LIQUID SCINTILLATION COUNTING

Volume 5

Edited by:
M. A. Crook
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LIQUID SCINTILLATION COUNTING

INCORPORATING WHOLE-BODY COUNTING
AND RADIOIMMUNOASSAY

Volume 5

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Preface

This volume records the proceedings of the fifth in the series of International Symposia organised by the Radiochemical Methods Group (Analytical Division of the Chemical Society). Invited plenary lectures were given by Dr. E. D. Bransome, Prof. J. Landon, Dr. L. Burkinshaw and Dr. D. L. Horrocks. Special thanks are due to Dr. D. Edgington who stepped in at the last moment to undertake a plenary lecture. Unfortunately no script of Dr. Edginton's talk was available for publication. The Chairmen of the five sessions were Dr. P. Johnson, Dr. D. Taylor, Dr. G. Ayrey, Dr. A. Ware and Mr. G. Sutton. In all about 200 delegates attended the Symposium, representing 20 different countries.

As is customary an excellent exhibition was mounted by various manufacturers:

Alrad	Lumac N.V.
Beckman RIIC	Nuclear Chicago
Berthold	Nuclear Data
E.S.I. Nuclear	Nuclear Enterprises
Fisons	Packard Instruments
Intertechnique	Pye Unicam
Koch Light	The Radiochemical Centre
Kontron	Wang Electronics
Laboratory Impex	Werner Zinsser
LKB Instruments	

It is a pleasure to record our thanks to the Mayor and Council of the City of Bath for the provision once again of such excellent facilities and delightful surroundings for the symposium.

Following a suggestion made previously and referred to in the preface to Volume 4, the title of the present Symposium was modified to "Scintillation Counting", which widened the scope of the meeting and allowed the introduction of a number of topics of particular interest at this time. In order to maintain continuity of the series, however, the title of this volume has been retained as "Liquid Scintillation Counting".

May 1978

M. A. Crook
P. Johnson

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SECTION I
SAMPLE PREPARATION

Chapter 1

Some Problems in Sample Preparation for Liquid Scintillation Counting and their Solutions

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INTRODUCTION

Our august predecessors who have been given this subject to discuss in the twice-yearly meetings at Brighton or Bath have tended to discuss one specific technique of sample preparation or have developed arguments for one admonition or another. Although one of us has authored more than his share of 'Warnings' and 'Pitfalls' we find it difficult to choose between admonitions, much as a fundamentalist preacher finds it hard to decide which of the many preoccupying sins committed in his parish should be the topic of next Sunday's sermon. Suffice it to say that we find that many of our colleagues in medical research (who are cautious, careful, and even cynical in other respects) are content to take a sample, drop it into a scintillation vial, add a commercial scintillator-solvent combination with a catchy name, press the start button on a liquid scintillation (LS) counter and treat the resultant numbers as items of immutable truth. The radioactivity of a sample is regarded as not at all subject to the same vagaries that beset the investigations which have produced the sample.

Thus, although a discussion of sample preparation at a meeting organized by the Radiochemical Methods Group should probably not be a recitation of problems and their solutions (or vice versa), such a recitation is the purpose of this paper. Although some new information is worth reviewing (e.g. for counting α particles and Cerenkov radiation), we still find ourselves preoccupied with the common sources of errors in LS counting. We therefore intend to remind our readers of the existence of these problems and when possible how to discover and avoid them.

We assume at the outset that a 'sample' encompasses the scintillation vial and anything put into it. That the composition of the total sample is important has been amply documented by Kalbhen and Rezvanie[1] who showed that using the same preparation of β-emitting nuclide, different problems were encountered with each of 17 scintillation cocktails, three emulsifiers, and two sample oxidation methods.[1] Little and Neary[2] have shown in addition that for each type of radioactive sample and solvent there are different optimal concentrations of scintillator.[2]

The possibility that using commercial mixtures of primary and secondary solvents will result in less than ideal counting conditions should be an important consideration for anyone doing LS counting. Unfortunately such thoughts and the consequent studies of methodology are strange to most laboratories. In the pious hope that knowledge of the problems of sample preparation is power, we discuss:

(a) disparity between impurity (chemical) and color quenching;
(b) the peculiarities of dioxane as a scintillation solvent;

(c) difficulties with alkaline samples;
(d) the effect of vials on counting efficiency;
(e) difficulties with heterogeneous samples (solid supports, emulsions).

