THE NATURE AND CONTROL OF REACTIONS IN BIOLUMINESCENCE

With Special Reference to the Mechanism of Reversible and Irreversible Inhibitions by Hydrogen and Hydroxyl Ions, Temperature, Pressure, Alcohol, Urethane, and Sulfanilamide in Bacteria

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I. INTRODUCTION

The purpose of this paper is to present a general theoretical discussion, together with fairly extensive new data from experiments, concerning the action of various factors which influence the activity of enzymes in living systems. Although numerous studies have been published dealing with various aspects of the subject under discussion, certain circumstances have provided a means of reaching a somewhat clearer and perhaps more comprehensive view than has been possible heretofore. The most important of these circumstances are, briefly, as follows:—

In the first place, the concept of a reversible denaturation of the protein catalyst has furnished a partial explanation of some familiar characteristics of the relation between temperature and observed reaction rates in biological systems; *e.g.*, the reversible decrease in apparent temperature coefficient of a given process, as the temperature is increased towards the optimum. Qualitatively, the reversible denaturation of proteins as a possible controlling factor in biological reactions has been recognized for some time by various investigators, especially protein chemists (Kunitz and Northrop, 1934; Anson and Mirsky, 1934 *a*, *b;* Mirsky, 1936; *cf.* also, Anson, in Schmidt, 1944, p. 407). Kunitz and Northrop found that crystalline trypsin could be reversibly denatured by raising the temperature or increasing the alkalinity. If the conditions favoring reversible denaturation were made extreme or held for some time the denaturation became irreversible. They also observed a pronounced optimum for pH in enzyme activity. These results are particularly interesting in connection with the present study because of the close parallelisms shown to enzyme behavior in the living cell.

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More recent evidence has indicated that the reversible denaturation has a wider significance than in relation to the usual temperature-activity curves alone. For, the equilibrium between active and inactive forms of the enzyme, in the only cases thus far adequately studied, is characterized by a large volume change, as well as by the high heat and entropy of reaction typical of such complex molecules as those of proteins (Eyring and Stearns, 1939). Consequently, this equilibrium becomes indispensable in accounting for not only the temperature effect but for two other seemingly diverse types of phenomena, namely, (*a*) the effects of hydrostatic pressure, and (*b*), the action of certain drugs in relation to both temperature and hydrostatic pressure. With regard to (*a*), the pressure effects on the equilibrium become increasingly apparent as the value of the equilibrium constant becomes larger, with rise in temperature. Under conditions where this constant is large, and the effects of pressure on other reactions are relatively small, pressures of a few hundred atmospheres may increase the over-all rate several-fold, by shifting the equilibrium in favor of the active form of the enzyme.

As for (*b*), since catalytically active and inactive forms of the enzyme exist in equilibrium, at least two types of reversible combinations of different drugs with a given enzyme may take place: (I) at bonds not involved in the denaturation equilibrium, and (II) at bonds made available though this equilibrium. Different effects of temperature and pressure on the inhibitions produced by the two respective types would be anticipated: With "Type I" the inhibition should ordinarily decrease with rise in temperature, as the enzyme-inhibitor compound is dissociated, and it should be relatively insensitive to pressure because the reaction is independent of the reversible heat denaturation of the protein. With "Type II," on the other hand, although a rise in temperature would again bring about a dissociation of the enzyme-inhibitor complex, it might at the same time increase, to an even greater extent, the bonds available for combination between the two molecules, and the net effect would thus be an increase in inhibition. Moreover, because of the large volume change involved in the denaturation equilibrium, the action of drugs which combine in a manner that is dependent upon the value of this equilibrium will generally be influenced by hydrostatic pressure. Some quantitative evidence and kinetic formulations apropos of the denaturation equilibrium in controlling the response of the luminescence system to temperature, pressure, and drugs have been set forth in recent studies (Johnson, Brown, and Marsland, 1942 *a*, *b;* Brown, Johnson, and Marsland, 1942; Eyring and Magee, 1942; Johnson, Eyring, and Williams, 1942; Johnson, Eyring, and Kearns, 1943; Johnson and Schneyer, 1944). The present paper amplifies and extends the previous work, by taking into account the irreversible as well as reversible inhibition of the enzyme, and also by including in the same general formulation additional factors, such as hydrogen ion concentration.

In the second place, the theory of absolute reaction rates (Eyring, 1935; Evans and Polanyi, 1935; Glasstone, Laidler, and Eyring, 1941) has provided a precise, modern physical-chemical basis for the understanding of rate processes similar to that available for equilibria. Thus, the theoretical significance of temperature, as well as the fundamental relation of hydrostatic pressure, to reactions in general have finally been clearly elucidated. An application of this theory to quantitative studies of biological processes has been made with some success, as in the investigations by the senior authors and their collaborators, referred to above (*cf.* also, Brown and Marsland, 1942; McElroy, 1943).

In the third place, the picture arrived at in this study has been greatly facilitated by particularly favorable experimental material. Luminescence furnishes a unique, natural indicator of its own reaction velocity, making it possible to record with both ease and accuracy the velocity at any instant. For, there is abundant evidence that under given conditions the intensity of the light provides a reliable index to the rate of the luminescent oxidation, and this circumstance makes possible the obtaining of accurate data during rapidly changing states of the system. Furthermore, because of the speed of observations, extensive experiments can be carried out with portions of the same suspension of bacterial cells, thus permitting unusually accurate comparisons of the effects of different factors, such as the relation of concentration of an inhibitor to both temperature and pressure. The investigations which constitute the background of our present study of bioluminescence, and which justify the more or less generally accepted assumptions on which certain aspects of the analysis are predicated, have been comprehensively treated in Harvey's recent monograph, Living light (1940) and review papers (Harvey, 1935; 1941). Subsequent investigations, and detailed points pertinent to the discussion, will be referred to later on.

It would hardly be possible to give fully adequate reference to the literature concerning the physical-chemical analysis of biological reactions, nor would an effort towards such an end be appropriate here. Certain modern studies, however, including some reviews and monographs, which for one reason or another have become more or less outstanding, might well be cited, without reference to whether or not we are fully in accord therewith. Among them are the following: Bělehrádek, 1935; Cattell, 1936; Clark, 1937; Crozier, 1924 *a*, *b*, 1926, and subsequent papers; Fisher and Öhnell, 1940; Henderson, 1930; Hoagland, 1936; Lineweaver and Burke, 1934; Meyer, 1927; Overton, 1901; Warburg, 1914, 1927; Warburg and Negelein, 1921, 1928; Warburg and Wiesel, 1912; Winterstein, 1926. Some quite recent studies in related fields appear to be in substantial accord with the ideas and treatment we have set forth (Winzler, 1943; Quastel, 1943; Goldstein, 1944).

II. THE BIOLUMINESCENT REACTION

(*a*) *In Extracts*

The more recent investigations on *Cypridina* extracts by Harvey and his associates (Harvey, 1941) have provided much suggestive evidence concerning the nature of the luminescent system and the manner in which it operates. In particular, the studies of Anderson (1933, 1935, 1936, 1937), Chase (1940, 1943), Chakravorty and Ballentine (1941), Johnson and Eyring (1944), and McElroy and Ballentine (1944), have furnished cogent information. As a result it now seems possible, through a critical examination of available data, to arrive at a more detailed picture of the mechanism of the reactions involved, although the chemical identity of the molecules remains to be established. The data on the effects of hydrogen ions in bacterial luminescence, presented in this paper, lend support to the existence of reactions which appear most probable on the basis of the previous evidence. Since a clear, and in so far as possible complete, view of the reactions influencing the production of luminescence is of obvious advantage in the analysis of the rate-controlling factors, the essential points in our present understanding are briefly set forth in the following paragraphs.

In accordance with a customary notation we will designate the comparatively heat-stable, dialyzable substrate, luciferin, as LH_2, and the heat-labile, nondialyzable enzyme, luciferase, as A. The luciferin from *Cypridina* has been greatly purified and concentrated. In the reduced state and in the absence of oxygen it may be kept indefinitely at room temperature. When oxygen, or certain other agents such as ferricyanide, are added, it undergoes an oxidation that is reversible by hydrosulfite, and is not accompanied by the production of visible light. The products do not remain long in a reversibly oxidized state, however, and fairly rapidly undergo a change leading to decomposition. The state of the luciferin may be determined simply by the addition of luciferase plus oxygen, in the presence of which reduced luciferin undergoes a luminescent oxidation while the reversibly oxidized, or decomposition products do not. In the luminescent oxidation, the total light emitted under given conditions is proportional to the amount of reduced luciferin supplied, and the intensity of the light at any moment is proportional to the product of the concentration of the reduced luciferin times that of the luciferase.

The behavior of luciferin towards oxidation shows that we must recognize at least three states of this stubstance: first, reduced luciferin, already referred to as LH_2, second, reversibly oxidized luciferin, which we will designate as L, and third, irreversibly oxidized, or in some manner destroyed, luciferin, which we will call L_1. In addition, from the fact that luciferin has been observed to luminesce under conditions which completely denature the enzyme, *viz.* in 95 per cent ethyl alcohol at 70°C. (Harvey, 1928) it is evidently necessary

to recognize an excited state of the luciferin.[1] Except under the aforementioned conditions, visible luminescence occurs only when LH_2 undergoes oxidation in the presence of both molecular oxygen and luciferase.[2] On the basis of spectrographic and certain other lines of evidence, as set forth below, it seems safe to conclude that in both cases the radiating molecule is the luciferin,[3] and since excitation occurs during oxidation we may designate the excited molecule as L*.

Further evidence that luciferin is the molecule that radiates in the bioluminescent oxidation derives from spectrographic studies with highly purified extracts (Chase, 1943). Concentrated solutions of reduced luciferin show an absorption maximum at 4350 Ångstrom units. The amount of luminescence which can be obtained on addition of luciferase plus oxygen is directly proportional to this absorption. On these grounds, the peak at 4350 Å is to be identified with LH_2. When the LH_2 solution is exposed to air, and the spectrograph quickly recorded, the peak at 4350 Å is seen to shift to 4650, and then gradually disappear. The maximum intensity of luminescence, at 4750 Å, is practically identical with the luciferin absorption peak at 4650 Å (Eymers and van Schouwenburg, 1937). This evidence suggests that the substance absorbing at 4650 may be identified with L. The disappearance of absorption in this region of the spectrum would then be associated with the formation of the substance we have designated as L_1.

Two additional facts of considerable significance in the spectrographic studies of Chase are, (1) that the luciferin apparently undergoes the same oxidative reaction in purified solution without the enzyme, as in the presence of the enzyme, though at a rate only about one hundredth as fast; and (2) that there is less absorption at 4650 Å in the presence than in the absence of the luciferase. From (1) it appears that the enzyme catalyzes the same oxidative reaction, with an accompanying luminescence, that occurs during auto-oxidation, without visible luminescence. The same oxidant results in both cases. From (2), if the compound absorbing at 4650 can be identified as L, it follows that in the enzyme preparation is a catalyst, whether luciferase or other agent,

[1] The evidence is not sufficient to conclude whether or not this is the same excitation that occurs in bioluminescence. It is an example of the luminescence corresponding to the characteristic fluorescence or phosphorescence of molecules generally (Lewis, 1944).

[2] In at least one instance luminescence has been observed under conditions excluding molecular oxygen. The mechanism in this case is not clear (Harvey and Korr, 1938).

[3] For some time it was thought that the radiating molecule was that of the enzyme, luciferase (Harvey, 1917). More recent studies (Harvey, 1944) have shed some doubt on the validity of the evidence on which the earlier conclusion was based. We favor luciferin as the molecule that radiates, for reasons given herein.

which speeds the disappearance of L. The rapid destruction of luciferin always accompanies light emission. Under conditions where there is rapid oxidation, as with a catalyst, relatively higher concentrations of the intermediate half reduced molecules are formed. These in turn presumably oxidize and reduce each other giving a completely reduced luciferin and an oxidized excited molecule in the process. One in a few hundred of the excited molecules then emits and is not destroyed,[4] while most of the remaining excited molecules are destroyed. Without an enzyme speeding the oxidation the concentration of half reduced molecules is much lower, so that instead of reacting so rapidly with one another the second hydrogen is removed by oxygen without luminescence, and without destroying the luciferin. This oxidized luciferin can now be reduced and when again oxidized with a catalyst luminesces.

This explanation does not require that the oxidation without a catalyst involve the removal of different hydrogens than are removed with the catalyst. Destruction arises only because the process proceeds by a different mechanism. In this way one understands Chase's observation that the same qualitative spectral changes with and without a catalyst occur. This was unclear if one supposed different hydrogens were being removed. It remains possible, of course, that with suitable reagents and conditions other hydrogens may be removed. In fact, the hydrogens of the luciferin redox potential have too little energy to provide the energy of luminescence upon oxidation. Thus, these hydrogens, without drastic modification of the molecule, could not be the important ones in luminescence.

With regard to the decomposition of L to L_1, it has been observed (Johnson and Eyring, 1944) that a concentrated preparation of luciferase (together with the other cold water extractives of powdered *Cypridina*), after being subjected to prolonged dialysis, and storage under aerobic conditions for about 2 years in the refrigerator, was capable of luminescing, without the addition of any new luciferin, simply by treating with hydrosulfite, then oxygen. These results were obtained with purified luciferase kindly supplied by Dr. A. M. Chase. One interpretation of this result, consistent with the facts already referred to, is that the L_1 came into equilibrium with L, an appreciable amount of which had not been removed by dialysis. When treated with hydrosulfite, reduction to LH_2 and subsequent oxidation by the enzyme in the presence of oxygen took place with light emission. Had the L continued to undergo irreversible decomposition, with a half life at this low temperature, of say a week, at the

[4] McElroy and Ballentine (1944) object to this suggestion of Johnson and Eyring (1944) that the excited luciferin molecules destroyed are those which do not luminesce, while those molecules which emit will be stabilized and can again be reduced and re-emit radiation. Their objection is that there is no evidence indicating that light emission is followed by reduction of the molecule and re-emission.

end of 2 years there would have remained 10^{-45} parts; *i.e.*, none of the original supply. The more likely explanation is that as the luciferin gets dilute the chance of collision of two half reduced molecules falls rapidly so that the degraduation of L to L_1 is much less complete than the above first order rate calculation indicates.

The possibility of reversal of the degradation L to L_1 could be determined by reducing large amounts of degraded luciferin and determining whether one gets proportionately more light upon adding hydrosulfite and oxygen. A negative result would provide compelling evidence of a falling off in the rate of degradation of L with time such as could only come from the reaction being of higher order with respect to half reduced luciferin or to the disappearance of an effective catalyst of destruction.

Through the kindness of Dr. L. Michaelis, some natural and synthetic compounds with one or more properties suggestive of a relationship to luciferin were made available to us. They included the following: glucosoalloxazine, *n*-methylalloxazine, α-araboflavin, 6,7-dimethyl-9, *l*-araboflavin, phenalloxanthin, phenazine, α-oxyphenazine, and pyocyanine. Each of these substances was tested for a capacity to luminesce under the same conditions that luciferin was observed to luminesce (Harvey, 1928); *i.e.*, in 95 per cent ethyl alcohol at 70°C. Sodium hydrosulfite was added to the alcoholic solution at room temperature, before heating to 70°. Aeration was accomplished by vigorous shaking of the test tube. No luminescence was observed under these conditions, or after the addition of oxidizing agents, including $KMnO_4$, H_2O_2, $K_3Fe(CN)_6$, $Ca(OCl)_2$, or H_2PtCl_6. Thus, although no luciferase enzyme preparation was available for testing a possible luminescent reaction with these compounds, the above results indicate that luciferin is not to be identified with any of the substances tested.

The chemical identity of luciferin remains to be established. From a consideration of spectrographic and other data the possibility has been noted (Eymers and van Schouwenburg, 1937; Doudoroff, 1938) that a flavoprotein is concerned in luminescence, and there is some evidence suggesting that luciferin might be a flavin prosthetic group of a specific enzyme (Johnson and Eyring, 1944). The absorption spectrum and emission spectrum of the *Cypridina* system, and the emission spectrum of many luminous bacteria approximate the fluorescent spectrum of riboflavin (Eymers and van Schouwenburg, 1937). These bioluminescent spectra are also closely related to the luminescent spectrum of 3-aminophthalhydrazide, which like the fluorescent alloxazine nucleus of the flavin has two amino groups adjoining carbonyl groups attached to a benzene ring. The general presence of yellow pigments in organs of luminescence, together with quantitative analytical evidence of an excessively high flavin content in the light organ of the firefly (Ball and Ramsdell, 1944) also indicate an intimate rôle of flavins in light emission. On the other hand,

in a micro analysis, Chakravorty and Ballentine (1941) failed to detect the presence of nitrogen in highly purified *Cypridina* luciferin. More recently, McElroy and Ballentine (1944) have obtained evidence that phosphate is liberated during the luminescent oxidation of purified luciferin by luciferase and associated substances in the cold water extracts of *Cypridina*. Anderson and Chase (1944) have expressed doubt that luciferin is to be identified with riboflavin, chiefly on the basis of the absorption spectra, and of the behavior of preparations containing luciferin with respect to oxidation-reduction potentials. Similar arguments have been advanced by McElroy and Ballentine. Korr (1936), Anderson (1936), and Chakravorty and Ballentine (1941) have noted the possibility that luciferin may be a polyhydroxy benzene. The study of oxidation-reduction potentials of flavoproteins by Michaelis, Schubert, and Smythe (1936) has shown that the semioxidized form comes into equilibrium in amounts of the same order of magnitude as the fully oxidized and fully reduced forms. Inasmuch as the data of Chase show no evidence of an intermediate form which might be interpreted as LH, in the oxidation of luciferin, (unless LH and LH_2 have the same absorption, which is not likely), the results of these two studies indicate that luciferin is not an ordinary flavin.

In the scheme of reactions set forth below, we have undertaken to systematize our knowledge and to provide a working hypothesis for interpreting the phenomena discussed in this paper. This hypothesis agrees in part with the mechanism of reactions suggested by Weiss (1938). It includes as a key intermediate, the semioxidized form of luciferin, LH.

In addition to the notation already given we will refer to the several reactions first by number, and later by either rate constants, k, or equilibrium constants, K, with subscripts to specify the reaction number. The backwards reaction is assumed to be small in comparison with the forwards reaction unless otherwise noted.

	With luciferase		Additional reactions that occur with and without luciferase
(1)	$AL + XH_2 \rightleftarrows ALH_2 + X$	(1′)	$L + XH_2 \rightleftarrows LH_2 + X$
(2)	$A + LH_2 \rightleftarrows ALH_2$		
(3)	$ALH_2 + O_2 \rightleftarrows ALH + HO_2$	(3′)	$LH_2 + O_2 \rightleftarrows LH + HO_2$
(4)	$ALH \rightleftarrows AL^- + H^+$	(4′)	$LH \rightleftarrows L^- + H^+$
(5) alpha	$AL^- + LH \rightarrow AL^* + LH^- \rightarrow AL + LH^- + h\nu$	(5′) alpha	$L^- + LH \rightarrow L^* + LH^- \rightarrow L + LH^- + h\nu$
(5) beta	$AL^- + LH \rightarrow AL + LH^-$	(5′) beta	$L^- + LH \rightarrow L + LH^-$
(5) gamma	$AL^- + LH \rightarrow AL_1 + LH^-$	(5′) gamma	$L^- + LH \rightarrow L_1$
(6)	$ALH + O_2 \rightarrow AL + HO_2$	(6′)	$LH + O_2 \rightarrow L + HO_2$
(7)	$AL + O_2 \rightarrow AL_1$	(7′)	$L + O_2 \rightarrow L_1$

With regard to this scheme of reactions, certain points call for specific mention. The more important include the following.

In the absence of luciferase, reaction (1) takes place either prior to the extraction of the luciferin, or is intentionally brought about, whenever the pre-

parations have to some extent undergone the auto-oxidation, by adding reductants, XH_2, such as hydrosulfite. In living systems glucose is an especially suitable hydrogen donator. Ordinarily, with purified luciferin stored in a reduced state, an oxidation, presumably reaction (3), occurs on exposure to oxygen. Either with or without luciferase, in a subsequent step HO_2 oxidizes LH or some other constituent. There is no evidence as to whether small amounts of H_2O_2 are formed. It is interesting to note, in this connection, that the addition of catalase, as well as certain other agents known to catalyze the destruction of hydrogen peroxide, will accelerate the luminescent oxidation of aminophthalhydrazide (Drew, 1939). In cells, catalase would undoubtedly prevent the accumulation of H_2O_2, and luminous bacteria have been shown to contain abundant catalase (van Schouwenburg, 1940).

The kinetic analysis of the "flash" of luminescence in bacteria, occurring when oxygen is suddenly admitted to cells that have been partially or completely anaerobic, indicates the presence of two successive first order reactions (Chance, Harvey, Johnson, and Millikan, 1940; Schoepfle, 1940). The first and second steps in (5) alpha are the two important rates. The first step appears first order because there is a large percentage of AL^- going with negligible percentage change in LH.

