THE NATURE OF ENZYME INHIBITIONS IN BACTERIAL LUMINESCENCE: SULFANILAMIDE, URETHANE, TEMPERATURE AND PRESSURE [1]

FRANK H. JOHNSON, HENRY EYRING, AND R. W. WILLIAMS
Physiological Laboratory, and the Frick Chemical Laboratory, Princeton University, New Jersey

SEVEN FIGURES

According to evidence recently obtained with luminous bacteria, a thermodynamic equilibrium between catalytically active and inactive forms of certain enzymes appears to exist normally within the living cell. This equilibrium has temperature and entropy characteristics resembling a protein denaturation. It is apparently a determining factor with regard to the optimum temperature for luminescence in a given species. In addition, it is sensitive to hydrostatic pressure and apparently also to certain narcotics. The quantitative data and theoretical considerations which support this hypothesis have been discussed at some length in previous papers (Johnson, Brown and Marsland, '42, a, b; Brown, Johnson and Marsland, '42; Eyring and Magee, '42; Johnson and Chase, '42).

The purpose of the present study has been to extend the data and theory concerning the temperature relations of bacterial luminescence intensity in order to elucidate further the significance of the phenomena observed in both normal and inhibitor-containing cell suspensions. Particular reference has been made to the action of sulfanilamide, whose inhibition of luminescence may be reversed to a large extent by increase in temperature. The action of urethane also has been investigated further, since its inhibition is partially or wholly reversible with increase in hydrostatic pressure. In this effect, the urethane inhibition of luminescence resembles that due to heat alone, which is also reversible, in part, by pressure. The action of all these inhibitory agents is related to the optimum temperature of luminescence in the specific organism concerned. The results of the present study make it possible to evaluate more clearly biological differences in the velocity-temperature relations of a given process, and to designate more precisely the action of added

[1] These studies have been aided, in part, by a grant to one of us (F. H. J.) from the Penrose Fund of the American Philosophical Society.

inhibitors. Furthermore, the simple formulations arrived at are of general use in testing the conformity of inhibition data to specific types of combination between enzyme and inhibitor.

METHODS

The various species of luminous bacteria were cultivated at their respective optimum temperatures on nutrient agar containing the optimum concentration of NaCl, as described in previous publications. Essentially the same method as heretofore was also used for measuring luminescence, with the aid of a modified Leeds and Northrup MacBeth illuminometer. A large amount of stock suspension was prepared in phosphate buffered NaCl and maintained at a low temperature for each experiment. The luminescence of 10 cc. portions, with and without inhibitor, respectively, was measured in a water bath. Frequent renewal of the specimens from the stock suspension were necessary at temperatures well above the optimum, because of the irreversible, thermal destruction which proceeds rapidly at the higher temperatures. With very short exposures, however, followed by immediate cooling, luminescence could be reduced to as little as 5% of the maximum, in an almost fully reversible manner on cooling. This important reversibility of heat diminution of luminescence intensity was confirmed for each species, and with each inhibitor.

THEORETICAL BASIS

The following considerations apply to changes in the velocity of an enzyme reaction arising from changes in the amount of active catalyst. In accordance with the evidence described in the previous publications, we will assume that the reversible heat diminution of luminescence intensity occurs through a reversible "denaturation" or inactivation of the luciferase, in which the native enzyme (A_n) goes to an inactive form, (A_d) with equilibrium constant K_1. The velocity (I) of the reaction catalyzed by the active enzyme is equal to a rate constant, k_2, times the product of the substrate (LH_2, luciferin) and the enzyme (A_n, luciferase) concentration. A proportionality constant (s) allows for the units employed. The above notation is appropriate to the luminescent system, in which the intensity of the light under ordinary conditions is evidently proportional to the over-all velocity of the reaction. The theory, however, is general. Thus, we may write:

$$I_1 = sk_2 [A_n] [LH_2] \quad (1)$$

Since the amount of A_n is normally assumed to depend on the equilibrium with an inactive form, A_d,

$$\frac{[A_d]}{[A_n]} = K_1 \tag{2}$$

Letting g represent the total luciferase, $A_n + A_d$, we have

$$A_n = g/1 + K_1 \tag{3}$$

and

$$I_1 = sk_2g[LH_2]/1 + K_1 \tag{4}$$

Let X represent the concentration of added molecules that combine reversibly with A_n or A_d. Let r represent the number of molecules combining with each molecule of A_n, and s the number combining with A_d. The following equilibria then obtain;

$$\begin{array}{ccc} r\,X + A_n & \overset{K_1}{\rightleftharpoons} & r\,X + A_d \\ \Big\uparrow\Big\downarrow K_2 & & \Big\uparrow\Big\downarrow K_3 \\ A_nX_r & \overset{K_4}{\rightleftharpoons} & A_dX_s + (r\text{-}s)\,X \end{array} \tag{5}$$

In the absence of X, this reduces to the simple equilibrium (2). In the presence of X, the total luciferase becomes

$$g = A_n + A_d + A_nX_r + A_dX_s \tag{6}$$

The several equilibria may be designated thus:

$$[A_d]/[A_n] = K_1;\ [A_nX]/[X]^r\,[A_n] = K_2;\ [A_dX]/[X]^s\,[A_n] = K_3K_1 \tag{7}$$

Hence,

$$g = [A_n] + [A_n]K_1 + K_2[X]^r\,[A_n] + K_3K_1[X]^s\,[A_n] \tag{8}$$

and

$$A_n = \frac{g}{1 + K_1 + K_2X^r + K_3K_1X^s} \tag{9}$$

Assuming that only A_n is catalytically active, let I_2 equal the new velocity in the presence of X. Then, from (4) and (9), omitting brackets,

$$I_2 = \frac{s\,k_2\,g\,LH_2}{1 + K_1 + K_2X^r + K_3K_1X^s} \tag{10}$$

and

$$\frac{I_1}{I_2} = \frac{1 + K_1 + K_2X^r + K_3K_1X^s}{1 + K_1} \tag{11}$$

This is the general form of the relation for the ratio of velocities before and after adding molecules which enter into a reversible combination with native and denatured forms of the enzyme. Among a diverse group of substances it might be expected that various types of combinations would take place. Some, for example, might combine without reducing catalytic activity. Others might reduce the velocity of the reaction by

forming complex equilibria with both native and denatured forms, with K_2 different from K_3, and r different from s. These possibilities could also be formulated, but they will not be considered further in this paper, since the examples specifically dealt with in the next section lend themselves largely to a simpler interpretation. We will, therefore, restrict the discussion to three possible types of inhibitor-enzyme combinations, as set forth below.

