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Conversion of Aldehyde to Acid in the Bacterial Bioluminescent Reaction†

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ABSTRACT: Bacterial luciferase catalyzes the emission of light in the presence of reduced flavine mononucleotide, oxygen, and a long chain aliphatic aldehyde. The role of the aldehyde and whether it directly participates in the chemical reaction have not been clear. In the present study, the function of the aldehyde in the luminescent system has been investigated using [1-^{14}C]decanal in a coupled enzyme system. The substrates for the bioluminescent reaction (aldehyde and $FMNH_2$) were generated in the reaction mixture by horse liver alcohol dehydrogenase and FMN reductase. The reaction products were quantitatively analyzed and the distribution of radioactivity has shown that aldehyde is exclusively converted to acid by bacterial luciferase. A linear relation between light emission and acid production was obtained with a quantum yield of 0.10 quanta emitted per molecule of acid produced. Investigations on two different experimental systems in which FMN reductase is absent and $FMNH_2$ is injected resulted in an identical yield of light per molecule of acid produced. These results thus support the proposal that aldehyde oxidation to acid provides the necessary energy for light emission in the bacterial bioluminescent reaction.

Bacterial luciferase catalyzes the emission of light at 490 nm in the presence of $FMNH_2$, O_2 and a long chain aliphatic aldehyde (Cormier and Strehler, 1953; Strehler *et al.*, 1954). Although the aldehyde is essential for high quantum yields, the fate of this molecule in the bioluminescent reaction is unknown. Investigations by a number of workers have shown that the total amount of light emitted is proportional to the amount of added aldehyde (McElroy and Green, 1955; Cormier and Totter, 1957; Lee, 1972; Shimomura *et al.*, 1972). It has been proposed that the aldehyde is converted to the corresponding long chain acid since this reaction would provide sufficient energy for the emission of a quantum of light at 490 nm (McElroy and Green, 1955). Recent support for this proposal has come from the detection by mass spectroscopy of acid in the bioluminescent reaction mixture (Shimomura *et al.*, 1972). However, it is important to establish that acid production does not occur in the absence of luciferase. Indeed, the slow turnover of luciferase ($t_{1/2}$ = 2 sec with decanal) and low yield of products have been the major reasons for the delay in identification of the chemical products of the reaction.

In the present studies, we have investigated the fate of the aldehyde in the bioluminescent reaction mixture and in controls missing a component essential for light emission. Acid production was observed and quantitated both in the controls and in the light emitting system. A net difference in the acid production between the bioluminescent reaction mixture and the controls was directly related to the total amount of emitted light. This result is in agreement with a recent communication of McCapra and Hysert (1973). Furthermore, the present studies show that acid is the only product arising from aldehyde in the bioluminescent reaction.

Experimental Section

Materials. Bacterial luciferase was isolated from *Photobacterium fisheri*, strain MAV, and purified according to the procedure of Gunsalus-Miguel *et al.* (1972). A specific activity of 0.8×10^{13} quanta/sec per mg of luciferase was measured from the maximal light intensity after injection of 1.0 ml of 5×10^{-5} M $FMNH_2$ (catalytically reduced with H_2 over platinized asbestos) into 1.0 ml of 0.02 M phosphate–0.2% bovine serum albumin (pH 7.0), containing luciferase and 10 μl of 0.1% dodecanal suspension. The protein concentration was determined spectrophotometrically on the basis of a specific absorbance coefficient of 0.94 (0.1%, 1 cm) at 280 nm.

FMN reductase was partially purified (100-fold) from *P. fisheri*, strain MAV. Activity measurements were based on the change in optical density at 340 nm on addition of the enzyme to 1×10^{-4} M NADH–5×10^{-6} M FMN, in 0.01 M phosphate buffer–0.1% bovine serum albumin (pH 7.0). One unit of enzyme activity is defined as the μmoles of NADH oxidized per min at 22–24°. The partially purified enzyme had a specific activity of 0.2 unit per unit absorbance at 280 nm. The enzyme was stored at 4° in 10^{-3} M dithiothreitol–0.1 M phosphate buffer (pH 7.0).

Horse liver alcohol dehydrogenase was purchased from Sigma Chemical Co. and dissolved in 0.1 M phosphate buffer (pH 7.0) prior to use. A specific absorption coefficient of 0.42 (0.1%, 1 cm) at 280 nm was used to calculate its concentration in mg/ml (Dalziel, 1958).

NAD and FMN were obtained from Sigma. Decanol (Sigma), decanal (Aldrich), and decanoic acid (Aldrich) migrated as single spots on thin-layer chromatograms (0.2 mm

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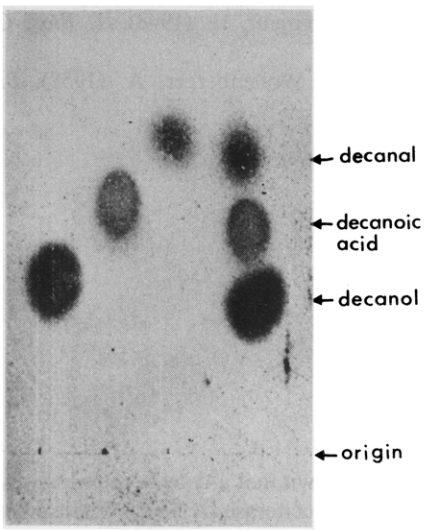

FIGURE 1: Thin-layer chromatography of decanal, decanol, and decanoic acid on silica gel in chloroform–ether (85:15).

silica gel N-HR/uv 254; precoated plastic sheets from Macherey-Nagel and Co.). Radioactive [1-^{14}C]decanol (4.6 Ci/mol) was purchased from International Chemical and Nuclear Corp. and all stock [1-^{14}C]decanol solutions were prepared by dilution into 0.1% Triton X-100. Phosphate buffers were made by mixing appropriate amounts of K_2HPO_4 and NaH_2PO_4. All other chemicals were of reagent grade.

