ACYLATION REACTIONS MEDIATED BY PURIFIED ACETYLCHOLINE ESTERASE II*

by

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The probability that acetylcholine esterase plays a role in the generation of the action potential[1] lends special interest to the study of the nature of this enzyme and of the reactions which it may mediate. In an earlier communication[2] the ability of the electric tissue esterase of *Electrophorus electricus* to mediate acylations of choline and hydroxylamine was noted. In the present report, factors which govern the rate and extent of these reactions are considered.

The specificity and affinity of purified electric tissues esterase for a wide range of substrates and inhibitors have been studied by NACHMANSOHN *et al.*[3,4] and more recently by AUGUSTINSSON[5,6]. An important function of the enzyme — the hydrolysis of esters as a function of p_H — has not been described previously. The manometric method of esterase assay is conveniently applicable within a narrow range of p_H. Characterization of the p_H function of the enzyme by the potentiometric technique for the determination of the acid reaction product would be feasible but laborious. A colorimetric method[7] for the assay of ester in the presence of excess of products of ester hydrolysis affords a convenient procedure for assay of esterase activity at any desired p_H. The method is applicable equally to measurement of both hydrolysis and synthesis of the ester and with its aid information concerning the p_H function of an esterase is easily obtainable.

METHODS

Acetycholine and propionylcholine were determined according to the procedure previously described[7]. Aliquots of 0.5 or 1.0 ml of the test solution containing 0.3 to 4.0 μM of ester were used for the determinations.

Acethyldroxamic and propionhydroxamic acid were measured in aliquots of 0.5 or 0.1 ml containing 0.3 to 4.0 μM. The samples were brought to p_H 1.0–1.4 with hydrochloric acid and then estimated colorimetrically with 1% ferric chloride essentially as in the method for the determination of acetylcholine[7].

The KLETT photoelectric colorimeter was used with green filter 54.

Enzyme

Acetylcholine esterase of the electric tissue of *Electrophorus electricus* was used. The enzyme was purified according to the method described by ROTHENBERG AND NACHMANSOHN[8]. The enzyme was dissolved in a medium of sodium phosphate 0.05 M, magnesium chloride 0.02 M, and sodium chloride 0.1 M at p_H 7.0 and stored in the cold at 4° C. Stock enzyme solutions were diluted into 2.8% gelatin freshly before use. In the hydrolysis experiments the final dilution of the enzyme solution was in the order of magnitude of one part in ten thousand; in the experiments on acylation a much higher enzyme concentration — an order of magnitude of one part in ten — was used.

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A. HYDROLYSIS OF ACETYLCHOLINE AS A FUNCTION OF p_H

An enzyme concentration assay curve is reproduced in Fig. 1. The hydrolysis-time curves in phosphate solution at p_H 7.4 depart from a straight line to a measurable extent only after about 30% of the substrate at an initial concentration of 4 μM per ml has been split. The plot of the initial reaction velocity against enzyme concentration in the range studied yields a straight line.

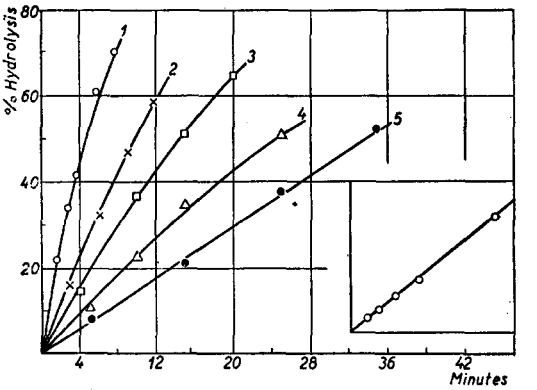

Fig. 1. Acetylcholine hydrolysis as a function of enzyme concentration. Mixtures contain 1.0 M potassium dihydrogen phosphate adjusted with sodium hydroxide to p_H 7.4, gelatin 0.07%, acetylcholine 4 μM/ml. Temperature 23° C. The p_H remained constant within 0.2 p_H units during the course of the hydrolysis. The non-enzymatic hydrolysis in these conditions was barely detectable. Curves 1–5 show findings with enzyme dilutions 1:4000, 1:8000, 1:12000, 1:20000 and 1:30000 respectively. In the inset, relative enzyme concentration is plotted on the abscisca and the corresponding relative initial reaction velocity on the ordinate.