Type 1. The inhibitor combines indiscriminately with both native and denatured forms of the enzyme: $K_2 = K_3$, and $r = s$

In this case, the general expression for I_2 simplifies to

$$I_2 = \frac{s\, k_2\, g\, (LH_2)}{(1 + K_1)(1 + K_2 X^r)} \tag{12}$$

Dividing I_1 by I_2 we obtain,

$$I_1/I_2 = 1 + K_2 X^r = 1 + e^{\frac{-\Delta H_2}{RT}} e^{\frac{\Delta S_2}{R}} X^r = 1 + e^{\frac{-\Delta F_2}{RT}} X^r \tag{13}$$

$$= 1 + e^{\frac{-\Delta E}{RT}} e^{\frac{-p\Delta V}{RT}} e^{\frac{\Delta S}{R}} X^r$$

Subtracting 1 from both sides of equation (13), and taking the logarithm, we have

$$\log_{10}(I_1/I_2 - 1) = \frac{-\Delta E_2}{2.303RT} - \frac{p\Delta V_2}{2.303RT} + \frac{\Delta S_2}{2.303R} + r\log_{10} X \tag{14}$$

Here ΔV is the increase in volume in going from the initial to the final state in the reaction, $A_n + rX \rightleftharpoons A_nX_r$. Since the slope obtained by plotting ΔF against p is ΔV, if we plot $\log_{10}(I_2/I_2 - 1)$ as ordinate against p in atmospheres as abscissae, the slope is $\Delta V/4.606T$. If in this slope the quantity RT be introduced in c.c. atmospheres per mole, the change in volume, ΔV will be expressed in cc's. per mole. If $\log (I_1/I_2 - 1)$ be plotted against the reciprocal of the absolute temperature, the slope of the line gives the heat of reaction, ΔH_2, for the equilibrium set up by the inhibitor with the enzyme. The expression

$$\frac{\Delta S_2}{2.303R} - \frac{p\Delta V_2}{2.303RT} + r \log X$$

reduces to the entropy if p is small, of the order of an atmosphere, and if (X) is equal to 1 molar. Finally, if $\log_{10}\left(\frac{I_1}{I_2} - 1\right)$ be plotted against $\log_{10}X$, the slope of the line is r, the number of molecules of the inhibitor combining with the enzyme.

Type II. The inhibitor combines only with the denatured form of the enzyme: $K_2 = 0$

Here,

$$A_n = \frac{g}{1 + K_1 + K_1K_3X^s}$$

$$I_2 = s\,k_2 \frac{g\,[LH_2]}{1 + K_1 + K_1K_3X^s} \tag{15}$$

Dividing as before,

$$I_1/I_2 = 1 + K_1 + K_1K_3X^s/1 + K_1 = 1 + K_1K_3X^s/1 + K_1 \tag{16}$$

whence,

$$\left(\frac{I_1}{I_2} - 1\right)\left(1 + \frac{1}{K_1}\right) = k_2X^s = e^{\frac{-\Delta H_3}{RT}}\, e^{\frac{\Delta S_3}{R}}\, X^s \tag{17}$$

taking the logarithm of both sides we obtain

$$\log_{10}\left(\frac{I_1}{I_2} - 1\right)\left(1 + \frac{1}{K_1}\right) = \frac{-\Delta H_3}{2.303RT} + \frac{\Delta S_3}{2.303R} + s\log_{10}X = \tag{18}$$

$$-\frac{\Delta E_3}{2.303RT} - \frac{p\Delta V}{2.303RT} + \frac{\Delta S_3}{2.303R} + s\log_{10}X$$

Thus, as before, by plotting the quantity on the left as ordinate successively against 1/T, p, and $\log_{10}X$ as abscissa, all the quantities on the right can be determined.

Type III. The inhibitor combines only with the native form of the enzyme: $K_3 = 0$

In the case, $A_n = g(1 + K_1 + K_2X^r)$, and hence,

$$I_2 = \frac{s\,k_2\,g\,(LH_2)}{1 + K_1 + K_2X^r} \tag{19}$$

Consequently,

$$\frac{I_1}{I_2} = \frac{1 + K_1 + K_2X^r}{1 + K_1} = 1 + \frac{K_2X^r}{1 + K_1} \tag{20}$$

whence

$$\left(\frac{I_1}{I_2} - 1\right)(1 + K_1) = K_2X^r = e^{\frac{-\Delta H_2}{RT}}\, e^{\frac{\Delta S_2}{R}}\, X^r \tag{21}$$

and

$$\log_{10}\left(\frac{I_1}{I_2} - 1\right)(1 + K_1) = \frac{-\Delta H_2}{2.303RT} + \frac{\Delta S_2}{2.303R} + r\log_{10}X \tag{22}$$

If $\log_{10}(I_1/I_2 - 1)(1 + K_1)$ be plotted successively against 1/T, p, and X, the slopes make it possible to calculate ΔH_2, ΔV_2 and r. Knowing these, ΔS_2 is readily calculated from (22).

It should be pointed out, as a corollary to Type III, that should a substance combine with the native form without reducing catalytic activity, it would be possible to influence the reaction governed by K_1 in a manner so as to yield more of the active enzyme. Possibly the stimulating effects of specific ions on certain enzyme reactions may be an example of such a combination. An increase in the over-all velocity of the reaction would result from influencing the possible denaturation equilibrium. The biologically interesting implications of this fact, how-

ever, will not be considered further in this paper for lack of experimental data in support of such a situation.

Certain changes in the optimum temperature of the reaction are implicit in the theory developed above. The theoretical basis for these changes has been formulated and will be given in a subsequent publication.

To test if an actual example fits type I, II, or III, successive plots are made of log $(I_1/I_2 - 1)$; log $(I_1/I_2 - 1)(1 + 1/K_1)$ and log $(I_1/I_2 - 1)(1 + K_1)$ against temperature. A straight line indicates agreement with the particular type. If pressure data are available, the plot may be made against pressure instead of temperature. In plotting log $(I_1/I_2 - 1)$ against 1/T or against pressure, if the ordinate tends to fall low only when 1/T is large or p is high, type II is indicated. In the same plot, if the ordinate tends to fall low when 1/T is small or p is small, type III is indicated. This follows from the correct form of the ordinate in the three cases, and the fact that K_1 is small for low temperatures and large for high temperatures; also, that K_1 is small for high pressures and large for low.

It is interesting and important to note that in each of the above cases, if log $(I_1/I_2 - 1)$ is plotted against log X (i.e., log. concentration of inhibitor) at constant temperature and pressure, a straight line relation results. Thus,

$$\begin{aligned}
&\text{Ia, for type I, } \log_{10}(I_1/I_2 - 1) = \log_{10} K_2 + r \log_{10} X = \log \text{constant} + r \log_{10} X \\
&\text{IIa, for type II, } \log_{10}(I_1/I_2 - 1) = \log_{10} \frac{K_1 K_3}{1 + K_1} + s \log_{10} X = \log \text{constant} + s \log_{10} X \qquad (23) \\
&\text{IIIa, for type III, } \log_{10}(I_1/I_2 - 1) = \log_{10} \frac{K_2}{1 + K_1} + r \log_{10} X = \log \text{constant} + r \log_{10} X
\end{aligned}$$

The slope of the line tells the number of molecules of X combining with the catalyst. Only in Ia, however, would the intercept of the line represent the logarithm of the equilibrium constant. In II a, if K_1 is very large, the constant obtained becomes K_3, while in IIIa, if K_1 is very small, the constant becomes K_2.

