

DIFFICULTIES IN COUNTING EMULSIONS OF  
 $^3\text{H}$  AND  $^{14}\text{C}$  LABELLED BIOMOLECULES

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*The difficulties in assessing the absolute counting efficiency of heterogeneous samples are outweighed by the convenience of this method of counting unknowns and by the availability of a myriad of commercial mixtures of nonionic and anionic surfactants "designed" for LSC. We have therefore retreated to an attempt to emphasize some of the less obvious pitfalls in such systems.*

*Triton X-100<sup>R</sup> (a polyphenoxy ethanol) is the single emulsifier (Rohm and Haas) most frequently used. Not only does water content of the sample have varying effects on counting efficiency which may not correlate with the visual clarity of the sample but changes in efficiency are not reflected by external standard channels ratios (ESCR) or by sample channels ratios (SCR). The use of an internal standard in such systems requires that the chemical and physical state of each unknown be mimicked perfectly, that the amount of water and of solutes not vary appreciably from sample to sample, and that addition of internal standard would not be itself affect the phase distribution or the size of the micelles in the emulsion; these conditions are almost impossible to fulfill. Although the addition of an anionic surfactant, (sodium dihexyl sulfosuccinate: Aerosol MA-80<sup>R</sup> (American Cyanamid) to a nonionic surfactant (Triton X-100<sup>R</sup>, Triton N-101<sup>R</sup>) results in greater stability and counting efficiency, aqueous samples still cannot be adequately assessed by ESCR or SCR. Addition of water and and solutes had a greater effect on the counting efficiency of tritium than on  $^{14}\text{C}$ ; tritium was also more sensitive to phase changes.*

*When some commercial surfactants exhibited a precipitous drop in  $^3\text{H}$  and  $^{14}\text{C}$  efficiency between 0.5 and 5% water content, as did the nonionic surfactants Triton X-100 or Triton N-101, others behaved in a manner similar to our contrived nonionic-anionic surfactant mixtures. It is thus apparent that accurate calculation of the relative radioactivity of emulsified aqueous samples depends on rigorous uniformity of sample preparations. Optimal proportions of surfactants, surfactant content, and water content will depend on the nature and amount of the solute being counted. The usual methods of quench correction in LSC must be examined very carefully, inasmuch as they will usually not be adequate for determining absolute counting efficiency.*

## INTRODUCTION

The practice of adding surfactants to form emulsions of aqueous samples with aromatic solvents such as toluene is especially popular in laboratories devoted to clinical assays or to biomedical investigation. Although warnings about some of the pitfalls have come from our laboratories (Sharpe and Bransome, 1973; Sharpe and Bransome, 1974; Bransome, 1976; Bransome and O'Conner, 1978) as well as from other workers (Van Der Laarse, 1967; Bush, 1968; Turner, 1968; Fox 1974; Fox, 1977; Noujaim et al., 1976; Horrocks, 1976), we continue to be surprised at how frequently our colleagues assume that they know the counting efficiency of samples - some of them gathered only after tedious hours of painstaking work - when they in fact, do not. We particularly note two common practices in emulsion or "sol-gel" counting: investigators frequently deduce the range of counting efficiency of their unknown samples from the marketing brochure for a scintillation "cocktail" and then assume that losses in efficiency are fairly uniform for "similar" unknown samples. More careful workers may on the other hand estimate counting efficiency from samples channels ratios (SCR) or from external standard channels ratios (ESCR), using quench correction curves derived from a series of sealed quenched standards labelled with the radioisotope of interest. They assume that changes in SCR and ESCR of samples in emulsions will adequately reflect variations in counting efficiency. The assumptions underlying either practice are frequently unfounded. Our purposes in this paper are not only to provide evidence that small changes in emulsion composition may have considerable effects on counting efficiency which will not be adequately mirrored by changes in appearance or by SCR or ESCR, but by providing examples of the behavior of a varie-

ty of surfactant preparations, to suggest to some guidelines.