TABLE I

ACETYLCHOLINE HYDROLYSIS IN PHOSPHATE SOLUTION AS A FUNCTION OF p_H IN THE ACID RANGE

The solutions contained a constant amount of enzyme, 0.07% gelatin, 0.1 M potassium phosphate, sodium hydroxide in varying amounts and acetylcholine chloride in a concentration of 4 μM/ml. The p_H remained constant during the course of the hydrolysis within 0.2 p_H units. Temperature 21° C. Non-enzymatic hydrolysis proved negligible in the conditions used. Control mixtures to which no acetylcholine was added failed to produce colour when examined with the reagent. The solutions remained clear and removal of the protein present in the reaction mixture was unnecessary.

p_H	Per cent hydrolysis at times (min)			
	10′	20′	30′	40′
7.8	17	32	45	57
7.4	16	31	45	55
6.8	15	28	39	49
6.3	13	25	35	44
5.8	9	17	23	30
5.5	—	13	—	—

Variation of esterase activity accompanied shift of p_H on the acid side of the scale in a range which is still of physiological interest. The course of the reaction in phosphate buffer is illustrated by the experiment recorded in Table I. It is evident that increase of p_H from 5.5 to 7.4 results in a progressive and marked rise of reaction rate in phosphate buffer. Between p_H 7.4 and 7.8 in phosphate and between p_H 7.6 and 9.4 in borate the enzyme-mediated hydrolysis exhibited a constant initial reaction rate. At p_H higher than 9.4 inactivation of enzyme occurred at 21° C., the inactivation was retarded considerably at 17° C. Non-enzymatic hydrolysis of the substrate was found to become relatively appreciable at p_H 9.2 and rose rapidly with further increase of the p_H (Table II). A summary of findings is presented in Fig. 2. The p_H range in which the acetylcholine

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TABLE II

ACETYLCHOLINE HYDROLYSIS IN BORATE SOLUTION AS A FUNCTION OF p_H IN THE ALKALINE RANGE

a) Reaction mixtures contained a constant amount of enzyme, acetylcholine chloride 4 μM/ml., 0.07% gelatin and 2 ml of SÖRENSEN borate buffer in 4 ml of final mixture. Temperature 21° C. p_H remained unchanged within 0.2 p_H units throughout the course of reaction. Non-enzymatic hydrolysis was negligible.

pH	Percentage hydrolysis at times (min)				
	5′	10′	20′	30′	35′
7.6	8	17	30	43	51
7.9	8	18	31	44	49
8.1	9	18	33	—	50

b) As in a) but with borate-potassium chloride-sodium carbonate solutions of ATKINS AND PANTIN[11] as the buffer. Enzyme was added to the reaction mixtures as the last component. By use of a high enzyme concentration and a rather low temperature for the incubation the relative role of the non-enzymatic hydrolysis could be kept to a minimum. The same device served also to prevent undue interference at highly alkaline p_H by progressive inactivation of the enzyme. The temperature was 17° C. p_H remained unchanged within 0.2 p_H units throughout the observed course of the reaction.

pH	Percentage hydrolysis at times (min)						
	3′	4′	6′	8′	9′	10′	12′
Total hydrolysis							
8.5	17	—	30	—	42	—	53
9.4	15	—	28	—	41	—	50
10.0	—	21	31	38	—	45	—
10.4	—	22	31	39	—	44	—
Non-enzymatic hydrolysis							
8.5	—	2	—	0	—	—	0
9.4	—	0	—	1	—	—	2
10.0	—	5	—	9	—	—	13
10.4	—	6	—	11	—	—	16
Enzymatic hydrolysis							
8.5	15	—	30	—	42	—	53
9.4	15	—	28	—	40	—	48
10.0	—	16	24	29	—	34	—
10.4	—	16	22	28	—	31	—

hydrolysis was essentially independent of p_H is relatively wide. The p_H function of the acetylcholine esterase from electric tissue differs in this respect from some other esterases which have been studied by GLICK[9].

The effect of addition of choline and acetate on acetylcholine hydrolysis has been studied in detail by AUGUSTINSSON[10]. It seemed of interest to ascertain whether p_H influences the role played by the hydrolysis products. In an experiment reported in Table III the effect of choline chloride (12.5 μM/ml) on the hydrolysis of acetylcholine (4 μM/ml) at three selected p_H values is shown. Choline proved to be about equally inhibiting at p_H 7.7 and 6.8; the choline was only about one-half as active an inhibitor at p_H 5.9. Acetate even in high concentrations (0.1 M) failed to inhibit acetylcholine hydrolysis by electric tissue esterase in phosphate solution either at p_H 5.5 or 7.7. Since

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moderation of the action of esterase inhibitors by way of regulation of p_H might be a matter of some practical as well as theoretical interest, further study of the p_H-dependence of esterase-inhibitor interactions appears desirable.

