

## Chapter 15

# Sample Preparation Techniques in Biochemistry with Particular Reference to Heterogeneous Systems

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### INTRODUCTION

It is generally agreed that for an accurate radioassay of any  $\beta$ -emitter, homogeneous scintillation counting techniques are essential. For labelled tissues, the most accurate method is undoubtedly the combustion technique; however, the combustion procedure can be impracticable for very large numbers of samples, e.g. from chromatographic columns, and from electrophoretic and centrifugation procedures. In order to achieve complete degradation of many biological samples, often mixed with salts, metal ions and macromolecules, it is often more convenient to employ complexing or degradative procedures. Where specific components are needed to be measured, such as a labelled macromolecule, lipid, or other solvent-extractable material, the pre-processing may be simplified by extraction or precipitation procedures, followed by disc or other heterogeneous counting methods.

Analytical procedures using paper, thin layer, or column chromatography, polyacrylamide or agarose electrophoresis, caesium chloride or sucrose gradient centrifugation, each benefit from specific short cuts which enable reproducible counting to be undertaken rapidly within each method.

I therefore propose to a) discuss some general aspects of the use of solubilizers, disc counting and suspension techniques; b) to describe some of the techniques found to be useful in analytical and preparative procedures involving chromatography, electrophoretic and centrifugation methods; c) to describe in greater detail some aspects of the technique of colloid scintillation counting.

### SOLUBILIZERS

I do not intend to review the field of solubilizers, as this has been covered by Rapkin<sup>1</sup> and others in several excellent reviews.

One of the problems associated with the use of the quaternary ammonium hydroxides such as Hyamine-10X, Soluene-100 or NCS is the phenomenon of chemiluminescence, especially noticeable with Soluene-100. The phenomenon is clearly associated with the presence of peroxides in the scintillating solvents, and dioxane is a notorious solvent in this respect. Herberg<sup>2</sup> noticed that the count rate due to chemiluminescence appeared to be

inversely related to the viscosity of the solution and also observed that it increased on exposure to light. Winkelman and Slater<sup>3</sup> showed that benzoyl peroxide, normally employed for colour bleaching, tended to produce an enormous count rate in the presence of Hyamine-10X or NCS. The recognition that peroxides were the cause of the trouble led to the use of hydrochloric acid or, more recently, 10% ascorbic acid (McClendon *et al.*)<sup>4</sup> to act as peroxide scavengers. We have found that the chemiluminescence due to peroxides in Dioxane–naphthalene–phosphor mixtures can be largely overcome by keeping the scintillant solution over granulated zinc, but this only seems to work with sodium hydroxide-induced chemiluminescence and not with Soluene-100. Winkelman and Slater<sup>3</sup> did find that hydrochloric acid reduced the count rate to low levels but higher than the minimum reached by natural decay of the chemiluminescence. It seems, therefore, that hydrochloric acid does not destroy the chemiluminescent reaction but simply slows it down to tolerable rates. Reducing the temperature of counting from 20°C to 8°C simply reduces chemiluminescence by 'one third', presumably again reducing the rate of reaction. For low activity counts, therefore, it may be necessary to allow decay, without hydrochloric acid treatment, for over a week. I am sure there is scope for the production of a solid peroxide scavenger that can be added to scintillant mixtures.

Both internal standard and channels ratio techniques are suitable for quench correction. The external standard ratio should be used with extreme care since it is not clear whether a solubilizer produces a true solution or whether it possesses some of the properties of an heterogeneous, colloidal system. Many commercial preparations are now being introduced by liquid scintillation firms, and I would like to appeal very strongly to avoid the use of secret recipes for these agents so that such factors as the advisability of using an appropriate quench correction procedure can be assessed by the user. It is not clear for example, if surfactants are used as well as quaternary bases, but without this knowledge the user could use processed information derived from automated systems employing external standard ratios which may have no meaning in colloid systems.

In our experience, most tissue samples can be radio-assayed very conveniently by using the perchloric acid–hydrogen peroxide degradation technique in the scintillation vial suggested by Mahin and Lofberg,<sup>5</sup> provided the mixture is heated at not more than 75°C and for longer than 30 min and the product blended into a toluene-based scintillant with 2-methoxyethanol.

I would now like to discuss certain aspects of heterogeneous or two-phase systems. The two phases may be either solid–liquid or liquid–liquid.

