

Chapter 1

Chemiluminescence as a Problem and an Analytical Tool in Liquid Scintillation Counting

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INTRODUCTION

Within the last 10 years the liquid scintillation counting technique has become the generally preferred method for the measurement of low energy β -emitting radioisotopes such as tritium, carbon-14, calcium-45 or sulphur-35. Due to its high sensitivity and efficiency and to the great progress which has been made in standardising the instrumentation and data handling equipment this technique has increased significantly in applicability and popularity.

Nevertheless there may occur certain reactions and factors which can disturb radioactive measurements and one of these factors is chemiluminescence.

In the past, chemiluminescence in liquid scintillation counting has been called the 'bête noir'.¹ Indeed it caused a great deal of trouble and has contributed to many errors in quantitative determination of radioactive material by the liquid scintillation technique. Only in recent years the cause and the physico-chemical nature of these luminescence phenomena, as well as the factors involved, have been thoroughly investigated.²⁻⁶ So today it is quite possible to calm down or to tame this 'bête noir' or to avoid any contact. In this respect the main part of this chapter will deal with the problems of chemiluminescence which may occur in liquid scintillation counting, and we shall report some hints and notes on how to overcome these problems in tracer experiments.

On the other hand chemiluminescence reactions are of interest and importance in organic and bio-organic chemistry and can be used for analytical purposes in non-radioactive assays with great benefit. For these assays liquid scintillation counters are quite adequate measuring devices permitting a highly sensitive quantification. Therefore a second and smaller part of this chapter is concerned with chemiluminescence as an analytical tool. I am sure that this note is of special interest for all researchers using liquid scintillation counters, as well as for the constructors and manufacturers of these instruments, because the analytical use of chemiluminescence reactions may extend to a broader application of liquid scintillation counters.

PART I

The basic concept of the liquid scintillation technique makes it necessary that the radioactive sample material is in *intimate* contact or in *actual solution* together with the phosphor solutes, in order to obtain maximum counting efficiency by maximum energy transfer and photon yield. The following solvents and solutes have most successfully been used in liquid scintillation counting:

SOLVENTS

TOLUENE, DIOXANE,
METHANOL, ETHANOL,
METHYL-, ETHYL-,
BUTYL-GLYCOL,
XYLENE,
TRITON X-100,
POLYGLYCOETHERS.

SOLUTES

PPO, POPOP,
BBOT, BIS-MSB,
PBD, DIMETHYL-POPOP,
BUTYL-PBD,
NAPHTHALENE,

From among the many liquid scintillation solutions which are used in various laboratories all over the world we have selected the following 17 cocktails (Fig. 1.) with qualitative and quantitative varying composition and we have investigated their sensitivity and reactivity for chemiluminescence reactions.

It can be seen from this list that many scintillation solutions contain dioxane in order to make them miscible with water containing samples.

Unfortunately the range of sample materials, especially of biological samples, that can be dissolved in such solvents is severely limited.

Besides other methods of sample preparation some basic solubilisers, as listed below, are used to digest or dissolve such biological materials as, amino acids, polysaccharides, nucleic acids, proteins, cells and entire tissues.

BASIC SOLUBILISERS

AQUEOUS OR ALCOHOLIC KOH OR NaOH
HYAMINE-10 X, NCS, PRIMENE 81-R,
PHENYLETHYLAMINE, SOLUENE-100

Although initially the quaternary ammonium bases were regarded as 'universal' solubilising agents, there are some limitations and disadvantages such as severe quenching, colour formation or chemiluminescence, which may occur by the use of HYAMINE, NCS or SOLUENE.

For many years investigators have observed spurious counts and high background levels in liquid scintillation counting, especially when radioactive biological materials solubilised by strong organic or inorganic bases were measured.

This 'photo-luminescent effect' was first attributed to proteins reacting with the quaternary ammonium bases.^{7, 8} In recent years the causes of the unwanted chemiluminescence reaction have been somewhat better understood and investigated and will be reported here. It should be mentioned that phosphorescence or static discharges are not involved here and are outside of the scope of this work.

