

RECENT ADVANCES IN SAMPLE PREPARATION

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Although new concepts in the preparation of samples for liquid scintillation counting have not emerged, some improvements have been made. We now have, for example, a range of solubilizing agents which are more effective and less quenching than Hyamine hydroxide and we can count several milliliters of aqueous material where, previously, we were restricted to a few microliters. In the area of combustion techniques we have traveled a route which started from tedium with a touch of hazard and is now firmly headed for automated convenience. What has also changed is the application of liquid scintillation counting to samples generated by current analytical techniques such as gel electrophoresis and radioimmunoassay. Certainly a new dimension to radioisotopic techniques has been added by the recognition that energetic beta-emitters such as ^{32}P can be counted directly in a liquid scintillation counter by Cerenkov radiation.

A sample for liquid scintillation counting consists of 4 components: 1) the solvent 2) scintillator 3) sample 4) the sample container or counting vial.

Solvents

Toluene remains the best general purpose solvent with regard to energy transfer efficiency, solubility of the scintillator, availability, uniformity and cost. The para and meta xylenes are more efficient solvents than toluene giving a 10% increase in pulse height. Since the various xylenes have different energy transfer efficiencies ensuring the uniformity of solvent composition adds to the cost of this system although xylene is used as the major solvent in some commercial preparations of ready-to-use cocktails. The solvent is one of the least efficient components of the counting system since 90 to 95 percent

of the energy theoretically available for photon production is lost during the energy transfer between the beta particle and the solvent. It is unlikely, however, that a new solvent will be discovered which exhibits markedly better energy transfer characteristics than those already available.

Scintillator

Although PPO is still widely used, a better choice is butyl PBD. The major difference is that butyl PBD is more resistant than PPO to concentration or self-quenching. This is illustrated in Fig. 1 which shows the effect on counting efficiency of increasing concentrations of scintillator on a tritiated toluene based counting solution quenched with 0.5 percent carbon tetrachloride. The concentration quenching observed with PPO can be partially alleviated by the addition of a secondary scintillator as discussed below. Butyl PBD shows essentially a flat response with an optimum concentration of around 25g/liter. Butyl PBD is, therefore, used in much higher concentrations than PPO and is more expensive to use.

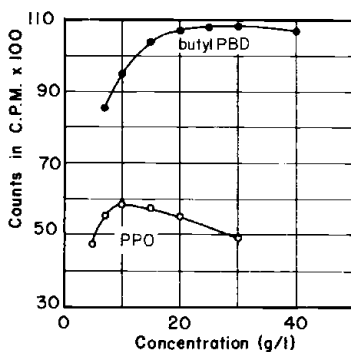


Fig. 1 Effect of increasing concentrations of PPO and butyl PBD on counting efficiency of tritiated toluene containing 0.5% CCl_4 .

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The most frequently used primary scintillators emit light with a wavelength in the region of 365 nanometers. For many years scintillation counters were equipped with photomultiplier tubes with a cesium-antimony photocathode exhibiting an S-11 spectral response curve. This is illustrated in Fig. 2 which shows that these tubes are maximally sensitive at 440 nanometers. It was common practice, therefore, to include a secondary scintillator in the sample to function as a wavelength shifter emitting light at approximately 410 nanometers. Alterations in design, however, have produced photomultiplier tubes which are most sensitive around 390 nanometers. The spectral response of two of these alkali tubes is shown in Fig.2.

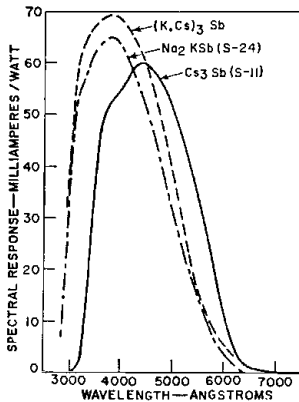


Fig. 2 Spectral characteristics of 3 photomultiplier tubes used in liquid scintillation counters (Courtesy of RCA)

It can also be seen that inclusion of a secondary scintillator, if it emitted light above 410 nanometers would actually reduce efficiency. PBBO, 2-(4'-biphenyl)6-phenylbenzoxazole, emits light at 397 nanometers and is potentially one of the more useful of the secondary scintillators currently available for optical matching

with the bialkali tubes. Whether or not a secondary scintillator should be included in a counting solution depends, therefore, on several factors and Bush and Hansen (1) have suggested that a secondary scintillator should be used only when:

1. the sample contains a compound which directly quenches the primary scintillator
2. the concentration of primary scintillator produces concentration quenching
3. the counter demonstrates better response at longer wavelengths
4. the counting sample has significant absorption in the near ultraviolet.

