Chapter 14

Correct Liquid Scintillation Counting of Steroids and Glycosides in RIA Samples: a Comparison of Xylene-based, Dioxane-based and Colloidal Counting Systems

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INTRODUCTION

The last major step in the radioimmunoassay (RIA) technique (before calculation of the results) is the measurement of the radioactivity. Steroids, heart glycosides, prostaglandins and other small labelled molecules are commonly ^{3}H-labelled and counted by the liquid scintillation (LSC) technique. The correct choice of a counting solution is important to the reliability of the assay.

RIA sample types counted in LSC

After separation of free and antibody-bound antigens by various means, one of them is counted. The fraction to be counted contains either a free labelled compound or the antigen–antibody complex in a buffer or plasma solution.

Type of scintillator solution

Some investigators use ingenious methods to extract the free labelled antigens directly in the counting fluid. Steroids have been extracted in toluene,[1] dioxane[2] has been used for buffer solutions and some investigators have used mixtures of Triton X-100 and toluene for aqueous solutions[3] and plasma.[4]

CRITERIA OF A LIQUID SCINTILLATION SYSTEM FOR CORRECT MEASUREMENT OF RIA SAMPLES

In RIA, the following parameters are important for accurate liquid scintillation counting.

Absence of chemiluminescence assures a counting of the samples soon after preparation and eliminates the need to count the samples for a second cycle.

Stability of count rate. The quench level in samples prepared with the same amount of buffer in scintillator solution is often the same, which makes it possible to compare samples without quench correction. This, of course, requires a stable count rate.

Dissolving properties for the sample. For samples with varying colours, a quench correction must be applied. For any type of accurate quench correction, a homo-

geneous sample is necessary. This can be obtained if proteins and the buffer can be dissolved completely in the scintillator solution.

Comparison of scintillator solutions

In this paper, the above-mentioned criteria are compared in xylene-based, dioxane-based and colloidal scintillation solutions for either bound or free antigens of different polarity.

Using colloidal scintillators with plasma and buffer samples, phasing or sedimentation of salt or proteins sometimes occurs. The influence of sedimentation or phasing on count rate stability and correct quench correction is illustrated by varying the ratio between the scintillator solution and a RIA sample containing a semi-polar steroid aldosterone.

EXPERIMENTAL

Materials

Insta-Fluor (xylene-based), Permafluor II (a dioxane-based modified Bray solution), Insta-Gel and Triton/toluene (33% Triton X-100 in toluene with 9 g Permablend III/L) were chosen as scintillation solutions (all from Packard Instrument Co.).

(1, 2, 6, 7 (*n*) - ^{3}H) Testosterone (Amersham), a non-polar steroid, (1, 2, 6, 7 (*n*) - ^{3}H) aldosterone (Amersham), a semi-polar steroid, and (G - ^{3}H) digoxine (Amersham), a polar glycoside, were chosen as examples of compounds of different polarity.

Methods

To obtain bound antigens, the labelled compounds of known activity were bound to antibodies (CEA, IRI, Sorin) in PBS buffer (0.100M phosphate-buffered saline with pH 7.4). The dilution of the antiserum was chosen in such a way that at least 80-90% of the antigens and antibody was bound. RIA plasma samples were prepared by adding 10% antigen-antibody complex in PBS buffer to normal plasma. Various amounts of these antigen-antibody complex solutions were pipetted into counting vials and 10 ml of the scintillation solutions were added. To obtain free antigens, identical samples were prepared without the antibody.

Liquid scintillation counting

Immediately after preparation, the samples were placed in a Model 3385 Tri-Carb with optimum ^{3}H setting and operating at 12 °C. The samples were counted a total of six times with 10000 counts accumulated each time during a 24-h period. The count rate stability and sample appearance was noted and quench correction was performed with the automatic external standard ratio (ESR) technique.

