ISOLATION, IDENTIFICATION, AND FUNCTION OF LONG CHAIN FATTY ALDEHYDES AFFECTING THE BACTERIAL LUCIFERIN-LUCIFERASE REACTION*

BY BERNARD L. STREHLER† AND MILTON J. CORMIER
(From the Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee)
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Although luminous bacteria are among the most accessible of light-producing organisms, until recently attempts to obtain the light-producing reaction in vitro have been unsuccessful. Owing partly to the development of ultrasensitive light-detecting apparatus such as the quantum counter (1), it has been possible in our laboratory to develop procedures required to obtain a sustained bright luminescence from extracts of the luminous bacterium Achromobacter fischeri and from the other nine strains of luminous bacteria tested (2). Since it was a simple matter to detect the minimal luminescence emitted when an acetonized powder from luminous bacteria was suspended in water, a systematic investigation of the effect of various physical and chemical factors soon led to the experimental production of a bright luminescence. Initially, we found that DPN1 or its reduced homologue was a factor which first became limiting for luminescence (3). Subsequently (4), at least two other factors were found to be necessary for optimal luminescence. One of these compounds is FMN; the other is a factor from hog kidney cortex which we have termed KCF.

McElroy and coworkers have confirmed the finding of a DPNH2 requirement for luminescence and they have also observed a requirement for FMN (5, 6). In addition, they observed that another factor obtained from the bacteria which they have considered analogous to firefly luciferin (7, 8) is necessary for luminescence. Their basis for this terminology was, by analogy to the inactivation of firefly luciferin, apparently the destruction of this heat-stable factor during luminescence. It is not at present clear whether this loss of activity is dependent on light emission or merely on removal by an unknown enzymatic action in the bacterial extracts.

Recently, we have reported in preliminary form (9) the identification of

* Work performed under contract No. W-7405-eng-26 for the Atomic Energy Commission.
† Present address, Institute of Radiobiology and Biophysics, University of Chicago, Chicago, Illinois.
1 The abbreviations used in this paper are as follows: DPN, diphosphopyridine nucleotide; FMN, flavin mononucleotide; KCF, kidney cortex factor; DPNH2, reduced diphosphopyridine nucleotide.
KCF as plasmal (10), or specifically, palmitic aldehyde. This paper describes the purification steps, identification procedures, and enzymology of palmitic and homologous fatty aldehydes in the bacterial luminescent reaction. Evidence will be presented concerning the site of action of these Feulgen-positive components in the sequence of reactions leading to luminescence.

**EXPERIMENTAL**

*Materials and Methods*

The light-measuring equipment consisted of a liquid nitrogen temperature photomultiplier (IP22) operated as a pulse-counting device (1). A convenient sample of the bacterial enzyme prepared as described earlier (4) was diluted 5:1 with phosphate buffer, pH 6 (0.1 m), and DPNH2 and FMN were added in excess. The effect of added factors was determined by the increase in luminescence. 10- to 200-fold increases in luminescence were observed.

DPNH2 and FMN were obtained from the Sigma Chemical Company, St. Louis, Missouri, defatted hog kidney cortex from the Viobin Laboratories, Monticello, Illinois, heptaldehyde from the Eastman Kodak Company, Rochester, New York, and decaldehyde from the Columbia Organic Chemical Corporation, Columbia, South Carolina. Octyl and undecyl aldehydes were prepared by a bichromate oxidation of the respective alcohols and steam distillation of the aldehyde (11). Pelargonic aldehyde was prepared by ozonolysis of oleic acid followed by decomposition with zinc and acetic acid of the ozonide and steam distillation of the aldehyde from the reaction mixture (12). Palmitic aldehyde was prepared from the acid chloride by a Rosenmund reduction (13).

