[CONTRIBUTION FROM THE DEPARTMENT OF AGRICULTURAL BACTERIOLOGY, UNIVERSITY OF WISCONSIN, AND DEPARTMENT OF BIOLOGY, PRINCETON UNIVERSITY]

Combined Influence of Temperature and Urethan on the Respiration of Rhizobium

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The successful application of the theory of absolute reaction rates to the quantitative interpretation of bioluminescence[2,3] encouraged this attempt to analyze bacterial respiratory processes in a similar manner. Such an analysis supports the views that: (a) the respiratory rate of "resting cells" of *Rhizobium trifolii* is affected by an equilibrium between catalytically active (native) and inactive (denatured) forms of the respiratory enzymes, an equilibrium of increasing influence from temperatures slightly below to those exceeding the normal optimum; (b) ethyl carbamate (urethan) inhibits respiration by promoting the reversible denaturation of one or more of these enzymes as well as an irreversible denaturation that is evident at the higher concentrations or temperatures.

Methods

Cultures of *Rhizobium trifolii*, Wisconsin strain 209, were grown for forty-eight hours at 30° on a yeast-water, mineral salts, agar base medium without added carbohydrate; suspensions of non-proliferating cells were prepared as described by Wilson.[4] Errors arising from variations among cultures harvested at different times were minimized by the use of a single refrigerated suspension for related experiments. Oxygen uptake was measured in a Warburg respirometer and expressed as $Q_{O_2}(N)$. The contents of the flasks were: 1.0 ml. $M/15$ mixed phosphate buffer (pH 6.5), 0.5 ml. neutral urethan solution (to give a final concentration of 0.167, 0.2, 0.25 and 0.33 M); 1.0 ml. bacterial suspension; 0.5 ml. $M/25$ glucose; and 0.15 ml. 20% potassium hydroxide in the inner cup. When urethan was omitted, water was added to bring the final volume of liquid to 3.15 ml. The rate of methylene blue reduction was determined in special Thunberg tubes by the method of Tam and Wilson.[5] The contents of the tubes were: 1 ml. 1:10,000 MB, 1 ml. $M/50$ glucose, 3 ml. mixed phosphate buffer (pH 8) and 3 ml. water. One ml. cell suspension and 1 ml. neutral urethan solution (to give a final concentration of 0.167, 0.20 and 0.25 M) were placed in the hollow stopper; when urethan was omitted, 1 ml. of water was added. The tubes were evacuated for three minutes with a water pump. Color intensity was measured with the Evelyn photometer using a 660 filter. The logarithm of ($\log I_0 - \log I_t$) plotted against time gives a straight line, the negative slope of which measures dehydrogenase activity. In this expression I_0 is the galvanometer reading after complete reduction, and I_t is the reading at any time, t.

Normal Relations Between Temperature and Respiratory Activity

Theory.—Temperature affects the respiratory enzymes of "resting" root nodule bacteria, as it does other enzymes, in two general ways. Suboptimal temperatures influence predominantly the rate of the enzyme-catalyzed reaction whereas superoptimal temperatures affect primarily the denaturation of enzymes. The fact that a suspension of *Rhizobium*, when kept for a limited time at temperatures below or slightly above the optimum, still respires as actively as a freshly prepared dilution of the stock suspension stored in the refrigerator suggests that at these temperatures enzyme inactivation, if it occurs at all, is entirely reversible. Designating the native and denatured state of a given enzyme as E_n and E_d, respectively, such a relationship can be represented by the following equilibrium with constant K_1

$$E_n \overset{K_1}{\rightleftharpoons} E_d \qquad (1)$$

Where this equilibrium between native and denatured forms of the enzyme exists, and other factors, such as substrate concentration, are not limiting, the following equation[6] (equation 24 in Johnson, Eyring and Williams[7]) should fit the data when the observed enzymatic activity is determined at various temperatures

$$I_1 = \frac{CTe^{-\Delta H\ddagger/RT}}{1 + e^{-\Delta H_1/RT}e^{\Delta S/R}} \qquad (2)$$

in which I_1 is enzyme activity, for example the rate of oxygen uptake or MB reduction, C is a constant,[8] $\Delta H\ddagger$ is the energy of activation for the enzyme-catalyzed reaction; and ΔH_1, ΔS_1 are the heat of reaction and entropy, respectively, for the denaturation equilibrium. The formulation applies to a given enzyme system, and is precisely applicable, therefore, to a single system. It is approximately correct when the over-all effects occur in the manner of a single system, as when one member of a series is largely limiting. In a complex process, the validity of the theory is tested by its conformity to the data.

