techniques of sample preparation for liquid scintillation counting

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TECHNIQUES OF SAMPLE PREPARATION FOR LIQUID SCINTILLATION COUNTING

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Preface

The use of weak beta-emitting radioisotopes in an increasing variety of investigative procedures, particularly in the biochemical field, has stimulated the demand for better methods of assaying those isotopes which are more conveniently incorporated into organic molecules employed as precursors.

The prime object of the present work is to bring together the many different ways that have been designed to prepare the samples in a form suitable for liquid scintillation counting. Although the prime object is to bring the weak beta-emitter into close molecular contact with the primary solvent in the system, the method of doing this is highly dependent on the nature of the sample being measured. The initial sample for assay can exist in any state and associated with many other components which exceed the concentration of the actual beta emitter by many orders of magnitude. Where the level is low, careful thought should be given to the possible means whereby the labelled material itself may be isolated or alternatively the contaminating materials removed from the labelled substance. Occasionally, either one of these processes may be effected by solvent extraction, but before doing so, the sample multiplicity must also be considered. The number of samples may be too great to allow even a simple process of this kind to be conducted within a reasonable time. A decision as to whether or not such a tedious extraction procedure should be contemplated will depend on the level of accuracy needed by the experiment.

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Such decisions are often overlooked before assay and many users of liquid scintillation instruments rely solely on the claims of the many commercial scintillation cocktails available, hence incurring unnecessary expense. Often in addition, incorrect application of such cocktails may produce anomalies which are thought to be associated with the experiment itself rather than with the counting conditions employed.

In every counting technique used, some form of 'quenching' (see § 1.2) will be experienced. This reduction of counting efficiency can usually be accurately assessed and allowed for in a number of different ways. However, there are certain situations where these correction procedures cannot be used. It is in these circumstances that anomalous results usually appear and are often unrecognised by the experimenter.

In many, and probably in most biochemical applications, it may not be essential to obtain an accurate assessment of the absolute level of isotope present. Reproducibility of sample counts by careful control of the sample preparation is often the goal to be achieved. The ability to conduct several hundred measurements with a reproducibility of one and often up to 5% between samples is often all that is necessary. The correct initial choice of counting method at the outset will be repaid by the avoidance of many hours of tedious and unnecessary sample preparation, which may otherwise be costly not only in time consumed but also in the preprocessing and scintillation materials used.

Most of the confusion arising from apparently conflicting data in the literature on sample preparation methods, appears to arise from the fact that many authors consistently refrain from stating the reference efficiency of the machine being used. It is thus not possible to determine from the work, whether the increased efficiencies apparantly obtained by his method is indeed an improvement or just a demonstration of the acquirement of a scintillation counter with improved engineering. Also since a judicious choice of window settings can appear to confer increased efficiency, open window data should be quoted in making such comparisons for single isotopes. This would avoid giving artifactual increases by quench shifts into lower windows.

It is suggested that for data on the measurement of low specific activity solutions an Instrument-corrected Merit Value (MIV) be used. Where there is a possibility of instability due to phase perturbations such as in heterogenous liquid scintillation counting, an instrument efficiency and stability corrected value (MISQ) be used. These terms are defined in greater detail in § 2.2 and Appx. IV.

Only a passing reference will be made to the scintillation equipment itself, since this has been the subject of several authoritative reviews. The choice of instrument is more often than not dictated by the money allotted to this aspect of the work programme. The instrument expense is usually related more to the cost of data-processing associated with it, rather than to the basic instrument; due consideration should be given to the number of users and samples to be assayed and whether or not some centralization of the facility is envisaged. In practice, an instrument holding 300 samples is a convenient size, since it will allow samples to be counted for 10-min periods over a week-end. Where shorter counting only is required, e.g. 2-min counts, it is clearly an advantage to purchase equipment with a higher potential capacity for samples.

Probably the most important point to bear in mind throughout all the applications of this technique is to be aware of the proportion of disintegrations that are being measured, and to force this to be as high and as constant as possible with the least effort.

