as separate titrations since it was found that the points corresponding to 0.5 and 1.5 equivalents of added base differed by over 3 pH units. The apparent ionization constants were taken as the slopes of the least squares lines in these regions according to the equation

$$(H^+) = K'(HA)/(A^-)$$

where (H^+) is furnished by the pH data and

$$\frac{(HA)}{(A^-)} = \frac{\text{(vol. of alkali for complete neutralization)} - \text{(vol. added)}}{\text{(volume of alkali added)}}$$

Essentially the same results are obtained by averaging the data derived from the following equation for each experimental point.

$$pK' = p\text{H} - \log((A^-)/(HA))$$

The pK' values thus determined were plotted against the square root of the ionic strengths of the corresponding solutions, as shown in Fig. 2.

A curve, Fig. 1, relating pH and the moles of alkali combined per mole of ampholyte was calculated at the ionic strength of a typical DMPi dihydrochloride titration by means of the expression

$$\frac{\text{moles of alk. comb.}}{\text{moles of ampholyte}} = \frac{(A^-)}{(HA + A^-)} = \frac{K_1'(H^+) + 2K_1'K_2'}{(H^+)^2 + K_1'(H^+) + K_1'K_2'}$$

The experimental points are shown plotted along this curve for comparison.

Enzymatic Rate Determinations.—Rate runs in the presence and absence of inhibitors were made as previously described.[3] Duplicate runs were carried out at each pH level for both the inhibited and uninhibited enzymatic hydrolyses, with agreement in rates in each pair of duplicates found to be within 5%. All AC hydrolyses were in the presence of 0.01 M Mg^{++} ion, with the total ionic strength for each phosphate buffer adjusted to 0.143 in final reaction mixtures by means of added sodium chloride. Initial substrate concentrations were fixed at the optimum value of 3.35×10^{-3} M, using twice-recrystallized acetylcholine chloride. The stock enzyme preparation used in these kinetic runs assayed at 2.75×10^5 μmoles AC hydrolyzed/hr./mg. protein/ml. and was obtained by fractionation of the electric organ of *Electrophorus electricus* according to the method of Rothenberg and Nachmansohn.[3] Enzyme solutions aged[11] in at least 1:100 dilution for a week or more were used as secondary stock solutions, from which final aliquots were taken for the actual runs.

DMPi solutions were freshly prepared before use, from redistilled free diamine.[3] Kinetic runs (at 25.12 ± 0.03° in a reaction volume of 3.20 ml.) employing inhibitor gave rates at the various pH levels which were compared to the corresponding hydrolytic activities of the uninhibited enzyme at these same pH values, in calculation of the respective percentage inhibition figures. Estimated errors in these v_i/v inhibition ratios were based on the observed precision of duplicate determinations.

Acknowledgment.—We are indebted to Mr. C. W. Coates and Dr. H. S. Polin for their generous aid in procurement of electric eel tissue and its initial extracts.

(11) For the desensitization of enzyme to Mg^{++} on aging see: S. L. Friess, I. B. Wilson and E. Cabib, THIS JOURNAL, **76**, 5156 (1954).

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Acetylcholinesterase: Enthalpies and Entropies of Activation[1]

BY IRWIN B. WILSON AND ENRICO CABIB[2]

RECEIVED MAY 18, 1955

The maximum velocities and Michaelis-Menten constants have been measured as a function of temperature for acetylcholinesterase and a series of acetyl esters of ethanolamine, namely, acetylcholine (I), dimethylaminoethyl acetate (II), methylaminoethyl acetate (III) and aminoethyl acetate (IV). The Michaelis-Menten constants do not change with temperature. Linear Arrhenius plots of the maximum velocities were obtained for the poorer substrates IV and III, but not for II and I which yielded smooth curves approaching very low energies of activation at higher temperatures. These data were interpreted in terms of the previously developed theory of enzyme hydrolysis, specifically in terms of a two-step hydrolytic process involving an acetyl enzyme. Appropriate kinetic relationships were derived and their consequences explored. It is possible to develop a theory of substrate inhibition based in part upon the hydrolysis of acetyl enzyme as a rate controlling step. In general the enthalpy and entropy values in this series are not sufficiently different for substrates II, III and IV to draw conclusions concerning the relationship of these quantities to the specificity characteristics of this enzyme. However, the values for substrate I are very different, in particular the entropy of activation is quite favorable whereas the enthalpy of activation is rather unfavorable compared to the other substrates.

