CHEMILUMINESCENCE

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Abstract. Factors causing chemiluminescence in liquid scintillation system are briefly reviewed and means of avoiding and suppressing it given. Evidence is presented to implicate singlet oxygen as a causative agent in chemiluminescence.

1. Introduction

Chemiluminescence and photoluminescence are sources of spurious events in liquid scintillation counting. These phenomena were first studied by Lloyd and his coworkers [1] and by Herberg [2]. Since then many reports have dealt with the avoidance and correction of these spurious counts in the measurement of sample radioactivity.

Although chemiluminescence and photoluminescence differ in their mechanism, they often appear to be closely linked in a liquid scintillation system. For example, the counting vials may be intensely photoluminescent while the sample may be either chemiluminescent or photoluminescent or both. Therefore, it may be useful to review briefly the photophysics of energy absorption.

2. Photophysics of Energy Absorption

Upon absorption of energy a molecule becomes excited and may emit light as luminescence or degrade the absorbed energy as heat upon de-excitation. Depending upon the origin of excitation, the luminescence may be classified as photoluminescence, sonoluminescence, chemiluminescence or thermoluminescence (Table I). Among these, the phenomena of photoluminescence of molecular crystals and scintillation solution systems have been employed to advantage for the detection of ionizing radiation. Salient examples are the use of sodium iodide crystals activated with thallium for measuring γ-ray radiation and the application of liquid scintillation systems for detecting all nuclear emissions. The time resolution requirement in pulse analysis has allowed only molecular systems which emit fluorescence with short decay times to be useful for nuclear detection. In liquid scintillation counting, fluors with lifetimes ranging from 96 nanoseconds for naphthalene to 1.1 nanoseconds for PBD [3] are available.

The photophysics of molecules upon absorption of light quanta or radiation has been treated by Birks [4] and others [5,6]. The absorption of light excites the molecule in which an electron is promoted from the highest bonding orbital to the lowest antibonding orbital. For aromatic hydrocarbons, such a transition is a π- π^* transition.

Once excited, the molecule in the excited state must dissipate its energy by internal conversion as heat or by radiative transition with light emission as prompt fluorescence or by intersystem crossing to yield the triplet state which then decays by emission of phosphorescence, delayed

Table I. Types of Luminescence

1. Chemiluminescence (chemical energy)

   Bioluminescence
   Electrochemiluminescence

2. Photoluminescence (radiation energy)

   Fluorescence - Prompt fluorescence

   Delayed fluorescence

   E-type (thermal repopulation)
   P-type (triplet-triplet annihilation)

   Phosphorescence

3. Sonoluminescence (thermal energy)

   Triboluminescence

4. Thermoluminescence (radiation and thermal energy)

fluorescence or heat or by dissociation into smaller molecular fragments or by chemical reaction with other molecules. The radiative transition from an excited singlet to the ground singlet state is spin-allowed and the light emission is termed fluorescence and is short-lived (10^{-9}-10^{-6} s), whereas the transition between the triplet state and the ground singlet state is spin-forbidden; the light emission is long-lived (10^{-2}-10^{2} s) and is known as phosphorescence. Owing the long lifetime, phosphorescence is more susceptible to quenching than fluorescence and is only observed in solids, frozen solutions or highly viscous liquids. The spectral maxima of absorption, fluorescence and phosphorescence spectra occur at increasing wavelength in an inverse relationship to their respective energies.

The excited molecules may also undergo energy transfer to other molecules by mechanisms of radiative, collisional, resonance, and exchange transfer. Resonance transfer operates by long-range dipole-dipole interaction (Förster mechanism) and can occur at molecular distances over 50-100 Å between donor and acceptor molecules. It is the predominant mechanism of energy transfer between solvent and solute molecules in liquid scintillation systems [7]. At high concentrations of solute, energy transfer is by short-range exchange forces [8]. Radiative transfer also occurs between the solvent and the solute molecules in liquid scintillation systems [9]. If the acceptor molecule has a higher quantum efficiency than the donor molecule the light output of the system will be enhanced. On the other hand, if the acceptor molecule converts the excitation energy into molecular vibrations which are eventually degraded as heat, the light yield will be decreased, and in such cases, the acceptors act as quenchers. In liquid scintillation systems, efficiency depends upon energy transfer; if the excitation energy transfer is terminated at the quencher molecule, efficiency will be reduced. If sources of excitation other than nuclear emissions are present and result in chemiluminescence, photoluminescence, electrostatic charges, etc., spurious counts will be observed.

3. Mechanisms of Organic Chemiluminescence in Solution

Many chemical reactions emit light but the quantum yields are low and the systems inefficient. With the use of more sensitive light detecting devices, an increasing number of organic reactions are found to be chemiluminescent [10,11].

Chemiluminescence is presumed to comprise one-photon events and can be discriminated against by coincidence gating when measuring sample radioactivity. Chemiluminescence is not subject to photo-reactivation.

The mechanism of organic chemiluminescence in solution involves three stages (i) preliminary reactions to provide the key intermediate, (ii) an excitation step in which the chemical energy of the key intermediate is converted into electronic excitation energy, and (iii) fluorescence emission from the excited product formed in the chemical reaction [10-13]. In reactions in which a fluorescent compound is added to enhance the chemiluminescent emission, an efficient energy transfer occurs and the resulting chemiluminescence is known as "sensitized" chemiluminescence. Chemiluminescence observed in liquid scintillation systems probably belongs to this category.

The mechanism by which chemical energy is provided for chemiluminescent reactions may be by (i) peroxide decomposition [14], (ii) electron transfer by cation-anion radical combination [15] and (iii) energy transfer from excited oxygen molecules and molecular pairs [16]. Some illustrations are given below:

Chemiluminescence derived from peroxide decomposition may be exemplified by the oxidation of oxalyl chloride or oxalic ester with hydrogen peroxide in the presence of 9,10-diphenylanthracene [17] which fluoresces at 400 nm and requires at least 71.5 kcal of energy for excitation. The key intermediates formed in these oxidation reactions are presumably monoperoxyoxalic acid HO.CO.CO.OOH and 1,2-dioxetanedione O=C-C=O . The latter has been identified by mass spectromet-
O-O ric measurement [18]. Decomposition of the key intermediate through a concerted process in which the cleavage of 2 or 3 bonds occurs simultaneously, provides instantaneous energy release for the electronic excitation of the fluorescent compound [19]. The peroxy acids and 1,2-dioxetanediones are key intermediates involved in the oxidation of triphenylimidazole (lophine) bis-acridinium compounds (lucigenin) and many others.

