ON THE FUNCTION OF ALDEHYDE IN BACTERIAL BIOLUMINESCENCE: EVIDENCE FOR AN ALDEHYDE REQUIREMENT DURING LUMINESCENCE FROM THE FROZEN STATE

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Since the initial discovery that long chain aldehydes were required for bacterial bioluminescence (Cormier and Strehler, 1953) numerous attempts to define the role of aldehyde have been made. Quantum yield measurements by Cormier and Totter (1957) and independent studies by McElroy and Green (1955) suggest that aldehyde is utilized by an FMN-dependent pathway during luminescence. On the other hand Strehler (1961) suggested that aldehyde may cycle in the reaction.

More recently, Hastings and Gibson (1963) have presented evidence for several intermediates during the oxidation of $FMNH_2$ by luciferase isolated from *Ph. fischeri*. These may be represented as follows:

$$\text{Enzyme} + FMNH_2 \longrightarrow \text{reduced enzyme (I)} \quad (1)$$

$$I + O_2 \longrightarrow \text{long-lived intermediate (II)} \quad (2)$$

$$II + RCHO \longrightarrow \text{enzyme} + \text{light} \quad (3)$$

$$II \longrightarrow \text{enzyme} + \text{heat} \quad (4)$$

Hastings, *et al.* (1966) have proposed that intermediate II decays in the presence of aldehyde, to give a luminescence of high quantum yield and that, in the absence of aldehyde, it decays to produce a luminescence of low quantum yield The aldehyde is regarded as exerting an effect on protein conformation rather than participating in the chemistry involved. Most of this notion was derived from observations by Hastings, *et al.* (1964) on bacterial bioluminescence from the frozen state. They found that upon initiating the reaction with $FMNH_2$ in the liquid state at 5°C, followed by quick freezing to 77°K, light is emitted, upon warming, at a characteristic temperature (-3 to -9°C) which is below the melting point of ice, and that the color of the light is essentially the same as that from solution. Furthermore, they found that although a 70-fold increase in quantum yield occurs in the presence of aldehyde at 21°C, this difference is minimized to within a factor of 3 in the frozen state.

If aldehyde is not required for luminescence *per se* then the above findings would represent a big advance in our understanding of the role of aldehyde. We therefore decided to reinvestigate the characteristics of luminescence from the

frozen state using luciferase preparations isolated from *Ph. phosphoreum* and *Ph. fischeri*.

METHODS

The organisms used in these studies were *Photobacterium fischeri* (ATCC No. 7744) and a strain of *Photobacterium phosphoreum* acquired from Dr. W. Terpstra. Both organisms were cultured in a medium containing per liter of deionized water: 5 gm. yeast extract; 5 gm. tryptone; 30 gm. NaCl; 6.5 gm. Na_2HPO_6; 0.73 gm. KH_2PO_4; 3 ml. glycerol; 1.5 ml. of Dow Corning Antifoam B. The pH of the media was adjusted to 7.0. The cells were cultured in a 100 gallon fermenter and harvested with a large refrigerated Sharples centrifuge. Acetone powders were prepared from the cells as previously described (Strehler and Cormier, 1953).

Luciferase was isolated from *Ph. fischeri* by slight modifications of the method described by Kuwabara, *et al.* (1965). These modifications included adsorption and elution from calcium phosphate gel, following the initial extraction, and chromatography through Biogel P-60 instead of through DEAE. Preparations of luciferase from lysed cells of *Ph. fischeri* were also prepared by the method of Hastings, *et al.* (1965). Luciferase from *Ph. phosphoreum* was prepared by the method of Eley, *et al.* (to be published).

Luciferase preparations from *Ph. fischeri* varied in specific activity from 5×10^{13} $h\nu$ $sec^{-1}mg^{-1}$ to 1×10^{14} $h\nu$ $sec^{-1}mg^{-1}$ whereas that from *Ph. phosphoreum* had a specific activity of 4×10^{12} $h\nu$ $sec^{-1}mg^{-1}$ which represents 35-fold purified enzyme. The 280/260 absorbancy ratio of the latter was 1.72.

Luminescence was measured with an apparatus previously described (Cormier, 1960) or with a MacNichol photomultiplier photometer (Chase, 1963). Luminescence units are expressed as quanta sec^{-1} or total quanta by use of light standards as described by Hastings and Weber (1963).

