

ON THE USE OF LIQUID SCINTILLATION COUNTERS
FOR CHEMILUMINESCENCE ASSAYS IN BIOCHEMISTRY

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ABSTRACT

In order to help interpreting the results obtained with commercial firefly luciferase preparations, their active components were assayed and the conversion of ATP studied with labelled nucleotides. A quenched scintillating solution containing tritium was used as a reference for photon counting. The use of scintillation counters for the assay and study of oxygenases and oxidases is discussed.

INTRODUCTION

About ten years have elapsed since the use of liquid scintillation counters was extended to the measurement of bioluminescence in the microassay of ATP with firefly luciferase. Although such instruments may have superfluous features they have ever since proven reliable and adequate not only for the assay of ATP and related substances with firefly luciferase, but also for that of flavine and pyridine nucleotides with the bacterial system. Several survey articles have covered the status of the art in recent years (1, 2, 3). In the present paper we wish to report more specifically on the use of scintillation counters for the study and assay of other biochemical reactions, involving oxidases and oxygenases, as well as on some recent experience with luciferases.

LUMINESCENCE MEASUREMENTS

A problem often encountered in luminescence countings is the lack of a stable source delivering single discrete photons. Such a source is essential when checking for the efficiency or reproducibility of scintillation counters and when optimizing the settings for high voltage, amplifi-

cation and discriminators. As mentioned earlier (2, 3), one cannot rely on the settings found adequate for the counting of tritium. The use of luminol as a standard has been described by Lee *et al.* (4). However, the concentrations used by these authors produce light levels much higher than those suitable for scintillation counters. Scaling down the reaction is not an easy matter because of the reagent background (5) and of the tricky character of the reaction. A stable luminescence source, based on the use of a carbon-14 source in a liquid scintillator, was developed by Hastings *et al.* (6,7). This kind of standard is quite adequate in conjunction with analog instruments but is not suitable as a reference source for photon counting because of the production of multi-photon flashes. The use of a light-emitting diode driven by a small mercury battery has been suggested by Stanley (3). When trying to solve the problem ourselves, satisfactory results were obtained by incorporating a tritium source in a liquid scintillator, quenched so as to emit only single photons. Adequate quenching is easily achieved by the addition of carbon tetrachloride until the number of counts registered with the coincidence switched on falls to 1%, or preferably less, of that registered with the coincidence left out. The number of two-photon pulses falling on either one of the photomultipliers is not exactly the same as the number of such pulses distributing themselves between the two photomultipliers (8). The number of coincidence pulses is therefore not perfectly representative but offers a sufficient approximation. A rather high degree of quenching is necessary to eliminate two-photon pulses ($> 99\%$) and the initial radioactivity should therefore be chosen in accordance. Care should also be taken to repeat the test when changing the high voltage or discrimination levels as the ratio of two-photon vs. one-photon pulses may increase with higher voltages or higher discrimination levels. The validity of the present method was checked by analyzing the energy spectrum of the emitted light. The setup used for this purpose was equipped with an RCA 8850 photomultiplier, specially designed for single-photon counting, and a SA41 400-channel analyzer. The quenched tritium spectrum was compared with the phosphorescence spectrum of a scintillation vial cap that had been exposed to intense light (see figure 1). This spectrum also matches that obtained for firefly bioluminescence. If desirable, the emission spectrum of the present reference solution could be made to match more closely that of the luminescent reaction being studied by the addition of a suitable secondary scintillator.

Any tritium-labelled compound available in the laboratory is in principle eligible for the preparation of a refe-

