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THE MECHANISM OF BIOLUMINESCENCE

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Bioluminescent organisms offer unexcelled opportunities for an experimental attack on mechanisms of energy liberation and utilization in biological systems. Although the production of light by living organisms is of widespread occurrence, an actual demonstration of the essential components of the chemical systems involved has been made in members of only five of some twenty-one groups of luminous forms which have so far been investigated.[1] From these five an enzyme, luciferase, and its substrate, luciferin, have been separated. Although similar substances have not been demonstrated in other organisms, such as luminous bacteria, it is assumed that the basic reactions of all bioluminescence involves an enzymatic reaction, in the presence of oxygen, between a "luciferin" and its complementary enzyme, a "luciferase." Only from the crustacean, *Cypridina hilgendorfii*, have quantities of partially purified components of the luminescent reaction been isolated. The detailed study of the nature of the luminescent reaction itself has been limited to this isolated system. Consequently the recent quantitative formulation of inhibitor action in luminous bacteria[2] has rested upon the transfer of the properties of the demonstrated luciferin-luciferase system of *Cypridina* to the bacterial luminescent system. The general importance of this inhibitor theory is further reason for making every effort to understand more thoroughly the mechanism of light production.

Properties of the Cypridina Luminescent System.—Any mechanism for the bioluminescent reaction in *Cypridina* must be compatible with the following experimentally determined properties of this system.

(*a*) Luciferin undergoes two oxidative reactions. One of these is a reversible non-luminescent oxidation-reduction with an E_0' of + 0.26 v. at pH 7.0.[3] The second is the following irreversible degradation of the side chain on the luciferin molecule:

$$RCOCH_2OH \rightarrow RCOOH^4. \quad (1)$$

The latter is involved in the luminescent reaction proper and accounts for the over-all irreversibility in the isolated system.

(*b*) Both the light-producing and the non-luminescent reactions show the same series of changes in the absorption spectra. The first change observed is a shift in an absorption maximum at 430 mμ to 470 mμ. This is followed by a disappearance of the 470 mμ band. These changes occur over 100 times more rapidly in the luminescent than in the non-luminescent oxidation.[5]

(*c*) Luciferin contains a ketohydroxy side chain and a hydroquinone ring system,[4] probably in either the anthraquinone or naphthaquinone series.[5] Nitrogen, sulfur and halogen are absent from the molecule.[4]

(*d*) The emission spectrum of luminescence reveals peaks at 4750 Å. and 5600 Å.[6] These peaks correspond to energies of 59,400 and 50,000 cal.

(*e*) There is a release of phosphate during the luminescent reaction. This is correlated with the presence of acid-labile phosphate in the luciferin preparation.[7]

Except for point (*e*) the properties here listed have been known for some time. Nevertheless, as will now be shown, no over-all reaction scheme has yet been proposed as the explanation of the bioluminescent phenomenon which is consistent with all these properties. Following an analysis of the various schemes, we shall propose a hypothesis which not only is in agreement with all known properties, but which promises to be of service in devising new attacks on the problem.

Kinetic Interpretation of Bioluminescence.—Numerous studies on the kinetics of the bioluminescent reaction have led to the formulation of the following mechanism:[8]

$$LH_2 \text{ (luciferin)} + A \text{ (luciferase)} \rightarrow ALH_2 \quad (2)$$

$$ALH_2 + {}^1/_2O_2 \rightarrow ALH_2O \quad (3)$$

$$ALH_2O \rightarrow A' \text{ (excited luciferase)} + L \text{ (oxidized luciferin)} + H_2O \quad (4)$$

$$A' \rightarrow A + h\nu \quad (5)$$

The essential feature of this scheme consists in the combination of luciferase and luciferin (E_0' + 0.26, pH 7.0) followed by a direct transfer of two hydrogens to oxygen. In such a transfer the over-all free energy change is approximately 24,000 cal. This is insufficient to account for the energy release on luminescence as demanded by the emission spectrum data (point (*d*)). Inasmuch as this scheme is based upon the analysis of the light emitting step, it fails to consider the possible occurrence of "dark reactions" leading up to the final emission of light. Therefore it is not surprising that

the points enumerated under (*a*), (*b*), (*c*) and (*e*) also remain unexplained by this scheme.

Johnson and Eyring[9] have attempted an elucidation of the nature of the luciferin molecule and the transformations undergone in the luminescent reaction. According to them "luciferin apparently contains both coenzyme (I or II) and a flavine prosthetic group, the former component providing a reductant, and the latter, after loose combination with its specific protein, comprising molecules excitable by oxidation." This hypothesis demands that the luciferin molecule possess a flavin component as an essential part. Contrary to Johnson and Eyring's observations, the chemical data do not support this assumption. First, it has been demonstrated that doubly purified luciferin does not contain nitrogen.[10] Second, independent of the nitrogen analysis, the flavin hypothesis is not in agreement with the spectroscopic data.[5] The three absorption peaks normally associated with known flavins are not to be found in the luciferin absorption spectrum. Further, it must be stressed that luciferin is initially in the reduced state (*a*), whereas the absorption bands of the flavin are characteristic of the oxidized molecule. Thus in the luminescent reaction the yellow color of luciferin disappears on oxidation rather than appearing (*b*) as would be expected if the substance were a flavin. Actually the absorption spectrum of luciferin shows a marked qualitative resemblance to certain naphtha- and anthraquinone derivatives.[5] Third, both Anderson and Korr place the redox potential near the hydroquinone-quinone system,[3] a value considerably more positive than those of known flavin systems. These data taken as a whole appear to invalidate the flavin hypothesis.[11]

