Hassón for helpful cooperation in the chromatographic experiments, Dr. Seymour Friess for the supply of crystalline acetylcholine and other chemical substances and fruitful discussions, and Prof. Carlos Chagas for his interest and encouragement.

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THE THERMAL INACTIVATION OF ACETYLCHOLINESTERASE

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SUMMARY

Thermal inactivation of acetylcholinesterase (Acetylcholine acetyl-hydrolase, EC. 3.1.1.7) from mammalian erythrocytes is accompanied by a decrease in the Michaelis constant K_s, while the substrate inhibition constant K_{ss} remains unchanged. The time course involves two first-order processes, suggesting (a) the presence of two enzymes, or (b) the formation of a second less active enzyme from the native enzyme both species inactivating independently. The second suggestion would explain the K_s and K_{ss} data, if the difference in the two enzymes resides in the spacing of the anionic and esteratic sites. A non-specific salt effect was found, salt stabilising the enzyme solution. The pH range for optimum thermostability is 6.5–7.5, and energies and entropies of inactivation were determined from temperature coefficients of the rate constants.

Abbreviation: AChE, acetylcholinesterase.

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INTRODUCTION

A study of the thermal inactivation of the enzyme acetylcholinesterase (Acetylcholine acetylhydrolase 3.1.1.7), has been undertaken as part of a general investigation of the temperature dependence of the enzyme-catalysed hydrolysis of its specific substrate, acetylcholine. As in a previously reported study of AChE inhibition[1], mammalian erythrocytes have been employed as a source of this enzyme. The influence of temperature, pH, and the presence of various ions on the inactivation process have been examined and a study has also been made of the effect of partial inactivation on the dissociation constants of the two complexes, which AChE forms with its substrate.

EXPERIMENTAL

Materials

Enzyme preparations

Haemolysate: The erythrocytes of fresh citrated ox blood were collected by centrifugation, and washed three times with a 0.9% (w/v) solution of NaCl. The washed erythrocytes were then lysed with an equal volume of distilled water.

MENDEL AND RUDNEY *preparation:* 500 ml of the haemolysate prepared as above were adsorbed on 70 g of acid-washed kieselguhr[2], allowed to stand for 1 h at 0°, and the kieselguhr filtered off. The resulting cake was dispersed in 100 ml of 0.001 N NaOH solution, and allowed to stand at 0° overnight. The solution was then filtered free of kieselguhr.

Stromatal preparation: Packed human erythrocytes were washed three times, by centrifugation, with 0.9% (w/v) NaCl solution, lysed with 4 times their own volume of distilled water, and the pH adjusted to 5.5–6 with 1 N acetic acid. The resulting stromata were separated by centrifugation, washed three times with ice-cold water saturated with CO_2, and allowed to settle overnight. The clear supernatant was siphoned off, and the residue freeze-dried.

For use 700 mg of this material was dissolved in 50 ml of water.

Substrate solution

Reagent-grade acetylcholine bromide was recrystallised twice from absolute ethanol, and stored under vacuum, in a desiccator. Fresh solutions were prepared daily and stored in a refrigerator when not in use.

Bicarbonate buffer

A 1% (w/v) solution of A.R. grade $NaHCO_3$ was used for all determinations, except those concerned with the effects of pH, where a 4% (w/v) solution was used.

Methods

Thermal inactivation

For most of the work, 2.0-ml aliquots of the enzyme preparation, contained in 25-ml conical flasks, stoppered with rubber bungs, were immersed in a thermostat

(controlled to within ± 0.05° of the required temperature) for the required length of time. Immediately upon removal from the thermostat the flasks were chilled in ice-water and then stored in a refrigerator until activity determinations were made.

To investigate the effect of pH, 5.0-ml portions of the haemolysate were mixed with 5.0-ml portions of a series of Veronal buffers[3]; 2.0-ml samples of each mixture were incubated for 1 h at 55°. Activities were also determined on the unincubated mixtures.

To investigate the effect of ions, 2.0-ml samples of a series of mixtures of the Mendel and Rudney preparation with equal volumes of various salt solutions, were incubated for 30 min at 55°.

The effect of dilution, both with water, and with a completely inactivated enzyme preparation, was studied by incubating 2.0-ml samples of the appropriately diluted material, for 10 min at 53°.