Sample Vials

The standard sample container has been the 22 ml low potassium glass vial. Recently low potassium vials have become available as well as a series of plastic vials. The usefulness of the smaller vial is illustrated in Fig. 3 which shows the effect of increasing the volume of the counting solution on the counting efficiency of tritium in a standard 22 ml vial and a 12 ml low potassium glass vial. The optimum volume for this counter is between 8 and 13 ml for the 22 ml vial and between 4 and 7 ml for the minivial. The small reduction in tritium efficiency of about 5 percent is due to altered geometry i.e. the increased distance between the smaller sample vial and the photo-multiplier tubes. In our laboratory we have switched almost completely to the use of minivials. The saving in cost is considerable since the vials themselves are cheaper and the amount of counting solution required is much less than for the 22 ml vial.

The background characteristics of some of these vials were investigated. Tritium background was determined in a wide tritium window where the ^3H efficiency was 60%. The carbon-14 background was determined in a narrow window set above the ^3H -spectrum i.e. at the conventional double-isotope setting. In this particular counter the carbon-14 efficiency above ^3H was 63%. Ten samples of each vial were counted twice for ten minutes each time and the results are summarized in Table I.

An interesting observation is that the major contributor to the carbon-14 background is the counter itself. The primary sources of these empty chamber counts are

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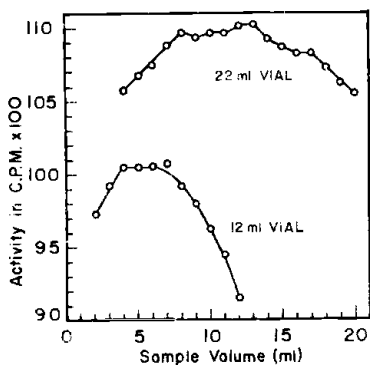


Fig. 3 Effect of counting solution volume on counting efficiency of tritium in toluene containing 8g butyl PBD/l in 12 and 22 ml low K glass vials.

contributions from the glass faces of the photocathode and from what is known as electroluminescence and arcing on the envelope of the photomultiplier tubes. Glass has a tendency to electroluminesce when subjected to an electrical gradient. This condition occurs between the pins of the photomultiplier tube which carry the high voltage and is increased by increasing humidity. In this respect, the refrigerated systems have an advantage over ambient units because the air within the cooled units is dehumidified by the cooling. Another possible source of empty chamber background is the lucite light pipe which is sometimes used to improve optical coupling between the sample and the photomultiplier tube. The lucite could be a source of Cerenkov radiation, but this is only conjecture.

For tritium the counting chamber is a minor contributor and most of the counts are due to the vial itself. Accordingly, there is a substantial reduction in the tritium background when plastic vials are used in place of glass vials. The E^2/b value for tritium in this particular counter is increased from around 130 to 330 by

Table I

SOURCES OF BACKGROUND COUNTS IN AIR-QUENCHED SAMPLES OF
TOLUENE CONTAINING 0.8% BUTYL-PBD

<u>Counting Conditions</u>	<u>³H Channel</u>	<u>C-Channel</u>
Tritium counting efficiency	60%	0.05%
Carbon-14 counting efficiency	20%	63%
<u>System</u>	<u>C.P.M.±S.D.</u>	<u>C.P.M.±S.D.</u>
22 ml low-K glass vial + 10 ml counting solution	27.19±1.91	18.57±1.72
12 ml low-K glass vial + 5 ml counting solution in polyethylene holder	17.70±1.25	16.33±1.82
20 ml polyethylene vial + 10 ml counting solution	11.76±0.91	18.40±1.53
7 ml polyethylene vial + 5 ml counting solution in plastic holder	8.94±0.95	15.34±0.77
Empty counting chamber	4.42±0.46	15.41±0.91

switching to miniplastic vials. For carbon-14 there is little or no change in background when plastic vials are used. The solvent contributes very little to the background.

Sample Preparation

Samples may be prepared by methods which result in homogeneous solutions or by procedures which give rise to heterogeneous systems (Table II). Cerenkov counting is also included here even though it does not involve any sample preparation.