Once the investigator is aware that he is confronted with a problem, it is often possible for him to solve it.

We have also elected to review, albeit briefly, significant recent advances in the use of LSC for the measurement of Cerenkov radiation, α emitters, and nuclides which are 'novel' as far as LSC is concerned: those which decay primarily by electron capture or by γ-ray emission.

COLOR VS IMPURITY ('CHEMICAL') QUENCHING

Even with sample homogeneity established, the question of whether acceptable quench-correction curves can be constructed from series of standards of different composition, remains. Neary and Budd[3] originally pointed out that while there was no divergence of color and impurity quench-correction curves for tritiated samples, the effects of significant color quenching on ^{14}C efficiency could not be predicted accurately from plots of sample channels ratios (SCR) or external standard channels ratios (ESCR) obtained with a series of colorless quenched standards.[3] The remedial strategy of making up suitable color-quenched standards[4] is complicated by the necessity of closely matching the absorbance spectra of the unknown samples (which may be quite complex) in the sensitive wavelength band of the photomultiplier tubes of the specific liquid scintillation counter being used.[5]

Decolorizing each sample will eliminate this problem of adequate standardization[4] which of course applies to counting any nuclide with emission energy above the tritium range, and to Cerenkov counting as well as standard LS counting. In the special case of the ferric ion which is known to be a strong color quencher, Blanusa[6] has shown that adding fluoride ion (as NaF) would eliminate most of the effect of the ion on counting efficiency.[6] Chemical decolorization will, however, by adding impurities to the sample, increase quenching and if peroxides are used with ^{14}C-labelled samples, radioactivity may be lost as $^{14}CO_2$. Combustion of the sample will yield colorless counting mixtures of known composition, but has not yet been shown to be a reliable methodology except for ^{3}H and ^{14}C and perhaps ^{35}S.[7] It requires, moreover, considerably more time and expense spent in sample preparation and the acquisition of automated sample oxidizers if many samples are to be prepared.

Several advances in LS counter design should eliminate or at least minimize the overestimation of the counting efficiency of colored samples by impurity quench-correction plots from instruments in which pulse height spectra are summed. One is a technique introduced by Laney into Searle Analytic (Nuclear Chicago) LS counters a few years ago: lesser pulse height analysis.[8-11] The other has been introduced even more recently to Beckman LS counters by Horrocks: the H number (H#) a measurement of the inflexion point of the Compton electron spectrum generated by a single γ-emitting external standard.[12] Either of these new electronic features, in addition, makes it possible to determine the absolute radioactivity of an unknown sample.

Painter[5] has recommended that the quenching in colored particulate samples be assessed by internal standardization. When samples are truly homogeneous and differences between the partition coefficients of added labelled standards and unknowns are not a problem, this is an excellent strategy. When LS counters which do not have lesser pulse height analysis are used to count colored samples, quenched series of internally standardized unknown samples should always be prepared to discover whether quench-correction by sample or external standard (Compton electron) channels ratio techniques will give acceptable counting accuracy. If samples are severely color-quenched, however, spectra are so distorted that there is no algorithm which can be used to predict efficiency.[13,14]

SAMPLES IN DIOXANE-BASED SCINTILLATION MIXTURES

Since Bray's article[15] of 1960 showed that aqueous samples were miscible with a solution of scintillator in dioxane, we have been concerned with either how much

water dioxane-based cocktails could tolerate, or with occasional chemiluminescence which is attributed to peroxide impurities. Wombacher and Reuter-Smerdka[16] have recently pointed out, however, that unexpected losses in efficiency may occur in dioxane mixtures with only small amounts of water.[16] The presence of metal ions (Ca^{2+}, Mg^{2+}, Cu^{2+}) and labelled compounds with which they may combine (e.g. nucleotides) may lead to the formation of heterogeneous samples: self-absorption thus may occur with no visible evidence of precipitation or of phase change. In such a situation decreased counting efficiency cannot be detected by comparing external standard and sample channels ratios or via internal standardization with $^{3}H_2O$ or ^{3}H-toluene, one of the 'tests' of sample homogeneity suggested by Bush[17] and Mueller.[17,18] This problem of 'photon' quenching was not encountered when identical samples were mixed with emulsions or thixotropic gels.[16]

As a method of precipitating plasma proteins, adding a portion (1:1) of 1,4-dioxane has recently been proposed[19] rather than ethanol which tends to be a significant quenching agent. The supernatant is then miscible with dioxane-based scintillation media.

