHIBITION BY CHOLINE BROMIDE

Rel. mol. concn. choline bromide	0.5	1.0	2.0	2.5	3.0
$K \times 10^2$ min.$^{-1}$	.55	0.46	0.29	0.26	0.23
$1/K \times 10^{-3}$	.18	.22	.34	.38	.44

Figure 1 indidates that $1/K$ is a linear function of the choline bromide concentration. On extrapolation to zero choline concentration, the line passes through 0.12×10^3. This extrapolated value of $1/K$ is that for acetylcholine hydrolyzing at 0.004 M concentration without added choline. The experimental value reported for K at this concentration in Table II is 0.84×10^{-2}, and thus $1/K$ is experimentally 0.12×10^3.

In several experiments, the velocity constant was found to be proportional to the serum concentration in the concentrations used. This proportionality between serum concentration and rate of hydrolysis has been demonstrated by several workers under different conditions.[7a,11]

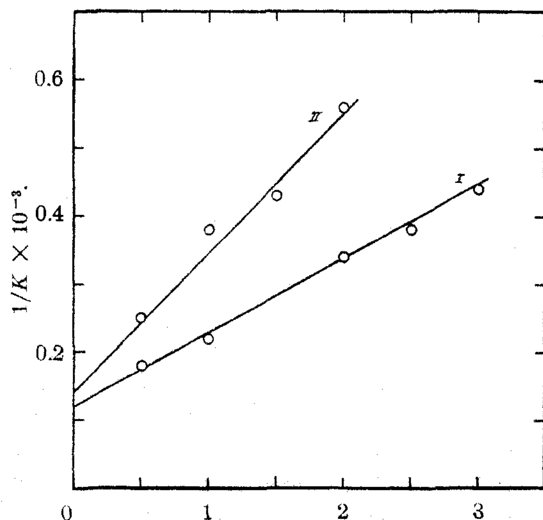

Fig. 1.—I, inhibition of hydrolysis of acetylcholine by choline; II, inhibition of hydrolysis of acetylcholine by ethoxycholine.

Discussion

The linear relationship (Curve I, Fig. 1) between $1/K$ and the choline bromide concentration at constant initial concentration of acetylcholine is expressible by the formula

$$K = 1/(K_bC_b + C)$$

where C is a constant, and C_b represents the concentration of choline bromide.

This equation is identical in form with one which can be derived by considering simultaneous adsorption of acetylcholine and choline ions on the surface of the esterase. Setting $C_{ads.}$ equal to the concentration of acetylcholine adsorbed on catalytic surfaces, one may write an expression for the rate of hydrolysis of acetylcholine

$$-dC_a/dt = K_1C_{ads.} \quad (1)$$

$C_{ads.}$ may be expressed as a fraction of the serum concentration

$$C_{ads.} = \sigma_aK_s[\text{Serum}] \quad (2)$$

where σ_a is the fraction of the available catalytic surface which is covered by acetylcholine ions, and K_s is a constant relating the catalytically active surface and the serum concentration.

(11) Matthes, *J. Physiol.*, **70**, 338 (1930).

Expressing σ_a for two components strongly adsorbed,[12] and combining (1) and (2) we obtain

$$-dC_a/dt = \frac{K_1K_sK_a\,[\text{Serum}]C_a}{K_aC_a + K_bC_b} \quad (3)$$

where C_a and C_b are the concentrations of acetylcholine and choline in solution, respectively. K_a and K_b, from their derivation, are the affinity constants of acetylcholine and choline for the esterase, respectively.

On combining constants, the unimolecular velocity constant K may, therefore, be expressed by

$$K = K'[\text{Serum}]/(K_aC_a + K_bC_b) \quad (4)$$

At constant serum and acetylcholine concentrations, this equation expresses the relationship between K and C_b obtained from Fig. 1, Curve I. The relationship between the velocity constant K and the variables which have been studied in the foregoing is also given by equation (4). Comparison indicates that this expression, derived for the velocity constant on the assumption of simultaneous adsorption of onium ions, is in agreement with the experimental data obtained.

Since, as will be shown, $K_a > K_b$, the slow rise in the unimolecular constant which was observed in Table I may be explained in terms of equation (4). The inverse proportionality between K and the initial acetylcholine concentration is required by equation (4), since C_b at the start of the runs is zero. Finally the proportionality between K and the serum concentrations, keeping other factors constant, is in agreement with the expression for K.

