BACTERIAL BIOLUMINESCENCE - IDENTIFICATION OF FATTY ACID AS PRODUCT, ITS QUANTUM YIELD AND A SUGGESTED MECHANISM

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Summary. By using radioactive decanal the direct transformation of this aldehyde to decanoic acid, with a quantum yield of 0.13, has been demonstrated. A mechanism analogous to that of other better understood bioluminescent reactions is proposed, leading to a product, as yet unisolated from the enzymic reaction, whose fluorescence spectrum is an excellent match for that of the in vivo luminescence.

The extensive examination[1, 2, 3] of the isolated bacterial luminescence system has resulted in the accepted outline shown. We wish to modify it, in accordance with the previous evidence, by suggesting that 'intermediates I and II' in Hastings' terminology[2] are the same enzyme bound $FMNH_2$ moiety.

$$FMNH_2 + \text{enzyme} \rightleftharpoons \text{enzyme} \cdot FMNH_2$$
$$\text{enzyme} \cdot FMNH_2 \xrightarrow{O_2} \text{enzyme} + FMN + H_2O_2$$
$$\text{enzyme} \cdot FMNH_2 + RCHO \rightleftharpoons \text{covalent complex}$$
$$\text{covalent complex} + O_2 \longrightarrow P^* + RCO_2H$$
$$P^* \longrightarrow P + h\nu \; ; \quad P \xrightleftharpoons{-H_2O} FMN$$

A lively controversy has surrounded the attempts to determine whether aldehyde exerts a purely catalytic role[2] or is transformed in the reaction.[4] If the aldehyde reacts, then the simplest product is the corresponding carboxylic acid, perhaps formed via the peracid. The most likely alternative reaction would involve enolistation and oxidation at the α-methylene group. We examined the second alternative fairly carefully, and found no evidence for it. We do not wish to

report these results in detail at present, since we have now established that the acid corresponding to that formed in a normal autoxidation of the aldehyde is the product. Some indication of the nature of the products of the reaction is available.[5]

Since the amount of product in the reaction is restricted to a very low level by the concentrations required, we labelled decanal with tritium at C-2 and thus were able to record the yield with some precision. Although recent work[6] strongly implies that acid is formed stoichiometrically, the direct measurement of the quantum yield with respect to acid formation is necessary before a mechanism can be written. We have suggested a mechanism compatible with observations in this system, analogous to all cases of bioluminescence for which a mechanism is reasonably well established. This mechanism also leads to a product excited state with excellent agreement around pH7 in fluorescence wavelength to that of the in vivo luminescence.

Methods and Results

Partially purified bacterial luciferase was prepared from a strain of Photobacterium phosphoreum, from Torry Research Station, Aberdeen, according to the method of Hastings and Riley.[7] For the quantum yield studies crystalline Achromobacter fischeri luciferase from Sigma Inc., provided the highest purity of enzyme obtainable with sufficient FMN reductase to allow continuous formation of $FMNH_2$ and consequent reaction of virtually all added aldehyde. Decanal obtained from Koch-Light Ltd., was purified as crystalline α-hydroxydecylhydroperoxide (HDHP) by the method of Rieche[8] and gives better results in the light reaction than do solutions of decanal. Aldehyde is generated in situ quantitatively from HDHP as shown in Table 1; HDHP itself is not a substrate for the reaction under anaerobic conditions. An earlier observation[9] that it was more effective than free aldehyde is probably the result of the higher purity of released aldehyde. (See Table) Increasing the concentration of hydrogen peroxide beyond the equivalent amount formed by the rapid dissociation, has no effect on either the rate or yield of light.

Quantum yields were measured against the standard luminol reaction[10] using an E.M.I. 9781B photomultiplier in a light-tight box, the photometer unit of an Aminco-Bowman spectrofluorometer and a Beckman recorder with Disc integrator. A typical reaction solution contained FMN, 4.0×10^{-6}M; NADH 2.38×10^{-3}M, luciferase, 10 n.mole (assuming a molecular weight of 80,000); phosphate buffer, pH 7.0, 0.083 M; [HDHP], 3.11μM. Bioluminescence and fluoresence spectra were

TABLE 1

Substrate	Absorbance at 20 sec.	Absorbance at 60 sec.		Total available decanal	% theoretical
HDHP	1.50	1.61	1.61	2.07×10^{-4} M	100
HDHP	1.50(93%)	1.59(98%)	1.61(100%)	2.07×10^{-4} M	100
decanal	1.25	1.37	1.37	1.76×10^{-4} M	85.6

Available decanal in buffer solution as measured by the absorption of the semicarbazone at 236 nm.