ALKALINE SAMPLES

The hydrolysis of labelled biological samples with strong base leads to problems if standard LSC solutions are employed: development of color quenching as a result of reactions of organic scintillators with alkali chemiluminescence,[20] and[21] (if organic acids are added to neutralize the base) severe impurity quenching[21] and phase separation.[1] Under certain circumstances addition of acid may even produce chemiluminescence.[1]

The use of a solubilizer tolerant to alkali (Biosolv BBS-2) has been reported[22,23] as a method which is successful in eliminating chemiluminescence (which presumably results from the oxidation of lipids in the sample)[24] or as unsuccessful in doing so.[25] Several years ago Neame reported some success with strong acidification, addition of water, and then of secondary solvent (Triton X-100).[20] Flindt-Eegbak[25] has recommended that Instagel TM (Packard Instrument Co.) be used to dissolve neutralized alkali hydrolyzates inasmuch as no significant chemiluminescence occurs. He has not provided data which would allow us to assess the accuracy of sample channels ratios for the calculation of efficiency, nor has he shown that chemiluminescence is totally eliminated.

In most circumstances, a sample of unknown composition (usually of biological origin) is hydrolyzed. The efficiency is compromised by impurity quenching and color quenching from complex absorption spectra. If the nuclide of interest is ^{3}H or ^{14}C, combustion of the sample is highly recommended.

PROBLEMS WITH VIALS

Permeation

The loss of scintillators and solvents from soft polyethylene vials over a few days or weeks may be appreciable.[26,27] Mueller[28] and others[29,30] have reported a continuous drift of external standard channels ratios over several days which is a consequence of progressive migration of scintillator into the vial walls. The extent of this migration is greater at ambient temperature than in refrigerated LS counters; Compton cpm accumulate in the lower energy range. As a result of this phenomenon (which is not observed with glass and nylon vials) small amounts of labelled samples will also be lost. Aqueous samples in emulsions may also be lost to a significant extent over a period of days[31] resulting in lowered counting efficiency. The conclusions to be reached from these observations are obvious:

1. Soft polyethylene vials should not be used to count samples dissolved in organic solvents.
2. If other plastic vials are used, some idea of the extent of permeation of the vial wall by organic scintillators may be obtained by determining the external standard channels ratio at several intervals over 24 h as suggested by Johanson and Lundqvist.[29] If the ESCR changes, then quench correction should be carried out by other approaches.
3. Whether the solvent is organic or aqueous, the vials glass or plastic, it is

likely that significant radioactivity will be lost from the counting solution if it is stored for several weeks. If there are (uncommon) circumstances that require such delays, the vial should be made of glass and securely flame sealed.

Sample adsorption to vial walls

A number of investigators[32] have reported a loss in the counting efficiency of labelled samples because of their adsorption to the walls of glass scintillation vials. Wigfield's laboratory in Canada has been active in the past few years in studying this phenomenon and have developed an algorithm (A) the 'Adsorption Shift'[33,34] which takes advantage of the pulse height shift (to lower photoelectron energy ranges) caused by the backscattering of nuclides adsorbed in 2π rather than 4π configuration. Sample channels ratios (SCR) are compared to external standard channels ratios (ESCR), the former being affected by adsorption and the latter not. Bush[17] has observed previously that quench correction plots of the two ratios vs counting efficiency diverged when heterogeneous samples were examined.

$$\underline{A} = \underline{a} (\log \text{ESCR} + \underline{b} - \log \text{SCR})$$

The constants a and b were obtained by determining the log ESCR and log SCR for a non-adsorbed labelled sample (e.g. hexadecane or toluene), knowing that an equation exists for the relationship: log SCR = a (log ESCR + b). With samples which are not adsorbed, A = 0.00; with maximal adsorption A is about 0.40.