The annihilation of positive and negative aromatic hydrocarbon radical ions generated by electrode processes gives rise to electro-chemiluminescence [15]. Oxidation and reduction of radical anions and cations with chemical oxidants and reductants, respectively, also lead to chemiluminescence. In this reaction, the excited singlet state of aromatic hydrocarbon is formed by electron transfer from the anion radical to the cation radical. Representative examples are phenanthrene and rubrene. Recent investigations indicate a pre-annihilation electro-chemiluminescence involving the formation of the triplet state by energy transfer [20,21].

Reaction in an alkaline solution of hydrogen peroxide with chlorine gas or hypochlorite ion generates singlet oxygen

($^1\Delta_g$) which gives rise to red chemiluminescence. When anthracene, acridine, eosin, fluorescein, quinine sulfate or aesculin are present in the solution reaction, $H_2O_2 + ClO^-$, a strong sensitized chemiluminescence of these compounds is observed. As the energy gap between the ground state and the first excited state in these compounds is greater than the electronic excitation energy from singlet oxygen ($^1\Delta_g$, $^1\Sigma_g^+$), it is infeasible to effect an energy transfer from the latter to the former. Khan and Kasha [16] have shown the occurrence of the excited oxygen molecular pairs ($2[^1\Delta_g]$, $[^1\Delta_g + {}^1\Sigma_g^+]$, $2[^1\Sigma_g^+]$) and their transition to the ground state can provide sufficient electronic excitation energy for the fluorescent compound in the reaction solution. The electronic energy levels of singlet oxygen and excited oxygen dimers are shown in Fig. 1.

Singlet oxygen is present in the air and can be generated from the reaction of sodium hypochlorite with hydrogen peroxide [22], from thermal decomposition of many organic ozonides [23], epidioxides [24], and linear hydrazides [25], from the reaction of potassium superoxide in water [26], and from microwave discharge through gaseous oxygen [27]. Singlet oxygen is also produced by biological and enzyme systems, such as xanthine oxidase [28], rat liver microsomes, NADPH and O_2 [29], human polymorphonuclear leukocytes upon phagocytosis [30] and adrenodoxin-reductase-adrenodoxin enzyme system [31].

4. Chemiluminescence in Liquid Scintillation System

Chemiluminescence is observed when an alkaline tissue solubilizer is added to a dioxane-based scintillator containing naphthalene [32]. It occurs to a lesser extent when the solvent dioxane is replaced with toluene. Treatment of the scintillation solvents, p-dioxane and toluene, by shaking with H_2O_2 increases the intensity of chemiluminescence. Alkaline tissue digest bleached with benzoyl peroxide or H_2O_2 causes more chemiluminescence than unbleached digests. These observvations focus attention on the culprit role of peroxide and excited oxygen.

The necessary conditions for chemiluminescence in some liquid scintillation systems appear to be the presence of peroxide and an alkaline tissue solubilizer such as Hyamine, Soluene, NCS, Protosol, etc. These cannot be used to explain the occurrence of chemiluminescence in emulsion-type liquid scintillators upon addition of water [33] and in samples containing tissue digests treated with perchloric or nitric acid [34].

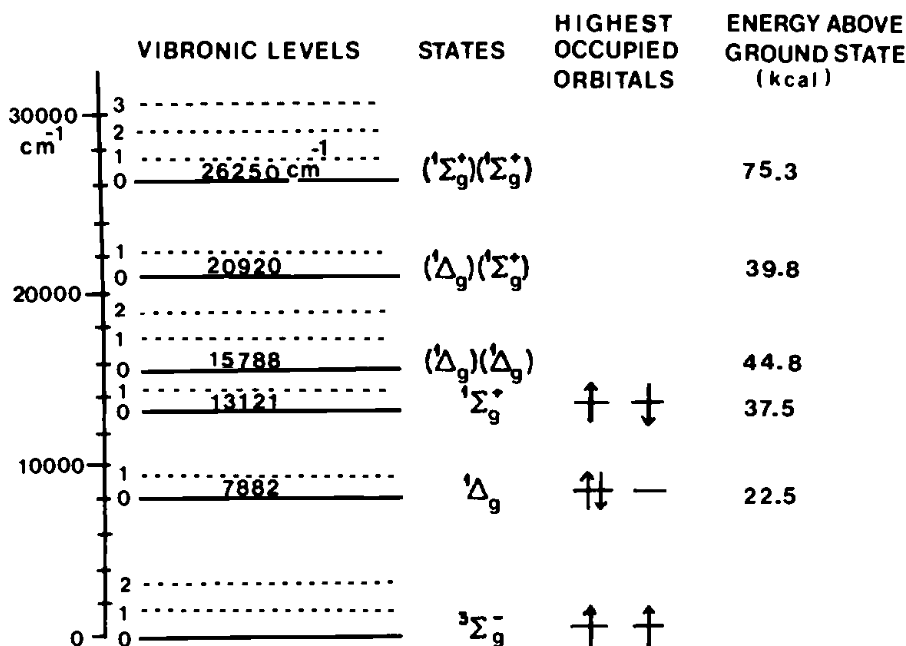

Figure 1. Electronic energy levels of singlet oxygen and excited singlet oxygen dimers (from Reference [26]).

The chemiluminescence-emitting species in liquid scintillators has not been identified. Because of the nature of the system, fluorescence emission may be due to either direct or sensitized chemiluminescence or both; fluorescent components in the system may participate in chemical reactions as well as in energy transfer reactions. In this connection, it may be mentioned that benzoyl peroxide causes more intense chemiluminescence than H_2O_2 because the reaction product of the former can participate in energy transfer more efficiently than H_2O.

5. Decay Rate, Pulse Height, Temperature Effect and Other Factors Affecting Chemiluminescene

Kalbhen [32,34,35] and Kearns [36] have measured the chemiluminescence spectrum in liquid scintillation systems. The pulse height spectrum changes with time and is related to the decay of chemiluminescence which is dependent upon the rate of chemical reaction and the lifetime of the fluorescing molecular species. Initially, the light pulses are of large amplitude and grow in intensity as the reaction proceeds. With the passage of time, the intensity of chemiluminescence diminishes with a concomitant shift of the pulse height towards the low energy end. At low-level chemiluminescence, the spectrum has a pulse height distribution overlapping the ^3H spectrum, thus rendering futile any effort to discriminate against chemiluminescent by adjusting the bias setting or by the use of channels-ratio method.

The low-level chemiluminescence may persist for many hours or even days to yield a count rate appreciably above the background. The decay is temperature dependent and proceeds faster at elevated temperature.