#### MATERIALS AND METHODS

For examples of tritium and  $^{14}\text{C}$  labelled compounds we used  $^3\text{H}$ -leucine (62 Ci/mMole) from Schwarz Mann Inc.,  $^{14}\text{C}$ -uracil (2 mCi/mMole) from the New England Nuclear Corp., and  $^{14}\text{C}$ -protein (100  $\mu\text{Ci/ml}$ ) from Amersham-Searle, each diluted in water. To determine differences in counting efficiency between dissolved and emulsified samples we used  $^3\text{H}$ -toluene ( $2.48 \times 10^6$  DPM/ml) and  $^{14}\text{C}$ -toluene ( $3.69 \times 10^5$  DPM/ml) from New England Nuclear. Fraction V Bovine Serum Albumin was obtained from Miles Laboratories. For our own counting solutions we employed Permablend I (Packard Instrument Co.) as a scintillator, 5.5 g/liter of toluene (Fisher Scientific, reagent grade) made up to volume with the surfactant of interest. Triton X-100 (a nonionic polyphenoxyethanol) and N-101 (nonylphenoxyethanol) were generous gifts of Rohm and Haas Inc. and Aerosol MA-80 (an anionic surfactant: dihexylsulfosuccinate) from American Cyanamid, Inc.

Aqueous samples in 25  $\mu\text{l}$  were added to counting vials already containing 10 ml of a scintillation-solvent-surfactant combination or to premixed counting "cocktails": HR<sup>R</sup>, GP<sup>R</sup>, EP<sup>R</sup>, and Filter-Solv<sup>R</sup> from Beckman Instruments. Merit<sup>R</sup>, Optisol<sup>R</sup>, Multisol<sup>R</sup>, and Scintisol<sup>R</sup> from Isolab, Inc., Handifluor<sup>R</sup> from Mallinckrodt, and Biofluor<sup>R</sup> from New England Nuclear. Additional distilled water was added as indicated. Experiments with Beckman Biosolv-BBS-2 and BBS-3 have been reported in a previous publication (Bransome and O'Conner, 1978). Following vigorous shaking, the samples were allowed to stabilize overnight and counted in a Beckman LS-230 at ambient temperature in wide  $^3\text{H}$  and  $^{14}\text{C}$  channels. Absolute counting efficiencies, SCR and ESCR have been plotted vs the %  $\text{H}_2\text{O}$  of each scintillation counting mixture. Several dynamic ranges of SCR and ESCR were employed in this work. The ESCR ratios referred to in this paper were based on Beckman Inc. (factory set) discriminator settings. We have not dealt with all of the further complications of sample solute (protein, sugar, salt) content in this paper, but remind the reader that most surfactant - water - organic solvent emulsions are remarkably intolerant of additional solutes. We do give some small attention however to some of the less predictable effects on counting efficiency of the presence of protein in aqueous samples.

## RESULTS

Despite the reiterated assumption that aqueous samples in emulsions should emulate homogenous (truly dissolved) samples as much as possible - i.e. that they should be small micelles in a single phase, we have noted that in many laboratories emulsified samples are allowed to separate into more than one phase and/or that gels or precipitates form in scintillation vials when samples are allowed to stand for more than a few hours. Without trying to determine how  $^3\text{H}$ - and  $^{14}\text{C}$  labelled aqueous samples are partitioned in such complex mixtures, we have once again addressed the question (already answered in the negative by Noujaim et al (1976), of whether the appearance of an emulsion was a reliable indication of counting efficiency.

Figure 1 provides several examples of the changes in appearance of the sample-surfactant-solvent mixtures which occurred as water content increased.

After adding Triton X-100 alone, Triton N-101 alone, or either plus Aerosol MA-80 (75%:25%) a mixture similar in be-

