QUANTUM EFFICIENCY DETERMINATIONS ON COMPONENTS OF THE BACTERIAL LUMINESCENCE SYSTEM

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INTRODUCTION

The nature of the components involved in the bacterial luminescence system, and the interaction of these components to produce light have now become relatively well understood owing to the work of a number of investigators[1-10].

On the basis of results obtained by the workers mentioned, the sequence of events that lead to light emission in bacteria may be summarized as follows:

$$\text{DPNH} \xrightarrow[\text{FMN}]{\text{DPNH Oxidase}} \text{FMNH}_2 \xrightarrow[\text{RCHO}]{\text{Luciferase}} \text{Light} + \text{unknown products.}$$
$$\text{O}_2$$

The cell-free extracts will reduce FMN*** in the presence of an FMN-dependent DPNH oxidase. The reduced FMN then reacts with the enzyme luciferase in the presence of a long-chain aldehyde and oxygen to produce light plus unknown products of the reaction. It should be pointed out, for the sake of future discussion, that there is good evidence for the existence of two sites for FMN on the surface of luciferase[8,9].

The identity of the energy-yielding reaction during bacterial luminescence, however, remains unknown, although McELROY AND GREEN[9] suggested that a peroxidation of an aldehyde to an acid might furnish the necessary energy. In addition, the fate of FMN during the luminescence reaction is still highly speculative.

Answers to these questions were sought in a determination of the quantum efficiencies of FMN and a long-chain aldehyde (dodecyl aldehyde in this case) in the bacterial luminescence system. The quantum efficiency for DPNH was also determined, since the percentage of the DPNH oxidized via a luminous pathway to produce light would be of interest.

MATERIALS AND METHODS

Acetone-dried powders of the luminous bacterium, *Achromobacter fischeri* (ATCC No. 7744) were prepared by methods described by STREHLER AND CORMIER[2]. Extracts of lysed cells of *A. fischeri* were prepared by methods described by McELROY *et al.*[5] and McELROY AND GREEN[9].

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*** The following abbreviations are used in this paper: DPNH, reduced diphosphopyridine nucleotide; FMN, flavin mononucleotide; $FMNH_2$, reduced flavin mononucleotide; RCHO, *n*-dodecyl aldehyde.

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Preparations C-1 and B-2 were 10% by weight water extracts of acetone-dried powders of *A. fischeri* acid precipitated—the former with 0.1 *N* acetic and the latter with 0.01 *N* HCl—and washed twice with dilute HCl (pH 4.0). Preparation C-4 was the 40–70% $(NH_4)_2SO_4$ fraction (nondialysed) of the supernatant from lysed cells of *A. fischeri*.

Reduced diphosphopyridine nucleotide was obtained from the Sigma Chemical Company, the riboflavin phosphate from the California Foundation for Biochemical Research, and the dodecyl aldehyde from the Matheson Company, Inc. The dodecyl aldehyde was vacuum distilled under 12 mm pressure, and the fraction boiling at 129–130° C was used.

Light was measured with a liquid nitrogen-cooled quantum counting device, described by STREHLER[11] that contained a 1P21 photomultiplier.

Quantum efficiency determinations were made in a light-tight tunnel approximately 300 cm long (from the photomultiplier to the end of the tunnel) that contained an optical bench for correct measurements of distance. The inside of the tunnel was painted dull black. The optics inside the tunnel with respect to the photomultiplier appeared to be satisfactory, since the inverse square law was obeyed over the distances used in the experiments.

In principle, the efficiency of the photomultiplier was determined as follows: The thermopile was calibrated from a National Bureau of Standards 50-watt lamp of known flux under conditions set by the National Bureau of Standards. From thermopile measurements, a secondary-standard lamp (a General Electric 18-amp, 6-volt, filament lamp operated at a constant voltage of 4 volts) was made. Two Corning glass filters (numbers 3387 and 4303) were placed in front of the housing that contained the lamp. The light coming through the filters covered most of the visible region of the spectrum and had a transmission peak at 490 mμ. Fig. 1 is a schematic diagram of the apparatus used in the determination of the efficiency of the photomultiplier.