For the investigation of the effects of partial inactivation on the dissocation constants of the enzyme–substrate complexes, a different technique was used. A vessel, consisting of 250 cm of 0.5-cm (internal diameter) glass tubing wound in a helix of nine turns, was used to incubate the stromatal preparation. A capillary jet was attached to the bottom of the helix, and so bent that the tip would just project over the edge of the thermostat, when the rest of the vessel was immersed. A piece of rubber tubing, closed by a Mohr's clip, was attached to the top of the helix. By this means, a sample of the vessel's contents was blown into a chilled tube, at the required time intervals. Incubation was carried out at 50°.

Activity measurements

Activities were measured by the Warburg manometric method. The bicarbonate buffer, the substrate solution and the enzyme preparation were saturated before use, with a mixture of N_2–CO_2 (95 : 5), the first two by bubbling the gas mixture through them, the third by agitating gently in a stream of the gas, for 10 min. The final bicarbonate concentration in each flask was 0.75% (w/v), in all except the samples concerned with the effect of pH, where twice this concentration was used. The final substrate concentration was 0.03 M in all cases except those concerned with the effects of partial inactivation on the dissociation constants, where a range of eight substrate concentrations (0.05–0.0005 M) was employed.

Manometers and flasks were flushed with the N_2–CO_2 mixture for 10 min, before transferring to the Warburg thermostat. Flasks were tipped after a period of 10 min equilibration, and manometer readings were made at 1-min intervals. Activities were determined at 20°.

The pressure readings, corrected for thermobarometer changes, were plotted against time, and the initial slopes determined (these plots are virtually linear for a considerable period when the substrate concentration exceeds 0.01 M). After multiplying the initial slopes by the appropriate flask factors, the activities were initially expressed as the b_{20} units of AUGUSTINSSON[4]. However, for convenience in subsequent calculation, the activities of partially inactivated samples have been expressed as fractions of the initial activity (taken as unity), for all except the stromatal samples.

RESULTS

The effects of pH, and the presence of inorganic ions, are illustrated in Figs. 1 and 2. The effects of dilution, with water and the denatured preparation, are given in Table I.

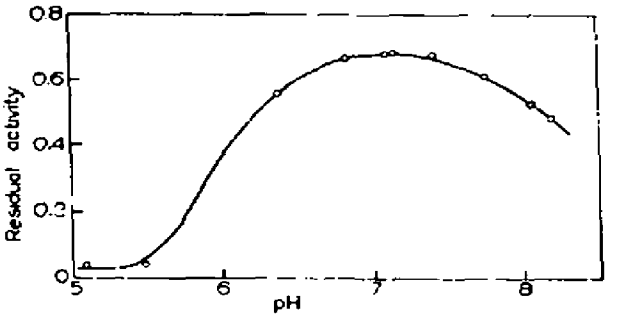


Fig. 1. Residual AChE activity as a function of pH. Haemolysate incubated with equal volume of buffer at 55° for 60 min

Figs. 3 and 4 illustrate the course of the inactivation of the haemolysate, and the Mendel and Rudney preparation, respectively. The curves show that the inactivation is adequately represented by an expression of the form

$$A = N_1 e^{-\lambda_1 t} + N_2 e^{-\lambda_2 t}$$

where A is the observed activity at time t, and N_1, N_2, λ_1 and λ_2 are constants evaluated by the method indicated by JOHNSON, EYRING AND POLISSAR[5]; their significance is discussed below.

TABLE I

EFFECT OF DILUTION, WITH WATER OR DENATURED MATERIAL, ON THE INACTIVATION OF AChE

Mixture (ml)		*Residual activity*	*Activity corrected to 100%*	*Mixture (ml)*		*Residual activity*	*Corrected to 100%*
Haemolysate	*Water*			*Haemolysate*	*Denatured material*		
2.0	0.0	0.839	0.839	2.0	0.0	0.839	0.839
1.8	0.2	0.740	0.822	1.8	0.2	0.740	0.822
1.6	0.4	0.660	0.825	1.6	0.4	0.666	0.833
1.4	0.6	0.570	0.815	1.4	0.6	0.585	0.835
1.2	0.8	0.495	0.825	1.2	0.8	0.509	0.847
1.0	1.0	0.412	0.825	1.0	1.0	0.423	0.840
		Mean =	0.822			Mean =	0.837