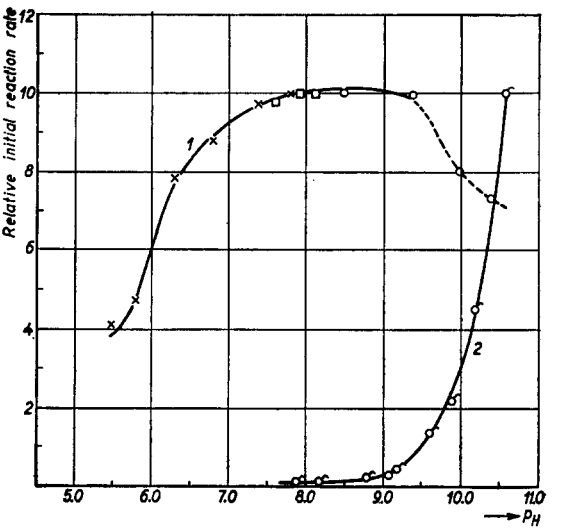

Fig. 2. Acetylcholine hydrolysis as a function of p_H. Curve 1: Hydrolysis of acetylcholine in the presence of enzyme. Relative initial reaction rates corrected for enzymatic hydrolysis are plotted on the ordinate. The curve is a composite of data given in Tables I and II. Values for p_H 7.8 in phosphate, and p_H 8.1 and 8.5 in borate are taken equal to 10. Curve 2: Hydrolysis of acetylcholine in absence of enzyme. Acetylcholine concentration 4 μM/ml. p_H was regulated with borate buffer. Initial reaction rates are plotted on the ordinate. The value for p_H 10.6 is taken equal to 10. The temperature was 21° C.

TABLE III

INFLUENCE OF CHOLINE ON ACETYLCHOLINE HYDROLYSIS AT DIFFERENT p_H VALUES

Reaction mixtures contained a constant amount of enzyme, acetylcholine chloride 4 μM/ml, choline chloride (or sodium chloride) 12.5 μM/ml, potassium phosphate 0.1 M, sodium chloride 0.05 M, magnesium chloride 0.02 M, gelatin 0.07% and different amounts of sodium hydroxide. Temperature 37° C.

p_H	Choline	Percent hydrolysis at times (min)						
		10′	20′	30′	40′	50′	60′	70′
7.7	—	20	40	58	71	—	—	—
7.7	+	—	15	22	32	39	—	—
6.8	—	20	38	53	65	—	—	—
6.8	+	—	15	21	—	39	—	—
5.9	—	—	—	22	—	38	—	49
5.9	+	—	—	16	—	26	—	38

B. SYNTHESIS OF ACETYL- AND PROPIONYLCHOLINE BY THE ACTION OF PURIFIED ACETYLCHOLINE ESTERASE

The equilibrium constant of esterification reactions favours strongly the reaction direction of hydrolysis[12]. Earlier investigators[13] observed that the pharmacological activity of choline is enhanced by incubation with acetate in the presence of crude tissue preparations of esterase. Demonstration of this synthesis and measurement of the equilibrium was greatly facilitated in the present work by the availability of the hydroxylamine method which could be applied to the determination of the ester in the presence of a large excess of the products of the hydrolysis.

Figs 3 and 4 analyse the effect of p_H on the equilibrium position of the hydrolysis of acetylcholine and propionylcholine respectively by the purified esterase. The approach to equilibrium at three selected p_H was realized in each case from both reaction directions.

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It is apparent that acid shift of p_H within the range studied displaces the equilibrium in the direction of synthesis. In the experiments of Figs 3 and 4 the speed of the approach to the equilibrium was found to be dependent upon the esterase concentration. To insure a close approach to the equilibrium in a conveniently short time, a much greater enzyme concentration than is conveniently used in a hydrolysis assay was taken.