Phase diagrams of Insta-Gel with the PBS buffer and various other buffers applied in the RIA technique were defined at various temperatures. This was done by adding 0-10 ml of buffer solution to 10 ml of Insta-Gel and recording the appearance after 16 h of stabilisation in water baths at 5, 7, 12, 21 and 25 °C.

RESULTS

Free antigen

Free testosterone (non-polar) was extracted out of the aqueous sample completely

by xylene and dioxane (Table 1). Free aldosterone (semi-polar) was extracted partially by xylene and totally by dioxane. Free digoxine (polar) was counted in dioxane with a yield of 90% but could not be counted in lipophilic xylene. In colloidal scintillators, the aqueous sample was dispersed homogeneously and all types of free antigens could be counted with a 100% yield.

Table 1. Recovery of free antigens with various polarity[a] in the xylene-based, dioxane-based and colloidal scintillation solution.

Scintillator solution	Free antigen recovered (%)		
	Testosterone	Aldosterone	Digoxine
Xylene (Insta-Fluor)	102	62	0
Dioxane (Permafluor II)	100	100	90
Colloid (Insta-Gel)	100	100	100

[a] 0.5 ml buffered solution (PBS) in 10 ml scintillator solution.

Antigen–antibody complex

Non-polar testosterone. Figure 1 shows the behaviour of testosterone in different scintillators for a sample load of 5% buffer (PBS). In the dioxane cocktail, testosterone in buffer was measured for 100%, based on ESR quench correction. The count rate was variable until testosterone was separated from its antibody and the salt from the buffer was sedimented out completely. In the two-phase solution of xylene (Insta-Fluor), the count rate was changing until the testosterone was separated and completely extracted. In the homogeneous colloidal scintillation solution (Insta-Gel), the radioactivity was measured at 100% recovery immediately and the count rate was constant.

Semi-polar aldosterone. Figure 2 shows the difference between the scintillators for aldosterone in a 5% aqueous buffer (PBS). In the dioxane cocktail, aldosterone was measured for 70–100%, depending on the time of measurement. In the xylene-based scintillation solution, it was counted for 24–62%, the recovery increasing with time. In the homogeneous colloidal scintillation solution (Insta-Gel), aldosterone was measured for 95%. If sedimentation was present in the colloidal scintillation solution or phasing occurred, results were different. The count rate changed with time in dioxane and in xylene, as shown in Fig. 3.

Polar digoxine. Figure 4 shows the performance of digoxine with 0.5 ml of buffer and 20% of plasma in various scintillation solutions. In dioxane, digoxine was counted for 80–90%. Digoxine could not be counted in xylene, but was measured for 100% in the homogeneous colloidal scintillation solution. A stable count rate was obtained for Insta-Gel within 30 min and for Permafluor II (dioxane) in 1 h.

Influence of phasing of colloidal scintillators on liquid scintillation counting of RIA samples. Various amounts of buffer (from 0.2 to 2.0 ml) in 10 ml of Insta-Gel and in 10 ml of Triton X-100/toluene resulted in samples of different appearances. The influence of this parameter on LSC was studied with buffer and plasma samples containing the aldosterone–antibody complex.