Coupled Assay System. A coupled assay system was used to investigate the products of the bacterial luciferase reaction. The bioluminescent reaction was initiated by the addition of 10 μl of a 8×10^{-4} M suspension of [1-^{14}C]decanol into 1.0 ml of 0.1 M phosphate buffer (pH 7.0) containing 10^{-3} M NAD, 3×10^{-6} M FMN, 0.01 mg of horse liver alcohol dehydrogenase, 0.002 unit of FMN reductase, and 0.5 mg of bacterial luciferase. The reaction mixture in a glass vial was placed in an enclosed chamber and the light intensity measured with a photomultiplier photometer (Mitchell and Hastings, 1971). The instrument readings were related to the absolute units of quanta/sec through the use of the luminol reaction as described by Lee *et al.* (1966). It should be noted that the use of the liquid light standard of Hastings and Weber (1963) gave light intensities 2.7 times higher than that obtained using the luminol reaction. The total quanta of light emitted was obtained by integrating the light intensity over the course of the reaction.

Extraction of Radioactive Products. The reaction was stopped by the addition of 5 μl of ethanol containing decanal (400 μg), decanol (500 μg), and decanoic acid (750 μg) and one volume of cold diethyl ether per volume of reaction mixture. The sample was vortexed for 30 sec and then centrifuged for 1 min in the clinical centrifuge resulting in a two-phase system with the protein precipitated at the interface of the aqueous and organic phase. Over 95% of the radioactivity was recovered in the ether phase with less than 3% remaining in the aqueous phase.

Thin-Layer Chromatography. An aliquot (5 μl) of the ether phase was applied to a thin-layer chromatogram (silica gel N-HR/uv$_{254}$ from Macherey-Nagel and Co.) that was previously activated at 120° for 45 min and prespotted with 1 μl of ethanol containing decanal (80 μg), decanol (100 μg), and decanoic acid (150 μg). The chromatogram was developed in chloroform–ether (85:15) until the solvent front had migrated 7.5–8 cm (~20 min). This solvent system completely resolves decanal, decanol, and decanoic acid (see Figure 1) which have mobilities of 0.60, 0.30, and 0.45, respectively, relative to the solvent front under these conditions. The chromatograms were then stained over iodine crystals for 15 min. The stained spots corresponding to decanal, decanol, and decanoic acid were immediately excised and added directly to 10 ml of Aquasol. An average of 95% of the applied radioactivity was recovered as decanol, decanal, and decanoic acid both in the complete reaction system and in controls missing one of the components. Less than 5% of the radioactivity was found on other parts of the chromatogram and was not located in any single position (*i.e.*, 2% at the origin, <2% with a mobility less than decanol, and <2% with a mobility greater than decanal).

The amount of [^{14}C]decanoic acid in the reaction mixture was calculated by multiplying the fraction of radioactivity as decanoic acid in the ether extract (dpm as decanoic acid on chromatogram/total dpm applied to chromatogram) times the total dpm in the reaction mixture just prior to ether extraction. This approach eliminates variations in the [^{14}C]decanol concentration and corrected for a small loss of radioactivity in the reaction mixture during the incubation period (30 min) both in the coupled system and in controls missing one of the components. The dpm as decanoic acid was converted to molecules of decanoic acid by multiplying by 6×10^{10} (*i.e.* $(6 \times 10^{23}$ molecules/mol)/$(2.2 \times 10^{12}$ dpm/Ci) (4.6 Ci/mol)).

Radioactivity Measurements. All samples were counted in 10 ml of Aquasol (New England Nuclear) in a Packard Scintillation counter at window settings of 50–1000 and a gain setting of 8%. An efficiency of 72% was measured using an internal standard of [^{14}C]toluene (New England Nuclear) for the excised chromatogram spots in 10 ml of Aquasol. An efficiency of 80% was obtained for ^{14}C for all other measurements.

Direct Analysis of Decanoic Acid. Samples could be directly analyzed for the amount of decanoic acid by addition of 1.0% (v/v) of concentrated NaOH to the reaction mixture prior to extraction with ether. This procedure makes the aqueous phase basic (pH >10) and prevents the extraction of decanoic acid by ether. The radioactivity remaining in the aqueous phase is then a direct measure of the amount of [^{14}C]decanoic acid in the reaction mixture. Analysis of the ether extract showed that only decanol was present in the organic phase. In some experiments, the basic aqueous phase was neutralized (pH <7) after ether extraction with concentrated HCl. The remaining radioactivity was then extracted with ether and analysis by thin-layer chromatography showed that only decanoic acid was present.