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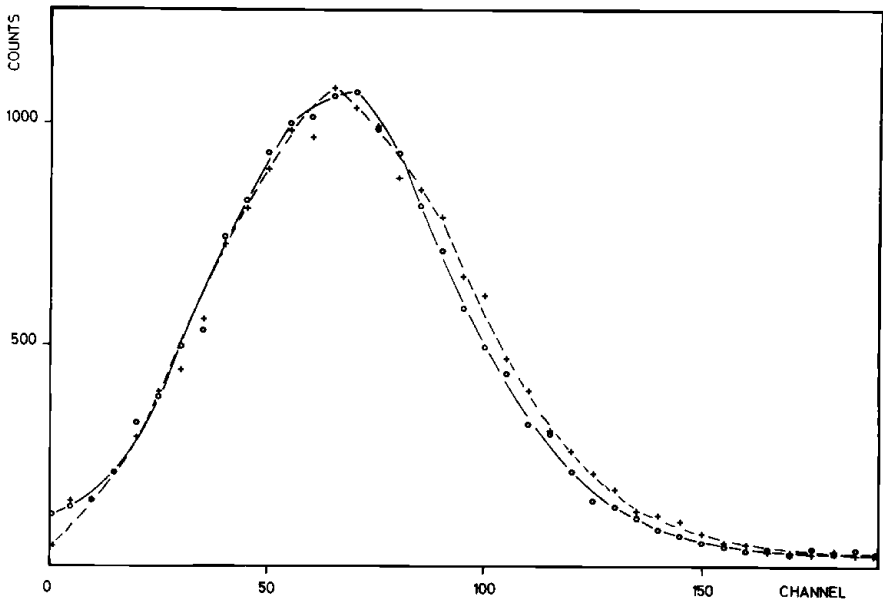


Fig. 1. Energy spectrum recorded with SA41 multichannel analyzer:
a) tritium in scintillating solution containing PPO and POPOP and quenched with CCl_4 (—)
b) phosphorescence of scintillation vial cap (---)

rence luminescence source. We wish however to stress the fact that when hydrophilic substances are dissolved in scintillating solutions containing emulsifying agents as Triton-X-100, temperature has to be controlled very carefully. Several authors have indeed shown the counting efficiency of emulsions to be rather sensitive to slight temperature changes (9). This sensitivity is still more apparent when single photons are counted with the coincidence left out, amounting to a 3 % increase of the count rate for a one centigrade drop. A lower degree of temperature dependence was observed when using tritium-labelled toluene in regular scintillating solutions.

Our luminescence standard allowed us to check our counting equipment for long-term stability as well as for short-lived fluctuations. For a Packard model 2002 counter the fluctuations observed over one minute periods were somewhat larger than statistically predicted (half of them exceed twice the standard deviation) but are not in general higher than 1 %. The absolute calibration of the standard solution just described would provide an easy tool for the estimation of the quantum efficiency of bio- and chemiluminescent reactions, and which can be used in precisely the same geometrical conditions as the sample being assayed.

LUMINESCENCE PRODUCED BY LUCIFERASES

The use of scintillation counters for bioluminescence measurements in analytical biochemistry has been discussed by Stanley (see present volume) and we shall therefore restrict ourselves to a few recent contributions from our laboratory.

First of all we wish to mention the work performed by E. Gerlo and J. Charlier (10) on the separation of the enzymes involved in bacterial bioluminescence. The MAV strain used for this purpose, kindly supplied by J.W. Hastings and at present identified as *Beneckea Harveyi*, was preferred to the wild type *Photobacterium Fischeri* because the luciferase is more easily separated from the FMN reductase (NADH-dehydrogenase). In the course of this separation it occurred to Gerlo and Charlier that two FMN reductases were actually present in the extract, specific for NADH and NADPH. These enzymes were separated by chromatography on DEAE-Sephadex and purified by a factor of 90 and 140. They were further identified by studying several of their characteristics: molecular weight, thermostability, relative specificity for NADH and NADPH, dissociation constants of the binary and ternary complexes (with FMN). These findings open new possibilities

for the selective assay of NADH and NADPH, which can be individually measured in the presence of a 50-fold or even higher excess of the other nucleotide.