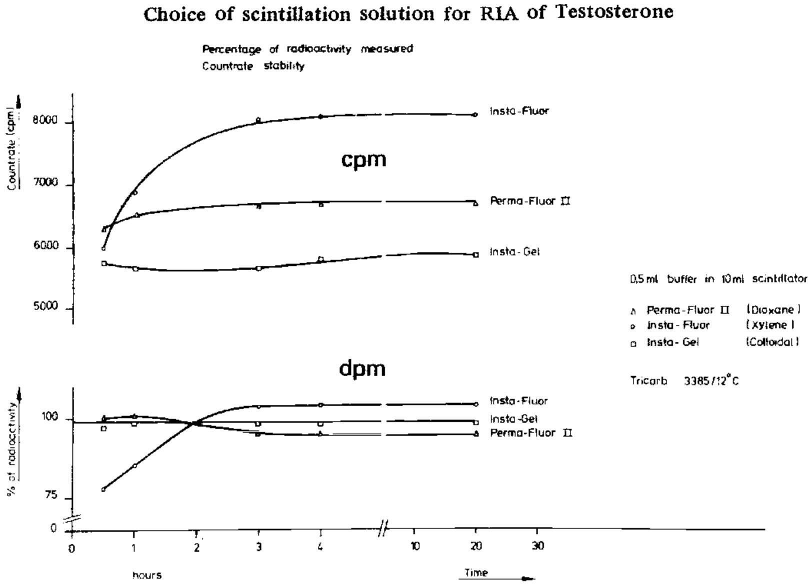

Fig. 1. Count rate stability and recovery of disintegrations min^{-1} of ^{3}H-testosterone-antibody complex in various scintillation solutions.

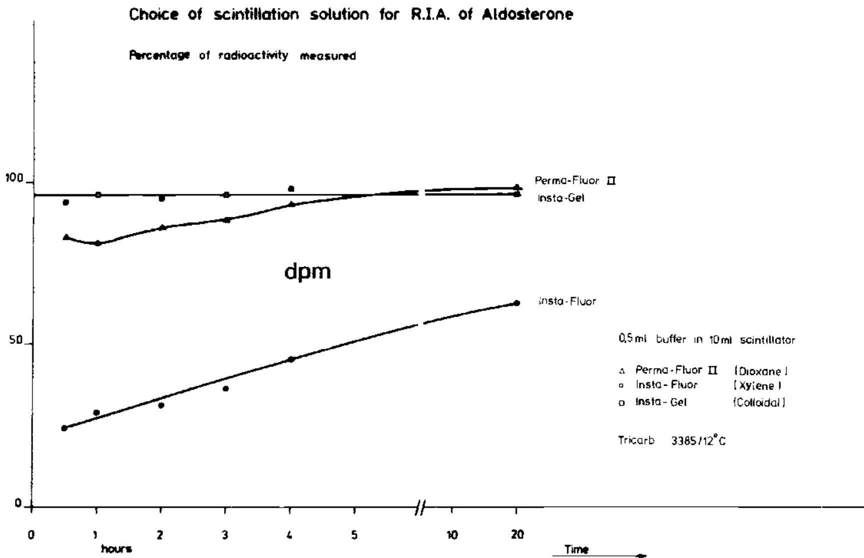

Fig. 2. Recovery of disintegrations min^{-1} of ^{3}H-aldosterone-antibody complex in various scintillation solutions.

Figure 5 presents the count rate stability and the percentage of the activity measured in Insta-Gel with various volumes of a buffer solution containing aldosterone bound to its antibody. The sample with sedimentation (0.2 ml) showed a 21% increase in the count rate in 20 h, and this was not correctable with the ESR quench correction method. Single-phase samples (0.5 and 1.0 ml) had a stable count rate which was