### Solid–liquid

The most efficient system for weak  $\beta$ -emitters is to use the liquid phase as fluor, although early attempts to use the solid phase as fluor have been tried, e.g. Pilot B beads.<sup>6</sup>

Since the development of counting  $\beta$  activity on filter paper in the early 1960's, it has been realized that glass fibre and membrane filters are especially efficient supports for precipitates, either as a pulp suspension or as discs. Discs are easy to use and need not employ more toluene-based scintillant than is necessary to wet the paper. Furthermore, as Pinter *et al.*<sup>7</sup> have shown, up to twenty-five discs can be counted in a single vial, with no reduction in counting efficiency. On a standard disc of GF/C Whatman, 24 mm diameter, about 0.2 ml of aqueous solution may be dried. Twenty-five discs would thus represent a total of 5 ml and may therefore make it possible to obtain significant counts from low specific activity material by simply adding more discs (see Fig. 1).

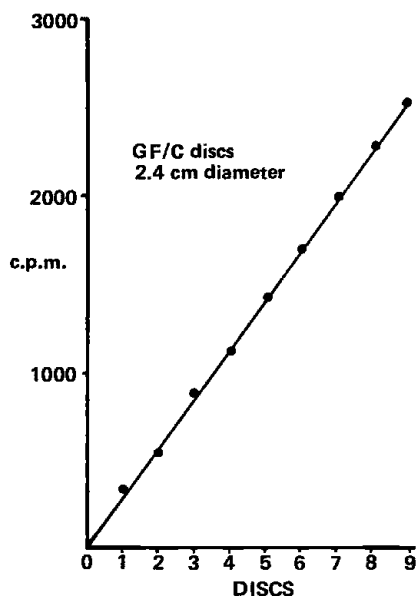


Fig. 1. Additive effect of counting glass fibre discs in the same vial. Each disc had 0.2 ml of tritiated thymidine solution dried on it.

A word about cost. A simple brass punch to cut out 24 mm circles from larger diameter glass fibre discs can reduce costs appreciably.

Gill<sup>8</sup> has examined the efficiency of a number of different materials as solid supports and has concluded that glass fibre is superior. Even scotch tape has been used recently to determine some parameters of the kinetics of epidermal proliferation of labelled hairless mice by stripping off the surface layers and counting adhering material in a toluene phosphor.<sup>9</sup>

The use of ion exchange paper, in particular DEAE in the form of discs was also described by Furlong *et al.*,<sup>10</sup> Breitman,<sup>11</sup> and Loftfield and Eigner<sup>12</sup> to distinguish charged from uncharged species in enzyme systems. It became clear that many assays could be undertaken, such as thymidine kinase,<sup>11</sup> hydroxamate formation,<sup>12</sup> galactose-1-phosphate from galactose,<sup>13</sup> adenosine diphosphate glucose pyrophosphorylase,<sup>14</sup> adenosine diphosphate creatine phosphotransferase<sup>15</sup> and glycerol kinase and hexokinase.<sup>16</sup> As an example, the thymidine kinase system first described by Breitman allowed 98% removal of thymidine and allowed 100% retention of the monophosphate. However, a study of this system has recently been undertaken by Roberts and Tovey<sup>17</sup> who have shown that the conditions of salt elution and paper drying are very critical for some enzyme assays.

### Thin layer chromatography

Thin layer chromatography has been widely used and the fumed silica product Cab-O-Sil has been successfully employed to improve the poor counting efficiency caused by geometry artifacts by preventing wall absorption of trace quantities of radioactive materials, thereby allowing nearly  $4\pi$  counting rather than  $2\pi$  counting.<sup>18</sup> When Cerenkov counting is used, however, e.g. with phosphorus-32, it is important to realize that Cab-O-Sil

can introduce abnormal quenching artifacts as reported by Wiebe *et al.*<sup>19</sup> recently. Polyacrylamide gels have also been used extensively, and methods of solubilization of these have been recently reviewed by Paus.<sup>20</sup> He concluded that gels up to 5% can be conveniently solubilized by heating to 60 to 65°C in Soluene-100, BBS-3 or NCS. However, it is worth noting that Soluene-100 apparently interacts with PBD fluors and considerable counting losses can occur.<sup>21</sup>