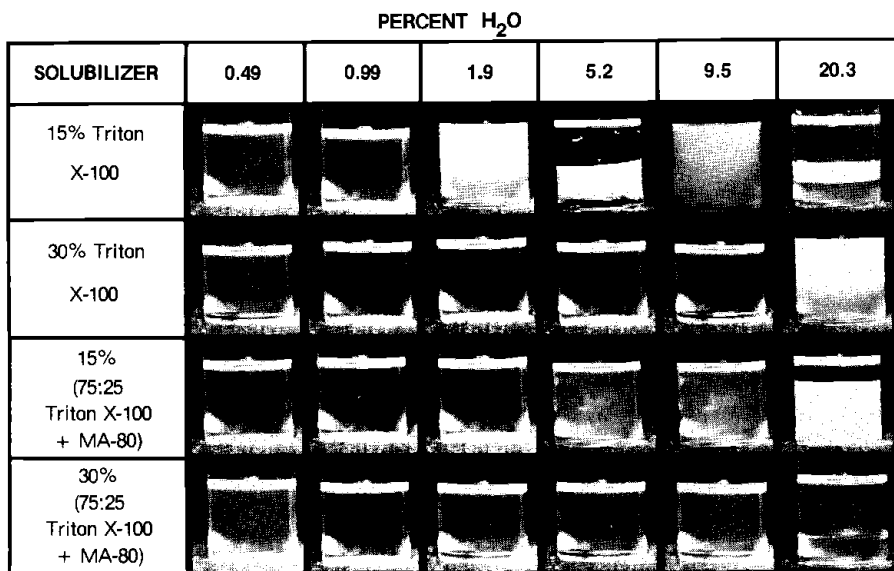


FIGURE 1. Examples of the appearance of the combination of toluene, scintillator, surfactant, and aqueous sample. See the text for details.

TABLE I. Effect of Water on the Appearance and Counting Efficiency of Selected Emulsions of Aqueous Samples Containing  $^3\text{H}$ -Leucine or  $^{14}\text{C}$ -Uracil.

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<u>Percent Water</u>									
	0.49	0.99	1.96	3.38	5.21	9.50	13.41	17.01	20.31
<u>15% Triton X-100 in Toluene</u>									
Appearance	D*	D	2P**	2P	3P	4P	2P	3P	4P
$^3\text{H}$ Efficiency	28.7	14.5	33.9	35.9	22.5	15.5	13.5	16.4	21.4
$^{14}\text{C}$ Efficiency	64.2	44.9	43.6	45.9	36.5	22.8	26.4	49.3	47.0
<u>30% Triton X-100 in Toluene</u>									
Appearance	C+	D	D	D	D	C	2P	2P	2P
$^3\text{H}$ Efficiency	45.7	44.7	25.7	40.0	44.1	40.8	35.0	25.8	28.7
$^{14}\text{C}$ Efficiency	63.3	61.7	57.1	55.8	57.5	51.4	48.4	43.7	43.7
<u>15% Triton X-100: MA-80 (75:25)</u>									
Appearance	D	D	D	C	O***	O	2P	3P	3P
$^3\text{H}$ Efficiency	44.6	46.0	52.1	52.8	51.7	47.3	45.8	35.3	27.6
$^{14}\text{C}$ Efficiency	69.0	69.0	68.5	69.9	69.5	66.8	63.8	55.0	41.6
<u>30% Triton X-100: MA-80 (75:25)</u>									
Appearance	D	D	C	C	C	C	C	C	2P
$^3\text{H}$ Efficiency	39.3	37.5	36.5	32.0	40.7	39.6	37.8	35.1	31.6
$^{14}\text{C}$ Efficiency	58.4	58.1	56.2	55.2	52.5	46.8	42.0	42.0	39.5

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\* D - Droplets: clear with droplets on the bottom of the vial

\*\* P - Number of phases

\*\*\*O - Opaque

+ C - Clear

(See Figure 1 for illustrations of appearance and the text for further details.)

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havior to many of the commercially available surfactant combinations to a toluene solution of scintillator in different proportions, we determined the counting efficiency of  $^3\text{H}$  leucine or  $^{14}\text{C}$ -uracil in the presence of progressively increasing volumes of water. Examples are shown in Table 1. The clarity of samples was best maintained with increasing water content

at higher concentrations of the surfactants as was counting efficiency, but the appearance and efficiency were not closely correlated in any single series. Figure 2 provides several illustrations of the potential for error in assuming that samples which have the same appearance (Figure 1) can be assumed to be equally efficient when counted.