Results

A reaction system that produces a high sustained level of light emission is advantageous for the investigation of the fate of the aldehyde in the bioluminescent reaction. A coupled assay system containing three enzymes, alcohol dehydrogenase, FMN reductase, and bacterial luciferase, was adopted for this purpose. These enzymes catalyze reactions 1, 2, and 3, respectively.

$$NAD^+ + [1\text{-}^{14}C]\text{decanol} \rightleftharpoons NADH + H^+ + [1\text{-}^{14}C]\text{decanal} \quad (1)$$

$$NADH + H^+ + FMN \rightleftharpoons NAD^+ + FMNH_2 \quad (2)$$

$$FMNH_2 + O_2 + [1\text{-}^{14}C]\text{decanal} \longrightarrow \text{light} + {}^{14}C \text{ product} + \ldots\ldots \quad (3)$$

TABLE I: Requirements for Light Emission in the Coupled Assay System.[a]

Sample	Quanta Emitted × 10^{-14}
Complete system	0.75
−Luciferase	0.0
−FMN reductase	0.04
−FMN	0.05
−Alcohol dehydrogenase	0.04
−NAD	0.0
−[1-^{14}C]Decanol	0.04

[a] The complete reaction mixture contained 3×10^{-6} M FMN, 10^{-3} M NAD, 8×10^{-6} M [1-^{14}C]decanol, 0.01 mg of alcohol dehydrogenase, 0.002 unit of FMN reductase, and 0.5 mg of bacterial luciferase in 1.0 ml of 0.1 M phosphate buffer (pH 7.0). The components were incubated for 30 min at 24°. The light intensity was recorded graphically, and then integrated to give total quanta emitted as described in the Experimental Section.

In this assay system, NAD, FMN, and [1-^{14}C]decanol are mixed with the three enzymes. Decanol is oxidized to decanal by alcohol dehydrogenase with the reduction of NAD to NADH. The NADH is then used by FMN reductase for the reduction of FMN. In this way, relatively constant levels of $FMNH_2$ and aldehyde can be maintained for the bioluminescent reaction catalyzed by bacterial luciferase (reaction 3). A system for the reduction of FMN is necessary since $FMNH_2$ is rapidly oxidized chemically (Gibson and Hastings, 1962). It is also particularly advantageous to maintain decanal at low and constant concentration since aldehydes are highly susceptible to chemical oxidation (Hastings *et al.*, 1963) and high concentrations of aldehyde inhibit bacterial luciferase (Hastings *et al.*, 1969).

Figure 2 gives a plot of light intensity *vs.* time for the complete assay system. The light intensity rises to a maximum of about 0.45×10^{11} quanta/sec within about 4 min after initiation of the reaction, and then decreases slowly over the next 20–30 min. Since the rate of light emission decays only 10–15% over the course of the reaction, $FMNH_2$ and decanal are therefore being maintained at relatively constant levels for the bioluminescent reaction. The total yield of light during the course of the reaction (30 min) is given in Table I. If any single component is omitted very low light yields are obtained.

The goal of this investigation was to determine the fate of the long chain aliphatic aldehyde in the bioluminescent reaction. Since [1-^{14}C]decanal is present in this assay system, the conversion of aldehyde to product(s) by bacterial luciferase would result in the product that contains the C_1 carbon of decanal being radioactively labeled. One proposal is that the aldehyde is converted enzymically to the corresponding long chain acid (*e.g.*, decanoic acid) (McElroy and Green, 1955).

Our investigations were therefore directed toward elucidating whether any radioactive compound, and, in particular, decanoic acid, was present in higher amounts in the complete system than in controls lacking any one of the assay components. Analysis of the reaction mixture was performed initially by extraction of all (>97%) of the radioactivity with ether and thin-layer chromatography on silica gel with a solvent system (chloroform–ether, 85:15) that resolves decanol, decanal, and decanoic acid (see Experimental Section). The results are given in Table II.

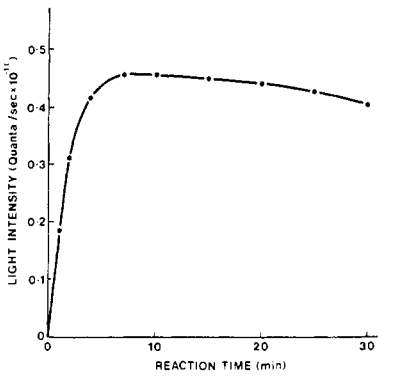

FIGURE 2: Light intensity as a function of time in the coupled enzyme system. Additional details are given in Table I.

The majority of the radioactivity is found only at the positions of decanol and decanoic acid after thin-layer chromatography both in the controls and in the complete system. Very little radioactivity is found at the position of decanal as would be expected if the aldehyde was maintained at a low level in the assay system. No radioactivity (<2%) was found at any other position on the chromatogram.

A significant difference in the percentage of radioactivity as decanoic acid can be observed between the complete reaction mixture and controls missing any of the components essential for light emission indicating that luciferase is converting decanal to decanoic acid and not to another radioactive product. The decanoic acid in the controls can arise by chemical oxidation of decanol and decanal to decanoic acid as well as by oxidation catalyzed by alcohol dehydrogenase (Hinson and Neal, 1972).