Introduction

Enzyme specificity can be readily analyzed in two categories: (1) specificity in binding the substrate and (2) specificity in the rate with which the enzyme–substrate complex undergoes reaction to yield products and regenerate the enzyme. With a number of enzymes, acetylcholinesterase amongst them,[3] it has been possible to recognize the binding features of a substrate (or competitive inhibitor) and define the corresponding forces which stabilize the enzyme–substrate (or enzyme–inhibitor) complex. It has thus been possible to explain binding. The second category, specificity in the hydrolytic process (in the case of hydrolytic enzymes), deals more directly with the catalytic mechanism. For this reason it is especially desirable to study the hydrolytic specificity for a series of similar substrates and to obtain data at different temperatures so that the enthalpies and entropies of activation may be compared and other mechanistic features possibly revealed. The present study with acetylcholinesterase and a series of acetyl esters of substituted amino alcohols substantiates the conclusion reached in other ways that an acetyl enzyme[4]

(1) This work was supported in part by the Medical Research and Development Board, Department of the Army, Office of the Surgeon General, Contract No. DA-49-007-MD-37, and in part by the Division of Research Grants and Fellowships of the National Institutes of Health, Grant No. B-573, United States Public Health Service.

(2) Supported by a Grant from the Rockefeller Foundation. Present address: Instituto de Investigaciones Bioquimicas, Fundacion Campomar, Julian Alvarez 1719, Buenos Aires, Argentina.

(3) I. B. Wilson, "The Mechanism of Enzyme Action," Ed. W. D. McElroy and B. Glass, The Johns Hopkins Press, Baltimore, Md., 1954, p. 642.

(4) I. B. Wilson, F. Bergmann and D. Nachmansohn, *J. Biol. Chem.*, **186**, 781 (1950).

TABLE I

THE MICHAELIS-MENTEN CONSTANT (MOLES/L.) AS A FUNCTION OF TEMPERATURE AT *p*H 7.00

	$K_m' \times 10^4$					
	15°	20°	25°	30°	Av. ± a.d.	
Acetylcholine	0.92	0.92	0.94	0.97	0.94	±0.02
Dimethylaminoethyl acetate	6.1	6.8	6.5	6.2	6.4	±0.2
Methylaminoethyl acetate	81	70	78	82	78	±4
Aminoethyl acetate	154	134	156	157	151	±8

occurs as an intermediate. The importance of the entropy of activation in determining the specificity of the enzyme toward acetylcholine as compared to the other substrates is brought out. As will be discussed later, the data also yield an insight into the marked substrate inhibition shown by this enzyme with acetylcholine as substrate. A new interpretation of a decrease in apparent energy of activation with rising temperature is presented involving a two-step hydrolytic mechanism.

Methods

Enzyme.—Acetylcholinesterase was prepared from electric tissue of *Electrophorus electricus* by the method of Rothenberg and Nachmansohn.[5] A preparation which hydrolyzed 3.2×10^4 μmoles of acetylcholine per hour per mg. of protein was used in all the measurements.

Substrates.—Acetylcholine bromide, substrate I, was a commercial product recrystallized from ethanol. Dimethylaminoethyl acetate hydrobromide (II), methylaminoethyl acetate hydrochloride (III) and aminoethyl acetate hydrochloride (IV) were prepared from the amino alcohols.[6,7]

Enzyme Activity.—Kinetic measurements were made with a slightly modified Beckman automatic titrator at *p*H 7.00 ± 0.02 and at temperatures held to ±0.03°. The reaction medium was 0.1 *M* NaCl with 0.1% gelatine and 1×10^{-4} *M* EDTA added as precautionary measures. In those cases where high concentrations of substrates were used, the concentration of NaCl was reduced so as to keep an ionic strength of 0.1 *M*. The volume of the reaction mixture (40 to 350 ml.) was kept sufficiently large so that in general only 2% of the substrate was hydrolyzed during the 5 minutes which were used for kinetic measurements.

Small blanks were caused by absorption of atmospheric carbon dioxide and in some cases by spontaneous hydrolysis of substrate. The absorption of carbon dioxide was usually eliminated by a slow stream of nitrogen.

The reaction was started by adding enzyme. The amount of hydrolysis occurring during the 4-minute interval, starting one minute after the addition of enzyme, was taken as the initial velocity. These values were reproducible to about 1%.