6. Means of Avoiding and Suppressing Chemiluminescence

Chemiluminescence may be diminished or eliminated by acidification of the quaternary-base-solubilized tissue digests with acetic or hydrochloric acid to a pH below 7.0 before addition of liquid scintillator. Oxidizing acids ($HClO_4$, HNO_3) or sulfuric acid (H_2SO_4) should not be used for neutralization because they may cause precipitate formation or high coloration and may not always succeed in eliminating the chemiluminescence; in fact tissue samples digested with $HClO_4$ or HNO_3 have been shown to luminesce [34]. Neame [37] recommends that the acidification and dilution of alkaline tissue digests be carried out in sequential steps followed by heating at

50°C and the final addition of liquid scintillator, in order to avoid chemiluminescence.

The presence of oxygen and peroxide in the scintillator or solvent may be scavenged by adding minute amounts of ascorbic acid or di-*t*-butyl-4-hydroxy-toluene (BHT) in combination with hydrochloric acid [36]. The enzyme catalase has been used to remove residual benzoyl or hydrogen peroxide in bleached tissue digests [38]. Hydrochloric acid and ascorbic acid have also been used to decompose residual H_2O_2 in bleached hemolyzed and jaundiced samples used in digoxin and T-3 uptake assays.

Affected samples may be stored at elevated temperature prior to counting to allow chemiluminescence to decay, thereby minimizing interference with normal counting. Use of a refrigerated spectrometer for sample counting may also serve to suppress chemiluminescence due to the slow rate of chemical reactions at low temperatures [36]. Light pulses from chemiluminescence may be discriminated against electronically by delayed coincidence; the photon monitor in the newer liquid scintillation spectrometer is based on this principle. Sample preparation by combustion may be practiced if all other means of eliminating chemiluminescence fail.

7. A Mechanistic Interpretation of the Cause of Chemiluminescence in Liquid Scintillation System.

Several unique features are associated with the phenomena of chemiluminescence in liquid scintillation system: (i) the liquid scintillator provides an ideal system for sensitized chemiluminescence to occur because of its highly efficient energy transferring process, (ii) the persistence of low-level chemiluminescence over a long duration indicates either a very slow or a continuing chemical reaction, (iii) the presence of peroxides is essential, and (iv) the chemiluminescence is enhanced in alkaline media.

With the assumption that chemiluminescence contributes to single photon events, we made the following observation in the "singles" or no coincidence mode (Table II).

(i) Glass counting vials after dark adaptation gave a consistent, repeatable singles count rate. Photoluminescence can be induced by brief exposure to light due to photo-reactivation; it decays rapidly within minutes to background.

(ii) Neat solvents including 30% H_2O_2 gave only slight or no additional singles count rate.

(iii) Tissue solubilizers such as Hyamine, Soluene, NCS, and Protosol yielded higher singles count rates than the solvents, and the intensity of the count rate appeared to be

Table II
Single-Events Rates of Liquid Scintillators and Accessories[a]

Sample[b]	Single-event rate[c] (cpm)	Coincidence rate (obs) (cpm)	Accidental Coincidence (calc)[d] (cpm)
Glass vial	1.09×10^4	28	
H_2O	1.15×10^4	38	
H_2O_2	1.90×10^4	-	
BBS-3	2.22×10^4	925	
BBS-3 + H_2O_2	7.17×10^4	811	1
NCS	2.2×10^5	--[e]	
NCS + H_2O_2	5.2×10^6	41	6760
Protosol + H_2O_2	3.7×10^6	55	4322
Soluene-100	5.6×10^4	20	1
Soluene-100 + H_2O_2	1.16×10^6	184	336
Triton X-100	3.7×10^6	91	3422
Benzoyl peroxide (1.5%) in toluene	1.2×10^5	44	4
Aquasol	1.71×10^5	--[e]	
Aquasol + H_2O_2	2.32×10^5	20	13
Monophase	2.0×10^5	54	10
Monophase + H_2O_2[f]	2.65×10^6	4985	1755
Monophase + N_2[g]	2.6×10^4[h]	49	
Monophase + air	7.1×10^4[h]	56	1
Monophase + O_2[g]	5.6×10^5[h]	352	78
Hypochlorite + H_2O_2[i]	1.4×10^5	24	5

a. All measurements were made on Beckman LS-9000 Spectrometer unless stated otherwise. The author is grateful to Dr. D.L. Horrocks of Beckman Instrument Co. for assistance.

b. All samples were low-potassium glass vials with aluminum cap. The sample volume was 10 ml, and 0.1 ml of 30% H_2O_2 was added. Samples were obtained commercially: Aquasol was from New England Nuclear; Monophase, Soluene-100 and Triton X-100 were from Packard Instrument Co.; NCS from Amersham/Searle; BBS-3 from Beckman Instrument Co.

c. Counted in single-photon or no coincidence mode.

d. Calculated according to the formula $2\,(N/2)^2$, where is the resolving time of the coincidence gate in minutes and N the observed single-event rate in counts per minute.

e. Not measured.

f. The content of the counting ampoule was bubbled with N_2 or O_2 gas before sealing.

h. Measurement was made on a Packard Model 3375 spectrometer.

i. The sample consisted of 10 ml of water and 0.1 ml of each reagent.

characteristic of the solubilizer. The emulsifier BBS-3 (neat) yielded a high rate on account of its high viscosity. The count rate was diminished when diluted with toluene. The decay rate of chemiluminescence in these agents appeared to be comparable. (Fig. 2).

(iv) The emulsifier-scintillator, Monophase (Packard), showed intense chemiluminescence after the addition of H_2O_2 (100/1, v/v). Accidental coincidence count rate was 3 to 4 times the value predicted by theory ($2\tau N_1N_2$). Measured pulse height spectrum indicated the presence of multiphoton events (Fig. 3). The underlying cause is not known.

(v) Aliquots of Monophase purged with N_2, air, and O_2 and sealed in counting ampoules gave singles count rate increasing in that order indicating the ability of oxygen to generate counts.

(vi) A mixture of sodium hypochlorite and H_2O_2 (0.1 ml/ 10 ml water) yielded a high singles count rate. The mixture is known to be chemiluminescent due to the presence of singlet oxygen ($^1\Delta_g$). The singles rate observed is presumably a combination of the reaction rate and the decay of singlet oxygen.

The above observations lead us to propose that singlet oxygen ($^1\Delta_g$) is the cause of chemiluminescence in the liquid scintillation system. Oxygen is ubiquitous and singlet oxygen has a radiative lifetime of about 45 minutes [39] but has a lifetime of about 0.1 sec in the air because of quenching by nitrogen [40]. Its lifetime in solution varies with the solvent. Oxygen is soluble in toluene, and its molecular pairs ($2[^1\Delta_g]$, $[^1\Delta_g + {}^1\Sigma_g^+]$, $2[^1\Sigma_g^+]$) can provide excitation energy to some fluorescent compounds in a step-ladder fashion. The implication of singlet oxygen as a source of excitation minimizes the difficulty of explaining the persistence of low-level chemiluminescence which remains above background rate over extremely long periods of time.

