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Reversible Inactivation of Enzymes at Low Temperatures. Studies of Temperature Dependence of Phosphatase- and Peroxidase-catalyzed Reactions

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RECEIVED SEPTEMBER 7, 1954

The liquid phase enzymic reactions studied exhibited pronounced deviations from the Arrhenius equation at sub-zero temperatures. The postulation of a reversible equilibrium between active and inactive enzyme satisfactorily accounted for the non-linearity of the Arrhenius plots. The inactivation of the enzyme at low temperatures is attributed to an increase in intramolecular hydrogen bonding.

It is now well-known that application of the Arrhenius equation to enzymic reactions both *in vivo* and *in vitro* gives rise to non-linear plots indicating increasing activation energies as the temperature is lowered. Claims of sharp breaks for plots of logarithm of the rate constant against the reciprocal of the temperature have been critically evaluated by Kistiakowsky and Lumry.[1] These authors have shown that such breaks cannot be demonstrated with the experimental evidence available; instead the experimental data give rise to smooth curves, although a considerable straight line portion exists for the higher temperatures used. In the specific case of the urease-catalyzed hydrolysis of urea in the presence of sulfite ion they were able to explain the curvature in the Arrhenius plot by postulating a reversible inhibition of urease by sulfite ion.

The widespread occurrence of non-linearity of Arrhenius plots for enzyme-catalyzed reactions suggests a behavior pattern inherent in enzymes. Kavanau[2] has proposed just such a behavior pattern. The enzyme is considered as existing in an active and an inactive form which are in reversible equilibrium with each other. The model Kavanau suggests is an inactive form which is not sufficiently unfolded, perhaps because of the formation of intramolecular hydrogen bonds. This is the reverse of the effect postulated for irreversible high temperature denaturation, the unfolding and breaking of hydrogen bonds. Kavanau presents evidence to show that the behavior of a number of enzyme systems is in accord with this interpretation. However, many enzyme systems do not exhibit anomalous behavior when data are plotted on the basis of the Arrhenius equation. The data are generally limited to the region above 0° for obvious reasons in aqueous media. Also, the examples of Kavanau are limited to hydrolytic enzyme reactions. Evidence in support of the suggestions of Kavanau based on data for an oxidizing enzyme as well as for a hydrolytic enzyme in the temperature range +20° to −30° using aqueous mixtures are presented herein.

Experimental

The direct spectrophotometric technique for obtaining initial reaction rates and the low temperature apparatus, described[3] previously, were used in this work.

Enzyme Solutions.—Dried bovine intestinal phosphatase (Armour Laboratories) was freshly prepared in aqueous solutions with 0.02 *M* ammonium hydroxide–ammonium chloride buffer, *p*H 9.9, for each experiment. The concentrations of phosphatase used in the 23% sodium chloride, 60% glycerol and 40% methanol systems were 1.3, 2.0 and 2.7 mg./ml., respectively. A crude turnip peroxidase solution free from catalase and polyphenolase activity was prepared[3] and stored in small glass vials at −20°. Enzyme solutions of varying activities were prepared by water dilution of the peroxidase solution. During the course of a rate study (4–8 hours) the enzyme solution being used was held at 0° to ensure stability.

Substrate Solutions.—The alkaline phosphatase substrate solution consisted of 9 ml. of one of the concentrated solutions (60% glycerol, 40% methanol or 23% sodium chloride solution) to which 1 ml. of 0.05 *M* disodium *p*-nitrophenyl phosphate was added.[4] The crystalline disodium *p*-nitrophenyl phosphate was stored at −10° and fresh solutions were prepared for each experiment. The peroxidase substrate solution consisted of 20 ml. of one of the concentrated solutions (60% glycerol, 5 *M* acetate buffer or 40% methanol solution) to which 1 ml. of 0.04 *M* guaiacol and 1 ml. of 1 *N* hydrogen peroxide were added.[5]

Reaction Mixture.—The reaction mixture consisted of 3 ml. of one of the substrate solutions plus 0.15 ml. of one of the enzyme solutions. The enzyme concentration used with each concentrated solution varied, but all data have been corrected to an equivalent enzyme concentration.