The results described in the above paragraph are of particular interest in connection with the work of Fisher and collaborators (Fisher and Ohnell, '40; Fisher and Stern, '42) who have plotted the log $\frac{\%\text{ uninhibited}}{\%\text{ inhibited}}$ against log concentration of the inhibitor and found that in many biological reactions straight lines obtain over a considerable range. Since the per cent uninhibited is the same as our I_2, and the per cent inhibited is the same as our $I_1 - I_2$, it is clear that they have plotted as ordinates, in our language, log $(I_2/(I_1 - I_2))$. This is simply the negative of log $\frac{I_1 - I_2}{I_2}$, which we plot. In this respect we are in essential agreement. From our previous discussion it is evident, however,

that depending on whether they deal with type I, II, or III, they may or may not be justified in interpreting their constants as equilibrium constants. Only an analysis based on the effects over an adequate range of temperature or of pressure can establish this fact. In the next section, application will be made to experimental data. The sulfanilamide inhibition conforms largely to type I, and the urethane inhibition to type II.

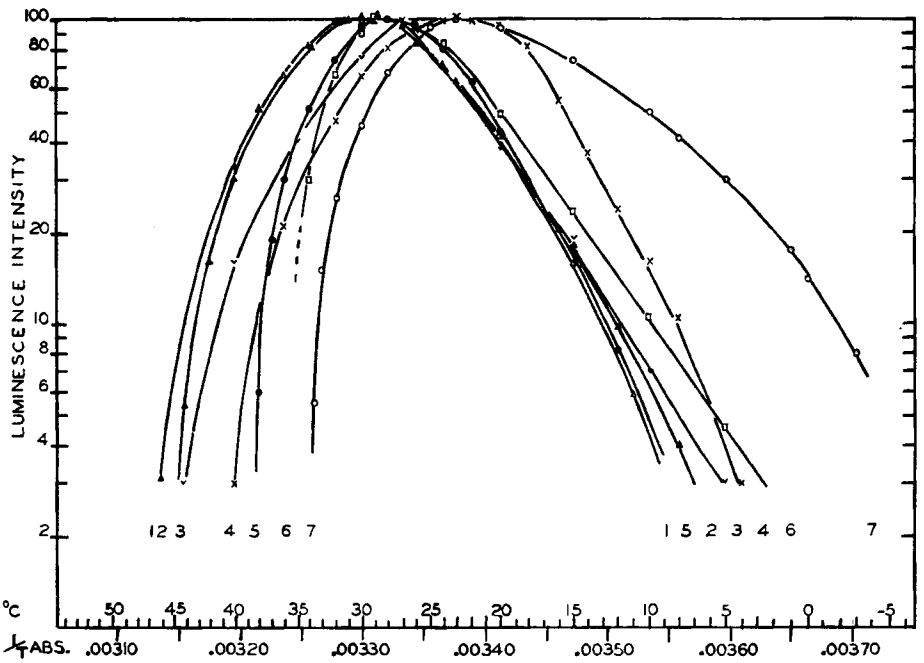

Fig. 1 Relation between temperature and luminescence intensity for various species of bacteria and a fungus mycelium. The maximum intensity has arbitrarily been taken as 100, and the data recalculated to conform to this scale. The lines represent the smoothed curves drawn through a multitude of experimental points. Curve 1, hollow triangles, Achromobacter Harveyi; curve 2, solid triangles, Vibrio phosphorescens; curve 3, open checks, Vibrio phosphorescens of Root ('32); curve 4, crosses, Bacterium phosphoreum of Morrison ('25); curve 5, solid circles, Achromobacter Fischeri; curve 6, squares, Panus stypticus; curve 7, open circles, Photobacterium phosphoreum.

RESULTS OF EXPERIMENTS

"Normal" temperature relations of luminescence in various species

In figure 1 the relation between luminescence intensity and temperature has been summarized for four different species of bacteria: A. Fischeri, A. Harveyi, P. phosphoreum, and V. phosphorescens. The maximum intensity has arbitrarily been taken as 100 in each case, in

order to have a similar scale for comparing the phenomenon in different organisms. Data for the mycelium of a luminous fungus, Panus stypticus,[2] are also included. Finally, two examples have been recalculated from figures previously published, and also included.[3] These are for Bacterium phosphorescens (Morrison, '25) and a strain of V. phosphorescens (Root, '32). The activation energies for luminescence in the various species ($\Delta H^{\ddagger}$ for k_2) are given in table 1, along with the heats of reaction and entropies for the denaturation equilibrium (ΔH_1 and ΔS_1 for K_1) and certain other data where available. In obtaining the normal activation energies ($\Delta H^{\ddagger}$) and heats of reaction of the denaturation (ΔH_1), the following equation for light intensity has been employed:

$$\ln I = \ln c'' - \ln 1/T - \frac{\Delta H^{\ddagger}}{RT} - \ln \left(1 + e^{\frac{\Delta S_1}{R}} e^{\frac{-\Delta H_1}{RT}}\right)$$

This equation is the logarithmic form of equation (4), derived as follows:

$$I = \frac{sk_2gLH_2}{1 + K_1} = \frac{c'k'}{1 + K_1} = \frac{c'' Te^{\frac{-\Delta H^{\ddagger}}{RT}}}{1 + e^{\frac{\Delta S}{R}} e^{\frac{-\Delta H}{RT}}} \qquad (24)$$

This equation is not adequate to give a perfect fit for theoretical curves in relation to experimental data. The position of the maximum is extremely sensitive to the exact values assigned to ΔH and ΔS. It is for this reason only that the values in table 1 are given to so many figures. The theoretical curves tend to be too sharp at the maximum. This means that the equation is too simple. Multiple equilibria between catalytically active and inactive forms of the enzyme would "round out" these curves. It would appear likely that multiple equilibria are indeed involved, and responsible, in part, for the shape of the curves.

The various curves in figure 1 are, in general, remarkably similar. They differ in two important respects: first, the position of the optimum on the absolute scale of temperature, and second, the slopes of the ascending as well as of the descending portions of the curves for different species. With regard to the optimum temperatures, it should be noted that the total range for these species amounts to some 10°C. As

[2] These data have not been published heretofore. They were obtained in connection with the work of Johnson and Lynn ('40) which has appeared in abstract.

[3] The recalculated curves are subject to considerable error in slope at the extremes of temperature, because of the difficulty in obtaining accurate values for these regions of the curves from the published figures. With regard to the identity of the several bacterial organisms, it is possible that the species studied by Root and Morrison, respectively, are more or less closely related to the strains employed in the present study. Sufficient facts are not available for deciding this point.

TABLE 1

Constants for the temperature-intensity relation of bacterial luminescence obtained in repeated experiments with various species, and in the presence of sulfanilamide and urethane, respectively.

