3. The inhibition of the growth of the mutant caused by L-isoleucine and L-valine is suppressed in a non-competitive way by the addition of either L-leucylglycine or glycyl-L-leucine.

4. Different explanations for the results obtained are considered, taking into account that the presence in the mutant of the peptidases that hydrolyse the peptides studied is not incompatible with a utilization of the peptides prior to their mere hydrolysis, for example, through transpeptidation reactions.

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Acetylcholine

1. HYDROLYSIS BY HYDROGEN AND HYDROXYL ION

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(*Received* 22 *November* 1951)

This paper presents a study of the acid- and alkali-catalysed hydrolyses of acetylcholine bromide, which may be written

(1) $CH_3COOCH_2CH_2N^+(CH_3)_3 + H_2O + H_3O^+ \rightleftharpoons CH_3COOH + HOCH_2CH_2N^+(CH_3)_3 + H_3O^+$,

(2) $CH_3COOCH_2CH_2N^+(CH_3)_3 + OH^- \rightleftharpoons CH_3COO^- + HOCH_2CH_2N^+(CH_3)_3$.

Our object has been to obtain kinetic data leading to frequency factors and activation energies, for comparison with similar data on the neutral hydrolysis catalysed by true and pseudo cholinesterases. A comparison of the data obtained here with published data on non-ionic esters, such as methyl acetate, has an intrinsic theoretical interest for reaction mechanisms, because of the positive charge on the acetylcholine ion. To date, the study of the non-enzymic hydrolysis of acetylcholine has been limited to obtaining the percentage non-enzymic hydrolysis in 20 min. at pH values near to 7 (Easson & Steadman, 1936). We shall discuss the equilibrium point of the reaction in a later paper, but in both cases it is far enough to the right for us to neglect the back reaction in our kinetic analysis.

EXPERIMENTAL AND RESULTS

The hydrogen-ion catalysis

Method. Commercial acetylcholine bromide was recrystallized from absolute ethanol until it reached a constant melting point of 143°. Conventional methods were used to follow the reaction. Hydrochloric acid catalyst solution (100 ml.) was brought to the thermostat temperature in a stoppered conical flask. Four concentrations of acid were used, 0·1, 0·075, 0·05 and 0·025 N-HCl. At zero time, a weighed amount of acetylcholine bromide was added and the flask shaken, so that the bromide rapidly dissolved. After varying time intervals (decided after preliminary experiments), 5 ml. portions of the reaction mixture were withdrawn and run into a known amount of standardized NaOH solution plus phenolphthalein, in a conical flask at 0°. The NaOH solution was calculated to neutralize the catalyst present. The solution was then rapidly titrated to the end point with further NaOH. If the titre at infinite time is r_∞ and at time t is r_t, the quantity of ester remaining unhydrolysed at time t is equivalent to $r_\infty - r_t$. The difficulties associated with the determination of r_∞ were avoided by the use of Guggenheim's (1926) method. If r_t be the titration value at time t and $r_{t+\tau}$ the titration value at time $t+\tau$, where τ is a suitable standard time interval, chosen to be at least twice the half-life of the reaction, then Guggenheim's method gives the first-order constant k as the slope of the

plot of $2{\cdot}303 \log_{10}(r_t - r_{t+\tau})$ against time t. A specimen plot in Fig. 1 shows the reaction is accurately first order in the ester concentration, as is the case for non-ionic esters. The first-order constants are given in Table 1.

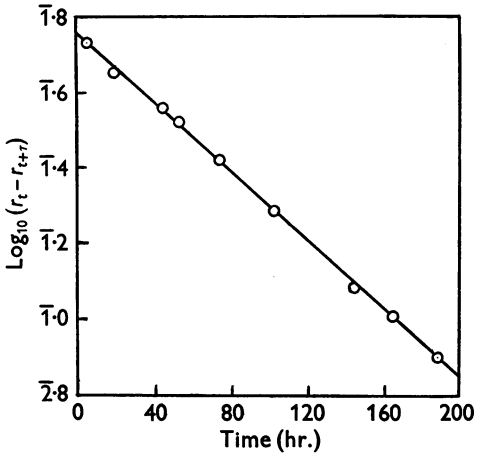


Fig. 1. First-order plot for 2·2718 g. acetylcholine bromide in 100 ml. 0·1 N-HCl at 25°.