It may also be postulated that alkalinization of the liquid sintillation medium with tissue solubilizers induces O_2 to form superoxide ion O_2^-, which may react with a cation to form additional singlet oxygen and increase the intensity of chemiluminescence. Polymeric molecules such as surfactants, etc. may also be postulated to exert a catalytic effect in enhancing the chemiluminescence; this may account for the multi-photon events observed in the mixture of Monophase and H_2O_2. The hypothesis role of oxygen in chemiluminescence is further strengthened by our observation that the presence of stannous chloride in the mixture of Monophase and H_2O_2 enhances the intensity of chemiluminescence, presumably due to the fact that the metal ion catalyzes the decomposition

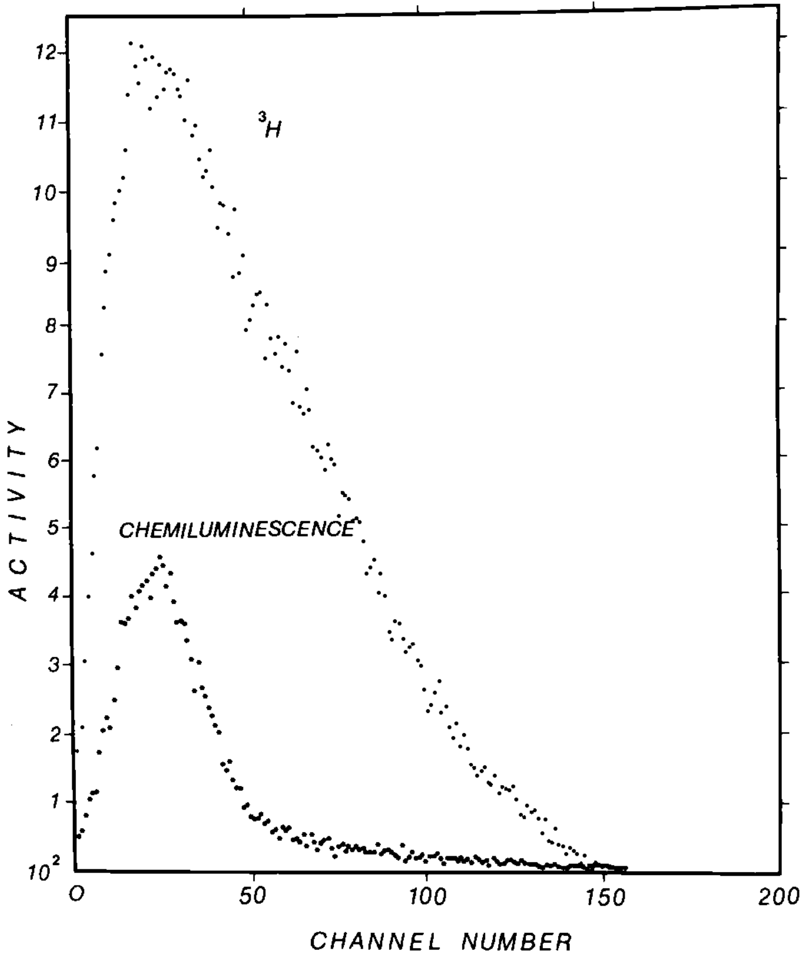

Figure 2. Pulse heights spectra of the chemiluminescent mixture (Monophase + H_2O_2) and ^3H.

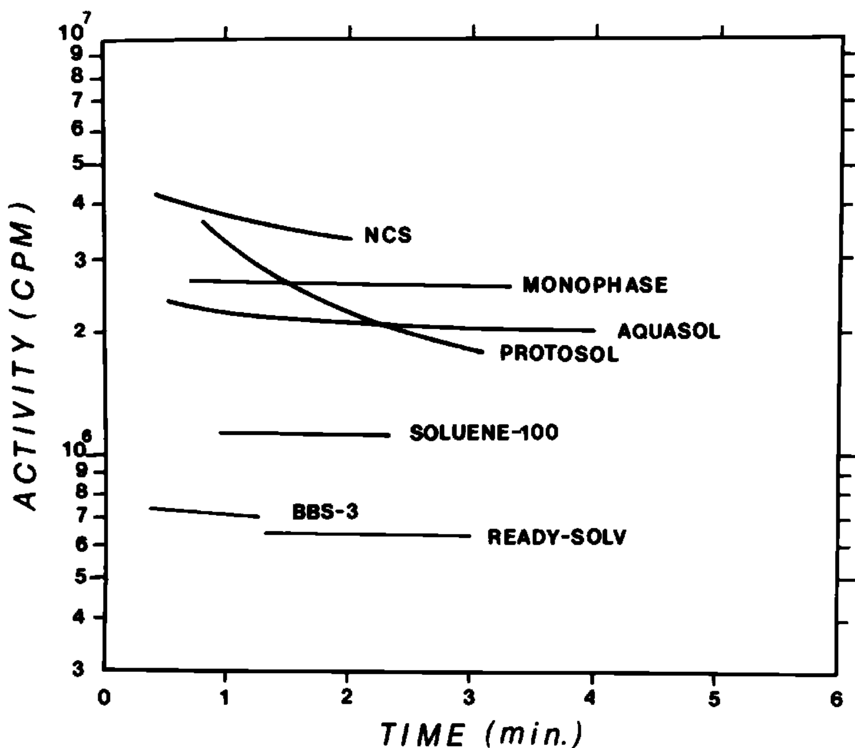

Figure 3. Decay of chemiluminescence in solution. Measurement began shortly after addition of 0.1 ml 30% H_2O_2 to the sample. The mixture (Monophase + H_2O_2) was aged for about 24 hrs.

Table III. Phosphorescence in Liquid Scintillation Components and Counting Vials.

Component	Phosphorescence
Scintillation solvents	
Toluene	(-)
Triton X-100	(+)
Triton X-100 + toluene	(±)
Liquid scintillators	
PPO in toluene	(-)
BBOT in toluene	(-)
Dioxane based scintillator	(+++)
PCS	(+)
Oxifluor	(+)
Insta-gel	(±)
Monophase	(++)
Solubilizers	
NCS, neat	(-)
Soluene, neat	(-)
Hyamine, neat	(-)
Counting Vials	
glass	(-)
Nylon	(++)
Polyethylene	(++)
White plastic caps	(++++)
Black caps*	(-)

*White plastic cap is spray-painted black.

of H_2O_2. This observation casts doubt on the effectiveness of $SnCl_2$ as an agent in suppressing chemiluminescence as previously reported [38].