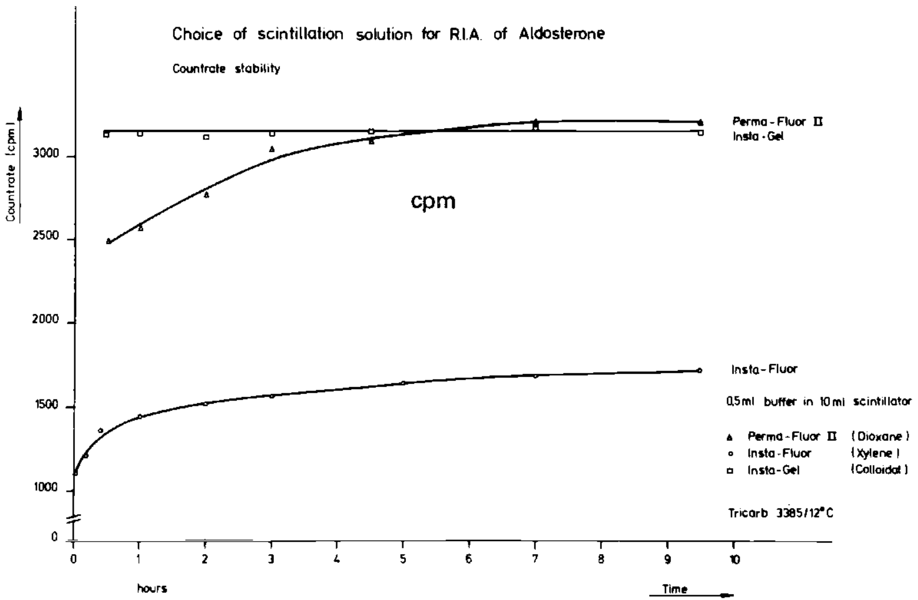

Fig. 3. Count rate stability of ^{3}H-aldosterone-antibody complex in various scintillation solutions.

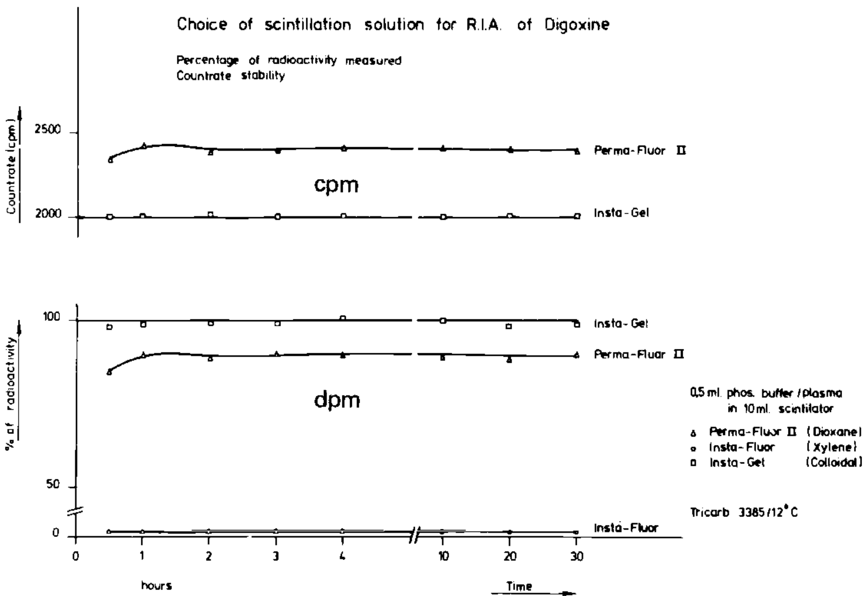

Fig. 4. Count rate stability and recovery of disintegrations min^{-1} of ^{3}H-digoxine in various scintillation solutions.