A second approach was used in these experiments to identify and quantitate the decanoic acid in the reaction mixture. In this experimental approach, the reaction mixture was made basic prior to extraction with ether. As a consequence, the decanoic acid was not extracted with ether and the radio-

TABLE II: Distribution of Radioactivity after Thin-Layer Chromatography.[a]

Sample	% Decanal	% Decanol	% Decanoic Acid
Complete system	<2	63	35
−Luciferase	<2	82	17
−FMN reductase	<2	84	14
−FMN	<2	80	19
−Alcohol dehydrogenase	<2	88	10
−NAD	<2	92	6

[a] Experimental conditions are idential with those given in Table I. At the completion of the reaction (30 min) the radioactivity (>97%) was extracted into ether and an aliquot was chromatographed on silica gel as described in Experimental Section. The percentage of each component is based on the total cpm recovered as decanal, decanol, and decanoic acid. Greater than 95% of the applied radioactivity was recovered after chromatography.

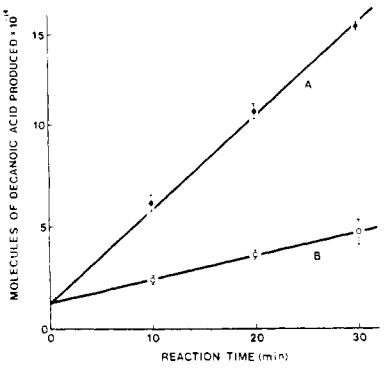

FIGURE 3: The production of decanoic acid as a function of time for A, the complete reaction mixture (●), and B, the control missing luciferase (○). The reaction was initiated by the addition of 10 μl of 8×10^{-4} M [1-^{14}C]decanol to 1.0 ml of 0.1 M phosphate (pH 7.0) containing 1×10^{-3} M NAD, 3×10^{-6} M FMN, 0.002 unit of FMN reductase, 0.01 mg of alcohol dehydrogenase, and ±0.5 mg of bacterial luciferase. Aliquots of 200 μl were taken from the reaction mixture at 10-min intervals and the reaction was immediately terminated by the addition of 5 μl of 6 N NaOH, and the basic solution was extracted with 1–2 volumes of cold diethyl ether. The radioactivity remaining in the basic aqueous phase was used to calculate the amount of decanoic acid that would be present in the 1.0-ml reaction mixture. Each point is the average of three independent experiments and the error bars represent the average deviation. The light intensity for the complete reaction mixture (A) was recorded graphically and corrected for decreases in light intensity due to the removal of the aliquots for analysis. The light intensities in quanta/sec were integrated over the specific time interval to give the total light emitted by a 1.0-ml reaction mixture.

TABLE III: Dependence of the Extraction of Decanol and Decanoic Acid on the pH of the Aqueous Phase.[a]

	Neutral (pH 7.0) Ether Extract	Basic (pH 10.5) Ether Extract	Basic (pH 10.5) Aqueous Phase[b]
Complete system			
% Decanol	63	95	3
% Decanoic acid	35	3	95
% Decanal	<2	<2	<2
− Luciferase			
% Decanol	82	97	<2
% Decanoic acid	17	<2	98
% Decanal	<2	<2	<2

[a] Experimental conditions are given in Table I and in Experimental Section. Over 97% of the radioactivity was extracted with ether from the neutral aqueous phase, whereas 65 and 83% of the radioactivity was extracted by ether from the basic aqueous phases of the complete system and the control missing luciferase, respectively. The percentages of each component are based on the total radioactivity recovered as decanal, decanol, and decanoic acid (>95% of applied radioactivity) after thin-layer chromatography. [b] The radioactivity remaining in the basic aqueous phase after ether extraction was analyzed by neutralization of the aqueous phase with HCl (pH <7), extraction of the radioactivity into ether (>95%) and analysis of the distribution of radioactivity in decanal, decanol, and decanoic acid after thin-layer chromatography (see Experimental Section).

activity remaining in the aqueous phase is a measure of the amount of decanoic acid. This conclusion is supported by the data given in Table III. If a reaction mixture known to contain 35% decanoic acid and 63% decanol (complete system) is first made basic (pH 10.5), then only 65% of the radioactivity is extracted by ether. Thin-layer chromatography and analysis of the distribution of radioactivity show that only decanol (>95%) and not decanoic acid is extracted from this sample (ether extract, pH 10.5). If the basic aqueous phase is neutralized (pH <7) after ether extraction, the remaining radioactivity (35%) can be now extracted from the aqueous phase with ether. Only decanoic acid (>95%) is present in this extract (aqueous phase, pH 10.5). An identical distribution of radioactivity is obtained for the reaction mixture missing luciferase (Table III), except this control contains a lower percentage of decanoic acid (17%).

The amount of decanoic acid produced in the bioluminescent reaction was thus measured either from the amount of radioactivity in decanoic acid after thin-layer chromatography of an ether extract of the neutral aqueous phase or by the amount of radioactivity remaining in the aqueous phase if it was made basic prior to ether extraction. A comparison of the amount of decanoic acid calculated by the two methods of analyses both for the complete system and the controls is given in Table IV. No significant difference exists between the amounts of decanoic acid estimated by these two methods of analysis for any sample.

A comparison of the amount of decanoic acid produced in the presence (A) and in the absence (B) of bacterial luciferase shows that at least twice as much decanoic acid is produced in the complete light emitting system as in the control missing luciferase. The controls in which luciferase and decanal are both present but $FMNH_2$ is not available for the bioluminescent reaction (−FMN reductase, −FMN) have the same amount of decanoic acid as the control in which $FMNH_2$ and decanal are present but luciferase is missing (−luciferase). This result shows not only that the aldehyde level is identical in these controls but that the level of decanoic acid is increased by luciferase only if $FMNH_2$, a necessary requirement for the bioluminescent reaction, is also present.