SPECIES	EXP.	INHIBITOR	CONCENTRATION (MOLAR)	ENERGY OF ACTIVATION	K_1 REVERSIBLE "DENATURATION"		K_2 EQUILIBRIUM WITH SULFANILAMIDE		K_3 EQUILIBRIUM WITH URETHANE [1]	
				$\Delta H\ddagger$	ΔH_1	ΔS_1	ΔH_2	ΔS_2	ΔH_3	ΔS_3
Vibrio phosphorescens	1	.....	....	28,400	91,200	300				
		Sulfanilamide	.003	31,400	87,000	284	—17,300	—39.6		
	2	.....	....							
		Urethane	.098						—70,000	—204
		Sulfanilamide	.002				—16,100	—35.8		
Photobacterium phosphoreum	1	.....	....	15,600	71,000	239.2				
		Sulfanilamide	.005	19,700	68,000	228.2	—12,200	—37.8		
	2	.....	....	22,200						
		Sulfanilamide	.0025	37,700			—16,980	—42.2		
	3	.....	....	22,500						
		Urethane	.07	18,800						
	4	.....	....	31,200						
		Sulfanilamide	.005	26,200			—14,400	—37.8		
		Urethane	.075	34,200					—56,000	—165
	5	.....	....	27,000						
		p-aminobenzoic acid	.0225	33,600			—20,300	—56.6		
	6	.....	....	13,200	70,000	234				
		p-aminobenzoic acid	.017	18,400	42,300	142	—18,200	—50		
Achromobacter Harveyi	1	.....	....	27,000	69,600	228				
		Sulfanilamide	.005	31,800	86,300	781	—13,500	—31		
A. Fischeri	1	.....	....	34,800	83,800	279				
		Sulfanilamide	.00038	34,700	86,600	286.6	—14,950	—31.7		
Bacterium phosphoreum (Morrison, '25)		Single experiment		22,440						
Vibrio phosphorescens (Root, '32)		(average of 16 experiments, 20 to 24 hour cultures)		25,620						
Vibrio phosphorescens (Root, '32)		(average of 15 exps.; 14-18 hr. cultures)		28,300						
Panus stypticus		(average of 2 experiments)		26,600						

[1] The data of van Schouwenburg ('38) indicate that three molecules of urethane combine with one molecule of the enzyme. This value was used in calculating the entropy of the urethane equilibrium. The value of one, found for sulfanilamide, was assumed to hold throughout, except for urethane.

for the slopes of the curves it is evident from table 1 that the differences among different species are hardly greater than the differences which may occur in the same species on different occasions. Thus, the values obtained for both the activation energy of luminescence, at low temperatures, and for the heat of reaction in the reversible inactivation, at high temperatures (relative to the optimum) vary in repeated experiments. Moreover, they vary under the influence of added substances, including urethane and sulfanilamide, as set forth below. Under the influence of inhibitors, the optimum temperature itself may shift over a range as great as that exhibited by different species. It should be emphasized that these variations are not the result of experimental error of measurements, nor of any apparent difference in procedure employed in repeated experiments. The equations developed in the preceding section furnish a theoretical basis for just such changes.

These facts bear on the critical evaluation of the significance of the slopes normally found both within a given species, and among distinctly different species. They indicate that the observed slopes of the lines are determined not only by the intrinsic properties of the luminescent system, such as might be observed with the extracted, pure substrate and enzyme, but also by the specific metabolic "landscape" of the reaction. In accounting for the observed differences, it might easily suffice if different chemical products of normal metabolism should combine reversibly with the enzyme, and thereby influence both the optimum temperature and the apparent activation energy of its reaction. It is possible that the true activation energy would in each case be the same, or nearly so, in isolated systems of the several organisms, if it could be measured under conditions involving a minimum of inactivating influences on the enzyme. Since the luminescent systems cannot be extracted as yet from bacteria or molds, only indirect evidence is available concerning their fundamental identity in different organisms. Spectrographic analysis of several of the luminescent species (Eymers and van Schouwenburg) is one line of evidence for a basic similarity in the light-emitting systems. The analysis of the sulfanilamide inhibition, described below, provides further evidence of interest in this as well as in more general connections.

The sulfanilamide inhibition of luminescence intensity

In figure 2, the relation between temperature and luminescence intensity for four different species of luminous bacteria, with different absolute temperature relations, is shown together with that for corresponding portions of the same respective cell suspensions containing

a moderately inhibitory concentration of sulfanilamide. It is at once apparent that in each case the curve for the sulfanilamide-containing suspension is related to the normal in such a manner that the per cent inhibition greatly decreases as the temperature increases towards the optimum and beyond. Associated with this is the tendency for the optimum temperature itself of the inhibited cells to shift to a slightly higher temperature than normal. The apparent activation energies

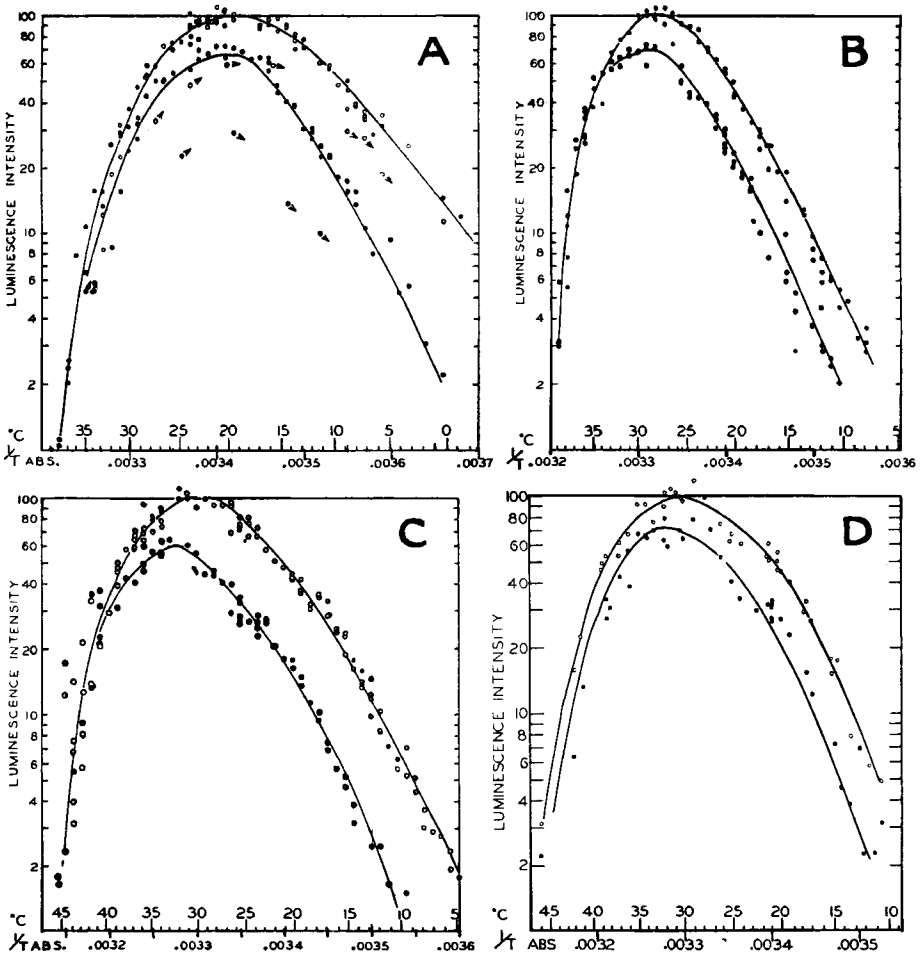

Fig. 2 Relation between temperature and luminescence intensity of cell suspensions with added sulfanilamide (solid circles) in comparison to a control portion of the same suspension (hollow circles). Figure A; P. phosphoreum; lower curve for suspension containing 0.0025 M. sulfanilamide. (The points with arrows were obtained on cooling the two respective suspensions. Some permanent heat destruction is evident, and a slight shift in optimum for the cooling curve. The latter effect has not been studied critically as yet). Figure B: A. Fischeri; lower curve with 0.00038 M. sulfanilamide. Figure C: V. phosphorescens; lower curve with 0.003 M sulfanilamide. Figure D: A. Harveyi; lower curve with 0.005 M. sulfanilamide.