(1) PPO POPOP Toluene	4 g 50 mg 1000 ml	(6) PPO bis-MSB Toluene	5 g 0.5 g 1000 ml	(11) PPO POPOP Naphthalene Ethylglycol Methanol Dioxane	4 g 0.2 g 60 g 20 ml 100 ml 1000 ml	(15) Toluene-based Scintillator solution for Soluene™ [Prototype from Packard]
[Bray, <i>Anal. Biochem.</i> 1, 279 (1960)]						
(2) PPO POPOP Toluene Methylglycol	2.6 g 80 mg 300 ml 500 ml	(7) PPO bis-MSB Naphthalene Dioxane	5 g 0.5 g 120 g 1000 ml	(12) PPO Methylglycol Toluene	6 g 600 ml 1000 ml	(16) Insta-Gel [Packard]
[Mahin and Lofberg, <i>Anal. Biochem.</i> 16, 500 (1966)]						
(3) PPO POPOP Naphthalene Dioxane	4 g 75 mg 120 g 1000 ml	(8) Butyl-PBD Toluene	7 g 1000 ml	(13) NE 233 (Toluene-based)		(17) PPO bis-MSB Toluene + Triton X-100 (2 + 1) 5 g 0.5 g 1000 ml
[Butler, <i>Anal. Chem.</i> 33, 409 (1961)]						
(4) BBOT Naphthalene Toluene Methylglycol	4 g 80 g 600 ml 400 ml	(9) Butyl-PBD Naphthalene Dioxane	7 g 100 g 1000 ml	(14) NE 250 (Dioxane-based)		
(5) PBD Naphthalene Dioxane	133 mg 33 g 1000 ml	(10) PPO POPOP Naphthalene Dioxane	7 g 50 mg 50 g 1000 ml			
[Nuclear Enterprises Ltd.]						

Fig. 1

METHODS AND RESULTS

In our experimental procedure for quantitative determination of the intensity of chemiluminescence reactions 1.0 ml of the basic solubilising agent was added to 10.0 ml of scintillation cocktail or solvent in the counting vial and the light impulses were measured 30 s later over a period of 6 s in a liquid scintillation counter, at a temperature of 10°C. If not otherwise mentioned the high voltage of the photomultipliers of the instrument were adjusted in the same manner as for carbon-14 integral counting.

The chemiluminescence measurements were performed with Nuclear Chicago liquid scintillation counters (three channel models 725 and Mark II) and two Packard instruments (Tricarb 3003 and Tricarb AAA 3380/544).

As bleaching agents hydrogen peroxide (30%) and benzoyl peroxide (saturated solution in toluene) were used. All reagents were of 'analytical grade'.

Whilst investigating 17 scintillation solutions it was found that dioxane containing cocktails gave highest chemiluminescence values while toluene or toluene-methylglycol based cocktails had minor chemiluminescence intensities.

An example of the duration and decay of chemiluminescence, shown in Fig. 2, indicates that the count rates are initially very high, fall exponentially over several orders of magnitude within the first 30 mins but remain elevated for several hours over the background baseline.

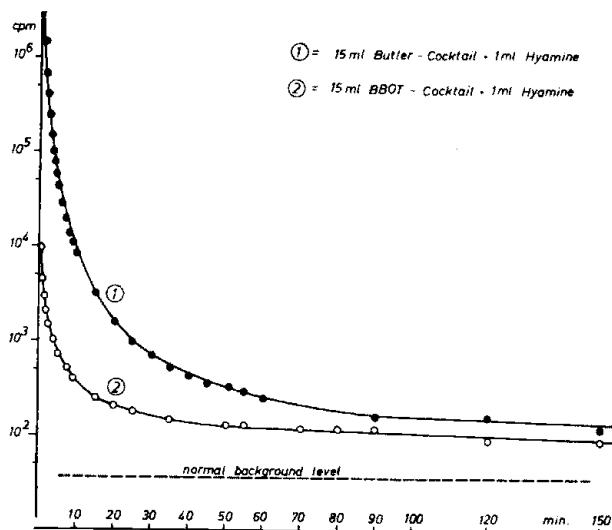


Fig. 2: Decay and duration of chemiluminescence reaction at 10°C in two scintillation mixtures containing 1.0 ml HYAMINE 10-X (counts on log-scale).