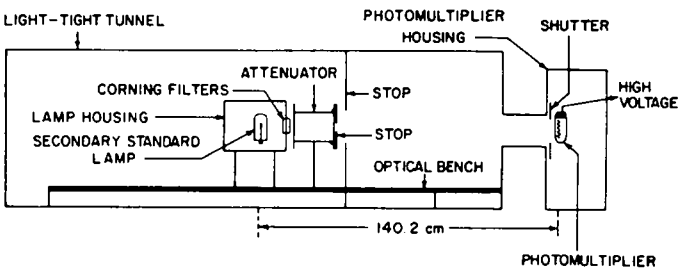

Fig. 1. Diagram of apparatus used in the determination of the photomultiplier efficiency.

Since the light from the lamp was still much too bright, under conditions used for operation of the photomultiplier, attenuation was necessary. For this purpose, a chemical filter was used that consisted of 57.8 g of $Cr_2(SO_4)_3$ plus 97.2 g of $CuSO_4$ per liter of solution. The attenuator solution obeyed Beer's law at the concentration used, and had an optical density of 0.78 per cm at 490 mμ. The light through the filter was almost monochromatic, with a very sharp transmission peak at 490 mμ. For example, the transmission at 10 mμ and at 20 mμ on either side of the peak was one-eighth and one-five hundredth, respectively, the transmission at the peak. The attenuator solution was placed into a lucite cell having a 10-cm light path.

Attenuation produced by the system described was calculated as follows:

Let $J\lambda$ = energy per unit wavelength from the secondary standard lamp (determined with the use of a Farrand monochromator and then corrected for dispersion),
F = transmission through the two Corning filters,
and $T\lambda$ = transmission through the attenuator.

Then the total light from λ_1 to λ_2 emitted from the secondary standard would be equal to

$$\int_{\lambda_1}^{\lambda_2} J\lambda F\lambda \mathrm{d}\lambda$$

The attenuation factor is then represented by the ratio of the two integrals:

$$\text{Attenuation} = \frac{\int_{\lambda_1}^{\lambda_2} J\lambda F\lambda T\lambda \mathrm{d}\lambda}{\int_{\lambda_1}^{\lambda_2} J\lambda F\lambda \mathrm{d}\lambda}$$

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The value obtained for the attenuation was $2.1 \cdot 10^{-9}$. We made allowance for any absorption of light by the lucite cell and water by making the original measurements of the flux from the secondary-standard lamp with the lucite cell plus water in front of the lamp.

From the attenuation factor calculated as described and the known flux of the secondary-standard lamp, the flux in watts times cm^{-2} could be calculated at any distance from the photomultiplier inside the light-tight tunnel. From the value thus obtained ($21.9 \cdot 10^{-16}$ watts cm^{-2} at 140.2 cm from the phototube), the number of quanta times sec^{-1} times cm^{-2} at the surface of the photomultiplier was then calculated, yielding a value of $5.4 \cdot 10^{2}$. After determination of the counts times $seconds^{-1}$ from the secondary standard, the efficiency of the photomultiplier (S_{490}) at 490 mμ was represented by the following ratio:

$$S_{490} = \frac{324 \text{ counts sec}^{-1}}{5400 \text{ quanta sec}^{-1} \text{ cm}^{-2}} = 0.06 \text{ counts cm}^2 \text{ quantum}^{-1}$$

The value of 0.06 is the number of counts produced by one quantum for the entire grid area on the phototube. It can be thought of as a sort of cross section of the grid area with respect to a quantum of light. Since the grid area was 1.89 cm^2, the efficiency in counts per quantum was 0.032.

Quantum efficiency determinations of the bacterial luminescence system were made at 133 cm from the phototube in 2.1-ml spherical glass vessels. The total quanta emitted from such a system could then be determined from the product of the expression:

$$\text{(Counts) (area of a sphere of radius} = 133 \text{ cm)} \left(\frac{\int_{\lambda_1}^{\lambda_2} \frac{C_\lambda}{S_\lambda} \, d\lambda}{\int_{\lambda_1}^{\lambda_2} C_\lambda \, d\lambda} \right)$$

where C_λ = counts as a function of wave length and S_λ = efficiency of the 1P21 phototube as a function of wavelength. The curve for C_λ was obtained from a determination of the bioluminescence energy distribution of a cell-free extract of *A. fischeri*, which had been determined with a Farrand monochromator, placed in front of the quantum counter in such a way that luminescence rates could be conveniently measured as a function of wavelength. Efficiency values for the phototube used in this calculation were arrived at as follows: The efficiency of the photomultiplier in counts times cm^2 times $quantum^{-1}$ at 490 mμ. was determined to be 0.06. From a photosensitivity curve for the 1P21 tube published by the Radio Corporation of America, we constructed a new curve by giving a value of 0.06 for the sensitivity of the tube at 490 mμ. Since the RCA curve gives a value of 0.76 for the relative sensitivity of the tube at 490 mμ, division of the published RCA value by 12.7 gives S_λ for any given wavelength.