To apply this equation, values for $\Delta H\ddagger$, ΔH_1, ΔS_1 and C are estimated, as follows: If the logarithm of I_1 is plotted against $1/T$, the apparent value for $\Delta H\ddagger$ is obtained by multiplying by 4.6 the slope of the line that best fits the experimental points from the low temperatures to where it deviates in approaching the optimum. The value of ΔH_1 is more difficult to establish,

(1) From the M.S. Thesis, University of Wisconsin, of H. Koffler, May, 1944. The experimental portion of this paper was supported in part by funds from the Rockefeller Foundation and the Wisconsin Alumni Research Foundation.

(2) Eyring and Magee, *J. Cell. Comp. Physiol.*, **20**, 169 (1942).

(3) Johnson, Eyring, Steblay, Chaplin, Huber and Gherardi, *J. Gen. Physiol.*, **28**, 463 (1945).

(4) Wilson, *J. Bact.*, **35**, 601 (1938).

(5) Tam and Wilson, *ibid.*, **41**, 529 (1941).

(6) Derivations of the equations in this paper are omitted since these, together with theoretical considerations, are readily available in references (2) and (3) and the recent paper by Johnson and Lewin, *J. Cell. Comp. Physiol.*, **28**, 47 (1946).

(7) Johnson, Eyring and Williams, *ibid.*, **20**, 247 (1942).

(8) This constant includes a proportionality constant, the term $e^{\Delta S\ddagger/R}$, and $\kappa\, k/h$, in which κ is the transmission coefficient, k is the Boltzmann constant, and h is Planck's constant.

but can be estimated by adding, disregarding signs, the value for $\Delta H\ddagger$ to the value obtained when the decreasing slope in the region between about 20 to 40% of the maximum rate is multiplied by 4.6. A more nearly correct value of ΔH_1 may be arrived at by calculating the whole curve, using values slightly higher and lower than the one first estimated, to see which value best fits the experimental points. The theory presupposes that the enzyme-catalyzed reaction obeys the Arrhenius equation over the entire temperature range and that denaturation interferes to an extent sufficient to yield a positive slope at higher temperatures. When $\Delta H\ddagger$ and ΔH_1 are determined, ΔS_1 is calculated from[9]

$$K_1 = e^{-\Delta H_1/RT} e^{\Delta S_1/R} \qquad (3)$$

At the optimum temperature (*i. e.*, when $I_1 = 100$) K_1 is estimated from[9]

$$K_{1\max.} = \frac{\Delta H\ddagger + 600}{\Delta H_1 - \Delta H\ddagger - 600} \qquad (4)$$

Once ΔS_1 is known, K_1 can be calculated for all other temperatures. Finally, C is determined by substituting these values of ΔS_1, ΔH_1 and $\Delta H\ddagger$ in equation (2).

Experimental.—Theoretical and experimental respiratory activities of *Rhizobium trifolii* 209 at various temperatures, in the presence and absence of urethan, are compared in Figs. 1 and 2. For oxygen uptake $\Delta H\ddagger$ and ΔH_1, calculated from the slopes of the 0.0 line in Fig. 1, were estimated to be 13,400 and 96,000 calories. The optimum temperature was 37° (*i. e.*, $I_1 = 100$ at $T = 310$). Accordingly from equation (4), $K_{1\max} = e^{-1.767}$. When this value is substituted in equation (3), $\Delta S_1 = 306.14$ entropy units, and from equation (2), $C = 0.3775\ e^{21.61}$. Accordingly, theoretical values for I_1 at various values of T in absence of urethan can be calculated from

$$I_1 = \frac{0.3775Te^{21.61}e^{-6,700/T}}{1 + e^{153.07}e^{-48,000/T}} \qquad (2a)$$

The corresponding values for MB reduction (Fig. 2) are: $\Delta H\ddagger$, 13,600 calories, ΔH_1, 126,000 calories, ΔS_1, 399.72 entropy units, C, $0.3613\ e^{21.79}$, optimum T, 312 (39°), and

$$I_1 = \frac{0.3613Te^{21.79}e^{-6,800/T}}{1 + e^{199.86}e^{-63,000/T}} \qquad (2b)$$