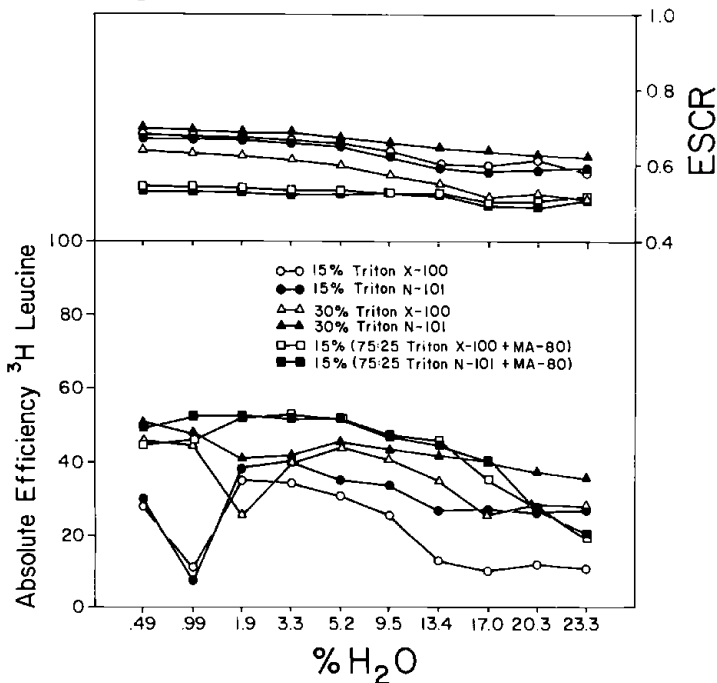


FIGURE 2. The effect of added water and surfactants on the efficiency of counting <sup>3</sup>H-l-leucine. 36,666 DPM were added in 25  $\mu$ l to each vial. (See text for discussion.)

Our preliminary conclusions are that significant variations in micelle size which are important in counting weak  $\beta$  emitters may not have visible effects. Neither SCR or ESCR (examples are shown in Table I and Figure 2) may be reliable indicators of losses of efficiency. To predict counting efficiency with confidence, we must examine the effect of sample composition and volume on the behavior of specific toluene, scintillator-surfactant mixtures. While we hope that the samples of our investigations of the performance of various surfactants will be helpful to workers who want to count aqueous samples in surfactant-toluene mixtures, we cannot overemphasize the importance of investigating the behavior of such systems using aqueous samples as similar as possible in composition to the anticipated unknowns.

*Non-Ionic Surfactants*

Triton X-100 was first introduced by Meade and Stiglitz in 1962 as a surfactant for liquid scintillation. Many laboratories are still using the formulation (30% Triton X-100 in an organic scintillation solvent) suggested by Patterson and Greene in 1965. Benson (1966) stated that with aqueous sample volumes of less than 2.5 ml per scintillation vial, Triton X-100 was completely satisfactory although there was a "steady decrease in counting efficiency with increasing sample size". Although 10 ml of this mixture is capable of holding up to 10% water in an apparent single phase, there is a marked loss in tritium efficiency in samples containing between 0.5 and 2% water, a range of sample volumes frequently encountered in biomedical work with small volumes. Although the physical appearance of some of these samples was clear, small droplets were frequently apparent upon closer scrutiny. We also observed a return to near maximum efficiency when the water concentration was increased to 5.2%. ESCRs did not give any hint of these fluctuations. Van Der Laarse (1967) in his warnings about Triton X-100 did not mention this problem which we have called the "non-ionic dip". It is characterized by a large loss in efficiency at low water levels followed by a partial or total recovery with further addition of water. Because of this unexpected behavior of 30% Triton X-100, we extended our investigations to cover other concentrations of Triton X-100 and of Triton N-101 as well. We observed the same behavior (e.g. Table I, Figure 2).

Turner (1968) reported his inability to achieve reproducible results with  $^3\text{H}$  and 30% Triton X-100. He suggested that Triton X-100 systems were satisfactory for counting in similar volumes of aqueous samples. We are in qualified agreement. Either nonionic surfactant (X-100 or N-101) system is acceptable for counting  $^{14}\text{C}$  but unacceptable for tritium. As Turner and others have pointed out, different batches of Triton exhibit varying degrees of quenching with some batches obviously discolored. If Triton X-100 is to be used as a surfactant, any given set of experiments should utilize the same batch of Triton and the batches with obvious discoloration should be avoided. Even if the surfactant is translucent the quenching properties of each batch should be checked.