Table II summarises the course of the inactivation of the stromatal preparation. The activities are expressed in the original b_{80} units, and from these, values for the maximum velocity V_{max}, and the dissociation constants, K_s and K_{ss} may be calculated from the velocity expression

$$v = \frac{V_{max}}{\frac{K_s}{(S)} + 1 + \frac{(S)}{K_{ss}}}$$

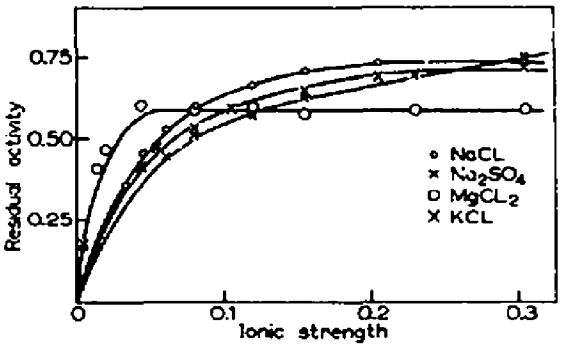


Fig. 2. Residual AChE activity as a function of ionic strength. Mendel and Rudney preparation incubated with equal volume of salt solution at 55° for 30 min.

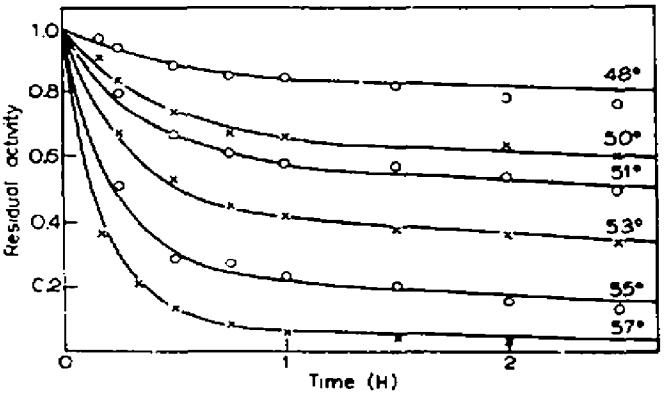


Fig. 3. Time course for the inactivation of haemolysate. Experimental points: theoretical curves calculated from equation in text.

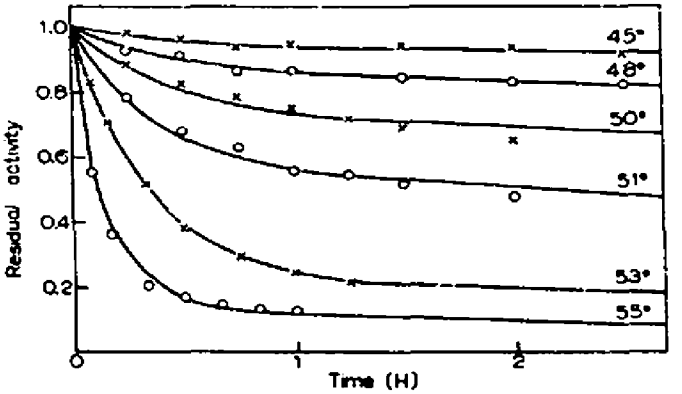


Fig. 4. Time course for the inactivation of Mendel and Rudney preparation. Experimental points: theoretical curves calculated from equation.

TABLE II

ACTIVITIES OF PARTIALLY INACTIVATED HUMAN STROMATAL AChE SAMPLES, MEASURED AGAINST A SERIES OF SUBSTRATE CONCENTRATIONS (b_{30} UNITS)

Substrate concn. (M)	Time of incubation (min)							
	0	10	20	30	45	60	75	90
0.05	161	92	74.5	67	56	52	47	42
0.025	199	116	98	81.5	73.5	64.5	58.5	50.5
0.015	235	141	115	96	80.5	77.5	68	62
0.01	262	154	125	108	92	84.5	75	66.5
0.00125	333 c	162	133	118	103	89.5	80	72
0.001	218.5	157	131.5	113.5	102	87.5	78.5	69.5
0.00075	198	146.5	125.5	105.5	98	83.5	70	65
0.0005	166.5	135	112.5	98.5	88.5	76	68	59.5

where v is the observed velocity, and (S) the substrate concentration[4]. Values for $V_{max.}$, K_s and K_{ss} may be obtained by an extension of EADIE's method[6], previously described[1]. Table III gives the values for these three constants.