Because some liquid scintillation counters are operated with a refrigerated system while others run at ambient temperature, it was of interest to know the influence of temperature on the degree of chemiluminescence. For the investigation of the temperature dependence, 10.0 ml of our Butler Cocktail (No. 3) and 0.5 ml HYAMINE were mixed and measured at different temperatures. The results are shown in Fig. 3 and demonstrate that the chemiluminescence reaction is more intensive at higher temperatures.

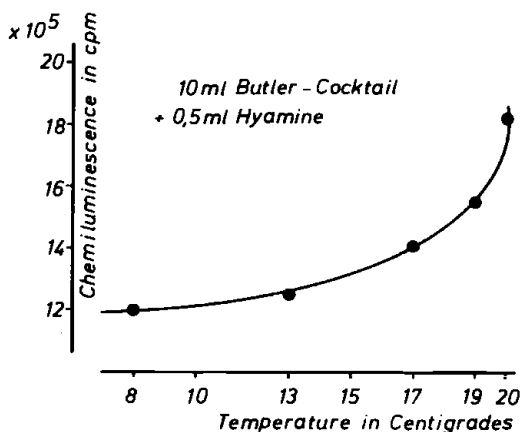


Fig. 3: Influence of temperature on the intensity of the chemiluminescence reaction.

It was further found that the different solubilising agents exerted quite different chemiluminescence intensities as may be seen from Table 1.

Table 1. Chemiluminescence of 0.5 ml Base and 10.0 ml Butler Cocktail.

HYAMINE	8.7×10^5 cpm
NCS	42.4×10^5 cpm
SOLUENE	61.2×10^5 cpm

This effect may be related to their different solubilising potency. While soft tissues and cells are easily digested by these solubilisers, harder materials such as cartilage, bone and collagen fibres will not dissolve completely. SOLUENE is the most potent of these solubilisers and in contrast to HYAMINE did not produce any colour formation.

As was demonstrated in our experiments the chemiluminescence reaction was always more pronounced when the solvents contained scintillator solutes. To investigate the effect and participation of solutes on the chemiluminescence reaction the photon yield of a mixture of dioxane and HYAMINE with increasing amounts of naphthalene dissolved in dioxane was measured. The results of this experiment are shown in Fig. 4.

Although a mixture of naphthalene and HYAMINE alone did not emit any light, there was a significant increase in chemiluminescence depending on the amount of naphthalene dissolved in the dioxane. These findings indicate that naphthalene itself may not react with HYAMINE, but it makes the dioxane more transparent, so that the photomultiplier of the counter will 'see' more light impulses. On the other hand it cannot be excluded that naphthalene (and this may be true for other scintillator solutes too) may participate in some, as yet unknown way in the chemiluminescence reaction. Naphthalene as well as the scintillator solutes may improve the energy transfer efficiency of the solvents.⁹

Investigations as to the cause and physico-chemical nature of chemiluminescence in liquid scintillation counting have made clear that organic peroxides react in an alkali