**Results**

*Preliminary Considerations*—The first evidence obtained concerning the necessity of a factor other than DPNH2 for luminescence of bacterial extracts was an early observation in our laboratory that a boiled acetonized powder would markedly increase the luminescence of a preparation that had been "exhausted" by continued luminescence in the presence of excess DPNH2 plus malate. After several hours of luminescence, the enzyme responded only weakly to further additions of DPNH2. At this point the addition of boiled bacterial extract produced a marked increase in luminescence. Even when a non-exhausted preparation was used, a 2- to 4-fold stimulation was obtained by adding boiled bacterial powders.

Certain lines of evidence suggested that this component is not diffusible. Chief among these was the failure of prolonged dialysis (5 days) to prevent a response to DPNH2. This suggested that either no dialyzable factors in
addition to DPNH₂ are required in order for some luminescence to occur or that other diffusible components, if required, are non-dialyzable. Addition of the concentrated dialysate to an exhaustively dialyzed preparation produced no increase in the luminescence.

Attempts were made to fractionate the boiled bacterial extracts in an effort to concentrate the activity therein. These attempts generally failed. Briefly, it was noted that the activity was carried along with the heat-precipitated residue of the boiled extracts, little if any activity remaining in the supernatant solution. Attempts to liberate a factor from this precipitate through 1 N acid digestion at 100° for 1 hour, digestion with papain, rattlesnake venom, ribonuclease, or lipase were unsuccessful.

One of the possibilities at this point was that the activity was due to some unspecific protein effect or to some other factor associated with the débris. To test this possibility a number of tissue extracts were prepared, including extracts of kidney cortex powders. In water extracts of this tissue, a potent stimulatory factor was present in the supernatant solution and in the precipitate. The most active extract was that obtained from hog and beef kidney cortex and beef liver. This supernatant solution was of an order of magnitude more potent than the boiled bacterial powders. The further steps in purification and identification of KCF are detailed in the next section.

Isolation and Identification—Various procedures were attempted during the purification of KCF, including ion exchange, charcoal chromatography, and counter-current distribution. Ion exchange on Dowex 1 and 50 was unsuccessful, owing largely to the elution difficulties encountered. Chromatography on charcoal was more promising. The active component adsorbed well on a charcoal column from a water-methanol solution and was eluted with chloroform with considerable purification. However, an attempted improvement in the chromatographic separation by a preliminary wash composed of equal volumes of methanol and chloroform, which removed much inactive colored material, was unsuccessful. The active material could not be removed from the charcoal with chloroform following the preliminary wash. Finally, a counter-current separation involving a partition between 10 per cent water-methanol and hexane was attempted. The partition coefficient of the crude material between these solvents was 1. However, on repeated partition and development, the activity was found concentrated in the first few tubes of the methanol phase, indicating that the partition coefficient changed during the purification process. Although considerable concentration of the active principle was achieved by this method, the procedure followed in large scale preparations is shown in Diagram 1.

5 kilos of kidney cortex powder were extracted two times with twice its
Kidney cortex powder

\[ \text{Chloroform} \]

Evaporate in presence of hexane

\[ \text{Extract with 10\% H}_2\text{O}-\text{methanol} \]

Hexane Methanol

\[ \text{Acidify, HCl; extract with hexane} \]

Hexane

Concentrate; add acetone until complete pptn.

Ppt. Acetone

Concentrate; add H\textsubscript{2}O, HCl

Ppt. Supernatant solution

Suspend in NaOH; ppt. with H\textsubscript{2}O; dissolve in minimal amount of acetone; cool to \(-20^\circ\)

Ppt. Supernatant solution

Concentrate and cool

Ppt. Supernatant solution

Add chloroform-methanol; concentrate and cool

Ppt. Supernatant solution

Dissolve NaOH; ppt. with HCl; wash with H\textsubscript{2}O

Purified KCF

**Diagram 1**

volume of chloroform. The chloroform was evaporated to a small volume (about 600 ml.) at room temperature in *vacuo* and a small amount of hexane (about 500 ml.) was added. The chloroform was then completely removed
in vacuo. The concentrated extract in hexane was partitioned between an equal volume of methanol (containing 10 per cent water) and hexane through seven counter-current stages with separatory funnels. The activity remained largely in the methanol-water layer, while copious amounts of colored impurities were removed in the hexane layer. The pooled methanol fractions (3 liters total) were acidified with HCl to about pH 2 to 3 and extracted three times with \( \frac{1}{3} \) volume of hexane (total volume about 3 liters).