It may be noted here that when the stromatal data are expressed as fractions of the activity at zero time, the values for $V_{max.}$ and of those samples measured against substrate concentrations between 0.05 and 0.01 M are virtually identical,

TABLE III

VALUES OF $V_{max.}$, K_s AND K_{ss} DURING THE COURSE OF THE INACTIVATION OF HUMAN STROMATAL AChE

Time of incubation (min)	$V_{max.}$ (millimin)	$K_s \times 10^4$ (M)	$K_{ss} \times 10^3$ (M)
0	331	5.2	3.7
10	197	2.7	
20	163	2.3	
30	137	2.1	
45	123	2.1	
60	108	2.2	
75	96	2.2	
90	85	2.2	

for the same period of incubation. For samples measured against lower substrate concentrations however, the fraction of residual activity apparently increases with decreasing substrate concentration. This indicates that the activities determined with 0.03 M substrate solutions, used throughout the rest of the present investigation, provide a satisfactory measure of enzyme activity.

DISCUSSION

The greater susceptibility of AChL to thermal inactivation at extreme pH values, is typical of most enzymes. It may be noted that the pH range of greatest

thermostability (pH 6.5–7.5) is narrower for AChE, than that (pH 5–8) for butyrylcholinesterase[7]. Since AUGUSTINSSON[8] has reported an isoelectric point in the acid region (pH 4.65–4.7) for AChE, the observed region of greatest thermostability for this enzyme is in agreement with the suggestion of JOHNSON, EYRING AND POLISSAR[9] that enzymes with an acid isoelectric point exhibit greatest stability at more alkaline pH values.

The effect of inorganic ions suggests that the activation process of the denaturation involves a decrease in charge on the enzyme. No specific ion effects are apparent, and no effect such as that described by LAUFFER[10] for the denaturation of tobacco mosaic virus, where small amounts of salt were found to increase the rate of denaturation, and larger amounts to decrease it.

It would be desirable to study the inactivation of AChE over a wide range of initial concentrations, but the limits set by the initial activity on the one hand, and the accuracy of the assay on the other, have precluded an extensive study of this point with the present source of AChE. However, over the small range studied, the variations in the activities observed are within the limits of experimental error, and dilution does not appear to affect the inactivation.

Again, it would be desirable to employ a pure enzyme preparation for a more extensive study of this process, but the results of CHASE[11], using a crude luciferase preparation are very similar to those of KUNITZ[12] using a highly purified preparation of a soy-bean trypsin inhibitor. It seems reasonable to conclude therefore, that the present results, which resemble both of these, give a meaningful picture of the inactivation of AChE.

The fact that the inactivation of AChE does not follow simple first-order kinetics is illustrated by the time courses (Figs. 3 and 4) and this is confirmed by the fact that a plot of log. activity against time is not linear (*cf.* ref. 24).

Although the data are few, the results for the dilution experiment suggest that the order with respect to concentration is unity. The order with respect to time is obviously not unity, and the falling off of the first-order constants may be explained in various ways:

(a) The inactivation is reversible, and an equilibrium is reached.

This explanation is ruled out by the fact that it is possible to inactivate AChE preparations completely, by prolonged heating.

(b) The product of inactivation serves to stabilise the remaining active material.

This possibility appears to be eliminated by the results obtained when incubation was carried out in the presence of denatured material.

(c) Two AChE enzymes exist, having different thermostabilities (and differing in other respects, see below).

There are quite a number of lines of evidence for the existence of two enzymes with AChE activity. ANDERSON AND PETHICA[13] have made this suggestion to explain their observation that haemolysis does not occur in ageing erythrocytes, until 50% of the AChE activity is lost. They quote HOWARD AND GRIEG[14] who have reported that no changes in permeability of erythrocytes occur, until half of the AChE activity has disappeared. BERGMANN AND SEGAL[15] from a study of the ratio of the concentration of hexamethonium to decamethonium necessary to produce a certain level of AChE inhibition in various tissues, have suggested the presence of two AChE enzymes. COHEN *et al.*[16] have advanced a similar hypothesis to explain their results

of inhibition experiments with physostigmine. DAVISON[17] has reported evidence for the existence of two AChE enzymes, differing in the stability of their organo-phosphorus derivatives. HARGREAVES[18] has recently found that after purification an AChE preparation from electric tissue showed two components in the ultracentrifuge. If this hypothesis is accepted, the thermal inactivation of AChE may be represented as:

$$A_1 \overset{k_1}{\rightarrow} I \overset{k_2}{\leftarrow} A_2$$

(It may well be that the inactive form I, produced from the active enzyme A_1, differs from that produced from the other active form, A_2, but in the present instance these cannot be distinguished, so the simplest formulation appears to be most appropriate). The expression for the residual activity, A, at time t, is given[19] by:

$$A = A_1 e^{-k_1 t} + A_2 e^{-k_2 t}$$

where A_1 and A_2 are the initial concentrations of the two active forms, and k_1 and k_2 are the respective velocity constants for their inactivation.