Table 1

(*a*) *First-order constants, k min.*$^{-1} \times 10^3$, *for the hydrolysis of acetylcholine*

HCl (N)	*t* (°) 25	37	46·1	60
0·1	0·181	0·603	1·82	3·61
	0·182	0·552	1·70	3·52
0·075	0·142	0·421	1·47	—
	0·134	0·442	1·63	2·65
0·05	0·0833	0·241	0·668	1·68
	0·081	0·258	—	1·41
0·025	0·035	0·108	—	0·783
	0·0391	—	—	—

(*b*) *The catalytic constant* k_{H^+} *l.mole*$^{-1}$ *sec.*$^{-1} \times 10^5$, *for the reaction*

t (°) ...	25	37	46·1	60
k_{H^+} ...	3	9·7	30	51·7

Results. If the reaction is first order in the concentration of hydrochloric acid catalyst, then the catalytic constant k_{H^+}, the second-order constant, is given by $k = k_{H^+}[H_3O^+]$, where $[H_3O^+]$ is the concentration of hydrochloric acid. Fig. 2 shows this to be so, within the experimental accuracy. Values of k_{H^+} are given in the last line of Table 1, their accuracy being about ± 5 %, with the exception of the point at 46·1°, which is less accurate for some reason.

Experiments were carried out at 25, 37, 46·1 and 60°. In Fig. 3, the results are plotted as $\log_{10} k_{H^+}$ against $1/T$. The line has been drawn according to the method of least squares, and the activation energy corresponding to the slope of this line is 16 570 cal./mole, with a probable error of 1522 cal./mole.

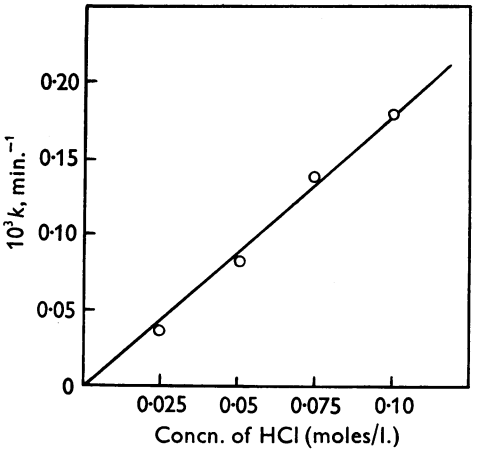


Fig. 2. Evaluation of k_{H^+}, the second-order velocity constant at 25°.

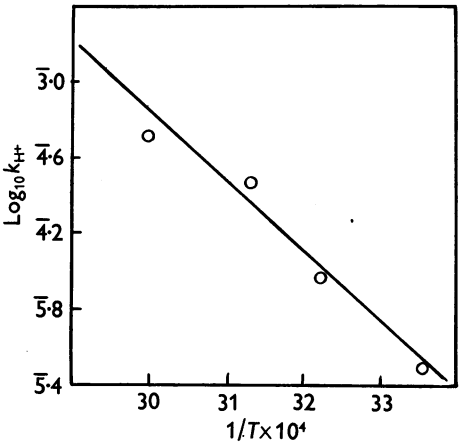


Fig. 3. Arrhenius plot for the hydrogen-ion-catalysed hydrolysis.

The Arrhenius equation is $k_{H^+} = Ae^{-E/RT}$, and from the intercept in Fig. 3, we obtain

$$A = 4{\cdot}7 + 10^7 \text{ l.mole}^{-1}\text{sec.}^{-1},$$

with a probable error of a factor of 10.

The hydroxyl-ion catalysis

Method. Although this reaction was rather rapid, it was found possible to follow it by the normal titration method. For a given experiment, 25 ml. of standardized NaOH solution were brought to the required temperature in a thermostat, together with a flask containing the acetylcholine solution. To start the reaction, 10 ml. of the acetylcholine were pipetted into the NaOH, the stop-watch being

started when half the solution had been transferred. The solution was vigorously shaken for a few seconds and after lapse of the appropriate time intervals, stopped by adding an excess of standardized HCl solution. The amount of alkali used up in the saponification was then determined by titration of the solution against standard NaOH solution, using phenolphthalein, until the first sign of a pink coloration (this, of course, rapidly disappeared as the small excess of alkali induced further saponification). Six experiments were made for each complete run, at a series of time intervals up to 120 sec., for temperatures 0, 10, 12·5 and 15°, and up to 20 sec. for 18 and 20°. The rather rapid technique necessary was secured by the co-operation of two workers. From the titration data we obtain a = initial concentration of NaOH; b = initial concentration of acetylcholine bromide; x = concentration of product at time t.