Many chemiluminescent mixtures show photoluminescence; even the mixture of ClO^- + H_2O_2 exhibits twice the singles count rate of the glass counting vial upon excitation. As photoluminescence occurs frequently in scintillation components and is related to the presence of luminescent impurities, it can be detected by activating the object under study directly with an electronic photoflash. Table III shows the phosphorescence of the scintillation components.

The singlet oxygen hypothesis provides a basis for further study on chemiluminescence in liquid scintillation systems. Photo-activation and counting of singles provides the means for detecting and measuring minute concentrations of luminescent impurities that are present in scintillation solvents, additives and fluors that may diminish the efficiency of a liquid scintillation system.

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Discussion

Dr. B.E. Gordon - Lawrence Radiation Laboratory (US)

I have some concern about two of the introductory talks, one by Dr. Wigfield and one by Mr. Ferris. I am actively concerned about the application of solubilizers to biological samples and also about the possibility that we have unknown systems, by which I mean that the composition and the exact structure is unknown.

I will talk about the adsorption problem first and then about solubilization.

It is true that you can do some tests to determine whether you have an adsorption problem. It is incorrect, if you have an unknown system and you observe adsorption, to apply a carrier addition approach because this approach will only work when you know the structure of the adsorbed molecule. I must also express my grave concern about the adsorption shift as a quantitative method. That will work I think again only when you know the exact structure of the molecule.

Now as to the problem of solubilization, I think a brief lecture in polymer physics is in order. It is well known that non-polar polymers such as polybutene irreversibly adsorb onto active surfaces when they exceed a certain molecular weight. This is because the possibility that all adsorbed points of the randomly coiled polymer have a low probability of being desorbed at the same time. As the molecular weight increases the probability decreases; all this occurs even if the heat of adsorption per monomer unit is small. It is clear then that the situation with polar molecules (i.e. proteins, DNA, tissues, etc.) is much worse. The molecule can be much smaller and still be irreversibly adsorbed because the heat of adsorption is much greater per unit monomer (e.g. amino acid). Imagine then a macromolecule of 1000 monomer units which has only 10% of its polar sites available for adsorption and the heat of adsorption is 2 k cal/mol. The molecule is adsorbed in a dynamic state with some groups desorbing and others adsorbing but the probability of all adsorbed groups coming off at once is exceedingly small. As long as one group is adsorbed, then the molecule is held close to the vial wall and 4 π geometry is not achieved. Looked at another way, the energy required to ensure simultaneous desorption of all 100 groups from the surface is 2 x 100 or 200 k cal/mol - greater than C-C bond strengths and so the molecule is irreversibly adsorbed.

When you apply the solubilizer, whether it be an acid or a base or a quaternary ammonium compound to that kind of a system and it is applied to a macromolecule, the weakness of the situation is that one has no idea of what the species are like. If one has no idea, then one cannot predict whether the measured count rates will be accurate. If any part of the system is adsorbed, and if the molecular weight is high enough, then by the explanation above some of this is irreversibly adsorbed and a correction cannot be made.

I would like to support Dr. Bransome wholeheartedly in pointing out that if the material you have to deal with is water soluble then you can count it in a detergent system. If it is fat or oil soluble it can be counted, and counted accurately I might say, in a toluene based fluor. All other materials as far as I am concerned must be burned. I have refereed some papers on this and I must say that if I am called on again, and if the sample preparation is by way of solubilization, I will have to turn in a negative report.

The advantage of combustion is not so much that the sample is converted into a form that is easily handled but that you know the molecular composition of the labelled species. When you know that, whether it be a hydrocarbon or a carboxylic acid or whatever, then you can deal with it. On the other hand, when the molecular composition is unknown our measurements are really fraught with danger.

The only advantage I can see in solubilization is that you handle the samples in parallel rather than in series as you do with combustion. Everything else I hear about it is in fact negative.

Dr. D.C. Wigfield - University of Victoria (CAN)

I agree with both points that Dr. Gordon made about adsorption problems. First of all, clearly if you do not know what the material is that you are counting, and if it does not have a well defined structure then this is one of the biggest limitations of the carrier dilution technique. Secondly, I also agree that the adsorption shift technique may be questionable when you do not know the chemical structure of the thing you are trying to count.

Dr. A.A. Noujaim - University of Alberta (CAN)

Dr. Wigfield, I have read your elegant articles concerning adsorption. However, the double ratio technique you describe applies only to ^{14}carbon. If I recall correctly, one of the compounds that is severely adsorbed

is glucose. Now glucose is used extensively in biochemistry, especially in the tritiated form. Is there a way by which we can predict adsorption of glucose or other tritiated compounds to glass?

Dr. D.C. Wigfield

If you are asking the question, is there a way to overcome adsorption for tritiated materials, I do not know of any except the classical way of getting rid of it by carrier addition or something like that.

If you are asking about the question of detection of adsorption which I think was your question, then I would think that carrier dilution and vial emptying might give you some kind of hint. We have not applied adsorption shift to tritiated materials.

Dr. E.D. Bransome Jr. - Medical College of Georgia (US)

When Dr. Wigfield published his first article on this topic we immediately went to try it with tritium, and as a matter of fact one of the things we used was tritiated glucose. It simply does not work. One loses the counts altogether.

Dr. K. Painter - Colorado State University (US)

I would like to comment on an additional couple of ways reported in our review on counting vials in the Sydney symposium (1973). When using glass vials siliconizing the walls helps. It is a messy procedure but almost always works for everything. Another possibility is pH change. The detergents are usually quite good at preventing things like glucose from adsorbing. Lastly, complexing agents, and there are many of these, 2-ethylhexanoate, the quaternary ammonium compounds etc. which are all used. There are about ten ways actually.

Dr. A.A. Noujaim

I address this question to Dr. Bransome. I would like to object to the use of the term DPM in publications because it relates to absolute activity while what we use when we measure samples is a secondary standard, ±5%. I am wondering whether we could use the term relative DPM, or relative CPM, or standardized CPM rather than absolute DPM as is implied at present.

Secondly, in our experience the use of different standards from different manufacturers unfortunately gave

different results. Is there any particular experiment that we should use to standardize those supposed standards?

Thirdly, I would like to hear some comment about the present status of counting ^{125}iodine in liquid scintillation counters.

Finally, how do we check current commercial solubilizers? Should we use some simple criterion such as the double ratio method to indicate the maximum permissible water load rather than the figure of merit which could be very deceptive?

Dr. E.D. Bransome, Jr.