The decanoic acid in the controls presumably arises from oxidation of both decanol and enzymically produced decanal. The controls in which decanal is not enzymically produced (−alcohol dehydrogenase, −NAD) have substantially lower amounts of decanoic acid than the other controls. This result is expected since decanoic acid can only arise in this case by chemical oxidation of decanol.

The net amount of decanoic acid produced by bacterial luciferase (A − B) is given at the bottom of Table IV. This result is the average difference between the amount of decanoic acid in two samples, one containing all the assay components (A) and the other missing luciferase (B), where the two experiments were conducted simultaneously. Such an approach gave the most consistent results for A − B since the level of decanoic acid both in controls and the complete system is higher if the stock [1-^{14}C]decanol solution is not freshly prepared.

The relationship between the amount of decanoic acid produced and the reaction time is given in Figure 3 for the complete system and the control missing luciferase. The amount of decanoic acid increases in both samples with time; however, the rate of production of decanoic acid is substantially more rapid in the complete light emitting system than in the control sample (−luciferase). A small amount of decanoic acid can be detected at the start of the reaction by the method

TABLE IV: Determination of the Amount of Decanoic Acid Produced in the Bacterial Bioluminescent Reaction.

	Method of Analysis[b]			
	Ether Extraction Followed by tlc and Analysis of ^{14}C in Decanoic Acid		Analysis of ^{14}C in Basic Aqueous Phase (pH >10) after Ether Extraction	
Sample[a]	Molecules of Decanoic Acid Produced $\times 10^{-14}$ ± Std. Dev.	No. of Determinations	Molecules of Decanoic Acid Produced $\times 10^{-14}$ ± Std. Dev.	No. of Determinations
Complete System (A)	17.4 ± 1.9	7	15.0 ± 0.8	8
−Luciferase (B)	8.1 ± 1.7	7	6.4 ± 2.1	8
−FMN reductase	6.6 ± 1.4	6	6.5 ± 1.3	5
−FMN	8.3 ± 2.8	6	7.3 ± 1.1	4
−Alcohol dehydrogenase	4.4 ± 0.7	3	4.0 ± 0.8	3
−NAD	2.6 ± 0.2	3	2.9 ± 0.7	3
A − B	9.3 ± 1.6[c]	7	8.6 ± 2.1[c]	8

[a] Experimental details given in Table I. An average of 0.9 ± 0.1 $\times 10^{14}$ quanta were emitted over the course of the reaction (30 min) in the complete system. Less than 0.06 $\times 10^{14}$ quanta were emitted over the same period in any control lacking one of the components. [b] Additional details are given in Experimental Section. [c] The standard deviation was calculated after subtraction of paired sets (A and B) of data where the experiments for the complete reaction system (A) and the control (B) were conducted at the same time.

of analysis used in this experiment (*i.e.*, from the radioactivity remaining in the basic aqueous phase after ether extraction). It should be noted, however, that this amount only represents 2% of the total added radioactivity (48 $\times 10^{14}$ molecules of [1-^{14}C]decanol in 1.0 ml).

A plot of the quanta of light emitted *vs.* the number of molecules of decanoic acid produced enzymically (A − B) is given in Figure 4. Included in these data are not only the results from Table II and Figure 3, but experiments in which low yields of light were observed. This result occurred if the decanol solutions were not freshly prepared or when the specific activity of the FMN reductase preparation declined. Higher light yields were restored in these cases by using either fresh decanol or by addition of a larger volume of FMN reductase (although the same amount of activity). A line through the origin with the minimum standard deviation fits the data with a slope of 0.095 ± 0.004[1] quanta of light emitted per molecule of decanoic acid produced. If the results for the two different methods of analysis for decanoic acid (○ and ●) are each fitted with a line by the method of least squares no difference in slope is observed.

The results in Figure 4 and Table IV show that (a) a linear relationship exists between the amount of light produced and the increase in acid production in the coupled system by luciferase, and (b) the increase in acid production by luciferase occurs only if a system for the reduction of FMN is also present. The absence of either the FMN reductase system (−FMN or −FMN reductase) or luciferase (−luciferase) results in an identical and substantially lower production of acid. These results provide evidence that bacterial luciferase, in a $FMNH_2$ dependent step, catalyzes the oxidation of aldehyde to acid.

An alternate model explaining these results, however, is still possible. If bacterial luciferase, in the presence of the FMN reductase system, raises the free aldehyde concentration, then a more rapid oxidation of aldehyde to acid could result *via* the same pathway(s) as in the controls (−FMN, −FMN reductase, −luciferase). Such a result cannot simply occur by aldehyde binding to a complex of luciferase and $FMNH_2$ since this would result in a lower free aldehyde concentration.[2] A possible mechanism which would explain an increase in the free aldehyde concentration is to propose that luciferase acts indirectly by decreasing the $FMNH_2$ concentration (either by binding and/or oxidation). Consequently, additional NADH would be oxidized by FMN reductase to reduce FMN. This, in turn, would result in more NAD being reduced to NADH by alcohol dehydrogenase with the conversion of alcohol to aldehyde. Consequently, the free aldehyde concentration would be raised in the complete system. Although this model is complex, the previous results do not eliminate this possibility. As a result, an experimental system(s) in which the FMN reductase system is absent was investigated.