Wigfield claims to be able to evaluate adsorption losses of ^{14}C and more energetic nuclides,[35] but no 'Adsorption Shift' is observed with the decreased counting efficiency of weak β emitters such as tritium,[36] and up to 20% of the ^{14}C radioactivity in a vial may be adsorbed before an 'adsorption shift' is detected.[37] With energetic isotopes such as ^{32}P, adsorption shifts may be encountered[38] which are not necessarily due to simple adsorption to vial walls but to Cerenkov radiation and to other as yet unknown phenomena.[39]

Two other tests may be performed: emptying the vial and counting it again with fresh scintillation medium is a simple and obvious tactic. Dilution with unlabelled carrier[32] is possible only if the specific carrier is available and if precipitation of the sample will not occur as a result. The chemicals which tend to be adsorbed significantly are high in oxygen content[38,40,41] and in specific activity. The critical concentration to saturate binding sites was found by Wigfield to be 2×10^{-6} M or 3×10^{-8} mol per 15 ml of counting solution. Solutions to this problem include:

(a) Using plastic vials. (As already noted, this may result in permeation of the sample through the vial wall.)
(b) Adding carrier. (This may not be practical).
(c) Emulsion counting.

The latter has been suggested as a strategy for eliminating ^{32}P orthophosphate adsorption[38] but has not to our knowledge been tested with other labelled compounds liable to adsorb to vial walls.

SAMPLES ON SOLID SUPPORTS

Our laboratory has already been heard from in warnings of the inaccuracies encountered when β emitting samples are counted on paper, cellulose ester filter discs, or glass fiber paper.[42] Recently Long et al.[43,44] have reiterated the message that 'it is necessary to dissolve the filter in order to obtain the true activity of the sample and to give the magnitude of error encountered...' Despite all this, many investigators assume that losses in counting efficiency are constant or can be calculated through the use of standard quench-correction procedures. Drying the filters first frequently results in the translucency of filters in scintillator solutions — an effect which improves counting efficiency but not to the same extent as dissolution of the filters. Samples are still heterogeneous unless the filter is dissolved or the sample is completely taken up from the filter into the scintillator medium. If the dissolution in incomplete the sample will be counted in two phases.