Reactions.—Alkaline phosphatase catalyzes the hydrolysis of disodium *p*-nitrophenyl phosphate to the sodium salt of *p*-nitrophenol and phosphate ion. The rate of hydrolysis was obtained by measuring the increase in absorbance with time at 400 mμ.[4] Liquid phase reaction rate studies were made over the temperature range +20° to −24° in the following concentrated media: 60% (w./w.) aqueous glycerol, 40% (w./w.) aqueous methanol, and 23% (w./w.) sodium chloride solution all of which were 0.1 *M* with respect to ammonium hydroxide–ammonium chloride buffer, *p*H 9.9.[6] In addition they all contained 0.0005 *M* magnesium ion. The magnesium was necessary as an enzyme activator.[4]

The reaction involved in the peroxidase system was an oxidation of guaiacol to tetraguaiacoquinone[7] and a concomitant reduction of hydrogen peroxide to water. The rate of formation of the tetraguaiacoquinone was followed by measuring the increase in absorbance with time at 430 mμ. Liquid phase rate determinations were made over the temperature range +20° to −30° in the following concentrated media: 60% (w./w.) aqueous glycerol, *p*H 6.1; 5 *M* sodium acetate–acetic acid buffer, *p*H 5.3; and 40% (w./w.) aqueous methanol which contained 0.25 *M* sodium acetate–acetic acid buffer, *p*H 5.5.

Non-enzymic Catalysis.—A non-enzymic catalyst, hemin, was used in place of peroxidase to catalyze the oxidation of guaiacol. Reaction rates were determined in 50% (w./w.) aqueous methanol and 60% (w./w.) aqueous ethanol both of which were buffered at *p*H 5.5 with 0.25 *M* acetate buffer. Over the temperature range studied, +20° to −30°, good conformance to the Arrhenius equation existed.

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

(2) J. L. Kavanau, *J. Gen. Physiol.*, **34**, 193 (1950).

(3) V. P. Maier and A. L. Tappel, *Anal. Chem.*, **26**, 564 (1954).

(4) A. Bessey, O. H. Lowry and M. J. Brock, *J. Biol. Chem.*, **164**, 321 (1946).

(5) J. D. Ponting and M. A. Joslyn, *Arch. Biochem.*, **19**, 47 (1948).

(6) R. Aschaffenburg and J. E. C. Mullen, *J. Dairy Res.*, **16**, 58 (1949).

(7) J. B. Sumner and G. F. Somers, "Chemistry and Methods of Enzymes," Academic Press, Inc., New York, N. Y., 1947, p. 217.

Results and Discussion

The experimental results obtained are shown in Figs. 1–3. A pronounced deviation from the Arrhenius straight line relationship exists in each case; however, no sharp breaks occur. That this deviation from the Arrhenius equation is due to some effect of the low temperature on the protein moiety of peroxidase is indicated by the results of the hemin-catalyzed oxidation of guaiacol. The composition of the hemin system differed from that of the peroxidase system essentially in only one component, the protein portion of the enzyme.

The treatment of the data given in Figs. 1–3 is similar to that given by Kistiakowsky and Lumry.[1]

Consider the equilibrium between active and inactive enzymes

$$E_a = E_i \quad (1)$$

$$[E_0] = [E_a] + [E_i] \quad (2)$$

$$K = [E_i]/[E_a] \quad (3)$$

where E_0 refers to the total enzyme and E_a and E_i refer to the active and inactive forms. The rate of formation of products in the region where the reaction is zero order with respect to substrate is given by

$$\frac{d[P]}{dt} = k[E_a] \quad (4)$$

If the equilibrium is such that substantially all of the enzyme is in the active form

$$\frac{d[P]}{dt} = k[E_0] \quad (5)$$

This condition will be met at the higher temperatures provided ΔH for the equilibrium is negative.

If the equilibrium is such that substantially all of the enzyme is in the inactive form

$$\frac{d[P]}{dt} = \frac{k}{K}[E_0] \quad (6)$$

This condition will be met at the lower temperatures for ΔH negative. Furthermore, an Arrhenius plot will give a straight line for the first condition and an activation energy, U. The second condition likewise gives a straight line on a Arrhenius plot with an activation energy, $U - \Delta H$. Since ΔH is negative, the activation energy corresponding to this condition will be greater than U by the absolute value of ΔH. In the intermediate region

$$\frac{d[P]}{dt} = \frac{k[E_0]}{K+1} \quad (7)$$

An inspection of the data shows that at the higher temperatures used, the first condition is indeed met. If the Arrhenius plot for this region is extrapolated to the lower temperature region, the difference between the extrapolated and experimental values is equal to log $(K + 1)$. An example of the application of this method is presented graphically in Fig. 4. The data show good agreement with the van't Hoff equation which is a necessary condition for our interpretation of the experimental results. A value of ΔH may be obtained from the plot as well as values of K over the temperature region studied. The values of K thus obtained were used