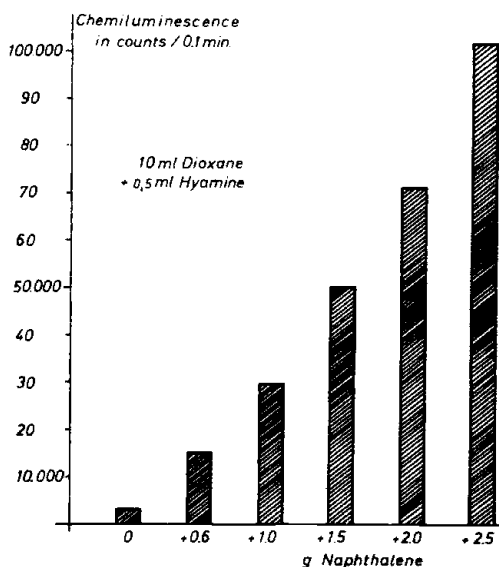


Fig. 4: Effect of naphthalene (dissolved in dioxane) on the intensity of chemiluminescence registered by a liquid scintillation counter.

medium to produce emission of light (see Hercules⁵ and Gundermann¹⁰). Neither proteins nor other substances from biological samples appear to be involved in this phenomenon. To demonstrate that chemiluminescence is due to peroxides present in dioxane and toluene as contaminants, these solvents were shaken with small amounts of hydrogen peroxide and measured after addition of HYAMINE.^{2, 3} The result was an intense stimulation of light emission. As reported by Winkelman and Slater,¹¹ the increase of chemiluminescence was even more pronounced, when benzoyl peroxide rather than hydrogen peroxide was used.

During sample preparation for liquid scintillation counting, solutions of digested tissues, blood or urine in HYAMINE or NCS very often become coloured depending on the haem or pigment concentration in the digested material. This colour increased with the temperature and duration of the solubilising process. To avoid colour quenching, bleaching agents, such as hydrogen peroxide,⁸ benzoyl peroxide^{12, 13} chlorine water,¹⁴ sodium borohydride¹⁵ have been used for decolourisation. Benzoyl peroxide was found to be the most satisfactory agent and the least quenching among those examined.¹²

Studies by Winkelman and Slater¹¹ and those in our laboratory have shown that mixtures of benzoyl peroxide, basic solubilising agents and scintillation cocktails will produce very intense chemiluminescence (Fig. 5). This can last not only for hours but for days and even weeks.

With regard to the fact that benzoyl peroxide may cause severe and long lasting chemiluminescence in scintillation mixtures containing *strong basic agents*, the use of this bleaching agent can be quite problematic.

Although there are quantitative differences in the intensity of chemiluminescence between basic solubilisers, such as HYAMINE, NCS, sodium and potassium hydroxide, our experimental results have indicated that an alkaline medium is essential for the

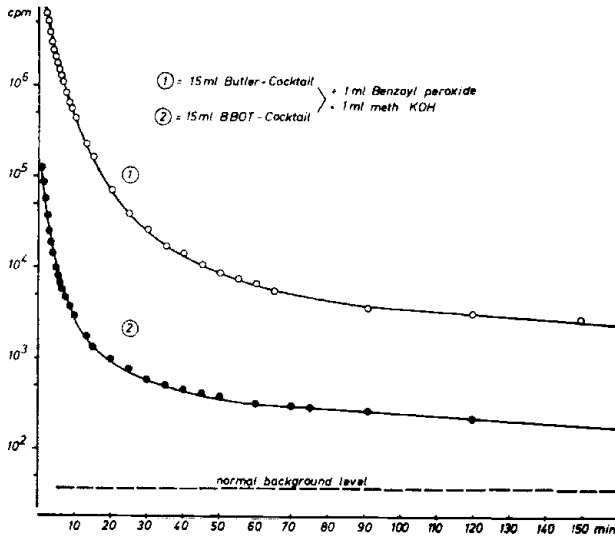


Fig. 5: Chemiluminescence decay curves of liquid scintillation mixtures containing benzoyl peroxide and methanolic KOH (counts on log-scale).

chemiluminescence reaction in standard scintillation solutions. Addition of acid to a neutral pH or lower than 7.0 will generally stop luminescence.