Either with or without luciferase, the relative amounts of reactions (5), alpha, beta, and gamma, may be influenced by various factors, such as the type of oxidant, the pH, presence of specific ions, etc. In the absence of luciferase, the amount of reaction (5) alpha must be extremely small, since no visible light ordinarily appears. The manner in which certain factors operate to influence the course of (5) can best be illustrated with the aid of a potential energy diagram as shown in Fig. 1. The fact that more total light is produced from a given amount of purified luciferin in the presence of the chloride ion, and less total light in the presence of thiocyanate and certain other ions (Anderson, 1937), presumably occurs through influencing the relative extent of these reactions. Variations in the amount of destruction of 3-aminophthalhydrazide during luminescent oxidation are known to occur as the result of certain conditions (Drew, 1939), and presumably the same mechanism is again involved since Drew has shown that the reactions corresponding to our (5) beta may actually occur as well as (5) alpha.

The difficulty of dialyzing the luciferase completely free of the luciferin (Giese and Chase, 1940; Johnson and Eyring, 1944) indicates that the combination must be a fairly strong one, in this way resembling the combination between the flavin prosthetic group and its protein enzyme described by Theorell (1935).

The rate-determining reaction in the extracted *Cypridina* system depends upon the state of the luciferin, as Anderson has shown. When the luciferin

is all present as LH_2, as when pretreated with sufficient hydrosulfite, reaction (2) is rate-determining at the start. As the oxidation proceeds, some of the LH_2 undergoes a gradual, dark auto-oxidation, and this, together with the molecules of L that have undergone light emission, may be reduced by agents present in the solutions. In competition with the reduction of L is the catalytic destruction of the L referred to in the previous paragraphs, but the net result is that during the latter part of the reaction the rate of reduction of L governs the velocity of the luminescent oxidation, and reaction (1) becomes the pace setter. Under these conditions, a long, dim afterglow may follow the more or

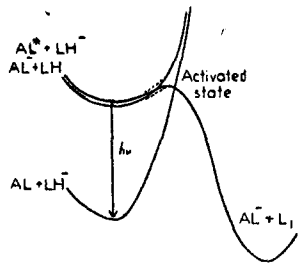


FIG. 1. Three possible energy states as ordinates are plotted against the reaction coordinate as abscissa. To the left of the activated state the distance between AL* and LH^- or AL^- and LH or AL and LH^- respectively is the abscissas. The amount of the attractions between the respective pairs indicated by the minima on the left is not known experimentally. Passing over the activated state may involve a reaction with O_2. The middle curve corresponds to the distance between AL^- + LH decreasing until in the dotted region there is an electron transfer when the system shifts to the upper level returning to the left as AL* and LH^-. AL* may then radiate becoming non-excited AL. On the other hand instead of forming excited AL* the system may pass irreversibly through the activated state or it may return unchanged as AL^- and LH.

less rapid, initial stage of the reaction. Even with highly purified preparations of luciferin, and dialyzed luciferase, a critical study of the data indicates that towards the end of the reaction the velocity constant decreases, as it would if reaction (1) replaced reaction (2) as the pace setter (*cf.* for example, the figures in Johnson and Chase, 1942). If XH_2 is present in large excess, then with either reaction (1) or (2) as the dominant reaction governing the over-all velocity, it should appear to be first order with respect to the concentration of luciferin added, and a plot of log-light intensity against time should give a straight line. Furthermore, both reactions (1) and (2) are slower than the remainder under ordinary circumstances. Thus, Chance, Harvey, Johnson, and Millikan (1940) found that in crude *Cypridina* extracts, the slowest reaction is the formation of ALH_2, the luciferin-luciferase complex that reacts with oxygen.

The reactions listed above are such as to make it appear that an important

function of the luciferase in catalyzing the reaction is to ionize the LH. Evidence for the rôle of ionization will be presented in connection with the relation of bacterial luminescence to pH.

(*b*) *The Bioluminescent Reaction in Bacteria*

Luminous extracts have not as yet been obtained from bacteria, and anything that disrupts the cell structure appears to destroy, at the same time, capacity for luminescence (Korr, 1935). Indirect evidence, however, from similar emission spectra and other sources, indicates that fundamentally the same luminescent system operates in bacteria as in *Cypridina* extracts. In the following discussion we will assume that the same reactions, set forth above for extracts, along with some additional reactions, occur also in bacteria. The available data indicate that in a large measure such an assumption is justified.

Non-proliferating bacteria aerated in buffered NaCl at favorable temperatures emit a constant over-all luminescence for periods of some time; minutes, or sometimes hours. Reaction (5) alpha is evidently proceeding at a constant rate, and since this depends on the slower reactions (1) and (2) we must conclude that LH_2 is being formed as fast as it is used up, provided there is no change in the activity of the enzymes. Reaction (1) is important in maintaining a continuous luminescence; *i.e.*, the over-all intensity is governed in part by the rate of reduction of L. This is shown by the effects of glucose on the "total light" of washed cell suspensions (Johnson, 1939), as well as by the investigations of the "flash" following oxygen deficiency, (Harvey, 1932; Johnson, van Schouwenburg, and van der Burg, 1939; Schoepfle, 1940, 1941). The "flash" has been studied in relation to the influence of substrate, time of anaerobiosis, temperature, and certain inhibitors. The evidence indicates that in the absence of oxygen, reaction (1) comes into an equilibrium depending on the amount of XH_2. The XH_2 may be formed from glucose either aerobically or anaerobically. When glucose is added anaerobically, the XH_2 accumulates over a period of time, progressively changing the equilibrium in favor of more ALH_2, as shown by the flash intensity when oxygen is admitted. The amount of A and of LH_2 as well as XH_2, however, are important in determining the steady state value of ALH_2, and hence the over-all rate of the luminescent reaction in the presence of oxygen. The situation is obviously complicated, and it is only because some of the reactions are important or fast in comparison with others, that an analysis may be made with respect to the over-all process. By changing the conditions, different reactions may be made more important than previously in rate control. Under ordinary conditions favorable to luminescence, when there is a constant intensity, the reactions are in a steady state. With oxygen not limiting, and with an excess of appropriate substrate, glucose in particular, the amount of XH_2 may be maintained essentially constant. The over-all reaction may thus be considered limited by reaction

(2), and the intensity of luminescence (I) is then given by the following equation, in which b is a proportionality constant:

$$I = b\, k_2\, (\mathrm{A})\, (\mathrm{LH_2}) \tag{8}$$

With regard to the action of inhibitors, it has been shown (Johnson and Chase, 1942) that certain drugs combine reversibly in the purified luciferin plus dialyzed luciferase system of *Cypridina*, retarding the rate of the reaction without affecting the total light produced. The combination presumably takes place with the enzyme, although kinetic evidence alone is not sufficient to distinguish between enzyme and substrate as the site of such a combination. The inhibitors studied in this regard include several sulfonamides, *p*-aminobenzoic acid, and ethyl urethane. By inference, numerous other drugs may act in the same way (Taylor, 1934), although some substances appear to combine with purified luciferin, reducing the total light (cyanide; Giese and Chase, 1940), and others evidently act by quenching (azide; Chase, 1942). In bacterial luminescence, for purposes of analysis, the luciferase may be considered the chief site of the effects of pressure, temperature, and drugs such as sulfanilamide and urethane. On the whole, the analysis has been largely satisfactory, although evidence of complicating factors is also apparent.

Omitting the derivations, which have been given in earlier publications, we arrive at the following expressions with regard to the action of inhibitors that enter into an equilibrium with the enzyme, independently of the reversible denaturation (Type I), or in relation to it (Type II). The notation is as follows: I_1 = intensity of luminescence before adding inhibitor; I_2 after adding inhibitor; equilibrium constants, K, and thermodynamic constants, ΔF°, etc., have their usual meanings, with subscripts referring to type of inhibition, *viz.* "1," for the normal reversible denaturation equilibrium, "2," for the equilibrium of luciferase with Type I inhibitor, and "3," for the equilibrium involving a Type II inhibitor; X = molar concentration of Type I inhibitor; r = number of molecules of Type I inhibitor combining with each enzyme molecule; U = molar concentration Type II inhibitor; s = number of molecules of Type II inhibitor combining with each enzyme molecule; p = hydrostatic pressure.

Type I

$$\left(\frac{I_1}{I_2} - 1\right) = K_2 X^r = X^r e^{-\Delta F_2^0/RT}$$
$$= X^r e^{-\Delta H_2^0/RT}\, e^{\Delta S_2^0/R} = X^r e^{-\Delta H_2^0/RT}\, e^{-p(\Delta V_2/RT)}\, e^{\Delta S_2^0/R} \tag{9}$$

Type II

$$\left(\frac{I_1}{I_2} - 1\right)\left(1 + \frac{1}{K_1}\right) = K_3 U^s = U^s e^{-\Delta F_3^0/RT}$$
$$= U^s e^{-\Delta H_3^0/RT}\, e^{\Delta S_3^0/R} = U^s e^{-\Delta H_3^0/RT}\, e^{-p(\Delta V_3/RT)}\, e^{\Delta S_3^0/R} \tag{10}$$

At this point it is perhaps useful to systematize, with the aid of a diagram (Fig. 2), our present information regarding the relation of the luminescent oxidation to the general respiratory pathway in bacteria in terms of the modern understanding of the mechanisms of cellular oxidations (Ball, 1944).

The scheme shown in Fig. 2 is a somewhat further specification of the one arrived at by van Schouwenburg (1938). Arrows point to the site of action of

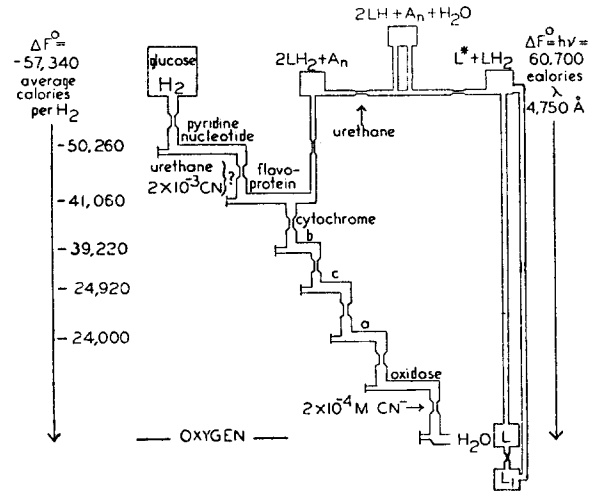


FIG. 2. Schema of the currently accepted sequence of respiration processes and on to which has been joined the luminescence process. The drawing is not to scale but we have indicated the free energy which each system liberates as two hydrogens (or two electrons + 2 protons) are liberated to form water. Constricted tubes indicate the chemical inertia of processes which may be much further inhibited by reagents written near the sensitive pathway. All the hydrogen or electron acceptors can enter into a variety of side reactions suggested by the closed off pathways to the left. The flavoprotein, in spite of its energy being lower than LH_2 will serve as a hydrogen transfer catalyst to LH_2 providing the hydrogens on the flavoprotein are not drawn off too rapidly by the cytochromes or by side reactions. Proportionate poisoning of oxidation and luminescence indicates action on an intermediate common to both processes. This intermediate is taken to be flavoprotein.

inhibitors, as indicated by the available data in this regard. Thus, the luminescent system shows an especial sensitivity to various narcotics, and is generally affected to a much greater extent than total oxygen consumption. Some of the narcotics have been shown to act on the extracted *Cypridina* system in comparable concentrations. On the other hand, the bacterial luminescent system is relatively insensitive to cyanide, which drastically reduces the rate of oxygen consumption, presumably by acting on the cytochrome oxidase. In high concentrations, cyanide reduces oxygen consumption and lumines-

cence proportionately, and under these conditions possibly affects a flavoprotein system. Some extracted flavoproteins may be inhibited by cyanide (Ball, 1939). The higher concentrations of narcotics like urethane, which considerably affect the rate of oxygen consumption as well as intensity of luminescence, very likely affect more than one system. However, from the presumed proximity of the luminescent system to the first steps in the dehydrogenation of the substrate, there is less opportunity for antecedent reactions to become pace setting in the process of luminescence than in the overall oxygen consumption. Under appropriate conditions, any one of the whole sequence of consecutive reactions involved in transfer of hydrogen from substrate to oxygen could become pace setting, but relatively few of these reactions precede luminescence. The measurement of light intensity, therefore, should provide a less complex, more direct approach in the living cell to the action of metabolic inhibitors which act upon those systems which influence its

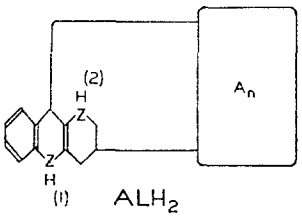


FIG. 3a

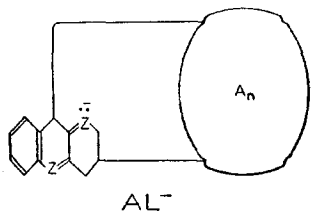


FIG. 3b

FIG. 3. (*a*) Hypothetical model of the luciferase molecule in the reduced state. The "*Z*" is for an unknown atom or group on which the oxidizable H occurs. (*b*) Hypothetical model of the luciferase molecule in the activated state.

activity. With regard to the actual light-emitting molecules, it will facilitate the discussion to use a tentative model, suggested both by the available data, and by the phenomena described in this paper. The model shown in Fig. 3 has obviously been influenced by Theorell's flavoprotein (Theorell, 1937), as well as by the structure of some molecules that give rise to pertinent chemiluminescences. It should be emphasized, however, that more evidence is yet needed before the structure of the prosthetic group can be specified. As indicated in Fig. 3 we assume that one hydrogen atom, number (1), is removed first to give LH, and then one proton, resulting in L^-. These assumptions are made to account for the fact that excessive concentrations of either hydrogen or hydroxyl ions interfere with the light-emitting oxidation, since H^+ would oppose the formation of L^-, while OH^- would remove the proton from LH, and either effect would retard the reaction.

III. EXPERIMENTAL METHODS

The data analyzed in this paper have been obtained exclusively in experiments with bacteria rather than extracts. Brief remarks should suffice with regard to

experimental methods, since fully detailed accounts have been published in the papers referred to earlier. Most of the experiments have been carried out with the psychrophilic, marine species, *Photobacterium phosphoreum*, which has the advantage that it produces a brilliant luminescence at room temperature. It was cultivated at 15° C. on nutrient agar containing 3 per cent NaCl, 1 per cent glycerol, and 0.5 per cent $CaCO_3$. The cells were placed in a solution which has been referred to as "PN," made by adding equal parts of M/2 NaCl and M/4 phosphate buffer at the desired pH. A stock suspension in PN was aerated for 20 to 30 minutes, after which it usually maintained a constant luminescence intensity, during continued aeration, for another half hour, more or less. Portions of the suspension, cooled at once, following initial aeration at room temperature, to between 3 and 5°, remained constant for some hours, and on rewarming to room temperature would recover their

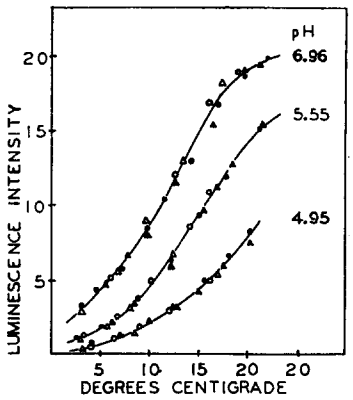


FIG. 4. Luminescence in relation to temperature in phosphate buffer and sodium chloride at three pH values. The repeated readings for each curve were obtained by cooling and warming the same suspension three successive times over the temperature range shown. The species of bacteria used for this and succeeding figures, except where specified otherwise, was *P. phosphoreum*.

former intensity. Such stock suspensions were always prepared for experiments that involved measurements of luminescence in a given portion over a period of more than 15 or 20 minutes. Actually, under conditions of temperature, etc., which do not bring about any appreciable destruction of the luminescent system, the intensity may remain constant for considerably longer periods than this. Fig. 4 shows that closely reproducible readings may be obtained with regard to the luminescence intensity at different temperatures, when portions of a given suspension, adjusted to different pH values, are warmed and cooled, over a considerable range of temperatures, three times in succession.

With the "fresh water" species, *Vibrio phosphorescens*, 0.9 per cent NaCl was used in the agar medium, and the PN was diluted with 2 volumes of distilled water. Because of the higher optimum temperature of this organism, cultures were incubated at 25° C., and the stock suspensions were maintained at about 15° C. Apart from these differences, the same procedures were followed with both species.

In most experiments, luminescence was measured by means of a photoelectric cell and D. C. amplifier, but in certain cases, such as very dim luminescence, visual photometry was employed with the aid of a modified Leeds and Northrup Macbeth illuminometer. The latter was always used for the experiments involving hydrostatic pressure.

Hydrostatic pressure at the desired temperature and other conditions was applied by means of a specially constructed bomb (Fig. 5), gold-plated to avoid contact of the bacterial suspension with physiologically active metals. The suspension was introduced through a gold-plated needle valve, and pressure applied from a hydraulic pump. Luminescence was viewed through a window of herculite, half an inch in diameter and of equal thickness. The entire bomb was held in a constant tempera-

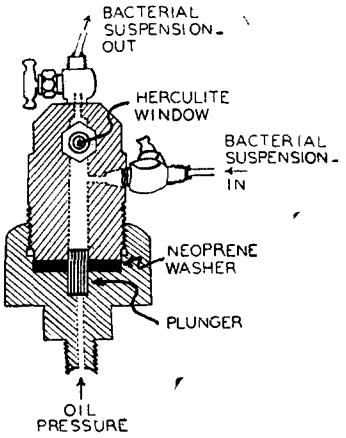


FIG. 5. Diagram of the gold-plated, high-tensile bronze pressure bomb. The bacterial suspension could be introduced and pressure applied within about 1 minute, with the bomb maintained at a given constant temperature in a water bath.

ture water bath. Temperature equilibration was rapid because of the small capacity, about 12 cc., of the bomb, and the large amount of conducting surface. Actual filling of the bomb and application of pressure could be completed within a few seconds.

IV. LUMINESCENCE IN RELATION TO pH

(*a*) *Influence of the Buffer System*

The relation between pH and the activity of a given enzyme system is influenced by the specific buffer system used in regulating the hydrogen ion concentration, because the equilibrium between the enzyme and the anion, as well as the cation, is significant in determining the over-all rate of catalytic action. The situation appears to be fundamentally the same as in the combination of hydrogen ions with wool, which as Steinhardt (1941) has shown varies

with the buffer system. In the luminescent system, the intensity at a given pH in some instances varies greatly with the buffer mixture (Table I).[5] A complete analysis of these relations would involve an extended study with the different systems. For present purposes, a full analysis with a single system is clearly more desirable.

Under most conditions phosphate appeared to be the best system for luminescence in bacteria. In approximately isotonic concentrations (M/4; *cf.* Johnson and Harvey, 1938) phosphate reduced the intensity of luminescence at acid pH values, although little effect was noticeable at neutrality. These relations are apparent in Fig. 6, which shows the effects of various phosphate concentrations, added to 3 per cent NaCl in order to maintain the osmotic pressure. The increasing "toxicity" of increasing concentrations of phosphate at a low pH is very evident. In Fig. 6 there is also apparent a tendency for luminescence to recover with time, following the intitial reduction in intensity on

TABLE I

Influence of the Buffer System on Luminescence of P. phosphoreum at Similar pH Values. Temperature 22°C.

	Buffer mixture								
	Phosphate M/2 NaCl 2 parts M/4 phosphate 2 parts			Phthalate M/2 NaCl 2 parts M/4 phosphate 2 parts M/4 phthalate 1 part			Acetate M/2 NaCl 2 parts M/4 phosphate 2 parts M/4 acetate 1 part		
pH	5.5	6.0	6.5	5.5	6.0	6.5	5.5	6.0	6.5
Intensity of luminescence	25.7	28.4	28.6	12.5	26.0	26.7	<1.0	<1.0	6.9

addition of the small volume of a concentrated cell suspension to the various solutions indicated. This recovery is due in part to the decreased concentration of inhibitory metabolites, present in the thick stock suspension, that occurs when the suspension is diluted. Other factors, not yet fully worked out must also be concerned, some of which are related to temperature. Thus, at higher than optimum temperatures, the irreversible decrease of luminescence which occurs logarithmically with time takes place faster with increase in hydrogen ions (Fig. 25). At lower and lower temperatures, however, there is an increasing tendency for the logarithmically declining luminescence intensity to come to a halt, and a recovery process to set in. The latter is obviously a different reaction than the one which brings about the initial reduction in luminescence. Phenomena of slow diffusion or differential permeability of membranes to ions (*cf.* Danielli and Davson, 1943) may be involved in these relatively slow after effects.