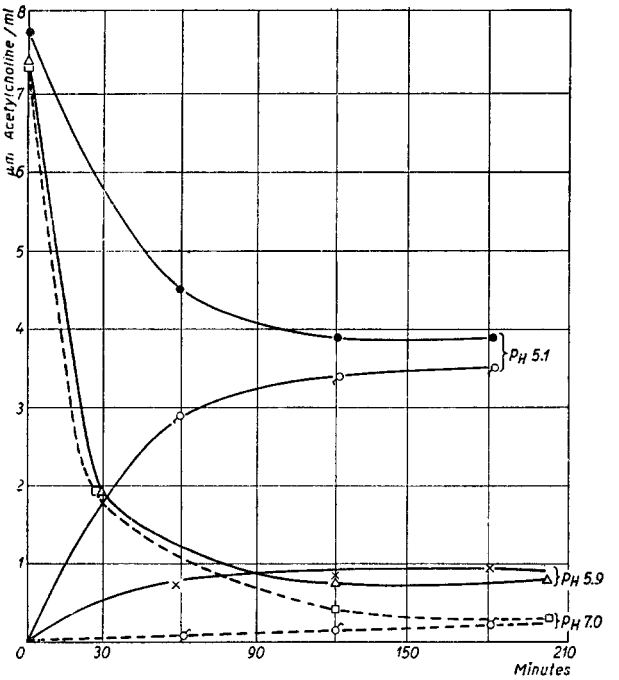

Fig. 3. Synthesis of acetylcholine as a function of p_H. Solutions were made with 1.15 g each of choline chloride and sodium acetate trihydrate at p_H 5.1 in a total volume of 6.0 ml, and with 1.21 g each of these substrates in the same total volume at p_H 5.9 and 7.0. p_H was set with hydrochloric acid and measured with a glass electrode in samples diluted for the purpose with three volumes of water. In one control mixture at each p_H, 8 μM of acetylcholine per ml was added at the outset. Enzyme was added in an amount per ml sufficient to effect hydrolysis of 2 g of acetylcholine chloride per hr in optimum conditions. Temperature 23° C. Ester was determined on aliquots of 0.5 ml. A standard curve was constructed with known acetylcholine amounts in the same medium. Care is taken in the ester determination to bring the p_H of the sample to 1.0–1.2 at the step prior to ferric chloride addition in order to avoid interfering colour by reaction between fatty acid and ferric chloride. In several cases, water was added to a reaction mixture in which the synthesis had come to a rest. A rapid shift of the equilibrium in the reaction direction of hydrolysis could then be observed. In the absence of either acetate, choline, or esterase, no ester formation was observed.

p_H	Concentration at equilibrium (molarity)					K	ΔF
	water (a)	choline (b)	acetic acid plus acetate (c)	acetic acid (d)	acetyl-choline (e)	$= \frac{(a \cdot e)}{(b \cdot d)}$	$= -4.58\,T \log \frac{55.5}{K}$
5.1	41	1.35	1.4	0.45	$3.7 \cdot 10^{-3}$	0.25	−3160
5.9	39	1.45	1.5	0.1	$1.0 \cdot 10^{-3}$	0.27	−3140

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The effect of p_H on the equilibrium might be interpreted as follows. On general grounds, it seems reasonable to suppose that the immediate product of ester hydrolysis is the undissociated acid molecule rather than its ion:

$$RCOOR' + H_2O \rightleftarrows RCOOH + R'OH \quad (1)$$

$$RCOOH \rightleftarrows RCOO^- + H^+ \quad (2)$$

where RCOOR' represents the ester and RCOOH and R'OH the acid and alcohol products of hydrolysis. Equilibrium in the synthesis will then be defined by the relationship:

$$K = \frac{[H_2O]\,[RCOOR']}{[R'OH]\,[RCOOH]}$$

where K is the NERNST equilibrium constant calculated from concentrations in molarity. As p_H is decreased, the concentration of the undissociated acid rises and an accompanying

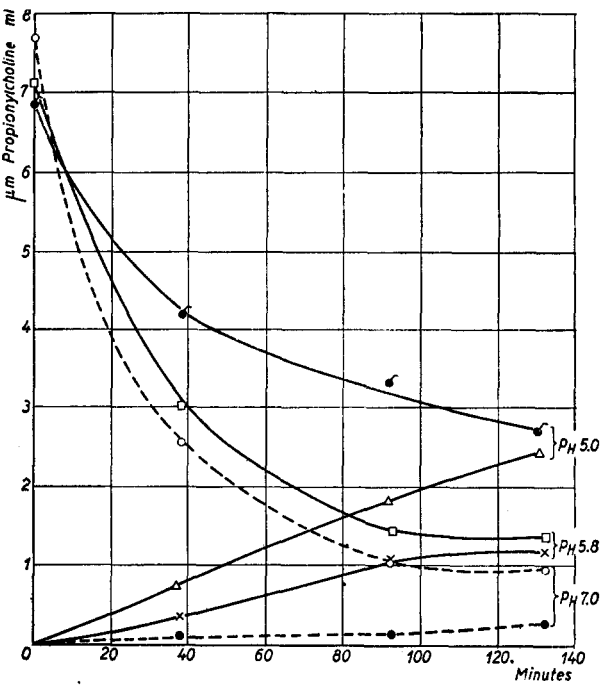

Fig. 4. Synthesis of propionylcholine as a function of p_H. Solutions were made with 1.21 g of choline chloride and 0.85 g of sodium propionate at p_H 7 and 5.8 in a total volume of 6.0 ml, and with 1.01 g of choline chloride and 0.71 g of sodium propionate at p_H 5.0 in the same total volume. Temperature 18° C. Procedure otherwise as described under Fig. 3.

p_H	Concentration at equilibrium (molarity)					K	ΔF
	water (a)	choline (b)	propionic acid plus propionate (c)	propionic acid (d)	propionyl-choline (e)	$= \frac{(a \cdot e)}{(b \cdot d)}$	$= -4.58\,T \log \frac{55.5}{K}$
5.0	41	1.2	1.25	0.52	$2.6 \cdot 10^{-3}$	0.17	−3350
5.8	39	1.45	1.5	0.15	$1.26 \cdot 10^{-3}$	0.23	−3170

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increase of ester concentration at equilibrium may be expected. The values found for the K of the choline esterifications approximated 0.2 within the limits of the experimental error*. The reasonably good constancy of the values for K despite the large variation of the absolute concentration of ester at equilibrium in the investigated p_H range supports the suggestion that undissociated acid rather than the anion enters into the equilibrium of the esterification.