The substrate ammonium ions (except I) are somewhat more dissociated at *p*H 7 than the amino alcohols which are products of the reaction and so it was necessary to add small corrections (1–8%) to the measured rates. Small corrections (<3%) were applied to acetylcholine measurements for substrate inhibition. The Michaelis-Menten constants (K_m') were obtained in duplicate from measurements at three concentrations which included the value of the constant within their range. The two values of K_m' differed by less than 5%. The maximum velocities (V_m) for a given substrate were calculated with K_m' as determined above from measurements in duplicate at one suitably high concentration at each temperature. All V_m measurements for a single substrate were made in one day and the first temperature measurements were repeated at the end of the series as a precaution to be sure that the activity of the preparation had not been altered. Calculations were based upon the usual equations

$$v = \frac{V_m S}{K_m' + S}; \quad V_m = kE^0 \qquad (1)$$

where E° is the total concentration of enzyme.

(5) M. A. Rothenberg and D. Nachmansohn, *J. Biol. Chem.*, **168**, 223 (1947).

(6) I. B. Wilson, *ibid.*, **208**, 123 (1954).

(7) C. W. Crane and N. H. Rydon, *J. Chem. Soc.*, 527 (1947).

Results

The Michaelis–Menten constants calculated from equations 1 are presented in Table I. There evidently is no trend in the values with respect to temperature and the average mean deviation of the values for all temperatures is about the same as for the individual values. Within our experimental error K_m' is independent of temperature between 15 and 30°. The same values were assumed to hold for 35, 10 and 5°. The value of K_m' for acetylcholine and human erythrocyte acetylcholinesterase has also been found to be independent of temperature.[8] The extent to which the ammonium ion-ester is methylated very greatly affects the value of this constant.

The values of k (Table II) were calculated from equations 1 using values of E° based upon an activity of 4.15×10^5 μmoles acetylcholine hydrolyzed per hour per mg. of protein and a molecular weight (taken as the equivalent weight) of 3×10^6 obtained by Rothenberg and Nachmansohn for for their purest preparation.[6] This preparation showed only one component during electrophoresis and ultracentrifugation. Evidently there is considerable uncertainty in the value of E° particularly since there may be as many as 50 enzyme sites per molecule.[9] While the absolute values of k and the entropies of activation may thereby suffer, no difficulty is involved in the comparative use of the data.

TABLE II

THE SPECIFIC RATE CONSTANT (SEC.$^{-1}$) AS A FUNCTION OF TEMPERATURE AT *p*H 7.00

	k			
°C.	Acetylcholine	Dimethylaminoethyl acetate	Methylaminoethyl acetate	Aminoethyl acetate
5	1.62×10^5	0.81×10^5		
10	2.12	1.07	0.412×10^5	
15	2.53	1.27	.530	0.470×10^4
20	2.96	1.50	.65	.60
25	3.32	1.75	.82	.87
30	3.52	1.95	.98	1.14
35	3.64	2.14	1.15	1.44

The usual Arrhenius plot of log k *versus* T^{-1} was attempted with the results shown in Fig. 1. Substrates III and IV evidently yield reasonably constant energies of activation, but the smooth curves obtained with substrates I and II yield energies of activation which decrease as the temperature is raised.

Discussion

A number of other investigators working with other enzymes have found cases in which the energy of activation is not independent of tempera-

(8) R. Shukuya, *J. Biochem. (Japan)*, **40**, 135 (1953).

(9) H. O. Michel and S. Krop, *J. Biol. Chem.*, **190**, 119 (1951).

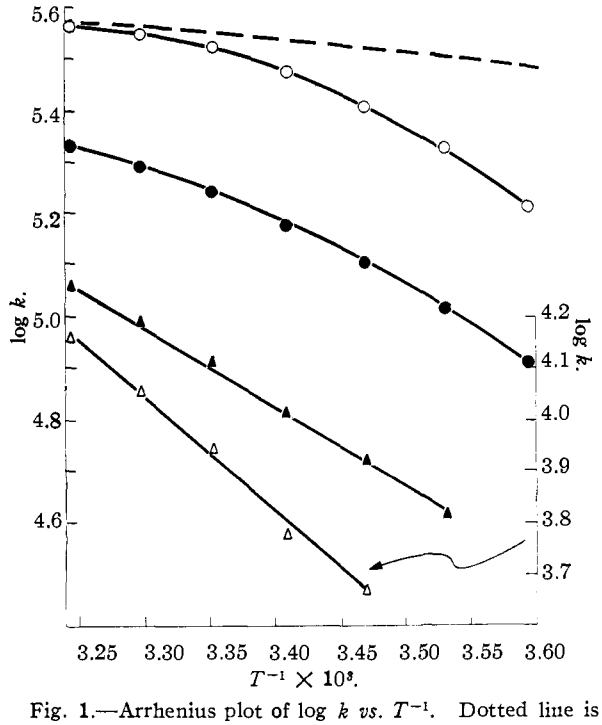

Fig. 1.—Arrhenius plot of log k *vs.* T^{-1}. Dotted line is drawn as an estimated asymptote to curve for substrate I and represents log k_4.

ture.[10] In some cases, as in the present study, the energy of activation declined at higher temperatures.[11] In general two explanations have been offered for this phenomenon. One was based upon irreversible enzyme denaturation which is, of course, higher at higher temperatures. The other explanation is based upon equations 1. If log v is plotted as a function of T^{-1} one should not expect a straight line because two temperature dependent parameters V_m and K_m are involved.