both measured on an Aminco-Bowman spectrophotofluorometer and are uncorrected for photomultiplier or monochromator response. Each spectrum was the average of a least six scans. Fluorescence quantum yields were obtained on the same spectrometer by the method of Parker.[11] The reaction was quenched at various times by injecting 100μl of 1.63×10^{-2} M $NaBH_4$ in 2N NaOH solution. The total light emitted was corrected for the light output in the absence of aldehyde. Blank reactions in which one component, i.e. one of enzyme, FMN, DPNH and HDHP, was omitted in turn, were also carried out. The appropriate substractions were made to either the light yield or acid yield. A further blank in which excess H_2O_2 was added to simulate the results of autoxidation of $FMNH_2$ showed that there was a very small increase in the yield of acid, which was taken into account. [2-^{3}H] - decanal was prepared from decanal by the method of Williams et al[12] and purified as $CH_3(CH_2)_8CH(OH)O_2H$, (HDHP) which had a specific activity of 6.56 m.Ci per mole. Radioactive counting was done on a 6500 A liquid scintillation counter by the method of Bray.[13]

The acid was isolated by adding a known amount of decanoic acid (BDH, 99% pure) to the reaction mixture before working up, and crystallising (three times) to constant activity as the S-Benzylisothiouronium salt. We had previously shown that exchange did not occur at C-2 in either the aldehyde or acid under the conditions of the experiment by performing the experiment with decanal in T_2O and isolating acid and unreacted decanal.

The quantum yield of fluorescence of 1-ribityl-2-oxo-3-carbomethoxy-1, 2-dihydro-6, 7-dimethylquinoxaline[14] (I) was measured in pure DMSO at 490 nm as 0.08 against quinine sulphate. Figure 2 shows the comparison between this spectrum

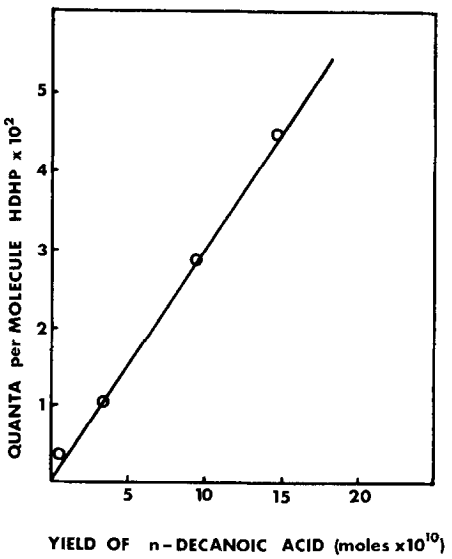


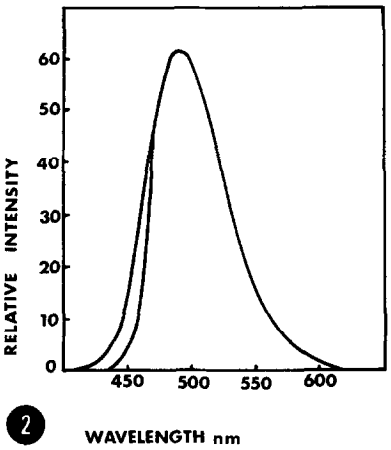
Fig. 1.

and that of an oxygenated suspension of luminescing P. phosphoreum. We have not as yet obtained the presumed emitter itself, but have established that substitution of the urea moiety in (I) by methoxyl has no significant effect on the fluoresence spectrum in related compounds, as was expected.

Discussion

By analogy with examples of efficient chemiluminescence, two reasonable general mechanisms for the bacterial system must be considered. Electron transfer chemiluminescence is not normally effective in aqueous solution, although recently[15] it has been shown that aldehydes of a particular structure apparently luminesce by this route. However, simple aliphatic aldehydes are unlikely sources of luminescence by this mechanism, since formation of an aliphatic acid in an excited state would require an impossibly high energy. Excitation of a flavin cation by electron abstraction by oxygen or a peracid from a flavin semiquinone would not yield sufficient energy (about 65 k.cal per mole). Peroxylauric acid in place of dodecanal gave no measurable light in the reaction.

A more likely consequence of the reduction of FMN is that the resulting $FMNH_2$ is now an effective nucleophile. Thus the second reasonable mechanism will involve nucleophilic attack by $FMNH_2$ on the aldehyde, and chemiluminescence by autoxidation and bond fragmentation. There are now more examples of this class of oxidative chemiluminescence than any other.[16] The fate



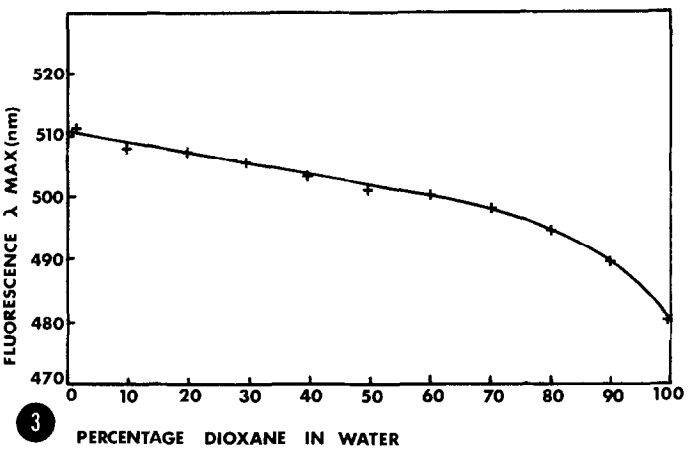


Figure 2

Comparison between the luminescence of whole cells of P. phosphoreum and the fluorescence of I (broader curve) in DMSO. The photomultiplier was adjusted sot that the intensities were equal.

