our extrapolated value of 2.423×10^{-6}, equation (16), at the lower temperature. Although the difference is small in view of the variation of D_0 over this temperature interval, the direction is that which would be anticipated from increased hydration at the lower temperature.

Acknowledgments.—The authors are deeply appreciative of the interest and suggestions of Dr. J. W. Williams throughout the progress of this research. They are also much indebted to Miss Ruth Christofferson, Miss Jean V. Thomas and Mrs. Dorothy C. Gosting for aid in performing calculations. The coöperation of Mr. Edwin Hanson in the design of the refractometer cell and the construction of the cell by him are gratefully acknowledged.

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Summary

Diffusion results obtained at 1 and at 25° using the Gouy interference method are reported for aqueous sucrose solutions at concentrations below 6 g./100 ml. These data provide further confirmation of the theory of this method. Specific gravities and relative viscosities were determined over the same concentration range so that tests of equations relating the concentration dependence of the diffusion coefficient to viscosity and thermodynamic factors might be made. Gordon's relation was found to hold with high precision for this system. Specific refractive increments for sucrose have been derived from the diffusion experiments and from a separate refractometer cell.

Additional theory utilizing the Airy integral has been presented as an improvement over the previous theory of Kegeles and Gosting for fringes farthest from the slit image.

MADISON 6, WISCONSIN RECEIVED JANUARY 18, 1949

[CONTRIBUTION FROM THE GIBBS LABORATORY, HARVARD UNIVERSITY]

Anomalous Temperature Effects in the Hydrolysis of Urea by Urease

BY G. B. KISTIAKOWSKY AND RUFUS LUMRY

Several investigations of the rates of biochemical processes have shown the effect of temperature to be more complex than would be expected on the basis of simple Arrhenius hypothesis. In particular, it has been claimed[1] that joined segments of straight lines are observed on plotting the logarithm of the rate constant against inverse temperature, with sharp breaks at "critical" temperatures. Crozier[1] has proposed the theory of "Master" or "Pacemaker" reactions which are supposed to account for the sudden changes in activation energy. This theory has been repeatedly discussed[2] but a brief analysis from the point of view of chemical kinetics will further clarify the situation.

A curvature in the line of an Arrhenius plot, providing it does not exceed, say, 100 cal./degree, finds ready kinetic or statistical interpretation,[3] but discontinuous changes in activation energy do not unless a first order phase transition of at least one of the reactants is assumed to occur. While such cannot be denied *a priori* for processes in living cells, they are inconceivable in homogeneous solutions of enzymes *in vitro*. Actually it is impossible to prove a discontinuity in activation energy from *rate measurements alone;* such measurements can at most establish that two straight segments of an Arrhenius plot are joined by a curve extending over a finite temperature range. In fact, experimental precision must rise to rather unusual heights in kinetic work to limit a suspected range of curvature to only a few degrees. For instance, if the two activation energies differ by 3,000 cal./mole, an extensive and statistically well planned series of tests, with a standard deviation of 1%, could restrict the curved region to within 2°, with a level of confidence somewhat greater than 90%. Contrasting these considerations with the precision and extent of published data, one begins to doubt altogether the reality of the majority of claimed anomalies. A few, however, do appear to be genuine and call for a kinetic interpretation.

Consider first the proposition that two competing mutually non-exclusive elementary reactions are involved. For instance, that what was thought to be a pure molecular species is in reality a mixture of two enzymes; or that each enzyme molecule has several active centers which catalyse the reaction with different activation energies; or that the reaction may proceed through the intermediate formation of two different "critical complexes." Any such mechanism may be readily ruled out because it demands that the activation energy increase with rising temperature, whereas in cited instances just the reverse is observed. Furthermore the curved region of the Ar-

(1) W. J. Crozier, *J. Gen. Physiol.*, **7**, 189 (1924), and other publications; I. W. Sizer, *ibid.*, **22**, 719 (1939); I. W. Sizer and B. S. Gould, *Enzymologia*, **8**, 75 (1940); I. W. Sizer and E. S. Josephson, *Food Research*, **7**, 201 (1943); I. W. Sizer, *Annals of Surgery*, **121**, 231 (1945); S. Darling, *Nature*, **160**, 838 (1947).

(2) W. J. Crozier, *J. Gen. Physiol.*, **7**, 189 (1924); A. C. Burton, *J. Cell. and Comp. Physiol.*, **9**, 1 (1937); M. F. Morales, *ibid.*, **30**, 303 (1947).

(3) V. K. La Mer, *J. Chem. Phys.*, **1**, 289 (1933). S. Glasstone, K. J. Laidler and H. Eyring, "The Theory of Rate Processes," McGraw-Hill Book Company, Inc., New York, N. Y., 1941.

rhenius plot would not be limited to only a few degrees, unless at least one of the activation energies was inordinately large: to limit the markedly curved region of the plot to only two degrees at 295° K., one of the activation energies would have to be greater by at least 200,000 cal./mole. The observed activation energy changes would produce by this mechanism only gently curved plots over the entire temperature range accessible to biochemical rate measurements.