It is apparent that both of these complications might be avoided. In our case the temperature was far too low to produce measurable inactivation in the 5-minute initial period during which measurements were made. Also, as has been mentioned, two of the substrates follow the Arrhenius law. In this study both V_m and K_m' and not simply v were measured as a function of T.

Considering for the moment V_m, certain plausible explanations have been advanced. For example, it might be considered that there is an equilibrium between various species of acetylcholinesterase molecules, some active, some inactive and some with varying degrees of activity (rapid reversible denaturation). If now one assumes that, as the temperature increases, the less active forms are favored, the phenomenon would be explained. However, this explanation does not appear to be satisfactory because two of the substrates have linear Arrhenius plots. It is therefore evident that no explanation will be suitable which does not involve the substrate. We thus do not further consider this explanation but pass to a new explanation.

(10) G. B. Kistiakowsky and R. Lumry, THIS JOURNAL, **71**, 2006 (1949).

(11) V. Massey, *Biochem. J.*, **53**, 72 (1953).

The theory of enzyme-catalyzed hydrolysis developed with this enzyme suggests an explanation of the curvature. According to this theory the enzyme–substrate complex reacts in two successive steps

enzyme–ester complex $\longrightarrow$ amino alcohol + acetyl enzyme

acetyl enzyme + $H_2O \longrightarrow$ acetic acid + enzyme

Thus the plot of log k_3 *vs.* T^{-1} should yield a single straight line only when one of the steps is rate determining at all temperatures. In the more general case, the results expected would be a straight line in the range of temperatures where one of the steps is rate determining and a different straight line in the range where the other step is the slower; the slopes being proportional to the corresponding energies of activation. The two lines would be joined by a curved portion in the temperature range where both rates are comparable. As the temperature is raised the rate approaches that which has the lower energy of activation. A two-step process must therefore yield a curve flattening toward higher temperatures.[12]

In any experiment we have only a relatively small temperature range available (in our case 30°), and we see, therefore, only a portion of the complete curve. It is our contention as discussed below, that our experimental temperature range happens to be that range in which the first step is rate controlling for substrates IV and III, but the range in which both steps are comparable, *i.e.*, the region of curvature, for the better substrates II and I.

According to the theory, in the hydrolysis of our series of *o*-acetylamino alcohols, the acetyl enzyme is a common intermediate. Since all the substrates are hydrolyzed at different maximum rates, the rate-controlling step cannot in general be the hydrolysis of acetyl enzyme. Thus the superiority of one substrate over another must be the consequence of a faster rate in the first step. As the first step increases in rapidity, *i.e.*, as we go in the series to better and better substrates, it must finally approach and possibly exceed the rate of the second step. Evidently, this has occurred in our series; the first step is essentially rate controlling for substrates III and IV, both steps are controlling for substrate II and for substrate I the second step is even slower than the first. Evidently, the hydrolysis of acetyl enzyme (dotted line Fig. 1) has a very low energy of activation.

The equations for this formulation

$$EH + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EHS \xrightarrow{k_3} ES' + ROH$$

$$ES' + H_2O \xrightarrow{k_4'} EH + CH_3COOH$$

assuming $d(ES)/dt = d(ES')/dt = 0$ yield

$$v = \frac{kE^0S}{(k_2/k_1 + k_3/k_1)/(1 + k_3/k_4) + S} = \frac{V_mS}{K_m' + S} \quad (2)$$

where

$$\frac{1}{k} = \frac{1}{k_3} + \frac{1}{k_4} \text{ and } k_4 = k_4'(H_2O)$$

(12) It is assumed in this argument that the individual steps of the hydrolytic process each obey the Arrhenius law.

and EH represents the enzyme, S the substrate, EHS the Michaelis–Menten complex, ES′ acetyl enzyme and ROH the appropriate amino alcohol. In this formulation the known pH dependence of both V_m and K_m' have not been included. Since at pH 7 the factors involved, which are of the form $(1 + H^+/K_a)$, are not very much greater than 1, this simplification is desirable.