A value for the ΔF of choline ester hydrolysis may be calculated from K with the aid of the relationship

$$-\Delta F = RT \ln \frac{55.5}{K}$$

whose derivation has been discussed recently by MEYERHOF AND GREEN[14]. $-\Delta F$ calculated in this manner was found to approximate 3200 cals. Although molarities rather than activities are used above to calculate K, it is believed likely that error from this cause in the value for ΔF does not exceed 10%**. It is noteworthy that the value for ΔF of hydrolysis of two choline esters is of an order similar to the observed in the case of several anionic esters[14].

The amount of the acetylcholine at equilibrium is minute in comparison to the concentration of the other participants of the system. However, it seems desirable in view of the great biological potency of acetylcholine to consider the possibility that esterase functions as an agent of acetylcholine synthesis *in vivo*, supplementing in this respect the role of choline acetylase. It has been demonstrated that acetylcholine esterase in the nerve axon is localized in the neuronal surface membranes[15]. The concentration of esterase substrates and the p_H prevailing in the membrane are unknown, but there is reason to believe that H^+ and choline$^+$ may be significantly higher at the membrane interface than in the surrounding milieu[16]. Specific binding of ester and sudden variation in p_H at the membrane with resulting shift of equilibrium are conceivable. For a local choline concentration of 0.01 M and a similar concentration of undissociated acetic acid, the value 0.2 for K leads to an equilibrium acetylcholine concentration of 0.06 micrograms per ml. An ester concentration of this order would be sufficient to produce major biological effects.

C. FORMATION OF HYDROXAMIC ACIDS

The ability of proteolytic enzymes to catalyse ester hydrolyses has been demonstrated by NEURATH and his coworkers[17]. The ability of O-acyl hydrolases-lipase[18] and esterase[2] to form hydroxamic acids by the condensation of fatty acid with hydroxylamine is an interesting counterpart to this situation in which a group of hydrolases catalyses both O- and N-acylation.

The effect of reactant concentrations on the rate of the formation of hydroxamic acid in the presence of the electric tissue esterase is shown by experiments summarized in Fig. 5. Within a wide range of reactant concentration the relation between reaction rate and reactant concentration remains almost linear. Reactant concentrations up to 0.75 M or higher failed to saturate the enzyme. Its affinity for acetate, propionate, and

* Inaccuracy in the measurement of p_H would exert a relatively large effect on the value of K. The computation of K for p_H above 6 suffers from an additional inaccuracy because the concentration of ester approached the limit of the ester determination as the p_H increased above 6.

** I am much indebted to Professor O. MEYERHOF for the discussion of this question.

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hydroxylamine may be concluded, therefore, to be of a much lower order than the affinity of the enzyme for acetylcholine. This conclusion has been further supported by the demonstration that neither acetate nor hydroxylamine significantly affect the rate of acetylcholine hydrolysis by the esterase. The substrate concentration-activity relationship observed in hydroxylamine acylation resembles that of neutral ester hydrolysis by the enzyme[3, 5].

The rate of reaction of acetate with hydroxylamine in the presence of esterase is very small as compared to the rate of hydrolysis of acetylcholine by a similar concentration of the enzyme, the relative magnitude of the rates being in the proportion of one to one or two thousand. The rate of hydroxamic acid formation, like the hydrolysis of acetylcholine, varied in a direct manner with the esterase concentration (see Fig. 6).

The specificity of electric tissue esterase in regard to the fatty acids which it can cause to condense with hydroxylamine is rather sharply defined (see Table IV). As in choline ester hydrolysis[3], a maximum is observed with acetic acid. A lower rate is found with propionic acid. The enzyme-catalyzed reaction observed with butyric acid was almost negligible. The findings with formic acid reveal a relatively large spontaneous reaction between formate and

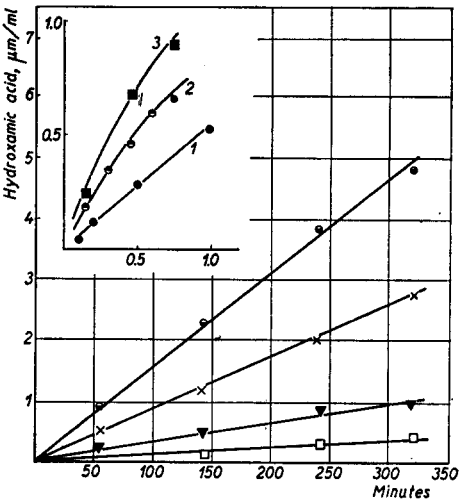

Fig. 5. Formation of hydroxamic acid as a function of reactant concentration. The reaction mixtures are 0.5 M as to sodium acetate and 1.0 M as to sodium chloride. p_H 6.8. 37° C. □, ▼, ×, ◒, — correspond to mixtures with 0.1, 0.2, 0.5, and 1.0 M hydroxylamine respectively. Curves 1 to 3 of the inset are not mutually comparable since they were obtained with different batches of the enzyme. Relative reaction rates are plotted on the ordinates and reactant concentrations in molarity on the abscissae. Curve 1 summarizes the detail of the main part of the figure showing the effect of variation of hydroxylamine concentration. Curves 2 and 3 show the effect of variation of acetate and propionate concentration respectively in the presence of 1.0 M hydroxylamine.