Mechanisms that appear to have lower activation energies at higher temperatures may be devised if consecutive as well as parallel elementary reactions, in brief stationary reaction chains, are postulated. But the requirement that the change from one over-all activation energy to another be completed within a range of very few degrees presents the same difficulties as mentioned above because stationary chain reactions give over-all rate expressions which are simple algebraic functions of the component rate constants. A term in such expressions does not overwhelm another, hitherto dominant term, upon a temperature change of only a few degrees, unless temperature coefficients of component rate constants are themselves extremely high. A proof that no stationary chain mechanism will produce sharp bends in Arrhenius plots, without involving very high individual activation energies, is impossible to adduce, but we do not believe that such chains exist.

Non-stationary (branching) chains would have to be of the degenerate[4] type to explain even qualitatively the non-explosive character of the biochemical reactions. But then they are ruled out because their essential attribute, the induction period, is not observed in enzymic reactions *in vitro*.

The possibility also exists that an enzyme may exist in two (or more) forms which transform reversibly or irreversibly into one another and which differ in their catalytic activities. If mobile equilibrium exists between two forms and the Arrhenius plot consists of two straight segments with a bend between them, the catalysis by one form must predominate below the bend, that by another above. Allowing for unavoidable experimental uncertainties in the determination of the Arrhenius plot, this means that one rate must gain at least a factor of ten over the other within the temperature range of the bend. If the latter extends over only two degrees, the heat of the equilibrium reaction must be at least 200,000 cal./mole and the entropy change (if the specific activities of the two forms are comparable, so that at the temperature of the bend they are present in comparable concentrations) at least 700 e.u. The value of the entropy change in the equilibrium reaction does depend on the specific activities of the two forms but whichever way one changes the assumptions, somewhere in the processes involved there occurs an abnormally large entropy change. The calculated minimum magnitude of these enthalpy and entropy changes is inversely proportional to the temperature range within which the bend of the Arrhenius plot is completed. If less confidence is placed in the accuracy of the published data on the sharpness of the bends, one arrives at figures which are entirely comparable to those which have been observed in thermal denaturation of proteins.[5] In fact, it has been repeatedly demonstrated that at higher temperatures the Arrhenius plots of enzyme catalyzed reactions show steep downward curvatures[6] owing to the thermal deactivation process. This phenomenon may be looked upon as a special case of the coexistence of two forms of the enzyme, one being completely inactive. Equilibria between active and inactive forms of enzymes have been qualitatively described also in numerous publications on their inactivation by various ions and by certain organic substances. In the case of Luciferase several cases of inhibition have been quantitatively investigated by Johnson and interpreted by him and by Eyring and their co-workers.[6] When the inactivation process is not reversible and its rate is slow, the resultant kinetics are complex, but need not concern us here. The other case, that of a fast and reversible deactivation, is readily amenable to treatment.[6] The reaction equation may be written generally as

$$n\mathrm{A} + m\mathrm{X} = i\mathrm{I} + k\mathrm{Y}$$

where A stands for an active center on an enzyme molecule and centers on n different molecules react with m molecules of an inhibitor to form i inactive centers and k molecules of some substance Y. We need not define the active center in detail except to note that it may consist of several active groupings in the enzyme, provided that they react as a unit in the inhibition reaction (*e. g.*, two –SH groups oxidizing to –S–S–). The above reaction is represented by an equilibrium constant

$$K = \frac{(\mathrm{I})^{i/n}(\mathrm{Y})^{k/n}}{(\mathrm{A})(\mathrm{X})^{m/n}} \qquad (2)$$

with

$$RT \ln K = -\Delta H + \Delta TS \qquad (3)$$

where ΔH is the heat of reaction per mole of these active centers. Then, at temperatures at which the active form is present practically exclusively this equilibrium has, of course, no effect on the kinetics of the enzymic reaction and therefore the activation energy has its true value, E. At temperatures at which the active form has become but a small fraction of the total, a constant activation energy will be also observed, equal to $E - \Delta H$. In the intermediate temperature range the activation energy changes, the Arrhenius plot is therefore curved and the shape of it depends somewhat

(4) Semenoff, "Chemical Kinetics and Chain Reactions," Clarendon Press, Oxford, 1935.

(5) Reviewed by I. W. Sizer, "Advances in Enzymology," Interscience Publishers, Inc., New York, N. Y., 1943, Vol. III, p. 35.

(6) *Cf.* Sizer, above; also F. H. Johnson, H. Eyring, R. Steblay, H. Chaplin, C. Huber and G. Gherardi, *J. Gen. Physiol.*, **28**, 463 (1944).

on the order (n) of the equilibrium reaction in the enzyme. It will be noted that an equilibrium between an active and an inactive form of the enzyme must always result in lower activation energy at higher temperatures[2]; it is the true activation energy when ΔH is negative and therefore the deactivation reaction predominates at low temperatures; it is $E - \Delta H$ if ΔH is positive (as in thermal denaturation reactions) and hence deactivation is promoted by a rise in temperature.

