XXIV. THE TEMPERATURE COEFFICIENTS AND ENERGY EXCHANGES OF THE CITRIC ACID DEHYDROGENASE OF CUCUMBER SEEDS.

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(Received January 1st, 1931.)

AMONG modern theories of chemical reaction the activation theory has proved to be one of the most fruitful not only in the hands of the physical chemists who were primarily responsible for its development, but also as a tool for the biochemist in his attempt to elucidate the mechanism of enzyme action. The theory postulates that an exceptionally high energy content is the most important of the criteria which determine the activated state preceding reaction, because the change of reaction velocity with changing temperature follows an expression of the same form as the law which expresses the variation with temperature of the number of molecules whose energy content exceeds an assigned value. The acceptance of this postulate has led to the view that in general the energy content of a molecule must be increased by an energy increment (the "critical increment" or "energy of activation") as a necessary prelude to chemical change of that molecule.

In terms of this theory the function of a catalyst is to aid the molecules of the reactant to attain the activated state—that is, to lower the critical increment. It is the examination of the way in which a heterogeneous catalyst lowers the critical increment of the molecules of a reactant which is germane to the problem of the mode of action of an enzyme. The action of the catalyst here can be described rather loosely as the straining of the substrate molecule, and this description has the merit of suggesting that the catalyst does work upon or yields energy to the molecule in activating it; but since it has become widely recognised that the forces in play at the boundary of any two phases are not purely physical but are chemical forces of valency, a more helpful picture of catalysis results from the legitimate postulate that the catalyst and reacting substance combine chemically, and that in the process the molecules of the reactant become activated.

This hypothesis may be applied to enzyme reactions in two ways. In the first method the measure of the critical increment may be ignored and attention may be concentrated on the changes wrought in the substrate molecule during activation, *i.e.* upon the straining of the molecule, which is most easily pictured as isomerisation. This mode of attack was employed by Quastel [1926]

and Quastel and Wooldridge [1927 and 1928] in studies of the enzyme systems of resting *B. coli*, although it is evident that Quastel did not regard the union of enzyme and substrate as a simple chemical reaction. The second method is to ignore the qualitative effect of activation on the substrate molecule and to examine the energy exchanges between the enzyme, the substrate and the surrounding system. This quantitative method has its own limitations—for instance it cannot lead us to a reason why citric acid dehydrogenase oxidises citric acid to acetonedicarboxylic acid while, in the absence of the dehydrogenase, reduction of the critical increment of citric acid by raising the temperature causes it to pass into aconitic acid—but it yields valuable data in other directions. The energy changes are measured indirectly by calculation from observations of the kinetics of the enzyme action, a fact of importance because at present the kinetics alone among the characteristic properties of an enzyme are amenable to exact quantitative measurement.

A rational description of the energy relations accompanying an enzymic reaction can be given on the assumption that the union of enzyme and substrate is a simple chemical reaction, following the hypothesis developed by Michaelis and Menten [1913] to account for the observed facts relating to the effect of substrate concentration on the rate of sucrose hydrolysis by saccharase, and subsequently applied with such marked success to the elucidation of many problems in the kinetics of this and of other enzymes. Their theoretical deduction of the relation between substrate concentration and initial reaction velocity for saccharase may be generalised in the following way. Let E represent the molecule of the enzyme, S the molecule of the substrate, and P the molecule of the product (or of one of the products if there are several). Then the enzyme and substrate are assumed to combine reversibly to form the compound ES which breaks down irreversibly into $E + P$, according to the equation $E + S \rightleftharpoons ES \rightarrow E + P$. At the commencement of reaction, the total molar concentrations of enzyme, substrate and the compound ES being represented by e , s and p respectively, the dissociation constant of the compound ES is given by

$$K_m = \frac{(s-p)(e-p)}{p} = \frac{s(e-p)}{p}$$

to a close approximation because s is supposed to be much greater than e ; and

$$p = \frac{es}{K_m + s}.$$

If the velocity constant of breakdown of ES to $E + P$ is written k , then the actual initial velocity of reaction

$$V = kp = \frac{k es}{K_m + s}.$$

This expression foretells the shape of curve obtained by plotting the initial velocity of reaction against substrate concentration to be a rectangular hyperbola, since V increases rapidly at first as s is increased from zero and then when s exceeds K_m further increase causes V to approach asymptotically the