TABLE IV

SUBSTRATE SPECIFICITY OF ELECTRIC TISSUE ESTERASE IN FORMATION OF HYDROXAMIC ACID

The reaction mixtures are 1.0 M as to hydroxylamine and 0.75 M as to the sodium salt of the fatty acid in 0.9 M solution of sodium chloride at p_H 6.2–6.4. Temperature 37° C. Propionhydroxamic, butyrhydroxamic, and acethydroxamic acid yield equivalent amounts of colour per mole with ferric chloride. The amount of the formhydroxamic acid is calculated on the same basis.

Enzyme addition	Fatty acid	Hydroxamic acid, μM/ml, at times in minutes		
		50	100	200
+	formate	1.3	2.4	
—	formate	0.7	1.3	
+	acetate	3.0	6.0	9.9
—	acetate	0.0	0.0	0.1
+	propionate	1.1	2.3	
—	propionate	0.0	0.0	
+	butyrate		0.3	0.6
—	butyrate		0.2	0.5

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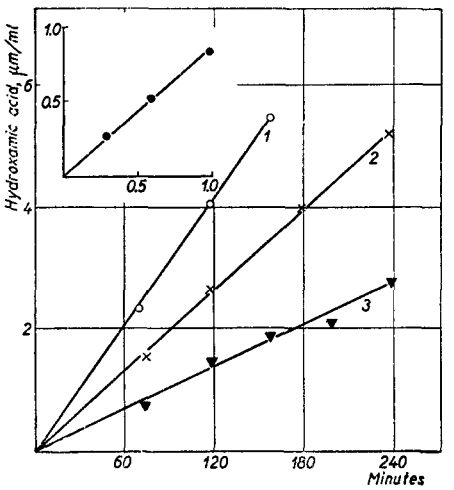

Fig. 6. Formation of hydroxamic acid as a function of esterase concentration. Reaction mixtures were 1.0 M as to hydroxylamine and sodium acetate in 1.0 molar sodium chloride. p_H 6.8. 37° C. Curves 1 to 3 correspond to relative enzyme concentrations 10, 6, and 3. In the inset the relative reaction rate is plotted on the ordinate and the relative enzyme concentration on the abscissa.

hydroxylamine, and some enzymatic catalysis of this reaction. Substitution of an α-amino group into acetate or propionate caused complete loss of their ability to condense with hydroxylamine in the presence of the enzyme.

The ability of the esterase to effect hydrolysis of acethydroxamic acid was examined at a substrate concentration of 3 μM/ml in phosphate buffer at p_H 7.4. Even with a great concentration of enzyme no hydrolysis of acethydroxamic acid was found, although acetylcholine added to the same reaction mixture was hydrolysed rapidly. An acyl transfer reaction between acethydroxamic acid and choline with resultant intermediary formation of hydrolysable acetylcholine could be excluded, since addition of choline to the same reaction mixture failed to evoke a disappearance of acethydroxamic acid.

The p_H dependence of hydroxylamine acylation by electric tissue esterase is illustrated by the experiment given in Table V. The reaction between acetate and hydroxylamine showed a peak in a range near p_H 6.3. The p_H function of hydroxylamine acylation by the esterase is thus very different from the p_H function of acetylcholine hydrolysis by the enzyme. The finding that the p_H dependence of hydroxylamine acylation and choline ester hydrolysis are quite different is consistent with an assumption, discussed later, concerning the mechanism of these two reactions.

TABLE V

FORMATION OF HYDROXAMIC ACID IN PRESENCE AND ABSENCE OF CHOLINE AT DIFFERENT p_H

Reaction mixtures are 0.5 M as to acetate and 1.0 M as to hydroxylamine in 0.9 M solution of sodium chloride at p_H specified with or without addition of 0.5 M choline chloride. In absence of choline addition, an equivalent amount of sodium chloride was added. The p_H was determined in aliquots with a glass electrode after four-fold dilution with water. Temperature 37° C. The formation of hydroxamic acid in absence of enzyme was negligible at p_H 6.3 and 5.3 and none was detected at p_H 7 and higher. The reaction time was 4 hours.

p_H	Choline	Hydroxamic acid, μM/ml
7.9	+	0.2
7.5	—	1.5
7.1	+	0.2
7.1	—	1.6
6.3	+	0.7
6.3	—	2.4
5.3	+	0.4
5.3	—	0.2

In the presence of choline, the rate of the acetylation of hydroxylamine by esterase

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acting at p_H 7.1 was reduced markedly (Fig. 7). The effect of p_H on the choline inhibition is illustrated by the experiment shown in Table V. As in the case of acetylcholine hydrolysis, the lowering of p_H reduced the inhibitory effect of choline. At p_H 5.3, a region in which the enzyme activity was rather low but still measurable, an activating effect by choline on hydroxamic acid formation was observed. The inhibitory effect of choline can be ascribed to its ability to combine with the enzyme at an active site[10]. An explanation of activation by choline may be found in the fact that at acid p_H the concentration of acetylcholine in the system is increased. It has been shown[7] that acetylcholine acetylates hydroxylamine rapidly at alkaline p_H and slowly at acid p_H, the rate being dependent on the concentration of the acetylcholine at constant hydroxylamine concentration. At p_H 7 the concentration of acetylcholine in the acetate-hydroxylamine-choline-system is negligible. The ability of choline to serve as an acetyl carrier at this p_H must therefore become very small.