(9) Dr. Eyring supplied the derivation of this to one of us (F. H. J.). From equation (2) one writes

$$\ln I = \ln C + \ln T - \Delta H\ddagger/RT - \ln (1 + e^{-\Delta H_1/RT} e^{\Delta S/R})$$

Differentiating with respect to T

$$\frac{d \ln I}{d T} = \frac{1}{T} + \frac{\Delta H\ddagger}{RT^2} - \frac{e^{-\Delta H_1/RT} e^{\Delta S/R}}{1 + e^{-\Delta H_1/RT} e^{\Delta S/R}} \cdot \frac{\Delta H_1}{RT^2}$$

Since

$$K_1 = e^{-\Delta H_1/RT} e^{\Delta S/R}$$

$$\frac{d \ln I}{d T} = \frac{1}{T} + \frac{\Delta H\ddagger}{RT^2} - \frac{K_1 \Delta H_1}{RT^2(1 + K_1)}$$

When K_1 is a maximum (optimum point of curve)

$$d \ln I/dT = 0$$

Then

$$0 = RT + \Delta H\ddagger - K_{1\max} (\Delta H_1)/(1 + K_{1\max})$$

or

$$K_{1\max.} = \frac{\Delta H\ddagger + RT}{\Delta H_1 - \Delta H\ddagger - RT}$$

or approximately

$$\frac{\Delta H\ddagger + 600}{\Delta H_1 - \Delta H\dagger - 600}$$

The agreement between theoretical and observed values of I_1 in the absence of urethan is remarkably good, on the whole, for the temperatures between 15–18° to 42–44°, indicating that the theory and data are in accord.

Temperature and Respiratory Activity in the Presence of Urethan

Theory.—It has been shown previously[3] that urethan inhibits bioluminescence by combining reversibly with the *denatured* form of luciferase with equilibrium constant designated K_3, or with the *native* form with equilibrium constant K_1K_3. The same product results in either case, and there is no physical way of distinguishing between the two

$$E_n \underset{}{\overset{K_1}{\rightleftharpoons}} E_d \qquad K_1K_3 \nwarrow \searrow \text{EU} \swarrow \nearrow K_3 \qquad (5)$$

The net effect of temperature on urethan inhibition is thus determined by the values of the equilibrium constants K_1 and K_3. Since urethan apparently combines with bonds which become available in the denaturation, it acts as if it promoted this reaction. Thus raising the temperature leads to an increase in inhibition, a lowered optimum temperature, and a decrease in the apparent activation energy of the over-all process. To test whether urethan affects oxygen uptake and MB reduction in this manner, we apply the following equations (17 and 18 in reference[7])

$$(I_1/I_2 - 1)(1 + 1/K_1) = K_3U^s = U^s e^{-\Delta H_3/RT} e^{\Delta S_3/R} \qquad (6)$$

$$\log\{(I_1/I_2 - 1)(1 + 1/K_1)\} = -\Delta H_3/2.3RT + \Delta S_3/2.3R + S \log U \qquad (7)$$

In these equations I_2 is the rate of oxygen uptake or MB reduction after the addition of urethan; ΔH_3, ΔS_3 are the heat of reaction and entropy, respectively, for the equilibrium with constant K_3, existing between the denatured respiratory enzyme and urethan; U is the molar concentration of urethan; and s, the number of urethan molecules combining with each denatured enzyme molecule.

A straight line, obtained when $\log\{(I_1/I_2 - 1)(1 + 1/K_1)\}$ is plotted against $1/T$ implies that urethan inhibits respiratory activity by the described mechanism. The slope of this straight line multiplied by 4.6 estimates ΔH_3. If $\log (I_1/I_2 - 1)$ plotted against $\log U$ gives a straight line, its slope measures the average number of urethan molecules combining with each enzyme molecule (*cf.* with equation 23 in reference[7]). At a given temperature, T, the value of K_1 can be calculated from equation (3); since ΔH_3, s, U and the experimentally determined values for $(I_1/I_2 - 1)$ are known, ΔS_3 can be estimated by substitution in equation (6). These values can then be used in equation (8) to calculate urethan inhibition at various temperatures and concentrations. Equa-