Figure 5 gives the light intensity after injection of $FMNH_2$ into either a solution containing the alcohol dehydrogenase system (horse liver alcohol dehydrogenase, [1-^{14}C]decanol, and NAD) and luciferase (curve C) or a solution containing only [1-^{14}C]decanol and luciferase (curve E). The light intensity rises rapidly to a maximum and then decreases rapidly since $FMNH_2$ is oxidized within the first few seconds (Gibson and Hastings, 1962) and a system for reduction of FMN is not present. The initial light intensity is higher in both systems than in the coupled system described previously (Figure 2); however, in less than 1 min after initiation of the reaction, sample E is emitting light at a rate substantially lower than in the coupled system. A higher light intensity is observed in sample C since alcohol dehydrogenase is present for the conversion of alcohol to aldehyde. An average of 0.34 $\times 10^{14}$ and 0.052 $\times 10^{14}$ quanta are produced in samples C and E, respectively, over the course of the 2-min reaction period.

The amount of acid present in these solutions was determined by measurement of the radioactivity remaining in the aqueous phase after addition of NaOH and extraction with

[1] The sample standard deviation of the regression coefficient.

[2] It could be argued that the bound aldehyde would also be oxidized, resulting therefore in an increased production of acid. However, this model is not distinguishable from the proposal that luciferase catalyzes the oxidation of aldehyde to acid in a $FMNH_2$ dependent step.

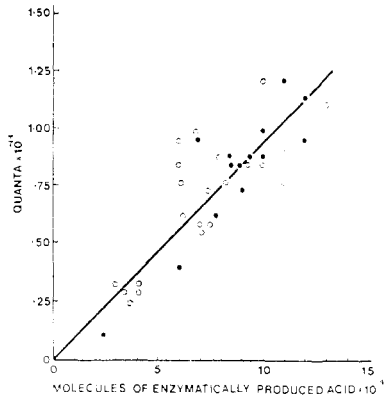

FIGURE 4: The relationship between the amount of light emitted and the acid produced by luciferase in the coupled assay system (see Table I). The amount of acid produced was estimated either from analysis of the radioactivity distribution after thin-layer chromatography (●) or from the radioactivity remaining in the aqueous phase if NaOH is added prior to extraction with ether (○). Additional details are given in the Experimental Section. Each point was obtained by plotting the total light emitted in a sample containing all the reaction components *vs.* the difference in acid production for this sample and a control missing luciferase. The data were obtained from the experiments described in Table IV and Figure 3.

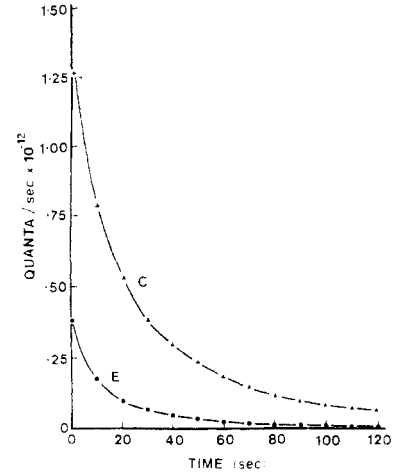

FIGURE 5: Light intensity (quanta/sec) as a function of time after injection of 0.5 ml of 5×10^{-5} M $FMNH_2$ into 1.0 ml of 0.1 M phosphate (pH 7.0) containing either 8×10^{-6} M [1-^{14}C]decanol, 10^{-3} M NAD, alcohol dehydrogenase (0.005 mg), and 0.5 mg of luciferase (C) or 8×10^{-6} M [1-^{14}C]decanol and 0.5 mg of luciferase (E).

ether. The results are given in Tables V and VI for samples C and E, respectively. The previous results have shown that only decanoic acid remains in the aqueous phase under these conditions (Table III). Control solutions in which luciferase (−luciferase) was absent or FMN was injected (−$FMNH_2$, +FMN) resulted in a substantially lower production of acid relative to the complete reaction mixture. A higher acid production is found in the controls for the experimental system described in Table V than the system described in Table VI since alcohol dehydrogenase is present in the former system and not only can convert alcohol to aldehyde but may catalyze the conversion of aldehyde to acid (Hinson and Neal, 1972).

The results on these two experimental systems, both of which differ from the coupled system described previously which contained FMN reductase, provide strong proof that luciferase converts aldehyde to acid. Furthermore, the possibility suggested earlier of luciferase removing $FMNH_2$ and indirectly increasing the free aldehyde concentration through the FMN reductase system is not possible in these two experimental systems. A comparison of the quantum yields obtained in all three systems is given in Table VII. A yield of 0.10–0.11 quanta per molecule of acid produced is obtained in all three systems.

TABLE V: Acid Production by Bacterial Luciferase in a System Containing Only One Coupling Enzyme (Alcohol Dehydrogenase).