[5] The assumption is made, of course, that the hydrogen ion concentration at the enzyme is the same, or proportional to, the concentration as measured in the medium.

(*b*) *Relation between Luminescence and pH of Phosphate-Buffered NaCl at Optimum Temperature*

From the point of view of analyzing the relation between pH and luminescence under given conditions, the most satisfactory results were obtained by adding a very small volume, generally less than 0.1 cc. of 6 N NaOH or 6 N HCl, respectively, to 10 cc. portions of an only moderately concentrated cell

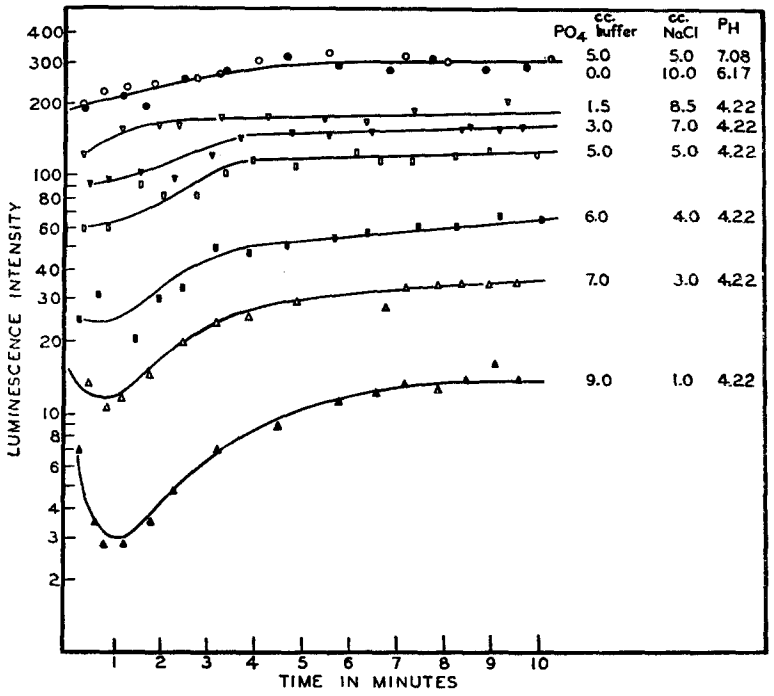


FIG. 6. The influence of phosphate concentration on luminescence intensity in approximately isotonic sodium chloride solution at various pH values. Semilogarithmic scale.

suspension in PN at an initial pH of 7.0. In this manner there was a minimum change in the suspension medium aside from the actual concentration of H^+ and OH^- ions. The acid or alkali was added by serological pipette while the suspension was being rapidly stirred, with an electric stirrer, or by vigorous bubbling of air, in order to assure a quick distribution of the ions. This precaution was found necessary. Figs. 7 and 8 show that the change in intensity which takes place within the first minute following such a procedure is fairly constant with time, although there is again a tendency for recovery provided the initial effect is not too drastic.

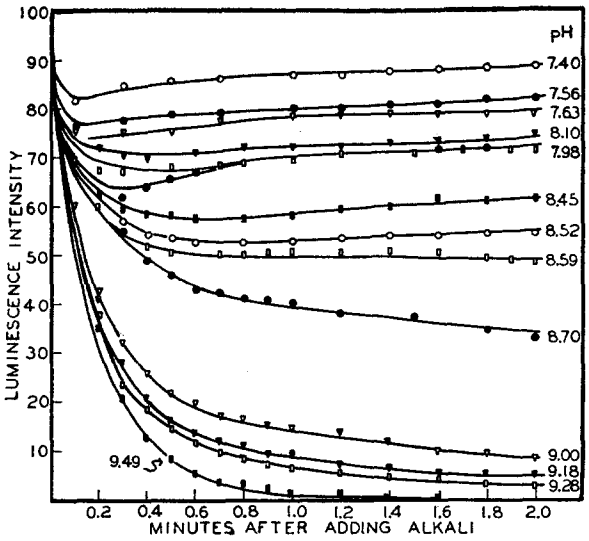


FIG. 7. The effect of changing the pH to the alkaline side of neutrality on the intensity of luminescence. Room temperature (approximately 22° C.).

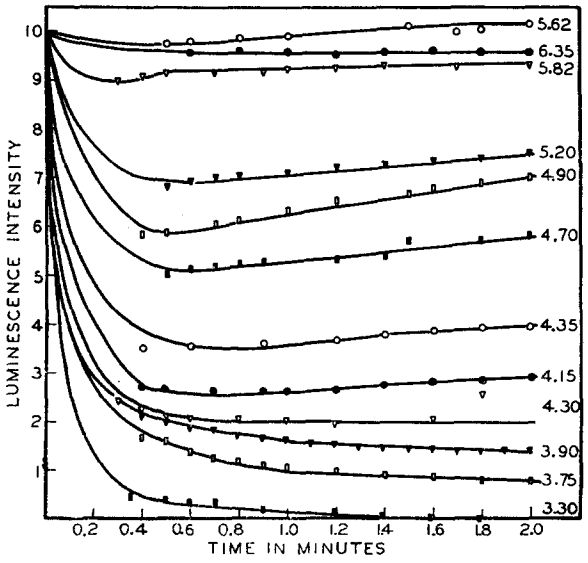


FIG. 8. The effect of changing the pH to the acid side of neutrality on the intensity of luminescence. Room temperature (approximately 22° C.).

The reversibility of the action of strong alkali is shown in Fig. 9. Similar effects were obtained when acid was first added, then neutralized. A certain amount of destruction is evident, as might be expected at the extremes of pH, but even after luminescence has decreased to about 1 or 2 per cent, the intensity quickly returns to nearly 70 per cent of its former value when the acid or alkali is neutralized. The effects are thus largely reversible, permitting analysis on the basis of equilibria. With regard to the relation between concentration and inhibition at a given temperature and hydrostatic pressure, we may take the maximum luminescence intensity as I_1, at the pH where this

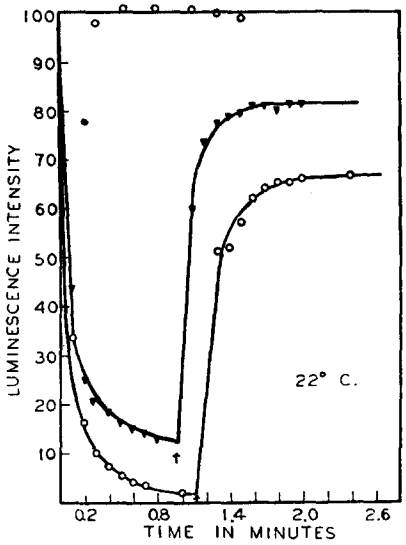


FIG. 9. The reversibility of the effect of strong alkali on the intensity of luminescence at 22° C. After introducing the alkali, at zero time, equivalent acid was added, at the time indicated by the arrows, to restore the original pH of approximately 7.

occurs, and the intensity at other respective pH as I_2. Using equation (9) or (10), we find that the $\log\left(\frac{I_1}{I_2} - 1\right)$ plotted against pH throughout the acid range between 3.6 and 6.4 results in a straight line of slope approximately 1 (Fig. 10). Assuming that the site of these effects of hydrogen ions is on the luciferase molecule, possibly on the luciferin as a prosthetic group, the slope of this line indicates that a ratio of one hydrogen ion to one luciferase molecule is involved in the equilibrium. Similar results are obtained in the corresponding analysis with respect to hydroxyl ions, throughout the alkaline range between pH 7 and pH 9, although a complication is encountered in the denaturation of the protein that occurs increasingly fast with rise in alkalinity, as well as temperature.

The relation between pH and luminescence intensity, throughout the range in which the enzyme does not undergo appreciable destruction, may be expressed by a single equation which assumes that one hydrogen or one hydroxyl ion per enzyme molecule is required to eliminate catalytic activity. Using K_5 to designate the constant for the equilibrium between hydrogen ions and the enzyme, and K_6 similarly for the hydroxyl ions, the values of these constants

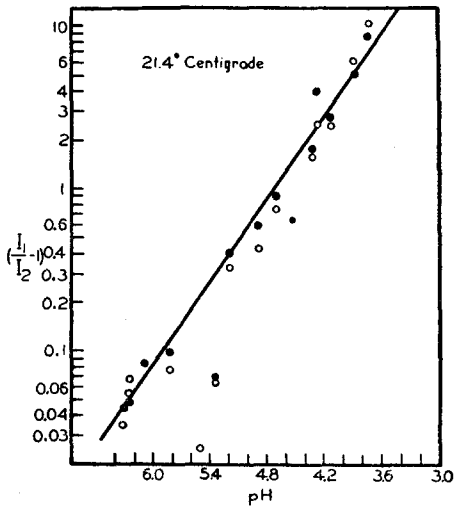


FIG. 10. An analysis of the data in Fig. 8, and similar experiments, as a Type I inhibition. The solid and open circles represent the intensity of luminescence 1 and 2 minutes, respectively, after adding the acid or alkali. The slope of the line is approximately one. Semilogarithmic scale.

may be estimated from the data available in Figs. 7 and 8. We thus arrive at the following equation:

$$\left(\frac{I_1}{I_2} - 1\right) = K_5\,(H^+) + K_6\,(OH^-) = 4.84 \times 10^4\,(H^+) + 4.8 \times 10^5\,(OH^-) \qquad (11)$$

In Fig. 11 the theoretical curve has been plotted for this equation. The points are from the experiments represented in Figs. 7 and 8. K_5 and K_6, of course, vary with the acid and base used. This indicates that the companion ions of (H^+) and (OH^-) combine with the enzyme.

At the extremes of pH shown in Fig. 11 there is evidence of inhibitory effects greater than would be expected from the simple equation (11), thus indicating that additional reactions, unfavorable to luminescence, occur. On the whole, however, the agreement between the experimental data and theory is satisfactory, showing that the possible complicating factors are not so prominently concerned as to obscure the primary mechanism. Equations (9) and (10)

provide a basis for the understanding of these effects. According to the smooth curve of Fig. 11, and the reactions (4) and (4′) assumed, the pK of ALH occurs at pH 4.95, and the pK of LH at 9.45.

(*c*) *The pH-Temperature Relation of Luminescence in the Acid Range*

At acid pH values which cause moderate and reversible diminutions in luminescence at the optimum temperature, the effect of the given pH changes greatly with change in temperature. At low temperatures the addition of acid results in a much greater inhibition than for the same pH at the higher

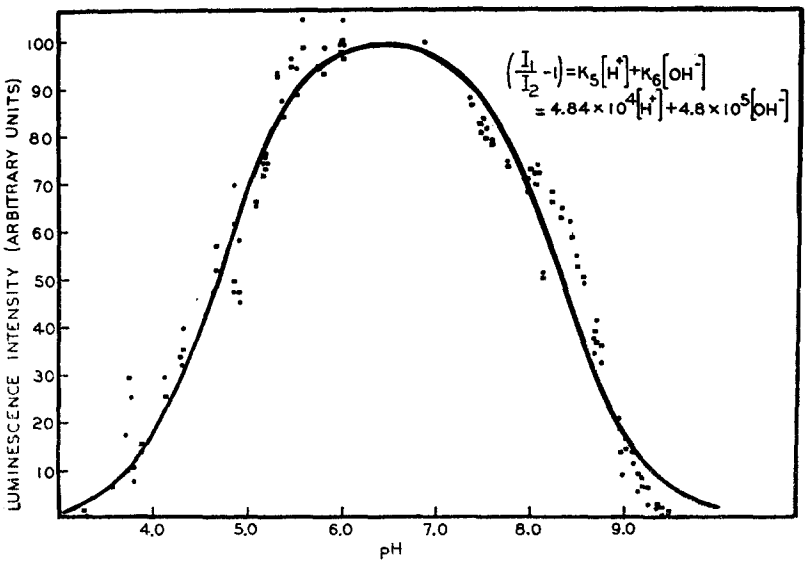


FIG. 11. Luminescence intensity *versus* pH, based on the intensity at 1 minute (squares) and 2 minutes (circles) after adding acid or alkali. The smooth curve has been drawn from the equation shown in the upper right hand margin of the figure.

temperatures (Fig. 12). Such a result is understandable in terms of the assumed reactions leading to luminescence. Thus, at low temperatures, relatively little of the ALH is dissociated (reaction 4), while at high temperatures the ALH may be assumed to be very largely dissociated. Thus, with hydrogen ions acting as the inhibitor, a rise in temperature decreases the inhibition by dissociating the inhibitory agent, just as in the case of sulfanilamide, which appears to act in very much the same manner. The action of sulfanilamide will be considered in more detail presently. The action of hydrogen ions may be considered either from the point of view of inhibitors in general, or from the point of view of the mechanisms controlling the activation energy for luminescence. The latter point of view is taken first, in the following paragraph.

The relation between the activation energy for luminescence and the pH of the medium[6] is evident in Fig. 12. At the pH of 5.05 the activation energy, calculated as a straight line relation over the range between 2° and 7° C., amounts to 40,900 calories, while at pH 6.92 it amounts to approximately half of this value, or 20,700, calculated in a similar manner. In both cases the heat of ionization constitutes a definite fraction of the over-all activation energy. At the optimum temperature and at neutrality, the ionization of ALH to AL^- must be nearly complete, as shown by the fact that K_5 (H^+), which measures the concentration of (ALH), is zero. At the optimum tempera-

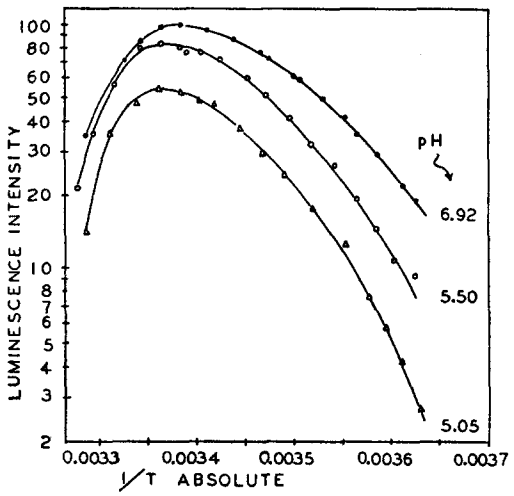


FIG. 12. The intensity-temperature relation of luminescence at acid pH values (*P. phosphoreum*). The activation energy for luminescence, indicated by the slopes of the lines at low temperatures, amounts to approximately 20,700 calories at pH 5.05 and 40,900 calories at pH 6.92. The difference of about 20,000 calories is attributed to the heat of ionization of the ALH in the process of activation. Semi-logarithmic scale.

ture and pH, however, the value of K_1, the equilibrium constant governing the reversible protein denaturation of the enzyme, is sufficiently large to cut down the apparent, or observed, rate of the luminescent reaction. Since the value of K_1 under given conditions can be only approximately determined, it becomes difficult to estimate with accuracy just what fraction of the change in the apparent activation energy is to be atrributed to the heat of ionization, and what fraction to the change in K_1 with temperature. At low temperatures, the difference of about 20,000 calories between the activation energies at

[6] The pH indicated in connection with the data shown in Figs. 12 and 15 are the pH values as determined at room temperature with the Beckman glass electrode pH meter.

pH 5.05 and pH 6.92 is clearly to be attributed largely to the ionization process, and by this route we are able to arrive at an estimate for the heat of ionization which checks roughly with the 14,000 calories heat of dissociation of ALH, when the hydrogen ions are considered as an inhibitor and analyzed in the appropriate manner (Fig. 13).

In Fig. 12, the influence of dissociation on the activation energy for luminescence is evident in two distinct trends of the curves. In the first place, at low temperatures, the slope is steeper at an acid pH, which requires more energy for ionization. In the second place, with any pH, the slope decreases

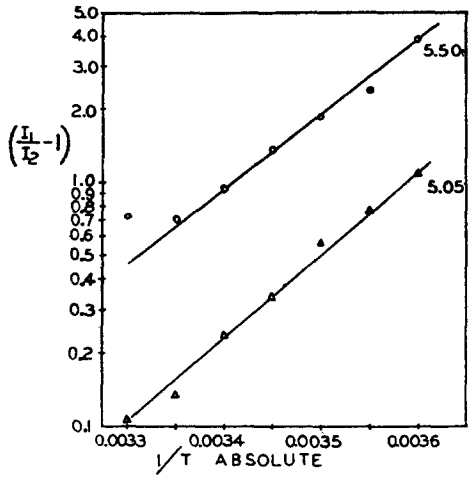


FIG. 13. Analysis of the data shown in Fig. 12, considering hydrogen ions as a Type I inhibitor. The slopes of these lines indicate a heat of reaction of about 14,000 calories for the equilibrium involving hydrogen ions. The 14,000 calories check roughly with the 20,000 calories indicated for the same process as judged by the slopes of the lines in Fig. 12. Semilogarithmic scale.

in going from the low temperatures toward the optimum. A part of this decrease in slope is brought about, according to our theory, by the increasing proportion of ionized over unionized ALH. In approaching the optimum, however, an additional factor becomes more and more significant in decreasing the slope of the curve; *viz.*, the increasing value of K_1 with rise in temperature in the denaturation equilibrium. Qualitatively, the picture is easily understandable on the basis of what has already been set forth. Quantitatively, it would be difficult to distinguish with accuracy all of the number of factors or reactions which operate to influence the apparent activation energy and heats of denaturation equilibria. Results obtained in experiments with hydrostatic pressure, described below, are consistent with the foregoing partly qualitative interpretations.

(d) The Relation of Luminescence to pH and Pressure at Constant Temperature

Fig. 14 shows the relation between luminescence intensity, hydrostatic pressure, and pH at a single constant temperature. At this temperature, as previously shown (Brown, Johnson, and Marsland, 1942) pressure up to some 500 atmospheres or more has little effect on luminescence at pH 7.3, although at lower temperatures pressure reduces, and at high temperatures pressure increases the intensity at the same pH. As the reaction of the medium is made more acid a pressure effect becomes more noticeable, acting in the direction of retarding luminescence. In connection with Fig. 14 it should be borne

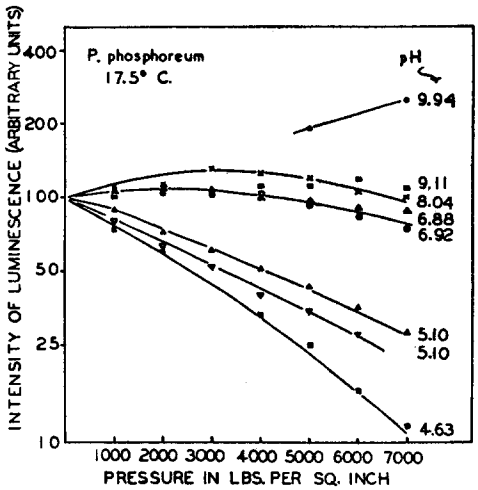


FIG. 14. The relation between the observed intensity of luminescence and hydrostatic pressure at various pH values at 17.5° C. At each pH, the intensity at normal pressure has been arbitrarily taken as equal to one hundred. The actual intensities at the different pH and at normal pressure, vary in the manner indicated by Fig. 11.

in mind that the actual intensity at different pH, particularly at the extremes, may be very different, although in this figure the intensity at normal pressure has arbitrarily been taken as 100 in each case, in order to show more clearly the effect of pressure alone. Thus, in a medium whose pH is 5.10, pressure readily reduces the intensity of luminescence, just as it does at a neutral pH at low temperatures. In both cases the ALH is relatively unionized, and when the ionization is increased by a rise in temperature, with the neutral solutions, or by a decrease in hydrogen ions at optimum temperature, the pressure effect largely disappears. It is evident, therefore, that the ionization is accompanied by a considerable volume change, amounting to about 71 cc. per gm. molecule as a mean value from the slopes of the duplicate determinations for pH 5.10, shown in Fig. 14. At low temperatures and neutral pH, the volume change of

activation, calculated earlier (Brown, Johnson, and Marsland, 1942) appeared to be 58 cc. at 0° C. The calculations of Eyring and Magee (1942) indicated 50 cc. at 0° C., and showed a temperature dependence for this $\Delta V\ddagger$[7] which can now be accounted for in part by the temperature dependence of the degree of ionization of ALH; *i.e.*, a strong effect of pressure in reducing the intensity of luminescence will be noted wherever the conditions are unfavorable for ionization of the ALH. Factors which increase this ionization might be expected to counteract the pressure diminution of luminescence.

It will be recalled that pressure also counteracts the reversible denaturation of the protein, thereby increasing the intensity of luminescence whenever the value of K_1 becomes significant. Thus, the disappearance of a pressure effect on luminescence in the neighborhood of the optimum temperature and in neutral solutions is due to two influences, *i.e.* the effects on K_1 and k_2, and for reasons already indicated it would be difficult to state much better than has been done previously (Eyring and Magee, 1942) the quantitative rôle of each in the total effect observed. The data indicate that the volume change accompanying ionization is largely responsible for the effect of pressure in increasing the activation energy.