limiting value $V_{\max} = ke$. Further, V will reach a figure equal to half the limiting value when $s = K_m$. Thus for an enzyme which follows the Michaelis equations, measurements of the initial rate of the reaction with varying substrate concentrations render it possible to calculate the value of the Michaelis constant K_m , which is one of the most characteristic constants of an enzyme. The assumption that the velocities of formation and dissociation of the enzyme-substrate compound are much larger than the velocity of its breakdown to the products of reaction is implicit in the above derivation; but there is evidence which suggests its validity [*cf.* Haldane, 1930, p. 41], and for the present it will be accepted as correct.

In considering the energetics of an enzyme action represented as above, there are three important and characteristic quantities which must be carefully distinguished. These are (1) the heat of formation of the compound ES , (2) the energy of activation, otherwise the critical increment, of the compound ES , which is necessary to enable it to react to yield the product P ; and (3) the heat of the reaction $S \rightarrow P$, which is the total heat of reaction of the complete process, and which is not of necessity related to the other heat quantities. The first quantity can be calculated when the equilibrium constant of the reaction $E + S \rightleftharpoons ES$ is known at two or more temperatures, with the aid of van't Hoff's reaction isochore

$$\frac{d(\log_e K)}{dT} = -\frac{Q}{RT^2},$$

where K is the equilibrium constant of the reaction, Q the heat of formation, T the absolute temperature, and R the gas constant. When an enzyme follows the Michaelis laws and the value of the Michaelis constant can be measured at different temperatures it is therefore possible to determine the heat of formation of the enzyme-substrate compound. The heat of activation of this compound can be calculated by the use of a similar expression due to Arrhenius, when the velocity constant k of its breakdown can be measured at two temperatures; and finally the heat of the catalysed reaction can be calculated by use of the isochore if the change of the total equilibrium with change of temperature is known. As the present considerations deal only with the initial stages of reaction this heat quantity is left out of account because it cannot be calculated from observations of the early stages of the reaction; attention has therefore been confined to the first two quantities and the relation between them.

The calculation of the first two of the separate heat quantities detailed above depends on the measurement of the temperature coefficients of the Michaelis constant and of the velocity constant k respectively, and since Haldane [1930, p. 38] has suggested that the characteristically low Michaelis constants of oxidising enzymes as compared with those of hydrolytic enzymes may indicate a fundamental difference between the two types, it is of immediate interest to discover whether the temperature coefficient of K_m is of the

same magnitude for enzymes of the two types¹. Before any generalisations can be made it will be necessary to obtain data about many enzymes, but as accurate data for the hydrolysing enzyme saccharase are already on record any figures for an oxidising enzyme will be of value for comparison. To obtain suitable data demands observations upon some typical oxidising enzyme, the desiderata which govern the choice being that the Michaelis constant is small and is susceptible of reasonably accurate measurement over some suitable temperature range.

Such an enzyme is the citric acid dehydrogenase described recently by Thunberg [1929]. Fig. 1 embodies data given by Thunberg in his paper, and

Fig. 1. The relation of initial velocity of reaction to substrate concentration for the citric acid dehydrogenase of cucumber seeds. Drawn from data given by Thunberg [1929].

it shows that the dehydrogenase has a low Michaelis constant (which can be read approximately from the graph as the substrate concentration at which the initial velocity reaches half its maximum value) and suggests that it should be possible to measure this constant with considerable accuracy. The curve deviates from the ideal rectangular hyperbola to a considerable extent at the lowest substrate concentrations because it is impossible with the technique available to measure at all accurately the initial rate of reaction with very small concentrations of substrate. The experimental method consists of the observation of the time taken for a standard small amount of the substrate to be oxidised, the concentration of the substrate at the commencement of the observation being known. It is thus implicitly assumed that the velocity throughout the observation remains constant at the initial value, and error is introduced because this can only be true with relatively high concentrations. Fortunately this difficulty vanishes if the lowest concentration at which

¹ Professor J. B. S. Haldane suggested this problem to the author, pointing out the importance which would be attached to the discovery of a high temperature coefficient of K_m for an oxidising enzyme.

accurate measurement of the reaction time is possible is less than the concentration at which the enzyme is half saturated by its substrate, because corrections can then be applied in order to calculate the true initial velocity from the apparent initial velocity of the reaction.