of the sulfanilamide-inhibited suspensions are also different from those in the normal. These differences are all consistent with the theoretical equations, which, when applied to the experimental data indicate that the sulfanilamide inhibition conforms fairly closely to type I. In figure 3 data obtained from figure 2 are plotted in the manner described for inhibition type I. The points are remarkably close to the same straight line over a considerable range of inhibition. In spite of the differences in the normal activation energies of luminescence, heats of reaction

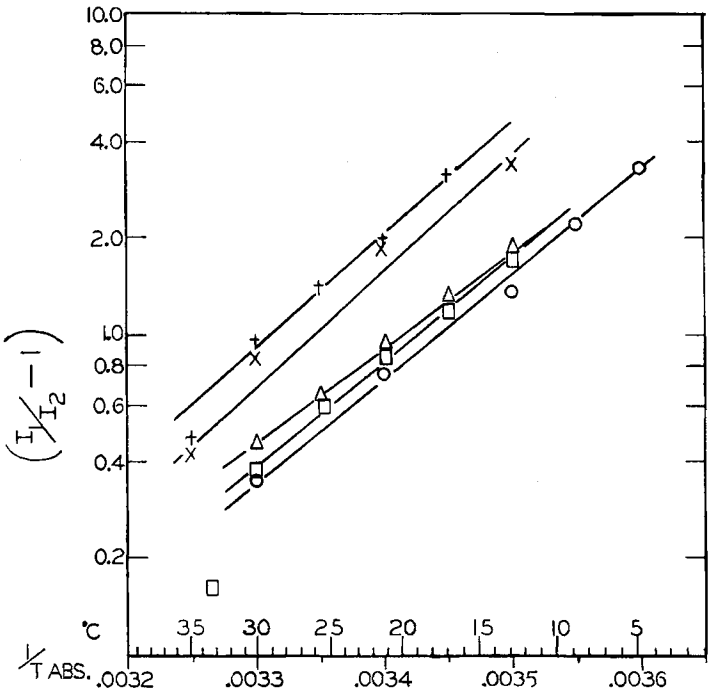

Fig. 3 Temperature relation of the sulfanilamide inhibition of luminescence in different species. The slopes of these lines indicate the heats of reaction for the equilibrium between the enzyme and sulfanilamide. V. phosphorescens, crosses (also, additional experiment, indicated by plus marks); P. phosphoreum, circles; A. Harveyi, triangles; A. Fischeri, squares.

of denaturation equilibria, and optimum temperatures of different species (fig. 2 and table 1), very similar slopes are obtained for the temperature relation of the sulfanilamide inhibition. This means that the heat of reaction of the sulfanilamide-enzyme equilibrium is practically identical, and consequently, the combinations likely take place at a very similar group on the enzyme molecule. Thus, we have evidence in addition to that obtained in spectral analysis that a closely related system is concerned in the luminescence of different species.

Studies with the extracted and purified luminescent system of Cypridina (Johnson and Chase, '42) have shown that the light-emitting system itself is directly affected. Both sulfanilamide and urethane cause a reversible inhibition. With regard to the site of combination in this system, numerous considerations make it appear extremely unlikely that it is the substrate, although direct evidence of combination with the enzyme is not available.[4] On the other hand, it might well combine with a prosthetic group rather than the actual protein of the enzyme;

[4] Kinetic data alone are not sufficient to establish this point. The following considerations show that a reversible combination of the inhibitor with either the enzyme or the substrate, respectively, could account for the changes in reaction velocity of the system in vitro, such as those described for luminescence. From equation (1) above, $I = sk\,(LH_2)\,(A)$. Letting (S) represent the concentration of inhibitor, and "n" the number of molecules combining with one molecule of enzyme (case 1) or of substrate (case 2) respectively, and letting the subscript "$_o$" refer to the initial concentration, we may consider the results of the two cases on the intensity of luminescence. Under given conditions, the intensity of luminescence is assumed proportional to reaction velocity as expressed in equation (1).

Case (1)

$$I = ek(LH_2)(A)$$

$$(LH_2) = (LH_2)_o - (L_1)$$

$$A + nS \rightleftharpoons A\cdot Sn$$

$$\frac{(A\cdot Sn)}{(A)(S)^n} = K_{(1)}$$

$$(A) + (A\cdot S_n) = (A_o)$$

$$(A) = \frac{(A_o)}{1 + (S)^n K_{(1)}}$$

$$I = \frac{ek[(LH_2)_o - (L_1)](A)_o}{1 + (S)^n K_{(1)}}$$

Case (2)

$$LH_2 + nS \rightleftharpoons LH_2\cdot Sn$$

$$\frac{(LH_2\cdot S_n)}{(LH_2)(S)^n} = K_{(2)}$$

$$(LH_2) + (L_1) + (LH_2\cdot S_n) = (LH_2)_o$$

$$(LH_2) + K_{(2)}(LH_2)(S)^n = (LH_2)_o - (L_1)$$

$$(LH_2) = \frac{(LH_2)_o - (L_1)}{1 + (S)^n K_{(2)}}$$

$$I = \frac{ek[(LH_2)_o - (L_1)](A_o)}{1 + (S)^n K_{(2)}}$$

Thus, whether we assume that the reversible combination takes place between inhibitor and enzyme, or inhibitor and substrate, we arrive at a similar equation. The intensity in relation to time, during the course of the reaction may be formulated as follows, in which "a" = total light at time = infinity. As usual, total light at $t = \infty$ is taken as a measure of the amount of LH_2 oxidized to L_1 by luciferase. A proportionality constant α is introduced to allow for the units of measurements. Thus, $\alpha\,(LH_2) = \alpha a$, and $(L_1) = \alpha x$. From the above,

$$I = \frac{dx}{dt} = \frac{ck[(LH_2)_o - (L_1)](A_o)}{1 + (S)^n K} = \alpha\,\frac{ck\,(a - x)\,(A_o)}{1 + (S)^n K}$$

whence, $\ln (a - x) = \ln a - \left(\frac{ck\,(A_o)}{1 - (S)^n K}\right) t$, or,

$\ln (a - x) = \ln a - gt$, in which g represents the slope of the line when $\ln (a - x)$ is plotted against time. This is the integrated form of the first order equation, $dx/dt = g\,(a - x)$. The results from experiments do, indeed, give straight lines, which, with given enzyme concentration, are parallel for different concentrations of substrate. Likewise, the change in slope resulting from sulfanilamide addition is independent of the substrate concentration, in accordance with experimental data. Dilution experiments demonstrating reversibility of the combination cannot distinguish between the enzyme and substrate as the combining molecules with the inhibitor, since both are present when the reversibility is tested. A final conclusion as to whether it is the enzyme or substrate molecule that combines with the inhibitor must, therefore, await more direct evidence than is available from kinetic data alone.

indeed, if the same prosthetic group in combination with genetically different protein-carriers, were concerned in the luminescence of different species, the results described above would be very nicely accounted for.