### Liquid-liquid

One of the earliest accounts of the use of emulsions in liquid scintillation counting was by Meade and Stiglitz<sup>22</sup> when they showed that the counting efficiency of body fluids and tissues could be improved by using the detergent Triton X-100 in conjunction with Hyamine-10X. Erdtmann and Herrmann<sup>23</sup> appreciated the possibility of exploiting emulsions for the measurement of  $\beta$ -emission which was later examined by Patterson and Greene<sup>24</sup> who suggested empirically derived compositions of Triton X-100 and toluene. The first systematic study of the system was undertaken by van der Laarse,<sup>25</sup> who whilst examining ground water samples, also described the appearance of all possible combinations of the three components by plotting on a triangular plotting system such as is used in phase composition research. The diagram is not strictly speaking a phase diagram since the whole system in all its areas is probably two-phase; however, we will refer to the diagrams as phase diagrams. They also employed a *figure of merit* to describe the efficiency, being the product of the absolute efficiency and the volume of aqueous phase used. The so-called *merit value* which I shall adopt is a product of the percentage efficiency and the percentage of aqueous solution present, based on a total volume of 10 ml. The value is, of course, based on the instrument used and to compare values between instruments it is necessary to divide by a comparative factor. I have not made use of this factor, and all measurements which I shall show were conducted on a Beckman LS 200 at ambient temperature operation, with a reference toluene standard efficiency of 45%.

The system consists of a range of transparency from crystal clear to completely opaque and also a range of viscosity from that of toluene to a thick gel which retains its position on inversion of the vial. These are separately assessed for each point of the diagram on an empirical scale of 0 to 4.

The clear areas represent submicroscopic colloidal droplets from 5 nm to 0.1  $\mu\text{m}$  whereas the opaque areas are true emulsions with a droplet range from 0.1  $\mu\text{m}$  to probably greater than 1  $\mu\text{m}$ . The mean range of  $\beta$ -particles from tritium in matter of unit density is approximately 0.56  $\mu\text{m}$  or, better still, 50% is absorbed within a thickness of 0.4  $\mu\text{m}$  (half value layer). The Triton X-100 apparently forms an organized double layer, in which the phenolic ends are associated within the droplet and the hydrophilic ethoxy chains extend out into the aqueous layer, interlacing with other chains arranged in the opposite direction.<sup>26</sup> The final efficiency of counting will then depend on a) the relative volumes of fluor-containing organic phase to the  $\beta$ -emitter containing aqueous phase, and b) the size of the fluor-containing droplet in relation to the path length of the  $\beta$ -emitter.

In practice, these parameters need not be assessed, since the areas of interest can be determined for an individual solution by determining the merit value for each point within the diagram using a polar tritium standard and the stability of the counts of each sample measured repeatedly over a reasonable counting period, 24 to 48 h. It is not possible to predict in detail the appearance or stability of the various parts of this diagram, but after constructing quite a number of these, several general points of interest emerge.

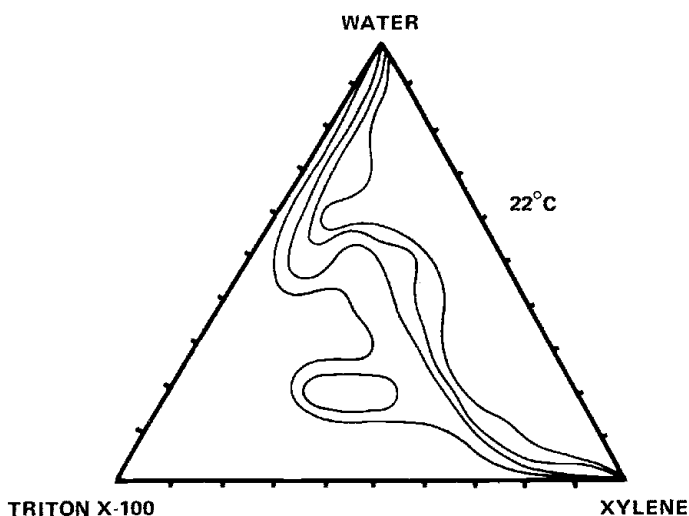


Fig. 2. The phase appearance of all combinations of water, Triton X-100 and xylene at 22°C. The areas outlined range from completely transparent (bottom left) to completely opaque (right hand edge).