The luminescence system on which the quantum efficiency determinations were made generally consisted of 0.2 ml of one of the enzyme preparations previously described, and DPNH, FMN, and dodecyl aldehyde. All components were added in excess except the one whose quantum efficiency was being determined. STREHLER *et al.*[6] showed that the only requirements for bacterial luminescence are luciferase, $FMNH_2$, a long-chain aldehyde, and oxygen. For determinations of quantum efficiency, however, use of $FMNH_2$ was impractical because of its rapid autooxidation and also because relatively high concentrations of FMN will inhibit the luminescence system[8]. Therefore, an $FMNH_2$-generating system that required only catalytic amounts of FMN was used. This system was the FMN-dependent DPNH oxidase, which was present in the enzyme preparations used. Thus the addition of an excess of DPNH would regenerate $FMNH_2$ continuously in the presence of a catalytic amount of added FMN.

RESULTS

In general, we determined the quantum efficiency of each component of the bacterial luminescence reaction by making the component in question limiting, and determining the total light emission from the addition of a known amount of this component.

The results from quantum efficiency determinations (expressed as molecules utilized per quantum emitted) for FMN, DPNH, and dodecyl aldehyde are presented in Table I. For the aldehyde determinations, three independent measurements were

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TABLE I

QUANTUM EFFICIENCY DETERMINATIONS FOR COMPONENTS OF THE BACTERIAL LUMINESCENCE SYSTEM*

Enzyme preparation	Molecules utilized per quantum emitted				
	Dodecyl aldehyde			FMN	DPNH
C-1**	43	40	40	0.28	2800
B-2				0.34	
C-4	20	19	23		

* Protocol for:

(1) Aldehyde quantum efficiency — 0.1 *M* phosphate buffer pH 7.0 (1.0 ml); FMN (2.3 μg); 1% egg albumin (0.05 ml); DPNH (4 μmoles); dodecyl aldehyde (0.05 μg); enzyme (0.2 ml); water to 2.1 ml.

(2) FMN quantum efficiency — 0.1 μg of FMN, 0.3 ml of a saturated water solution of dodecyl aldehyde, and 0.15 ml of BAL (0.15 *M*) were added. All other components were the same as described under (1).

(3) DPNH quantum efficiency — 20 μg of DPNH and 0.3 ml of a saturated water solution of dodecyl aldehyde were added. All other components were the same as described under (1).

** The procedures used in making the different enzyme preparations are given under MATERIALS AND METHODS.

made with each of two enzyme preparations to show the reproducibility of the results. Apparently, enzyme preparation C-4 was more efficient in the utilization of aldehyde than preparation C-1 (see MATERIALS AND METHODS for a discussion of the enzyme preparations).

During the aldehyde determinations, the luminescence rate decayed linearly with time. It was important that we know whether this decay was proportional to the amount of aldehyde that disappeared. Since the amounts of aldehyde used in these determinations were too small for chemical assay, indirect enzymic methods were used. In this procedure, which made use of the luminescence system, we followed the decay of the luminescence rate to one-half the initial value, and at that point added one-half of the initial aldehyde concentration. When this was done, the luminescence rate immediately returned to the original level. In several repetitions of this cycle, quantum efficiency determinations for each half cycle were about the same (see Fig. 2). These results suggest that the decay of the luminescence rate was proportional to the amount of aldehyde utilized during the experiment. Another important point in the aldehyde determinations is that a correct value for the quantum efficiency can be approached only with high concentrations of luciferase. Since at one-twentieth the enzyme concentration used in these experiments, a further decrease in the enzyme concentration by a factor of 4 caused a concomitant 10-fold decrease in the quantum efficiency, concentrations of enzymes were used that tolerated a several-fold variation in enzyme concentration without significantly affecting the efficiency values obtained.

Depending on the enzyme preparation used (Table I), about 20–40 molecules of aldehyde are utilized per quantum of light emitted. These efficiency values can vary, depending on a number of factors, *e.g.*, the concentrations of luciferase and aldehyde, already discussed. Other factors, *e.g.*, the presence of competing reactions for aldehyde utilization, that may affect the value obtained for the efficiency will be discussed.