We are interested in comparing k_3 for the different substrates. If we had been able to continue the curves to very low temperatures, the rate-controlling step would in all cases have been the formation of the acetyl enzyme and we should have measured k_3 directly. However, it is possible to get reasonable values of k_4 (which is the same for all these substrates) by drawing an asymptote for the acetylcholine curve in Fig. 1. Values for k_3 may then be calculated from equation 3. In Fig. 2, log k_3 has been plotted as a function of T^{-1}. (Only three points for acetylcholine have been used since the errors in the other points would be expected to be high, *i.e.*, k_4 and k differ by only a few percentage. It will be noted that the apparently mild alteration in structure between substrates I and II produces a very great difference in k_3. This much greater specificity of the enzyme toward substrate I is consistent with their comparative interaction with other members of the acetylcholine system, but is obscured in the over-all rate. For substrate I, k_3 and k_4 are equal at about 3°, but for substrate II equality does not occur until about 25°.

But this visual selection of the function k_4 has been made in an undefined manner and we may expect that a range of linear log k_3 and linear log k_4 functions could be found which would enable a good synthesis of the function in accordance with the relationship expressed in equation 2. These different functions may have considerably different enthalpies and entropies of activation. The range of selection of possible log k_4 functions is limited by certain considerations: (1) the same k_4 function must be suitable for all substrates, essentially only I and II; (2) the energy of activation must be less than that of k for substrate I. For the range 25–35° this energy of activation is 1.7 ± 0.4 kcal./mole (the error corresponds to somewhat greater than 1% in k). Thus the energy of activation should be in the range 0–2.5 kcal./mole; (3) restricted to such low energies of activation for k_4 it is not possible to obtain linear log k_3 values for substrate II, unless log k_4 values are selected below about 5.65 at 35°. Since log k_4 must exceed log k for substrate I there is a lower limit of 5.56.

TABLE III

VALUES OF k_3 (SEC.$^{-1}$) AT 25°, pH 7.00 CALCULATED FROM EQUATION 3 AND THE CORRESPONDING ACTIVATION DATA

Also included are the values for k_4, the specific rate constant for the hydrolysis of the acetyl enzyme.

	k_3(25°)	$\Delta H^{\pm}$	$\Delta S^{\pm}$
Acetylcholine	1.5–3.4 × 10^6	14–19 × 10^3	+(16–34)
Dimethylaminoethyl acetate	3.3–3.5 × 10^5	6.7–8	−(6.5–10.5)
Methylaminoethyl acetate	1.1 × 10^5	8	−9
Aminoethyl acetate	9 × 10^3	9.5	−9
Acetyl enzyme (k_4')	6–8 × 10^3	0–1.5	−(41–46)

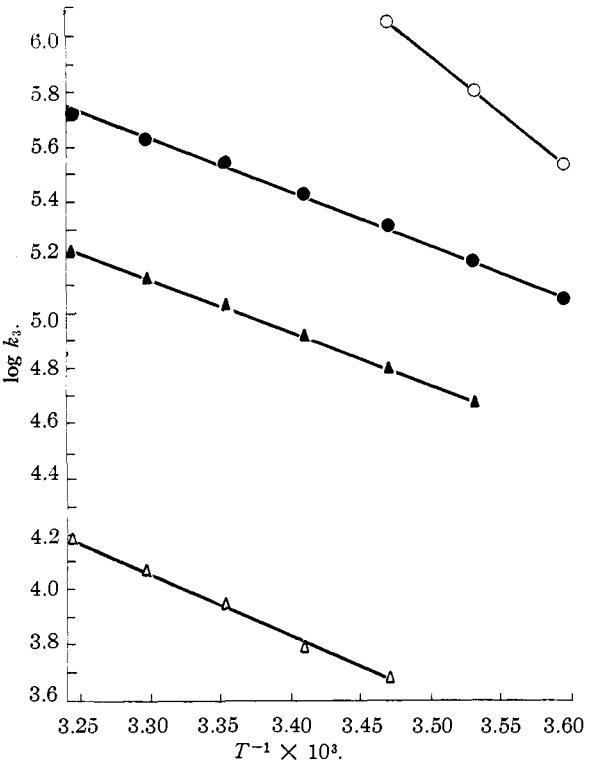

Fig. 2.—Arrhenius plot of log k_3 *vs.* T^{-1}: k_3 is calculated from the experimentally observed k and the estimated k_4.

Working within these confines, ranges of possible enthalpies and entropies of activation have been obtained and are presented in Table III.