In figure 4 the log $(\frac{I_1}{I_2}-1)$ is plotted against log concentration of sulfanilamide for portions of a single suspension of bacteria at various temperatures. From the previous discussion, the slope of the straight lines represents the number of molecules of sulfanilamide combining

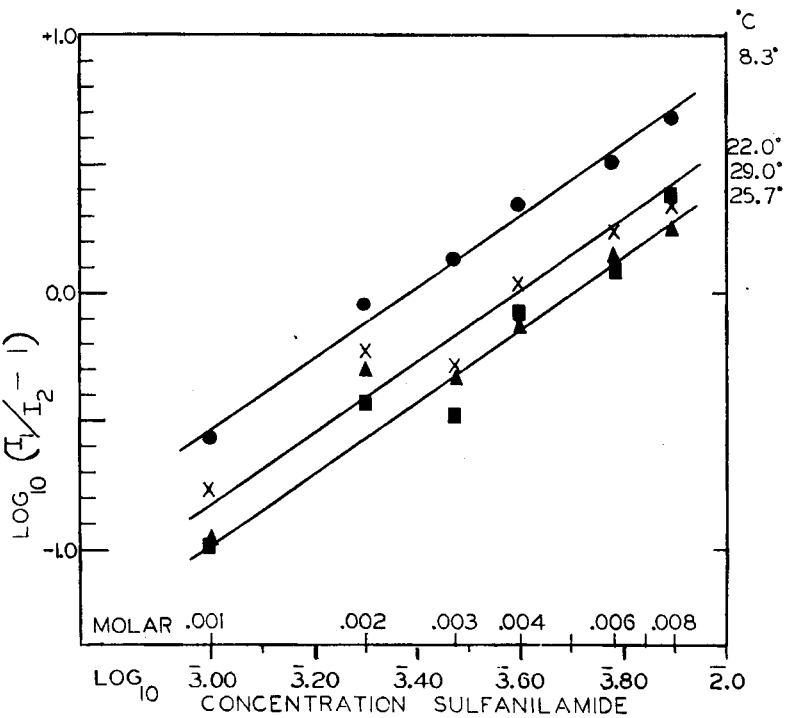

Fig. 4 Relation between concentration of sulfanilamide and inhibition of luminescence in P. phosphoreum at different temperatures. The slope of lines relating log $(\frac{I_1}{I_2}-1)$ to log concentration indicates that approximately 1 molecule of sulfanilamide combines with 1 molecule of enzyme..

with one molecule of the enzyme. The slopes are clearly independent of temperature. The actual values indicate that approximately one molecule of sulfanilamide combines with one molecule of the enzyme.

The temperature relations of luminescence inhibited by p-aminobenzoic acid are very similar to those obtaining in the case of sulfanilamide. Figure 5 represents a typical experiment. Table 1 gives the values of ΔH_2 and ΔS_2 calculated in the same manner as with sulfanilamide.

It will be noted from table 1 that the heat of reaction for the combination of p-aminobenzoic acid with the enzyme appears to be somewhat higher, by some 4,000 calories, than that for sulfanilamide. It might be expected, therefore, that it would combine some 1000 times more readily than sulfanilamide with the enzyme. Yet the concentration of p-aminobenzoic acid required to cause a moderate inhibition is much higher than the concentration of sulfanilamide. This may mean that

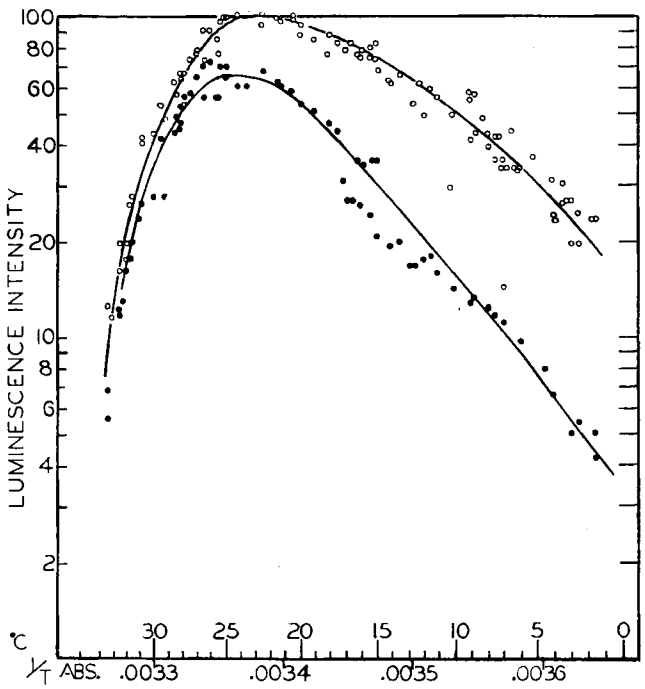

Fig. 5 The temperature-luminescence intensity of a suspension of P. phosphoreum containing 0.017 M. p-aminobenzoic acid (solid circles), as compared with a control portion of the same suspension (hollow circles).

these calculated differences in heats of reaction are not real, especially since the exact values of ΔH are extremely sensitive to slight errors in the experimental curves. On the other hand, the differences may indeed be real. This would indicate that p-aminobenzoic acid loses considerably more entropy in being adsorbed than does sulfanilamide. Such a situation would be understandable if, for example, only ions of p-aminobenzoic acid were adsorbed, while neutral molecules of sulfanilamide are adsorbed. The exact explanation must await further experiments. Certain points, however, are worthy of mention in regard to the action of these drugs on luminescence, and on bacterial growth, respectively.