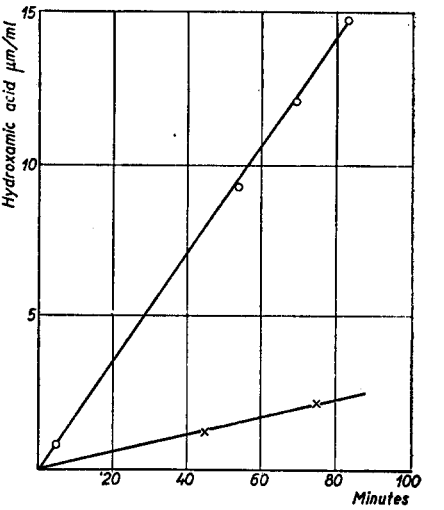

Fig. 7. Effect of choline on formation of hydroxamic acid. The reaction mixtures are 1.0 M as to hydroxylamine and 0.75 M as to sodium acetate in 0.9 M solution of sodium chloride at p_H 7.1. Temperature 37° C. O, mixture without choline; X, mixture with 0.9M choline chloride. The reaction in absence of enzyme is negligible in both cases. The inhibitory effect of choline was unaffected by the choline concentration in the range of 0.1 to 0.9 M.

The inhibitory effect of choline on hydroxylamine acylation and the finding[2] that incubation of the enzyme with specific inhibitors—prostigmine and tetraethylpyrophosphate—abolishes the ability to catalyse hydroxamic acid formation support the view that the same enzyme ,and possibly the same prosthetic group, effects both acetylcholine hydrolysis and hydroxamic acid formation. But the reaction of hydrolysis of acetylcholine is reversible, while that of hydroxylamine acylation appears to be irreversible. Choline shows a fairly marked affinity for the enzyme, whereas hydroxylamine shows little or no affinity. The possibility has therefore to be considered that the role of esterase in hydroxylamine acylation is confined to the activation of the carboxylic acid reactant, and that a terminal reaction between activated carboxylic acid and hydroxylamine is spontaneous and irreversible. In the case of choline acylation it is assumed that the esterase may activate the two reactants.

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SUMMARY

1. Some general properties of ester hydrolysis and synthesis by the purified acetylcholine esterase of the electric tissue of *Electrophorus electricus* have been investigated with the aid of a simple colorimetric technique for the determination of an ester in the presence of its hydrolysis products.

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2. The hydrolysis of acetyl- and propionylcholine by the esterase have been shown to be reversible. The equilibrium of the reaction was found to be characterized by the ratio:

$$\frac{[\text{acetylcholine}]\ [\text{water}]}{[\text{choline}]\ [\text{RCOOH}]} = K$$

where RCOOH represents the undissociated form of the carboxylic acid.

3. The possibility that esterase plays a part in synthesis of acetylcholine at the neuronal membrane surface has been discussed.

4. The condensation of fatty acids with hydroxylamine by the action of the esterase has been investigated in respect to its dependence on reactant concentration, enzyme concentration, carboxylic acid structure, and p_H.

5. Acethydroxamic acid was not hydrolysed by the esterase either in the presence or absence of choline. The reaction of hydroxamic acid formation, unlike ester hydrolysis by the enzyme, thus appear to be irreversible.

6. Condensation of acetate with hydroxylamine in the presence of esterase acting at p_H 6.3 and above was markedly inhibited by choline.

7. A reaction mechanism which could explain some of the differences observed between the catalysis of choline ester hydrolysis and that of hydroxamic acid formation by the same esterase has been discussed.

RÉSUMÉ

1. Quelques propriétés générales de l'hydrolyse et de la synthèse des esters par l'acétylcholine estérase purifié du tissu électrique de *Electrophorus electricus* ont été étudiées à l'aide d'une technique colorimétrique pour la détermination d'un ester en présence de ses produits d'hydrolyse.

2. On a montré que l'hydrolyse de l'acétylcholine et de la propionylcholine par l'estérase est réversible. L'équilibre de la réaction est caractérisé par le quotient:

$$\frac{[\text{acétylcholine}]\ [\text{eau}]}{[\text{choline}]\ [\text{RCOOH}]} = K$$

où K représente la forme non dissociée de l'acide carboxylique.

3. La possibilité que l'estérase joue un rôle dans la synthèse de l'acétylcholine à la surface de la membrane neuronale a été discutée.