Chemiluminescence reactions in liquid scintillation mixtures are of quite low energy and may for this reason interfere even more in tritium counting. To demonstrate this a mixture of 1.0 ml benzoyl peroxide, 1.0 ml NCS and 15.0 ml solution D (Butler

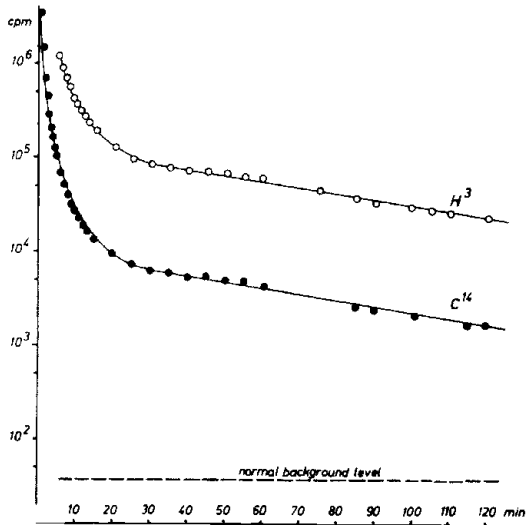


Fig. 6: Chemiluminescence curves of a mixture of 1.0 ml benzoyl peroxide, 1.0 ml NCS, and 15.0 ml Butler Cocktail measured at carbon-14 and tritium settings of the counter (counts on log-scale).

Cocktail) was measured at a high voltage setting of the counter for carbon-14 and tritium. As can be seen from Fig. 6, the chemiluminescence is much higher when measured in the tritium range, although the decay curves are quite similar in shape.

Until recently, chemiluminescence was known only to occur in an alkaline medium but, as we will see later, it is possible to reduce or eliminate chemiluminescence by just neutralising or acidifying the alkaline digest.

A few months ago during a comparative investigation¹⁶ on sample preparation methods, solutes and solvents for liquid scintillation counting, a chemiluminescence was

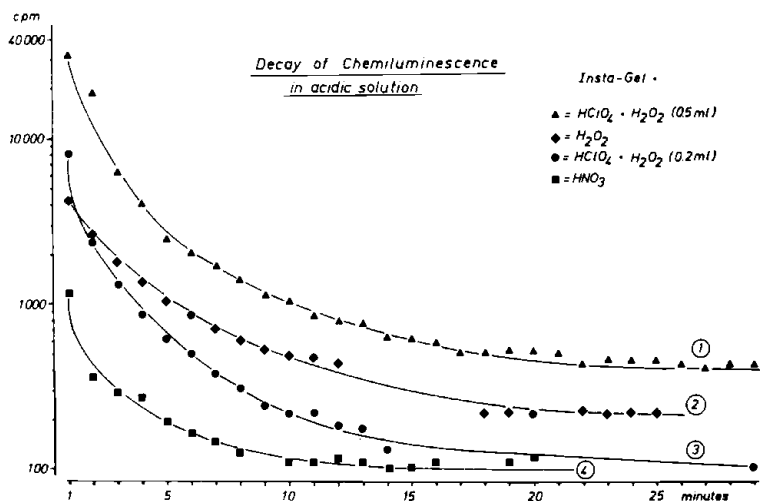


Fig. 7: Chemiluminescence from oxidising acids in Insta-Gel.