Measurement of Affinity

Inverting equation (4) at constant serum concentration, we obtain

$$1/K = (K_aC_a/K'') + (K_bC_b/K'') \quad (5)$$

This is a linear equation expressing the relationship between $1/K$ and C_b at constant acetylcholine concentration, the plot of which is shown in Curve I, Fig. 1. The slope of this line is given by K_b/K'' and the intercept on the $1/K$ axis by K_aC_a/K''. Therefore

$$K_b/K_a = C_a(\text{slope})/\text{intercept} \quad (6)$$

Equation (6) allows evaluation of K_b/K_a, the relative affinities of choline and acetylcholine for the esterase, in terms of the slope, intercept, and acetylcholine concentration used. It is thus possible to measure the relative affinities of choline

(12) Taylor, "Treatise on Physical Chemistry," Vol. II, D. Van Nostrand Co., New York, N. Y., 1931, p. 1077.

and acetylcholine for the esterase. This method also was extended to measure the affinities of other choline derivatives relative to acetylcholine.

Experimental.—The inhibition of the hydrolysis of acetylcholine by the ethyl ether of choline (ethoxycholine bromide), and the butyl ether of formocholine (butoxyformocholine bromide), was studied by the procedure previously outlined for choline bromide. The data obtained for ethoxycholine are presented in Table IV and those for butoxyformocholine in Table V. Since K is not affected appreciably by the production of choline during a run, the effect of the relatively small amount of the latter compound produced during twenty-minute hydrolysis may be disregarded.

TABLE IV

HYDROLYSIS OF ACETYLCHOLINE IN THE PRESENCE OF ETHOXYCHOLINE

Rel. mol. concn. of ethoxycholine bromide	0.5	1.0	1.5	2.0
$K \times 10^2$ min.$^{-1}$	.40	0.26	0.23	0.18
$1/K \times 10^{-2}$	.25	.38	.43	.56

TABLE V

HYDROLYSIS OF ACETYLCHOLINE IN THE PRESENCE OF BUTOXYFORMOCHOLINE

Rel. mol. concn. of butoxyformocholine bromide	0.0625	0.125	0.25	0.5
$K \times 10^2$ min.$^{-1}$	.51	.27	.18	.118
$1/K \times 10^{-2}$	.20	.37	.56	.85

The values of $1/K$ for acetylcholine, in the presence of varying concentrations of ethoxycholine, have been plotted in Fig. 1, Curve II. In Fig. 2, $1/K$ has been plotted against the concentration of butoxyformocholine. Table VI presents the values of K_b/K_a and the affinities of these compounds and of choline relative to that of acetylcholine as calculated by slope and intercept measurements of the plots in Figs. 1 and 2.

TABLE VI

RELATIVE AFFINITIES TO ACETYLCHOLINE

	K_b/K_a	Relative affinity
Acetylcholine bromide	...	100
Choline bromide	0.87	87
Ethoxycholine bromide	1.48	148
Butoxyformocholine bromide	11.1	1110

Discussion

The applicability of the Langmuir adsorption equilibrium to the study of enzyme systems was first pointed out by Langmuir.[13] Hitchcock[14] has proved the formal identity between the formulation of the Langmuir adsorption equilibrium and the law of mass action. This has also been referred to by Lineweaver and Burk.[15] There seems, however, to have been little direct study of enzyme systems from this point of view. In the present study, the Langmuir adsorption equilibrium has been employed to describe the kinetics of the hydrolysis of acetylcholine, and the effects of various inhibitors. Although it is not suggested that this is the only possible treatment of this system, it allows, however, measurement of affinities in higher substrate concentrations than is possible using the Michaelis equation.[6]

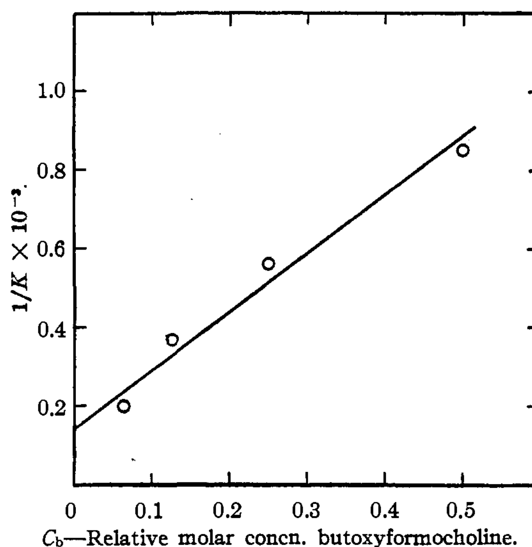

Fig. 2.—Inhibition of hydrolysis of acetylcholine by butoxyformocholine.