As far as firefly luciferase is concerned it should be observed that most routine assays are still performed with commercial enzymes that are impure. The practical implications of this on the specificity and the time-course of the luminescence have been reexamined very thoroughly in a recent paper by Lundin and Thore (11). In our opinion interpretation of the results is often impeded by the fact that the enzyme content of luciferase preparations is not known. They may further contain varying amounts of luciferine. In order to characterize commercial preparations in this respect a simple method was used for the rapid chromatographic fractionation and the fluorimetric titration of the enzyme with dehydroluciferine. One ml samples, containing the equivalent of ca. 10 mg firefly organs, are brought on a 1.28 x 10 cm Sephadex G-25 column (particle-size 50-150 μ) and eluted successively with glycyglycine buffer, pH 7.4, 0.025 M, and pure water to recover the luciferine (the present technique was adapted from Nielsen and Rasmussen, 12). Fractions 1.5 ml in size are analyzed for their fluorescence and, after addition of ATP, Mg^{++} and luciferine, for their bioluminescence (see figure 2). The first peak shows a spectrum typical for proteins and contains all of the luciferase together with contaminating enzymes. The second peak, identified by its strong fluorescence at 414 nm is well separated but was not identified thus far. The fractions containing luciferase are pooled and aliquots titrated with dehydroluciferine in an Aminco spectrofluorimeter, as described by DeLuca and McElroy (13). Dehydroluciferine was prepared by refluxing 10 mg of luciferine in one ml of 1 N sodium hydroxide for 4 hours (14) and isolated by chromatography on Sephadex G-25 under conditions similar to those described above.

The firefly extracts tested with the present method were purchased from Sigma (cat.nr. FLE-50) and from Boehringer (cat.nr. 15480). Assays performed on the luciferase fraction gave similar figures for the enzyme contents of both preparations, i.e. 3.8 and 2.7 x 10⁻⁷ equivalents per 100 mg organs. The figures were also similar when expressed as a function of the protein content, based on the optical density at 280 nm. Luciferine was estimated by the spectrophotometric assay of the pooled fractions obtained upon elution with water. The fluorescence excitation and emission spectra were also recorded and found typical for luciferine. Higher figures were obtained for Sigma than for Boehringer relative to the enzyme content (the molar ratios were 1.0

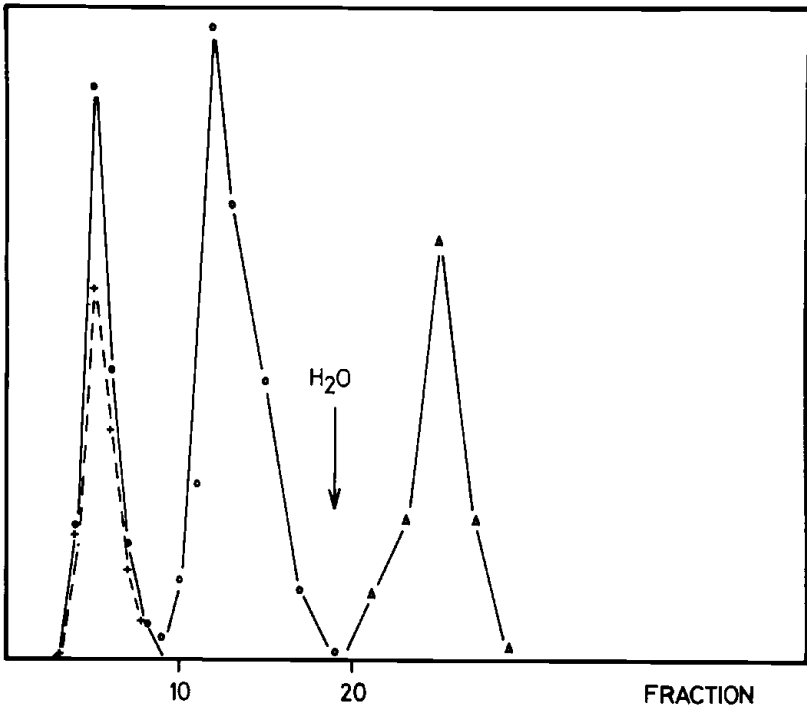


Fig. 2. Chromatography of commercial firefly luciferase.
 Ordinates (arbitrary units):
 Fluorescence: (*) excit. 280 nm em. 350 nm
 (o) " 348 " 414
 (Δ) " 330 " 530
 Bioluminescence: (+)

and 0.2 respectively). It should on the other hand be mentioned that the Boehringer preparation is perfectly soluble while that of Sigma contains suspended material which sticks on top of the column, or sediments upon centrifugation. Part of the luminescence was found to be associated with this precipitate. Due to the limited number of batches at hand no systematic tests could be performed up to now and the present results may therefore not be statistically valid. From the figures obtained it may nevertheless be concluded that the amount of enzyme routinely used for the assay of ATP with scintillation counters is several orders of magnitude higher than that of ATP. End-product inhibition is therefore not likely to occur under the usual conditions of assay and is at least not responsible for the gradual decrease of the luminescence. The complex time-course of the luminescence cannot be accounted for either, on the mere basis of the exhaustion of the substrate. Experiments performed with ^{14}C -labelled adenine nucleotides on the enzyme fraction obtained by our procedure have indeed shown the radioactivity of ATP to appear rather rapidly in ADP. The observed facts are in favour of an equilibrium between the several adenine nucleotides, which might account for the rapid initial decrease and the long tailing of the luminescence curve obtained with unpurified luciferase preparations.