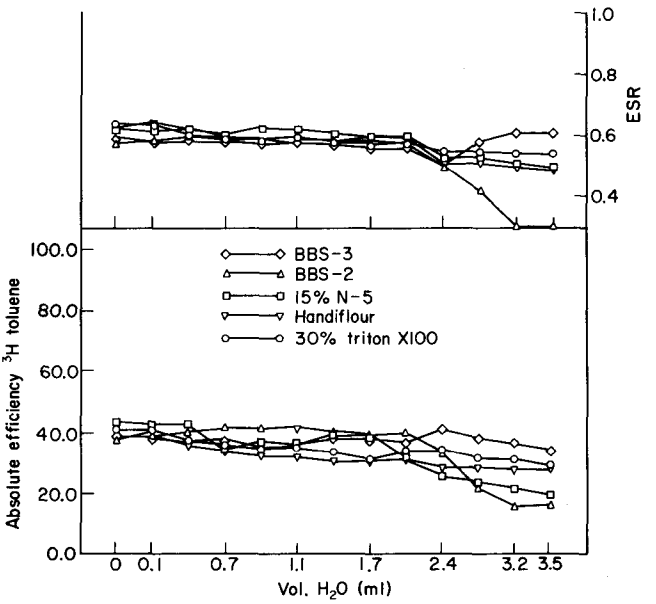

Fig. 1. The effect of added water and surfactants on the efficiency of counting ^{3}H-toluene which was obtained from the New England Nuclear Corporation, Boston, Massachusetts, U.S.A. 22 000 dpm were added to each vial. Radiolabelled 50 μl samples were added to a 10 ml counting solution consisting of toluene and 5.5 g l^{-1} Packard Instrument Co. Permablend (*91% PPO, 9% POPOP*) (except when Handifluor-Mallinckrodt™ was involved). The surfactants indicated in the figures were added to the volume. Each ml of water is thus representative of the percent volume if multiplied by 10 (e.g. 3 ml = 30% H_2O). Counting was carried out at ambient temperature with a Beckman LS-230 counter using an optimal wide channel for the isotope of interest. External standard channels ratios were calculated from the Compton electron spectra of a ^{137}Cs source using fixed channels. Biosolv BBS-2 and BBS-3 were obtained from Beckman Inc., Triton X-100™ from the Rohm and Haas Co., N-5 Handifluor™ from Mallinckrodt Inc. N-5 is a mixture of 94% nonylphenoxy ethanol and 6% sodiumdihexylsulfosuccinate prepared in our laboratory.

The use of tissue solubilizers to dissolve filters is inadvisable, inasmuch as considerable color quenching usually ensues.[45] Beckman Inc. have developed an emulsion system — Filter Solv™ which will dissolve cellulose acetate, cellulose nitrate or mixed ester filters.[43,44] It is worthwhile remembering that the sample may remain precipitated and may therefore be heterogeneous despite the dissolution of the filter. We thus find ourselves in disagreement with the statement of Apelgot and Duquesne[46] that 'it suffices simply to have a contact between the β particles and the liquid scintillator'.

Neither automatic quench-correction techniques nor internal standardization should be employed. External standard channels ratios are completely insensitive to losses of efficiency because of the 2π configuration of the sample. Sample channels ratios are unreliable inasmuch as the SCR may drop when samples are dissolved from filters while the count rate increases.[44] With a weak β emitter such as tritium no SCR vs efficiency plot should be employed to correct for absorption of βs; with more energetic nuclides a relationship between SCR and lost efficiency may be plotted but almost always with unacceptably high variance.[36] With energetic nuclides such as ^{36}Cl and ^{32}P, on the other hand, there may be significant losses of efficiency because the sample is positioned less than 1 cm (the approximate maximal range of ^{32}P in toluene-based scintillation fluid) from the vial wall.[47,48]

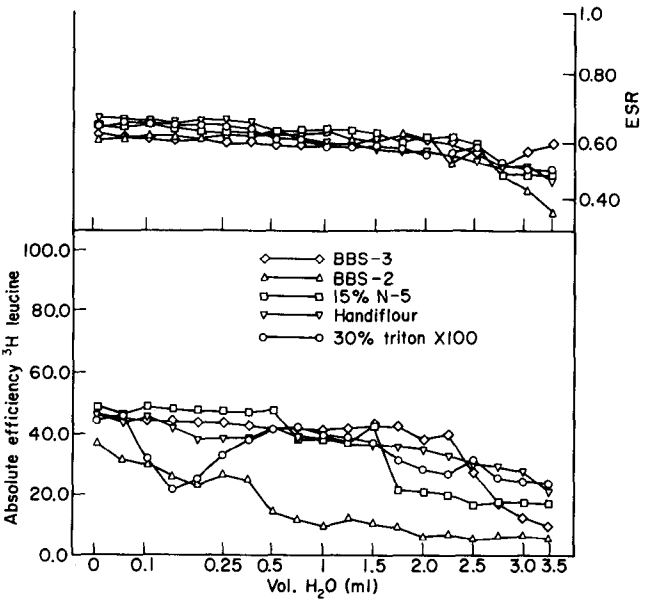

Fig. 2. The effect of added water and surfactants on the efficiency of counting 4,5-^{3}H-L-leucine (New England Nuclear, 62 Ci mol^{-1}). 36 666 dpm were added to each vial. See the legend to Fig. 1 for further details.