At increasingly acid pH, the pressure effect tended to become less and less readily reversible on release of pressure. At the quite acid pH of 4.63 the pressure diminution of luminescence became slightly greater with increase in pressure, and the effects were not appreciably reversible, on release of pressure, over short periods of observation. The irreversible, or only slowly reversible effects are possibly in the protein portion of the enzyme, and may be similar to the changes which take place when the viability of bacteria is reduced and proteins are permanently denatured by high pressures (Hite, Giddings, and Weakley, 1914; Larson, Hartwell, and Diehl, 1918; Giddings, Allard, and Hite, 1929; Bridgeman, 1931; Cattell, 1936; Matthews, Dow, and Anderson, 1940.

One further point calls for mention in connection with Fig. 14. It is apparent that although pressure causes no appreciable increase in brightness at this temperature and slightly alkaline reactions, pronounced increases may occur at the same temperature but in extremely alkaline media. At pH 9.94 the luminescence was fairly rapidly and irreversibly extinguished, but the application of pressure immediately increased the intensity at that moment. The irreversible destruction of luminescence by alkali will be discussed later. The point of present interest is that the increase in luminescence under the influence of pressure in quite alkaline solution appears to be the result of a pressure effect on the process of protein denaturation, rather than any effect on the

[7] In accordance with the notation usually employed in connection with the Theory of absolute reaction rates, the double dagger (‡) is used to indicate the ΔF^0, ΔH^0, ΔE^0, ΔV^0, and ΔS^0, respectively, in the equilibrium between the normal and activated states of reactants in a rate process, in distinction to the usual thermodynamic constants of equilibria between initial and final states.

dissociation of ALH. It would seem that, in general, the protein of the enzyme is stable in acid solution, but undergoes denaturation in alkaline solution. Some highly purified proteins have been found to share this characteristic (Northrop, 1939).

(e) The pH-Temperature Relation of Luminescence in the Alkaline Range

The intensity of luminescence in relation to temperature is shown in Fig. 15 for suspensions in NaCl solutions buffered by phosphate over the range

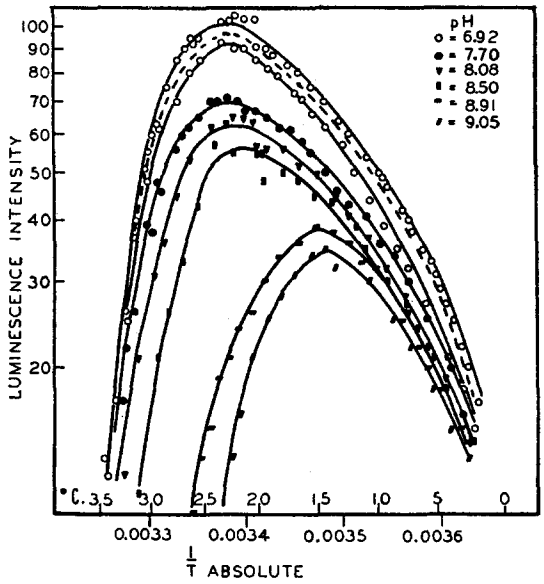


FIG. 15. The intensity-temperature relationship of luminescence at alkaline pH values. The duplicate curves drawn through the open circles were obtained with portions of the same suspension about 1½ hours apart. The dotted line was taken as the mean value in analyzing the data with respect to temperature (Fig. 16). Semi-logarithmic scale.

between pH 7 and 9. In general the inhibition is less at low temperatures. At reactions more alkaline than pH 8, there is a marked tendency for the maximum intensity of luminescence to occur at lower temperatures. From the relation of hydroxyl ions to the luminescent reaction as already discussed, it would follow that the initial effect of increasingly alkaline reactions, beyond a pH of 7.0, consists in an effect on the LH, slowing the light-emitting reaction. This effect itself will have a temperature coefficient, inasmuch as changes in temperature will influence the degree of ionization of the reactants taking part in luminescence.

In addition to the effect of the OH ions on the LH of reaction (5), the protein of the enzyme at optimum temperatures undergoes a reversible denaturation

at high alkalinities, as shown by the action of pressure. Thus, two distinct reactions are affected by increasing concentrations of hydroxyl ions, both in the direction of decreasing the intensity of luminescence. The first effect, on the ionization of essential reactants, takes place most prominently between pH 7.0 and pH 8.5. Beyond a pH of 8.5, the same reaction is still affected, but is not responsible for the major fraction of the further inhibition, which is now largely due to the denaturation of the protein. Accompanying the reversible phase of this denaturation is the irreversible denaturation. Thus, if we exclude the not altogether unlikely possibility that the protein of more than one enzyme involved in luminescence is also affected, there are at least three reactions which are influenced in the same direction of inhibiting luminescence by increasing the alkalinity beyond pH 7.0, namely, the ionization of LH, the reversible protein denaturation, and the irreversible protein denaturation. A possible source of error in the opposite direction conceivably takes place at the high temperatures. Bodine and Allen (1938) have shown that brief exposure to high temperatures, or to any of several protein denaturants, results in an activation of certain oxidative enzymes. Some indication that a similar effect might occur in the luminescent system was encountered in observations on the reversibility of the high-temperature inhibition of luminescence in alkaline solutions. For it was frequently found that when a suspension was heated briefly to a temperature well beyond the optimum, thereby diminishing the luminescence to around 10 per cent, it would then return, on cooling quickly, to an intensity somewhat higher than its previous maximum. The true significance of this increase is not fully clear on the basis of the data at hand, and it apparently does not constitute an important source of error. It is extremely unlikely that the reversibility of the temperature diminution of luminescence results from an almost perfect balance between an activation and an irreversible denaturation of the total number of enzyme molecules.

With the various sources of error and possible complications pointed out above, it is obvious that an analysis of the data in a family of curves, such as those given in Fig. 15, can only be approximate. The formulations that have been derived for relatively simple situations are useful, first of all, in showing whether or not the measured process is too complicated to analyze in such manner. If not, the formulations are useful in estimating the heats of reaction, etc., with an accuracy depending on both the precision of the data and the extent to which it is complicated by other reactions. Fig. 16 gives the temperature analysis for the curves in Fig. 15, and shows that the situation is indeed complicated by more than one reaction. The prominent up-turning of the curves in the high-temperature range, especially in the case of the high pH also, must represent multiple inhibitory reactions. At the lower temperatures, where the actual inhibition caused by a given OH^- concentration is much less, the lines become straight and parallel. There is a tendency, of course, for the

lines to become straight at low temperatures, where the values of $1/K_1$ become extremely large in comparison with the other terms in the expression used for calculating the points on the ordinate. The slope of the lines, however, is determined both by the equilibrium constant K_1, and by the value of the expression $\left(\frac{I_1}{I_2} - 1\right)$ at each temperature. The slopes of the straight line portions of the curves in Fig. 16 indicate a rather large heat of reaction, of about 60,000 calories. Most of this heat represents the Δ H of the reversible denaturation of the protein, while a much smaller fraction is to be attributed to the heat of ionization in the luminescent reaction (5). The mechanism of denaturation by hydroxyl ions will be referred to again in connection with the action of urethane and alcohol.

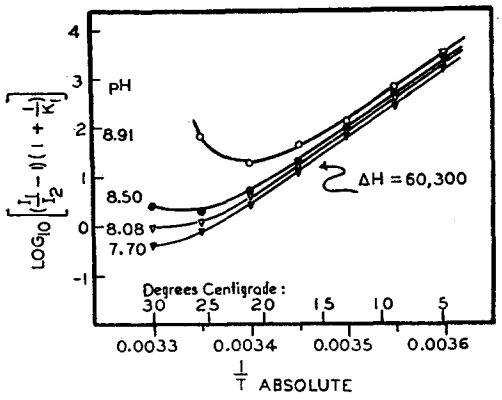


FIG. 16. Analysis of the data shown in Fig. 15

V. THE ACTION OF SULFANILAMIDE

(a) *Inhibition in Relation to Concentration and Temperature*

Previous studies have already indicated that the sulfanilamide inhibition of luminescence is of the kind we have termed "Type I." Equation (9) applies, and since the combination of enzyme and inhibitor evidently evolves heat, the inhibition will in general decrease with rise in temperature and concomitant dissociation of the enzyme-inhibitor complex. At temperatures beyond that of the normal optimum, where the reversible denaturation equilibrium constant K_1 rapidly increases and brings about increasing diminutions in the over-all intensity of luminescence, a practically complete dissociation of moderate concentrations of the inhibitor may occur. The nature of these effects is such that a slight shift of the temperature of maximum luminescence takes place in the direction of the higher temperatures. A simple differential equation has been derived to estimate the amount of this shift (Johnson and Eyring

1943) but the calculations yield values which are considerably too high for the results obtained in experiments, *i.e.* the actual shift in temperature of the maximum is less than that which would be predicted, given the constants K_1 and K_2, and the concentration of inhibitor added. A part of the error undoubtedly arises from the sensitivity of the equation to experimental error, and the difficulty of obtaining sufficiently precise values for the equilibrium constants K_1 and K_2 respectively. Probably the largest error, however, is due to over simplification of the equation, which does not take into account all the factors which may influence the observed result. For example, the changing tempera-

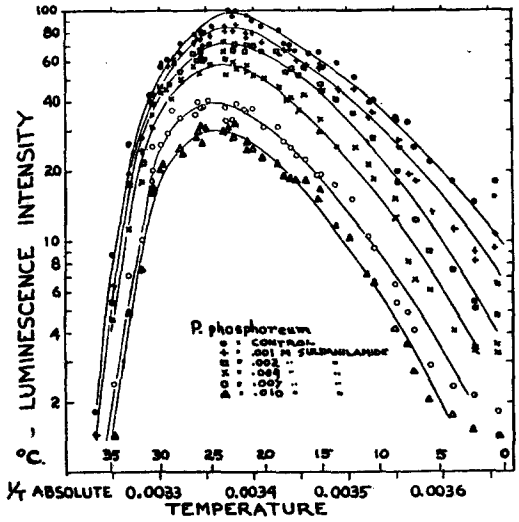


FIG. 17. The intensity-temperature relationship of luminescence in corresponding portions of the same suspension of *P. phosphoreum* containing different amounts of sulfanilamide as indicated. Note the tendency of maximum luminescence to occur at slightly higher temperatures with increasing concentrations of sulfanilamide. Semilogarithmic scale.

ture coefficient of activation energy, resulting from the varying degrees of ionization at different temperatures, was not taken into account. Thus, while the picture is again qualitatively clear with reasonable certainty, some of the quantitative predictions relating to the more detailed aspects are difficult to make with satisfactory accuracy.

Figs. 17 and 18 show the results of an experiment with portions of a single suspension of cells in which the effects of several concentrations of sulfanilamide were measured over a wide temperature range. The family of curves in these figures may be used as the basis for analyzing both the relation between concentration of sulfanilamide and inhibition of luminescence at constant temperature and inhibition at a constant concentration of sulfanilamide. The results

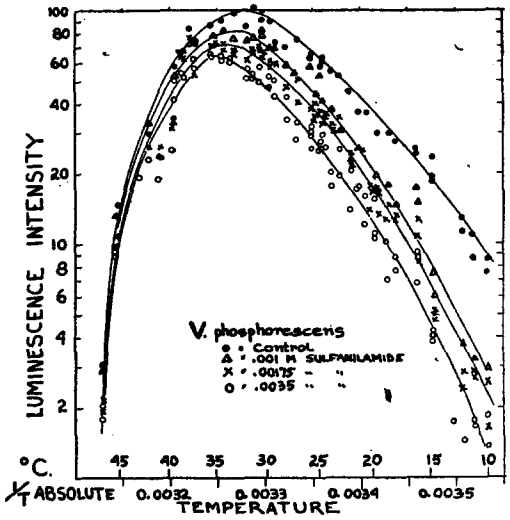


FIG. 18. The intensity-temperature relationship of luminescence in corresponding portions of the same suspension of *Vibrio phosphorescens* containing different amounts of sulfanilamide as indicated. Note the different position of the normal maximum intensity of luminescence in this species, as compared to that of *P. phosphoreum*, with respect to absolute temperature. A similar change in the temperature of maximum luminescence, however, is caused by the addition of sulfanilamide.

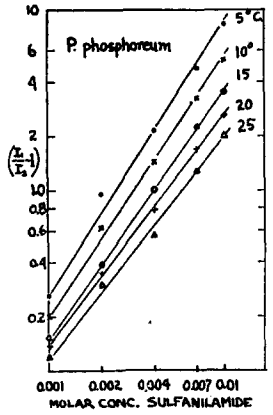


FIG. 19. Analysis of the data shown in Fig. 17 with respect to the concentration of sulfanilamide at different given temperatures. The slopes of the lines lie between 1 and 1.5. Log-log scale.

of these analyses for Fig. 17 are given in Figs. 19 and 20, respectively. The slopes of the lines in Fig. 19 vary only slightly with temperature and indicate that the ratio of sulfanilamide-enzyme molecules is somewhat greater than one.

In many experiments dealing with the effects of concentration alone, however, the slope was found to be very nearly equal to one. It would appear, therefore, that while complicating factors and experimental error give rise to some variation, the actual ratio may be considered to be one, just as in the case of hydrogen ions. Further resemblance between the effects of sulfanilamide and hydrogen ions is evident in the heats of reaction, calculated from the slopes of the curves in Fig. 20. These heats, which are strikingly similar throughout the range of sulfanilamide concentration of 0.001 to 0.01 molar, amount to

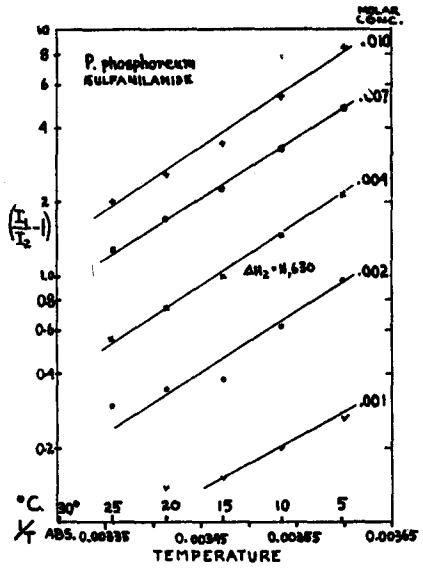
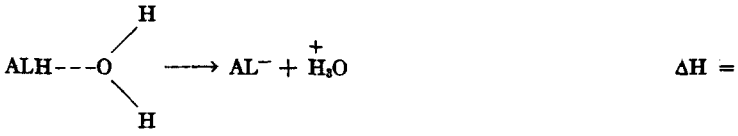


FIG. 20. Analysis of the data shown in Fig. 17 with respect to temperature for different given concentrations of sulfanilamide. The slopes of these lines indicate a heat of reaction of about 11,600 calories, as compared with 14,000 calories for hydrogen ions.

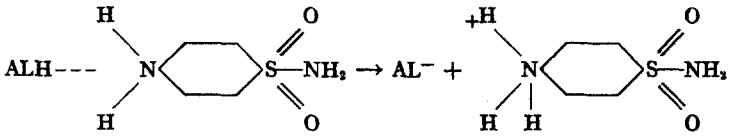
about 11,600 calories. This value is somewhat less than the average of 14,070 calories obtained by a similar analysis with hydrogen ions instead of sulfanilamide. Some previously reported estimates of the heat of reaction of the sulfanilamide equilibrium, based on the effects of one concentration of the drug at different temperatures in each of several species of luminous bacteria (Johnson, Eyring, and Williams, 1942), are higher than the present value of 14,070 calories for the hydrogen ion dissociation. Small differences in these heats of reaction are difficult to determine with certainty.

A reasonable explanation of the foregoing results is as follows: In the absence of sulfanilamide, reaction (4) yields the necessary AL^-, with an absorption of

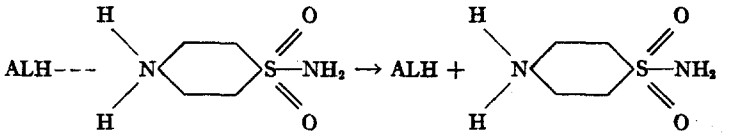
14,000 calories. When sulfanilamide is added, it seeks out the ionizing hydrogen, and raises this ionization energy (*cf.* Equation 13) to a larger value, blocking ionization. This inhibits formation of AL^- and therefore light emission. Reactions (12) and (13) will presumably involve about the same volume change. The lack of a pressure effect on (14) shows this involves no volume change.



$$\Delta H = 14{,}000 \quad (12)$$



$$\Delta H > 14{,}000 \quad (13)$$



$$\Delta H > 14{,}000 \quad (14)$$

The effect of temperature in relation to either hydrogen ion formation (13) or to sulfanilamide dissociation (14) will be fundamentally the same, since in both cases the heat of reaction is largely that which is associated with the ionization.

(*b*) *Relation to p-Aminobenzoic Acid and to Growth*

Because of the very extensive study that has been made of *p*-aminobenzoic acid (PAB) in relation to the sulfanilamide inhibition of growth (Woods, 1940, and numerous subsequent papers) the action of this drug in luminescence assumes particular interest, especially if the proposed luminescence reaction scheme makes it possible to reach a somewhat clearer picture of the mechanism involved in the effects on growth.

With respect to growth, low concentrations of PAB and closely related derivatives are able to overcome relatively high concentrations of sulfanilamide, and this effect depends only on the ratio, not the absolute concentrations, of the two drugs. Moreover, the phenomenon is very general, occurring in all cases where sulfanilamide inhibits growth under otherwise favorable conditions for the organism concerned. The PAB antagonism of sulfanilamide is lessened, or eliminated, however, under certain conditions; *e.g.*, at temperatures

slightly above the optimum (Lee, Epstein, and Foley, 1943; Lee and Foley, 1943). It is evident that the two drugs under ordinary conditions, act "competitively" at the same site, a probability that is strengthened by steric considerations of the closely similar molecular configurations, and even more so because of their having amino groups of similar basicity. Thus, it is generally believed that PAB itself (or a closely related compound) is an essential substance in normal metabolism which is displaced by sulfanilamide, thereby leading to an inhibition in the rate of growth. On the grounds of this assumption, plus an experimentally demonstrated relation between the growth-inhibitory properties of various sulfonamide derivatives and the electro-negativity

TABLE II

Luminescence Intensity, in Arbitrary Units, of P. phosphoreum and A. fischeri, Respectively, Suspended in Phosphate-Buffered NaCl Solution, pH 7.3, Containing Sulfanilamide Alone and in Addition to Various Concentrations of p-Aminobenzoic Acid (PAB). Room temperature, Approximately 20°C.

Molar concentration of sulfanilamide	Controls: No PAB, no sulfanilamide (duplicates)		No PAB	0.001 molar PAB, times fraction indicated in addition to sulfanilamide									
				× 1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512
A. fischeri													
0.00015	22.7	20.	14.8	12.8	12.9	13.0	15.0	14.8	15.9	16.5	15.7	14.7	14.5
0.00025	19.0	18.6	12.6	11.5	11.4	12.3	10.2	12.	10.4	11.6	12.	12.4	12.3
0.00160	18.	18.2	4.9	4.4	4.3	4.1	4.8	4.4	4.3	4.4	4.5	4.1	4.3
P. phosphoreum													
0.0025	21	19.2	11.5	10.7	13.5	9.5	11.7	12.5	10.8	10.4	11.6	11.7	10.5
0.005	22	23.	8.6	8.4	7.8	7.3	7.4	8.7	9.1	8.1	8.3	8.6	8.6
0.010	20.2	19.5	3.2	3.1	3.3	3.6	3.8	3.1	3.2	2.9	3.0	2.9	3.3

of the sulfone group, Bell and Roblin (1942) have advanced a theory regarding the type of derivative which might be expected to inhibit growth in fundamentally the same manner as sulfanilamide.

In luminescence, as in growth, PAB and sulfanilamide both appear to act at the same site on the critical enzyme concerned but unlike the situation with respect to growth, PAB either has no effect on luminescence beyond the limits of experimental error, with or without sulfanilamide simultaneously present, or causes an inhibition (Table II; *cf.* also, Johnson and Chase, 1942; Johnson, Eyring, and Williams, 1942; Johnson, Eyring, and Kearns, 1943). The inhibitory concentration of PAB in luminescence is roughly 10 times the concentration of sulfanilamide needed to cause the same effect. Although, as numerous papers have shown, high concentrations of PAB will also inhibit growth, this

inhibition can hardly involve the same site through which PAB counteracts the sulfanilamide inhibition of growth. The PAB inhibition will be considered later.