These considerations led the writer to attempt the measurement of the temperature coefficients of K_m and of the velocity of reaction for citric acid dehydrogenase in order to gain an insight into the energy exchanges occurring during its action.

EXPERIMENTAL METHODS.

All observations were made by the method of Thunberg [1928]. The dehydrogenase was allowed to act on its substrate in a Thunberg vacuum tube, and the hydrogen acceptor used was methylene blue. Each individual observation consisted in noting the time taken for the reduction of a standard amount of methylene blue under carefully controlled conditions of temperature, p_H , substrate concentration, enzyme concentration, etc.

The enzyme solutions employed in this work were prepared from seeds of the cucumber (*Cucumis sativa*) which were obtained from Messrs Weibull of Landskrona, Sweden, and bore the trade name "Västeras Grön Druv." For each series of observations a sufficient quantity of the seeds was shelled to give 5 g. of kernels, which were then ground in a mortar with 25 cc. of 0.87 % dipotassium hydrogen phosphate for 20 minutes. The milky liquid so obtained was centrifuged for 10 minutes at a speed of approximately 4000 r.p.m., at the end of which time it had separated into three layers. The bottom layer contained solid residue, the second layer which was still rather milky in appearance contained the active material, and the top layer consisted of the seed fats. The aqueous layer was separated from the others and was ready for immediate use.

In a single experiment a series of Thunberg vacuum tubes was prepared each containing 0.4 cc. of a solution of potassium citrate of known concentration. To each tube in turn were added 0.3 cc. of a 1 in 60,000 solution of methylene blue and 0.5 cc. of the enzyme solution; the tubes were then evacuated at the water-pump for 90 seconds, closed and immersed in a water-bath of which the temperature was kept constant within 0.05°. The concentrations of the potassium citrate solutions added at first were so graded that the complete reaction mixtures formed a series in which the citrate concentration varied from 4 mM to 400 mM, and other factors were constant. The time for the complete decoloration of the methylene blue in each tube was observed.

This method is similar in essentials to the one employed by Thunberg [1929] for the estimation of citric acid by means of this dehydrogenase, and the following comments may be made on it.

(1) As the total volume of reaction mixture in each tube is only 1.2 cc., it is essential that the tubes be thoroughly dried before use.

(2) The reaction is accelerated by fats and oils, so that the reaction mixture must not under any circumstances be allowed to come into contact with the grease lubricating the tap at the top of the tube. It is an advantage to put the whole of the enzyme preparation in a small Büchner flask and evacuate the vessel for a few minutes before making up the reaction mixtures, as the amount of frothing in the individual vacuum tubes during evacuation will be greatly diminished thereby. For the same reason the vacuum tube must be completely immersed in the thermostat. If the top of the tube is above the water level of the bath, distillation will occur within it and water from the reaction mixture will condense within the cooler tap and come into contact with the tap grease as it runs down the tube into the reaction mixture again. The reduction time of the methylene blue may be decreased by 50 % if this precaution is not taken.

(3) Evacuation by a good water-pump for 90 seconds reduces the oxygen concentration in the tube sufficiently.

(4) A fresh enzyme solution must be prepared for each series of observations, and kept at 0° if not used immediately.

(5) The measurements must be made at the optimum p_H of the system [cf. Nelson and Bloomfield, 1924].

(6) It was found that the results obtained always contained some irregular variations which could not be eliminated, therefore in the determination of the temperature coefficients the writer, after becoming conversant with the technique of the observations, performed a series of five experiments at 25° with different samples of active material, and from the readings calculated the mean value of K_m at that temperature. In the same way a mean value of K_m at 35° was measured and the temperature coefficient of the constant was determined from the two mean values.

EXPERIMENTAL RESULTS.