To construct a diagram, a series of 36 vials are arranged in a vial box in a triangular pattern and starting from each corner, rows of successively increasing volumes of the three components are dispensed so that the total volume in each vial is 10 ml. The toluene component contains 0.5 ml of a concentrated fluor solution (2.67 g PPO, 0.067 g POPOP in 50 ml toluene) the rest of the volume being pure toluene. The vials are shaken thoroughly and a visual assessment of transparency and viscosity is made (see Fig. 2). The backgrounds are assessed and the vials spiked with 10  $\mu$ l standard tritiated water, shaken and recounted several times by recycling over a period of 24 h. Comparison of these counts enables one to assess counting stability. The merit values are ascertained for the 2nd or 3rd cycle, plotted on the diagram and contours drawn corresponding to increments of merit value of 100 (see Fig. 3). A point can then be selected which combines high merit value with good counting stability in a transparent or opalescent region. The optimal value is then obtained by making up several samples of this mixture with varying proportions of concentrated fluor-toluene solution, to ascertain the highest merit value. All these stages were conducted for a series of solutions used in biochemical work.<sup>27</sup>

Turner<sup>28</sup> has pointed out that with the toluene : Triton X-100 (2 : 1) : water system, a discontinuity in appearance and efficiency occurs as water is added. This discontinuity may be predicted on examining the diagram for toluene : Triton-X : water (Fig. 4). The use of a different detergent, e.g. Triton N-101, one of the nonyl-phenol series shows a slightly different phase diagram (see Fig. 5), but has fewer merit value contours than that of the X-100 diagram. Incidentally, BBS-3 : water : toluene is also different, but optimal values are lower than those obtained with the Triton X-100 : water : toluene system (see Fig. 6). The value of this detergent, however, is said to be with high salt conditions, and these are currently being assessed in this way. The appropriate optimal concentrations and merit values are summarized in Table 1. An example of the unpredictability of the phase diagram

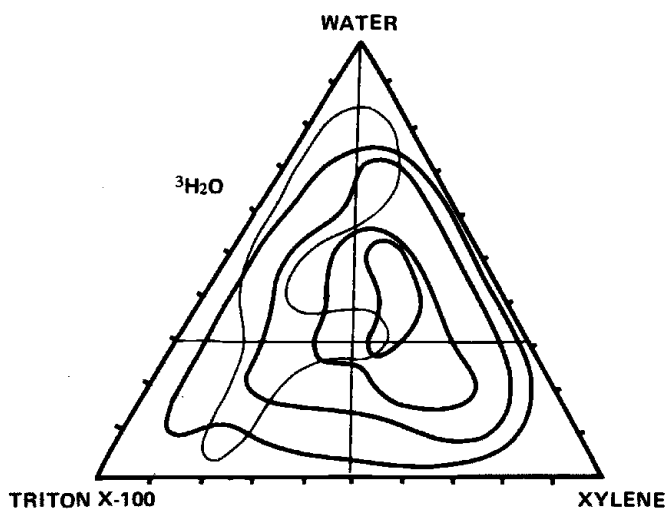


Fig. 3. The merit value diagram for the water : Triton X-100 : xylene system. Each contour (thick line) represents an increment of 100 starting from the outside. The fine line encloses the area of comparative counting stability and the point of intersection of the vertical and horizontal lines represents the final point chosen for counting. The final composition of the scintillant used will therefore be the ratio indicated by the base line. The ratio of this mixture to the aqueous solution involved would then be indicated by the vertical line.

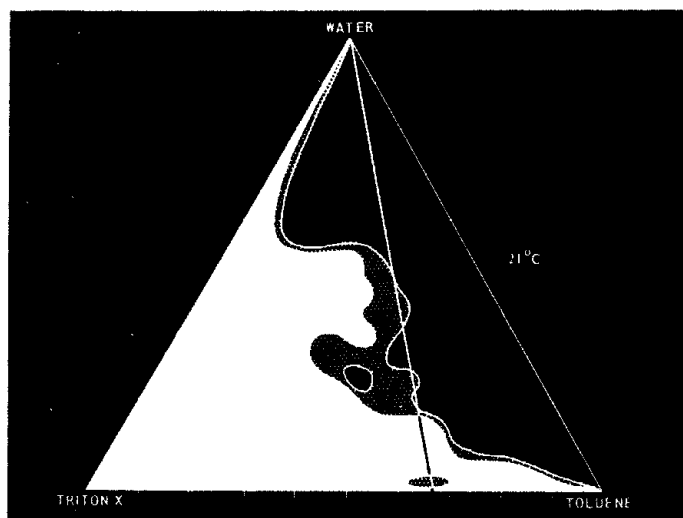


Fig. 4. A 'phase diagram' for the system water : Triton X-100 : toluene. (See legends to Figs. 2 and 3 for interpretation).