Luciferase from *Ph. phosphoreum* was routinely assayed at 25°C and the assay components consisted of: enzyme, 0.05 ml.; BSA, 1.2 mg.; 0.01 M Na_2HPO_4-KH_2PO_4 buffer, pH 7.0; dodecanal, 0.25 ml. of a saturated solution; and $FMNH_2$, 1.5×10^{-5} M. The final volume was 1.2 ml. and the luminescence was initiated by injecting $FMNH_2$ into an otherwise complete system. The assay for *Ph. fischeri* luciferase was the same as that above with the exception that a final concentration of 1×10^{-3} M dithiothreitol was incorporated into the assay. Experiments involving bioluminescence from the frozen state were performed using the method of Hastings, *et al.* (1964).

Dodecanal, obtained from K & K Laboratories, Plainview, New York, was further purified by gas chromatography prior to its use in these experiments.

RESULTS

Measurements were made of the total quanta produced at 25°C upon initiating

the reaction with $FMNH_2$. Similar measurements were made by initiating the reaction with $FMNH_2$ at 5°C followed by rapid freezing in liquid nitrogen to 77°K as described by Hastings, *et al.* (1964). The frozen mixture was then placed in front of a photomultiplier at room temperature and as the mixture gradually warmed the total quanta emitted from the frozen state were determined. The results of such experiments are shown in Table I in which case luciferase isolated from *Ph. phosphoreum* was used. It can be seen from Table I (experiments A and B) that the quantum yield both at 25°C and in the frozen state is highly dependent upon aldehyde. In each case the quantum yield is increased approximately 1000-fold by the addition of aldehyde. This is probably related to the fact that luciferase preparations from *Ph. phosphoreum* routinely have very low blanks, *i.e.* very little light is produced in the absence of added aldehyde. In fact with some preparations, quantum yields have been increased 10,000-fold by the addition of aldehyde.

Also shown in Table I (experiments C and D) is an experiment in which the level of aldehyde was adjusted such that luciferase was 5 per cent saturated according to initial rate measurements. The final concentration of aldehyde was 5×10^{-7} M. When such a concentration of aldehyde is added to the assay, the quantum yield at 25°C is increased only by a factor of 5 over that of the "without aldehyde" blank. However, in the frozen state a relatively large

TABLE I

Effect of dodecanal on the total quanta produced at 25° and in the frozen state by *Ph. phosphoreum* luciferase

Experiment	Conditions	Without Aldehyde	With Aldehyde	With Aldehyde (5×10^{-7}M)
A	25°C	1.2×10^{8}	1.0×10^{11}	
		1.0×10^{8}	0.8×10^{11}	
		1.2×10^{8}	1.2×10^{11}	
B	Frozen	5.6×10^{8}	5.2×10^{11}	
		9.5×10^{8}	5.7×10^{11}	
		6.3×10^{8}	5.4×10^{11}	
C	25°C			5.8×10^{8}
				6.0×10^{8}
D	Frozen			2.4×10^{10}
				2.6×10^{10}

The numbers above correspond to individual determinations and each experiment was done in triplicate or duplicate as indicated.

increase (35 fold) in quantum yield occurs over that of the "without aldehyde" blank. A similar phenomenon is observed using luciferase preparations prepared from *Ph. fischeri*.

Table II shows the results of experiments that are analogous to those reported in Table I except that luciferase isolated from *Ph. fischeri* was used The results in Table II indicate that the dependence of the quantum yield on aldehyde concentration from the frozen state is determined by the starting material from which luciferase is isolated. For example, if one uses relatively fresh cells, lyses them, and prepares luciferase, results are obtained equivalent to those reported by Hastings, *et al.* (1964). However, if cells are used that have been kept at -20°C for three years, a greater dependence of quantum yield upon aldehyde concentration is observed both at 25°C and in the frozen state. Analogous results are obtained when acetone powders of fresh cells are the source of luciferase as shown in Table II.

From our own data, as well as that reported by Hastings, *et al.* (1964), it appears that the higher the blank (aldehyde ?) the less the observed dependence of quantum yield on added aldehyde both at 25°C and in the frozen

TABLE II

Effect of dodecanal on the total quanta produced at 25° and in the frozen state by *Ph. fischeri* luciferase

Conditions	Without Aldehyde	With Aldehyde
Luciferase from lysed cells, 3 years old:		
25°C	1.3×10^9	4.4×10^{11}
	1.4×10^9	3.8×10^{11}
	1.3×10^9	3.4×10^{11}
Frozen	1.6×10^9	0.8×10^{11}
	1.7×10^9	1.0×10^{11}
	2.4×10^9	1.2×10^{11}
Luciferase from acetone powders:		
25°C (average of 3 determinations)	3.2×10^{12}	5.7×10^{14}
Frozen (average of 3 determinations)	3.1×10^{13}	7.9×10^{14}