Results. The data fit the second-order law, examples being given in Fig. 4.

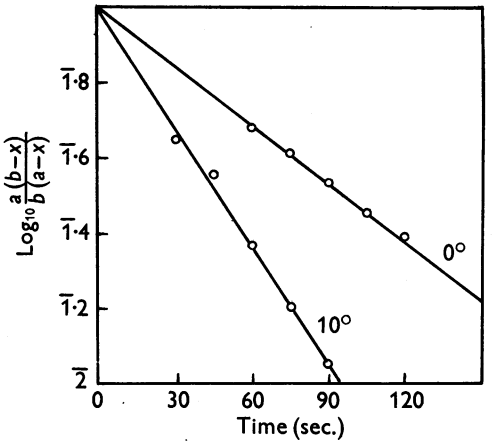


Fig. 4. Saponification of acetylcholine bromide at 0 and 10°.

Two or more runs were done at each temperature, and second-order constants k_{OH^-} calculated as below are given in Table 2.

$$k_{OH^-}=\frac{2\cdot303}{t(b-a)}\log_{10}\frac{a(b-x)}{b(a-x)}.$$

To obtain the activation energy E, we have plotted average values of k_{OH^-} as $\log_{10}k_{OH^-}$ against $1/T°K$ in Fig. 5. The result is $E=12\,180$ cal./mole, with a probable error of 240 cal./mole.

The equation for the absolute rate is obtained as

$$k_{OH^-}=1\cdot0\times10^{9}\,e^{-12\,180/RT}\ \text{l.mole}^{-1}\text{sec.}^{-1}.$$

The probable error on the A factor is a factor of 3·8.

DISCUSSION

In Table 3 we compare our data with the similar data on the hydrolysis of the non-ionic esters. It is apparent that the hydrogen-ion catalysis has a closely similar activation energy for acetylcholine, methyl acetate and ethyl acetate. The acetylcholine hydrolysis goes more slowly, the difference residing apparently in a smaller A factor. To discuss the matter further would require a critical discussion of the errors involved in the determination of E in the investigation concerned. It is interesting that 60 % aqueous acetone behaves very similarly to water as a solvent in this reaction.

Table 2. *Second-order velocity constants,* k_{OH^-}, *l.mole*$^{-1}$*sec.*$^{-1}$, *for the alkaline hydrolysis of acetylcholine*

Temp. (°)	Initial [AChBr] (mole/l.)	Initial [NaOH] (mole/l.)	$k_{OH^-}\times10^{1}$	$k_{OH^-}\times10^{1}$ (average)
0	0·046366	0·10413	2·044	2·018
	0·061913	0·09017	1·992	
10	0·034068	0·089942	4·294	4·573
	0·036967	0·089942	4·262	
	0·03781	0·03781	4·861	
	0·035375	0·036861	4·874	
12·5	0·04882	0·069776	5·493	5·375
	0·052396	0·063798	5·257	
15	0·049613	0·065283	6·860	6·588
	0·038717	0·061502	6·316	
18	0·03624	0·085547	8·406	8·175
	0·044136	0·085547	7·943	
20	0·042776	0·071285	9·491	9·506
	0·046005	0·071285	9·521	

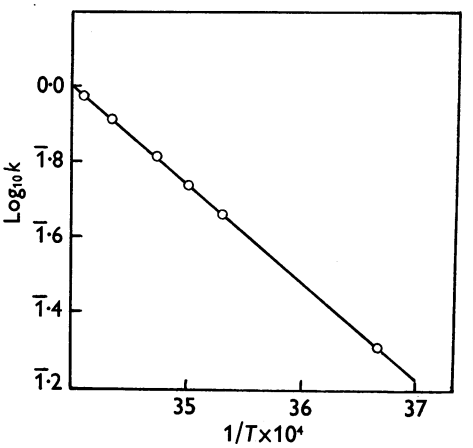


Fig. 5. Arrhenius plot for the hydroxyl-ion hydrolysis.