An explanation of the irreversibility of the luminescent reaction was also proposed by the same authors.[9] According to their scheme "the excited molecules radiate and are not destroyed but others failing to radiate are destroyed by their absorbed energy." Regardless of the specific structure of the luciferin molecule, their hypothesis, nevertheless, implies that those molecules which emit light are reducible to luciferin again by simple reduction. But such a reversible oxidation is not experimentally demonstrable, and it has been shown that the luminescent reaction involves an irreversible degradation (Eq. 1).

Proposed "Dark Reactions" in the Luminescent Reaction.—The direct oxidation of two hydrogen atoms of glucose makes available 57,340 cal.;[9] however, from what is known of stepwise oxidation in biological systems it is doubtful that such a direct energy release is available for light emission. This essential energy requirement may well be provided by preceding "dark reactions" involving the degradation of the ketohydroxy side chain (Eq. 1) which is an essential step in the luminescent reaction. The pathway of this degradation may be postulated, in its simplest form, to occur by the following reaction steps (R represents the ring structure):

$$RCOCH_2OH \rightarrow RCOCHO + 2H \quad (6)$$

$$RCOCHO + H_2O \rightarrow RCOCOOH + 2H \quad (7)$$

$$RCOCOOH \rightarrow RCHO + CO_2 \quad (8)$$

$$RCHO + H_2O \rightarrow RCOOH + 2H \quad (9)$$

The removal of the hydrogen on the ring structure is probably the last of a series of reactions which make the light emitting molecule "energy rich." Calculations on the basis of analogous reactions which occur in cell oxidation[12] reveal that the energy release in the postulated series of reactions can be sufficient to provide the additional energy necessary for light emission.

This postulated mechanism is in agreement with available chemical information about the luciferin molecule (*c*) and provides an explanation of the irreversible nature of the luminescent reaction *in vitro* (*a*). At the present time there is insufficient knowledge of the structure of luciferin to make possible detailed interpretation of the absorption spectrum. However, the general nature of the luminescent and non-luminescent reactions as divulged by these spectral measurements does not contravert this mechanism. As has been pointed out earlier, the reversible oxidation of luciferin is presumably an oxidation-reduction of the ring structure.[3]

The absorption spectrum data suggest that the luminescent and non-luminescent reactions go through the same reaction chain. The question then arises as to how the energy from the side-chain oxidation is preserved to satisfy the energy requirements for light emission. From what is known of energy transformations in biological systems one might suspect that phosphate is concerned. If this is the case, the difference between the luminescent and the non-luminescent reactions would be that between phosphorolysis and hydrolysis. In the former the energy would be preserved as phosphate bond energy[13] whereas in the latter the energy would be lost as heat. The luminescent reaction here postulated is essentially a reversal of the reactions which have been proposed to take place in photosynthesis.[14] With such a mechanism, equations (3) to (5) of the kinetic scheme would be valid. The postulated series of reactions would merely replace equation (2).

Release of Phosphate on Luminescence.—Assuming the above mechanism, luciferin preparations might well contain measurable quantities of some "energy rich" phosphorylated molecules. As will now be shown, an acid-labile phosphate is readily detected in the luciferin preparation available to us.[15] Although it is difficult to rule out the possibility that the phosphate released was not actually present as a contaminant, the fact that phosphate is released (*vide infra*) during the luminescent reaction supports the conclusion that luciferin does indeed contain "energy rich" phosphorylated

groups. The experimental data upon which these remarks are based may be presented as follows:

(1) Acid-labile phosphate. Doubly purified *Cypridina* luciferin, prepared by the method of Anderson,[16] was kindly supplied by Dr. A. M. Chase. One milligram of dry luciferin was dissolved in 15 ml. of 1 *N* hydrochloric acid. Aliquots were hydrolyzed at 100°C. for 7 and 30 minutes. Phosphate was determined by the method of Berenblum and Chain.[17] Inorganic phosphate initially present was 3.5, 3.7 γ/mg. luciferin preparation. This increased to 23.8, 24.5 γ/mg. at 7-minute hydrolysis. On 30-minute hydrolysis 22.4 γ/mg. was found. Hydrolysis under hydrogen for 7 minutes yielded 22.8 γ/mg.

(2) Phosphate release on luminescence. To 1.5 ml. of the above luciferin solution which had been brought to pH 7.4, 0.1 ml. of luciferase solution was added. The reaction was stopped by the addition of trichloroacetic when the major portion of light emission had taken place ($2^1/_2$ minutes). A control was run by adding trichloroacetic acid prior to the addition of the enzyme. The initial inorganic phosphate was found to be 9.4 γ/mg. luciferin preparation. After the luminescent reaction the inorganic phosphate had increased to 18.8 γ/mg. luciferin preparation.