Values for the energy, enthalpy and entropy of activation have been calculated using the formulation of the absolute reaction rate theory,[13] thus

$$\log k_3 = E/2.3RT + \text{constant}$$

$$\Delta H^{\pm} = E - RT$$

$$\Delta S^{\pm} = \Delta H^{\pm}/T - 2.3R\left[\log k_3 - \log \frac{KT}{h}\right] \quad (3)$$

These values are for the change: enzyme–substrate complex to activated complex for the reaction which produces the acetylation of the enzyme.

A comparison of these values indicates that it is doubtful if any conclusion can be drawn concerning the relative importance of entropy and enthalpy terms in determining the specificities of maximum velocities for substrates II, III and IV. However, it is clear that for substrate I the enthalpy of activation is very much higher than for the others and this is in the wrong direction for determining specificity. Evidently, the specificity is determined by the much more favorable entropy of activation. It has been noted that the observed Michaelis–Menten constant K_m' (equation 1) does not vary with temperature for any of these substrates. It is of interest to see how that part of the observed Michaelis–Menten constant which corresponds to the more conventionally interpreted Michaelis–Menten constant

$$K_m = \frac{k_2}{k_1} + \frac{k_3}{k_1}$$

(13) S. Glasstone, K. J. Laidler and H. Eyring, "The Theory of Rate Processes," McGraw–Hill Book Co., New York, N. Y., 1941.

which is given from equation 3 by

$$K_m = K_m' [1 + k_3/k_4] \quad (4)$$

varies with temperature. In Fig. 3 log $(1 + k_3/k_4)$ is plotted against T^{-1}, again for the visual asymptote of Fig. 1. Values for substrate IV are very small and therefore have not been included.

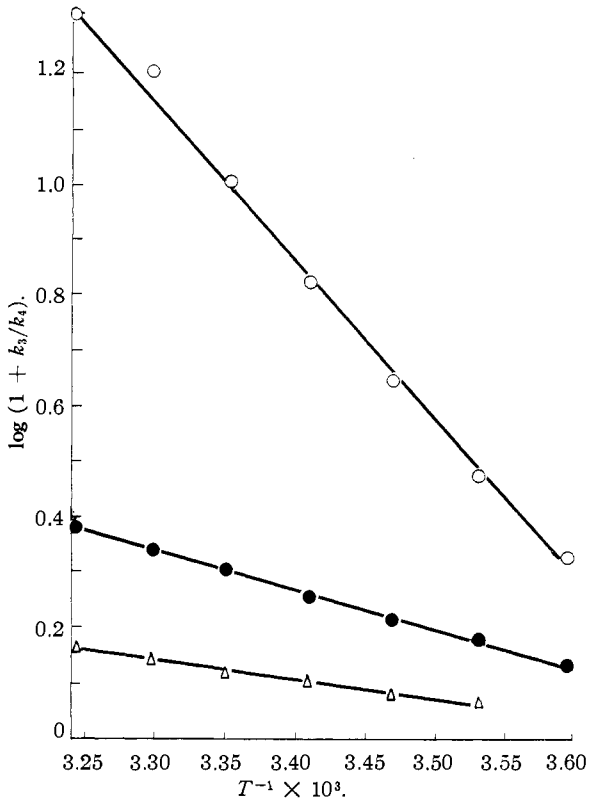

Fig. 3.—Arrhenius plot of log $(1 + k_3/k_4)$ *vs.* T^{-1}.

The values of k_3 for substrate I were obtained by extrapolation in Fig. 2. That straight lines are obtained indicates that either one term in K_m dominates or that the energies associated with each term are about the same (or a combination of both circumstances). It would appear that since K_m varies in an opposite direction from k_3 and that the K_m values roughly follow the general pattern for the binding of inhibitors, then K_m may to a large extent be determined in these cases by the enzyme–substrate dissociation constant k_2/k_1. Because of the indications that we may be dealing with the enzyme–substrate dissociation constant, approximate ΔH and ΔS values (for association) have been tabulated for K_m (Table IV). If we could correctly interpret K_m as the equilibrium constant for the complex of I with the enzyme, it would appear that this complex has a very different structure from that of the others. The enthalpy values for substrates II, III and IV change only slightly with different selections of k_4. Since our errors in measurement of k correspond to only ± 0.3 kcal., these differences would appear to be significant and indicate increasing energies of binding corresponding to additional methyl groups.