On the following pages it will be shown that the anomalies of the temperature coefficient of an enzymic reaction are due to the former type of equilibrium. The experiments here described were initiated with the broad objective of testing the authenticity of claimed "breaks." The experiments were therefore confined to one of the better investigated reactions *in vitro*, the hydrolysis of urea by urease, an enzyme crystallized and otherwise investigated by Sumner.[7] In a series of papers Sizer[8] has shown that urease catalysis in buffered but sulfite-free solutions has a constant activation energy of about 8700 cal.; that at a sulfite concentration of approximately 0.035 M the Arrhenius plots show a break at about 22°, the activation energy being 8700 cal. above and 11,700 cal. below this temperature. At still higher sodium sulfite concentrations the higher activation energy is observed over the entire accessible temperature range. These observations, whose consistency was good but not complete, were made with the enzyme from different sources and in different states of purity. It was suggested that the oxidation–reduction potential of the solutions is involved in the change of activation energy because Sizer had also observed that in solutions containing strong oxidizing agents, such as ferricyanide ion, the activation energy was changed to the higher value.

Experimental

The original objective of these experiments was the determination of the temperature interval over which the Arrhenius plot is curved, the presumption being that it is straight both above and below this region. To be able to narrow the curved region to a few degrees requires accurate and numerous rate data and the spontaneous deactivation of stock solutions of the enzyme calls for a special design of the experiments. We did not attempt to maintain the enzymic activity constant throughout the course of the experiments but compared instead almost simultaneous rate measurements at different temperatures on aliquot portions of the same enzyme solution, handled in the most reproducible manner known to us. Since a statistical analysis of the problem had shown that doing pairs of rate measurements would require an excessively large accumulation of data for a statistically significant result, three thermostats were employed and triplet experiments were run. A fairly extensive preliminary series of experiments, which we shall not reproduce here to save space, convinced us that there was no break under conditions specified by Sizer, the Arrhenius plot being straight above 22° and gradually curving to higher activation energies at lower temperatures. The design of the experiments was therefore changed and for the data reported below the three thermostats were set at temperatures differing by 5 to 10° from one another and thus relatively oriented were moved over the accessible temperature range, the result being a series of temperature coefficients over overlapping temperature intervals.

The thermostats were water-filled, with liquid cooling coils, electric heating elements and automatic temperature controls such that the temperatures could be maintained constant to ± 0.01° over the short times (ten to fifteen minutes) involved in individual experiments. Temperatures were read with Beckmann thermometers calibrated against a Bureau of Standards calibrated mercury thermometer.

After extensive experimentation with procedures involving volumetric determination of carbon dioxide evolved in the hydrolysis of urea, this technique was discarded because of the uncertainties caused by the fairly high solubility of carbon dioxide in buffered and nearly neutral solutions. It proved to be more accurate to determine the ammonia formed. The Nesslerization technique of Sumner and Hand,[9] with only minor modifications, proved to be reasonably satisfactory, though leaving something to be desired because of the inaccuracies introduced by the use of the Fisher filter photometer to measure the transmission of Nessler solutions. Most of the standard deviation of individual data, about 2%, was due to the use of this instrument.

An individual experiment started with the pipetting of 1 cc. of a urea–phosphate buffer solution into one compartment of the reaction flask, urea concentration being so chosen (3%) that after mixing with the urease solution and indeed throughout the entire reaction period the rate of urea hydrolysis would be independent of the urea concentration, that is, the reaction would be on the plateau of the Michaelis curve. One cc. of a urease solution containing from 0.1 to 0.6 Sumner unit[9] of enzymic activity was pipetted into another compartment of the reaction flask. These compartments were made by sealing two 40-mm. long thimbles of 14-mm. glass tubing to the closed end of an 80 mm. × 17 mm. test-tube so as to form an inverted Y with a 130° angle between the thimbles. The filled reaction flask was then immersed in one of the thermostats. After allowing five minutes for the solutions to reach thermal equilibrium, the reaction was started by rapidly tipping the flask back and forth so that the two solutions would run from one compartment to the other. Following one minute of this mixing procedure the flask was left in the bath for the remainder of the reaction time, a total of four minutes, but occasionally three to five minutes. Reaction was stopped with 1 cc. of 1 N sulfuric acid which was blown very rapidly from a pipet into the flask. The acid was thoroughly mixed with the reactant solutions by hand shaking, the whole being then transferred with suitable washings into a 250-cc. volumetric flask, to which was added 5 cc. of Nessler reagent. The transmission of the resulting solution for light of about 4250 Å. wave length was determined after a ten-minute waiting period. Exacting reproducibility in the operations and a

TABLE I

COMPARISON OF THERMOSTATS

Data in horizontal rows secured in the same run.

Bath 1, mg. NH_3-N/min.	Bath 2, mg. NH_3-N/min.	Bath 3, mg. NH_3-N/min.
0.0657	0.0678	0.0672
.0668	.0663	.0655
.0668	....	.0668
....	.0678	.0670
.0660	..	.0652
.0663	.0673	.0662
σ (single) .0006	.0009	.0009
σ (mean) .0003	.0005	.0004

(7) J. B. Sumner and G. F. Somers, "Chemistry and Methods of Enzymes," Academic Press, New York, N. Y., 1947, 2nd ed.

(8) I. W. Sizer, *J. Gen. Physiol.*, **22**, 719 (1939); *J. Biol. Chem.*, **132**, 209 (1940); *J. Bacteriol.*, **41**, 511 (1941).

(9) J. B. Sumner and D. B. Hand, *J. Biol. Chem.*, **76**, 149 (1928).

number of minor precautions were necessary to maintain standard deviations of individual runs at or below 2%. That such standard deviations were achieved is shown by the data of Table I which compares experiments performed in all three thermostats set to identical temperatures.