<table>
<thead>
<tr>
<th>Compound</th>
<th>C</th>
<th>( D_{\text{M.m.p.}} )</th>
<th>( \Sigma ) calculated</th>
<th>M</th>
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<tr>
<td>Heptanal</td>
<td>0.01440</td>
<td>1.035</td>
<td>21,131</td>
<td>294</td>
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<tr>
<td>Nonanal</td>
<td>0.00750</td>
<td>0.486</td>
<td>20,865</td>
<td>322</td>
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<tr>
<td>Palmital</td>
<td>0.00788</td>
<td>0.377</td>
<td>20,093</td>
<td>420</td>
</tr>
<tr>
<td>KCF</td>
<td>0.00794</td>
<td>0.388</td>
<td>20,696†</td>
<td>424‡</td>
</tr>
</tbody>
</table>

\[ \Sigma = \frac{M}{(C \cdot L)D} \]  
\[ C = \text{concentration}, \quad D = \log I/I_0, \quad M = \text{molecular weight}, \quad L = \text{length of absorption cell (1 cm.)}, \quad \Sigma = \text{molecular extinction coefficient}. \quad \lambda_{\text{max.}} = 355 \text{ m} \mu. \]

* Dinitrophenylhydrazine derivative.
† Average of three readings above.
‡ Calculated.

After concentrating the hexane solution to 300 ml., 6 volumes of acetone were added, the inactive precipitate was removed, and the acetone-hexane solution was concentrated to about 50 ml. The active material was precipitated from the acetone solution by adding 6 volumes to 0.1 N HCl. The precipitate was suspended in NaOH and reprecipitated by adding HCl. This precipitate was washed thoroughly with distilled water and dissolved in a minimal amount (about 40 ml.) of hot acetone. On cooling the acetone to \(-20^\circ\), a dark brown inactive precipitate was formed. The solution containing the active material was removed by decantation and was again concentrated to about 20 ml. and cooled. The inactive pre-
The precipitate was again removed. The solution was concentrated to 15 ml., diluted with \( \frac{1}{2} \) volume of chloroform and methanol, concentrated, and cooled, and the active precipitate was collected. This precipitate was suspended in NaOH, precipitated with HCl, and washed repeatedly with water. This light cream-colored material, which produced a 5-fold increase in luminescence at 0.6 \( \gamma \) per ml., was used for the quantitative tests and for the preparation of the derivatives. The total purification of the original chloroform extract was about 300-fold and the yield was 1.5 gm. of purified KCF per 7 kilos of starting material.

A number of preliminary qualitative determinations were made on the most highly purified samples available. No sulfur was detectable. Phosphorus was not present.

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**Fig. 1.** Effect of palmitic aldehyde concentration on luminescence. 0.1 ml. of a 2 per cent aqueous extract of A. fischeri (prepared by extracting 2 gm. of acetonized bacteria with 100 ml. of water and centrifuging as under "Materials and methods") was mixed with 1.5 ml. of 0.1 M phosphate buffer, pH 7.0, 1000 \( \gamma \) of DPNH\(_2\), and 4 \( \gamma \) of FMN, and the light intensity was determined. Palmitic aldehyde was then added as indicated and light intensity measured immediately after each addition. The upper curve shows the palmitic aldehyde concentration plotted against light intensity (upper and left-hand scales), while the lower curve is a plot of the reciprocal of the light intensity (1/V) versus the reciprocal of the molarity (lower and right-hand scales).
phorus was present in small quantities, but its concentration decreased as the purification of the material increased. Nitrogen also was present in very small amounts and decreased with increased purification. On the other hand, a strongly positive fuchsin aldehyde test was given by the crude as well as by the purified preparation, suggesting that the compound in question might be an aldehyde.