*Addition of Anionic Surfactant*

We will not summarize our reexamination of a series of anionic surfactants inasmuch as Aerosol MA-80 in combination with Triton X-100 or N-101 clearly had the most desirable attributes. Even a very small percentage of MA-80 when added

to Triton X-100 or N-101 eliminated the "nonionic dip", stabilized the behavior of the emulsion up to approximately 10% water, and increased the tritium counting efficiency (Figures 2 and 3).

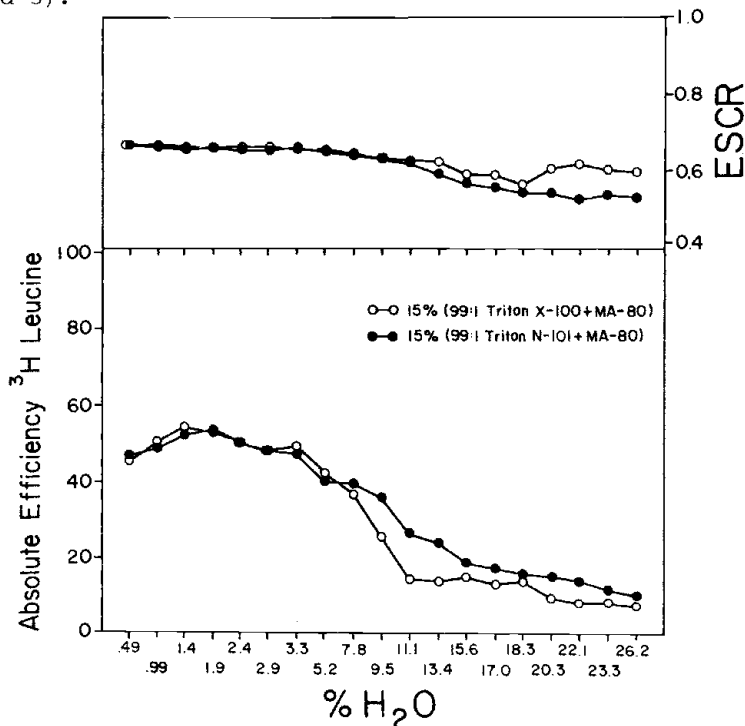


FIGURE 3. Elimination of the "nonionic dip" by a small amount of anionic surfactant. <sup>3</sup>H-l-leucine 36,666 DPM. Triton X-100:MA-80 (99:1)

Data from this laboratory have already been published which describe counting <sup>3</sup>H and <sup>14</sup>C in the presence of increasing water content using 15% surfactant "N-5" in toluene (94% Triton N-101: 6% MA-80) (Sharpe and Bransome, 1974). To discover the optimum mixture for counting <sup>3</sup>H-leucine, and <sup>14</sup>C uracil, we combined Triton X-100 or Triton N-101 with Aerosol MA-80 so that the entire range from 100% Triton to 100% MA-80 was covered in 10% increments in the presence of increasing amounts of water. Examples of the results of experiments in which the surfactant mixture was 10% to 85% in toluene are shown for <sup>3</sup>H-leucine in Figure 4. The results for counting <sup>14</sup>C-uracil were similar: with either nonionic surfactant the best efficiencies were observed in the region of 5-35% MA-80. We have arbitrarily chosen the 75:25 combination for illustrative purposes (eg. Figures 2,5,6).