In explanation of these phenomena, it appears that in luminescence, PAB and sulfanilamide go on the same position, and both block the enzyme reaction. In growth they also combine through their basic amino groups competitively (Bell and Roblin, 1942) and the sulfanilamide blocks growth while low concentrations of PAB permit growth (though not luminescence) even in the presence of sulfanilamide. In growth, we assume that each of these drugs combines through its amino group, with an ionizable hydrogen on the enzyme. This blocks ionization of the enzyme. However, PAB can overcome this effect by ionization of its carboxyl group, while sulfanilamide cannot. It therefore seems reasonable to look for the different effect of these drugs on growth in this circumstance. Bell and Roblin's theory deals with a complementary aspect of the problem; *i.e.*, the relative efficiency of drugs to form the addition compound between the basic amino group and the ionizable hydrogen and the enzyme. Their theory is not concerned with explaining why the PAB-enzyme compound permits growth. According to our view the ability of the drug-enzyme compound simply to ionize permits growth, whereas luminescence requires not only the ionization, but also the giving up of a particular electron which apparently neither the PAB-enzyme nor the sulfanilamide-enzyme complex can do.

Derivatives of PAB might be expected to exhibit varying degrees of either an antisulfanilamide action or a bacteriostatic effect of their own, according to their ability, first, to be adsorbed properly, and second, to prevent or to permit the functioning of the enzyme. This view is in accord with the data of Wyss *et al.* (1943).

From the foregoing considerations it seems evident that the sulfanilamide inhibitions of luminescence and growth do not take place on identical enzymes, although they may take place on very similar ones. Among a number of distantly as well as closely related species of luminous bacteria the relative sensitivity of the "growth system" and the "luminescence system" might be expected to vary considerably. Such a variation among different species does not necessarily result from a different equilibrium constant for the enzyme-sulfanilamide complex in growth, or in luminescence, respectively. There is evidence to show that the sulfanilamide-luciferase equilibrium is characterized by closely similar heats and entropies of reactions among several species of luminous bacteria (Johnson, Eyring, and Williams, 1942). The observed differences in sensitivity among different species—that is, the wide differences in per cent inhibition of luminescence by a given concentration of the drug acting on different species under the same conditions of temperature, pressure, pH, etc.—may be accounted for purely on the basis of the relation of the sul-

fanilamide equilibrium to the normal, specific temperature-velocity curve of the organisms concerned. Thus, the equilibrium between sulfanilamide and the growth enzyme among different species likewise may be identical, involving the same bonds, in all cases, while the specific susceptibility in any one case depends not only on the particular relation between temperature, etc., and rate of reaction of the growth enzyme in the organism concerned, but also the possible production of metabolites which, like *p*-aminobenzoic acid, may displace sulfanilamide and substitute for the normal group whose functional activity has been abolished.

The general effect of sulfanilamide on the growth of luminous bacteria, together with observations on the luminescence of the cultures that develop

TABLE III

Growth (G) and Luminescence (L) of Broth Cultures of Different Species of Luminous Bacteria in Relation to Sulfanilamide Concentration

Molar concentration of sulfanilamide	*P. phosphoreum**		*P. sepiae*		*P. splendidum*		*A. fischeri*		*A. harveyi*		*P. pierantoni*		*V. albensis*		*V. phosphorescens*	
	G	L	G	L	G	L	G	L	G	L	G	L	G	L	G	L
0.04	−	−	+	−	+	−	+	−	+	−	−	−	−	−	−	−
0.02	+	−	++	−	++	−	++	−	++	−	−	−	−	−	−	−
0.01	+	+	++	−	++	−	++	−	++	−	−	−	−	−	−	−
0.005	+	++++	++	+	++	−	+++	−	+++	−	−	−	−	−	−	−
0.0025	++	+++	+++	++	++	−	+++	−	+++	−	+	−	−	−	−	−
0.00125	++	+++	++++	+++	+++	+	+++	−	+++	−	+	−	++	+	+	−
0.00063	++	+++	++++	+++	++++	++	+++	−	+++	−	++	+	++	++	++	+++
0.00032	++	+++	++++	+++	++++	+++	+++	+	++++	+	++	++	+++	+++	++	+++
0.00016	++	++	++++	+++	++++	+++	+++	++	++++	++	++	++	+++	+++	+++	+++
0.00008	++	+	++++	+++	++++	+++	+++	+++	++++	++	++	++	+++	+++	+++	+++
0.0	++	+	++++	+++	++++	+++	+++	+++	++++	++	++	++	+++	+++	+++	+++

* Incubated 53 hours at 15° C. All other species incubated 28 to 30 hours at 25° C.

in various concentrations of sulfanilamide, is summarized in Table III for all the well authenticated species available in this investigation.[8,9] It will be noted that in some cases abundant growth took place over a wide range of concentration of sulfanilamide which prevented luminescence. In other cases, the appearance of visible growth was accompanied by visible luminescence.

[8] These studies were aided by a grant from the Penrose Fund of the American Philosophical Society.

[9] Cultures of the species listed under the genus *Photobacterium* were obtained in 1939 from the Delft Collection, through the kindness of Professor A. J. Kluyver. The culture of *Vibrio albensis* was obtained from the same source. This culture was almost indistinguishable from that of *V. phosphorescens*, kindly supplied from the collection of Professor Malcolm Soule. The species given under the genus *Achromobacter* are identical with the ones studied bacteriologically by Johnson and Shunk (1936).

Thus, among different species the systems limiting growth and luminescence respectively evidently have different thresholds of susceptibility to sulfanilamide. The susceptibility, of course, may rest upon complex factors, as indicated in the previous paragraph.

The relation of PAB to the sulfanilamide inhibition of growth of luminous bacteria is illustrated for *Vibrio phosphorescens* in Table IV. The effects appear to be fundamentally the same as those generally encountered among non-luminous bacteria; *i.e.*, relatively small amounts of PAB will overcome the growth inhibition resulting from much larger concentrations of sulfanilamide. Furthermore, once growth took place, the cells

TABLE IV

Relation between p-Aminobenzoic Acid and Sulfanilamide in the Inhibition of Growth (G) and Luminescence (L) of Broth Cultures of Vibrio phosphorescens at 25° C.

Concentration of PAB molar	0.01		0.005		0.0025		0.00125		0.000625		0.00031		0.00015		0	
Molar concentration of sulfanilamide	G	L	G	L	G	L	G	L	G	L	G	L	G	L	G	L
0.04	−	−	−	−	−	−	−	−	±	−	−	−	−	−	−	−
0.02	−	−	++	−	++	−	++	−	++	−	+++	−	+++	−	−	−
0.01	++	−	++	−	++	−	++	−	+++	+	+++	+++	+++	++	−	−
0.005	++	−	++	−	++	−	++	−	+++	++	+++	++++	+++	+++	−	−
0.0025	±		−		++	−	++	−	+++	+++	+++	++++	+++	++++	−	−
0.00125	+	−	++	−	++	−	++	−	+++	+++	+++	++++	+++	++++	+	+
0.00062	+	−	+++	−	++	−	++	−	+++	+++	+++	++++	+++	++++	++	++
0.00031	+	−	++	−	++	−	++	−	++	+	+++	++++	+++	++++	++	++++
0.00015	±	−	++	−	++	−	++	−	+++	+++	+++	++++	+++	++++	+++	++++
0.0	+	−	++	−	++	−	++	−	+++	++++	+++	++++	+++	++++	+++	++++

were either luminous or non-luminous, in the different concentrations of sulfanilamide, in much the manner that would be expected from Table III. These results again point to the conclusion that the enzyme systems limiting the the two processes, growth and luminescence, are not identical.

The inhibitory effect of PAB on growth is also apparent in Table IV. In fact, in high concentrations, PAB may not only inhibit growth by itself, but may show an additive effect with sulfanilamide. These inhibitory effects of PAB on growth must occur in some manner quite different from that by which this drug antagonizes the effects of sulfanilamide. In its inhibitory action PAB may combine either with a different enzyme essential to growth, and with relatively slight affinity for the drug, or with the same enzyme originally involved, but at a different site. Although no studies have yet been published that give sufficiently accurate and extensive data to enable an analysis of growth

in relation to temperature and concentration of the drug, on a basis similar to the one used in the present study with luminescence, there are experiments showing that, under given conditions, at temperatures above the normal "optimum," PAB tends to lose its capacity to antagonize sulfanilamide, and at high temperatures becomes increasingly inhibitory of itself (Lee, Epstein, and Foley, 1943). At a temperature above normal optimum, the growth-inhibiting potency of sulfanilamide, likewise, appears to increase greatly (White and Parker, 1938). At lower temperatures, there is a range in some cases through which the retardation of growth rates by sulfanilamide becomes less as the temperature is raised towards the optimum. Thus there is good reason to believe that both sulfanilamide and PAB act in a different manner at temperatures below and above the normal optimum. Moreover, the fact that the action of sulfanilamide at temperatures above the optimum evidently has a high temperature coefficient suggests that in this case the mechanism is similar to that of numerous protein-denaturing agents such as urethane, alcohol, and others, which will be discussed in detail in the next sections. Their inhibitions on a given system occur with a high temperature coefficient, and may be scarcely detectable, except in relatively high concentrations of the drug, at low temperatures.

Other significant aspects of the sulfanilamide inhibition of growth, and of its antagonism by PAB and other agents which act in a similar or different manner, can only be referred to briefly in this paper. The growth-stimulating effects of low concentrations of sulfanilamide, for example (Finklestone-Sayliss, Pain, and Patrick, 1937; Johnson, 1942; Lamanna, 1942; Green and Bielschowsky, 1942; Lamanna and Shapiro, 1943) take place by a mechanism that is not clear, but which reasonably involves the same type of phenomenon as the non-specific antagonism of sulfanilamide by urethane. The antagonism by urethane of the sulfonamide inhibition of luminescence has been considered quantitatively at some length (Johnson, Eyring, and Kearns, 1943). Qualitatively the same antagonism at appropriate temperatures and concentrations, has been demonstrated with respect to the growth of a number of common bacterial species in unpublished work in this laboratory.[10] These results suggest that a similar mechanism is involved in each case, namely, that of complex formation between urethane and sulfanilamide, thereby reducing the effective physiological activity of each when the two are simultaneously present.

Another important aspect, that of "drug-fastness," must involve still other mechanisms, which may or may not be associated with an increased production by the organisms of PAB-like metabolites (Landy, Larkum, Oswald, and Streighthoff, 1943). With luminous bacteria, serial transfer of broth cultures in media containing increasing concentrations of sulfanilamide, resulted in an

[10] A number of experiments in this connection have been carried out in this laboratory by Mr. Perry G. M. Austin, Jr.; *cf.* also, Weinstein and McDonald, 1945.

increased resistance to sulfanilamide not only of growth, but also of luminescence, as shown by the data in Table V.

TABLE V

Adaptation of Luminous Bacteria (A. fischeri) to Growth and Luminescence in Sulfanilamide-Containing Media

Serial transfers at frequent intervals were made for several weeks in media in which sulfanilamide was gradually increased. Cross-inoculations were then made in all media. Growth and luminescence recorded after 1 day at 25°C. Data represent average of two duplicate tubes in each case.

Highest concentration of sulfanilamide in serial transfer	Growth (mm. galvonometer deflection in photocell densimeter)				Luminescence (arbitrary units)			
	Molar concentration of sulfanilamide in medium				Molar concentration of sulfanilamide in medium			
	0.0	0.003	0.005	0.01	0.0	0.003	0.005	0.01
0.0	25	12	8	11	5.5	—	—	—
0.003	21	12	9	10	1.9	<1.0	—	—
0.005	20	18	14	9	8.2	3.3	1.0	—
0.010	27	27	36	11	8.9	6.4	3.2	<1.0

VI. TEMPERATURE DENATURATION OF THE ENZYME SYSTEM

(a) *Relation between the Reversible and the Irreversible Denaturation*

By "denaturation" we mean the loss of one or more of the most characteristic properties of a given protein, usually occuring with an excessively high entropy and heat of reaction or activation energy, typical of this class of complex organic molecules (Eyring and Stearn, 1939). The actual changes that take place in the molecule in going from the "native" to the "denatured" state may be many and varied, and under different conditions the same molecular structure no doubt undergoes denaturation in somewhat different manners (Schmidt, 1944; Neurath, 1944). In certain cases the denaturation, *e.g.* as judged by the loss of catalytic activity, is quantitatively reversible in highly purified preparations of crystalline proteins (Anson and Mirsky, 1934). The stability of the denatured form which is in equilibrium with the native form varies with the conditions of pH, solvent, etc.

In the present instance, the decreasing intensity of luminescence above the optimum temperature takes place with the high entropy and heat of reaction characteristic of a protein denaturation, and has therefore been interpreted as such. The pressure effects indicate that it is accompanied by a large molecular volume increase. It is quantitatively reversible, when, under optimal conditions, the temperature is raised momentarily sufficiently high that the intensity of luminescence falls to as little as 1 or 2 per cent of its former maxi-

mum. At these higher temperatures, however, permanent destruction also rapidly occurs, with a high temperature coefficient. Thus, there is both a reversible and an irreversible phase to the denaturation process in bacterial luminescence. The purpose of the following experiments has been to clarify and elucidate somewhat the interrelation of these phases under both "normal" conditions and in the presence of certain inhibitors.

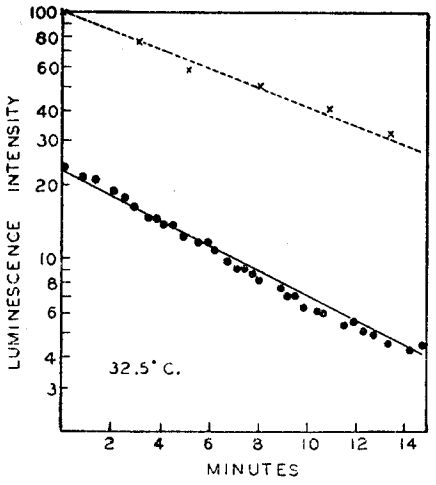


FIG. 21. The destruction of the luminescent system of *P. phosphoreum* at 32.5° C. roughly 10° above the normal optimum temperature for luminescence. The intensity at 22.5° C. and zero time has arbitrarily been taken as equal to one hundred. The crosses, through which the dotted line has been drawn, represent the intensity to which the luminescence returned when cooled from 32.5° C. to 22.5° C. at the time indicated. The experiment was carried out with eight to ten tubes, containing corresponding portions of the suspension, maintained at 32.5° C. Successive tubes were removed and cooled to obtain the points on the upper line. Note that the slopes of the two lines are almost parallel, indicating that most of the logarithmic decrease of luminescence with time at this temperature is not reversible with cooling. Semi-logarithmic scale.

Under the usual experimental conditions, bacterial luminescence remains constant for some time at the normal optimum temperature. At higher temperatures it undergoes a more or less rapid, logarithmic decrease with time. This decrease, that takes place with time at the higher temperatures, is almost entirely irreversible, as shown by Fig. 21. When portions of a suspension maintained at a constant temperature well above the optimum are cooled back to the optimum the extent of reversibility decreases almost in proportion with the time decrease. The lines are not precisely parallel in Fig. 21, for the slope of the upper line is somewhat less than that of the lower, as if the

amount of permanent destruction were somewhat less than the decrease with time would indicate. This recalls the possibility that some of the enzyme molecules, or their precursors, might be activated by above optimum temperatures, by OH^- ions, etc., which were mentioned in (IV, *e*). The lines are so nearly parallel, however, that the slope of lower line may be considered a fair index of the rate of irreversible denaturation. The error in such an assumption would amount to no more than the difference in the slopes, which is evidently only slight. At different constant temperatures, the slopes of the lines thus provide a basis for estimating the activation energy for the

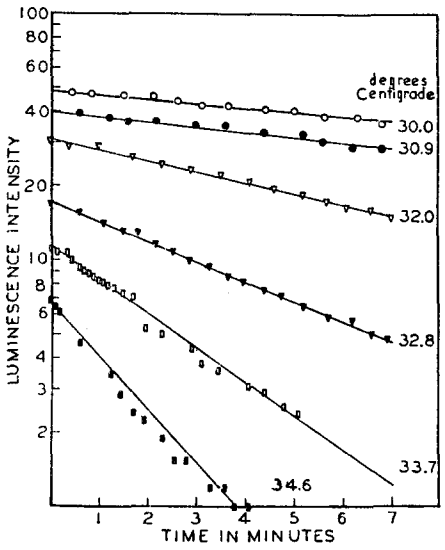


FIG. 22. The rate of destruction of the luminescent system of *P. phosphoreum* at temperatures above the normal optimum for luminescence. Semilogarithmic scale.

irreversible denaturation. Fig. 22 gives an example of the change in slopes with change in temperature. These slopes have been calculated in reciprocal seconds, and the logarithm of the resulting velocity constant has been plotted against the reciprocal of the absolute temperature in Fig. 23. The latter figure shows the results of three different experiments under similar conditions, carried out several months apart.

With regard to the significance of the data in Fig. 23, it will be noted that, in the first place, the points of the separate experiments lie fairly well on a straight line in each case, indicating that a first order reaction is taking place. Furthermore, these lines are all practically parallel, though there appears to be a slight difference in the intercept on the different occasions. The line in Fig. 23 was drawn by inspection, as representing the best average value

of the available data. The slope of this line would indicate that the irreversible denaturation has an activation energy of about 90,000 calories. Obviously, the activation energy could represent a reaction through which either the native or reversibly denatured enzyme, or both forms simultaneously, undergo destruction. Equation (15) gives the light intensity under these conditions. The notation is as follows: (A_0) = total luciferase; (A_{0i}) = total initial luciferase when time (t) equals zero; K_1 = equilibrium constant between native and reversibly denatured luciferase; k_n = rate constant for the destruc-

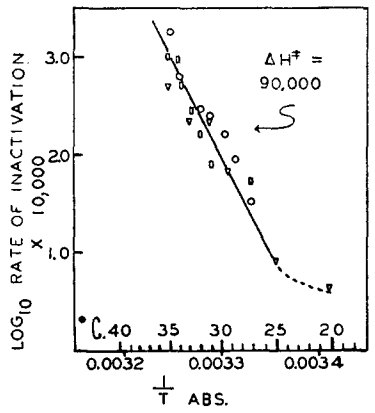


FIG. 23. The rate of destruction of the luminescent system, calculated in reciprocal seconds, from the slopes of the lines in Fig. 22 and similar experiments, as a function of temperature. The triangles, circles, and rectangles represent data from repeated experiments carried out several months apart. The point at 20° C., which falls too high, probably represents destruction through different reactions from those at the higher temperatures.

tion of native luciferase; k_d = rate constant for the destruction of the reversibly denatured luciferase; and the other symbols have their usual meaning.

$$I = \frac{bk(LH_2)(A_0)}{1 + K_1} = \frac{bk(LH_2)(A_{0i})}{1 + K_1}\, e^{-\left(\frac{k_n + k_d K_1}{1 + K_1}\right)t} \tag{15}$$

It is apparent from this equation that the slopes of the lines in Fig. 22 give the values for $\left(\frac{k_n + k_d K_1}{1 + k_1}\right)$ at the respective temperatures. In Fig. 23, the logarithm of $\left(\frac{k_n + k_d K_1}{1 + K_1}\right)$ is plotted against $1/T$. This is evidently a straight line relation over a range of almost 1000 in reaction rate constants. A straight line can be obtained over a wide range only if $k_n = k_d$. This, however, would mean that the reversible and the irreversible denaturation involve breaking different bonds. The point at low temperature in Fig. 23 connected

by a dotted line to the others involves complications due to the excessively long periods required to measure it. Since both denaturations involve extensive breaking of similar bonds it is to be expected that temperature, pressure, and reagents which promote one will promote the other, except for this divergence at below-optimum temperatures, referred to again in a later paragraph.

(*b*) *Relation to pH*

At optimum temperatures, or somewhat below, and with the experimental conditions ordinarily employed, the luminescent enzyme system appears to be stable throughout the acid range down to about pH 4.0. The pressure

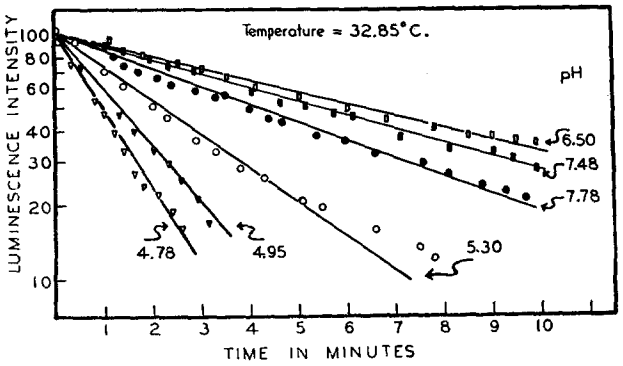


FIG. 24. The destruction of the luminescent system at 32.8° C. at different pH values. Semilogarithmic scale.

effect, referred to earlier, seems to indicate that the protein is stable in the alkaline range also up to about pH 9.0 at these temperatures. At tempertures above the optimum, however, the rate of the logarithmic decrease in luminescence with time varies markedly with pH, as shown in Fig. 24. When the velocity is calculated in reciprocal seconds from the slopes of these lines, and plotted as the logarithm against pH, the curve shown in Fig. 25 results. From this figure it is evident that there is a pH which is most favorable for the stability of the system, and this pH practically coincides with the pH of maximum luminescence at optimum temperature. It quite probably represents the isoelectric point of the protein. The data at hand, however, are not sufficient for a detailed interpretation of the denaturation process in relation to PH. The reaction is undoubtedly one which is subject to a complex interplay of various factors which require extensive investigation before a fully clear picture may be drawn. At present, it seems justified to conclude that the slope of the line at low pH values, in Fig. 25, provides an index, though somewhat rough, to the number of hydrogen ions concerned

in the denaturation at this temperature. A tangent to the curve in this region has a slope of 3.5, which may be considered as a first approximation of the number of hydrogen ions per protein molecule undergoing denaturation.