In an attempt to use a chemical step in the purification, the 2,4-dinitrophenylhydrazone was prepared (14). Originally, it had been intended to decompose the dinitrophenylhydrazone and thus to remove other non-aldehyde impurities from the isolated material. However, the dinitrophenylhydrazone was a crystalline material, and, after several recrystallizations from hot ethanol-water, gave a sharp melting point of 104–105°. The derivative was orange-yellow in color. On examining the literature for 2,4-dinitrophenylhydrazones of aldehydes (14, 15) that melt in the region 100–105°, it was found that the extrapolation of the melting point curve for the homologous series of aldehydes starting with C\textsubscript{1} and proceeding to C\textsubscript{18} went through a minimum (100°) at C\textsubscript{9} and that thereafter the melting points of the derivatives were approximately 105°. At this point, a sample of heptanal which was available was tested and found to give a lumines-
cence stimulation approximately one-thirtieth of the most highly purified KCF, on a weight basis. A number of other long chain aldehydes were prepared. The C_7, C_8, C_9, C_{10}, C_{11}, and C_{16} derivatives were obtained as indicated above.

The molecular weight of the dinitrophenylhydrazone of KCF was determined by measuring the extinction per unit weight of the derivative and comparing this with the extinction of the dinitrophenylhydrazones of aldehydes of known chain length. The molecular weight found by this means was 424. Elementary analysis data appear in Table I. The synthetic palmitic aldehyde derivative produced no depression of melting point when mixed with the corresponding derivative of the isolated aldehyde.

Effect on Luminescence—The effect of the addition of KCF or synthetic palmitic aldehyde on the luminescence of A. fischeri extract is illustrated quantitatively in Fig. 1. The Michaelis-Menten dissociation constant for palmitic aldehyde and KCF is about $3.6 \times 10^{-6}$ M.

Time-Course of Luminescence on Addition of Palmitic Aldehyde—In order to determine something about the sequence of reaction of the various components required for extract luminescence, the time-course of luminescence
after addition of palmitic aldehyde has been studied by a modification of the techniques used earlier by Chance et al. (16). The time required for a

![Graph of luminescence vs concentration of different aldehydes](image)

**Fig. 4.** Effect of concentration of different aldehydes on luminescence. To 0.1 ml. portions of a 2 per cent aqueous extract of _A. fischeri_ were added 1.5 ml. of 0.1 M phosphate buffer (pH 7.0) + 1000 γ of DPNH₂ + 4 γ of FMN, and the luminescence was measured immediately. The various aldehydes were added as indicated and the light intensity was determined after each addition. A = heptyl aldehyde, B = octyl aldehyde, C = pelargonaldehyde, D = decyl aldehyde, E = undecyl aldehyde, F = palmitic aldehyde.

<table>
<thead>
<tr>
<th>Table II</th>
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<td>Comparison of Apparent Dissociation Constants and Activation Energies in Presence of Various Aldehydes</td>
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</table>

<table>
<thead>
<tr>
<th>Aldehyde</th>
<th>Vₓ (arbitrary)*</th>
<th>Kₓ x 10⁶ M</th>
<th>ΔH†</th>
</tr>
</thead>
<tbody>
<tr>
<td>C₇</td>
<td>28,000</td>
<td>84.0</td>
<td>17,700</td>
</tr>
<tr>
<td>C₈</td>
<td>66,700</td>
<td>20.8</td>
<td>18,000</td>
</tr>
<tr>
<td>C₉</td>
<td>59,000</td>
<td>13.3</td>
<td>17,700</td>
</tr>
<tr>
<td>C₁₀</td>
<td>133,000</td>
<td>4.1</td>
<td>17,150</td>
</tr>
<tr>
<td>C₁₁</td>
<td>52,000</td>
<td>2.36</td>
<td>17,700</td>
</tr>
<tr>
<td>C₁₆</td>
<td>81,000</td>
<td>3.56</td>
<td>29,300</td>
</tr>
</tbody>
</table>