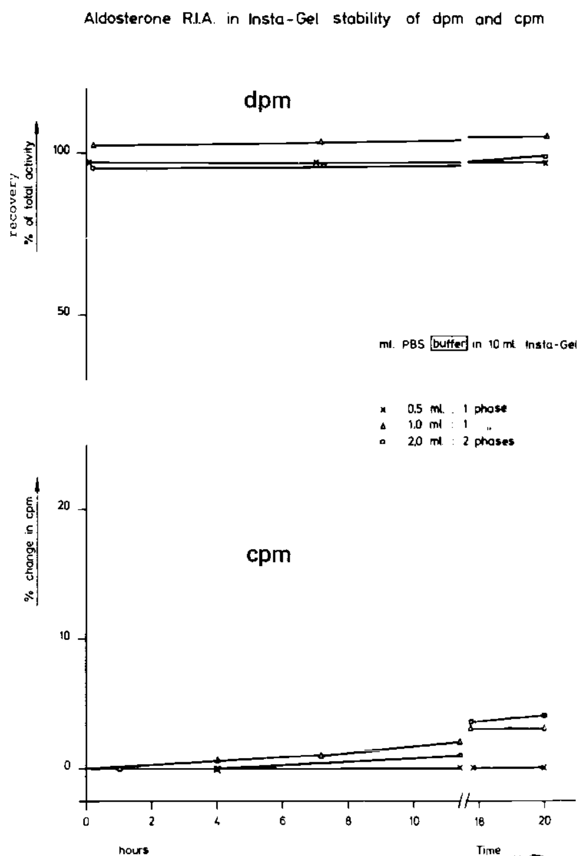

Fig. 5. Count rate stability and recovery of disintegrations min^{-1} in Insta-Gel with various volumes of buffer (PBS) solution containing ^{3}H-aldosterone–antibody complex.

correctable with the ESR method. The phasing samples (2.0 ml) showed a slow increase in the count rate, due to a slow extraction of free aldosterone out of the hydrophilic layer into the lipophilic layer. Figure 6 shows the same parameters for the Triton/toluene scintillator with various amounts of a buffer solution containing the aldosterone–antibody complex. The sample containing sedimentation (0.2 ml) had an unstable count rate. Calculation of disintegrations min^{-1} of this sample provided incorrect values. The single-phase sample maintained a constant count rate and its activity could be calculated correctly with the ESR method.

Plasma solutions with the aldosterone–antibody complex in Insta-Gel showed an increasing count rate with time (as shown in Fig. 7). This change in count rate was, in all single-phase samples, completely corrected by the ESR quench correction method.

The results showed that the sample appearance in colloidal scintillation solutions determines the accuracy of the liquid scintillation counting of RIA samples. In homogeneous samples, the count rate was stable for buffer solutions and quench correction with the ESR method could be applied.

The sample appearances of Insta-Gel with commonly used RIA buffers are presented as phase diagrams in Fig. 8. The filled areas indicate sample percentages

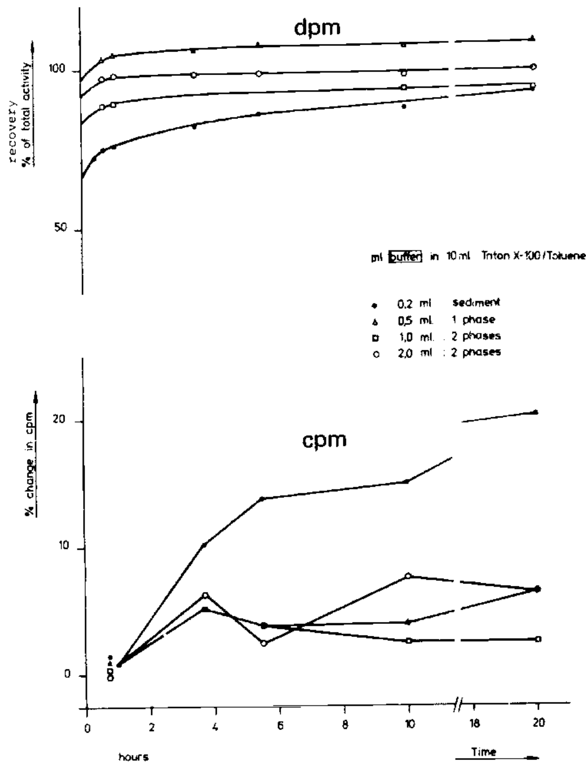

Fig. 6. Count rate stability and recovery of disintegrations min^{-1} in Triton X-100/toluene with various volumes of buffer (PBS) solutions containing ^{3}H-aldosterone-antibody complex.

providing homogeneous counting solutions; 5 to 10% sample load of buffer for all investigated buffers in Insta-Gel results in homogeneous counting solutions.

COMMENTS AND CONCLUSIONS

In this study, the commonly used scintillation solutions were compared for counting RIA samples. These samples had either free labelled antigens or antigens bound to an antibody in an aqueous buffer or plasma solutions. In toluene scintillator (Insta-Fluor), only non-bound, non-polar antigens could be counted. Non-polar, bound antigens required a long time for dissociation of the antigen-antibody complex before they could be counted. The toluene probably extracted the lipophilic antigen out of the water layer of the phasing liquid scintillation sample.