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List of abbreviations

(excluding those chemicals referred to in Appendix II, table 1)

ANDA	7-amino naphthalene-1,3-disulphonic acid
ATP	adenosine triphosphate
BHT	di-t-butyl-4-hydroxy toluene
cpm(s)	counts per minute (second)
CEA	carcino embryonic antigen
Ci	Curie
DEAE	diethylaminoethyl
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dpm(s)	disintegrations per minute (second)
EDTA	ethylenediamine tetra-acetic acid
ESR	external standard ratio
eV	electron volts (KeV = 1000 eV, MeV = 10^6 eV)
FMN	flavin mononucleotide
HeLa	a human cell line
LSC	liquid scintillation counting
NAD	nicotinamide adenine dinucleotide
PCA	perchloric acid
PSD	pulse shape discrimination
RNA	ribonucleic acid
RPH	relative pulse height
SCR	sample channels ratio
TLC	thin layer chromatography
TCA	trichloracetic acid

Introduction

Throughout the book, a simple coding system has been employed to designate the scintillant mixture being used. Such a coding is explained in Appendix II and the detailed compositions are listed in Tables 3 to 5 of this Appendix. The use of this coding system enables the qualitative composition of the scintillant to be recognized. Slight variations in the proportions of the components present are sometimes, but not often, carefully evaluated by the authors who proposed them. Most of the compositions are empirically derived and a rigorous assessment of the relative proportions of primary solvents, scintillants and blenders may reduce the number of really useful compositions even further.

My special thanks are due to my sister, Miss Mary Fox, for the very careful typing of the manuscript and to my wife and parents for checking. Thanks are also due to my scientific friends and colleagues who have suggested additional items, as well as the deletion of some.

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General principles of liquid scintillation spectrometry

1.1. Scintillation mechanism

The basic process of converting the energy derived from a beta particle to photons which can be detected efficiently by photomultiplier systems has been the subject of many detailed and authoritative works. Apart from technical broadsheets prepared by many of the liquid scintillation spectrometer manufacturers, a number of standard works has also been written. Most important of these are by Birks (1964, 1970) and Gundermann (1968). Edited collections of papers delivered at symposia devoted to different aspects of liquid scintillation spectrometry include those edited by Bell and Hayes (1958), Horrocks and Chin Tzu Peng (1971), Bransome (1970), Dyer (1971) and Crook et al. (1972).

Only a broad outline of the scintillation process will be given here, in as sufficient detail as is necessary only to help to understand the ways in which poor sample preparation techniques can introduce error or decrease the efficiency of counting.

Stage 1: The energy derived from a beta particle is first absorbed by the aromatic solvent molecule known as the *primary solvent*. Ninety % of the energy will be dispersed in the primary solvent molecule in exciting the bonding 'sigma' electrons and will be lost from the scintillation process as vibrational energy and heat. About 10% of the absorbed energy, however, will excite the more fluid and excitable pi electron system which exists around the molecules and raise them to higher excited states. The process is represented by eq. (1.1)

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$$\text{beta-emitter } (E) + \text{solvent } (X) \rightarrow \text{excited solvent } (X^{+}{}^{+}\cdot\cdot) \quad (1.1)$$

where E is the energy of the beta particle and X is that of the solvent. The suffix $+ +..$ represents varying degrees of excited states present in the solvent molecule after the initial excitation energy has been dissipated. The many higher excited energy states of the pi electron system soon dissipate their excess energy ($X^{+}\cdot\cdot$) as *internal conversion energy* and the excited energy descends almost entirely into the first excited singlet state (X^{+}) according to eq. (1.2).

$$(X^{+}{}^{+}\cdot\cdot) \rightarrow (X^{+}) + \text{internal conversion energy} \quad (1.2)$$

The number of such excited molecules, A, is related to the nature of the solvent itself and to the energy of the beta particle by the equation

$$A = sE \quad (1.3)$$

(where s is the solvent conversion factor).

The value of s is such that one excited molecule results from approximately 100 eV of beta particle energy deposited. For the purposes of comparing different solvents from this point of view, it is convenient to regard toluene as having an 's' value of 100. Table 2 Appx. II lists these values for a number of solvents. The data give some insight into the excitability of different solvents and together with the solubility data of a primary solute, can suggest suitable combinations of primary solute and primary solvent. However, there are several other factors to be taken into consideration first.