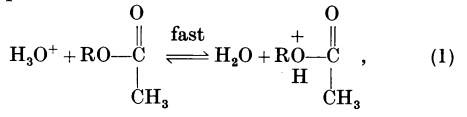
In the case of the hydroxyl-ion reaction, the acetylcholine reaction at 0° has a value of k_{OH^-} about 10 times that for ethyl acetate and methyl acetate, and the results show rather definitely that this is due to a larger A factor.

The hydrolysis of esters is supposed to take place in consecutive steps, the basic ideas being formulated by Lowry (1925, 1927), and recent developments discussed by Watson (1941) and Day & Ingold (1941). In the form set forth by the latter

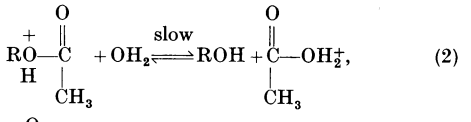
Table 3. *Comparison of kinetic data for ester hydrolysis*

Ester	Catalyst	Solvent	k (l.mole^{-1} sec.$^{-1}$)	Reference
Acetylcholine	H_3O^+	H_2O	$4{\cdot}7 \times 10^7\, e^{-16\,570/RT}$	Present paper
Methyl acetate	H_3O^+	H_2O	$3{\cdot}86 \times 10^8\, e^{-16\,920/RT}$	Lamble & Lewis (1914) Moelwyn-Hughes (1947)
Ethyl acetate	H_3O^+	H_2O	$1{\cdot}66 \times 10^8\, e^{-16\,830/RT}$	Taylor (1915) Moelwyn-Hughes (1947)
Methyl acetate	H_3O^+	60% (v/v) aq. acetone	$4{\cdot}36 \times 10^7\, e^{-16\,250/RT}$	Newling & Hinshelwood (1936)
Acetylcholine	OH^-	H_2O	$1{\cdot}0 \times 10^9\, e^{-12\,180/RT}$	Present paper
Ethyl acetate	OH^-	H_2O	$3{\cdot}79 \times 10^7\, e^{-11\,660/RT}$	Potts & Amis (1949)
Methyl acetate	OH^-	60% aq. acetone	$3{\cdot}7 \times 10^7\, e^{-11\,500/RT}$	Newling & Hinshelwood (1936)

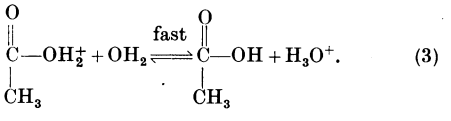
authors, the hydrogen-ion catalysis goes through three steps:



(1)



(2)

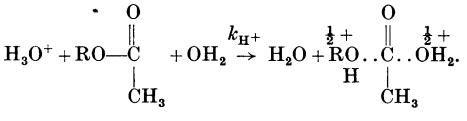


(3)

The observed velocity constant k_{H^+} will then be $K_1 k_2$ where K_1 is the equilibrium constant for reaction (1), that is, k_1/k_{-1} where k_1 and k_{-1} are the rate constants of forward and reverse steps (1), and k_2 is the rate constant of forward step (2), which determines the rate. For consideration of the energy change ΔH^*, and the entropy change ΔS^* in this process (Glasstone, Laidler & Eyring, 1941), as defined by

$$\Delta H^* = E - RT, \quad A = \frac{kT}{h}\, e^{\Delta S^*/R},$$

where E is the experimental activation energy, we may put steps (1) and (2) together and consider the changes in going from the three reactants to the activated complex of reaction (2)



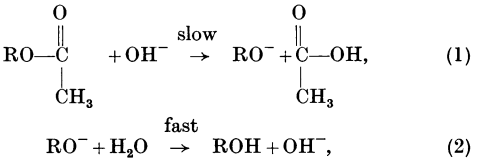
The evidence is that on changing from R as choline with a positive charge to R as methyl or ethyl with zero charge, that the rate of this process k_{H^+} is slightly increased. This is qualitatively what we might expect on the basis of electrostatic charges repelling each other.

Christiansen (1924) inserted a term for the electrostatic energy of two reacting ions into the expression for the activation free energy and Moelwyn-Hughes (1936, 1947) has given formulae for A which show that we shall expect a lowering of A in this case below that for the ion-neutral molecule reaction. Glasstone *et al.* (1941) have given the equivalent formulation of ΔS^* in terms of activated complex theory. The latter formulation is

$$\delta\Delta S^* = -10 z_a z_b \text{ cal. deg.}^{-1} \text{ mole}^{-1}$$

for the difference between the ion-ion and ion-neutral molecule reactions. Since $z_a z_b = 1$, we expect a lowering of the A factor by e^{-5}, i.e. 10^{-2}, but there are reasons for believing this term may be nearer unity when the ionic charges may resonate over several atoms as in the present case.