Pure urease is difficult to prepare and is extremely unstable in solution. Furthermore Sizer, in his three papers on the anomalous temperature behavior of urease, found that the state of purity of the enzyme was not related to the presence or absence of the break in the Arrhenius plot. Impure urease was therefore used for most of the experiments described in this paper. One brief series of experiments was carried out using a once-recrystallized sample of urease prepared in a manner similar to that of Sumner and Dounce.[10] These experiments on the considerably purer material confirmed Sizer's conclusion since both preparations gave the same unusual Arrhenius plot.

Urease solutions were prepared by Mr. J. F. Ambrose, of this Laboratory, as follows: Jackbean meal was extracted with distilled water, the *p*H of this solution was adjusted to 5.3 with acetic acid and the resulting precipitate which contains very little urease was removed by centrifuging. Aliquots of the remaining solution were diluted with glass-distilled water a few days before use to secure solutions for individual experiments. All solutions were stored at 0° at which temperature the crude concentrates were stable for one or two months. The dilute solutions had varying stability some being stable to 20% for one or two weeks, others losing as much as 10% per day. The crude concentrate had enzymic activities of about 50 Sumner units per cc., the pure preparation about 2200 Sumner units per cc.

In experiments requiring the presence of sodium sulfite the aliquots of crude or pure urease preparations were diluted with the appropriate sulfite solution which had previously been prepared from a stock solution with the following composition: Na_2SO_3, 2.54 g.; $NaHSO_3$, 0.57 g.; glass distilled water, 96.89 g. (0.272 m. in total sulfite ion, *p*H 7.0). All chemicals other than the urease preparations were of C. P. grade, recrystallization having been proven to have no effect on the results. The stock solution of sulfite had to be prepared frequently due to the oxidation of sulfite by air. The half-cell oxidation-reduction potentials, E_h, as determined by using a Beckmann *p*H meter Model G fitted with a platinum electrode, increased from +0.250 volt to about +0.300 volt for the dilute urease solutions which contained 0.068 *M* sulfite ion during the period they were considered suitable for use. A 15° lowering of temperature produced a reduction of only about 0.015 volt in this potential, a change comparable to that which took place during the course of a run. Unsulfited urease solutions had oxidation-reduction potentials of about +0.400 volt as did the urea-buffer solutions.

Though the conditions used by Sizer were generally duplicated in this work, it was found that the phosphate buffer he used partially precipitated below 10° with consequent large changes in *p*H. A weaker buffer without this defect was, therefore, generally used:

3.0 g. Urea · *p*H = 6.97 ± 0.03 at 25°
1.4 g. KH_2PO_4
6.4 g. $Na2HPO_4{\cdot}7H_2O$
89.2 g. H_2O

This buffer had such a capacity that the change in *p*H during any run was never greater than 0.2 unit and generally considerably less. At the upper end of the temperature range, the original buffer of Sizer was used. It has the same *p*H but about twice the buffering capacity. Control experiments showed that the effect of the resulting changes in ionic strength on enzyme activity was very slight. The concentration of urea in the reaction mixture (1.5%) was that reported by Sumner[7] to give a reaction of zero-order in urea at *p*H 7. A number of experiments were conducted to show that this was true and to show that the urea concentration did not change sufficiently during a run to take the reaction off the zero-order plateau. These experiments also served as a proof that the change in *p*H during a run was not sufficient to alter the rate of reaction. The results of a typical experiment of this type are shown in Table II. Ammonia-free water for the dilutions which preceded Nesslerization was prepared by passing ordinary distilled water through a column of an exchange resin, Amberlite IR-100, in its acid form, the water then being stored in contact with some of the resin. The blanks with this water were highly reproducible and low. Control experiments identical with the other experiments except that the acid was added immediately, provided the necessary correction to the Nessler transmission measurements. The transmission was measured against water blanks and the result, corrected for the control experiments, was interpreted in terms of milligrams of ammonia-nitrogen per cc. by comparison with a calibration curve. This curve was prepared from the transmissions of control samples to which were added known amounts of ammonium chloride. It was necessary to calibrate each new preparation of Nessler reagents. It was also found that light transmission varied with ionic strength so that different concentrations of buffer or sulfite required different calibration curves.

TABLE II

INSENSITIVITY OF REACTION RATE TO CHANGES IN *p*H AND UREA CONCENTRATION DURING A RUN

mg. of ammonia nitrogen produced per minute at 28°

Duration of runs	
4 min.	6 min.
0.0672	0.0668
.0670	.0668
.0654	.0655
.0670	.0668
.0660	.0663
.0652	.0657
.0663	.0664
σ (mean) .0004	.0003

The Nessler reagent[11] was made with glass-distilled water.

The Results

Although the order of the reaction is not of direct concern to us, studies of the dependence of the rate on urea and urease concentrations and on the duration of the run were made. They showed that within the limit of precision of the method (about 1%) the rate was independent of the urea concentration, was proportional to the urease concentration and remained constant over the duration of the runs, providing urease samples with enzymic activities not much greater than 0.7 Sumner unit per cc. (hydrolyzing about 5% of the total urea in five minutes at 35°) were used.