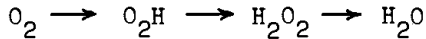
CHEMILUMINESCENCE INITIATED BY OXIDASES AND OXYGENASES

In the past years study of chemiluminescence in biochemistry has often concentrated on a series of reactions catalyzed by specific "luciferases". Beside the fact that these reactions show fascinating biological aspects, their specificity and high quantum yield make them suitable for a wide array of analytical applications, for which liquid scintillation counters are now routinely used. Bioluminescence is, however, not restricted to these specific cases and low level luminescence has been observed for many years in living cells not suspected of containing any luciferase. Light emission has been reported in mitochondrial preparations, in microsomal extracts, in the course of phagocytosis (see English et al., present volume), also. Although oxidation reactions have long been considered responsible for these phenomena, interest in these reactions has recently increased, due to several important discoveries:

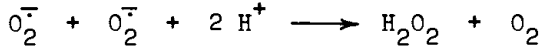
- the involvement of superoxide ions (O_2^-) in several biochemical reactions and physiological processes;
- the ubiquitous presence of superoxide dismutase (SOD) in the cells of aerobic organisms (15, 16).

New insights are developing as far as the direct involvement of oxygen in biological oxidations is concerned and the classification of some of the responsible enzymes might even have to be reconsidered. A comprehensive and critical survey of these enzymes is to be found in a publication of Hayaishi(17).

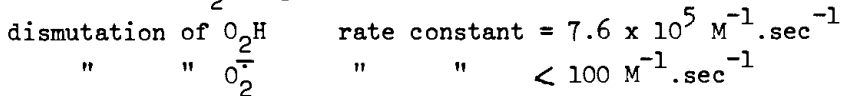
The reduction of O_2 concomitant with the oxidation of a substrate molecule can be considered to occur in discrete steps:



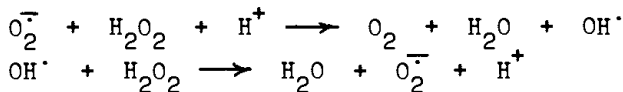
If O_2 is not fully reduced to water, as occurs with cytochrome oxidase, O_2 and H_2O_2 are likely to appear as intermediates. Superoxide radicals are rather unstable and will disproportionate spontaneously according to the following reaction: (18)



The speed of this reaction is, however, much dependent on the dissociation of O_2H ($pK = 4.8$): (19)



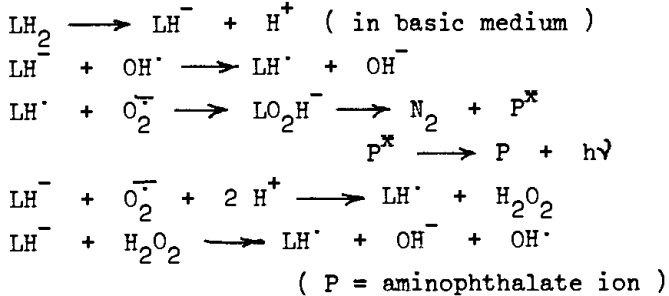
These facts account for the existence of superoxide ions at physiological pH and probably also for the toxicity of oxygen. The presence of superoxide dismutase, which catalyzes the dismutation of these ions, is therefore essential for the survival of aerobic cells. The study of reactions involving superoxide ions may be complicated by their interconversion in the presence of H_2O_2 (produced or not by the dismutation of superoxide radicals) according to the Haber-Weiss mechanism (20):



Hydroxyl radicals are potent oxidants and might therefore be the molecular species ultimately responsible for the toxicity of oxygen.