Roepke[6] has shown, by application of the Michaelis–Menten theory, that choline and several of its derivatives exhibit competitive inhibition of acetylcholine hydrolysis. The derivation of the expression presented above for the unimolecular velocity constant requires this type of inhibition and in the calculation of the affinities of ethoxycholine and butoxyformocholine, it has been assumed, on the basis of Roepke's results with other derivatives, that these compounds exhibit competitive inhibition.

As has been pointed out by Roepke[6] and others, cholinesterase is a very specific agent, exhibiting a highly selective action on esters of choline. Since it has been suggested by Clark,[16] Renshaw,[3] and others that adsorption is involved in the mechanism whereby onium derivatives exert their

(13) Langmuir, THIS JOURNAL, **38**, 222 (1916).

(14) Hitchcock, *ibid.*, **48**, 2870 (1926).

(15) Lineweaver and Burk, *ibid.*, **56**, 658 (1934).

(16) Clark, *J. Physiol.*, **61**, 530 (1926); **64**, 123 (1927).

physiological activity, it might be assumed that the affinities of choline derivatives for the specific surface of cholinesterase would be related to the intensity of the physiological activity which they exhibit. This would involve the assumption that the physiological receptors, upon which these compounds exert their activity, are similar in structure to the esterase. The results do not, however, justify this conclusion, and, in fact, there is ample physiological evidence that this is not true.

As Roepke has found for other derivatives, there is no relationship between the relative affinities for the esterase and the physiological activity of the compounds studied. It was found that butoxyformocholine has eleven times the affinity of acetylcholine, yet it is relatively inactive. Choline possesses 87% of the affinity of acetylcholine, but it is in the order of 1/10,000 as active in lowering the blood pressure.[17]

Although competitive adsorption on the surface of cholinesterase does not possess any physiological significance in itself, it does furnish a striking example of ionic exchange on a protein surface by derivatives here considered. Kahane and Lévy[18] and Broun and Scheiner[19] have found that acetylcholine is fixed to the non-ultrafilterable constituents of serum in an inactive form, which is resistant to the hydrolytic action of cholinesterase. It is suggested, on the basis of the present work, that the mechanism of this fixation is an ionic adsorption. Benzak[20] and Chang and Gaddum[21] believe that acetylcholine is stored in the animal body in the form of an "inactive complex." There is reason to believe that this is an adsorption complex.

In a paper to be published shortly, evidence is presented showing that infusion of ethoxycholine into laboratory animals liberates into the blood stream a substance with the properties of acetylcholine. Assuming that acetylcholine is stored naturally in an adsorption complex, the mechanism of this release might well be a simple exchange adsorption on some highly specialized surface analogous to the competitive adsorption on the surface of the esterase.

The authors wish to express their appreciation for the advice and criticism of Mr. Irving Welinsky of this department.

Summary

1. An equation was arrived at experimentally for the velocity of acetylcholine hydrolysis by cholinesterase in the region of maximum velocity. This equation was also derived by assuming simultaneous adsorption of acetylcholine and choline on the surface of the esterase.

2. A method was presented for measuring the relative affinities of choline derivatives for the surface of the esterase. The relative affinities of acetylcholine, choline, ethoxycholine, and butoxyformocholine were measured.

3. As has been found by Roepke, it was shown that the relative affinities for cholinesterase bear no relationship to the physiological activities which these compounds exhibit. The significance of the competitive adsorption on the surface of the esterase and its significance in the liberation of acetylcholine by more stable derivatives are discussed.

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(17) Preliminary experiments indicate that the triethyl analog of ethoxycholine, $(Et_3NCH_2CH_2OEt)^+$, has an affinity three times that of acetylcholine, while ethoxycholine itself, $(Me_3NCH_2CH_2OEt)^+$, has an affinity only 1.6 times that of acetylcholine. On the other hand, the triethyl analog shows none of the typical stimulating action of acetylcholine, while ethoxycholine is a marked parasympathetic stimulator.

(18) Kahane and Lévy, *Compt. rend.*, **202**, 781 (1936).

(19) Broun and Scheiner, *Bull. soc. chim. biol.*, **201**, 1046 (1935).

(20) Benzak, *J. Physiol.*, **82**, 189 (1934).

(21) Chang and Gaddum, *ibid.*, **79**, 255 (1933).