Sample	Molecules of Decanoic Acid Produced $\times 10^{-14}$ ± Std. Dev.	No. of Determinations
Complete (C)[a]	5.6 ± 0.9	9
−Luciferase (D)	2.4 ± 0.4	10
−$FMNH_2$, +FMN	2.4 ± 0.3	3
C − D	3.2 ± 1.0	

[a] The complete reaction mixture (C) contained exogenously added [1-^{14}C]decanol, NAD, $FMNH_2$, alcohol dehydrogenase, and luciferase. The reaction was initiated by the addition of 10 μl of 8×10^{-4} M [1-^{14}C]decanol to 1.0 ml of 1×10^{-3} M NAD, 0.1 M phosphate (pH 7.0), containing 0.5 mg of luciferase and 0.005 mg of alcohol dehydrogenase. After 1 min, 0.5 ml of 5×10^{-5} M $FMNH_2$ was injected and the light intensity recorded as described in Figure 5. An average of $0.34 \pm 0.02 \times 10^{14}$ quanta was emitted after correction for the light emitted (0.015×10^{14} quanta) in the absence of added decanol. The reaction was stopped by the addition of NaOH and extracted with ether as described in Experimental Section except that the aqueous phase was washed three additional times with ether. The total number of molecules of decanoic acid was calculated from the radioactivity remaining in the aqueous phase.

TABLE VI: Acid Production by Bacterial Luciferase in the Absence of Coupling Enzymes.

Sample	Molecules of Decanoic Acid Produced $\times 10^{-14}$ ± Std. Dev.	No. of Determinations
Complete (E)[a]	1.30 ± 0.18	7
−Luciferase (F)	0.79 ± 0.15	6
−$FMNH_2$, +FMN	0.85 ± 0.05	2
E − F	0.51 ± 0.24	

[a] The complete reaction mixture (E) contained exogenously added [1-^{14}C]decanol, $FMNH_2$, and luciferase. The reaction was initiated by the addition of 10 μl of 8×10^{-4} M [1-^{14}C]-decanol to 1.0 ml of 0.1 M phosphate (pH 7.0), containing 0.5 mg of luciferase. After 1 min, 0.5 ml of 5×10^{-5} M $FMNH_2$ was injected and the light intensity recorded for 2 min as described in Figure 5. An average of $0.052 \pm 0.007 \times 10^{14}$ quanta were emitted after correction for the light emitted (0.015×10^{14} quanta) in the absence of added decanol. The reaction was stopped and the amount of decanoic acid analyzed as described in Table V.

Discussion

The determination of the identity of the products of the bioluminescent reaction requires methods for analysis of extremely low amounts of material. This is necessary since bacterial luciferase has a very low turnover number. As a consequence, a radioactive label in the aldehyde is necessary to follow the fate of this molecule in the bioluminescent reaction.

In the present experiments, [1-^{14}C]decanol was converted to [1-^{14}C]decanal by horse liver alcohol dehydrogenase in a coupled system containing luciferase and FMN reductase. This system permits luciferase to continually turn over since the substrates of the bioluminescent reaction ($FMNH_2$ and aldehyde) are being maintained by FMN reductase and alcohol dehydrogenase.

The application of this coupled system to study the function of the aldehyde has a number of advantages as well as disadvantages. First, radioactive decanol, specifically labeled with ^{14}C in the C_1 position, is readily available as a pure and stable liquid. Consequently, the functional end of the aldehyde, the carbonyl group, can be specifically followed in the bioluminescent reaction. Secondly, the added decanol provides a large reservoir and thus permits the maintenance of a low and constant concentration of decanal by alcohol dehydrogenase. The equilibrium between alcohol and aldehyde in a NAD–NADH coupled system at pH 7 is largely in favor of the alcohol (Sund and Theorell, 1963). This is advantageous since high concentrations of aldehyde would inhibit luciferase (Hastings *et al.*, 1969) and furthermore would result in a more rapid rate of chemical autoxidation of the aldehyde.

The major disadvantage of this system is the possibility of a shift in the free aldehyde level in a coupled enzyme system. An increase in the aldehyde concentration in the complete system over that in the controls might then result in a more rapid rate of acid production through a pathway not catalyzed by bacterial luciferase. This possibility was eliminated by investigations on two other systems which did not contain FMN reductase and in which $FMNH_2$ was injected. Since the light intensity decreased rapidly in these systems due to the absence of a $FMNH_2$ generating system, the reaction was only followed for 2 min. It might be noted that analysis for the production of acid could be accomplished within 5 min after completion of the reaction and differences as small as 30 pmol could be detected. The experimental results in Table VII showed that all three experimental systems, which contained either two (alcohol dehydrogenase and FMN reductase), one (alcohol dehydrogenase), or no coupling enzymes, gave an identical yield of light per mole of acid produced. This result also suggests that luciferase, itself, and not small amounts of contaminating enzymes in the luciferase preparation is catalyzing the production of acid. Furthermore, increased acid production with the luciferase preparation is only observed on addition of reduced FMN and not oxidized FMN (*e.g.*, see Table VI). Consequently, if a contaminating enzyme in the luciferase preparation was responsible for the increased acid production it must be $FMNH_2$ dependent. To our knowledge, no other enzyme that catalyzes the oxidation of aldehyde is known to be dependent on reduced FMN. In addition, activity measurements on the luciferase preparation with alcohol or aldehyde and NAD (or NADP) did not result in the reduction of these coenzymes.

The detection of acid by mass spectroscopy in a reaction mixture containing bacterial luciferase has been reported by Shimomura *et al.* (1972). However, evidence is required to show that acid is not produced in the absence of luciferase and that a relationship exists between acid production and light emission. A recent communication by McCapra and Hysert (1973) has provided evidence in this regard, although quantitative and qualitative analysis did not exclude the possibility that other products had arisen from aldehyde. In addition, these studies used a single enzyme preparation containing both luciferase and FMN reductase and consequently their separate functions in the bioluminescent reaction mixture could not be determined.