SAMPLES IN EMULSIONS

Surfactants combined with the usual scintillators and organic solvents (toluene or xylene) have become extremely popular for counting βs in aqueous samples. Such solutions are able to hold water in micelles; the physical character of the micelles is affected by the amount of water added, the solutes present, and the temperature.[49] The use of Triton X-100 (a non-ionic surfactant) was first recommended 15 years ago[49] and still receives considerable attention (despite some variability between lots as to capacity for water and potential for chemiluminescence).[50,51] Most commercial surfactants are, however, combinations of non-ionic surfactants and lesser amounts of anionic surfactants.[52-59] There are a number of excellent reviews, including those by Fox[18] and by Mueller[18] in previous volumes of this series. We have already pointed out that surfactants may act as scintillators and that counting standards should thus contain the surfactant, solvent and scintillator employed for the preparation of practical samples.[60,61]

In the present review, we seek to emphasize that there may be considerable inaccuracies in applying automatic (ESCR and SCR) quench-correction techniques if the samples are functionally homogeneous (i.e. if the nuclides are counted in '2π' rather than '4π' configuration, virtually cutting counting efficiency in half). The greatest problem is that phase separation may occur before a visible change in the sample (e.g. see Ref. 52). If emulsions do break into two distinct phases, each has its own character as a scintillation medium, the partition of the radionuclide(s) being measured in either phase being a function of the solubility of the labelled sample in water in the organic solvent. Emulsions are also sensitive to varying amounts of other solutes and to the specific surfactants employed; their physical behaviour is thus difficult to predict unless the nature and amount of labelled unknown samples are extremely reproducible.

The experiments summarized in Figs 1-4 showed (in confirmation of many other studies) that the ESCRs could not predict counting efficiencies and that organic (^{3}H and ^{14}C-labelled toluene) standards could not predict the efficiencies observed when aqueous samples were counted. That different surfactants behave differently is also evident; what is not immediately evident is that degradation in counting

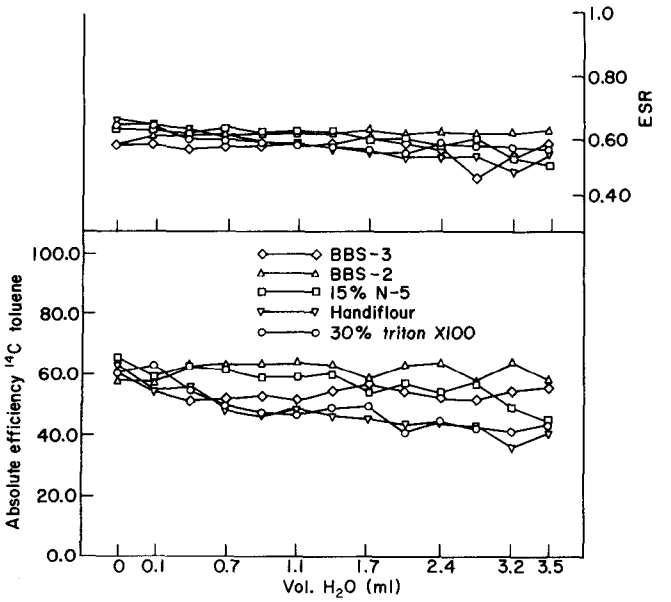

Fig. 3. The effect of added water and surfactants on the efficiency of counting ^{14}C-toluene (New England Nuclear). 20 600 dpm were added to each vial. See the legend to Fig. 1 for further details.

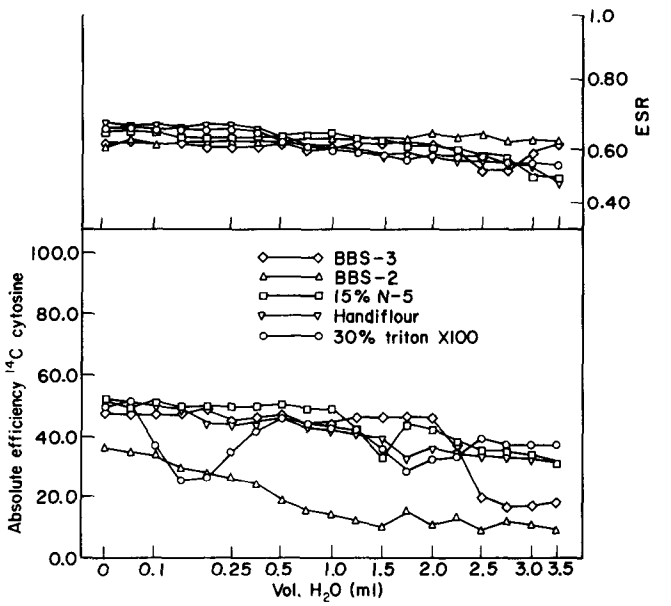

Fig. 4. The effect of added water and surfactants on the efficiency of counting 2-^{14}C-cytosine (New England Nuclear, 5 Ci mol^{-1}). 36 666 dpm were added to each vial. See the legend to Fig. 1 for further details.