* Maximal luminescence attainable when a particular aldehyde is added.

maximal response following the addition of aldehyde is of the order of 0.2 second (see Fig. 2).
Effect on Respiration—Palmitic aldehyde increased the rate of respiration as well as the luminescence of the extracts at low oxygen tension (below 7.6 mm.). Fig. 3 depicts the effect of oxygen tension on both the luminescence and respiration in the presence and absence of palmitic aldehyde.

Fig. 5. Effect of temperature on the luminescence rate when limiting concentrations of different long chain aldehydes are used. To 0.5 ml. portions of a 2 per cent aqueous extract of _A. fischeri_ in 0.1 M phosphate buffer, pH 7.0, were added 1000 γ of DPNH₂ + 4 γ of FMN + half saturating levels of the aldehyde in question. The mixture was brought to the desired temperature and the luminescence measured immediately. Total volume = 2.3 ml. A = undecyl aldehyde (ΔH‡ = 17,700); B = pelargonaldehyde (ΔH‡ = 17,700); C = octyl aldehyde (ΔH‡ = 14,800); D = heptyl aldehyde (ΔH‡ = 17,200); E = decyl aldehyde (ΔH‡ = 17,200); F = palmitic aldehyde (ΔH‡ = 29,300).

The Michaelis-Menten dissociation constants as derived from the half saturation values are about 2.8 × 10⁻⁶ M in the absence of aldehyde and 0.5 × 10⁻⁶ M in its presence for luminescence, and 6.1 × 10⁻⁶ and 2.0 × 10⁻⁶ M for oxygen consumption under similar conditions.

At higher oxygen tensions the aldehyde does not affect the respiration rate, although it produces a pronounced increase in luminescence. Even at low oxygen tensions the aldehyde produces this effect on luminescence.
Generality of Effect—Nine strains of luminous bacteria, in addition to A. fischeri, kindly furnished by Dr. C. B. van Niel, were tested for their response to KCF. All strains responded to the addition of palmitic aldehyde by emitting a markedly increased luminescence. In one strain, the crude extracts possessed a marked KCF-destroying activity.

Effect of Homologous Compounds—The effect on luminescence of a series of compounds homologous to the 16-carbon aldehyde has been determined and is indicated in Fig. 4. They have been plotted by the Lineweaver-Burk (17) method and the calculated concentration at half saturation is given in Table II. Likewise, the effect of these other aldehydes on the activation energy (apparent) for the luminescent reaction has been measured and is indicated in Fig. 5.

DISCUSSION

Qualitative tests have not demonstrated large amounts of long chain aldehyde in our acetone-dried bacterial powders, and, for this reason, it has not been shown conclusively that this type of compound is actually involved in the luminescence of the intact bacteria. The extremely low concentration of fatty aldehyde required for the activation of the bacterial systems and its long persistence suggest that the aldehyde is acting as a catalytic factor rather than as a stoichiometric reagent. The fact that microgram quantities of aldehyde will sustain luminescence for considerable periods (up to 24 hours) indicates that it is very slowly consumed during luminescence, if at all. Since the aldehyde by itself is incapable of eliciting a luminescent response, and indeed is not a substrate for the DPN-coupled dehydrogenase present in the system, it seems unlikely that it is acting as a hydrogen donor. The close similarity in the time-course of luminescence following addition of oxygen and aldehyde suggests that the aldehyde may be involved in oxygen activation in some manner (see also Michaelis-Menten dissociation constant for oxygen in presence and absence of aldehyde).