Figure 3

The variation in λ max. of I in dioxan with increasing concentration of added H_2O.

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of aldehyde must be known with certainty before a mechanism may be proposed. By the method described we have obtained conclusive evidence that the quantum yield of the reaction is linearly related to the yield of acid. We also minimised errors owing to trivial autoxidation during work-up and long reaction times. The slope of the straight line in figure 1 gives this quantum yield as 0.126 ± 0.023. Our particular enzyme preparation required 30 min. for complete exhaustion of the aldehyde used, and later points were unreliable owing to varying amounts of autoxidation unrelated to the light reaction. Since the quantum yield with respect to FMN is about 30,[17] any proposed mechanism must allow for regeneration of FMN. The one we suggest in Figure 4 has ample analogy in simpler reactions of enamines and aldehydes, and is in every way acceptable as a probable organic mechanism. The equation shown predicts that compound (I, R^1=D-ribityl phosphate, R^3=NH.CO.NH_2) should fluoresce around 490 nm in the usual solvents at neutral pH. This is in fact, the wavelength observed for (I, R^1=D-ribityl, R^3=OCH_3) in DMSO, and the agreement in shape between the curves for fluorescence and bioluminescence is rather good (Figure 2). By using varying amounts of water in dioxan, the whole range of emission maxima from around 480 nm to 510 nm observed in bacterial luminescence can be

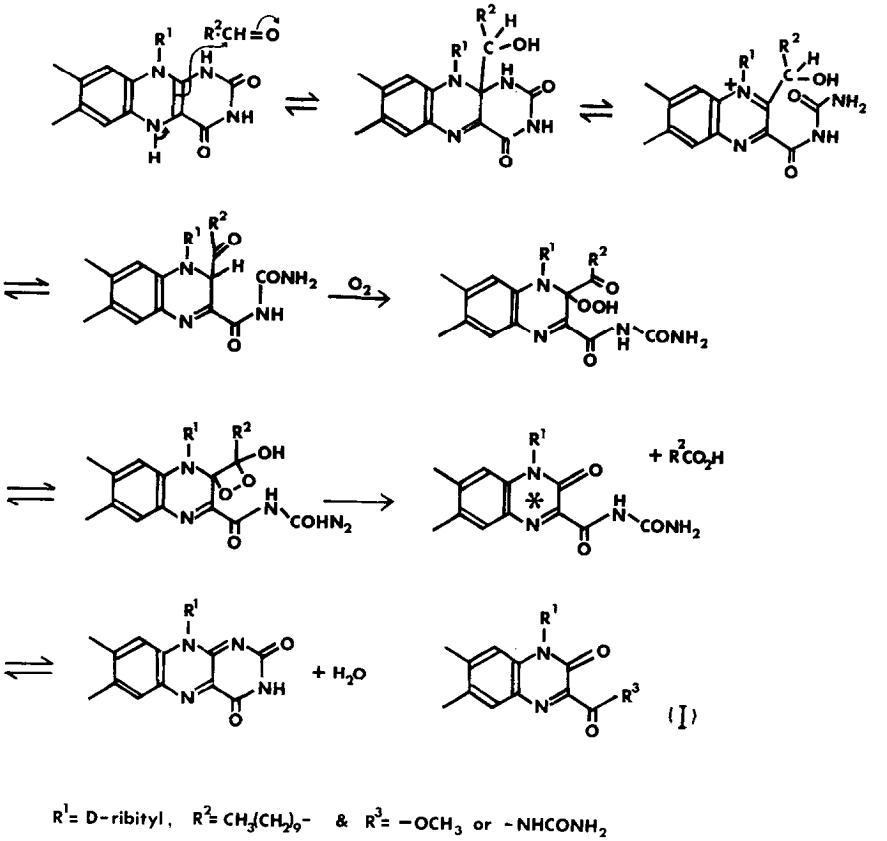

Fig. 4.

obtained, (see Figure 3). However, the minimum quantum yield allowable in the proposed emitter must be greater than 0.126. We find a value of 0.08 in DMSO. If we accept a recent value[6] of 0.17 for the bioluminescence quantum yield with respect to aldehyde, then the discrepancy is greater. Nevertheless, since enhancements of fluorescence efficiency on binding to an enzyme often greatly exceed the modest 3-fold increase required here,[18] we feel that the mechanism and product suggested must be considered seriously. We realise that the absolute value of the bioluminescence quantum yield will vary with the preparation of the enzyme used, but the method described provides conclusive evidence of the direct relationship between light emission and yield of acid.

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