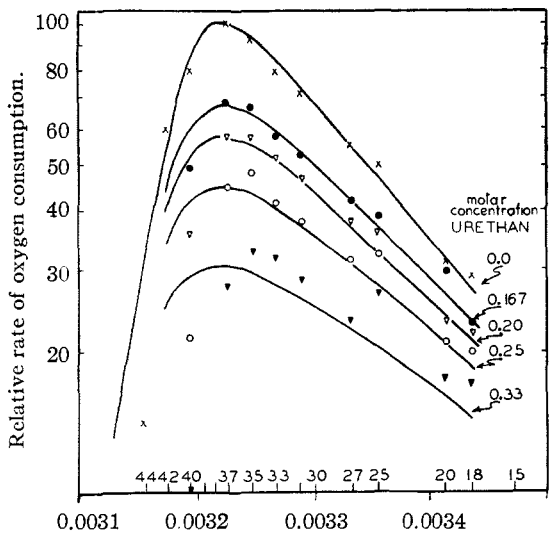

Fig. 1.—Relation between temperature and rate of oxygen uptake by *Rhizobium trifolii* in the presence and absence of urethan. The maximum intensity ($Q_{O_2}(N)$ = 635) has been set equal to 100 and the remaining data adjusted to this scale. In both Figs. 1 and 2 the experimental points represent the average of at least three determinations. The theoretical curves (solid lines) were calculated as described in the text.

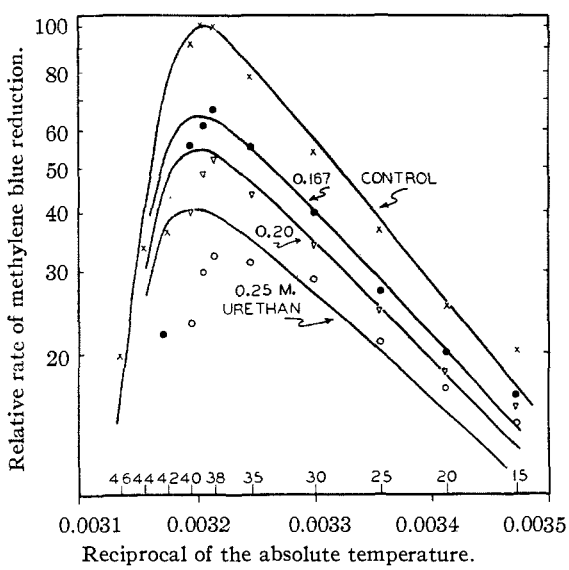

Fig. 2.—Relation between temperature and rate of methylene blue reduction by *Rhizobium trifolii* in presence and absence of urethan.

tion (8) is obtained by solving equation (6) for I_2

$$I_2 = \frac{I_1}{1 + \frac{K_1 K_3 U^e}{1 + K_1}} = \frac{I_1}{1 + \frac{e^{-\Delta H_1/RT} e^{-\Delta S_1/R} e^{-\Delta H_3/RT} e^{\Delta S_3/R}}{1 + e^{-\Delta H_1/RT} e^{\Delta S_1/R}} U^e} \quad (8)$$

With the aid of equations (2) and (8), I_1 and I_2, the respiratory rates at various temperatures in the presence and absence of urethan can be predicted with a fair degree of accuracy. Inasmuch as these equations are based on the premise that all reactions involved are completely reversible, calculated values of I_1 and I_2 agree with experimental values only under conditions which exclude irreversible denaturation. At temperatures well above the optimum, or at lower temperatures in the presence of high concentrations of urethan,

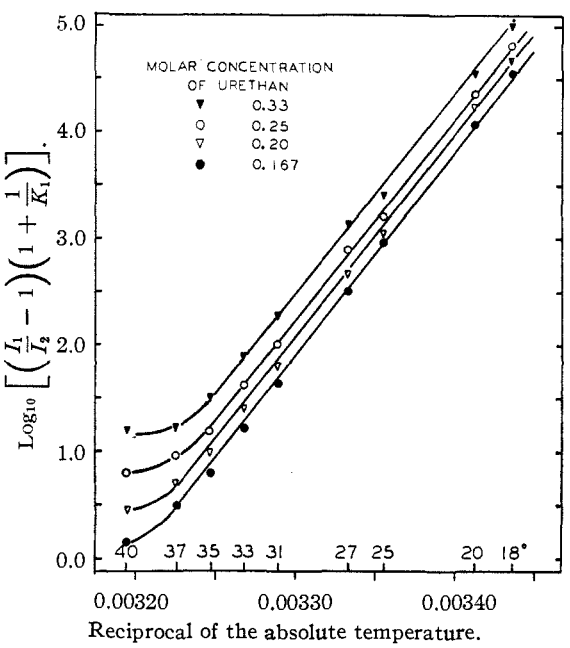

Fig. 3.—Urethan inhibition of oxygen uptake by *Rhizobium trifolii* as a function of temperature plotted according to equation 7.