From a comparative biochemical point of view, it is interesting that all of the factors required for bacterial extract luminescence thus far, except the enzymes, are obtainable from non-luminescent sources. Flavin mononucleotide and DPN are well known components of respiratory systems, while the long chain fatty aldehyde discovered by Feulgen and his collaborators, apparently in this case, performs the same respiratory function. Whether a similar function is extant in the tissues from which they have been obtained is an unanswered question at the present time. The wide-

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2 That a factor analogous to KCF is operative in intact bacteria is indicated by the fact that the effects of high hydrostatic pressures on the luminescence of intact bacteria and extracts are similar only when the latter are supplemented with KCF.
spread occurrence of these compounds and the acetals which give rise to them is suggestive of a general function, perhaps, in addition to their rôle in lipide metabolism. Among the possible mechanisms for action of these components in respiratory activity and luminescence are (a) through the intermediate "aldehyde-oxygen intermediates" which are reportedly formed by the addition of molecular oxygen to the free aldehydes and which have been observed to catalyze autoxidations (18-20) and (b) through peroxide-aldehyde addition products which have also been observed (indirectly) (21, 22) and which are effective oxidants. Whether such compounds might have a lower activation energy for some respiratory function than free unbound oxygen or peroxide cannot be answered conclusively at the present time.

From the evidence presented here and the kinetic data to be published elsewhere, the accompanying scheme for the function of the various intermediates in luminescence is postulated. According to this mechanism,

\[
\begin{align*}
XH_2 + DPN &\rightarrow DPNH_2 + X \\
DPNH_2 + FMN &\rightarrow FMNH_2 + DPN \\
E + FMNH_2 + O_2 &\xrightarrow{[KCF]} FMN \cdot H_2O_2 \cdot E \cdot KCF \\
FMNH_2(O_2?) \cdot E \cdot H_2O_2 \cdot KCF &\rightarrow FMN^* + 2H_2O + (O_2?) \\
FMN^* &\rightarrow FMN + h\nu
\end{align*}
\]

the function of the various intermediates is as follows: The DPN functions as a source of respiratory hydrogen; the flavin mononucleotide may serve a double function, first, as a peroxide-generating system, and, second, as a peroxide-oxidizing system. This type of luminescent reaction would be consistent with the obligatory intervention of oxygen in most luminescent reactions and the observed chemiluminescence of flavins (23), as well as with Drew's elegant chemical studies of the luminescence of 3-aminophthalhydrazide (24).

Some suggestion of a factor, other than those listed, operating in bacterial luminescence lies in the capability of boiled bacterial powders to elicit a slightly increased luminescence, even in the presence of an excess of the enumerated factors. However, it is impossible at present to rule out a purely physical effect resulting from some unknown component in the boiled bacterial extract.

**SUMMARY**

It has been possible to obtain in pure form a compound from hog kidney cortex powder, called KCF, which will produce a 100- to 200-fold increase in the luminescence of bacterial extracts in the presence of excess DPNH₂,
FMN, and oxygen. This factor has been identified as palmitic aldehyde by elementary analysis, molecular weight, and mixed melting point of the dinitrophenylhydrazine derivative.

Data have been presented concerning the time-course of luminescence following the addition of palmitic aldehyde, the saturation curve for palmitic aldehyde, and other homologous compounds, as well as some general information on the enzymology of this compound and its interactions with oxygen in this system. It is suggested that this compound is a cofactor in the oxygen activation system and is not serving as a substrate in the conventional sense.

We wish to thank Dr. D. G. Doherty for a number of enzymes used in this work and Dr. C. B. van Niel for nine of the strains of the luminous bacteria used. We also wish to thank Dr. John R. Totter and Dr. M. I. Dolin for many helpful suggestions during the course of the work and in the preparation of the manuscript.

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