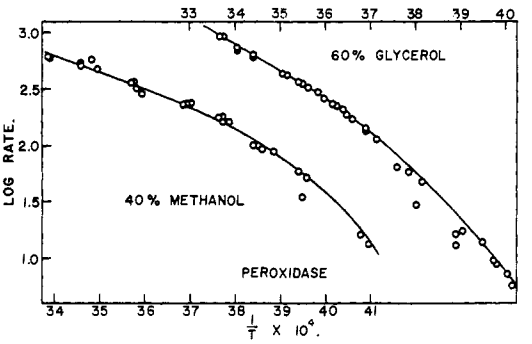

Fig. 1.—Arrhenius plots showing the experimental data (circles) and the theoretical curves (solid lines).

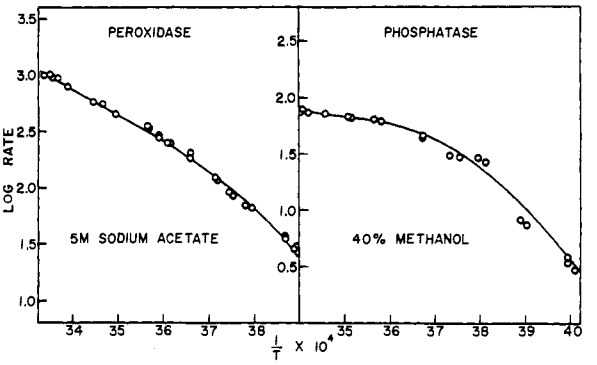

Fig. 2.—Arrhenius plots showing the experimental data (circles) and the theoretical curves (solid lines).

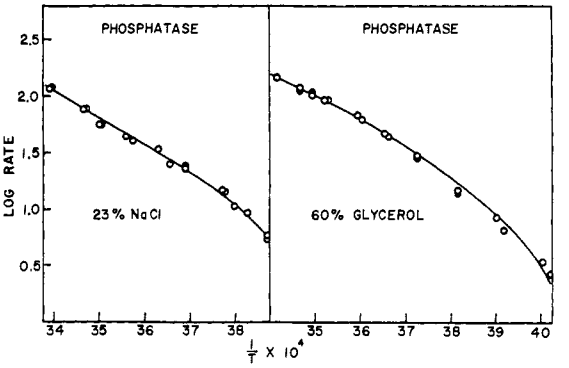

Fig. 3.—Arrhenius plots showing the experimental data (circles) and the theoretical curves (solid lines).

to extrapolate the higher temperature Arrhenius plot to the lower temperature region. The curves of Figs. 1–3 are theoretical ones based on the above interpretation. The points shown are the experimental values.

Thermodynamic values calculated for the equilibrium at $-10.0°$ are given in Table I. The last 3 values in the table relate to available data on the reversible denaturation of proteins. The difference in sign for $\Delta H°$ and $\Delta S°$ for these proteins as compared to that for peroxidase and phosphatase is compatible with the concept of hydrogen bond breaking for the high temperature denaturation and hydrogen bond forming for the low temperature reversible inactivation of enzymes. Further, if $\Delta H°$ is ascribed to the energy difference of hydrogen

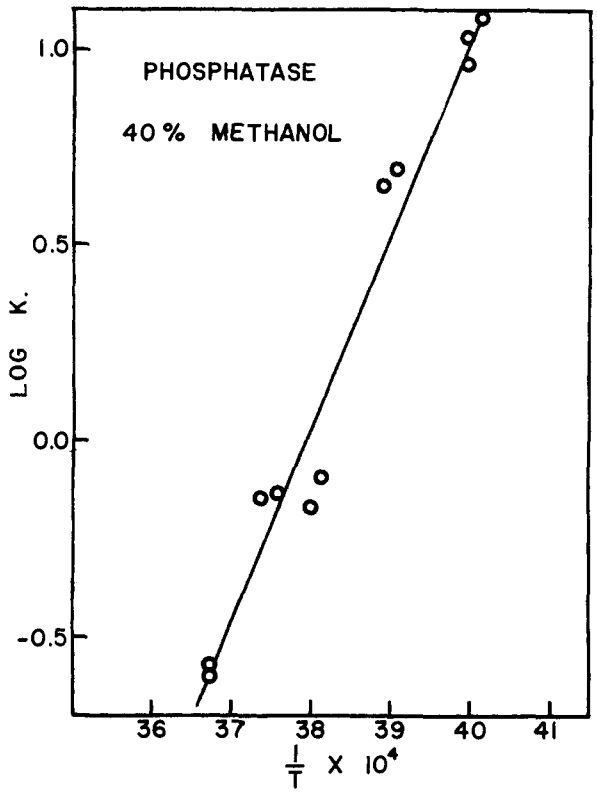

Fig. 4.—Dependence of K, the equilibrium constant for inactivation, on temperature.