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Because of the influence that various components may have on the aldehyde efficiency, one cannot predict the value for the efficiency within a living cell. The important observation is that whatever the efficiency value might be, it did not fall below 20 with the best enzyme preparation tested. In addition, evidence to be presented indicated that the aldehyde added to preparation C-4 was being oxidized by luciferase, not by other enzymes in the preparation. These results are in agreement with evidence presented by McElroy and Green[9], who found that the relative total light emission was proportional to the amount of aldehyde added, suggesting that the aldehyde was utilized during the luminescence process.

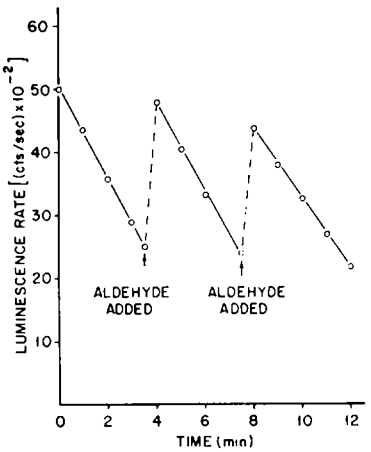

Fig. 2. The relation of luminescence rate to the disappearance of dodecyl aldehyde. Protocol: Enzyme preparation C-4 (0.2 ml); 0.1 *M* phosphate buffer pH 7.0 (1.0 ml); FMN (2.3 μg); 1% egg albumin (0.05 ml); DPNH (4 μmoles); water to 2.1 ml. Initially, 0.05 μg of dodecyl aldehyde was added. At the points indicated 0.025 μg of dodecyl aldehyde was added.

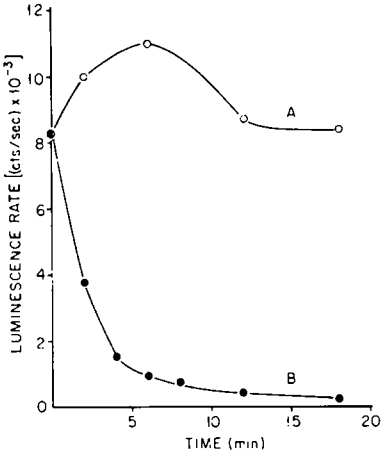

Fig. 3. The effect of a preparation of bacterial luciferase on the disappearance of dodecyl aldehyde. The reaction mixture consisted of enzyme preparation C-4 (0.12 ml), 0.1 *M* phosphate buffer pH 7.0 (1.0 ml), FMN (2.3 μg), 1% egg albumin (0.05 ml), DPNH (4 μmoles), dodecyl aldehyde (0.05 μg), and water to 2.1 ml. Curve A = incubation of all components minus the enzyme for the periods of time indicated. At the end of the incubation period, the enzyme was added and the initial rate of luminescence determined. Curve B = enzyme added at zero time, and the rate of luminescence followed as a function of time.

Table I also shows that the FMN was not destroyed during the luminescence of *A. fischeri* extracts. In the C-1 enzyme preparation, FMN produced 3.6 quanta of light per molecule of FMN present and 2.9 quanta in the B-2 preparation. In each of these experiments it is important to point out that the reaction was stopped at the end of 10 hours, and at that point the luminescence rate was still proceeding at 60% the initial value. Since the experiment was long, we added 2,3-dimercaptopropanol (BAL) to stabilize luciferase. Addition of BAL necessitated slow aeration of the mixture to maintain an optimum oxygen tension. Thus the reaction was allowed to proceed only to the point at which one might safely assume that FMN was indeed recycling in the luminescent reaction. Since it was difficult to remove the last traces of FMN from the enzyme, controls were run in which no FMN was added to the system. The total light emission from a system containing no FMN was, in every case,

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about 7% of that obtained in the FMN experiments, and the corresponding correction was made by subtraction of this blank from the value obtained in the complete system.

In enzyme preparation C-1, it was determined that the oxidation of 2800 molecules of DPNH gave rise to one quantum of light. Presumably, a large percentage of the hydrogens of FMN went directly to oxygen as a result of reduced FMN autooxidation. If this situation approximates that occurring within the cell, luciferase is not very significant as part of an over-all oxidative pathway.