Para aminobenzoic acid is extremely potent in antagonizing the sulfanilamide inhibition of bacterial growth (Woods, '40; Rubbo and Gillespie, '40, '42) and subsequent investigators. The weight of the evidence indicates that the former or a related compound may be concerned in a metabolic reaction normally essential to growth, and that the latter compound exerts its inhibitory effects by combining competitively at the same catalytic site. If the heat of reaction of p-aminobenzoic acid in this hypothetical combination is slightly higher than that of sulfanilamide, and the entropy change approximately equal, it is entirely understandable why such a small concentration would be needed to antagonize the sulfonamide inhibition, i.e., for each approximately 1,300 calories higher, the former should combine ten times more readily and thus allow the reaction to proceed in spite of the presence of an excessive number of sulfanilamide molecules. This is on the assumption that p-aminobenzoic acid is the normal substrate or "metabolite" in growth. In luminescence, however, presumably involving a different enzyme, even if p-aminobenzoic acid combined with the luciferase preferentially in the presence of sulfanilamide, the sulfonamide inhibition would not be lessened since p-aminobenzoic acid is not identical with luciferin, the normal substrate, and cannot take its place in the reaction. The only result that would be expected of the combined action of p-aminobenzoic acid and sulfanilamide in this case would be an increased inhibition. Such an effect is, indeed, observed (Johnson and Chase, '42). It is possible, of course, that both p-aminobenzoic acid and sulfanilamide are chemically related to luciferin, and all three compete for the same site on the luciferase. Furthermore, while the metabolic relationships of the light-emitting system are not known, it is possible that this system may be normally involved in growth. The peculiar property of light-emission itself is essential neither to growth nor to respiration, and may be manifest only under certain conditions. The possibility of a non-luminous functioning of luciferin in normal respiration has already been discussed (Johnson, van Schouwenburg, and van der Burg, '39). A rôle in growth is entirely speculative. The possible rôle of p-aminobenzoic acid in growth also remains to be elucidated.

The urethane inhibition of luminescence

In contrast to the effects of sulfanilamide, urethane causes the optimum temperature of luminescence to shift slightly in the direction of the lower temperatures (fig. 6). With suitable concentrations of the two inhibitors added to portions of the same suspension of bacterial

cells, it is possible to obtain respective optimum temperatures differing by 10°C., without greatly inhibiting the absolute intensity below that of the normal suspension. It will be recalled that the total range in optimum temperatures among different species, figure 1, is only some 10°C. When the temperature relations of the urethane inhibition are

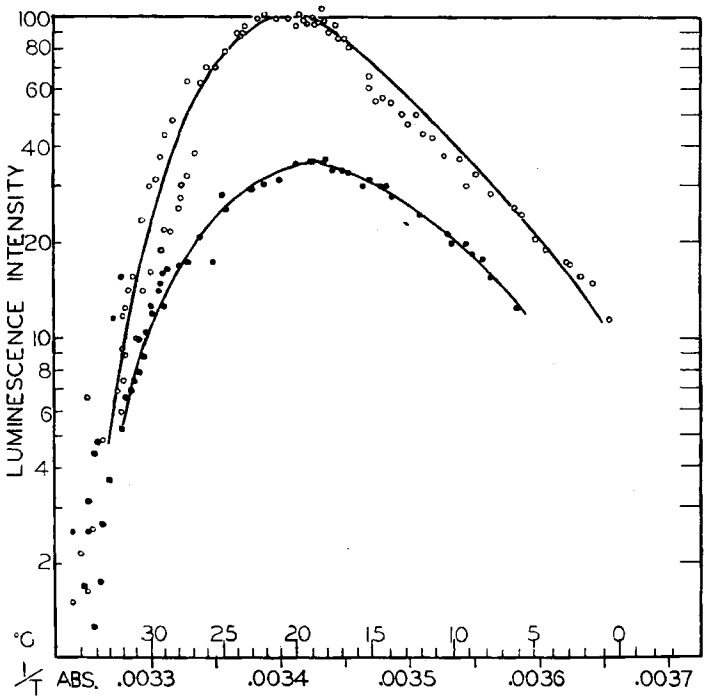

Fig. 6 The temperature-luminescence intensity of a suspension of P. phosphoreum containing 0.07 M. urethane (solid circles) in comparison with a corresponding portion of the same suspension without added inhibitor (hollow circles).

plotted in the same manner as sulfanilamide, the peculiar curve indicated by dotted lines in figure 7, results, showing that the data do not conform to type I. When the same values of $(I_1/I_2 - 1)$ are multiplied by $(1 + 1/K_1)$ and the log of the product plotted against 1/T, the straight line shown in figure 7 results. Thus, the data conform to inhibition type II. The values for K_1 are obtained from the normal curve, in the high temperature range. The straightness of the line in figure 7 provides substantial evidence of the correctness of the theory. The straight-line relation holds over a range of some 2,000 times on the ordinate. It would appear, therefore, that urethane combines with the denatured form of the enzyme in the reversible denaturation equilibrium, and in so doing, causes the equilibrium to shift to the right,

decreasing the amount of catalytically active enzyme, and consequently, the velocity of the reaction and observed intensity of luminescence. This conclusion is altogether in accord with expectations on the basis of the pressure effect in reversing both the temperature and urethane inhibitions, reported earlier. The effect of the urethane is substantially to

TABLE 2

Data for calculation of the heat of reaction (ΔH_3) in the equilibrium between 0.07 M. urethane and the "denatured" form of the enzyme in luminescence.

($K_1 = e^{\frac{-70{,}000}{RT}} e^{\frac{234}{R}}$; values of I_1 and I_2 from fig. 6)

$\frac{1}{T}$ ABS	I_1	I_2	$\frac{I_1}{I_2} - 1$	$\frac{1}{K_1}$	$\left(\frac{I_1}{I_2} - 1\right)\left(1 + \frac{1}{K_1}\right)$
0.00330	21	11.5	1.82 — 1	$10^{-0.6}$	1.03
0.00335	69	24	2.87 — 1	$10^{0.11}$	4.28
0.00340	100	34	2.94 — 1	$10^{0.87}$	16.30
0.00345	86	34	2.53 — 1	$10^{1.63}$	66.9
0.00350	56	26	2.15 — 1	$10^{2.39}$	282.9
0.00355	35	18	1.95 — 1	$10^{3.15}$	1340.4

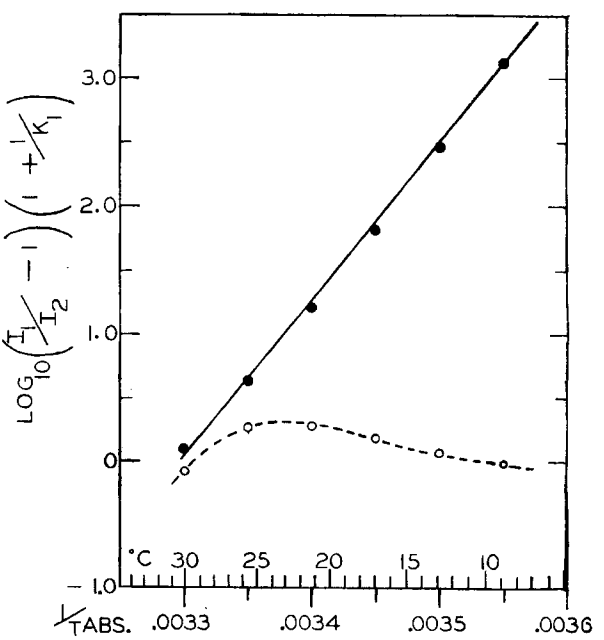

Fig. 7 Temperature relations of the urethane inhibition of luminescence in P. phosphorcum. The data from figure 6 and table 2, plotted as described in the text for inhibition type II (combination of the inhibitor with only the "denatured" form of the enzyme) are shown by the solid circles and straight line. When plotted according to type I (combination of the inhibitor with native and "denatured" forms of the enzyme indiscriminately), the curve shown by the dotted line results.

lower the temperature for reversible denaturation. The shift of the optimum temperature is thus understandable. It is also understandable that the heat and entropy values of the equilibrium formed by urethane with the enzyme (table 1; ΔH_3 and ΔS_3) are very high, amounting in some cases to as much as 70,000 calories since three molecules combine with the enzyme molecule in this case. Furthermore, unlike sulfanilamide, the values observed are by no means identical in the various species. On the other hand, they are related to the heat of reaction for the normal denaturation. These facts are all consistent with the interpretations advanced.