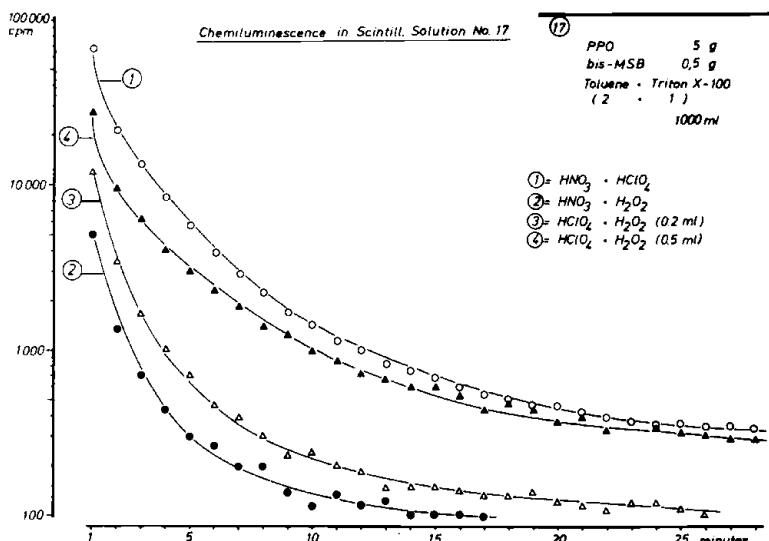


Fig. 8: Chemiluminescence from oxidising acids in emulsion cocktail No. 17 (see Fig. 1).

observed to occur when some biological material after wet combustion with perchloric acid and hydrogen peroxide were mixed with the emulsion cocktail Insta-Gel or the scintillation solution of Patterson and Greene.¹⁷

As may be seen from Fig. 7, further studies confirmed that the liberation of molecular oxygen from oxidising acids (such as nitric or perchloric acid) or from hydrogen peroxide in the presence of surface active gelifying agents (polyglycol ethers) can result in long-lasting chemiluminescence.

Quite similar effects were found in the toluene-Triton X-100 based cocktail of Patterson and Greene, as shown in Fig. 8.

CONCLUSION

To eliminate and overcome problems of chemiluminescence it is at first necessary to differentiate between chemiluminescence reactions in homogeneous, toluene or dioxane based cocktails and those in emulsion cocktails. For homogeneous scintillation solutions we recommend the following points:

1. In contrast to dioxane which is notorious for the formation of peroxides on contact with air, toluene-based scintillation mixtures generally contain much less peroxides and should be used preferentially in connection with basic solubilisers such as NaOH, KOH, HYAMINE, NCS.
2. The use of peroxides as bleaching agents should be avoided in basic solubilisers.
3. If biological samples are used, which had been digested in basic agents the samples should be neutralised to pH values equal or lower than 7.0 by the addition of acid. It should be mentioned however, that acid may increase quenching and may not always be sufficient to eliminate chemiluminescence.¹¹
4. All counting samples of a pH higher than 7.0 should be checked for chemiluminescence effects in order to avoid counting errors. If chemiluminescence is found in the prepared counting sample it is advisable to store the sample at room, or higher temperatures until the luminescence has decayed to a tolerable level.
5. If dioxane-containing scintillation solutions have to be applied, it is advisable to use acidic solubilising agents or the perchloric acid/hydrogen peroxide technique of Mahin and Lofberg¹⁸ for the digestion of biological materials.

When using emulsion cocktails in combination with the wet combustion technique of Mahin and Lofberg¹⁸ or with other oxygen liberating acids we recommend a pre-alkalised (i.e. Insta-Gel containing 1 N sodium hydroxide) scintillation solution which brings the pH of the final mixture to a value of 5 to 6. This can be done without remarkably decreasing the counting efficiency.

Houtman¹⁹ recommended BHT (di-*t*-butyl-4-hydroxy-toluene) as an antioxidant to reduce or eliminate chemiluminescence. Our results however, shown in Fig. 9, indicate that it is quite impossible to eliminate the luminescence completely by the addition of BHT even at concentrations up to 1 g per 10 ml scintillation solution; but as can be seen, BHT even contributes to chemical quenching, we therefore do not support the idea of Houtman.¹⁹