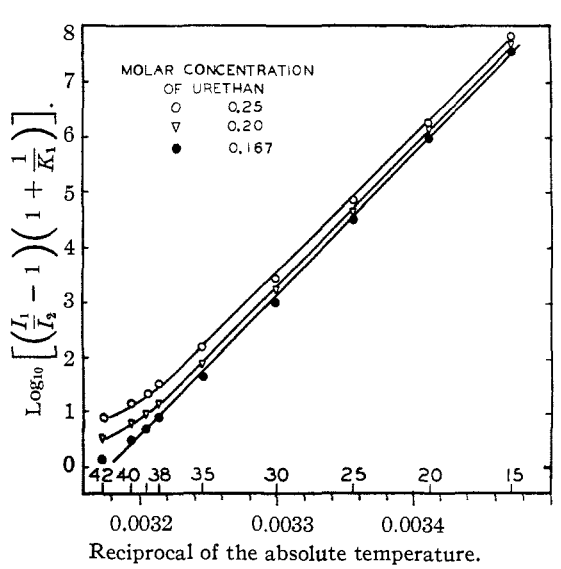

Fig. 4.—Urethan inhibition of methylene blue reduction by *Rhizobium trifolii* as a function of temperature.

differences between the theoretical and observed temperature–activity curves are to be expected, because determinations with reference to the reversible denaturation alone measure, to some extent, also an irreversible denaturation.

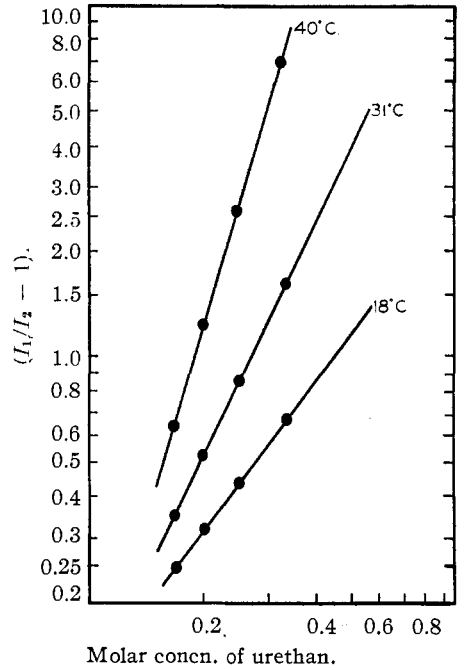

Fig. 5.—Inhibition of oxygen uptake by *Rhizobium trifolii* at different temperatures as a function of urethan concentration.

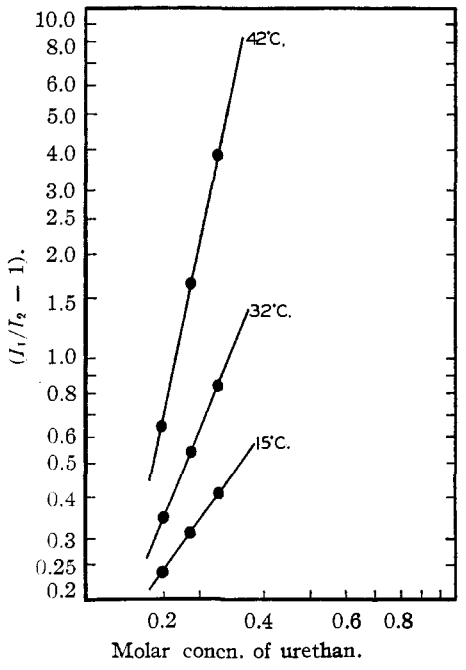

Fig. 6.—Inhibition of methylene blue reduction by *Rhizobium trifolii* at different temperatures as function of urethan concentration.