At low temperatures the luminescence system tends to be destroyed by reaction (5γ), which gradually decomposes the luciferin, rather than the protein enzyme. This type of slow destruction is very likely related to the phenomena studied at length in an earlier investigation (Johnson, 1939) and a different reaction from that involving the protein itself. According to this view, in Fig. 23, the point which, at lowest temperatures, falls too high for the straight line at higher temperatures, possibly represents such a destruction of luciferin.

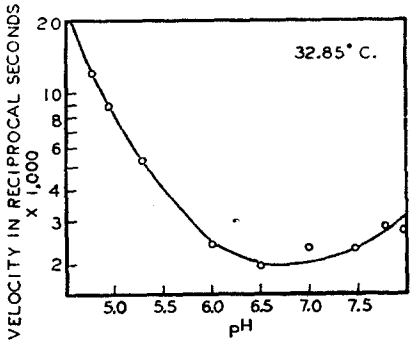


FIG. 25. The velocity of destruction at 32.8° C. as a function of pH, computed from the slopes of the lines in Fig. 24. Semilogarithmic scale.

(*c*) *Pressure versus Rate of Denaturation*

The influence of hydrostatic pressure on the reversible denaturation has already been considered at some length in the previous publications, and has been amplified, with reference to the importance of hydrogen ion concentration, in the present work. At a favorable pH, pressures up to between 500 and 600 atmospheres appear to cause little destruction, since the effects are readily reversible. At the same pH, but at temperatures causing a progressive destruction of luminescence, the application of pressure will retard this irreversible denaturation. A volume increase of activation, $\Delta V\ddagger$, therefore, must occur in this reaction. Data from experiments are shown in Figs. 26 and 27.

In Fig. 26 the decrease in luminescence with time at 34° C. and a pH of 7.3 is shown for different pressures. The slopes of these lines have been taken as a measure of the denaturation reaction rate, and the logarithm of the resultant velocity constants has been plotted against pressure in Fig. 27. Data from similar experiments at different constant temperatures are also included

in Fig. 27. It will be recalled that under the conditions of these experiments, luminescence first undergoes a considerable decrease in intensity through the reversible effects of above-optimum temperatures; *i.e.*, through influencing the value of the equilibrium constant, K_1. A further reduction then occurs with time, more or less rapidly according to both temperature and pressure, an increase in the former accelerating, and in the latter retarding this reduction, as shown in the figures.

Experimental difficulties, *e.g.* that of measuring by visual photometry a dim luminescence constantly decreasing in brightness, have made it impossi-

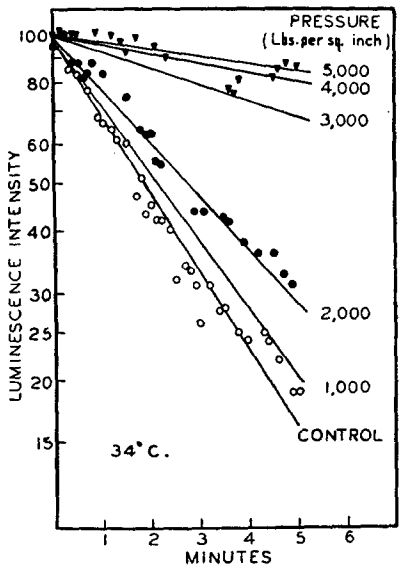


FIG. 26. The relation of the rate of destruction of the luminescent system to hydrostatic pressure. Semilogarithmic scale.

ble to obtain fully as accurate data with regard to the irreversible as with the reversible pressure-temperature effects. Thus the points appear somewhat scattered, but two relations appear evident in Fig. 27: (1) for pressures up to about 3000 pounds per square inch the lines are approximately parallel, and (2) at the higher pressures, particularly at higher temperatures also, there is a tendency for pressure to have less and less influence in slowing the net rate of the reaction.

In interpreting these relations, it seems clear in regard to (1) that the volume increase of activation is approximately the same over the range of temperature studied, covering a several-fold difference in reaction velocities. Taking the slope of the line for 32° C. as a measure of $\Delta V\ddagger$, the data in Fig.

27 indicate that the volume increase amounts to about 71 cc. per gm. molecule in going from the normal to the activated state. This value is almost the same as that of 64.6 cc. at 35° C., calculated by Eyring and Magee (1942) for the volume increase in going from the initial to the final state in the reversible denaturation equilibrium.

As for the second of the above relations, the changing slope at higher pressures indicates among other things that the assumed picture is oversimplified, and that several reactions may be affected. In fact, with pressures higher than those shown in Fig. 27, it might be anticipated that a destructive action of pressure itself would become increasingly apparent, with the consequence that the over-all destructive process, as measured by luminescence alone,

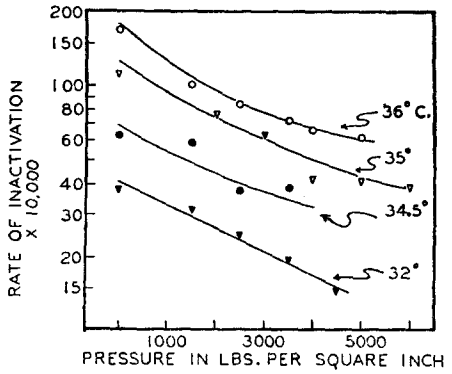


FIG. 27. The relation between the rate of destruction and hydrostatic pressure at various temperatures. The points in this figure were calculated from the slopes of the lines in experiments similar to Fig. 26. Semilogarithmic scale.

would become accelerated. In this event, of course, the curves would go through a minimum, of which there is already some evidence. Further studies with higher pressures would be necessary to resolve this question. The decrease in slope with increase in pressure is encountered again, and discussed, in connection with experiments concerning the alcohol inhibition of luminescence. An additional factor which should be taken into account for a more precise interpretation and calculation of the present results is the changing amount of A_n under the different conditions of temperature and pressure. For, while the slopes referred to above serve as a first approximation, the calculation of $\Delta V\ddagger$ should take into account k_n for different values of p and T. The data are sufficient to indicate clearly, however, that there is quite an appreciable volume change of activation in the irreversible denaturation reaction, and that this volume change is of the same order as that involved ordinarily in the reversible denaturation equilibrium.

VII. THE ACTION OF URETHANE

(a) *The Time Course of Inhibitions by Type I and Type II Agents*

The straightness of the line in Fig. 23 leads to the conclusion, as noted earlier, that the reversible denaturation is independent of the irreversible one, although similar factors promote each. Thus the velocity, v, of denaturation can be written as $v = k_n (A_0)$ where A_0 is the sum of native and reversibly denatured luciferase concentration but not irreversibly denatured. The rate

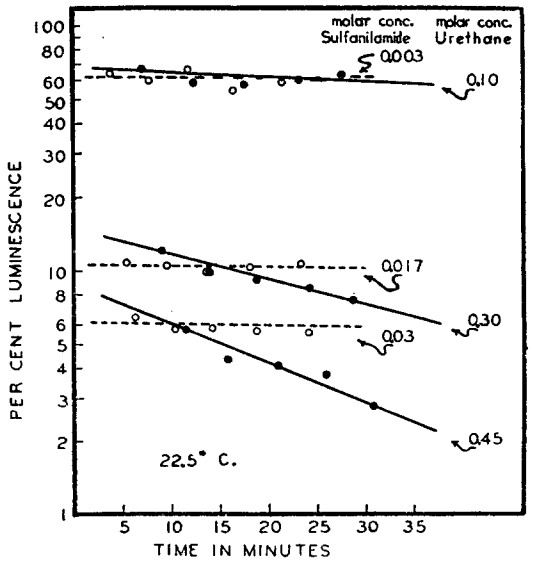


FIG. 28. Time course of inhibition by sulfanilamide and urethane respectively, in concentrations that cause initially similar diminutions in luminescence intensity. Semilogarithmic scale.

constant k_n will depend on temperature, pressure, and narcotics in much the same way as the equilibrium constant for reversible denaturation. This is because preliminary to the actual destruction of primary bonds there will be the same sort of opening of secondary bonds as occurs in reversible denaturation. Since Type I inhibitors, according to our view, combine with no bonds involved in reversible denaturation it is to be expected that they will not effect k_n of the irreversible denaturation. On the other hand Type II inhibitors, such as urethane in luminescence, break secondary bonds and so will increase k_n. Fig. 28 illustrates this effectiveness of urethane in speeding decomposition, and the absence of a similar effect of sulfanilamide. The results of the experiment in Fig. 28, were in fact anticipated. Although such a difference in the time course of inhibition by various

drugs acting on a given process has been frequently observed, the mechanism involved has, by and large, remained obscure (Quastel, 1943).

The slope of the line in Fig. 29 indicates that an average of one and a half more molecules of urethane are combined with the activated molecule undergoing irreversible denaturation than with the normal molecule.

With urethane and inhibitors of similar type a complication is likely to appear in the analysis of the relation between concentration and effect, as

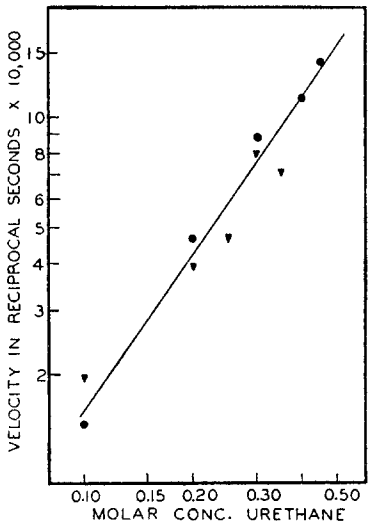


FIG. 29. Analysis of the data from two experiments similar to that shown in Fig. 28. The triangles and circles indicate repeated experiments carried out several months apart. The slope of this line is approximately 1.5. According to equation (10), this would indicate a ratio of about 1.5 between urethane and enzyme molecules in the destruction reaction concerned. Log-log scale.

well as temperature and effect, due to the superposition of an irreversible on a reversible denaturation. To some extent the irreversible reaction may be kept of minor importance by making quick observations, and an effort to this end has been made in carrying out the experiments described in the following discussion.

(*b*) *Relation of the Inhibition to Temperature and Urethane Concentration*

Figs. 30 and 31 give the relation between luminescence intensity and temperature, for two different species, *Vibrio phosphorescens* and *Photobacterium phosphoreum*, respectively, with various concentrations of urethane. The former of these species has a less brilliant luminescence, difficult to measure accurately. The points in Fig. 30 are, therefore, unusually scattered. The

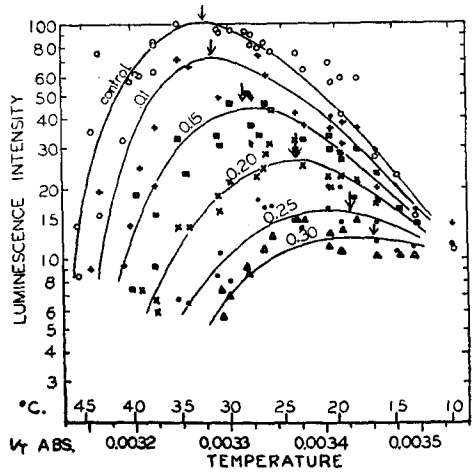


FIG. 30. The intensity-temperature relationship of luminescence in corresponding portions of a suspension of *V. phosphorescens* containing different concentrations of urethane. The relatively dim luminescence of this species is responsible for the unusually scattered points, determined by visual photometry. The smooth curves were drawn by inspection. The arrows indicate the calculated position of the optimum temperatures, according to the estimated equilibrium constants. Semilogarithmic scale.

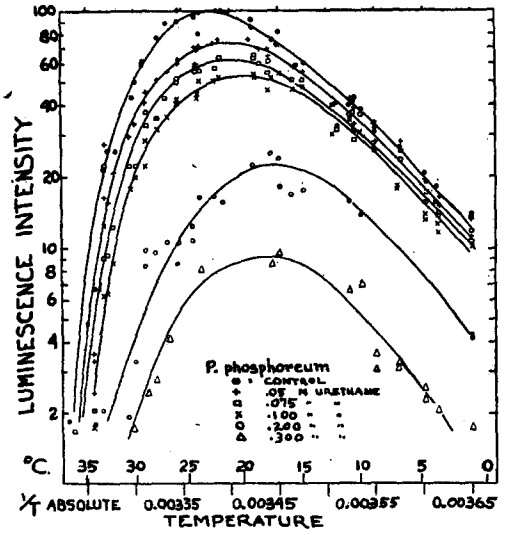


FIG. 31. The intensity-temperature relationship of luminescence in corresponding portions of a suspension of *P. phosphoreum* containing different concentrations of urethane. Semilogarithmic scale.

smooth curves have been drawn by inspection, and the analysis has been based on these lines.

At any given temperature, if the control without added urethane is taken I_1, and the urethane-containing suspension as I_2, then a plot of the logarithm of $\left(\frac{I_1}{I_2} - 1\right)$ against the logarithm of molar concentration for different drug concentrations, gives a fairly straight line, as in the case of sulfanilamide, but with considerably steeper slope. Fig. 32 shows such a plot for several different temperatures, and it will be noted that the slopes increase with rise in

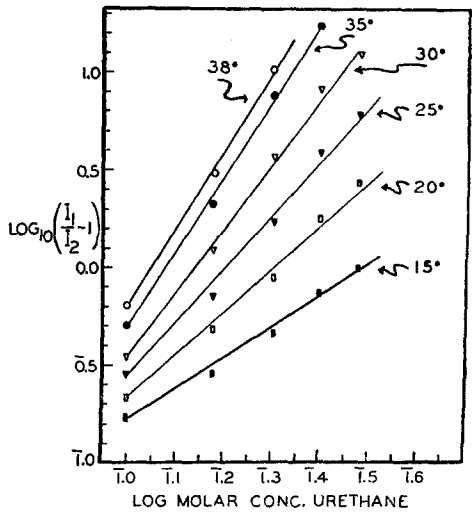


FIG. 32. Analysis of the relation between concentration and inhibition, at different temperatures, from the data given in Fig. 30. The significance of the increasing slopes of the lines with increasing temperature is discussed in the text.

temperature. Although the full explanation for the change in slope with rise in temperature cannot be given on the basis of the data available at present, certain points are worthy of brief discussion.

In the first place, the fact that the slopes of the lines in Fig. 32, as well as in numerous similar analyses, are not in all cases represented by whole numbers indicates the possibility that more than one type of combination takes place between the urethane and the protein. The observed slope would thus represent the average of two or more ratios of urethane-enzyme molecules, in the respective equilibria. Evidence for similar phenomena in the action of alcohol, as shown later, is apparent in the changing slopes in a corresponding analysis with respect to the relation between inhibition and concentration of alcohol at different hydrostatic pressures. The formulation given in equation (10) for Type II inhibitors may be readily extended, as follows,

to include more than one equilibrium between the inhibitor and enzyme. Qualitatively a basis is thus provided for interpreting the phenomena observed, but it is quantitatively somewhat tedious to apply. In the equations below, K_1 refers as usual to the normal reversible denaturation. The equilibrium between the enzyme and urethane, K_3, is now represented by two different equilibria, designated by a prime and double prime, respectively. Letting A_0 represent the total luciferase, we may write the equation for luminescence intensity and proceed with the formulation as before.

$$I_1 = \frac{bk_2}{1 + K_1} (A_0)(LH_2) \tag{16}$$

$$(A_0) = (A_n) + (A_d) + (A_d U_{s'}) + (A_d U_{s''}) \tag{17}$$

$$(A_0) = (A_n) + (A_n) K_1 + K_3' (U)^{s'} (A_n) + K_3'' (U)^{s''} (A_n) \tag{18}$$

$$(A_n) = \frac{(A_0)}{1 + K_1 + K_3'(U)^{s'} + K_3''(U)^{s''}} \tag{19}$$

$$\frac{I_1}{I_2} = \frac{1 + K_1 + K_3'(U)^{s'} + K_3''(U)^{s''}}{1 + K_1} \tag{20}$$

$$\left(\frac{I_1}{I_3} - 1\right) = \frac{K_3'}{1 + K_1} (U)^{s'} + \frac{K_3''}{1 + K_1} (U)^{s''} \tag{21}$$

From equation (21) it is obvious that if the values of K_3' and K_3'', and of s' and s'' are different from zero, the slopes of lines analyzed according to the simpler formulation for a single equilibrium will in general vary with either temperature or pressure. The net effect, of course, will be approximately indicated by the slopes of the lines obtained in the plot of $\log \left(\frac{I_1}{I_2} - 1\right)$ against log urethane concentration; *i.e.*, with increasing slope, the average ratio of drug-enzyme molecules increases. With rise in temperature the weaker of of two equilibrium combinations would lose in importance as compared with the stronger. If the more exothermal K is associated with the larger s, the number combined will seem to rise with temperature as in Fig. 32.[11]

[11] In the literature are a number of examples in which the quantitative effects of urethane, studied at only one temperature and pressure on complex processes such as growth or respiration, have been analyzed in a manner corresponding to that used in the present instance, and have shown more or less pronounced departures from a straight line relation (*e.g.*, Fisher and Stern, 1942). Although there is an understandable tendency to interpret such results in terms of an effect of the drug on more than one distinct system, the data have generally been inconclusive on this point. When a drug is capable of combining with an enzyme at more than one site with different bond strengths, equation (21) is obtained. With appropriate values of the K's and s's the complications that have been observed are obtained.

The temperature analysis of the curves in Fig. 30 is given in Fig. 33, for the several concentrations of urethane. In contrast to the analysis of the sulfanilamide inhibition (Fig. 20), the lines in Fig. 33 are not largely straight throughout, but curve markedly towards greater inhibition in the region of the higher temperatures, much as in the analysis of luminescence intensity against temperature at alkaline pH. For reasons already indicated, this departure from the simplest straight line relation might be anticipated in

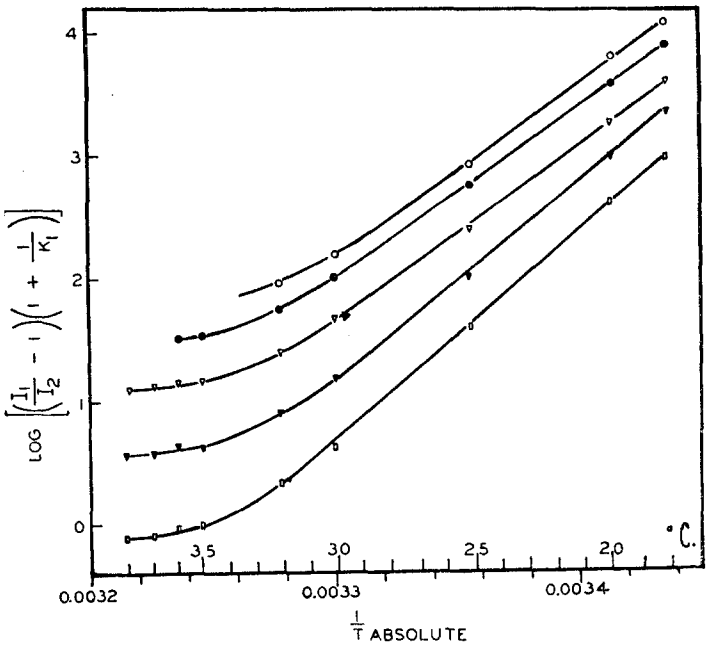


FIG. 33. Analysis of the data shown in Fig. 30 as a Type II inhibition, with respect to temperature, for the several concentrations of urethane.

connection with substances which cause an irreversible effect along with their reversible influence on the denaturation equilibrium, and in proportion to the time of exposure during which the rate process of the destruction is allowed to proceed. The values of $\log\left[\left(\frac{I_1}{I_2}-1\right)\left(1+\frac{I}{K}\right)\right]$ thus tend to be too high at the low values of $1/T$, and a bend occurs in the curve.

The straight line portions of the curves in Fig. 33 enable an estimate of the heat of reaction of the urethane-enzyme equilibrium. The average value amounts to about 65,000 calories, which is close to 60,300 calories found for the ΔH in the denaturation equilibrium at high concentrations of hydroxyl ions.