Homogeneous Systems

Whenever possible samples should be counted as homogeneous systems. The isotope is then in intimate contact with the solvent and the scintillator, there is no self-absorption and various methods of quench correction such as channels ratio and the external standard ratio methods can be applied. The five subgroups listed under homogeneous counting are self-explanatory. In this area of

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Table II

SAMPLE PREPARATION METHODS FOR LIQUID SCINTILLATION COUNTING

Homogeneous Samples

1. Toluene soluble samples
2. Polar solvent systems for aqueous samples
3. Systems for cationic and basic samples
4. Systems for anions, proteins and acidic samples
5. Combustion methods

Heterogeneous Samples

1. Samples on solid supports
2. Emulsions
3. Gels
4. Solid scintillators

Cerenkov Counting

1. Direct counting of beta emitters with energies greater than 263 Kev in water

sample preparation there has been little that is new. There are a variety of solubilizing agents now available which are more effective than Hyamine hydroxide. The most commonly used are NCS, Soluene-100, Protosol and the Biosolves. One of the problems that has arisen with the use of the solubilizing agents is the difficulty in determining whether the resultant sample is a true solution. In some cases the sample may become adsorbed onto the walls of the counting vial or small particles may be present which are not detected by a visual inspection. These phenomena are evidenced by a steadily decreasing count rate or a change in the sample channels ratio. Bush (2) has devised an ingenious method for detecting the homogeneity of solution. For homogeneous solutions there is a curve linear relationship between the sample channels ratio and the external standard channels ratio. The external standard channels ratio measures the counting efficiency of

the solution whereas the sample channels ratio measures the efficiency of the sample irrespective of its solubility. For a partially soluble sample the sample channels ratio gives a different efficiency than the external channels ratio as illustrated in Fig. 4. Points which deviate from the curve were shown to be heterogeneous samples by virtue of adsorption or precipitation.

Another problem encountered with basic organic solubilizing agents is that the use of butyl PBD as the scintillator results in the development of a yellow color in the sample. The scintillator of choice, under these circumstances, is PPO. With most of these solubilizers the usual precautions against chemiluminescence should be observed i.e. neutralizing the digest with acid before the addition of the counting solution.

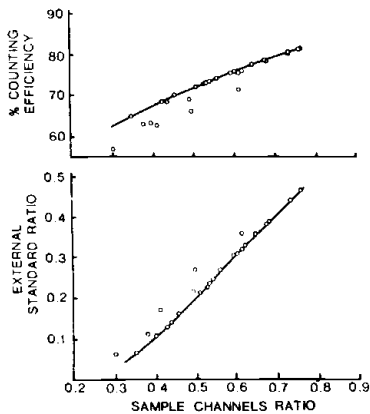


Fig. 4 Double-ratio plot of determining homogeneity of a counting solution (2).

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Combustion Techniques

One solution to most problems associated with the sample preparation is to oxidize all samples to a common material such as carbon dioxide and water. Combustion techniques, therefore, have wide application. The procedure should also be rapid, simple and quantitative and automated combustion may prove to be such a method. Our experience with the early Packard apparatus, model 305, indicated that recoveries were near quantitative for ^3H and about 95% for ^{14}C . Almost any type of biological material can be combusted but the early instrument had mechanical problems and was difficult to maintain in working order. An improved and more automated version of the oxidizer, model 306, has recently become available. Recoveries are stated to be $99 \pm 1\%$ for both isotopes and memory is less than 0.05 percent. For dual isotope combustion the contamination of ^3H with ^{14}C is less than 0.02% and contamination of ^{14}C with ^3H is less than 0.001%.

The automated combustion apparatus originally designed by Peterson in 1969 forms the basis for both the new Packard and the Intertechnique combustion units, the latter called the Oxymat, (3). This is illustrated in Fig. 5 and represents, in essence, an automated version of the classical catalytic combustion method used in organic chemistry.

Heterogeneous Systems

Samples on Solid Supports

The important factor to remember when counting samples on support media such as filter paper or scrapings from thin layer plates is that the sample should be soluble or insoluble. Partial solubility will make any measurement meaningless because the soluble fraction is counted with 4π geometry whereas the insoluble portion is counted with 2π geometry. For experimental samples the contribution from each fraction will be unknown.

Emulsion Counting

One of the limitations of toluene-based counting solutions has been the inability to accommodate milliliter quantities of aqueous samples. The best known cocktail for this purpose has been Bray's solution which is a dioxane-based counting solution which could hold over

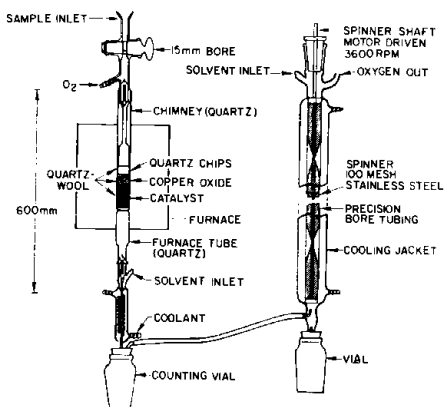


Fig. 5 The Peterson Combustion apparatus (3).