TABLE IV

A FACTOR OF THE MICHAELIS-MENTEN CONSTANT (MOLES/LITER) (EQUATION 5) CALCULATED FROM EQUATION 6 AT 25°, *p*H 7.00 AND THE THERMODYNAMIC DATA CORRESPONDING TO THE POSSIBLE INTERPRETATION THAT THESE VALUES REPRESENT EQUILIBRIUM CONSTANTS

	$K_m \times 10^4$ (25°)	ΔH	ΔS
Acetylcholine	4.2–9.5	−(8.2–12.8)	−(14–29)
Dimethylaminoethyl acetate	12–13	−3.2	+2.5
Methylaminoethyl acetate	98–101	−1.6	+3.7
Aminoethyl acetate	160	−0.3	+7.5

This enzyme shows marked substrate inhibition when acetylcholine is the substrate. While a reasonable explanation for this phenomenon has been proposed[14] another explanation is suggested by the concept of a two-step hydrolytic process. According to the theory, the active site of this enzyme is made up of two subsites, one which interacts with the ester group and is therefore called the esteratic site, and a second negatively charged or anionic site which binds and orients substituted ammonium structures. The anionic site still persists, though perhaps somewhat sterically hindered, in the acetylated enzyme. The acetyl enzyme might therefore be expected to be able to bind an acetylcholine molecule at this site. It is not hard to imagine circumstances under which this would inhibit the hydrolysis of acetyl enzyme. This enzyme appears to be organized so as to interact most readily with small molecules by attacks along the line of the anionic-esteratic sites. This is shown by the fact that quaternary ions inhibit the hydrolysis of all esters and also inhibit the reactivation of diethylphosphoryl enzyme by noncharged reactivators as well as by reactivators containing a substituted ammonium function. If we assume then that the attacking water molecule in order to utilize the catalytic mechanism must approach from the direction of the anionic site, we have a plausible explanation of substrate inhibition.

This theory can be put into quantitative form by adding to the previous formulation

$$ES' + S \rightleftarrows ES'\cdot S;\ K_s'$$

and solving subject to the same steady state conditions to yield

$$v = \frac{k_3 E^0 S}{K_m + S\left(1 + \frac{k_3}{k_4}\right) + \frac{k_3 S^2}{k_4 K_s'}} \quad (5)$$

The requirement for substrate inhibition ($S > K_m$) is that S be comparable to $K_s'(1 + k_4/k_3)$. This relationship points up the importance of k_4/k_3 in determining substrate inhibition.

The present interpretation of the curvature of the Arrhenius plot when acetylcholine is the substrate in terms of the hydrolysis of acetyl enzyme as a rate-controlling step has certain consequences concerning the free energy of hydrolysis of the acetyl enzyme. Evidently rather large fractions of the total enzyme may at any instant be acetylated and since this fraction may not exceed the value corresponding to equilibrium of the reaction (ester + enzyme → acetyl enzyme + amino alcohol) it appears that the equilibrium constant for this reaction must be 1 or larger. The free energy of hydrolysis of acetyl enzyme would be,

(14) E. A. Zeller and A. Bisseger, *Helv. Chim. Acta*, **26**, 1619 (1943).

therefore, not much larger and perhaps considerably smaller than that of the hydrolysis of acetylcholine, namely, 3 kcal./mole.[15] The energy of activation for the hydrolysis of acetyl enzyme is evidently (Fig. 1) very low. The value for the estimated asymptote is 1200 cal./mole, and the corresponding entropy of activation is exceedingly high. The very unfavorable entropy of activation suggests a very loose structure for the acetyl enzyme and a high degree of ordering of the protein and the reactant water molecule in the transition state.

(15) S. Hestrin, *Biochim. Biophys. Acta*, **4**, 310 (1950).

The energies of hydrolysis and activation are of only limited help in seeking the chemical identity of the acetylated group because there may well be concomitant changes in the protein structure which very greatly alter the energy values from those corresponding to reactions of the same functional groups in simple compounds. This may be just the role of the protein.

The explanation offered here of decreasing energy of activation with increasing temperature may be of general applicability, particularly in those cases where substrate inhibition is also observed.

NEW YORK, N. Y.

[CONTRIBUTION FROM THE NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES, NATIONAL INSTITUTES OF HEALTH]

Stevioside. III. The Anomeric 2,3,4,6-Tetra-*O*-acetyl-1-*O*-mesitoyl-D-glucopyranoses and their Behavior with Alkali[1,2]

BY HARRY B. WOOD, JR.,[3] AND HEWITT G. FLETCHER, JR.