Experimental.—Figures 3 and 4 show the relationship between temperature and urethan inhibition of oxygen consumption and MB reduction, respectively, plotted according to equation (7). The relationship deviates from a straight line at temperatures above the optimum; the greater the urethan concentration the greater the deviation. The slope changes in the direction of additional inhibition suggesting that, at the higher temperatures, an irreversible denaturation accompanies the reversible inactivation. These effects resemble those observed in the action of urethan on luminescence. In the present study, the slopes of the straight line portions indicate a ΔH_3 of $-85{,}000$ calories for urethan acting on oxygen consumption, and $-118{,}000$ calories on MB reduction.

Figures 5 and 6 illustrate typical relationships between concentrations of urethan and inhibition of oxygen consumption and MB reduction, respectively, at three temperatures. Similar lines were obtained for the other experimental temperatures. The slopes of the lines for oxygen consumption vary uniformly from 1.36 at 18° to 3.20 at 40°, and for MB reduction from 1.28 at 15° to 3.88 at 42°. Since these slopes are numerically equal to s—the average number of urethan molecules combining with one enzyme molecule—it is evident that this number increases with a rise in temperature. The interpretation is likely the same as that given to the corresponding observation in luminescence—that more than one equilibrium is established between urethan and enzyme. An appropriate equation has been derived,[3] but its application is laborious because ΔH, ΔS and s, of the separate equilibria involved in the net result are difficult to estimate. The theoretical predictions of the rates of oxygen consumption and of MB reduction can be made with some accuracy, however, assuming only a single equilibrium, with an average value of s and the values for ΔH_3 obtained from Figs. 3 and 4. Variations in the assumed value of s, from 1.5 to 3, cause only 1 to 2% variation in estimating ΔS_3, but markedly influence the course of the theoretical curves calculated for various concentrations of urethan.

For respiration, s was taken as 2.25. The entropy, ΔS_3, was then calculated from the experimentally observed values of I_1 and I_2 (0.2 M urethan at 300° K).

$$(I_1/I_2 - 1) = 0.455 \qquad (0.2)^{2.25} = e^{-3.62}$$
$$K_1 = e^{-96{,}000/600} e^{306.14/2} = e^{-6.93} \tag{3a}$$
$$(1 + 1/K_1)(I_1/I_2 - 1) = 466 = e^{6.15}$$
$$e^{6.15} = e^{-3.62} e^{85{,}000/600} e^{\Delta S_3/R} \tag{6a}$$
$$\Delta S_3 = -263.8 \text{ e. u.}$$

Similarly for methylene blue reduction, at 308° K. and 0.2 M urethan, when s was taken as 2.5,

$$(I_1/I_2 - 1) = 0.77 \qquad (0.2)^{2.5} = e^{-4.02}$$
$$K_1 = e^{-126{,}000/616} e^{399.72/2} = e^{-4.68} \tag{3b}$$
$$(1 + 1/K_1)(I_1/I_2 - 1) = 84.0 = e^{4.43}$$
$$e^{4.43} = e^{-4.02} e^{118{,}000/616} e^{\Delta S_3/R} \tag{6b}$$
$$\Delta S_3 = -366.2 \text{ e. u.}$$

All the unknown constants of equation (8) having been calculated from the experimental data, the family of curves relating respiratory activity to temperature in the presence of various concentrations of urethan can be determined. Uptake of oxygen depends on temperature and concentration of urethan according to

$$I_2 = \frac{I_1}{1 + \dfrac{e^{-48{,}000/T} e^{153.07} e^{-42{,}500/T} e^{-131.9}}{1 + e^{-48{,}000/T} e^{153.07}} \cdot U^{2.25}}$$
$$= \frac{I_1}{1 + \dfrac{e^{-5{,}500/T} e^{21.18}}{1 + e^{-48{,}000/T} e^{153.07}} U^{2.25}} \tag{8a}$$

in which I_1 has already been calculated by equation (2a). Similarly, for the reduction of MB:

$$I_2 = \frac{I_1}{1 + \frac{e^{-63,000/T}e^{199.86}e^{-59,000/T}e^{-183.1}}{1 + e^{-63,000/T}e^{199.86}} \cdot U^{2.50}} = \frac{I_1}{1 + \frac{e^{-4,000/T}e^{16.76}}{1 + e^{-63,000/T}e^{199.86}} U^{2.50}} \quad (8b)$$

in which I_1 has been calculated by equation (2b).