bonded compared to unbonded structures, presumably 4–7 kcal. per mole of hydrogen bond,[8] one finds an entropy change of 12–22 e.u. per mole of hydrogen bond broken in the proteins and (−16)–(−28) e.u. per mole of hydrogen bond formed in the enzyme inactivation.

(8) A. E. Stern, *Adv. Enzymol.*, **9**, 25 (1949).

TABLE I

FREE ENERGIES, HEATS AND ENTROPIES FOR THE EQUILIBRIUM BETWEEN ACTIVE AND INACTIVE ENZYME (T = 263°K.) AND FOR REVERSIBLE DENATURATION OF SOME PROTEINS

Enzyme and solvent	$\Delta F°$, kcal.	$\Delta H°$, kcal.	$\Delta S°$, e.u.
Peroxidase 40% methanol	0.770	−18.9	− 75
Peroxidase 60% glycerol	.294	−16.8	− 65
Peroxidase 5 M sodium acetate	.452	−21.8	− 85
Phosphatase 40% methanol	− .050	−22.3	− 85
Phosphatase 60% glycerol	.440	−17.8	− 68
Phosphatase 23% sodium chloride	.764	−25.6	−100
Trypsin[9] (50.0°)	−1.27	67.0	213.1
Chymotrypsinogen[10] (47.2°)	−1.44	99.6	316
Soy bean trypsin inhibitor[11] (50.0°)	−0.95	57.3	180

(9) A. E. Mirsky and M. L. Anson, *J. Gen. Physiol.*, **17**, 393 (1934).

(10) M. A. Eisenberg and G. W. Schwert, *ibid.*, **34**, 583 (1951).

(11) M. Kunitz, *ibid.*, **32**, 241 (1948).

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NOTES

The Preparation of Alkyl ψ-Oxatriazoles[1]

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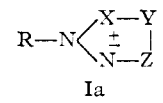
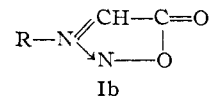
RECEIVED SEPTEMBER 7, 1954

A class of heterocyclic compounds (I) which has contributed a certain amount of interest in connection with structural requirements is probably best known by the special case, the sydnones. The proposed *meso*-ionic representation (Ia)[2] and the more recently suggested use of a coördinate covalent bond between the two ring nitrogen atoms in sydnones (Ib)[3] have indicated the complex nature of this ring system.

An additional member of this class of heterocycles



Ia



Ib

X = >CH, >CR or >N

Y-Z or Z-Y = $-O-\overset{\overset{O}{\|}}{C}-$, $-N-\overset{\overset{O}{\|}}{C}-$, $-N-\overset{\overset{R}{|}}{C}-\overset{\overset{O}{\|}}{C}-$ or $-O-\overset{\overset{N-R}{\|}}{C}-$

in which X = −N− and Y-Z = $-O-\overset{\overset{O}{\|}}{C}-$ has been known since 1933.[4] Certain aryl ψ-oxatriazoles (III)[5] were prepared by treating nitroform with an appropriate diazonium salt.[4,6] Alkyl ψ-oxatriazoles now have been obtained from the action of

(1) This research was supported by the Office of Ordnance Research, U. S. Army, under Contract No. DA-01-009-ORD-331.

(2) W. Baker, W. D. Ollis, V. D. Poole, J. A. Barltrop, R. A. W. Hill and L. E. Sutton, *Nature*, **160**, 366 (1947).

(3) J. C. Earl, *Chem. and Ind.*, 746 (1953).

(4) G. Ponzio, *Gazz. chim. ital.*, **63**, 471 (1933).

(5) The use of the prefix, ψ, to indicate the hybrid nature of the corresponding sydnones (ψ-oxadiazoles) was suggested by W. Baker, W. D. Ollis and V. D. Poole, *J. Chem. Soc.*, 307 (1949).

(6) A. Quilico, *Gazz. chim. ital.*, **63**, 912 (1932).