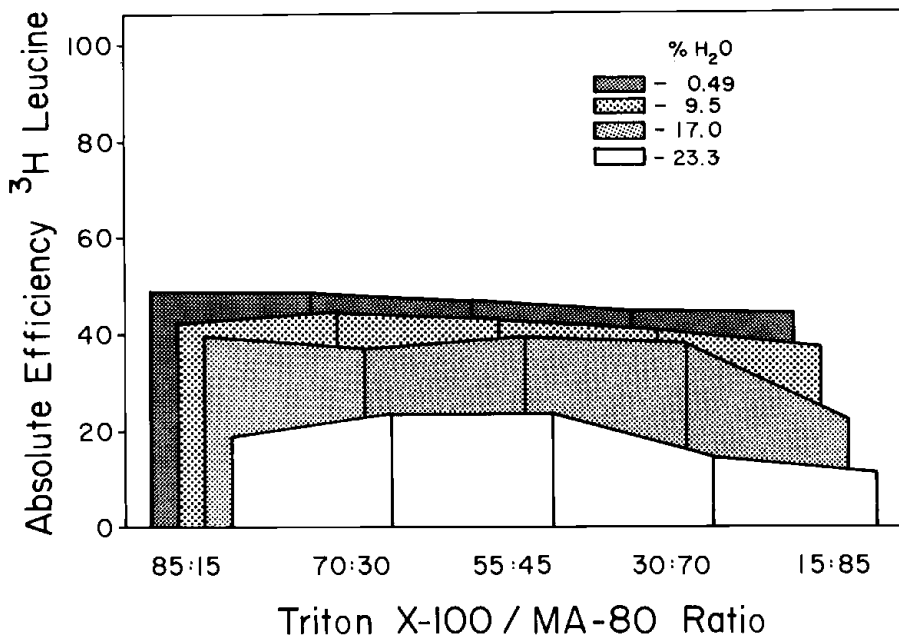


FIGURE 4. Effects on <sup>3</sup>H efficiency of 15% Triton X-100: MA-80 in various ratios. (<sup>3</sup>H-leucine 36,666 DPM per vial)

The anionic surfactant in combination with Triton N-101 has less tendency to allow droplet formation with volumes of water than with Triton X-100. No other gross physical differences between X-100 and N-101 combinations with MA-80 were apparent except for the fact that at high water concentrations the Triton X-100: MA-80 combinations did not form three phase systems. Either nonionic-anionic combination resulted in greater physical stability of the sample than the nonionic surfactant alone. At very low water concentrations, N-101: MA-80 combinations seemed to offer slightly greater tritium efficiencies, perhaps because of less droplet formation. For <sup>14</sup>C counting the X-100:MA-80 and N-101:MA-80 combinations both offered higher efficiency and less fluctuations with water than the nonionic surfactant alone. Whereas either combination at 10 and 15% of sample volume resulted in stable <sup>14</sup>C efficiencies through 9.5% water slope and a discernible ESCR, a 30% surfactant concentration resulted in a gradual decrease of <sup>14</sup>C efficiency with increasing water and a flat insensitive ESCR.

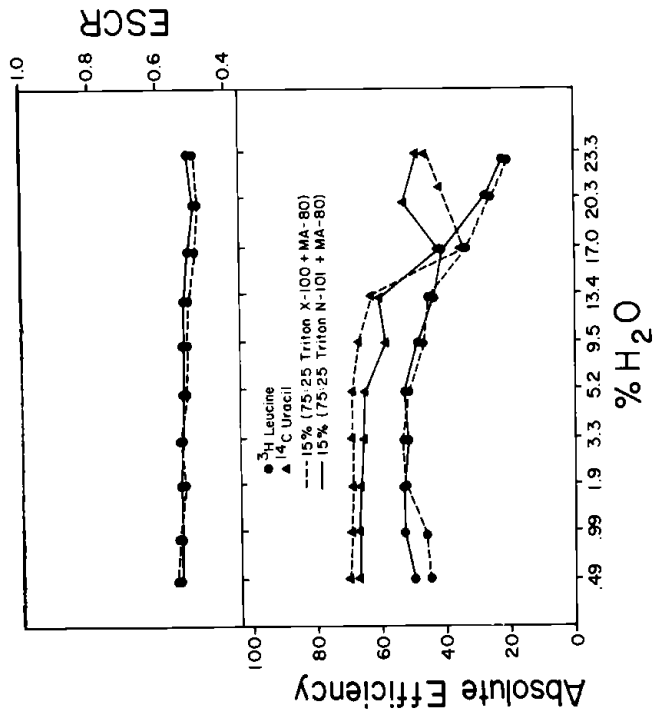


FIGURE 5. The effect of added water on the efficiency of counting <sup>3</sup>H-l-leucine 36,666 DPM per vial and <sup>14</sup>C-uracil 27,234 DPM per vial. The surfactant was 75:25 mixture of nonionic (Triton X-100) and anionic (Aerosol MA-80), 15% in toluene.