In Bray solutions (dioxane), non-bound polar and non-polar antigens provided reliable results. The antigen-antibody complex dissociated in dioxane,[5] generating a free antigen to be counted after a stabilisation time varying from 1 to 3 h. However, in Bray solutions the sample load was limited to 9-10% and proteins were poorly soluble.

Colloidal scintillators (Insta-Gel) were generally applicable. Samples could be measured within a short time after preparation. An antibody-antigen complex in

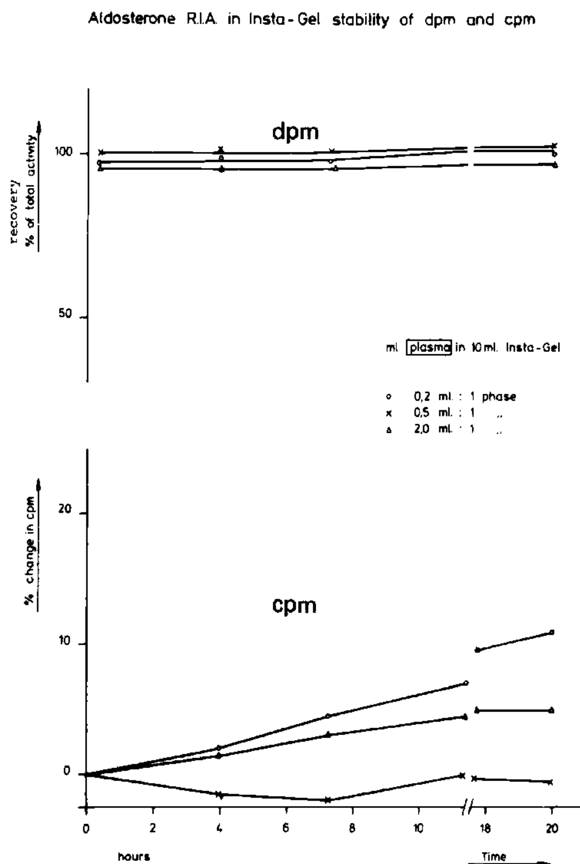

Fig. 7. Count rate stability and recovery of disintegrations min^{-1} in Insta-Gel with various volumes of plasma containing ^3H-aldosterone-antibody complex.

buffer exhibited a constant count rate and existing variations in the count rate for plasma samples could be corrected with the ESR quench correction method. Colloidal scintillators had a higher sample capacity than Bray solutions, thus requiring less scintillator. Because two-phase colloidal samples gave erroneous results, the sample volume had to be adjusted to produce homogeneous samples. In determining this volume, phase diagrams appeared very useful. Phase diagrams of colloidal scintillators varied for different RIA buffers, and even for Triton X-100 there could be variations from batch to batch for the same buffer.[6] Chemiluminescence was not evident in any of the investigated samples.

In our laboratory, colloidal scintillators have been developed for RIA buffers or plasma without phasing or sedimentation. Their sample holding capacity is higher than the commonly used scintillators, thus allowing the use of less scintillator solution. Comparative tests with other scintillator systems will be reported elsewhere.

SUMMARY

In this study the commonly used scintillation solutions were compared for liquid scintillation counting of RIA samples containing free or bound antigens of