Table II shows the effect of varying levels of FMN on the rate at which dodecyl aldehyde was utilized during the luminescence reaction. It can be seen that, although the total light emission per 0.0125 μg of aldehyde remained essentially constant, the time required for utilization of this amount of aldehyde changed by a factor of 10 from the lowest to the highest level of FMN used. At the three different levels of FMN, the luminescence rate remained a linear function of the aldehyde concentration at the level of aldehyde used in these experiments. The suggestion from these results that $FMNH_2$ is required for the utilization of dodecyl aldehyde during luminescence is supported by two observations made by STREHLER *et al.*[6]: (1) that $FMNH_2$, a long-chain aldehyde, luciferase, and oxygen, are the only required components for bacterial luminescence and (2) that in the presence of the aldehyde, $FMNH_2$ was oxidized at a much faster rate than in its absence. Furthermore, since the aldehyde quantum efficiency did not change significantly over a 10-fold change in reaction time, most of the aldehyde was probably being oxidized by luciferase, at the concentrations of luciferase used, and not by other enzymes in the extract.

TABLE II

EFFECT OF FMN ON THE RATE OF DODECYL ALDEHYDE UTILIZATION*

FMN added (μg)	*Total quanta $\times 10^{-12}$ per 0.0125 μg of dodecyl aldehyde*	*Reaction time (min)*
0.1	1.2	20.0
0.3	1.8	11.8
1.0	1.9	1.9

* Protocol: C-4 enzyme (0.1 ml); 0.1 *M* phosphate buffer pH 7.0 (1.0 ml); DPNH (4 μmoles); dodecyl aldehyde (0.025 μg); 1% egg albumin (0.05 ml); water to 2.1 ml. FMN added as indicated.

The conclusion drawn from Table II—that reduced FMN was required for the utilization of aldehyde—was further substantiated by experiments in which the aldehyde was incubated for 15 minutes in the presence of the complete luminescence system, and the total light emitted was determined at the end of the incubation period. When the preincubation was carried out in the absence of FMN, the total light emitted after the addition of FMN was two to four times that produced in the former experiment. The additional light production when FMN was absent during preincubation lends support to the suggestion that $FMNH_2$ is necessary for utilization of the aldehyde.

The enzyme was also shown to be essential for aldehyde utilization (see Fig.3). In these experiments, the aldehyde was incubated for varying periods with all components of the luminescence system present except the enzyme. At the end of the incubation periods, the enzyme was added, and the initial luminescence rate (curve A)

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was determined. In curve B, the enzyme was added at zero time and the rate of luminescence followed as a function of time. Thus it can be seen that only when the enzyme is present during incubation of the complete system will the initial rate of luminescence be decreased, indicating that the aldehyde disappears only in the presence of the enzyme. Furthermore, the quantum efficiency of the aldehyde, when the enzyme was added after 17 minutes' preincubation with all other components of the system, was the same as that for the aldehyde in the system to which enzyme was added at zero time. The enzyme was presumed to be stable during incubation since the addition of more aldehyde after 17 minutes restored the luminescence rate to the initial level. We do not know why the initial luminescence rate increased upon incubation, but the effect appeared to be real since the experiment was reproducible.

DISCUSSION

McELROY AND GREEN[9] proposed that the energy-yielding process prerequisite to light emission in extracts of luminous bacteria involved the peroxidation of a long-chain aldehyde. This proposal was based on the fact that the energy from the luminous process is equivalent to about 60 kcal/Einstein whereas the peroxidation of 1 mole of aldehyde to the corresponding acid produces 75 kcal. The latter reaction produces more than enough energy for the excitation process. Since the direct oxidation of the aldehyde by molecular oxygen (according to McELROY AND GREEN) does not produce enough energy to support the luminescent reaction, it seems reasonable to suppose that a peroxidation of aldehyde could furnish the energy for the process, as they have suggested.

The results presented in this paper now establish that the aldehyde is indeed used up during the luminescence reaction. Presumably, the peroxidation of aldehyde to the corresponding acid furnishes the energy for the reaction as proposed. In addition, it has been established that FMN is not destroyed during the light reaction, although it is assumed that FMN is the molecule that becomes excited. Since FMN is not utilized during the luminescence process, any significant luminescence pathway that does not result in a regeneration of FMN is ruled out. Furthermore, on the basis of the experiments presented, $FMNH_2$ seems necessary for the utilization of the aldehyde during luminescence.