Using the enzyme in phosphate solution as described above, it was found that at the end of the period of observation the p_H of the reaction mixture as determined by means of the quinhydrone electrode was always 7.84 ± 0.03 . On account of the necessity of measuring the temperature coefficient at the optimum p_H of the system, a preliminary examination of the p_H -activity relationship of the dehydrogenase was made in which the initial velocity of reaction was determined at different hydrogen ion concentrations, all measurements being made with substrate concentrations high enough to ensure that the enzyme was fully saturated by its substrate. The activity was found to rise from a minimum in acid solutions (the enzyme was precipitated from solution below p_H 3.5) to a maximum at about p_H 7, and in more alkaline solutions the activity remained constant until a p_H above 10 was reached. This result is expressed in the curve shown in Fig. 2. All experiments carried out with the enzyme in dilute secondary phosphate solution were therefore done within the optimum activity range of the system.

The reduction time of the methylene blue in each of the tubes of all ten experiments is given in Table I. To calculate the value of K_m from the figures for each experiment it is first necessary to work out the initial velocity of reaction V at each concentration of citrate, relative to the initial velocity $V_{\max}$ which is attained when the citrate is sufficiently concentrated to saturate the

Fig. 2. The p_H -activity curve for the citric acid dehydrogenase.

enzyme fully. The velocity of reduction of the methylene blue in each tube is proportional to the reciprocal of the reduction time, therefore the reciprocal of each of the reduction times recorded in each experiment has been tabulated and multiplied by 100 for convenience. The velocity of oxidation of the citrate in any tube is taken as proportional to the difference between the velocity of reduction of methylene blue in that tube and the velocity of reduction of methylene blue in the blank tube of the experiment (which contains no citrate). Finally, as in each experiment it was found that the initial velocity reached a maximum when the citrate concentration was 200 mM , the initial velocity at this concentration is taken as unity and the initial velocity at each of the other concentrations is expressed as a fraction of the initial velocity with 200 mM citrate, under the title $\frac{V}{V_{\max}}$. The successive figures obtained in this way from Exp. 1 are set out in Table II as an example of the procedure adopted to calculate values of $\frac{V}{V_{\max}}$ at each concentration in each experiment. In Table III the whole of the figures for $\frac{V}{V_{\max}}$ obtained in the complete series are collected together.

To find the heat of activation of the enzyme-substrate compound it was necessary to measure the reaction velocity with citrate concentrations large enough to saturate the enzyme completely; readings were taken both at 25°

Table I.

Reduction of methylene blue by citric acid in presence of citric acid dehydrogenase at p_H 7.84. Each tube contained 0.5 cc. of enzyme solution in 0.87 % secondary potassium phosphate, 0.4 cc. of potassium citrate solution of varying concentrations, and 0.3 cc. of a 1 in 60,000 solution of methylene blue.

Exp. No.	Temp.	Reduction time in minutes									
1	25°	120	115	92	92	100	63	32	27	23	27
2	"	71	70	54	64	64	54	30	27	21	23
3	"	56	70	44	64	47	41	*	21	21	24
4	"	76	85	76	81	83	59	43	38	37	31
5	"	99	93	98	92	89	69	75	37	33	30
6	35°	61	60	60	53	58	41	24	26	26	24
7	"	52	55	50	43	41	36	22	22	23	22
8	"	64	64	44	*	54	35	22	19	19	19
9	"	76	72	64	66	54	49	26	24	22	22
10	"	71	65	55	57	59	46	31	29	27	26
Citrate mM		0	4	8	16	20	40	80	100	200	400

* Indicates no figure obtained.

Table II.

Calculation of $\frac{V}{V_{max}}$ at each concentration for Exp. 1.										
Citrate mM	0	4	8	16	20	40	80	100	200	
Reduction time minutes	120	115	92	92	100	63	32	27	23	
$\frac{100}{\text{Reduction time}}$	0.83	0.87	1.09	1.09	1.0	1.58	3.11	3.7	4.35	
$V = \frac{100}{\text{Reduction time}}$	-0.83	0	0.04	0.26	0.26	0.17	0.75	2.28	2.87	3.51
$\frac{V}{V_{max}}$	0	0.01	0.07	0.07	0.05	0.21	0.64	0.82	1.0	

Table III.

Values of $\frac{V}{V_{max}}$ for all tubes of Exps. 1 to 10, calculated from the reduction times given in Table I.