Regarding your comments about DPM, there are no acceptable absolute standards. It seems to me that for some isotopes the National Bureau of Standards in the United States might be encouraged to expand its efforts and activities to some degree. One might therefore be able to relate experimental findings to one of those absolute or 'agreed upon' standards.

I would submit that provided we define what we mean by DPM that too would be probably acceptable. I think your implied criticism is correct and that few of us do make such a definition.

Regarding solubilizers, it is quite obvious from your work, and some of our own observations that back you up though not as elegantly, that the double ratio plot is not a significantly rigorous procedure. It is certainly not rigorous enough to make sure that a sample loaded with surfactant is homogenous.

Dr. Bush-Mueller's suggestion that the same isotope be put in tracer amounts in both phases, that is organic solvent and water, seems in some cases to provide an additional criterion with a little more power. The truth of the matter is that we do not know enough, and not enough work has been done in this area to really answer the question. I am sure you know that and you are asking the question to ensure that there is continuing attention to the problem.

As far as ^{125}iodine counting is concerned, one can count the conversion electrons, but one must be aware that the usual quenching effects on counting efficiency will be somewhat more dramatic than that observed for tritium which has a similar pulse height spectrum. It is a reasonable technique but it must be carried out with even more care than counting the usual beta emitters.

Dr. A.A. Noujaim

Dr. Noakes, I must agree totally that combustion is the method of choice and those people who have been solubilizing

tissue samples for years know the problems that are involved in preparing tissue samples for liquid scintillation counting. However, we have one problem which is not completely solved. We lack an absolute biological standard in order to compute the true recovery of the combustion system. What is the present status regarding the availability of such a standard right now?

Dr. J.E. Noakes - University of Georgia (US)

I understand that the instrument companies have prepared some acetate type paper which is labelled with ^{14}carbon and tritium of known activity. These small pieces of paper are put into the combustion apparatus and burned so that recovery may be measured. This, however, does not account for the differences between the acetate paper and biological materials.

Mr. E. Polic - Packard Instrument (US)

We have developed a technique for our oxidizers. We make a comparative check with a solution that we have developed. You can combust it and also prepare a sample without combustion and make a comparison between the two to check the recovery. This procedure is described in the operation manuals of our oxidizers.

We encountered the same problem of finding a suitable standard as implied in Dr. Noujaim's question, and that is why we went to a comparative method.

Dr. K. Painter

I'd like to suggest that before we get everyone converted to the combustion technique that in a clinical lab it is clearly not the answer until more work is done.

I list below a few disadvantages of the combustion technique when you need to process large numbers of samples.

i) cost per sample
ii) processing time per sample
iii) carry-over or memory
iv) capital expense
v) other nuclides.

Thus, we should not leave this conference with the idea that combustion is a panacea. I do not think that solubilization is a method that is free from faults either, but until such time that there is a method to do large numbers of samples quickly and inexpensively, I do not think that combustion will catch on.

Dr. E.D. Bransome Jr.

Since I am one of the advocates of combustion I should like to remark that I am not so sure that Dr. Painter's objections to combustion in the clinical labs are really terribly cogent right now because there are not many clinical procedures using liquid scintillation counting, although the future is probably going to offer many more. I will ask what about other isotopes, some of which have already been mentioned at this meeting; Plutonium isotopes present in bone, ^{35}Sulphur and a variety of labels that have not been proven to carry over, or to be recovered well in the presently available combustion systems. I do know that there are some ways to achieve combustion of samples that are not tritiated or containing ^{14}carbon and make this comment to hopefully elicit some information on the subject.

Dr. J.E. Noakes

It is unfortunate that Dr. Kisieleski is not here because he has done quite a bit of work with some of the other isotopes in combustion. Talking with him earlier, he said he has worked with ^{35}sulphur and ^{125}iodine and that the recoveries were not at all quantitative, about 80% in one case.

I would say that for isotopes other than tritium and ^{14}carbon that there is a big question which will have to be worked on. For ^{59}iron I might point out that the Searle 6550 combustor, which uses a planchet to hold the sample, collects any residue which is not combustible on that planchet. This might be a way of collecting a third isotope in the sample since it would not be carried over into the trapping mechanism. This will have to be looked at as I do not think there is any data available on this at present.

Regarding sample throughput, the combustor is well automated and takes between one minute and one minute and a half to process a sample which is quite fast. But it does require an operator even in an automated stage to tend the samples and place them in the combustor and cap the vials afterward. Looking at liquid scintillation counters with say a three-hundred sample capacity, reveals that it is the combustor that limits throughput. On the other hand it is possible we might see another generation of combustors to come out in which we would combust the samples, transfer directly the combustion products into a flow cell to be counted, and then discharge the waste into a receptacle. In this way we could do combustion and counting in a very rapid

mode. Maybe this will occur, but right now we are at the single sample combustion stage. If we compare this to the Schoniger flask which took ten to twenty minutes per sample, then the combustors of today are a great improvement, and certainly expedite the preparation of samples.

Dr. S. Apelgot - Institut du Radium (F)

The only trouble with the combustion technique is memory. Ordinarily one does not know the activity of experimental samples and if you happen to burn one of low activity after one of high activity you can have some difficulty.

One difficulty with the application of the solubilization technique to tritiated compounds in vivo is that one of the metabolic products is water. When you neutralize the samples after solubilization there is an increase in temperature which may vaporize that water.

Dr. J.E. Noakes

The memory of the available combustion units is low because there is a steam purge through the system to drive any residual material over into the collecting vial. Then after the delivery of the cocktail is completed, there is a second steam purge which cleans the whole system out. Typical recovery for tritium might be 99%, and this is valid for real samples as well as filter paper. I would say that the manufacturers have done a pretty good job in eliminating the memory. There is some spillover when doing dual labelled samples but this is very much less than 1%.

Dr. S. Apelgot

In my work with glass fibre filters I needed an accurate technique to check my own methods and I therefore used combustion. We came to the conclusion that it was necessary to burn an additional glass fibre filter between samples to be sure there will be no contamination from one sample to the next. Even 1% from a very high activity may give you enough contamination to cause large errors if the activity of the next sample is very low.

Dr. B.E. Gordon

The main concern for memory in the Packard 306 is in the tritium channel because the memory in the ^{14}carbon channel is extraordinarily low. If one burns a tritium sample containing 100,000 DPM then the memory after the

purging process that Dr. Noakes has described is only five counts per minute above background. We regard that as an acceptably low value. If anybody is concerned about those five counts per minute when I would suspect that the samples would have to be pre-screened.

I would like to ask a question of Dr. Noujaim. I do not understand the question about wanting to burn a biological standard in the oxidizer as compared to any standard. If a sample is burned to carbon dioxide and water, it eludes me as to why you are concerned about the original composition so long as it is burned in a quantitative way. We burn both biological samples and hydrocarbons and have not had any problems. I wonder if you would respond to that.