As mentioned before, the standard deviation of individual measurements was approximately 2% in nearly 80% of all runs. In the remaining 20% much larger deviations were observed, always in the direction of lower rates. Notwithstanding various changes of technique and added precautions we were unable to eliminate these deviations. In Table III, which is a summary of all final data, the activation energies which stand alone or are uppermost, when two values are given, are the averages of the activation energies calculated from

(10) A. L. Dounce, *J. Biol. Chem.*, **140**, 307 (1941).

(11) American Public Health Association, "Standard Methods for the Examination of Water and Sewage," New York, N. Y., 1933.

TABLE III

SUMMARY OF ACTIVATION ENERGIES, E

Enzyme Sumner units	Sulfite concn., M	ΔT_1, °C.	$E_1 \pm \sigma$,[a] cal./mole	ΔT_2, °C.	$E_2 \pm \sigma$,[a] cal./mole	ΔT_3, °C.	$E_3 \pm \sigma$,[a] cal./mole
Varied	0.000	5–20	8,850 ± 50	10–20	8,830 ± 130		
Varied	.034	5–20	~13,000			5–10	~15,500
Varied	.034			10–20	11,160 ± 165		
Varied	.000	20–30	8,510 ± 160				
Varied	.034	20–30	8,840 ± 265				
0.58	.034	0–7	14,750 ± 210	7–15	12,000 ± 550	0–15	13,150 ± 365
.58	.034	5–13	12,950 ± 185	13–20	10,500 ± 220	5–20	11,950 ± 190
.72	.034	11–20	10,900 ± 111				
.58	.034	13–22	10,450 ± 164				
.19	.034	24–31	8,850 ± 790	31–39	9,100 ± 600	24–39	8,750 ± 275
.58	.034	20–24	8,350 ± 670	24–31	8,200 ± 425	20–31	8,300 ± 405
.25	.034	20–24	10,150 ± 465	24–31	9,500 ± 92	20–31	9,650 ± 138
		20–24	9,250[b]		8,850[b]		8,980[b]
.25	.034	23–28	8,950 ± 570	28–36	8,750 ± 410	23–36	8,840 ± 295
.15	.136	22–28	9,600 ± 660	28–36	9,660 ± 360	22–36	9,700 ± 250
			10,500		9,700		10,100
.15	.136	5–12	15,400 ± 1000	12–20	12,500 ± 465	5–20	13,500 ± 153
			14,250		12,500		13,400
.25	.034	5–12	12,900 ± 470	12–20	10,050 ± 445	5–20	11,600 ± 210
			12,700		10,700		11,650

[a] The ± quantity following most of the "activation energies" is the standard deviation of the mean from that value of the "activation energy." [b] Average of preceding two listings.

the individual runs, the large deviations, however, not being included in the averages when greater than three standard deviations of a single observation, calculated from all data. It was sometimes possible to perform several experiments before the enzyme solution had undergone deactivation sufficient to be observable in the rate measurements. In these cases activation energies were computed from the averages of the rates thus making a more efficient use of the data. Where pairs of activation energies are recorded in Table III, the lower value was secured in this way and these values have been used when available for plotting the Arrhenius curve, Fig. 1. It should be noted however that this selection of values has no influence on the conclusions reached in this paper.

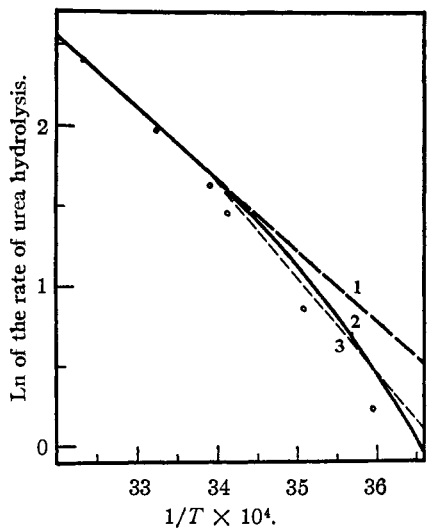

Fig. 1.—Plot of the rates of urea hydrolysis.

Table III shows that in sulfite-free solutions the activation energy is constant and equal to 8830 cal. In the presence of 0.034 M total sulfite (mixture of sulfite and bisulfite ions) the activation energy has the same value at temperatures above about 20°, but gradually increases to about 15,000 cal. near 5°. In the presence of 0.136 M sulfite the activation energy starts to increase at higher temperatures, so that virtually no straight portion can be found on the Arrhenius plot (noticeable thermal deactivation occurred at about 40°). Although a comparison of rate data not belonging to the same set of runs is complicated by the instability of urease stock solutions, our original data, most of which is summarized in Table III, are sufficient to establish two additional points: (1) addition of 0.034 M sulfite does not affect the absolute rate of reaction at higher temperatures and hence depresses it at lower; (2) this effect is reversible in the sense that at a definite temperature an enzyme solution shows a definite activity in the presence of sulfite ions, regardless of its previous thermal history.