efficiency usually could be detected with a lesser amount of added water than it took to achieve a visible phase separation and that, as Wagstaff and Ware[56] have pointed out, such drops in counting efficiency predict phase separation. In most of the emulsions (especially with Triton X-100) a certain volume of water is necessary to achieve optimal efficiency.[51,62]

Comparison of ESCR vs efficiency and SCR vs efficiency plots is usually (but not always) a sufficient practical test of sample homogeneity if ^{14}C or more energetic βs are to be counted. How reliable this is depends on the LS counter being used. Lesser pulse-height analysis, an electronic feature of some LS counters, has, as has been noted above, been helpful in eliminating differences between color and impurity quench-correction curves. The accuracy of comparing ESCR and SCR quench-correction curves to assess sample heterogeneity is, however, much less certain than with summed pulse-height analysis.[9] For tritiated samples the 'double ratio' plot is insensitive to heterogeneity: cpm are simply lost. Internal standardization with the same radiolabelled substance as that in the unknown samples is one remedy, but addition of more solute may then significantly change the physical character of the emulsion. Making up standards with nuclides which are restricted to the aqueous phase on one hand and to the organic phase on the other (e.g. ^3H_2O and ^3H-toluene) and comparing efficiencies as suggested by Mueller[18] is a useful and fairly stringent test. It is also worthwhile remembering that the surfactants have their individual quenching properties. Laney, for example, has pointed out that Triton X-100 exhibits more color quenching than other surfactants despite visual similarity of samples containing either secondary solvent.[9]

CERENKOV COUNTING

Cerenkov radiation is emitted when a charged particle exceeds the speed of light in a transparent medium. It is almost completely independent of the chemical nature of the medium, and is thus unaffected by 'impurity' quenching. Samples are, however, subject to color quenching and should be quench-corrected by internal standardization if possible.

There have been two very recent excellent reviews of the measurement of Cerenkov radiation in liquid scintillation counters.[63,64] The threshold energy for βs in water is 264 keV but can be increased by addition to the solution of a compound with a high effective atomic number. Methyl salicylate, for example, has been shown by the Radiopharmacy Group at the University of Alberta to increase counting efficiency dramatically, partly as a result of its high refractive index and partly because of its properties as a wavelength shifter.[65]

If a wavelength shifter with the properties of a scintillator (e.g. dimethyl POPOP, 4 methyl-umbelliferone) is added to the sample, then the independence from impurity quenching is mitigated. Ross has recenty introduced a quartz counting vial with an isolated sealed external compartment containing a wavelength shifter, which matches the spectrum of emitted light to higher wavelengths necessary for good photomultiplier response and thus increases counting efficiency.[64]

Gamma-emitting isotopes may be counted by the Cerenkov effect of the Compton electrons they generate at low (1.5-10%) efficiencies. Despite some suggestions in recent journals that samples adsorbed to filters of charcoal can be reliably counted by measurement of Cerenkov radiation, samples not in solution will suffer self-absorptive losses which cannot be monitored. Samples for Cerenkov counting should, therefore, always be in solution.

ALPHA COUNTING

That α particles should be counted with liquid scintillation equipment was evident more than 20 years ago.[66] Although the energy of α emissions is high (generally between 4 and 6 MeV) compared to that of β emitters, the range of αs is short, and the fluorescence quantum yield is much less than for βs — about 10%. Standard LS counting techniques can be employed if the α-emitting nuclide is in the same phase as the organic scintillator, if the α activity is at least several hundred cpm, and if no β- or γ-emitters contaminate the sample. Since the presence of impurities in the sample will quench and shift α-energy spectra, α peaks are in