Superoxide radicals can trigger the production of light in several ways. First of all their dismutation will produce oxygen in the singlet excited states Δ_g or Σ_g^+ . The energy of transition to triplet oxygen in the ground state corresponds to light emission in the red region (760 or 1260 nm) which is normally not registered by regular photomultipliers. Emission may however be detected through the formation of excited dimers (oxygen excimers). It has further been observed that the luminescence due to superoxide ions could be enhanced by bicarbonate anions. This effect has been

ascribed by Stauff (18) to the recombination of intermediary carbonate radicals. Much higher amplification can still be obtained by the addition of luminol (LH_2) which, according to present knowledge would react in the following way (21, 22):



As may be deduced from the above equations and from the Haber-Weiss reaction, superoxide radicals will be able to generate luminol luminescence by themselves, contrary to H_2O_2 . This last substance will, however, enhance the luminescence when superoxide ions are already present. All these facts should be carefully born in mind when using luminol for the study of the luminescence induced by oxidases and oxygenases.

Superoxide radicals are produced in a variety of biochemical systems (xanthine-oxidase, aldehyde-oxidase, flavoprotein oxygenases, aso) while other reactions will preferentially generate hydrogen peroxide (D-amino-acid oxidases, glucose oxidase aso.). The luminescence associated with these reactions or which can be induced by the addition of luminol, has become a very sensitive tool, not only for analytical purposes, but also for the study of the basic mechanisms involved in these reactions. In the last instance the appearance of specific oxygen intermediates can often be inferred from the influence of adequate inhibitors on the time-course of the luminescence. Examples of such inhibitors are SOD (superoxide dismutase) for superoxide radicals, catalase for hydrogen peroxide, scavengers like ethanol for hydroxyl radicals also. As for the reactions involving luciferases, scintillation counters have proven very useful for the kinetic study of such biochemical systems. Because of the interconversion of the oxygen intermediates the results should nevertheless be interpreted very cautiously and the answer will not always be straightforward.

The systems tested in our laboratory involve xanthine-oxidase, horse-radish peroxidase and reduced FMN. The xanthine-oxidase system is well known for the production

of superoxide radicals and its luminescence has been studied by means of scintillation counters by Arneson (23) and by Schaap *et al.* (24). While our own experiments with luminol and inhibitors were in progress similar work was reported by Hodgson and Fridovich (22) showing the inhibitory effect of both catalase and superoxide dismutase on the luminescence. We could further observe a cooperative effect between these inhibitors as well as the inhibitory effect of ethanol. Spectrophotometric recording of the urate formation shows that

- the formation of superoxide radicals is a result of the enzymatic reaction and inhibition of the luminescence does not interfere with the actual oxidation of xanthine;
- the luminescence produced can be turned to use as a very sensitive method of assay for xanthine or xanthine-oxidase.

In all these experiments the enzymatic reaction is performed at pH 10.2 in order to fit with the pH requirements of the luminol reaction.

The reaction products of the xanthine-xanthine oxidase system (uric acid and hydrogen peroxide) happen to be substrates for horse-radish peroxidase. When mixing both enzymes a coupled system was obtained which produced a luminescence increased by a factor of 5-10 compared with that obtained for xanthine-oxidase alone.

Reduced FMN is very unstable and reoxidizes spontaneously with a half-life of the order of a second. Because of the known production of hydrogen peroxide in this reaction it seemed interesting to study the effect of inhibitors on the luminescence produced by the addition of luminol (25). FMN was reduced with dithionite according to Gerlo and Charlier (10). Inhibition was observed for both superoxide dismutase and catalase.

All our luminescence measurements were performed with either a Packard (model 2002) or an Intertechnique (model SL20) scintillation counter.

The use of oxygenase induced chemiluminescence for analytical purposes is best illustrated by the method described by Bostick and Hercules (26) for the automated determination of glucose with immobilized glucose oxidase. Hydrogen peroxide, but no superoxide radicals, is produced in this reaction and potassium ferricyanide has therefore to be added to the luminescent system. Another feature of this method is the fact that the oxidation of glucose and the luminescent reaction are occurring sequentially with the intercalation of a pH jump. The method would seem to be applicable to other substrates yielding hydrogen peroxide in neutral solution.

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