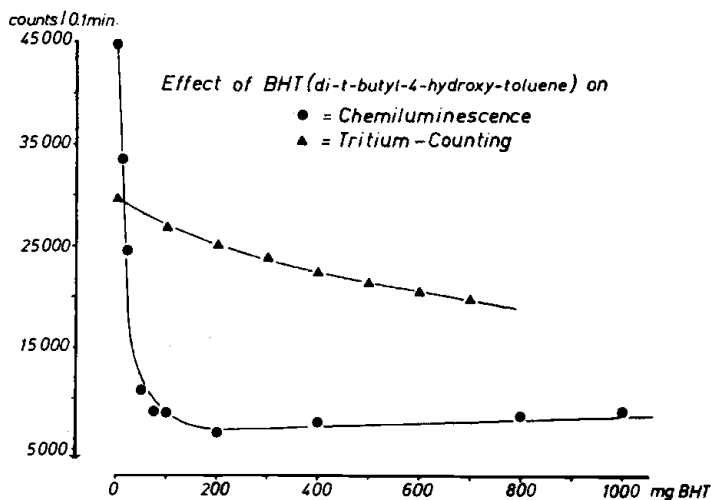


Fig. 9: Influence of BHT on luminescence and tritium counting.

PART II

Chemiluminescence as an analytical tool

Since the development of highly sensitive photodetectors and photomultipliers about 20 years ago, chemiluminescence phenomena in organic chemistry have been extensively studied. They have been found to occur in a great number of organic and bio-organic reactions. Although the reaction mechanism of many light emitting chemical processes is not yet completely understood, the various factors that may determine the quantity and efficiency of the light emission have been investigated and are relatively well known.¹⁰

Quantitative microchemical assay

The intensity of the luminescence reaction, for example, which occurs in a mixture of luminol and hydrogen peroxide, is known to be dependent on the concentration of metal salts that catalyse the reaction. Based on this and other chemical reactions, many procedures have been developed which can be used for micro-assays of various compounds. Some substances which may be determined by a chemiluminescence method are listed below:

CHEMILUMINESCENCE ASSAYS (See Ref. 4)

INORGANIC

IRON, COPPER,
COBALT, CADMIUM,
VANADIUM, OZONE,
CYANIDES, H₂O₂

ORGANIC

α-AMINO ACIDS,
ATP,
OXIMES,
PHENOLES,
ORGANOPHOSPHORUS
COMPOUNDS (TABUN, SARIN)

Many other compounds such as organic peroxides, glucose, vitamin C, nitroaniline, aniline, resorcinol, pyrogallol, methyl-, ethyl- and propylalcohol may be determined by chemiluminescence using the luminol reaction.²⁰

Under optimal conditions, the sensitivity of this new analytical method can exceed even activation analysis, which may suggest the importance of chemiluminescence methods for future research. Although, with their highly sensitive photomultipliers and their excellent counting electronics, liquid scintillation counters are most useful measuring devices for these methods, the applicability of these instruments has so far been quite underestimated and unrecognised.

Bioluminescence assay

Following the suggestion of Tal *et al.*,²¹ in our laboratory we have elaborated a routine method for the quantitative microdetermination of adenosine triphosphate (ATP) using the bioluminescence reaction with firefly enzyme and a liquid scintillation counter as a photodetector.²² With this instrument, the sensitivity of ATP assay is greatly increased and the costs for enzyme are markedly reduced. Essentially similar procedures for ATP determination were developed and extensively studied by Schram²³ and by Stanley and Williams.²⁴ In July 1970 at the San Francisco LSC Meeting these authors presented a bioluminescent assay for the specific determination of FMN (flavin mononucleotide) and NADH (reduced nicotinamide adenine dinucleotide).^{25, 26}

In recent years we have done several thousand ATP determinations with a liquid scintillation counter. I am quite sure that other analytical methods based on chemi- or bioluminescence reactions can easily be performed with this instrument. I hope that these enlarged possibilities of application will be included in the design of future liquid scintillation counters and in the experimental methodology of investigators who use counters.

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DISCUSSION

M. Krichevsky: What is the effect of changing discriminator settings in rejecting chemiluminescent pulses? Also pyruvic acid or salts thereof might be considered as scavengers for peroxides in chemiluminescent situations as α -ketoacids react quantitatively in an oxidative decarboxylation.

D. A. Kalbhen: In answer to your first point, this is possible only for unquenched carbon-14 samples. As to your second point, we have not tried this but it seems to be a good idea for future investigation.