Values for A_1, A_2, k_1 and k_2 are given in Table IV. Figs. 3 and 4 show that this

TABLE IV

VALUES OF A_1, A_2, k_1 AND k_2 CALCULATED TO FIT THE EXPERIMENTAL DATA

Mechanism $A_1 \overset{k_1}{\rightarrow} I \overset{k_2}{\leftarrow} A_2$

Temp.	Haemolysate			
	A_1	A_2	k_1 (h^{-1})	k_2 (h^{-1})
48°	0.15	0.85	2.5	0.025
50°	0.33	0.67	3	0.05
51°	0.40	0.60	3.5	0.07
53°	0.53	0.47	4.5	0.13
55°	0.72	0.28	5.5	0.25
57°	0.90	0.10	6.5	0.45

Temp.	MENDEL AND RUDNEY preparation			
	A_1	A_2	k_1 (h^{-1})	k_2 (h^{-1})
45°	0.06	0.94	2	0.01
48°	0.13	0.87	2.5	0.025
50°	0.24	0.76	3	0.05
51°	0.42	0.58	3	0.08
53°	0.76	0.24	3.25	0.11
55°	0.84	0.16	8	0.30

expression provides a satisfactory representation of the experimental data. From a plot of log. k against the reciprocal of the absolute temperature (see Fig. 5) values for the apparent energies of activation may be obtained from the slopes. (For this purpose, and for subsequent calculations, only values obtained from the inactivation of the haemolysate have been used, because those for the MENDEL AND RUDNEY preparation, whilst generally similar, show a wider scatter.) From the apparent activation energy, values for the heats, free energies and entropies of activation may be calculated in the usual way[20], and these are listed in Table V. The values for ΔH^* and ΔS^* for the inactivation of the less stable form A_1 seem rather low for such

TABLE V

VALUES FOR ΔH^*, ΔG^* AND ΔS^* FOR THE INACTIVATION OF AChE, ASSUMING THE EXISTENCE OF TWO AChE ENZYMES

Mechanism $A_1 \underset{k_2}{\overset{k_1}{\rightleftharpoons}} I \underset{k_4}{\overset{k_3}{\rightleftharpoons}} A_2$

Temp.	k_1			k_3		
	ΔH^* (cal)	ΔG^* (cal)	ΔS^* (cal·degree^{-1}·mole^{-1})	ΔH^* (cal)	ΔG^* (cal)	ΔS^* (cal·degree^{-1}·mole^{-1})
48°	23 160	23 530	−1	67 360	26 480	127
50°	23 160	23 540	−1	67 360	26 190	127
51°	23 160	23 530	−1	67 360	26 060	127
53°	23 150	23 510	−1	67 350	25 790	127
55°	23 150	23 510	−1	67 350	25 540	127
57°	23 150	23 540	−1	67 350	25 290	128

a reaction[21]. The values of ΔH^* and ΔS^* for the inactivation of the more stable form A_2 are of the expected magnitude, as are the values of ΔG^* for both forms. There remains one other explanation of the experimental data:

(d) One AChE enzyme exists, but thermal inactivation proceeds by more than one route.

This explanation has been advanced in a number of cases of protein denaturation, or enzyme inactivation. Several mechanisms can account for the form of the experimental results[11] thus:

$$I \xleftarrow{k_1} A \underset{k_3}{\overset{k_2}{\rightleftharpoons}} X$$

Here the active form A gives rise to an inactive material I by an irreversible process, and a second product X by a reversible process. X may or may not possess activity,