Stage 2: The energy stored in one primary solvent molecule as excitation energy, does not remain there, but by a combination of thermal movement and other diffusion changes, the excited energy is transferred to an adjacent molecule and forms a short-lived dimer in doing so, a so-called 'excimer' (for a detailed treatment see Horrocks 1971). The energy is very rapidly transferred from molecule to molecule by this *solvent–solvent energy transfer process.* This process is highly efficient and takes place in approximately one picosecond (10^{-12} sec). Provided that the molecules are in close proximity to one another,

i.e. no blending or quenching molecules interrupt the process, the transfer is also quantitatively very efficient and can be represented by

$$X_a^+ + X_b \rightleftharpoons (X_a - X_b)^+ \rightleftharpoons X_a + X_b^+ + \text{vibrational relaxation energy} \quad (1.4)$$

Stage 3: The process of solvent–solvent interaction by excimer formation and dissipation continues until contact with a primary solute molecule (Y) occurs, into which energy is preferentially transferred by a solvent–solute energy transfer process. Since the first excited singlet state of the latter is usually slightly lower than that of the former, there is again some energy loss in the transfer process, represented by

$$X_b^+ + Y \rightleftharpoons X_b + Y^+ \quad (1.5)$$

The efficiency of this transfer process is indicated by the solvent–solute energy transfer quantum efficiency (f). This value is the fraction of excited solvent molecules which succeed in transferring their energy to the primary solute and is affected only by the concentration of the primary solute in the solvent. For most of the combinations normally used in scintillation counting the value of 'f' is close to 1.0. The number of excited primary solute molecules (B) arising from 'A' number of excited primary solvent molecules is then given by

$$B = fA \quad (1.6)$$

Stage 4: An excited primary solute molecule, e.g. diphenyl oxazole (PPO), liberates within a few nanoseconds (solute fluorescence lifetime) of its excitation, a fluorescence photon, with a relatively high efficiency (solute fluorescence quantum efficiency = q). The wavelength of the photon emitted will depend on many structural features of the primary solute itself as will also the efficiency with which it is emitted. There is also some evidence to suggest that there is a similar solute–solute energy transfer process involving excimers similar to that between primary solvent molecules, and could be represented as in eq. (1.4).

$$Y_a^+ + Y_b \rightleftharpoons (Y_a - Y_b)^+ \rightleftharpoons Y_a + Y_b^+ + \text{vibrational relaxation energy} \quad (1.7)$$

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If this process terminates in light production however, the reaction will be represented as follows

$$Y_b^+ \rightarrow Y_b + h\nu \ (h = \text{Planck's Const.}, \nu = \text{frequency of emitted light}) \tag{1.8}$$

The final number of photons emitted (P) is closely related in amount to the number of excited solvent molecules produced initially, provided that the quenching level is nil

$$P = qB = sfQE \tag{1.9}$$

However, one of the effects of quenching molecules (see § 1.2) is to decrease the number of photons emitted per original excited event and hence the number of photons per event will thereby decrease. Furthermore, it is worth noting at this stage, that the higher the energy of the original beta particle, the greater the number of photons eventually produced, but the effect of quenchers is to reduce the effectiveness of such events in producing photons. Hence the *proportion* of light quanta will be reduced by the same proportional amount, whether the source of the photon emission was a weak or a strong beta-emitter. This fact is used in the 'external standard method' i.e. using a gamma-emitter to produce Compton electrons which in turn are used to determine the degree of quenching of a soft beta-emitter.

Stage 5: The final stage of the liquid scintillation process is a modification of the light spectrum produced to match more closely the spectral sensitivity of the photomultipliers used. The proportion of overlap of the fluorescence spectrum from the primary solute, with the sensitivity spectrum of the photomultipliers is known as the matching factor (m). Owing to an improvement in the design of the photocathodes of the photomultiplier tubes, the degree of overlap has considerably increased, i.e. better matching factors are obtained. In the earlier instruments, the addition of a secondary solute or wave-shifter, improved the matching factor by causing the finally emitted light to overlap more completely the less sensitive photomultiplier tubes in use at the time. The secondary solute is a similar aromatic fluorescent molecule to that of the primary solute, but is usually used