Discussion

A comparison between the theoretical and experimental points in Figs. 1 and 2 indicates essentially good agreement at suboptimal temperatures, and at the lower concentrations of urethan. At temperatures above the optimum, especially in higher concentrations of urethan, the predicted rates are generally too high. These facts suggest that the discrepancies are, in part at least, due to an additional inhibition, possibly an irreversible denaturation, that is not taken into account by the theory. Possibly for the same reason, the experimentally observed lowering of the optimal temperature in the presence of urethan is not predicted by the calculated curves, although such a prediction in change in optimal temperatures would result with a greater difference in the constants ΔH_1 and ΔS_1 in comparison with ΔH_3 and ΔS_3, respectively. It may be significant also that the experimental points at 18° in Fig. 1 and at 15° in Fig. 2 are high in comparison with the theoretical curve. The interpretation is uncertain, however, and would require further studies through a range of still lower temperatures.

The similarity between $\Delta H‡$ values for oxygen uptake and MB reduction (13,400 and 13,600 calories, respectively), in addition to the observation that the inhibition of both processes by a given concentration of urethan and at a given temperature is nearly the same, suggests that the anaerobic dehydrogenase is very largely the limiting system in the total oxygen consumption and that the action of urethan is primarily on that system. Differences in the values of ΔH_3 and of ΔS_3 for oxygen uptake and MB reduction possibly originate, in part, to differences in *p*H of the medium used. In view of the relatively anaerobic conditions which exist in the nodules, the importance of dehydrogenases in the respiratory mechanism of root nodule bacteria is plausible. A fraction of the total oxygen consumption probably goes through a urethan-insensitive system as the inhibition of oxygen uptake was somewhat less than that of MB reduction.

Summary

Respiratory experiments with *Rhizobium trifolii* 209 suggest that urethan inhibits its uptake of oxygen and reduction of methylene blue by influencing a denaturation equilibrium that exists between native and denatured enzymes. At suboptimal temperatures urethan promotes the reversible denaturation of one or more critical enzymes, thus decreasing the concentration of active catalysts. As the temperature is increased beyond a threshold value that varies with the concentration of urethan, this reversible denaturation is increasingly accompanied by an irreversible denaturation, and the inhibition by urethan becomes progressively more pronounced.

The fact that this interpretation agrees satisfactorily with the quantitative implications of the theory of absolute reaction rates provides further evidence for the applicability of that theory to general biological problems.

MADISON, WISCONSIN RECEIVED NOVEMBER 29, 1946

[CONTRIBUTION FROM THE SCIENTIFIC LABORATORIES, FREDERICK STEARNS & COMPANY, DIVISION OF STERLING DRUG INC.]

Preparation of Some Primary and Secondary β-Cyclohexylalkylamines[1]

BY BERNARD L. ZENITZ, ELIZABETH B. MACKS AND MAURICE L. MOORE

Since it has been well established that the β-phenylethylamine skeleton is closedly associated with sympathomimetic activity, it was of interest to determine how this relationship would be affected by a replacement of the phenyl nucleus by a cyclohexyl radical.

Though a large number of compounds containing a β-cyclohexylethylamine grouping have been prepared in the antispasmodic field,[1,2,3,4] only a comparatively few of them have been pharmacologically investigated for sympathomimetic activity.[5,6,7]

Therefore, we prepared for further physiological study the series of β-cyclohexylalkylamines listed in Table I. Of this series, the syntheses of I, II, III and IV only have been described previously.[1,8,9,10]

(1) Presented before the Division of Medicinal Chemistry at the American Chemical Society meeting, Chicago, Illinois, September 13, 1946.

(1a) Blicke and Monroe, THIS JOURNAL, **61**, 91 (1939).

(2) Blicke and Zienty, *ibid.*, **61**, 93 (1939); **61**, 771 (1939); **61**, 774 (1939).

(3) Blicke, U. S. Patent 2,180,344, Nov. 21, 1939.

(4) Heyn, U. S. Patent 2,278,123, March 31, 1942.

(5) Gunn and Gurd, *J. Physiol.*, **97**, 453 (1940).

(6) Shonle and Rohrmann, New York Meeting of the American Chemical Society, Division of Medicinal Chemistry, 1944.

(7) Lands, Lewis and Nash, *J. Pharmacol. Exptl. Ther.*, **83**, 253 (1945).

(8) Wallach, *Ann.*, **353**, 284 (1907).

(9) Coleman and Adams, THIS JOURNAL, **54**, 1932 (1932).

(10) Levene, Mikeska and Passoth, *J. Biol. Chem.*, **88**, 27 (1930).