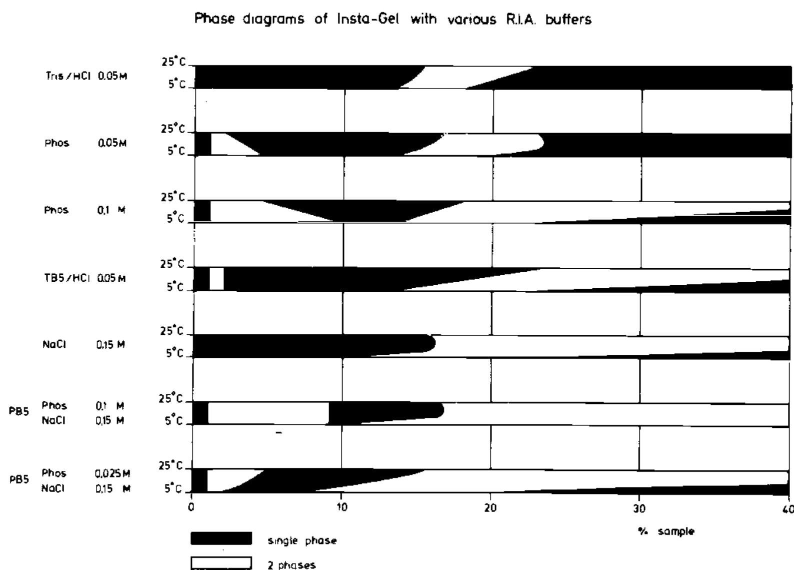

Fig. 8. Phase diagrams of Insta-Gel with various buffer solutions at temperatures of 5°C to 25°C.

different polarity.

Absence of chemiluminescence, stable count rate and good dissolving properties of the labelled compound were required for counting RIA samples. Xylene or toluene scintillators met these requirements only for non-bound, non-polar antigens.

Dioxane-based scintillation solutions could be applied for free antigens of each polarity. The antibody-antigen complex was counted after a stabilisation time needed for dissociation of the complex. The sample capacity of dioxane was limited and proteins were poorly soluble in dioxane.

Colloidal scintillators could be used for either bound or non-bound antigens in plasma or buffer solution. Variations in count rate in plasma samples could be corrected by the external standard ratio quench correction method, provided the solution was homogeneous.

Two-phase colloidal scintillators could provide varying count rate and erroneous quench correction. Bound antigens could be counted in dioxane with limited sample capacity after a stabilisation time for dissociation varying from 1 to 3 h.

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DISCUSSION

D. I. Chapman: One of the problems in counting large numbers of samples is the prohibitive cost of commercial scintillators. To reduce the volume of scintillant one either has to reduce the volume of sample, which means discarding counts, or find a scintillator which will accept large volumes of aqeuous sample. Figure 8 of your paper indicates that Insta-Gel will accept up to 40% of certain aqueous buffers. Are these mixtures really homogeneous solutions or are they heterogeneous? Are they fluids or are they gels?

H. Spolders: The mixtures are really homogeneous for liquid scintillation counting. At ambient temperatures, the samples with aqueous buffer are viscous fluids, but at sub-ambient temperatures they are gels.

N. Lucas: With regard to Triton X-100 systems, preparation of a single phase is difficult, but if a batch of Triton X-100 is assayed, results can be stabilised. Normally, Triton X-100/toluene can accommodate a 10% sample. Samples should be counted at 6–8°C and allowed to equilibrate for 20–30 min before counting.

D. I. Chapman: May I disagree with Dr. Lucas? My experience is that batch to batch variation of Triton X-100 does occur, and on standing a precipitate may settle out. Acceptable material can be obtained by decanting clear material from the top of the drum, the residue being discarded or used as a washing-up detergent.

N. Lucas: The precipitate can be removed by warming – it apparently dissolves.

H. Spolders: If precipitation occurs in drums with large volumes of Triton X-100, warming can cause chemical changes which are responsible for chemiluminescence. Triton X-100 changes not only from batch to batch but also large quantities are inhomogeneous. To obtain reproducible results from Triton X-100 in large drums, they should be shaken well before use, which is rather difficult to do and does not change the final poor performance.

V. Tarkkanen: Heating does not actually remove the reason for the poor and variable sample holding characteristics of Triton X-100 based scintillator systems. This is based on the fact that the polymerisation process involved in production of Triton does not produce an equal product and the higher molecular weight homologs which do interfere cannot be removed by heating. The same applies to the peroxides present in varying amounts in Triton causing alterations in background.