20 percent water at -50°C as a homogeneous sample (4). A variety of similar dioxane-based cocktails have been formulated over the years for counting aqueous solutions. The main drawback with these cocktails is that micro precipitation or adsorption on the vial walls may occur because salt solutions are not easily accommodated. The problem of counting of relatively large volumes of aqueous radioactive solutions has been met by the use of non-ionic emulsifying agents, the most popular being the surfactant Triton X-100. The chemical formula of Triton X-100 is shown in Fig. 6. A surfactant such as Triton X-100 is composed of a hydrophilic end which is attracted to water and to polar compounds and a hydrophobic end, which is attracted to non-polar solvents and compounds such as toluene. In the Triton series of surfactants, the hydrophilic end is, of course, the ethoxy end. The longer the polymer, the greater the solubility in water. Its solubility in water is inversely proportional to temperature - that is, the higher the temperature, the less water it can hold.

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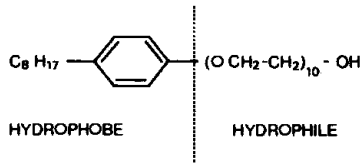


Fig. 6 Chemical formula of Triton X-100.

Increasing aqueous salt concentrations also reduces its miscibility. The Triton X-series are those which have 8 carbons on the hydrophobic end and the N-series are those which have 9 carbons.

The Triton surfactants have been formulated with toluene or xylene to hold as much as 50 percent water as a gel for counting. The Triton surfactants are probably used in the commercial products such as Insta-gel, Aquasol and PCS which are widely used for aqueous solutions. These chemicals are able to hold water as micelles as illustrated in Fig. 7. Water is held by the hydrophilic end of the Triton molecule while the hydrophobic end, represented by the tails, holds the micelle in the toluene. The water droplet represents the dispersed phase and the toluene, the external phase. The character of the micelle is a function of the amount of water being held and the micelle can be very small - less than five-hundredth of a micron - or can range over 1 micron in diameter. As the contribution of the dispersed phase increases, the optical

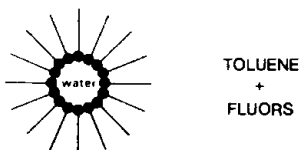


Fig. 7 A hypothetical model of a Triton X-100/water micelle in toluene.

characteristics change. In Table III the size of the micelle is related to the physical appearance of the emulsion. The maximum range of a tritium beta particle is 6 microns and the average range is 1.2 microns. This means that the tritium should be counted with solution efficiency in all but the milky emulsion. Since carbon-14 has about ten times the range of tritium, carbon-14 should be counted at solution efficiency with even the heaviest emulsions. Emulsion counting, then, appears to be a very reasonable way to prepare and count aqueous radioactive samples. However, the evidence is not at all clear that this is a simple technique. Emulsion counting requires strict adherence to a defined regimen if reliable data are to be generated.

Fig. 8 is a type of phase diagram prepared by Turner some years ago showing the effect of increasing water content on the counting efficiency of ^{14}C glucose and tritiated water in a toluene-Triton X-100 system. The percent value of the Y-axis refers to the efficiency of emulsion counting compared to that in a homogeneous solution.

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Table III

PARTICLE SIZE AND EMULSION APPEARANCE

<u>Macroglobules</u>	<u>Two Phases May Be Distinguished</u>
Greater than 1 micron	Milky white emulsion
1 to 0.1 micron	Blue-white emulsion
0.1 to 0.05 micron	Gray semi-transparent
0.05 micron & smaller	Transparent

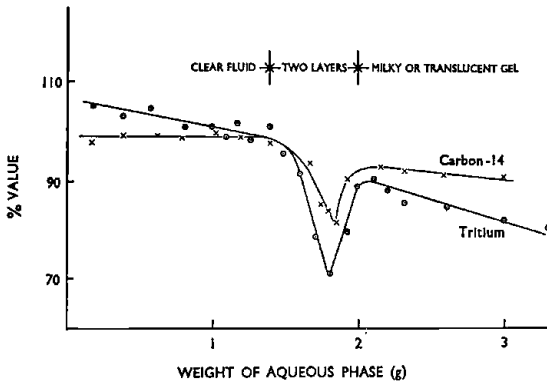


Fig. 8 Emulsion counting of D-glucose-¹⁴C (U) and tritiated water using toluene/Triton X-100, 2:1 (v/v). Reprinted from (5) by permission of The Radiochemical Centre Ltd.