Since the temperature intervals over which the activation energy data were obtained overlap, Table III may be used to construct the complete Arrhenius plot of the reaction, with and without sulfite ions, just as though all the runs had been made on enzyme solutions of identical activity. Where the plot is curved, successive adjustments must be made in drawing the curve but otherwise the procedure is straightforward in view of the additional observations mentioned above. The result of the construction, Fig. 1, shows that the

true curve for the unsulfited reaction (curve 1) is a straight line corresponding to an activation energy of 8830 cal. in close agreement with Sizer's finding of 8700 cal. In the presence of 0.034 *M* sodium sulfite curve 2 results, whereas curve 3 is Sizer's interpretation of his data with this concentration of sulfite. At higher temperatures the agreement between curve 2 and Sizer's published data is well within the combined errors of the two investigations but at lowest temperatures Sizer's straight line is considerably above our curved plot. Professor Sizer has privately informed us that his data, both published and unpublished, showed lower reaction rates in the neighborhood of 0° than could be fitted to his straight line. Thus, allowing for Sizer's estimate of the accuracy of his data as ±5%, no disagreement in fact remains.

If the curved Arrhenius plot is caused by a reversible inhibition of urease by sulfite, quantitative information on this equilibrium is derivable from Fig. 1 since at any temperature the distance between line 1 and line 2 is the logarithmic ratio between the total and the active enzyme. Hence one may calculate for various temperatures the values of a partial equilibrium constant from which reference to sulfite ions is omitted. This has been done for $n = 1$ (*cf.* eqn.(1)). The results of these calculations are shown in Fig. 2 which is so plotted that the slope of the line is equal to the (negative) heat of reaction. One would expect that this reaction heat remains constant over the small temperature interval involved, if the proposed interpretation of the Arrhenius plot as a reversible inhibition of urease is correct. This is indeed reasonably well the case, judging from Fig. 2, although the highly indirect nature of this plot makes the data rather inaccurate. That the order of the inhibition reaction is first with respect to urease is not proven by Fig. 2. However, several, otherwise similar, experiments in Table III were run at different concentrations of the enzyme and yet the observed activation energies were identical. Under the conditions of the experiments this indicates that the inactive fraction of the enzyme does not depend on enzyme concentration, or that the inactivation reaction is first order in enzyme. For such reaction the plot of Fig. 2 gives a heat of reaction equal to $\Delta H = -29{,}000$ cal. per mole of active centers as defined earlier but the precision of this figure is very low. The order of reaction in sulfite ions is in principle calculable from a comparison of experiments with 0.034 and 0.136 *M* sulfite concentrations, but the data of Table III and Fig. 1 lead to uncertain results, the order changing with temperature. It is approximately 2 at 20° and about 1 at 12°, but these figures should be taken with considerable caution because of the difficulties of fitting the 0.136 *M* curve to the other on account of its curvature persisting to higher temperatures. Since the influence of improper fit of the two curves on the calculated order of the reaction decreases with decreasing temperature, first order appears to be the more probable.

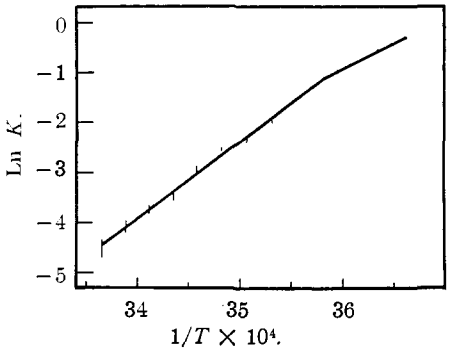

Fig. 2.—Plot of the inhibition equilibrium constant

The uncertainty about the number of sulfite ions involved in the deactivation of an active center as well as lack of positive knowledge of chemical structure of such centers precludes a positive identification of the chemistry of the inhibition equilibrium. It is not excluded that the reaction involved is similar to the combination of several organic sulfonic acid ions with serum albumin investigated by I. Klotz[12] and his co-workers. On the other hand, it has been repeatedly suggested that sulfhydryl groups play a preeminent role in the activity of hydrolytic enzymes. Thus Hellerman[13] has shown that upon reaction of porphyrinden or *p*-chloromercuribenzoate with some seventeen most reactive sulfhydryl groups of urease the activity of the enzyme remained unaltered but was totally destroyed upon the reaction of the remaining seventeen available sulfhydryl groups on the same molecule. It is most unlikely that sulfite ions remove sulfhydryl groups by oxidizing them to the dithionic groups, –S–S–, but an observation of Clarke[14] on cystine suggests a possible mechanism of the inhibition. He found the following reactions to take place

$$\mathrm{COOHCH(NH_2)CH_2SSCH_2CH(NH_2)COOH + Na_2SO_3 \rightleftharpoons}$$
$$\mathrm{COOHCH(NH_2)CH_2SSO_3Na + COOHCH(NH_2)CH_2SNa}$$
$$\mathrm{+ H_2O + H^+ \longrightarrow 2COOHCH(NH_2)CH_2SH + H_2SO_4}$$

of which the first is rapid and reversible, while the second is very slow in nearly neutral solutions. Presumably a very similar reaction is responsible for the effect of bisulfite ions on keratin and several other proteins investigated by Lindley and others.[15] Because of presence of atmospheric oxygen and other (unintentional) oxidizing agents an equilibrium may exist in solutions of urease between the reduced (active) and the oxidized (inactive) forms of the enzyme, which is far on the side of the active form unless strong oxidizing agents are deliberately added. In presence of sul-

(12) I. M. Klotz, THIS JOURNAL, **68**, 2299 (1946).
(13) L. Hellerman, F. P. Chinard and V. R. Deitz, *J. Biol. Chem.*, **147**, 443 (1943).
(14) H. T. Clarke, *ibid.*, **97**, 235 (1932).
(15) H. Lindley, *Biochem. J.*, **42**, 481 (1948).

fite ions the reaction investigated by Clarke may take place, removing the oxidized form and thus forcing more of it to be formed from the active enzyme.