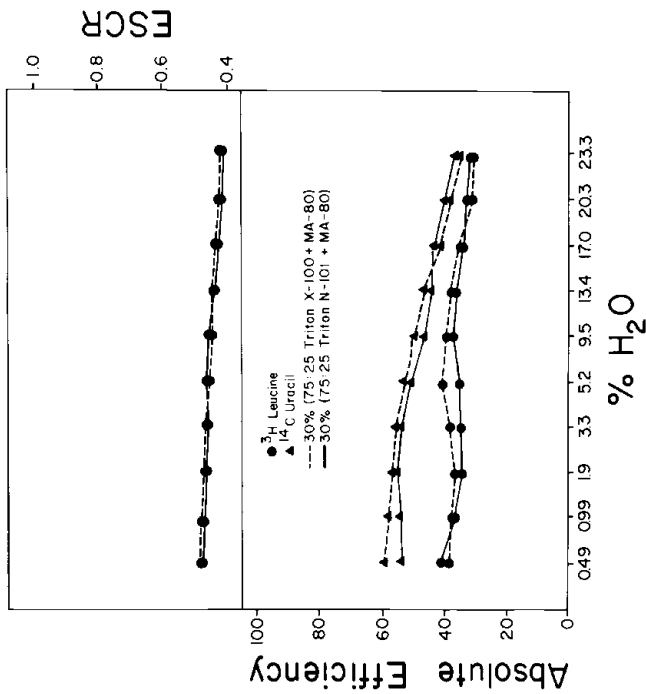


FIGURE 6. The effect of added water on the efficiency of counting <sup>3</sup>H-l-leucine 36,666 DPM per vial and <sup>14</sup>C-uracil 27,234 DPM per vial. The surfactant was a 75:25 mixture of nonionic (Triton X-100) and anionic (Aerosol MA-80), 30% in toluene.

*Samples Containing Protein.* While we do not present the data in this paper, one finding worth attention has come from similar experiments (designed to simulate biological fluids) carried out with solutions of 1% BSA or saline rather than distilled water. When samples were counted in the presence of protein, the efficiency of counting  $^3\text{H}$ -leucine or  $^{14}\text{C}$ -uracil tended to drop off less rapidly above 17% water than with pure aqueous samples, suggesting that the protein probably aided in the formation of smaller micelles or stable emulsions,

#### *Examples of Commercial Surfactants*

Our selection of commercially available surfactants is intended to be illustrative. Most cannot be strictly compared in counting efficiency to our Triton X-100:MA-80 and Triton N-101:MA-80 combinations because of differences in the concentration of scintillators. We have already commented (Bransome and O'Conner, 1978) on the use of Beckman Biosolv BBS-2 and

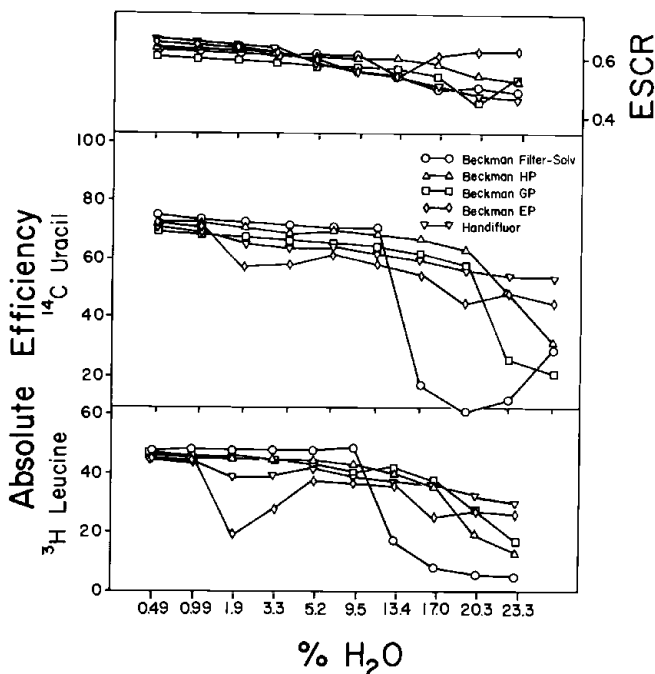


FIGURE 7. The effect of added water on the efficiency of counting  $^3\text{H}$ -l-leucine 36,666 DPM per vial and  $^{14}\text{C}$ -uracil 27,234 DPM per vial. The surfactants were obtained from commercial sources. See the text for details.