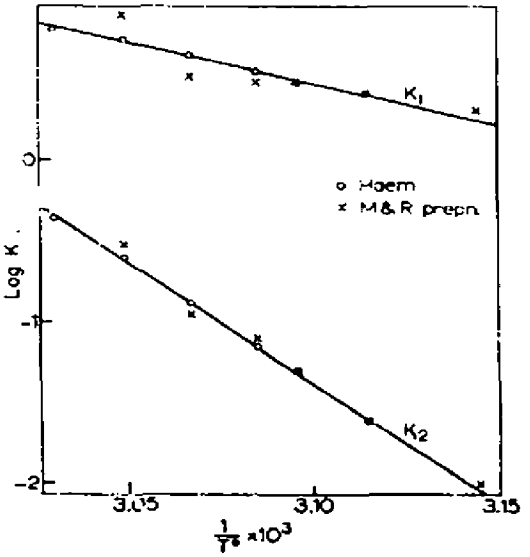


Fig. 5. Arrhenius plots for k_1 and k_3. Haemolysate and Mendel and Rudney preparation.

but the expression for the residual activity A, at time t, is given by the equation:

$$A = N_1 e^{-\lambda_1 t} + N_2 e^{-\lambda_2 t}$$

where N_1, N_2, λ_1 and λ_2 are constants. The significance of λ_1 and λ_2 differs according to whether X is an active or an inactive form. CHASE[11] has pointed out that if X is inactive, there should be some recovery of activity in partially inactivated samples, on cooling. No such recovery was observed in the case of AChE, so it would appear that X is an active form. This was the conclusion reached by WRIGHT AND SHOMAKER[32] in the case of the denaturation of diptheria antitoxin by urea. The mechanism may therefore be more appropriately represented as:

$$I \xleftarrow{k_3'} A_1 \underset{k_3'}{\overset{k_2'}{\rightleftharpoons}} A_2$$

and in this case the velocity constants are given by:

$$k_1' = \frac{\lambda_1 N_1 + \lambda_2 N_2}{N_1 + N_2}$$

$$k_2' = \frac{\lambda_1 \lambda_2}{k_1'}$$

$$k_3' = \lambda_1 + \lambda_2 - k_1' - k_2'$$

Since the expression for the residual activity has the same form as that for the mechanism involving two AChE enzymes, the values for N_1, N_2, λ_1 and λ_2 are the same as those for A_1, A_2, k_1 and k_2, respectively, given in Table IV. From the equations given above, the values of the three velocity constants, k_1', k_2' and k_3' may be calculated, and values for the apparent activation energies obtained from a plot of log. k' against the reciprocal of the absolute temperature (see Fig. 6). Hence values for the heats, free energies and entropies of activation may be calculated, and these are listed in Table VI.

It may be noted that the value of ΔS^* for the conversion of the original active form A_1 to the other A_2 is large and negative. A comparable value has been calculated by JOHNSON, EYRING AND POLISSAR[23] for the renaturation of luciferase, from the data of CHASE[11]. Perhaps more unexpected is that the reversion of A_2 to A_1 should involve a large positive value of ΔS^*, and yet yield an active enzyme. This suggests

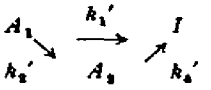
TABLE VI

VALUES OF ΔH^*, ΔG^* AND ΔS^* FOR THE THREE REACTIONS OF THE WRIGHT AND SHOMAKER MECHANISM

Mechanism $I \xleftarrow{k_3'} A_1 \underset{k_3'}{\overset{k_2'}{\rightleftharpoons}} A_2$

Temp.	k_1'			k_3'			k_2'		
	ΔH^* (cal)	ΔG^* (cal)	ΔS^* (cal degree^{-1} mole^{-1})	ΔH^* (cal)	ΔG^* (cal)	ΔS^* (cal degree^{-1} mole^{-1})	ΔH^* (cal)	ΔG^* (cal)	ΔS^* (cal degree^{-1} mole^{-1})
48°	61 760	24 690	116	−7980	23 680	−99	45 160	25 290	62
50°	61 750	24 240	116	−7980	23 840	−99	45 160	25 480	61
51°	61 760	24 110	116	−7980	23 910	−98	45 160	25 480	61
53°	61 750	23 900	116	−7980	24 060	−98	45 150	25 420	61
55°	61 750	23 720	116	−7990	24 420	−99	45 150	25 340	61
57°	61 750	23 720	116	−7990	24 660	−99	45 150	25 090	61

that an alternative mechanism, mentioned by WRIGHT AND SHOMAKER[22] may be more appropriate.