It has been mentioned already that several other Arrhenius plots are quite similar to that described by Sizer for urease. We believe that the interpretation offered here—and also suggested by Morales—a reversible inhibition of an enzyme by one or more of the constituents of the solution, holds true for them as well. The data on the rate of heart beat of rats described by Crozier, Pincus and Renshaw[16] and shown in Fig. 3 are a good example. It is very evident that a straight line at higher temperatures, followed by a curve at lower temperatures (drawn for a reaction heat of −42,000 cal., if the reaction involved is of first order) describes the data extremely well and it avoids all the grave theoretical difficulties arising from a belief in breaks or sharp bends in Arrhenius plots. Thus one must conclude that reversible enzyme inhibitions play an important role in biological processes at low temperatures. Their specificity should provide an excellent tag to be used in an identification of specific enzymes controlling the overall rate of observable complex processes in living cells.

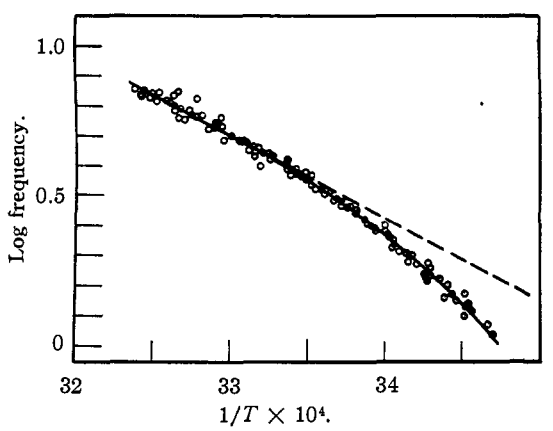

Fig. 3.—Temperature dependence of the frequency of rat heart beat.

In conclusion we would like to return to the uninhibited catalysis by urease. For this reaction the present measurements provide an accurate value of the activation energy, 8830 cal. On the other hand Sumner[7] has determined that 1 g. of purest enzyme hydrolyses 0.0158 mole of urea per second at 20° under conditions which insure that the reaction is of zero order in urea concentration. Applying the Michaelis mechanism of enzyme catalysis to urease

(1) enzyme + substrate ⟶ compound k_1
(2) compound ⟶ enzyme + substrate k_2
(3) compound ⟶ enzyme + products k_3

(16) W. J. Crozier, G. Pincus and B. Renshaw, *J. Gen. Physiol.*, **18**, 491 (1934).

$$(4)\quad -\frac{d(\text{substrate})}{dt} = \frac{k_3(\text{substrate})(\text{enzyme})}{(\text{substrate}) + (k_3 + k_2)/k_1}$$

we see that those are conditions when the entire active enzyme is present as the compound with the substrate and that therefore the information refers to the rate constant k_3. Since Sumner has also determined the molecular weight of urease as 483,000, we are able to compute the absolute reaction rate per mole of urease and so, in conjunction with the above value for the activation energy, to compute the entropy of activation for the third process, in which the compound between enzyme and substrate forms a critical complex prior to forming reaction products. The result is −12.5 e. u., with water, which undoubtedly takes part in the formation of the complex, being taken at unit activity in the liquid state at the concentration in which it exists in these dilute solutions. The above calculation, however, assumes that from one mole of the enzyme one mole of the compound is formed; in other words that a urease molecule has only one active center combining with one molecule of urea. If urease molecules have m such identical independent active centers, each of which combines with a molecule of urea, then the absolute rate per mole of compound is m times slower than assumed above and hence the entropy change per mole of compound (or of urea) is $-12.5 - R \ln m$ e. u. If the centers are not identical, one may still conclude that the entropy change, on the average, is more negative than −12 e. u.

Summary

Claims that Arrhenius plots of biochemical processes show sharp breaks at critical temperatures are rather numerous in literature. A kinetic analysis shows, however, that their existence is highly unlikely and suggests therefore that previous experimental data have not been correctly interpreted. One of the reactions for which a break has been claimed is the hydrolysis of urea by urease in the presence of sulfite ion. It is shown by extensive measurements of the temperature coefficients of this reaction, that no sharp break exist in the Arrhenius plot of the reaction even in presence of sulfite ions. The plot is a straight line, with an activation energy of 8830 cal. at all temperatures when sulfite ions are absent and no strong oxidizing agents are present, but in the presence of sulfite ions the rate of reaction at lower temperatures falls off more rapidly, so that the apparent activation energy gradually rises to about 15,000 cal. near 5°. Higher sulfite concentrations intensify the effect. These deviations from linearity of the Arrhenius curve are shown to be due to a reversible inhibition of urease by sulfite ions, the heat of the inhibition reaction being roughly determined to −29,000 cal. per mole of active centers on the urease molecules.