BBS-3 (apparently nonionic and anionic surfactant combinations) as surfactants and the evidence that our N-5 mixture and BBS-3 are themselves scintillators with low fluorescence quantum yields (Bransome and Sharpe, 1973). The solutions have been divided into two arbitrary groups in Figures 7 and 8.

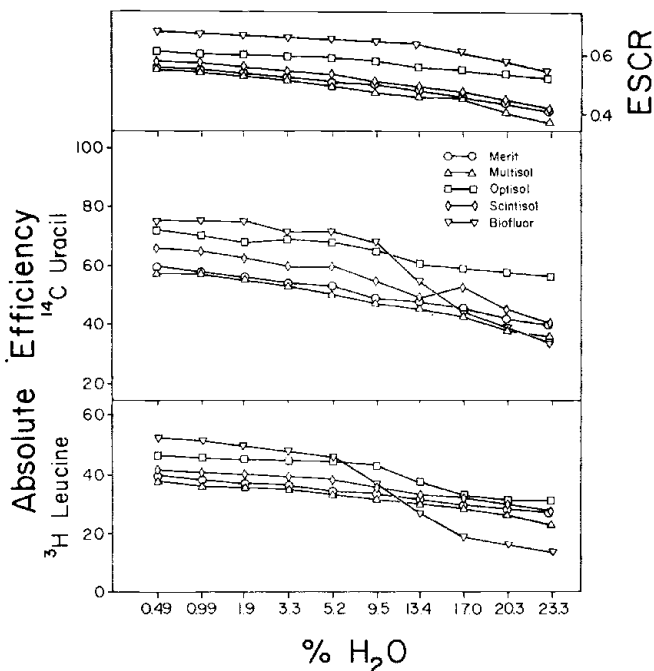


FIGURE 8. The effect of added water on the efficiency of counting <sup>3</sup>H-l-leucine 36,666 DPM per vial and <sup>14</sup>C-uracil 27,234 DPM per vial. The surfactants were obtained from commercial sources. See the text for details.

When the efficiency of counting <sup>3</sup>H-leucine was studied it was apparent that Beckman EP (Econo-fluor) exhibits a loss of counting efficiency in the same range of water concentration as that in which the "nonionic dip" had been observed; indeed, Triton X-100 is listed as being a constituent of EP. Just as MA-80 has removed the "dip" in our mixtures, so it will with Beckman EP; in this case however there is a concomitant loss (albeit less than 10%) in <sup>3</sup>H efficiency. Handifluor was the only other solution utilized which evidenced a similar loss in efficiency. It was noted that the Isolab solutions tolerated water better, especially at higher concentrations, and yielded

ESCR plots which were more sensitive. Differences in efficiencies with the Beckman Inc. mixtures were not discernible with ESCR plots. When  $^{14}\text{C}$  labelled protein was counted under similar identical conditions to  $^{14}\text{C}$ -uracil, differences in behavior were noticed in some of the counting solutions. For example, the loss in efficiency of Beckman EP was greatly exaggerated and Merit, Optisol and Scintisol evidenced a smaller decrease in efficiency at similar water concentrations. ESCR plots gave little indication of the losses in efficiency. These findings should serve as a reminder that the composition of the sample needs to be as standardized as much as possible no matter what surfactant combination is used.

#### CONCLUSIONS

Our findings emphasize the necessity for careful standardization of samples and of surfactant content in counting emulsified aqueous samples in toluene. Subtle changes in phase distribution may have dramatic effects on tritium counting especially. To provide our readers with some independence from the commercial surfactants marketed for scintillation counting, we have recounted some of our own experiments with several nonionic:anionic combinations of chemicals available in industrial quantities. If it is difficult to standardize unknown samples or if they have a high salt content, we still advise other sorts of sample preparation such as combustion of  $^3\text{H}$  or  $^{14}\text{C}$  labelled samples (Bransome, 1976). We have not been very concerned with counting other radionuclides (Sharpe and Bransome, 1974) in emulsions, but have recently noted that it is possible to extract alkalimetal salts into toluene with novel organic chemicals which are not surfactants. There are thus additional possibilities for the preparation of aqueous samples for LSC.

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