Dr. A.A. Noujaim

My question relates to my concern about the completeness of the combustion. We have no control over the temperature which may change from time to time. If there is an incomplete combustion we may not get a complete recovery. We may not obtain carbon dioxide for example.

Dr. J.E. Noakes

If you look at the combustors they are very versatile. You can regulate the oxygen flow, you can regulate in some cases the temperature of the reaction, you can use extra fuel material to slow the reaction down, you can determine how long you want it to combust, there are a lot of variables that you can adjust. If you combust a sample that burns very fast and it does not get enough oxygen then you will get a dirty burn and you will have problems. We are not saying that the combustion units that are on the market today, and I think I speak for all of them, are completely automated so that you can throw anything in and expect to get a clean burn every time. I think you have to apply a rational approach just as in any analytical technique.

If you have a lot of samples that are very similar then once you have set the conditions up appropriately then you can expect to get consistent results. You can adjust all the flow rates, what size sample you want, what type of cocktail, the combination of cocktails using unlabelled material.

If you change the nature of the samples then you must go back and find new optimal operating conditions. There is some operator expertise that goes with it too.

Dr. D. Horrocks - Beckman Instruments (US)

I must defend myself from some of Dr. Bransome's earlier comments and to talk a little bit about two things. First of all about the 'H' number as compared to the limitations of External standard channels ratio (ESCR) and sample channels ratio (SCR) methods. Secondly, I will provide a little information on ^{125}iodine counting in liquid scintillation systems.

Concerning this latter topic, just this last month a paper of mine has been published in Nuclear Instruments And Methods (volume 133 page 293 - 1976) on "The measurements of ^{125}iodine by liquid scintillation methods". I have analyzed the mode of decay of ^{125}iodine as it relates to the emission of auger electrons and conversion electrons which would be detected in a liquid scintillation system. Also I tried to get some estimate of the contribution of the x-rays and γ-rays to the counting efficiency of ^{125}iodine in a typical liquid scintillator. In this paper we have done some studies on the counting efficiency with different types of quenching, and also have made a measurement of the pulse height spectra.

The spectrum of ^{125}iodine exhibits two peaks spread over a range of pulse heights somewhat wider than that usually obtained for tritium. So the ^{125}iodine will have an appreciable counting efficiency even for such a degree of quenching that would have reduced the tritium counting efficiency to zero. This is because for ^{125}iodine we have a fair number of electrons which have energies greater than the end point energy of tritium. We also found out that in a twelve millilitre volume of liquid scintillator less than eight percent of the x-rays or γ-rays interacted with the scintillation cocktail at all. They comprised a very small contribution to the counting efficiency.

Dr. E.D. Bransome Jr.

In our work, some of which is represented in an Analytical Biochemistry Article published in 1973, when we were looking at high levels of quenching we were no longer able to resolve the two peaks of ^{125}iodine. I have no argument with what you have to say about being able to completely quench tritium and still get some ^{125}iodine counts. However, the quench correction curve was rather different in our hands than for tritium and with high levels of quenching we found a rather more precipitous fall and this is what I was referring to earlier. This was probably due to the loss of the peak at lower pulse heights, but we couldn't tell which because of lack of resolution.

Dr. D. Horrocks

In the article I referred to there is a figure showing the effect of both colour and chemical quenching on the pulse height spectra of ^{125}iodine in a liquid scintillation system. The effect of quenching is rather similar to increasing the lower threshold and indeed over the initial levels of quenching the ^{125}iodine count rate will decrease quite dramatically because you are eliminating a fair number of the low energy electron events. At higher levels of quenching you will have a more gradual decrease. We constructed some quench curves using something similar to an 'H' number and they were practically linear. Perhaps in the ESCR or the SCR methods you may very well get some funny quench correction curves but by using this method it straightens things out and gives a very nice line.

Now I would like to clear up some uncertainty with regard to the limitations of methods of quench correction which are based on a measurement of pulse height and I think Mr. Laney will agree with this since Searle uses a similar technique. When you use these techniques you are not as limited in the capability of being able to make measurements over a much wider range of quenching as you would be using the ESCR or SCR methods. Also, if you use the ESCR or SCR methods you can get (almost) any shape of curve you want depending upon how you choose your windows. Therefore, it is not difficult to understand how people in different parts of the world have a hard time comparing results when you consider the variability in ESCR or SCR calibration curves. Now if you are measuring a pulse height, or an average pulse height, you are measuring something which is invariant. Thus, quench correction curves will be reproducible from lab to lab.

The ESCR method requires two counting windows to obtain the necessary ratio values and these windows may be selected in many ways. Typically you might choose two overlapping windows, one (window A) covering a wide range of pulse heights and a second (window B) that covers a narrower range of pulse heights because its lower level discriminator is set at a higher value. The required ratio may then be computed as the ratio of the counts in those two windows due to some external standard source. Now at high levels of quenching the range of pulse heights due to the external standard may all fall below the lower level of window B giving a ratio (B over A) of zero. At even higher levels of quenching you will also get a ratio of zero and you can no longer interpolate the sample efficiency from the quench correction

curve. Now if you measure a pulse height value you do not have that limitation. Limitations only arise when the compton spectrum is completely removed which is a quench factor of some four-hundred, and much greater than the twenty or thirty that we can obtain with the ESCR or SCR methods.

Also, recently we examined a problem in using the ESCR method when counting in plastic vials. We found that as the plastic vial aged with the liquid scintillator in the vial, the scintillation cocktail and the sample and the solutes would diffuse into the plastic wall. In doing so, it converted the plastic wall into a plastic scintillator because it deposited the scintillators into the matrix. When the external standard was brought up next to the vial compton electrons were created in both the scintillation cocktail and in the plastic vial wall. Since the additional scintillators due to the plastic wall are detected as a distribution of counts at small pulse heights, the spectrum of the external standard becomes distorted by an increase in counts at lower pulse heights.

Thus, you might get additional counts in channel A but not in B, giving an external standard ratio which changes with time as the diffusion takes place. Depending on how the windows have been selected the ratio may start to change either as soon as the sample is prepared or it may start to change up to three days later. That is, the higher the lower level of window A, the longer it will take for the diffusion to cause a problem. But the more you raise the lower level of window A, so the lower level of window B must be raised a corresponding amount and the range of quenching that can be handled by the ESCR method is thereby reduced.

One point I want to make is that none of the additional counts I have mentioned occur in the region of the compton edge. Thus, the 'H' number is not influenced by this effect.

Dr. K. Painter

I guess one could summarize Dr. Horrock's comments by saying that the ESCR method that has been thrust upon us by commercial people for ten years has now fallen into disrepute.