Exp. No.	$\frac{V}{V_{max}}$									
1	0	0.01	0.07	0.07	0.05	0.21	0.64	0.82	1	
2	0	0	0.12	0.04	0.04	0.12	0.55	0.65	1	
3	0	0.12	0.16	0.08	0.11	0.22	—	1	1	
4	0	0.1	0	0.05	0.06	0.23	0.74	0.98	1	
5	0	0.03	0	0.04	0.06	0.22	0.57	0.81	1	
6	0	0.01	0.01	0.11	0.04	0.37	1.15	1	1	
7	0	0.04	0.03	0.16	0.2	0.35	1.08	1.08	1	
8	0	0	0.2	—	0.07	0.35	0.81	1	1	
9	0	0.03	0.09	0.07	0.18	0.25	0.86	0.97	1	
10	0	0.06	0.18	0.15	0.13	0.33	0.80	0.89	1	
Citrate mM		0	4	8	16	20	40	80	100	200

and at 35° with the same enzyme preparation. These measurements were made on tubes which contained 0.5 cc. of enzyme solution, 0.3 cc. of 1 in 60,000 methylene blue and 0.4 cc. of a solution of potassium citrate, which made the citrate concentration of the reaction mixture 400 mM. The ratio k_{35}/k_{25} was found to be 1.61. Repetitions of the observation with other enzyme preparations gave values of 1.70 and 1.64; and one observation over

the wider temperature range from 15° to 35° gave the figure 2.71 for the ratio of the maximum velocities of reaction at these two temperatures.

In addition to the measurement of the temperature coefficients, some observations on other qualitative and quantitative properties which have not yet been fully investigated may be reported briefly. The fatty layer which separates on centrifuging the cucumber seeds ground up in phosphate possesses the power of accelerating the action of the enzyme: a fact which has also been noted by Thunberg (private communication to the author). Citraconic acid accelerates the action slightly, but on the other hand aconitic acid inhibits competitively, just as was found for the citric acid dehydrogenase of liver by Bernheim [1928]. Finally it was noticed that under certain conditions the enzyme preparation is much more slowly destroyed on standing than is suggested by Thunberg [1929] and other workers of his school, *e.g.* Scherstén [1929]. This was investigated in order to ensure that the destruction of the enzyme at 35° was not so great as to cause serious error in the measurement of the temperature coefficients. In a typical experiment it was found that under the standard conditions specified above, 0.5 cc. of a freshly prepared enzyme solution caused the reduction of 0.4 cc. of 1 in 60,000 methylene blue in presence of 400 *mM* citrate in 49 minutes, while after the enzyme had been kept at 35° for one hour it brought about an identical reduction in 54 minutes. The destruction of the enzyme was therefore slight. As it is to be expected that the destruction in presence of the substrate occurs more slowly, the error so introduced into the measurement of the temperature coefficients is negligible.

Calculation of the energy exchanges.

(1) *The heat of formation of the enzyme-substrate compound.* The first step is to calculate the value of K_m at 25° and at 35°. From the data of Table III it can be seen that the figures for $\frac{V}{V_{\max}}$ are extremely scattered for concentrations less than 40 *mM* and cannot be used in the determination of the temperature coefficient. This indeed is to be expected because the concentration of methylene blue in the reaction mixtures is approximately 10 *mM* (reckoning the molecular weight as 400), and serious errors are introduced when the concentration of the methylene blue approaches that of the citrate. The figures for $\frac{V}{V_{\max}}$ are however definitely greater at 35° than at 25° for 40 *mM* and 80 *mM* citrate solutions; and the figures for 100 *mM* solutions show plainly that the enzyme is saturated by its substrate at lower concentrations of citrate at 35° than at 25°. The form of the calculation by which K_m is derived from the figures for $\frac{V}{V_{\max}}$ magnifies greatly the experimental errors if $\frac{V}{V_{\max}}$ is greater than 0.5, therefore the calculations which follow are based on the $\frac{V}{V_{\max}}$ figures for the 40 *mM* citrate solutions in each experiment.