RECEIVED JULY 14, 1955

Treatment of 2,3,4,6-tetra-*O*-acetyl-1-*O*-mesitoyl-β-D-glucopyranose (VI) with alkali gives 1,6-anhydro-β-D-glucopyranose (II, levoglucosan) while the anomeric ester, 2,3,4,6-tetra-*O*-acetyl-1-*O*-mesitoyl-α-D-glucopyranose (X) yields no detectable quantity of this anhydride. The bearing of these facts on the configuration of the ester-linked D-glucopyranose residue in stevioside is pointed out and the analogy between these substances and the anomeric phenyl D-glucopyranosides with respect to their behavior with alkali is discussed.

In the first paper of this series,[2a] concerned with the structure of the three D-glucose moieties in the remarkably sweet natural glucoside, stevioside, it was shown that one of the D-glucose residues was joined to the large aglucon by esterification at its C_1-position with a highly hindered carboxyl group of the aglucon. At the time of this earlier work there was no evidence bearing upon the configuration of the C_1-carbon involved in the ester linkage; the object of the present paper is to describe further research designed to elucidate this question.

When stevioside (I) is heated with strong alkali the ester-linked D-glucose moiety is split off as levoglucosan (II, 1,6-anhydro-β-D-glucopyranose) and, upon acidification, the remainder of the molecule appears as steviolbioside (III). This transformation is a remarkable one; no other C_1-linked sugar ester of a carboxylic acid has previously, to the authors' knowledge, been cleaved to give a glycosan. Sugar esters normally hydrolyze through nucleophilic attack of the OH^- on the carbon of the carbonyl with acyl–oxygen scission occurring at the O—C=O linkage. In the case of the ester of a sterically hindered acid the carbonyl carbon is relatively inaccessible and the other C–O linkage is broken. If the ester is at C_1 of an aldose, the sugar fragment initially formed might be the same as that which has been postulated[4] as an intermediate in the alkaline cleavage of phenolic β-D-glucopyranosides to levoglucosan. Now it is well known that phenyl β-D-glucopyranoside readily gives levoglucosan when treated with alkali while its anomer, phenyl α-D-glucopyranoside, does not.[5] If the analogy of a C_1 sterically hindered ester to phenyl D-glucoside is a valid one we would predict that such a β-C_1-ester of glucose would give levoglucosan when treated with alkali but its anomer, an α-C_1-ester, would not. To test this point, the simplest and best-studied sterically hindered acid, mesitoic acid (VIII, 2,4,6-trimethylbenzoic acid) was chosen and ways were sought to obtain the two anomeric 1-*O*-mesitoyl-D-glucopyranoses.

When silver mesitoate (V) was condensed with tetra-*O*-acetyl-α-D-glucopyranosyl bromide (IV), a crystalline product giving appropriate analytical values for a 2,3,4,6-tetra-*O*-acetyl-1-*O*-mesitoyl-D-glucopyranose was obtained. From the mode of preparation and the fact that the rotation of the substance is small ($[\alpha]^{20}D$ +4.3° in chloroform)[6] the product is in all probability the β-anomer VI. Deacetylation with methanolic ammonia gave 1-*O*-mesitoyl-β-D-glucopyranose in crystalline form.

The synthesis of the α-isomer was initially attempted through condensation of tetra-*O*-acetyl β-D-glucopyranosyl chloride with silver mesitoate, but the only product isolated was found, as might be predicted, to be identical with that obtained earlier from the α-halide. Similarly, mesitoylation of 2,3,4,6-tetra-*O*-acetyl-α-D-glucose failed to give a new isomer. Attention was then turned to a method which was devised by Helferich and Schmitz-Hillebrecht[7] for the synthesis of the ace-

(1) Presented before the Division of Carbohydrate Chemistry at the 127th Meeting of the American Chemical Society, Cincinnati, Ohio, March 31, 1955.

(2) (a) Stevioside, I: H. B. Wood, Jr., R. Allerton, H. W. Diehl and H. G. Fletcher, Jr., *J. Org. Chem.*, **20**, 875 (1955); (b) stevioside, II: E. Mosettig and W. R. Nes, *ibid.*, **20**, 884 (1955).

(3) Chemical Foundation Fellow, 1953–1955.

(4) R. U. Lemieux and C. Brice, *Can. J. Chem.*, **30**, 295 (1952).

(5) E. M. Montgomery, N. K. Richtmyer and C. S. Hudson, THIS JOURNAL, **65**, 3 (1943).

(6) Rotations are specific rotations for the D line of sodium at 20°; concentration is expressed in g. of substance per 100 ml. of solution.

(7) B. Helferich and E. Schmitz-Hillebrecht, *Ber.*, **66**, 378 (1933).