Dr. J.A.B. Gibson - AERE Harwell (UK)

I would like to support what Dr. Horrocks has to say about the 'H' factor being independent of energy. The following table shows that the relative quenching factor

which I call 'G' is the same for a wide range of isotopes at the same quenching level. In fact this tends to support all that Dr. Horrocks has said.

Relative Quenching Factor For A Wide Range Of Electron And Beta Ray Energies

Isotope	Type	Energy Beta Max KeV	Relative Quenching Factor G
^{3}H	beta spectrum	18.5	0.80
^{14}C	beta spectrum	115.0	0.82
^{137}Cs	compton edge	478.0	0.82
^{137}Cs	conversion line	625.0	0.80
^{36}Cl	beta spectrum	707.0	0.81
^{207}Bi	conversion line	980.0	0.85

Mean relative quenching factor = 0.82 ± 0.02 (from J.A.B. Gibson. Int. J. Appl. Rad. Isotopes 18, 681, 1967).

Dr. E.D. Bransome Jr.

I would like to respond to one of Dr. Horrock's first comments. When I talked about the potential limitations of the new methods of quench correction I did in no way refer to the dynamic range, but to the validity of these methods as methods of standardization. I would like to re-emphasize that I think that the limitations, as with the older methods of ESCR and SCR, are going to be related to sample homogeneity, and this is what I was referring to. I think for the group present here this is a very obvious matter but for the majority of instrument buyers it will be an additional problem.

Mr. E. Polic

Some of Dr. Horrock's comments on dynamic range depend on the radionuclide used for the external standard. You can get around some of these problems by using a higher energy radionuclide for the external standard.

In Dr. Painter's introductory remarks he had a table which showed figures of merit for various cocktails. I think one should be cautious about comparing those figures of merit because they were taken over several years and because there has been a lot of instrumentation changes that occurred over that period of time. If you really

want to make a comparison of those cocktails you should measure them all in a single experiment using a single instrument, instead of taking data from a ten year period.

I liked the bibliography you sent around during your introductory talk but it looks like there is a void between 1957 and 1969, and there were several liquid scintillation symposia during that period. There was one in 1960 (Albuquerque),[1] there were a number of symposia that were organized by Packard and New England Nuclear, and Plenum Press published four volumes on the subject. I think they were called 'advances in tracer methodology' and are still available.[2] New England Nuclear had a number of good application articles in their Atom Light which is no longer available.

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[1] Daub, Guido H.; Hayes, F. Newton; and Sullivan, Elizabeth, Proceedings of the University of New Mexico Conference on Organic Scintillation Detectors, U.S. Government Printing Office, TlD-7612 (1960). (1960 Symposium, Albuquerque).

[2] Rothchild, Seymour, Advances in Tracer Methodology, Volume 1 (1963), Volume 2 (1965), Volume 3 (1966), Volume 4 (1968), Plenum Press.

Mr. T. Horan - W.W. Cross Institute (CAN)

I wish to address my question to Dr. Peng, concerning the singlet oxygen generating system of hydrogen peroxide/ sodium hypochlorite. Upon the mixing of these two chemicals there is a red flash due to the production and electronic relaxation of singlet oxygen. This red flash has been measured at 634 nanometres and lasts for one tenth of a second. We have measured a much longer lived emission from this system. However, the wavelengths do not match the emission pattern expected for singlet oxygen. I ask how you can be absolutely certain that this chemiluminescence is that produced by singlet oxygen. If it is singlet oxygen, why is there a shift in the observed wavelengths from the expected wavelengths?

Dr. C.T. Peng - University of California (US)

I can answer your question by giving you some of the absorption spectrum of, for instance, the hydrated electron. You have a solvent effect where the absorption maximum changes due to this effect. In the case of singlet oxygen

it has been reported in the chemical literature that hypochlorite with hydrogen peroxide would give singlet oxygen. I think this has been studied extensively by a number of schools, (especially Kasha and Khan) who have given some of the absorption spectra of these molecular species. Of course the light that you observe at 600 nanometres is probably associated with a very rapid decay. We have used the new Beckman spectrometer with which you can measure the decay of the single photon, but this is a very complicated reaction because what we are observing is the rate of the reaction between the two reactants and also the decay of the singlet oxygen. The singlet oxygen itself has a radiative lifetime of forty-five minutes. But in air the proportion of singlet oxygen is not known and it has a lifetime of approximately one tenth of a second, because of the quenching by nitrogen molecules. The singlet oxygen has a variable lifetime in different solvents. For instance, in carbon disulphide, I think Kearns has measured the lifetime in to be about seven-hundred microseconds. So far as your observation is concerned, I have not come across anybody measuring this directly, that is for singlet oxygen using this oxidation mixture. I should mention that singlet oxygen is not only generated by the reaction of hypochlorite and hydrogen peroxide, it has been observed in a number of reactions. Also, microwave discharge through gaseous oxygen and some of the enzyme systems also give rise to singlet oxygen.

I think Dr. Schram mentioned FMN and NADH systems where oxygen is involved. Probably those peaks, the λ_{max}, are due to singlet oxygen. I would say that what you observed probably is due to a slow component of the singlet oxygen.

Dr. W. Reid - University of Saskatchewan Hospital (CAN)

Do the commercial liquid scintillation counters provide a suitable means of comparing the observed accidental coincidence rate to that which is calculated from the singles rate? That is, can we really eliminate the pulse height analysis part of the machine? Can you test the machine with truly incoherent light? I think perhaps one can by perhaps introducing a light leak or a small flashlight.

Dr. C.T. Peng

All the information that I presented in that table earlier indicates the current state of the art. As I

understand it, the new Beckman machine is able to give you the singles rate and also at the same time give you the accidental coincidence rate. We have made some measurements with a Packard machine by switching between the singles and coincident modes and noticing the difference. As far as I know, Dr. Ross has shown that fluorescence of a glass vial which has been excited with coherent light is good as a means of monitoring the scintillation purity of components.

Dr. S. Apelgot

I would like to return to the topic of glass fibre supports. I have worked for many years with this technique under different experimental conditions and I feel it is a good technique because it is reproducible, and samples are stable. It is true that I have to construct special calibration curves, but whenever I try homogenous solutions I have greater difficulties and I return to fibre glass supports. Even with tritium, and even if the labelled compounds are not dissolved by the liquid scintillator, I believe that the fibre glass supports provide a superior technique.

Dr. E.D. Bransome

In reply to Dr. Apelgot's comment all I can say is that in our hands we encountered sufficient difficulties with a variety of samples, even those prepared on non-absorptive supports to render the technique undesirable from our point of view.