Since at a substrate concentration s ,

$$V = \frac{kes}{K_m + s},$$

and since the limiting value $V_{\max.} = ke$, it follows that

$$\frac{V}{V_{\max.}} = \frac{s}{K_m + s},$$

and therefore

$$K_m = s \left(\frac{V_{\max.}}{V} - 1 \right).$$

From each experiment of the series K_m has been calculated by means of this formula, and the figures are given in Table IV.

Table IV.

The Michaelis constants calculated from the data of Table III.

Exp. No.	1	2	3	4	5	6	7	8	9	10
K_m	150	293	142	134	142	68	74	74	120	81

Although these results are scattered, there is no overlapping of the figures for the first five experiments at 25° with those for the last five at 35°, and it is clear that there is a considerable shift of K_m due to a temperature change of 10°. The mean value at 25° is 172 and at 35° it is 83; a decrease which corresponds to a notable heat of reaction.

Before accepting these as the best available figures the errors they involve must be discussed. First there are the random errors of measurement which are partially eliminated by taking the mean values at each temperature and are probably quite small. Secondly, errors are introduced by a real difference between the Michaelis constants of different enzyme preparations. The figures for Exps. 2 and 9 vary from the means for their respective groups so much as to decide this point. Thirdly, in each figure is incorporated an error due to the relatively large concentration of methylene blue which it is necessary to use in the reaction mixture. The first and second errors are not serious because they affect the figures for K_m at both temperatures to the same extent, and will not therefore alter the ratio $K_m^{35^\circ}/K_m^{25^\circ}$ significantly. On the other hand, the third error varies in magnitude as the Michaelis constant changes, and as the change of Michaelis constant with temperature here recorded is much greater than has heretofore been discovered, it is necessary to show that the change cannot be due to this error distorting the results. The treatment which follows here shows that the error introduced is quite small, and although the true values of K_m are all rather smaller than those given in Table IV the ratio of the mean value at 35° to the mean value at 25° is almost unaffected.

The error is due to the fact that the concentration of the methylene blue is so large that it causes the citrate concentration to fall by an appreciable amount during the reaction. If t_0 be the reduction time of the blank tube and t_{40} the reduction time of the tube containing 40 *mM* citrate in any experiment, then in the tube containing the citrate the number of millimols of citrate reduced by the "blank process" is about $10 \times t_{40}/t_0$, since the initial concentration of methylene blue is approximately 10 *mM*. The number of millimols reduced by the citrate is therefore $10 (1 - t_{40}/t_0)$, and the concentration of the citrate must fall by this amount during the reaction. Thus for experiment 1 this

quantity is $10(1 - 63/120)$, or 4.75 mM . In calculating K_m it is necessary therefore to take into account that the citrate concentration fell from 40 to 35.25 mM , and this can be done with sufficient accuracy by substituting for the initial concentration the average concentration during the experiment, *i.e.* 37.6 instead of 40 . Then the corrected value of K_m is $37.6 \left(\frac{V_{\max}}{V} - 1 \right)$ and this is $37.6 \left(\frac{1}{0.21} - 1 \right)$, or 141 , instead of 150 , which is the uncorrected figure. In Table V the corrected Michaelis constants for each experiment are tabulated.

Table V.

The figures for the Michaelis constant corrected for the fall of citrate concentration during the reaction.

Exp. No. K_m (corr.)	1	2	3	4	5	6	7	8	9	10
	142	281	137	130	137	65	71	70	115	78
	Mean value K_m at 25° 165 mM .									
	,, ,, ,, 35° 80 ,,									

From these figures the heat of formation of the enzyme-substrate compound is calculated by substitution in the integrated form of the reaction isochore. The expression

$$\frac{d(\log_e K)}{dT} = - \frac{Q}{RT^2}$$

can be integrated on the assumption that Q is constant, giving

$$\log_e \frac{K_2}{K_1} = \frac{Q(T_2 - T_1)}{RT_1 T_2},$$

where K_2 and K_1 are the equilibrium constants at T_2 and T_1 respectively. The heat of reaction is generally assumed to be constant over a small temperature range such as 10° , so by substitution, since the equilibrium constant of the forward reaction $E + S \rightarrow ES$ is the reciprocal of the Michaelis constant:

$$2.3 \log_{10} \frac{K_m^{25^\circ}}{K_m^{35^\circ}} = \frac{Q}{1.99} \times \frac{(308 - 298)}{298 \times 308},$$

therefore
$$2.3 \log \left(\frac{165}{80} \right) = \frac{Q}{1.99} \times \frac{10}{298 \times 308}$$

and
$$Q = + 2.3 \times 0.3144 \times 1.99 \times \frac{298 \times 308}{10} = + 13460 \text{ cal.}$$

The heat liberated by the combination of citric acid dehydrogenase and citric acid is $13460 \text{ cal. per g. molecule of citric acid}$.