(c) *Shift in "Optimum Temperature"*

In several instances considered thus far the inhibitor has caused an obvious change in the temperature at which the maximum luminescence intensity is observed. Thus, the normal optimum is shifted to slightly higher temperatures in the presence of inhibiting concentrations of hydrogen ions (Fig. 12) or of sulfanilamide (Figs. 17 and 18), while it is altered to lower temperatures in the presence of unfavorable concentrations of hydroxyl ions (Fig. 15) or of urethane (Figs. 30 and 31). The apparent activation energy for luminescence is increased by the former two, and decreased by the latter two agents, in the manner that has already been discussed. The apparent heat of reaction in the denaturation equilibrium, which at best can only be approximated, is likewise affected, to a greater extent by the urethane than the sulfanilamide type. Complicating factors that enter into the control of the over-all, observed rate of luminescence or other processes in living cells, make it impossible to arrive at very exact values for the activation energy, denaturation equilibrium, and other thermodynamic or rate constants of reactions that influence the process in question.

From the theory that we have already described, it follows that the optimum temperature will shift in the manner that is qualitatively apparent in the figures. Formulations may be readily derived for predicting quantitatively the amount of this shift, but the accuracy of the predictions is extremely sensitive to the experimental error as well as to any complicating influences within the cell, such as an irreversible effect in addition to the reversible equilibrium established with a given enxyme by an inhibitor, or an effect of the inhibitor on more than one system. With urethane, better than with sulfanilamide the constants that fit the curves predict with some accuracy the amount of the change in the optimum temperature, showing that in some cases the possible complicating factors are not of overshadowing significance, and results are in fair agreement with the simple theory. The theoretically predicted changes in optimum temperature, indicated by the arrows in Fig. 30, were arrived at through the following equations.

$$I = \frac{bk_2(LH_2)(A_0)}{1 + K_1 + K_1K_3U^e} \tag{22}$$

$$\ln I = \ln((b)(LH_2)(A_0)) + \ln k_2 - \ln(1 + K_1 + K_1K_3U^e) \tag{23}$$

At the maximum, the change in luminescence intensity with respect to temperature is zero. Hence, at constant pressure,

$$0 = \frac{\partial \ln I}{\partial T} = \frac{1}{T} + \frac{\Delta H_2^{\ddagger}}{RT^2} - \frac{\frac{\Delta H_1}{RT^2}K_1 + \frac{\Delta H_1 + \Delta H_3}{RT^2}K_1K_3U^e}{1 + K_1 + K_1K_3U^e} \tag{24}$$

Multiplying through by $RT^2\,(1 + K_1 + K_1K_3U^s)$, and dividing by $RT + \Delta H_2\ddagger = \Delta H_2'$, we obtain:

$$1 + K_1 + K_1K_3U^s = \frac{\Delta H_1}{\Delta H_2'}K_1 + \frac{\Delta H_1 + \Delta H_3}{\Delta H_2'}(K_1K_3U^s) \tag{25}$$

$$1 + \left(1 - \frac{\Delta H_1}{\Delta H_2'}\right)K_1 = \left(\frac{\Delta H_1 + \Delta H_3}{\Delta H_2'} - 1\right)K_1K_3U^s \tag{26}$$

The change in optimum temperature is obtained by plotting y and z against temperature where

$$y = 1 + \left(1 - \frac{\Delta H_1}{\Delta H_2'}\right)K_1 \tag{27}$$

and

$$z = \left(\frac{\Delta H_1 + \Delta H_3}{\Delta H_2'} - 1\right)K_1K_3U^s \tag{28}$$

as shown in Fig. 34.

The temperature at which luminescence reaches a maximum intensity in different concentrations of U occurs at the intersection of y and z.

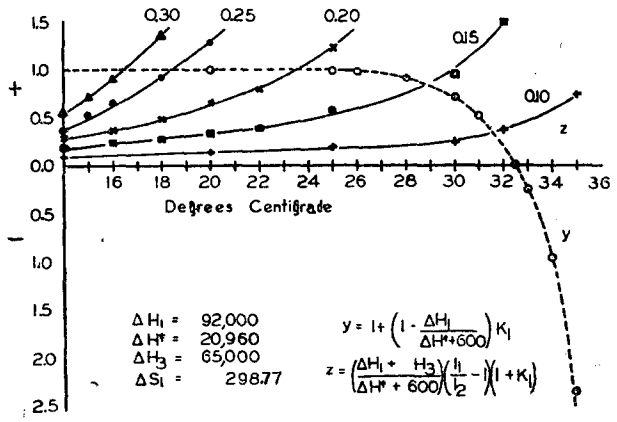


FIG. 34. Equations and data used in calculating the shifts in optimum temperature shown in Fig. 30.

VIII. THE ACTION OF ALCOHOL

In a previous publication (Johnson, Eyring, and Kearns, 1943) it was shown that, at low temperatures, the primary aliphatic alcohols containing from one to five carbon atoms appear to affect bacterial luminescence in a similar manner, though with different potencies. On plotting the logarithm

of $\left(\frac{I_1}{I_2} - 1\right)$ against the logarithm of alcohol concentration, a series of practically parallel lines resulted, with the intercept on the abscissa generally at lower concentrations the longer the carbon chain of the alcohol. The slope of these lines indicated that a ratio of between two and three molecules of alcohol per enzyme molecule is formed in the equilibrium. Furthermore, at 22° C. the inhibition by a given concentration was greater than at 5° C.

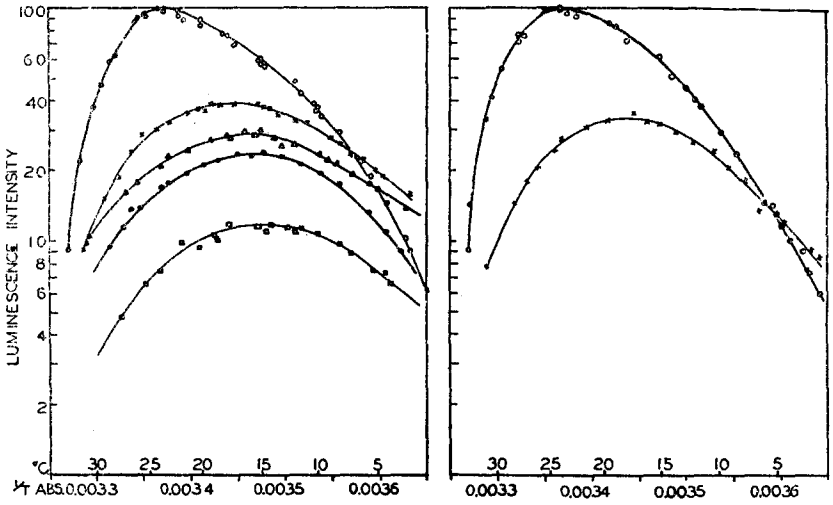


FIG. 35. The intensity-temperature relationship of luminescence in corresponding portions of a suspension of *P. phosphoreum* containing various concentrations of alcohol. The two graphs are for repeated experiments carried out on different days. Note the "stimulation" of luminescence by low concentrations of alcohol at low temperatures. Semilogarithmic scale. Open circles, control; crosses, 0.4 M alcohol in figure at left, 0.5 M in figure at right; triangles, 0.5 M alcohol; solid circles, 0.6 M alcohol; squares, 0.8 M alcohol.

These earlier results suggested that the simple alcohols act in much the same manner as urethane. Like urethane, they were found to enter into a loose complex formation with sulfanilamide, forming a combination sufficiently strong that, under appropriate conditions of temperature and concentrations, a mutual antagonism of their physiological inhibitions was evident. In the present study further data have been obtained with respect to the significance of temperature, and especial attention has been given the effects of hydrostatic pressure, in relation to both temperature and concentration.

(a) The Effect of Alcohol in Relation to Concentration and Temperature

The relation between temperature and luminescence intensity for suspensions of cells containing various concentrations of alcohol is shown in Fig. 35.

It is apparent that these results are similar, in certain distinct respects, to those obtained with urethane. The inhibitor causes a decrease in apparent activation energy, a decrease in apparent energy of denaturation, and a shifting of the normal optimum to lower temperatures. A "stimulating" effect of slight concentrations of alcohol at the lower temperatures, sometimes encountered with urethane, is also apparent in Fig. 35. This latter phenomenon is qualitatively similar to the urethane antagonism of sulfanilamide, as investigated by Johnson, Eyring, and Kearns (1943). The interpretation

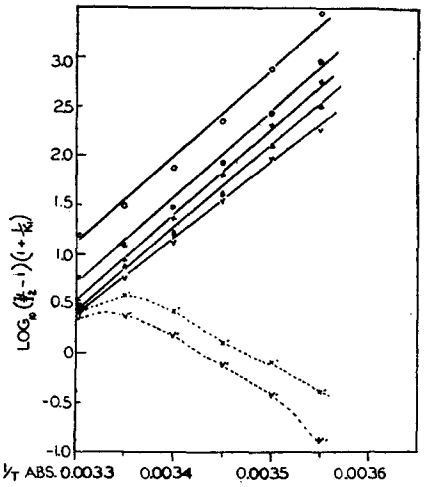


FIG. 36. Analysis of the data shown in Fig. 37 with respect to temperature. The solid lines represent $\log_{10}\left[\left(\frac{I_1}{I_2}-1\right)\left(1+\frac{I}{K_1}\right)\right]$ against $1/T$, appropriate to Type II inhibition, and the broken lines represent $\log_{10}\left(\frac{I_1}{I_2}-1\right)$ against $1/T$, appropriate to Type I inhibition. The inhibition appears to conform to the former type.

is thus suggested that the stimulatory effects in the present instance take place through the combination of the alcohol with some naturally occurring metabolite which ordinarily exerts a slightly retarding influence on the overall rate of the luminescent reactions. The major effect, that of inhibition at the higher concentrations and temperatures, has been analyzed in the same manner as with urethane, and the results plotted in Fig. 36. The dotted lines show the results of attempting an analysis according to the formulations for the sulfanilamide type of luminescence inhibitor. The urethane type is the one to which the data conform, as evidenced by both the direction of slope and linearity of the lines. The fact that the lines are almost linear indicates that the effect of the alcohol is, for the most part, either on the pace setting reaction

or on a preceding reaction with similar temperature coefficient. The fact that they are not completely linear, but rather tend to curve in the direction of greater inhibition at the higher temperatures, shows that complicating effects, such as those discussed earlier, and also as evidenced by a stimulatory action at low temperature, are present in this case also. The slopes of the line, however, again permit an estimate of the heat of reaction in the alcohol equilibrium, amounting to approximately—37,000 calories. This value is considerably less than that for urethane, and for hydroxyl ions.

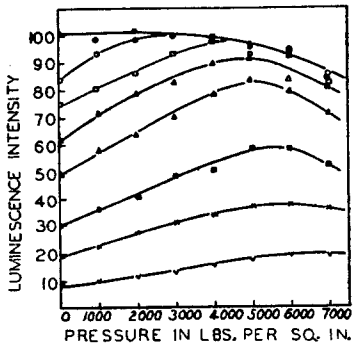


FIG. 37. Luminescence intensity of *P. phosphoreum* as a function of alcohol concentration and hydrostatic pressure. The intensity of the control, without alcohol and at normal pressure, is arbitrarily taken as equal to one hundred. Note the complete elimination of the apparent inhibition of alcohol in low concentrations by hydrostatic pressure. Note, also, the shift in observed optimum pressure with increasing concentrations of alcohol. The concentrations of alcohol, represented by successive curves, from top to bottom of the figure are as follows: 0 (control), 0.2, 0.4, 0.5, 0.6, 0.8, 1.0, and 1.5 molar. The temperature was 17.5° C. throughout.

(*b*) *Inhibition in Relation to Concentration and Pressure*

In studying the relation between pressure and inhibition of luminescence by different concentrations of alcohol at a given constant temperature, advantage may be taken of the fact that, in the neighborhood of the normal optimum temperature, pressure has very little effect on the observed intensity of luminescence, except when certain inhibitors are present. In Fig. 37 the relation between pressure, concentration of alcohol, and luminescence intensity is shown with respect to the control without alcohol, and at atmospheric pressure, arbitrarily taken as 100. The pressure effects were readily reversible, on release of pressure.

Noteworthy among the things that are qualitatively apparent in Fig. 37 are the following. In the first place, it will be noted that in the absence of added alcohol, pressure alone causes only a slight diminution in luminescence, beginning at about 4,000 pounds per square inch, and increasing to about 14

per cent at 7,000 pounds. With an initial addition of alcohol, however, resulting in a final concentration of 0.2 M, luminescence is reduced to around 16 per cent, but this inhibition becomes less on application of hydrostatic pressure, and disappears at about 2,000 pounds. With increasing concentrations of alcohol pressure again tends to counteract the inhibition, but if the alcohol concentration is higher than about 0.4 M, the inhibition is not completely abolished, for the high-pressure diminution of luminescence, observed in the absence of alcohol, now becomes more significant than the effect of pressure in eliminating the alcohol inhibition. Thus, with increasing concentrations of alcohol there is a very pronounced shift in the pressure at which maximum luminescence is observed.

The data in Fig. 37 include two variables, concentration of alcohol and hydrostatic pressure, other factors such as temperature, pH, etc., remaining constant. The general formulations which have been derived may be applied in an analysis from the point of view of either of these variables. An analysis of the relation between inhibition and concentration, for different pressures, is shown in Fig. 38.

In agreement with the earlier study that was carried out at normal pressure, Fig. 38 shows that a fairly steep slope of 2.8 is obtained. At higher pressures the slope becomes greater, amounting to 3.1 at 3,000 pounds, 3.8 at 4,000 pounds, and 4.0 at 7,000 pounds. It is difficult to see how this change in slope could represent an increase in the ratio of alcohol to enzyme molecules if only a single equilibrium between the two were involved. On the basis that there is more than one equilibrium between the two molecules, however, the phenomenon may be readily accounted for, in the manner discussed for the action of urethane at different temperatures.

In analyzing the data of Fig. 37 from the point of view alternative to the above, *viz.* with reference to the relation between the effects of constant concentrations of alcohol and different hydrostatic pressures, it becomes necessary to calculate the appropriate values of K_1 for each pressure. These values may be obtained from the relation:

$$\Delta F^0_{p_2} = \Delta F^0_{p_1} + \int_{p_1}^{p_2} \overline{\Delta V}\, dp \tag{29}$$

At the temperature of 17.8°C., used in this experiment, the value of K_1 varies between 0.03404 and 0.0295, or only about 1.15 times, throughout the range between normal pressure and 7,000 pounds. The results of the analysis are shown in Fig. 39.

There is a pronounced change in slope of the curves in Fig. 39 with increasing concentrations of alcohol, and a tendency to depart from a straight line relation that is very apparent at the higher pressures. It is evident, therefore, that the action of alcohol involves more than one equilibrium. Complications

such as those already discussed in connection with the temperature-concentration relation of the urethane inhibition, and the action of pressure on the rate of irreversible denaturation are evidently concerned in this case also.

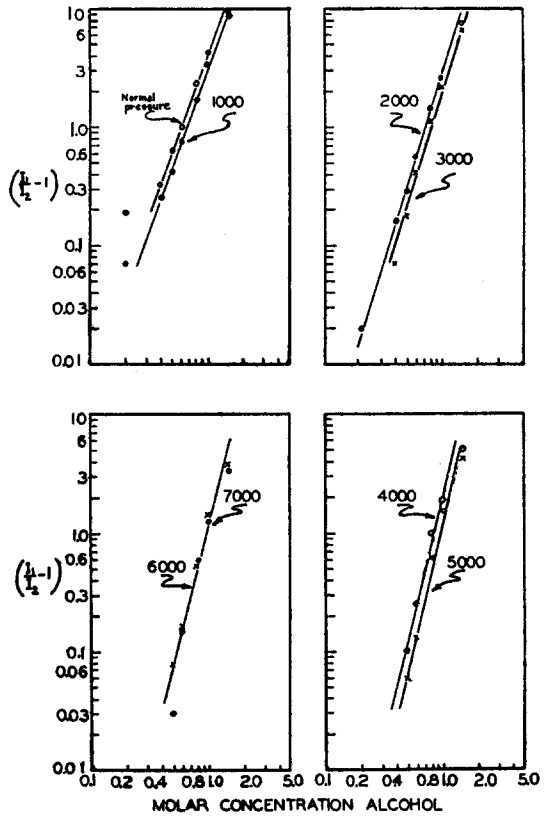


FIG. 38. Analysis of the data shown in Fig. 37 concerning the relation between inhibition and alcohol concentration at different given hydrostatic pressures. Note the increase in the slope at the lines from about 2.8 at normal pressure to 4.0 at 7,000 pounds per square inch. Log-log scale.

Moreover, the fact that a stimulatory effect of alcohol is observed at low temperatures and concentrations shows that there are additional reactions which necessitate an extension of the theory. The data in Fig. 39, while not permitting an exact analysis of the molecular volume changes involved in the effects of alcohol on the luminescent system, are useful in showing that the volume changes are large. In addition, the agreement of the analysis with the simple theory is sufficiently good to indicate that the complicating reactions are of secondary importance. A somewhat clearer view of the chief mechanism

is obtained through a study of the effects of a given concentration of alcohol in relation to both temperature and hydrostatic pressure, as discussed below.

(c) *The Inhibition in Relation to Pressure and Temperature*

The pressure effect on luminescence after addition of alcohol to give a final concentration of 0.5 molar is shown for four different temperatures in Fig. 40.[12]

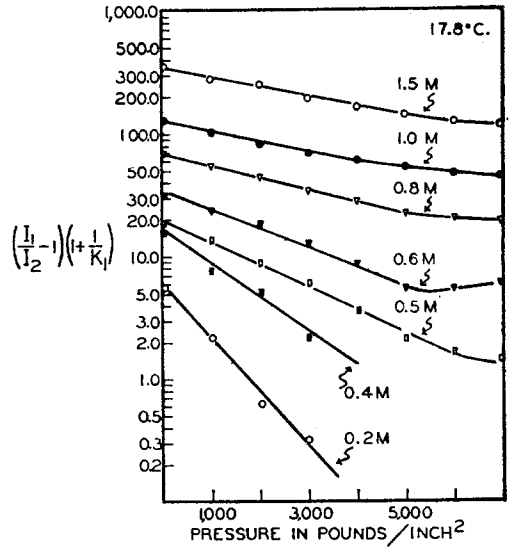


FIG. 39. Analysis of the data shown in Fig. 37 with respect to pressure and each of the given concentrations of alcohol.

In this figure the luminescence of cells without added alcohol and at normal pressure has again been set arbitrarily equal to 100, and the remaining points multiplied by the appropriate factor for this scale. It should be recalled that the intensity actually observed at normal pressure is very different at the several temperatures. The direction of the pressure effect, however, as well as its influence on the per cent inhibition, is most readily apparent in this plot. Thus, at temperatures below the normal optimum, such as 11.1° C., luminescence in the control continually diminishes as the pressure is raised, thereby opposing the volume increase of activation in the rate process. At this temperature the value of K_1 is too small for pressure to counteract, through its influence on the denaturation equilibrium, the effect on the rate process. If alcohol is added, however, an equilibrium is established that is accompanied by a volume increase of the enzyme, and a decrease in catalytic activity. The

[12] This figure is remarkably like the one obtained in a similar study with regard to the action of quinine (Johnson and Schneyer, 1944).

net effect of pressure thus becomes altogether different. At 11° C., a concentration of 0.5 molar alcohol causes just enough reversible denaturation that the effect of increasing pressures in slowing the light-emitting reaction is counterbalanced by the effect of supplying more of the catalytically active enzyme, through the denaturation equilibrium. Consequently, in the alcohol-containing cells there appears to be no effect of pressure under these conditions.

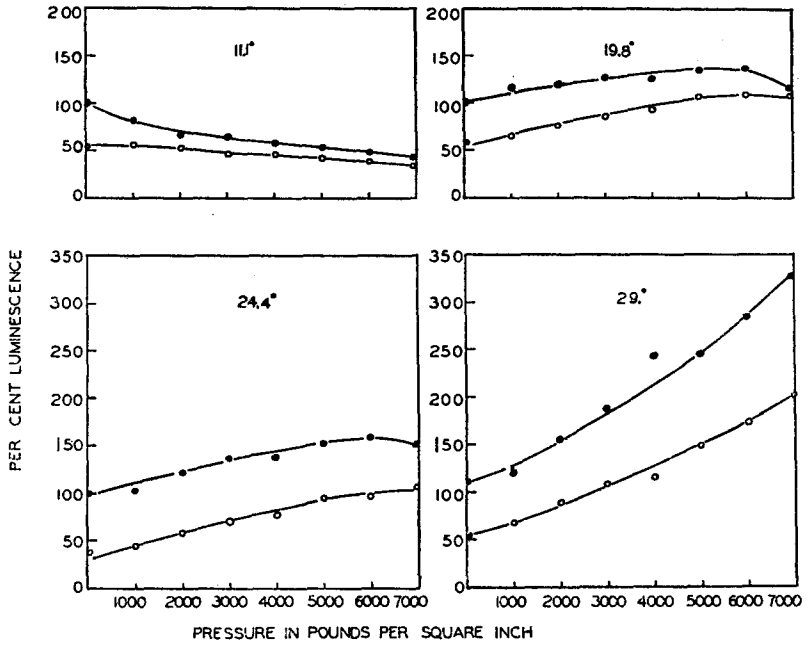


FIG. 40. The effect of pressure on the inhibition of luminescence by 0.5 molar alcohol at different temperatures. The intensity at normal pressure, without alcohol, has arbitrarily been taken as equal to one hundred at each temperature.