The over-all bacterial luminescence process, starting with DPNH, can now be expressed somewhat more completely as:

$$\text{DPNH} + \text{FMN} \xrightarrow{\text{DPNH oxidase}} \text{FMNH}_2 + \text{DPN} \qquad (1)$$

$$\text{RCHO} + \text{O}_2 + \text{FMNH}_2 \xrightarrow{\text{Luciferase-FMN}} \text{RCOOH}(?) + \text{FMN} + \text{H}_2\text{O} + \text{light} \qquad (2)$$

DPNH is visualized as being formed through a variety of metabolic pathways. Then in reaction (1), the reduced DPN, in the presence of an FMN-dependent DPNH oxidase, generates reduced flavin. The $FMNH_2$ formed in reaction (1) then reacts with the aldehyde and oxygen, in the presence of a luciferase-FMN complex, to give the products shown in reaction (2). The FMN regenerated [equation (2)] can now recycle and again generate reduced flavin as a result of the first reaction. The exact nature of the product, or products, of aldehyde oxidation will have to await chemical

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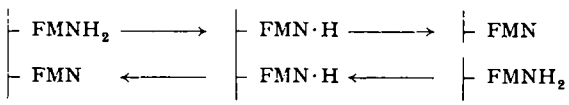
identification. Luminous processes leading to the formation of the corresponding alcohol have apparently been ruled out by McELROY AND GREEN[9].

It is not known whether both flavin molecules on luciferase must be reduced before excitation and subsequent light emission. Since FMN is generally assumed to be the excited molecule in the luminescence reaction, its transformation to a free radical could be a prerequisite for the excitation process. A precedent for the occurrence of free radicals in flavoprotein catalysis was made by BEINERT[12], who presented evidence for the existence of flavin adenine dinucleotide free radicals during the oxidation of fatty acids by flavoproteins.

Such a mechanism is schematically outlined; here there exist on the enzyme surface one molecule of reduced and one of oxidized FMN that could be in equilibrium thus:



The aldehyde could conceivably be attached to the enzyme through the aliphatic chain in such a way as to bring the aldehyde group between the flavin free radicals to form a resonating complex. The two flavin radicals, in the presence of oxygen, could favor the formation of an organic peroxide that in turn might be expected to react with the aldehyde leading to its peroxidation. The energy liberated by the peroxidation of the aldehyde might then be channeled into one of the flavin molecules to bring about an excitation state, which could lead to the emission of a quantum of light.

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SUMMARY

Quantum efficiency determinations for dodecyl aldehyde, FMN, and DPNH were made, various enzyme preparations from the luminous bacterium, *Achromobacter fischeri* being used. From such measurements, it was shown that FMN was not destroyed during luminescence but that dodecyl aldehyde disappeared during the reaction. The lowest value found for the aldehyde quantum efficiency was 20. In one enzyme preparation, about 0.036% of the DPNH was utilized for light production whereas the bulk of the compound was oxidized via other pathways. Reduced flavin mononucleotide and the enzyme, luciferase, were found to be necessary for the disappearance of the aldehyde in the luminescence reaction. The quantum efficiency data support a proposal by McELROY AND GREEN[9] that the peroxidation of dodecyl aldehyde (or of long chain aldehydes in general) furnishes the necessary energy for the excitation of FMN, which subsequently leads to light production. A working hypothesis describing the mechanism has been presented.

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CELLULAR DESTRUCTION AND PROTEIN BREAKDOWN INDUCED BY EXPOSURE TO X-RAYS

II. FURTHER STUDIES USING THE CONCEPT OF THE DYNAMIC GLYCINE POOL*

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The first paper of this series[1] was concerned with an assessment of tissue breakdown subsequent to exposure to X-irradiation by means of measuring the extent of dilution of ^{14}C-activity in the "free glycine fraction"[1] of rats given glycine-2-^{14}C after exposure to X-rays. The present communication deals with another approach to the problem of tissue breakdown, using again the concept of the dynamic glycine pool as defined by ARNSTEIN AND NEUBERGER[2]. In the present study, however, the rate of release of radioactive glycine into this pool is used as a measure of breakdown of proteins labeled extensively with glycine-2-^{14}C prior to exposure of rats to X-rays. In adopting the release of ^{14}C-labeled amino acid residues from tissue proteins as an index of radiation damage to tissue it is assumed that tissue proteins liberated from cells destroyed by ionizing radiation are rapidly catabolized by intracellular proteases or other catabolic reactions. In this way, increased amounts of ^{14}C-glycine and of other ^{14}C-labeled glycine precursors should appear in the "free glycine pool" assessible by means of the isotope concentration in the glycine moiety of urinary hippuric acid. Furthermore, it is assumed that ^{14}C-labeled glycine originating from tissue proteins is the only major source contributing ^{14}C-activity to glycine in urinary hippuric acid. This assumption appears justified in view of the fact that at the time of irradiation,

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