Except where indicated the numbers above correspond to individual determinations and each experiment was done in triplicate.

state. This relationship is shown in Figure 1. In Figure 1, we have plotted the ratio of the observed quantum yield with aldehyde added to that without aldehyde added in the frozen state *vs.* a similar ratio determined at 25°C. Points a and b represent luciferase preparations from old lysed cells and fresh acetone powders respectively and the data is taken from Table II. Point c represents data taken from Hastings, *et al.* (1964). Note in Figure 1 that there is a direct relationship between the level of the blank, determined at 25°C, and that of the aldehyde dependence on the quantum yield from the frozen state. In fact the extrapolated line suggests that if one prepared luciferase with a quantum yield ratio at 25°C of about 70, one would find an aldehyde independent quantum yield in the frozen state.

There is an additional unexplained factor which tends to reduce the dependence of quantum yield on aldehyde when using luciferase prepared from lysed cells. In the presence of aldehyde, the quantum yield in the frozen state is less than that at 25°C by a factor of about 3. This is not true when using acetone powders as the source material.

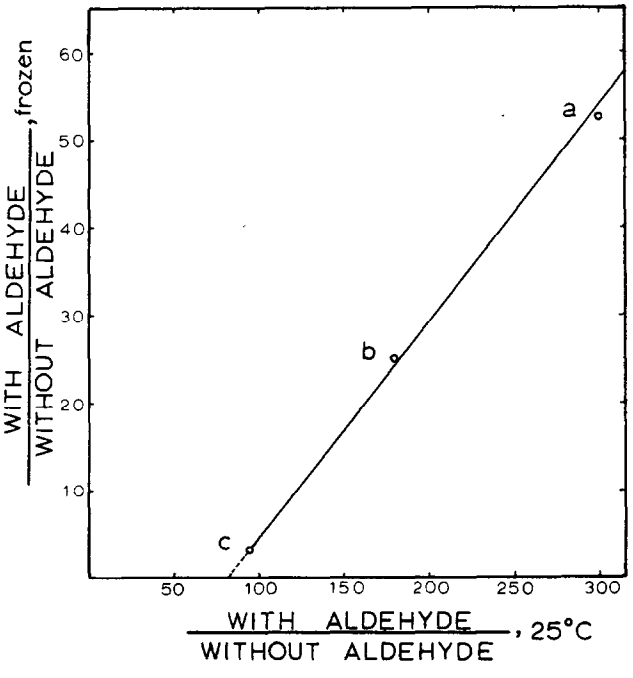

Figure 1. Relationship of the quantum yield ratios (with aldehyde/without aldehyde) at 25°C and in the frozen state to the type of luciferase preparation used. Point a refers to luciferase isolated from cells that were stored for 3 years at -20°C and then lysed. Point b refers to luciferase isolated from fresh acetone powders while point c refers to data reported by Hastings, *et al.* (1964).

DISCUSSION

The evidence presented shows that the dependence of the quantum yield on aldehyde is related to the level of the natural "aldehyde" blank in the luciferase preparations. The greater the level of this presumably natural aldehyde, the less the dependence of the quantum yield upon added long chain aldehydes. This effect we believe is due to reactant puddling in the frozen state (Wang, 1965). We base this proposal on data in Table I (experiments C and D) and on the fact that we have observed reactant puddling upon freezing in a -15°C deep-freeze by following the rate of formation of Shiff's bases between dodecanal and various compounds. Under these conditions, the Shiff's base forms much more readily and efficiently than it does at 25°C.

It has been difficult to isolate the natural aldehyde from luminous bacteria (Hastings, *et al.*, 1964). However, if Strehler (1961) is correct - that aldehyde functions catalytically - then it may be synthesized in very small amounts and may never be leached out of the cells due to binding to proteins, autooxidation, etc. In fact Rogers and McElroy (1955), working with aldehyde-less mutants of *Eh. fischeri*, have presented evidence which suggest that protein-bound aldehyde is released upon irradiation with ultra-violet light.

We suggest that aldehyde is not involved in changing the conformation of luciferase, for which there is no direct evidence, but that its role may be that of forming part of the emitting complex. This proposal is based on model experiments with aldehydes to be reported elsewhere. The finding that 2-decenal is a competitive inhibitor of the light reaction (Spudich and Hastings, 1963) is also consistent with this view.

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