In the present experiments a coupled enzyme system was used where FMN reductase and luciferase were individually purified. A quantum yield[3] of 0.10 was obtained for acid production on the basis of the luminol reaction (Lee *et al.*, 1966). This result is in excellent agreement with the quantum yield previously reported by Lee (1972) for aldehyde. The present results thus directly support the proposal of McElroy and Green (1955) that aldehyde is converted to acid by bacterial luciferase as part of the light emitting reaction. Furthermore, these results show that acid is the only product obtained from aldehyde in the bioluminescent reaction.

TABLE VII: Quantum Yield of Bacterial Luciferase.[a]

Experimental System[b]	Molecules of Decanoic Acid Produced $\times 10^{-14}$	Quanta Emitted $\times 10^{-14}$	Quantum Yield
Table IV	9.0[c]	0.9	0.10
Table V	3.2	0.34	0.10_6
Table VI	0.51	0.052	0.10_2

[a] The quantum yield was calculated by dividing the quanta of light emitted by the molecules of decanoic acid produced. [b] The experimental data were obtained from Tables IV, V, and VI and give the net number of molecules of decanoic acid produced in the light emitting system after correction for the acid production in a control without luciferase. [c] The average number of molecules of decanoic acid determined by the two methods of analysis (see Table IV).

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[3] A quantum yield of 0.27 would be obtained if the liquid light standard of Hastings and Weber (1963) is used for conversion of the instrument readings to quanta/sec.

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Spin-Labeled Ribonuclease A. Selective Incorporation of a Nitroxide Spin Label Sensitive to Active-Center Geometry†

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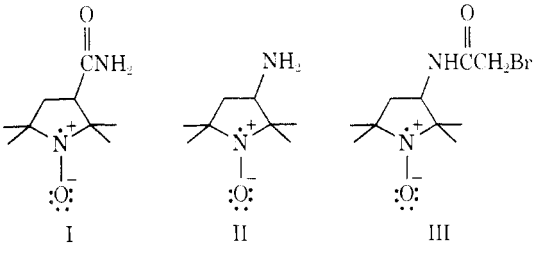
ABSTRACT: The reaction of *N*-(1-oxyl-2,2,5,5-tetramethyl-3-pyrrolidinyl)bromoacetamide with bovine pancreatic ribonuclease A has been investigated at pH 5.50. The two principle spin-labeled products, 3-SLHis-105-RNase A and a *S*-SLMet-RNase A, are slowly formed indicating that no special rate enhancement effects such as electrostatic positioning of the spin label for reaction, etc., are manifest. Furthermore, under the conditions used, no spin labeling occurs at histidine-12 or histidine-119. The site of alkylation in the 3-SLHis-105-RNase A derivative has been assigned to the number three imidazole nitrogen of histidine-105 by application of the subtractive Edman degradative procedure (one step) to a carboxyl-terminal tryptic peptide (residues 105–124) which was isolated. The 3-SLHis-105-RNase A has a specific enzymatic activity of ~85% that of native RNase A with both 2′,3′-cyclic cytidylic acid and yeast tRNA as substrates and exhibits active-site chemistry identical with the native enzyme. The evidence suggests that the covalently attached spin label is sensitive to active-center geometry and may therefore be useful in elucidating the conformational properties of the molecule.

Of the many methods utilized in investigating the structure of macromolecules in solution, those involving magnetic resonance have proved valuable. One of these, the spin-label technique (Stone *et al.*, 1965; Hamilton and McConnell, 1968; Griffith and Waggoner, 1969; McConnell and McFarland, 1970), has been used frequently because of its applicability to the study of a variety of biological systems. An investigation of bovine pancreatic ribonuclease A by the spin-label approach has been undertaken in this laboratory for several reasons. The primary sequence of RNase A is known (Smyth *et al.*, 1963), the tertiary structure of the molecule has been elucidated by X-ray diffraction (Kartha *et al.*, 1967), and many enlightening chemical and physical modifications of the enzyme have been accomplished (Barnard, 1969). In short, because of the vast amount of information already available concerning the enzyme, a study of RNase A by the spin-label method should provide highly interpretable results.

Shortly after our studies were initiated, Smith (1968) reported that the bromoacetamide nitroxide spin label III,



along with several other spin labels, had been employed to investigate conformational aspects of RNase A. In his studies, however, Smith dealt with the total reaction mixtures and did not separate these mixtures or attempt to identify the components; accordingly, the conclusions reached from these studies were considered preliminary. The present communication describes our investigation of the reaction of the spin label III with RNase A at pH 5.50 and the isolation and characterization of a ribonuclease A derivative spin labeled at histidine.

Experimental Section

Materials. Bovine pancreatic ribonuclease A (EC 2.7.7.16) (type IIA), trypsin (EC 3.4.4.4) (dicycohexylcarbodiimide-treated type XI), and cyclic 2′,3′-cytidylic acid, sodium salt, were purchased from Sigma Chemical Co., yeast tRNA (lot 6602) was obtained from Schwarz BioResearch, Inc., and an affinity resin for ribonuclease A [Sepharose coupled with 5′-

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