4. La condensation des acides gras avec l'hydroxylamine sous l'action de l'estérase a été étudiée en ce qui concerne sa dépendance de la concentration de la substance réagissante et de l'enzyme, de la structure de l'acide carboxylique et du p_H.

5. L'acide acétylhydroxamique n'a pas été hydrolysé par l'estérase ni en présence ni en absence de choline. Ainsi la formation de l'acide hydroxamique, contrairement à l'hydrolyse d'un ester par l'enzyme, semble être irréversible.

6. La condensation d'acétate avec l'hydroxylamine en présence d'estérase à un p_H de 6.3, est considérablement inhibée par la choline.

7. Un mécanisme de réaction a été discuté qui pourrait expliquer certaines différences observées entre l'hydrolyse d'un ester cholinique et la formation d'acide hydroxamique catalysées par la même estérase.

ZUSAMMENFASSUNG

1. Einige allgemeine Eigenschaften der Esterhydrolyse und -synthese durch gereinigte Acetylcholinesterase aus dem elektrischen Gewebe von *Electrophorus electricus* wurden untersucht und zwar mit Hilfe einer einfachen kolorimetrischen Arbeitstechnik zur Bestimmung eines Esters in Gegenwart seiner Hydrolyseprodukte.

2. Es wurde gezeigt dass die Hydrolyse von Acetyl- und Propionylcholin durch die Esterase reversibel ist und dass das Reaktionsgleichgewicht durch den Quotienten

$$\frac{[\text{Acetylcholin}]\ [\text{Wasser}]}{[\text{Cholin}]\ [\text{RCOOH}]} = K$$

charakterisiert ist, wo RCOOH die nicht dissoziierte Form der Carbonsäure darstellt.

3. Die Möglichkeit wurde erörtert, dass Esterase bei der Acetylcholin-Synthese an der Oberfläche der Neuronmembrane eine Rolle spielen könnte.

4. Die Kondensation von Fettsäuren mit Hydroxylamin unter der Einwirkung der Esterase wurde in Bezug auf die Abhängigkeit dieser Reaktion von der Konzentration der reagierenden Substanz und des Enzyms, sowie von der Struktur der Carbonsäure und dem p_H untersucht.

References p. 327.

5. Acetylhydroxamsäure wurde durch die Esterase weder in Gegenwart noch in Abwesenheit von Cholin hydrolysiert. Es scheint also, dass die durch das Enzym katalysierte Hydroxamsäurebildung zum Unterschied von der Esterhydrolyse irreversibel sei.

6. Die Kondensation von Acetat mit Hydroxylamin in Gegenwart von Esterase bei p_H 6.3 wurde durch Cholin stark gehemmt.

7. Ein Reaktionsmechanismus, welcher einige Unterschiede zwischen der katalytischen Cholinesterhydrolyse und der Hydroxamsäurebildung unter Einwirkung derselben Esterase erklären könnte, wurde erörtert.

REFERENCES

1 D. NACHMANSOHN, *Bull. Johns Hopkins Hosp.*, 83 (1948) 463.
2 S. HESTRIN, *J. Biol. Chem.*, in press.
3 D. NACHMANSOHN AND M. A. ROTHENBERG, *J. Biol. Chem.*, 158 (1945) 653.
4 D. NACHMANSOHN AND M. A. ROTHENBERG, E. A. FELD, *J. Biol. Chem.*, 174 (1948) 247.
5 K. AUGUSTINSSON, *Arch. Biochem.*, in press.
6 K. AUGUSTINSSON AND D. NACHMANSOHN, *J. Biol. Chem.*, 179 (1949) 543.
7 S. HESTRIN, *J. Biol. Chem.*, in press.
8 M. A. ROTHENBERG AND D. NACHMANSOHN, *J. Biol. Chem.*, 168 (1947) 223.
9 D. GLICK, *J. Gen. Physiol.*, 21 (1938) 289.
10 K. AUGUSTINSSON, *Acta Physiol. Scand.*, 15 (1948) Suppl. 52.
11 *Methoden der Fermentforschung*, Georg Thieme Verlag, Leipzig, Vol. 1 (1941) 783.
12 R. AMMON, *Handbuch d. Enzymologie*, Akademische Verlagsgesellschaft, Leipzig 1940, p. 350.
13 R. AMMON AND H. KWIATKOWSKI, *Pflügers Arch. ges. Physiol.*, 234 (1934) 269.
14 O. MEYERHOF AND H. GREEN, *J. Biol. Chem.*, 178 (1949) 655.
15 E. J. BOELL AND D. NACHMANSOHN, *Science*, 92 (1940) 513.
16 J. DANIELLI, *Proc. Roy. Soc.*, 122 B (1937) 155.
17 S. KAUFMAN, H. NEURATH, AND G. SCHWERT, *J. Biol. Chem.*, 177 (1949) 793.
18 F. LIPMANN, *Advances in Enzymol.* Vol VI, Interscience, New York 1946, p. 257.

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