D. S. Glass: Since chemiluminescence is a low energy phenomenon the problem can be at least brought to light using the double ratio technique (see E. T. Bush, *Int. J. Rad. Isotopes* **19**, 1147 (1968) and also D. S. Glass, *Proceedings of the 2nd International Symposium on organic scintillators and liquid scintillation counting*, San Francisco, July 1970 for the application of this method).

D. A. Kalbhen: The channels-ratio technique is an excellent device to detect chemiluminescence if quenching is not too high and discriminators are set for this purpose.

B. W. Fox: I have three points: (a) to what extent do metals influence the chemiluminescence from biological samples from dioxane/alkali mixtures? (b) referring to the *p*-aminosalicylate chemiluminescence first falling from a maximum and then rising again to a maximum after about 6–7 hr. Have you an explanation for this? (c) may not oxygen and a metal be necessary for chemiluminescence?

D. A. Kalbhen: (a) Metals do influence chemiluminescence intensity. (b) The phenomenon may be due to reaction products which may reduce or increase quenching. (c) This is true for ATP-bioluminescence dependent upon Mg^{2+} and oxygen.

D. A. Kalbhen: As to your first point, the intensity of chemiluminescence depends mainly on the peroxide content. In answer to your second: Yes, different scintillators have effects of light output or transmittance (or wave length shifting) so there are different counts for the same chemiluminescence reactions.

B. Scales: Does the chemiluminescence intensity correlate with different scintillators or is it only a function of the solvents and the pH used? Also, have you examined the chemiluminescence intensity of one solvent sample using different scintillators?

C. P. Bond: Have you ever tried using stannous chloride to 'kill' chemiluminescence as advocated by Beckman Instruments? We have found that this virtually eliminates chemiluminescence at least under some circumstances.

D. A. Kalbhen: We have not yet tried this.

J. H. Bates: I presume you carry out mixing of solutions in the absence of light to preclude phosphorescence effects?

D. A. Kalbhen: Yes, but the reduced light of an osmium bulb does not produce significant phosphorescence.

J. A. B. Gibson: How do you allow for the time dependence of the chemiluminescence in ATP estimation?

D. A. Kalbhen: It is advisable (and most users do) to start light measurement exactly 30 s after mixing the reagent and sample, i.e. the enzyme and ATP.

L. Schutte: When measuring chemiluminescence, did you use the coincidence technique? One might expect a higher efficiency when using single photomultipliers.

D. A. Kalbhen: We used *in* and *off* coincidence circuitry. Since chemiluminescence is a *single* photon event, you get much higher pulse rates when coincidence is *off*.

R. Evans: Did you use standardised illumination conditions during the preparation of the various cocktails for chemiluminescence measurement?

D. A. Kalbhen: Yes, it was necessary to avoid lamps which emit u.v. light. We used the reduced light of osmium bulbs.

B. Legg: Is it not possible to overcome the problem of chemiluminescence of peroxides in strong base simply by the addition of a reducing agent or by heating after the decolourising step in the sample preparation?

D. A. Kalbhen: This is possible, but will not completely eliminate chemiluminescence in most cases (see Ref. 11 to this paper).

W. Kolbe: Is there also an influence of temperature on the decay time of chemiluminescence reaction?

D. A. Kalbhen: Yes. The decay is faster at higher temperatures, so it is advisable to store samples obtained at 20–70° for decay.

J. B. Birks: (i) The fluorescence lifetime of liquid toluene of the highest commercial purity is 26 ns but the removal of residual peroxides increases this to 39 ns. (ii) Although spurious counts due to chemiluminescence can be eliminated by allowing the reaction to proceed to completion, the reaction by-products will quench the scintillation efficiency.

D. A. Kalbhen: (i) This may be due to catalytic reactions of contaminants. (ii) Very little is known about reaction intermediates or end-products occurring during chemiluminescence. An increase as well as a decrease in quenching has been observed.