(2) *The heat of activation of the enzyme-substrate compound.* The mean of the three figures obtained for the ratio of maximum reaction velocity at 35° to the maximum velocity at 25° is 1.65 , and by substitution in the expression

$$E = \frac{RT_1 T_2}{T_2 - T_1} \log_e \frac{k_2}{k_1},$$

the energy of activation is found to be $9139 \text{ cal. per g. molecule}$.

DISCUSSION.

The observations described above lead definitely to the conclusion that when the citric acid dehydrogenase combines with its substrate a large amount of heat is set free, and although the figure given is not accurate, it can be said with confidence that the heat of formation of the enzyme-substrate compound is about 12,000 or 15,000 cal. per g. molecule. This is a fairly large figure, and larger indeed than the heat of activation of the enzyme-substrate compound, which is about 9000 cal. per g. molecule. As far as the writer is aware the only other enzyme for which both the corresponding energy values have been determined is yeast saccharase. Euler and Laurin [1920] measured the heat of formation of the saccharase-sucrose compound and concluded that it was about 2000 cal. per g. molecule. On the other hand, Nelson and Bloomfield [1924] in the course of a very careful study of the kinetics of this enzyme found that the heat of formation of the enzyme-substrate compound was zero within the limits of experimental error. The heat of activation of the saccharase-sucrose compound was found by them to be 8400 cal. per g. molecule at 30°, in agreement with the figures obtained by Euler and Laurin and other workers.

It is evident that although the energy of activation of the enzyme-substrate compound is of the same order for yeast saccharase and for citric acid dehydrogenase at 30°, the heat of formation of the compound at the same temperature is quite large for the dehydrogenase and zero for the saccharase. Whether this distinction is a general one between oxidising and hydrolysing enzymes cannot be decided until similar data are available for many other enzymes of both classes, but the possibility must be born in mind. If it holds, it will take its place with the generalisations that hydrolyses proceed with much smaller heats of reaction than oxidations, and that the Michaelis constants of oxidising enzymes are much smaller than those of hydrolysing enzymes.

The fact that in the case of the citric acid dehydrogenase the heat of formation of the enzyme-substrate compound is greater than the energy of activation of the same compound, suggests that the action of the enzyme may be pictured in terms of the energy exchanges as follows: the enzyme molecule unites with a molecule of substrate and heat energy is thereby released which can immediately be absorbed by the enzyme-substrate compound. This absorption of energy by the compound serves to activate it and it then passes from the activated state to the final state of enzyme molecule + molecules of the product or products. Such a sequence of energy exchanges could not however apply when an enzyme-substrate compound has a heat of formation less than its energy of activation, as is the case with saccharase.

SUMMARY.

The Michaelis constant of the citric acid dehydrogenase of cucumber seeds has been measured at 25° and at 35°. At 25° it is $1.65 \times 10^{-4} M$, and at 35° it is $8 \times 10^{-5} M$. Thus the temperature coefficient $Q_{10} = 0.48$ at 30° and

the heat of formation of the citric dehydrogenase-citric acid compound as calculated from the temperature coefficient is 13460 cal. per g. molecule.

The temperature coefficient of the velocity of the reaction catalysed by the dehydrogenase has also been measured and $Q_{10} = 1.65$ at 30° . From this the calculated energy of activation of the citric dehydrogenase-citric acid compound is 8790 cal. per g. molecule.

These figures are contrasted with the corresponding ones for yeast saccharase and the significance of the contrast is discussed briefly.

My thanks are due to Professor T. Thunberg for advice concerning the use of the dehydrogenase, and to Professor J. B. S. Haldane for many valuable suggestions. I wish also to thank Sir F. G. Hopkins for much help and encouragement.

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