It is shown also that an inhibition equilibrium accounts quantitatively for the temperature coefficient of the frequency of heartbeat of rats investi-

gated by others and so it is made highly probable that this explanation applies to the great majority of anomalies previously described. Their common occurrence indicates that the low-temperature, reversible, inhibition of enzymes plays an important role in biological processes, both *in vivo* and *in vitro*.

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[CONTRIBUTION FROM THE GEORGE HERBERT JONES LABORATORY OF THE UNIVERSITY OF CHICAGO]

Electrolysis of Solutions of *m*-Dinitrobenzene in Liquid Ammonia

BY J. D. FARR, C. C. BARD AND G. W. WHELAND

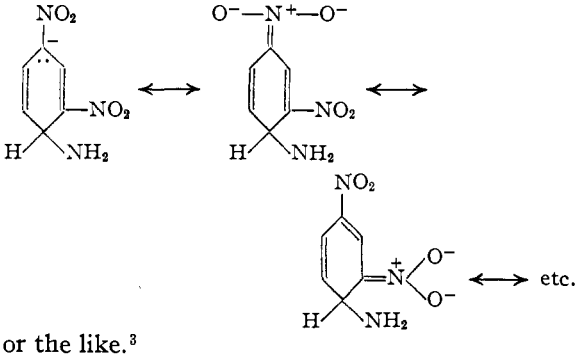
When *m*-dinitrobenzene is dissolved in liquid ammonia, there is obtained a deep purple solution which is a good conductor of the electric current. Other aromatic compounds with two or more meta nitro groups behave similarly; moreover, the ammonia solvent can be replaced by hydrazine or by ethylenediamine without obvious change in the properties of the solution.

The state of the dissolved nitro compound has been considered by several investigators.[1,2] Thus, by means of transference experiments in liquid ammonia, Field, Garner and Smith[1] showed that the color resides in an organic anion; on prolonged electrolysis, the solution surrounding the cathode ultimately consists of only the pure solvent. These authors reported, however, that there is no evolution of gas at either electrode and that, after the electrolysis, the nitro compound can be recovered unchanged. On the basis of these observations, they concluded that the ionization equilibrium in ammonia must be representable by some such equation as

$$C_6H_4(NO_2)_2 + 2NH_3 \rightleftarrows [C_6H_4(NO_2)_2]^- + 2NH_3^+$$

Thus, the cation and anion, when discharged at the corresponding electrodes, become identical, respectively, with the solvent and the uncharged solute. If, on the other hand, the cation were the ammonium ion, NH_4^+, hydrogen would have to be evolved at the cathode, or else the nitro compound would have to be reduced (or both). This same conclusion was supported also by Walden.[2]

In the work reported below, the electrolysis of the solution of *m*-dinitrobenzene in liquid ammonia was carefully re-examined. It was found that, contrary to the results of Field, Garner and Smith, hydrogen is evolved at the cathode, nitrogen is evolved at the anode, and the nitro compound is extensively reduced. It is therefore evident that the possible existence of an *ammonium* salt in the solution can again be considered. Although conclusive evidence regarding the constitution of this salt is lacking, a reasonable suggestion is that the anion may have the resonating structure



or the like.[3]

Experimental

Apparatus.—The electrolyses were carried out independently by two of the authors (J. D. F. and C. C. B.), with different apparatuses; an interval of approximately three years elapsed between the two sets of measurements. In the first series of measurements (J. D. F.), the apparatus was that shown in Fig. 1. In the second series of measurements (C. C. B.), the apparatus differed from the foregoing only in the use of the electrolysis cell shown in Fig. 2.[4] The two arms A of this cell were connected to the vacuum line by a common junction at the same point at which the cell D of Fig. 1 was attached.

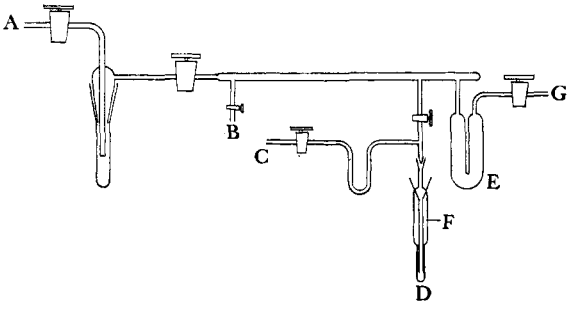


Fig. 1.—Apparatus used in the electrolysis of solutions of *m*-dinitrobenzene in liquid ammonia: A, to pumping system; B, to McLeod gage; C, to Töpler pump; D, electrolysis cell; E, U-tube; F, Pt electrode; G, ammonia inlet.

Materials.—The ammonia used as solvent was a commercial product, stored over sodium in steel cylinders.

(1) M. J. Field, W. E. Garner and C. C. Smith, *J. Chem. Soc.*, **127**, 1227 (1925).

(2) P. Walden, *Z. physik. Chem.*, **A168**, 419 (1934).

(3) *Cf.* N. V. Sidgwick, T. W. J. Taylor and W. Baker, "The Organic Chemistry of Nitrogen." Oxford University Press, Oxford, 1937, pp. 259 ff., for a discussion of the presumably analogous complexes formed between polynitro compounds and alkali alkoxides.

(4) For a somewhat similar cell, see S. Goldschmidt and F. Nagel, *Ber.*, **64**, 1744 (1931).