[1] The numerous studies on bioluminescence are discussed by E. Newton Harvey, *Living Light*, Princeton University Press, Princeton, 1940.

[2] See Johnson, F. H., Eyring, H., and Kearns, W., *Arch. Biochem.*, **3**, 1–31 (1943).

[3] Korr, I. M., *Jour. Am. Chem. Soc.*, **58**, 1060 (1936); Anderson, R. S., *Jour. Cell. Comp. Physiol.*, **8**, 261–276 (1936); Harvey, E. N., *Ann. Rev. Biochem.*, **10**, 531–552 (1941).

[4] Chakravorty, P. N., and Ballentine, R., *Jour. Am. Chem. Soc.*, **63**, 2030 (1941).

[5] Chase, A. M., *Jour. Cell. Comp. Physiol.*, **15**, 159–172 (1940); *Jour. Biol. Chem.*, **150**, 433–445 (1943).

[6] Eymers, J. G., and van Schouwenburg, K. L., *Enzymol.*, **1**, 107–119 (1936).

[7] This paper.

[8] Chance, B., Harvey, E. N., Johnson, F., and Milliken, G., *Jour. Cell. Comp. Physiol.*, **15**, 195–215 (1940).

[9] Johnson, F., and Eyring, H., *Jour. Am. Chem. Soc.*, **66**, 848 (1944).

[10] The previously reported analysis for nitrogen[4] was of sufficient sensitivity to detect between 3–5 per cent nitrogen. This would mean that if the luciferin preparation was more than 20 per cent pure, a positive nitrogen test should have been obtained on the above flavin hypothesis.

[11] Data from other sources and unpublished observations on the correlation of flavin and luminescence in living systems would indicate that a flavin is probably concerned in the resynthesis of the luciferin molecule once it has been oxidized during luminescence. Doudoroff, M., *Enzymol.*, **5**, 239–243 (1938); Brooks, G., *Compt. Rend.*, **210**, 228–230 (1940); Ball, E. G., and Ramsdell, P. A., *Jour. Am. Chem. Soc.*, **66**, 1419–1420 (1944).

[12] Kalckar, H. M., *Chem. Rev.*, **28**, 71–178 (1941).

[13] Lipmann, F., *Advances in Enzymology*, Vol. 1, 99–162 (1941).

[14] Ruben, S., *Jour. Am. Chem. Soc.*, **65**, 297–281 (1943); Emerson, R. L., Stauffer, J. F., and Umbreit, W. W., *Am. Jour. Bot.*, **31**, 107–120 (1944).

[15] In testing for acid-labile phosphate in the purified preparations it was assumed that

the organisms were preserved under such conditions that some of the luciferin was partially oxidized and consequently remained "energy rich."

[16] Anderson, R. S., *Jour. Gen. Physiol.*, **19**, 301–305 (1935).

[17] Berenblum, I., and Chain, E., *Biochem. Jour.*, **32**, 295–298 (1938).

ON THE THEORY OF THE TENSION OF AN ELASTIC CYLINDER

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In the accepted theory of the stretching of an elastic cylinder by a uniform traction in the direction of its axis, it is assumed that the cylinder which is granted to be elastically isotropic when the applied traction is zero, remains elastically isotropic after the traction is applied. We do not hold this assumption reasonable and we present here a theory of the traction of an elastic cylinder in which allowance is made for the lack of isotropy caused by the applied traction. Since we have very little definitive experimental knowledge of the effect of stress upon the elastic constants of a medium, our theory must be qualitative rather than quantitative. We take the point of view of our previous note[1] and so do not attempt to relate the energy of deformation to the (remote) unstressed state but, rather, consider merely the change of energy as we pass from any *stressed* position of the medium to a neighboring position in which the stress has been changed by an infinitesimal amount.

Taking our z-axis parallel to the axis of the cylinder, we assume that the stress is a uniform traction parallel to this axis so that the only non-vanishing component of the stress tensor is T_{zz} and we shall denote this simply by T. When T is changed to $T + \delta T$ any point (x, y, z) of the cylinder will be displaced to $(\xi, \eta, \zeta) = (x + \delta x, y + \delta y, z + \delta z)$. We follow the classical theory in assuming that δx is a function of x alone, that δy is a function of y alone and that δz is a function of z alone. This is only a rough approximation to what actually happens, since it implies that straight lines parallel to the axis of the cylinder are deformed into such and that plane cross-sections are deformed into such. As a consequence of this assumption the strain matrix e is diagonal with components $e_{xx} = \partial\delta x/\partial x$, $e_{yy} = \partial\delta y/\partial y$; $e_{zz} = \partial\delta z/\partial z$ (infinitesimals higher than the first order being neglected). We denote by ρ the density of the medium when under traction T and by ϕ the energy of deformation per unit mass when the traction is $T + \delta T$. In order to connect $T + \delta T$ and e we expand $\rho\phi$ as a function of the strain-components as far as terms of the second order. The first order terms in this expansion reduce to Te_{zz} since the stress, when