The pattern shown here is typical of emulsion counting systems. With a low water content, there is a clear phase followed by a region where phase separation occurs. As more water is added, the phase then changes into a milky or translucent gel. The clear phase has been described by Turner (5) and Benson (6) as a true solution while Van der Laarse (7) classifies all phases as suspensions of water in toluene. As the concentration of water is increased, the counting efficiency of tritium decreases because of the quenching effect of water. When the first phase change occurs, phase separation into an aqueous and organic layer is observed and there is a sharp fall in counting efficiency because the tritium stays in the aqueous phase whereas the scintillator remains dissolved in the organic phase. Only the tritium beta particles at the interface between the organic and aqueous layers have sufficient energy to penetrate the organic layer and interact with the scintillator. As more water is added, an emulsion is formed which consists of sub-micron water particles dispersed in the organic phase, as already described. Since the size of the water particles or micelles is less than the mean free path of the average tritium beta particle, the beta particles can interact efficiently with the scintillator with the consequent proportionate reduction by self-absorption in the number of beta particles escaping into the organic phase resulting in a drop in counting efficiency. For carbon-14 the sample can be counted at constant efficiency in the clear phase and near-constant efficiency in the emulsion phase due to the more energetic nature of carbon-14 beta particles.

A critical problem in emulsion counting that arises as a consequence of the partitioning of solutes between the organic and aqueous phases is the determination of counting efficiency. As a general rule, for emulsions, the proper internal standard can give the most accurate results. The internal standard should have the same partition coefficient for the emulsion system as that of the material being counted and, for tritium, this would normally be tritiated water. For ^{14}C , it should be an aqueous standard solution of the material being counted. In any case, the internal standard must not be labeled toluene or hexadecane which are insoluble in the aqueous phase. For a large number of samples, the sample channels ratio method for efficiency determination is convenient and

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useful if carefully done but the external standard cannot be used to monitor counting efficiency because the Compton electrons produced by the gamma source interacting with the sample vial and its contents are not attenuated by the dispersed water particles.

There are several properties of these emulsions that need serious consideration. First, the various phases are temperature dependent, as is the counting efficiency. One report documents the fact that the counting efficiency of a Triton X-100/toluene/water emulsion increased linearly 10 percent with decreasing temperature from about 17°C to a maximum at about 4°C (8). Lower temperatures did not increase the counting efficiency further. Also, emulsions were found to give reproducible results only if they were first heated to 40°C and then allowed to cool without shaking to 4°C and then stand for 2 to 4 hours. Emulsions prepared simply by shaking vigorously at room temperature, though clear in appearance were found to be unstable as judged by lower and more inconsistent counting data.

Some of the requirements for emulsion counting are:

1. Purity of the surfactant. The different batches of commercial surfactants are not always uniform and may contain luminescent impurities as well as quenching agents.
2. All samples should have the same volume and composition, i.e., the same aqueous sample volume in all cases.
3. All samples should be prepared in the same way, i.e., heat to 40°C and allowed to cool without further agitation.
4. All samples should be equilibrated a minimum of 2 hours in the counter to stabilize the gel phase and temperature before counting since the counting properties of emulsions are temperature-sensitive. Dark-adaptation will also reduce any phosphorescence or chemiluminescence.
5. Counting efficiency should be determined either by sample channels ratio or the internal standard method. External standardization should not be used.

Cerenkov Counting

Cerenkov radiation is generated when a charged particle travels through a medium faster than the speed of light through that medium. Theoretically, a beta emitter must have an energy greater than 263 to be detected by Cerenkov

counting in water and this eliminates tritium, carbon-14 and sulfur-35. The minimum energy threshold, however, is a function of the refractive index of the medium as discussed elsewhere in this volume. The major application of Cerenkov counting for biological scientists is in the counting of ^{32}P in aqueous solution. The sample size is limited only by the capacity and geometry of the counting chamber. Disadvantages of the method are that the counting efficiencies are lower than would be obtained if the sample were counted by more conventional means and sensitivity to color quenching. Counting efficiencies can be improved by the addition of a wavelength shifter and color quenching can, in many instances, be removed by chemical treatment.

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