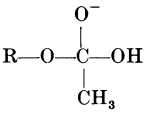
The steps suggested for the hydroxyl-ion catalysis are



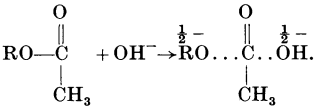
(1)

(2)

where (1) may be split into an initial rapid reversible formation of the species



and its slow decomposition. In any event, the relevant change is from the primary reactants to the activated complex below



Exactly the same argument applies as above, only, since $z_a = 1$ and $z_b = -1$, we shall expect an increase in A factor of about 10^2 as an upper value.

Thus, we conclude that the rather small differences evidenced in the hydrolysis of acetylcholine and a non-ionic ester such as methyl acetate fit in with what we know about the slow steps and activated complexes in these reactions.

SUMMARY

1. The kinetics of hydrolysis of acetylcholine bromide by hydrogen ion and hydroxyl ion have been followed by conventional methods. The bimolecular velocity constants respectively are, in l.mole^{-1}sec.$^{-1}$,

$$k_{H^+} = 4{\cdot}7 \times 10^7 \, e^{-16\,570/RT}$$

and $$k_{OH^-} = 1{\cdot}0 \times 10^9 \, e^{-12\,180/RT}.$$

2. These data are very similar to those for non-ionic esters, such as methyl acetate. The A factor for k_{H^+} is about 5 times smaller than for such esters, while for k_{OH^-} it is about 20 times larger. These differences find an explanation in the ionic charge of the activated complex.

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Acetylcholine

2. THE HEAT OF HYDROLYSIS

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(*Received* 22 *November* 1951)

A recent hypothesis of nerve conduction (Nachmansohn, 1945, 1950) supposes that the depolarization of the nerve membrane is brought about by acetylcholine, and that the polarized state is renewed by the hydrolysis of the acetylcholine by its esterase. The synthesis of acetylcholine is supposed to involve the high-energy phosphate bond in acetyl phosphate, in turn synthesized from creatine phosphate. In support of these views, there is the evidence that in *Electrophorus electricus* the energy release calculated for the observed creatine phosphate hydrolysis approximately equals the energy of electrical discharge. Nachmansohn has, however, pointed to a discrepancy in the energy balance of the postulated recovery process. Meyerhof & Schulz (1935) found $\Delta H_1^0 = -10\,700$ cal./mole for the neutral hydrolysis of creatine phosphate, from which Lipmann (1941) has calculated $\Delta G_1^0 = -10\,000$ cal. Lipmann (1946) subsequently corrected his calculated value for the free energy of hydrolysis of phosphopyruvate by 4700 cal. and since his creatine phosphate figure was based on the phosphopyruvate figure, it is appropriate to apply this correction to creatine phosphate, which now brings its free energy of hydrolysis to $-14\,700$ cal. Within the accuracy of the calculations this may be taken as 15000 cal. If the ΔG_1^0 for acetylcholine synthesis is similar to the values for other esters, it will be close to zero cal. and there will, therefore, be a loss of 15000 cal./mole in the recovery process. As a first step in an investigation of ΔG_1^0, we have measured the heat of alkaline hydrolysis $-\Delta H_2^0$, from which we can calculate the value for hydrolysis by neutral molecules $-\Delta H_1^0$, and thus obtain an estimate of ΔG_1^0. Later we hope to obtain the equilibrium constant K, and thus $\Delta G_1^0 = -RT \ln K$, using hydrogen ion as a catalyst. Since this work was completed, Hestrin (1950) has published a value for K and ΔG_1^0, using the enzyme-catalysed reaction. We shall consider this work in the discussion.

Thermochemistry of the hydrolysis

The hydrolysis of acetylcholine by cholinesterase at physiological pH may be written:

$$CH_3COOCH_2CH_2N^+(CH_3)_3 + 2H_2O \rightarrow CH_3COO^- + H_3O^+ + HOCH_2CH_2N^+(CH_3)_3 \quad (\Delta H_1^0)$$