In comparison with the control, of course, the inhibition continues to decrease with rise in pressure, and the nearly horizontal line representing luminescence intensity of the alcohol suspension is approached by the continually falling line of the control. The same general mechanism is responsible for the net effects of pressure observed at the other temperatures, both in the control and the alcohol-containing suspensions; *i.e.*, in all cases the difference between the retarding and accelerating effects of the pressure respectively, on at least the two reactions. At temperatures considerably above the optimum, *e.g.* 29° C., at which the value of K_1 is large and much of the enzyme is in the reversibly denatured condition, pressure causes a striking increase in the intensity of luminescence, both in the control and in the alcohol-containing suspension.

Since the actual intensity at this temperature, in comparison with the intensity at the normal optimum, has already been diminished by the above-optimum temperature, the several hundred per cent increase that occurs on application of pressure does not cause the luminescence intensity ever to exceed, to any considerable extent, the normal maximum.

An approximate analysis of the data in Fig. 40 with respect to the alcohol inhibition is shown in Fig. 41. A more precise treatment would involve calculating the appropriate values of K_1 at each temperature as well as each

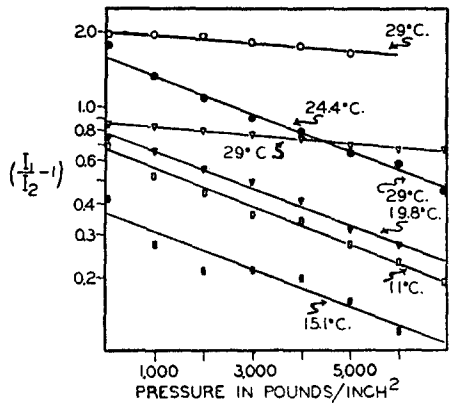


FIG. 41. Analysis of the data shown in Fig. 40 with regard to the effect of pressure on the alcohol inhibition at different temperatures. This analysis would be more precise if the values of $\left(\frac{I_1}{I_2} - 1\right)$ on the ordinate were multiplied by $\left(1 + \frac{1}{K_1}\right)$. Although the slopes in general are not greatly altered, the line for 29° C. becomes practically horizontal when the values of K_1 for the different pressures are taken into account. The two lines shown for 29° are from separate experiments.

pressure, and multiplying each point obtained for $\left(\frac{I_1}{I_2} - 1\right)$ by the proper value of $\left(1 + \frac{1}{K_1}\right)$, as before. This procedure has been carried out only for the curve for 29°. This curve, already nearly horizontal with respect to the abscissa, becomes almost perfectly horizontal when the values of K_1 are taken into account. Although we again have evidence of other reactions that enter into the measured results and thereby tend to complicate the picture that has been drawn on the assumption of the simplest conditions, the data appear adequate and sufficiently clear to justify a somewhat more detailed interpretation with regard to the chief mechanism, as follows: In the absence of alcohol, we have as usual

$$I_1 = \frac{bk_2(A_0)(LH_2)}{1 + K_1} \tag{30}$$

When alcohol in molar concentration (U) is added, this becomes

$$I_2 = \frac{bk_2}{1 + K_1 + (U)^s K_3 K_1} \tag{31}$$

Dividing equation (30) by equation (31),

$$\frac{I_1}{I_2} = 1 + \frac{K_3 K_1 (U)^s}{1 + K_1} \tag{32}$$

$$\left(\frac{I_1}{I_2} - 1\right)\left(1 + \frac{1}{K_1}\right) = K_3(U)^s \tag{33}$$

Letting the superscript (0) represent K at normal pressure,

$$K_1 = K_1^0 e^{-p\Delta V_1} \quad \text{and} \quad K_3 = K_3^0 e^{-p\Delta V_3}, \tag{34}$$

Substituting these expressions for K's, and taking the logarithm,

$$\ln\left(\frac{I_1}{I_2} - 1\right) = \ln\left(\frac{K_3 K_1}{1 + K_1}\right) + s \ln U = \ln\left(\frac{K_3^0 K_1^0}{1 + K_1^0 e^{-p\Delta V_1}}\right) + \left(\frac{-\Delta V_3 - \Delta V_1}{RT}\right) p + s \ln U \tag{35}$$

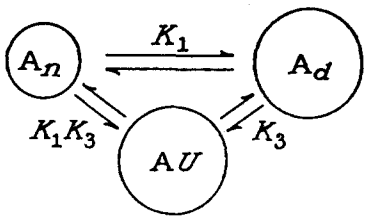
$$\ln\left(\frac{I_1}{I_2} - 1\right) = \ln K_3^0 K_1^0 - \ln (1 + K_1^0 e^{-p\Delta V_1/RT}) + \left(\frac{-\Delta V_3 - \Delta V_1}{RT}\right) p + s \ln U \tag{36}$$

From equation (35), it is apparent that at temperatures where K_1 is small in comparison with 1, then the $\ln\left(\frac{I_1}{I_2} - 1\right)$ gives the approximate value of $\ln (K_3 K_1)$ plus $s \ln U$. The expression $s \ln U$ remains constant for a given concentration of alcohol. As the value of K_1 becomes large in comparison with 1, then $\left(\frac{K_1}{1 + K_1}\right)$ tends to cancel out, and the $\ln\left(\frac{I_1}{I_2} - 1\right)$ gives the approximate value of $\ln K_3 + s \ln U$. Thus at relatively low temperatures, such as 11.1° and 19.8° C., where the value of K_1 is small, the slopes of the lines in Fig. 41 give the sum of the volume changes of the normal denaturation equilibrium, K_1, and the equilibrium K_3 characterizing the reaction between the alcohol and the protein. At the relatively high temperatures, on the other hand, the slope represents the volume change almost wholly with respect to K_3.

Since in Fig. 41 the slope of the line at 29° is so nearly horizontal, it follows that the value of ΔV_3 must be extremely small in comparison with ΔV_1. In other words, the volume change responsible for the observed pressure effects on the alcohol inhibition is confined practically altogether to the changes which this drug causes in the normal denaturation equilibrium, K_1. Thus, the volume change indicated by the slopes of the lines for the lower temperatures, in Fig. 41, should yield values for ΔV that correspond to the values obtained from the effects of pressure, at higher temperatures, on suspensions to which no alcohol

has been added. An exact comparison of the values obtained in the two cases is hardly possible, inasmuch as the temperature dependence of ΔV_1, pointed out in the study of Eyring and Magee (1942), as well as other facts, show that the situation is more complicated than the theory takes into account. However, the value of ΔV_1 at 35° C., according to the results of Eyring and Magee, is 64.6 cc. The data from the alcohol inhibition, in Fig. 41, as based on the slope of the curve at 24° C., which is practically parallel to the others at the lower temperatures, indicate a ΔV_1 of approximately 62.6 cc. Although the closeness of this striking agreement is no doubt partly fortuitous, it is in line with the theoretical expectations.

Diagrammatically, we might picture in simplest terms the equilibria primarily concerned in the relation of temperature and pressure to the inhibitory action of alcohol as follows:—



There is no physical way of distinguishing whether the alcohol-enzyme compound is formed as a result of a combination of this drug with the reversibly denatured form of the enzyme, according to the equilibrium constant, K_3, or with the native form of the enzyme, according to equilibrium constant K_3 times equilibrium constant K_1. The volume change and end result are the same in either case.

With regard to the mechanism by which alcohol combines with the protein and promotes the reversible denaturation, the general similarity between the observed effects of alcohol and urethane suggests that the action is fundamentally the same in the two cases.

IX. DISCUSSION

The significance of this study has several interrelated aspects, which are perhaps worth considering very briefly from the points of view of (1), the phenomenon of bioluminescence in particular, (2), the mechanism and control of biological oxidative reactions in the living cell and in extracts, and (3), the physical chemistry of inhibitions caused by drugs and other factors that act upon various enzyme systems.

With regard to the problem of luminescence, the results obtained in experiments with the various factors that influence the rate, or intensity, of this process in bacteria have made it possible to schematize, in somewhat greater

detail and clarity than previously, certain of the fundamental reactions concerned. The hypotheses advanced take into account the previously reported data derived from studies of kinetics, as well as the chemical and physical properties of the system, and provide a kinetic basis for interpreting several more or less obscure phenomena; *e.g.*, the relation between the luminescent and non-luminescent oxidation of luciferin. The final identity of the molecules that participate in the light-emitting system, and their rôle in cellular oxidations remain to be established, although the theoretical chemistry of the reactions has been examined in some detail, and has been brought to a stage satisfactory for an analysis of rate-controlling influences, as well as for a partial understanding of the fundamental processes involved.

In undertaking to elucidate more clearly the luminescent oxidation itself, it has been necessary to consider the action of various factors, such as hydrogen ion concentration, temperature, and pressure, both separately and in relation to each other. Some of the phenomena observed have been typical of those familiarly encountered in the study of these factors in relation to diverse biological processes. Presumably the basic mechanisms involved in many other cases will be the same as in this one. Some new relationships, such as that between the pH and the effect of pressure on the reaction velocity of an enzyme system, have been studied for the first time. The results have contributed to a somewhat more complete picture of events in the catalytic system during the process of electron transfer, and of the intimate mechanism involved in specific inhibitions and activations of such systems; *e.g.*, by sulfanilamide and *p*-aminobenzoic acid, respectively.

Finally, in investigating and accounting for the action of various drugs, etc., which influence the rate of the over-all process of luminescence, new formulations have been derived appropriate to the phenomena in question. These formulations are of general applicability for the purpose of testing the conformity of data from experiments to certain basic mechanisms. In luminescence, most of the observed effects appear to involve one chief site, and there are indications that this site is the luciferase molecule itself, in which the luciferin possibly occupies the rôle of prosthetic group to the protein enzyme. The theory accounts for two distinct types of inhibitions, in relation to temperature, pressure, hydrogen ion concentration, and constancy with time: Type I, caused by substances which combine with, or in place of, the prosthetic group, independently of the reversible protein denaturation; and Type II, caused by drugs which combine in a manner that promotes the reversible denaturation of the protein. In the latter type an irreversible reaction usually takes place along with the reversible change. It is of particular interest to note that, in this latter group, are a number of "lipoid-soluble narcotics" whose fundamental mode of action has long remained obscure. There is increasing evidence, however, to indicate that the effects of these substances, even in complex

phenomena such as narcosis of nerves, are mediated through oxidative enzyme systems (Quastel, 1943), apparently not very different from the luminescent system. The theory outlined in the present study, therefore, should be of use in this connection also.

In the literature are numerous examples of phenomena qualitatively similar, in one or another respect, to those described in this paper. Unfortunately, the quantitative data are generally insufficient for an analysis on the basis of the present theory. This is generally true of the data with respect to temperature, and almost wholly true with regard to pressure. Further and more complete studies with adequate reference to the several variables of pH, temperature, pressure, and concentration of specific inhibitors or activators, are needed in order to understand the theoretical chemistry concerned. In so far as studies of this kind contribute to the solution of biological and related problems they may be considered eminently worth while. The theory advanced herein will perhaps aid the progress of such investigations.

The authors wish to acknowledge, with much appreciation, the cooperation of Dr. E. N. Harvey in providing much of the apparatus used for measuring luminescence, and for studying hydrostatic pressure effects. Indebtedness is also gratefully acknowledged to Dr. L. Michaelis for the special compounds tested for a possible identity with luciferin. The pressure bomb was gold-plated through the kindness of Dr. Thon. Valuable assistance in the pressure experiments was rendered by Dr. Leon Schneyer, and in some of the other experiments by Miss Nina Zworykin. Indebtedness is also acknowledged for valuable discussions with Dr. Harvey, Dr. Chase, and Dr. McElroy.

X. SUMMARY

On the basis of available data with regard to the chemical and physical properties of the "substrate" luciferin (LH_2) and enzyme, luciferase (A), and of kinetic data derived both from the reaction in extracts of *Cypridina*, and from the luminescence of intact bacteria, the fundamental reactions involved in the phenomenon of bioluminescence have been schematized. These reactions provide a satisfactory basis for interpreting the known characteristics of the system, as well as the theoretical chemistry with regard to the control of its over-all velocity in relation to various factors. These factors, here studied experimentally wholly with bacteria, *Photobacterium phosphoreum* in particular, include pH, temperature, pressure, and the drugs sulfanilamide, urethane, and alcohol, separately and in relation to each other. Under steady state conditions of bacterial luminescence, with excess of oxidizable substrate and with oxygen not limiting, the data indicate that the chief effects of these agents center around the pace setting reactions, which may be designated by the equation:

$$A + LH_2 \rightarrow ALH_2$$

following which light emission is assumed proportional to the amount of the excited molecule, AL*.

The relation between pH and luminescence intensity varies with (*a*), the buffer mixture and concentration, (*b*), the temperature, and (*c*), the hydrostatic pressure.

At an optimum temperature for luminescence of about 22° C. in *P. phosphoreum*, the effects of increasing or decreasing the hydrogen ion concentration are largely reversible over the range between pH 3.6 and pH 8.8. The relation between luminescence intensity and pH, under the experimental conditions employed, is given by the following equation, in which I_1 represents the maximum intensity, occurring about pH 6.5; I_2 the intensity at any other given pH; K_5 the equilibrium constant between hydrogen ions and the AL^-; and K_6 the corresponding constant with respect to hydroxyl ions:

$$\left(\frac{I_1}{I_2} - 1\right) = K_5\,(H^+) + K_6\,(OH^-)$$

The value of K_5, as indicated by the data, amounts to 4.84×10^4, while that of K_6 amounts to 4.8×10^5.

Beyond the range between approximately pH 3.8 and 8.8, destructive effects of the hydrogen and hydroxyl ions, respectively, were increasingly apparent. By raising the temperature above the optimum, the destructive effects were apparent at all pH, and the intensity of the luminescence diminished logarithmically with time. With respect to pH, the rate of destruction of the light-emitting system at temperatures above the optimum was slowest between pH 6.5 and 7.0, and increased rapidly with more acid or more alkaline reactions of the medium.

The reversible effects of slightly acid pH vary with the temperature in the manner of an inhibitor (Type I) that acts independently of the normal, reversible denaturation equilibrium (K_1) of the enzyme. The per cent inhibition caused by a given acid pH in relation to the luminescence intensity at optimum pH, is much greater at low temperatures, and decreases as the temperature is raised towards the optimum temperature. The observed maximum intensity of luminescence is thus shifted to slightly higher temperatures by increase in (H^+).

The apparent activation energy of luminescence is increased by a decrease in pH. The value of $\Delta H\ddagger$ at pH 5.05 was calculated to be 40,900 calories, in comparison with 20,700 at a pH of 6.92. The difference of 20,200 is taken to represent an estimate of the heat of ionization of ALH in the activation process, and compares roughtly with the 14,000 calories estimated for the same process, by analyzing the data from the point of view of hydrogen ions as an inhibitor. The decreasing temperature coefficient for luminescence in proceeding from low temperatures towards the optimum is accounted for in part by the greater degree of ionization of ALH.

At the optimum temperature and acid reactions, pressures up to about 500 atmospheres retard the velocity of the luminescent oxidation. At the same temperature, with decrease in hydrogen ion concentration, the pressure effect is much less, indicating a considerable volume increase in the process of ionization and activation.

In the extremely alkaline range, beyond pH 9, luminescence is greatly reduced, as compared with the intensity at neutrality, and under these conditions pressure causes a pronounced increase in intensity, presumably by acting upon the reversible denaturation equilibrium of the protein enzyme, A.

Sulfanilamide, in neutral solutions, acts on luminescence in a manner very much resembling that of hydrogen ions at acidities between pH 4.0 and pH 6.5. Like the hydrogen ion equilibrium, the sulfanilamide equilibrium involves a ratio of approximately one inhibitor molecule to one enzyme molecule. The heat of reaction amounts to about 11,600 calories or more in a reversible combination that evidently evolves heat. Like the action of H ions, sulfanilamide causes a slight shifting of maximum luminescence intensity in the direction of higher temperatures, and an increase in the energy of activation.

The effect of sulfanilamide on the growth of broth cultures of eight species of luminous bacteria indicates that there is no regular relationship among the different organisms between the concentration of the drug that prevents growth, and that which prevents luminescence in the cells which develop in the presence of sulfanilamide. *p*-Aminobenzoic acid (PAB) antagonizes the sulfanilamide inhibition of growth in luminous bacteria, and the cultures that develop are luminous. When (PAB) is added to cells from fully developed cultures, it has no effect on luminescence, or causes a slight inhibition, depending on the concentration. With luminescence partly inhibited by sulfanilamide, the addition of PAB has no effect, or has an inhibitory effect which adds to that caused by sulfanilamide. Two different, though possibly related, enzyme systems thus appear to limit growth and luminescence, respectively. The possible mechanism through which both the inhibitions and the antagonism take place is discussed.

The irreversible destruction of the luminescent system at temperatures above that of the maximum luminescence, in a medium of favorable pH to which no inhibitors have been added, proceeds logarithmically with time at both normal and increased hydrostatic pressures. Pressure retards the rate of the destruction, and the analysis of the data indicates that a volume increase of roughly 71 cc. per gm. molecule at 32° C. takes place in going from the normal to the activated state in this reaction. At normal pressure, the rate of destruction has a temperature coefficient of approximately 90,000 calories, or about 20,000 calories more than the heat of reaction in the reversible denaturation equilibrium. The data indicate that the equilibrium and the rate process are

two distinct reactions. The equation for luminescence intensity, taking into account both the reversible and irreversible phases of the reaction is given below. In the equation b is a proportionality constant; k' the rate constant of the luminescent reaction; A_0 the total luciferase; A_{0i} the total initial luciferase at time t equals 0; k_n the rate constant for the destruction of the native, active form of the enzyme; k_d the rate constant for the destruction of the reversibly denatured, inactive form; t the time; and the other symbols are as indicated above:

$$I = \frac{bk'(LH)(A_0)}{1 + K_1} = \frac{bk'(LH)(A_{0i})}{1 + K_1} e^{-(k_n + k_d K_1/1+K_1)t}$$

For reasons cited in the text, k_n evidently equals k_d.

Urethane and alcohol, respectively, act in a manner (Type II) that promotes the breaking of the type of bonds broken in both the reversible and irreversible reactions and so promotes the irreversible denaturation. This result is in contrast to the effects of sulfanilamide, which at appropriate concentrations may give rise to the same initial inhibition as that caused by urethane, but remains constant with time.

The inhibition caused by urethane and alcohol, respectively, increases as the temperature is raised. As a result, the apparent optimum is shifted to lower temperatures, and the activation energy for the over-all process of luminescence diminishes. An analysis for the approximate heat of reaction in the equilibrium between these drugs and the enzyme, indicates 65,000 calories for urethane, and 37,000 for alcohol. A similar analysis with respect to the effect of hydroxyl ions as the inhibitor gives 60,300 calories.

The effects of alcohol and urethane are sensitive to hydrostatic pressure. Moderate inhibitions at optimum temperature and pH, caused by relatively small concentrations of either drug, are completely abolished by pressures of 3,000 to 4,000 pounds per square inch.

At optimum temperature and pH, increasing concentrations of alcohol caused the apparent optimum pressure for luminescence to shift markedly in the direction of higher pressures. Analysis of the data with respect to concentration of alcohol at different pressures indicated that the ratio of alcohol to enzyme molecules amounted to approximately 4, at 7,000 pounds, but only about 2.8 at normal pressures. This phenomenon was taken to indicate that more than one equilibrium is established between the alcohol and the protein. A similar interpretation was suggested in connection with the fact that analysis of the relation between concentration of urethane and amount of inhibition at different temperatures also indicated a ratio of urethane to enzyme molecules that increased with temperature in the equilibria involved.

Analysis of the data with respect to pressure and the inhibition caused by a given concentration of alcohol at different temperatures indicated that the

volume change involved in the combination of alcohol with the enzyme must be very small, while the actual effect of pressure is apparently mediated through the reversible denaturation of the protein enzyme, which is promoted by alcohol, urethane, and drugs of similar type.

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