GENERAL IMPLICATIONS

It would appear clearly established that in luminescence of bacteria, the urethane inhibition proceeds through influencing a denaturation equilibrium of the enzyme normally present. The fact that the mode of action appears to be a combination of the urethane with the denatured form is interesting in connection with the action of non-specific inhibitors in general. The pressure study reported earlier would place alcohol, chloroform, ether, and novocaine in the same class of inhibitors as urethane. The earlier distinction on the basis of pressure effects between this group, and another which included sulfanilamide, p-aminobenzoic acid, barbital, and chloral hydrate, has been generally substantiated by the present study. It should be emphasized, however, that a more detailed study of many other inhibitors, including those in the above lists, would likely reveal some whose effects do not conform so clearly as sulfanilamide and urethane to type I or to type II, respectively, but are intermediate; i.e., may set up complex equilibria in which different combinations are formed with the native and denatured forms of the enzyme. Furthermore, divergences from expected relations, in any complex system such as the environment of the living cell, may result from a change in mechanism. Theoretical type III, in which the inhibitor combines only with the native form of the enzyme, was not illustrated in the present study. Finally, it should be pointed out that the theory developed is concerned with the fundamental nature, and not the site, of the inhibitions. The actual metabolic reaction which is most susceptible to the action of the inhibitor, and which may be responsible for most of the physiological changes observed in a given instance, would have to be delimited by other evidence. The present theory is general, and would apply to various enzyme systems.

The foregoing results are of especial interest in connection with a critical view of the significance of temperature relations of a biological

process under normal conditions. Since the apparent activation energy of luminescence in a given species normally varies somewhat, and may be influenced by added substances which affect the enzyme, the question naturally arises as to the significance of the observed values. Likewise, since the optimum temperature of the same process may be altered, by adding substances causing a reversible inhibition, over a range fully as great as that which is normally observed with diverse species, the possible effects of normal metabolites might be of critical importance in this connection also. Certain products of metabolism most likely do enter into a reversible combination with various enzymes within the normal living cell, affecting their over-all activity to a greater or lesser extent. All such equilibria thus established are potentially important factors in determining the observed reaction of the system to temperature and pressure. Actually, therefore, the true activation energy of the catalytic reaction itself, e.g., the oxidation of luciferin by luciferase, will in general be somewhat different from that observed, except in an isolated, purified system. In some cases the values that would be obtained with the pure systems might be closely approximated by measurements carried out on the living cell. On the other hand, the fact that added substances cause such variations as described in this paper make it clear that all measurements of temperature or pressure relations of enzyme activity in the living cell are made against the particular metabolic "landscape" of the cells in question. This landscape varies not only in different species, but within the same species in the presence of inhibitors, or under different physiological conditions, as, perhaps, age of the culture. The action of any newly added inhibitor will be in some measure conditioned by the equilibria already present involving the enzyme. By appropriate methods of analysis, these other equilibria may sometimes be "cancelled out," and a basic similarity noted, as in the uniform value for the heat of reaction of the sulfanilamide combination in different species, in spite of diverse other temperature relations. The temperature, or the pressure effects are of the utmost importance in arriving at a clear picture of the action of inhibitors in general, while the influence of normal metabolites on the enzyme is very likely equally important in determining the over-all velocity and the normal optimum temperature of the system.

The authors wish to acknowledge the faithful and capable technical assistance of Mr. G. J. Gherardi in carrying out the experiments with the bacteria.

SUMMARY

On the basis of theoretical considerations, mathematical expressions are derived for the reversible inhibition of enzyme activity by temperature, pressure, and in general, substances which enter into a reversible combination with the catalyst. The temperature and pressure effects are assumed to be mediated through an equilibrium normally present between active and "denatured" forms of the enzyme, with equilibrium constant K_1.

Expressions are formulated for obtaining the equilibrium constants in three possible types of enzyme-inhibitor combinations, as follows: (I_1 is the rate of the uninhibited, and I_2 of the inhibited reaction). Type I, indiscriminate combination of the inhibitor with native and "denatured" forms of the enzyme: $\log_{10}\ (I_1/I_2 - 1) = -\frac{\Delta E}{2.303RT} - \frac{p\Delta V}{2.303RT} + \frac{\Delta S}{2.303R} + r \log_{10} X$. Type II, combination of the inhibitor with only the "denatured" form of the enzyme: $\log_{10}\ ((I_1/I_2 - 1)\ (1 + 1/K_1)) = -\frac{\Delta E}{2.303RT} - \frac{p\Delta V}{2.303RT} + \frac{\Delta S}{2.303R} + s \log_{10} X$. Type III, combination of the inhibitor with only the active form of the enzyme: $\log_{10}\ ((I_1/I_2 - 1)\ (1 + K)) = -\frac{\Delta E}{2.303RT} - \frac{p\Delta V}{2.303RT} + \frac{\Delta S}{2.303R} + r \log_{10} X$. In these expressions, the energy of reaction ΔE plus the energy change of the reactants, $p\Delta V$, equals the heat of reaction, ΔH. In any case, a plot of $\log (I_1/I_2 - 1)$ against log molar concentration (X) reveals the number of inhibitor molecules (r) or (s) combining with one molecule of the active or "denatured" forms, respectively, of the enzyme in a given reaction.

Application of these formulations to experimental data obtained with diverse species of luminous bacteria indicates that the sulfanilamide inhibition of luminescence conforms largely to type I, with a ΔH of approximately — 15,000 calories, and a ΔS of about — 35 entropy units (calories per degree) for all species. Approximately one molecule of sulfanilamide combines with each enzyme molecule. The p-aminobenzoic acid inhibition of luminescence appears to be fundamentally similar to that of sulfanilamide.

The urethane inhibition of luminescence conforms largely to type II, and indicates a ΔH of — 56,000 to — 70,000 calories and ΔS of — 165 to — 204 E. U. in the species analyzed. Unlike the constants for sulfanilamide, these depend on the "normal" temperature relations of luminescence in the particular species. The data of van Schouwenburg ('38) indicate that approximately 3 molecules of urethane combine with each enzyme molecule.

By addition of these inhibitors to cell suspensions of a single species, both the apparent activation energy and the normal optimum temperature of luminescence may be experimentally shifted to as great an extent as is normally encountered in different species.

The bearing of these considerations and data on a critical evaluation of the temperature relations of biological processes in general is discussed.

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