Here, $k_3' = 0$, and A_2 is transformed directly into an inactive form. (Again this may differ from that produced directly from A_1, but the two forms cannot be distinguished here.) WRIGHT AND SHOMAKER have pointed out that when $k_3' = 0$ and $\lambda_1 t \gg \lambda_2$, the values of k_1' and k_2' are unchanged, and $k_4' = \lambda_2$. When log. λ_2 (*i.e.* log. k_4') is

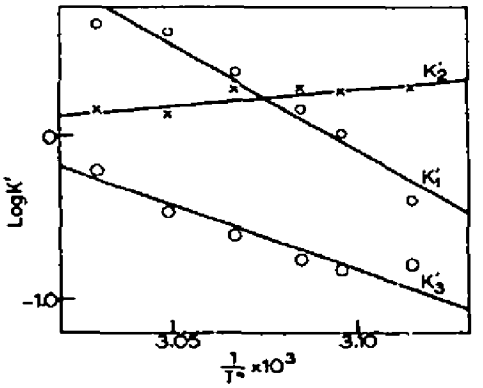
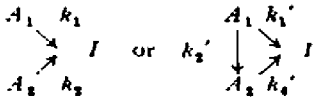


Fig. 6. Arrhenius plots for k_1^1, k_2^1 and k_3^1 (Haemolysate).

plotted against the reciprocal of the absolute temperature (see Fig. 5), values for E_a^*, ΔH^*, ΔG^* and ΔS^* may be calculated as before (see Table VII). It will be seen that the two inactivation steps both involves large positive values of ΔH^* and ΔS^*.

From these considerations it would appear that the choice of the explanation of the data lies between the existence of two AChE enzymes, and the mechanism last described:



Either of these would be consistent with the observation that K_s, the dissociation constant of the normal enzyme-substrate complex, changes during the initial stages of the inactivation. In the first case, the more thermostable form A_2 would possess a lower value of K_s, whilst in the second, the active form A_2 produced from the native enzyme A_1 would possess a lower value of K_s.

It is of interest to consider the implications of this latter possibility. If the view originally advanced by ADAMS AND WHITTAKER[24] is correct, K_s is the dissociation constant for a complex in which a single substrate molecule is bound both at the esteratic site (where hydrolysis occurs) and at an anionic site (where the N^+ atom of the substrate is held by a negative charge on the enzyme surface). K_{ss} is the dissociation constant of a substrate-inhibited complex, where two molecules are bound, one at the esteratic and one at the anionic site. Since K_s decreases on partial inactivation, binding is increased. If the esteratic and anionic sites are situated at

TABLE VII

VALUES OF $\Delta H^{\ddagger}$, $\Delta G^{\ddagger}$ AND $\Delta S^{\ddagger}$ FOR THE THREE REACTONS OF THE MODIFIED WRIGHT AND SHOMAKER MECHANISM

Mechanism I : $A_1 \xrightarrow{k_1'} A_2 \xrightarrow{k_2'} A_3 \xrightarrow{k_3'} I$

Temp.	k_1'			k_2'			k_3'		
	$\Delta H^{\ddagger}$ (cal)	$\Delta G^{\ddagger}$ (cal)	$\Delta S^{\ddagger}$ (cal degree^{-1} mole^{-1})	$\Delta H^{\ddagger}$ (cal)	$\Delta G^{\ddagger}$ (cal)	$\Delta S^{\ddagger}$ (cal degree^{-1} mole^{-1})	$\Delta H^{\ddagger}$ (cal)	$\Delta G^{\ddagger}$ (cal)	$\Delta S^{\ddagger}$ (cal degree^{-1} mole^{-1})
48°	61 760	24 690	116	7980	23 680	99	67 360	26 480	127
50°	61 760	24 240	116	7980	23 840	99	67 360	26 190	127
51°	61 760	24 110	116	7980	23 940	98	67 360	26 060	127
53°	61 750	23 900	116	7980	24 060	98	67 350	25 790	127
55°	61 750	23 720	116	7990	24 420	99	67 350	25 540	127
57°	61 750	23 720	116	7990	24 660	99	67 350	25 290	128

other than the optimal distance apart for maximal binding in the native enzyme, but assume optimal spacing in the A_3 form, K_s would decrease in the way observed. Since the two molecules of the substrate-inhibited complex are bound separately, a small variation in the spacing of the two sites need not affect K_{s_2}, and this is what is actually observed.

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