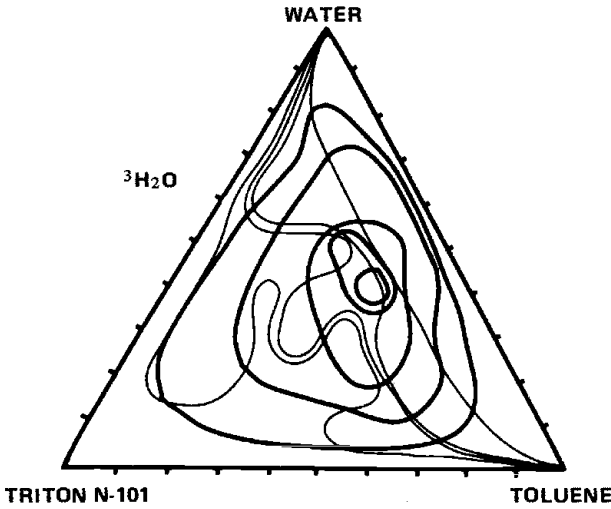


Fig. 5. A combined phase (fine lines) and merit value plot (thick lines) of the water: Triton N-101: toluene system. (See legends to Figs. 2 and 3 for interpretation).

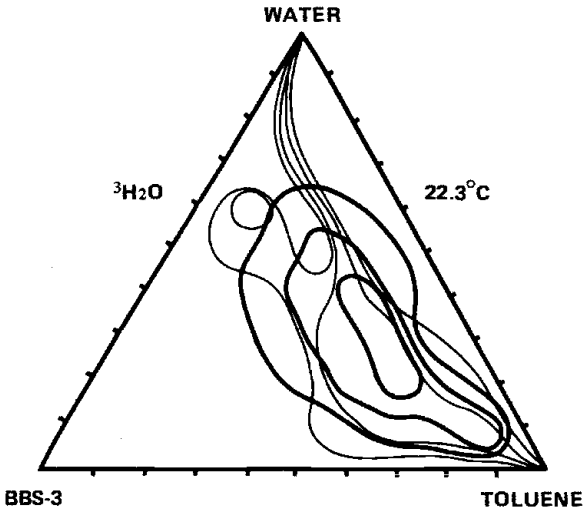


Fig. 6. A combined phase/merit value diagram for the Biosolve (BBS-3): water: toluene system, using PPO as fluor. (See legends to Figs. 2 and 3 for interpretation).

is shown by the 5% sucrose: toluene: Triton X-100 system.<sup>27</sup>

The most efficient region is within one of the 'islands' of clarity. The diagrams for comparable concentrations of fructose, glucose and maltose are quite different.

If sucrose gradients are being conducted, 0.5 ml fractions can be diluted with water for neutral gradients or an appropriate dilute hydrochloric acid solution for alkaline sucrose,

Table 1. Surfactant/solvent mixtures for tritiated water.

Surfactant	Solvent	Phosphor Mix		Tritium Counts		
		Phosphor	Water	Merit Value	Efficiency	Fluor
Triton N-101 ( 2)	Toluene ( 1)	6	4	510	12.5	PPO (opaque)
Triton N-101 ( 4)	Xylene ( 3)	7	3	378	12.6	PPO bis MSB
Triton X-100 (13)	Xylene (17)	7	3	409	13.6	PPO
BBS-3 ( 2)	Toluene ( 3)	5	5	273	5.5	PPO
Triton X-100 ( 5)	Benzyl alcohol (2)	7	3	130	4.3	PPO
Triton X-100 ( 1)	Toluene ( 1)	6	4	560	14.0	PPO

to bring the total volume to 5 ml. 5 ml of a toluene : Triton X-100 (2 : 3) : phosphor mixture is then added and shaken. If the experiment involves the use of direct cell lysis to examine labelled DNA and its precursors, the TCA precipitable DNA can be assessed by glass fibre disc methods and the data subtracted from a duplicate Triton-X : toluene system to give the distribution of acid soluble oligonucleotides on the sucrose gradient. With caesium chloride, a similar dilution of 0.2 ml fractions with 1.8 ml distilled water and after optical density measurement, is added to 8 ml of toluene : Triton X-100 phosphor (1 : 1) containing PPO and can then be counted with a merit value of 264.

Another feature of the colloid system can be seen in assaying chromatography eluates involving gradients. In the particular case of 1 M to 3 M hydrochloric acid elution of nucleotides on chloride anion exchangers, optimal counting conditions can be found for both 1 M and 3 M hydrochloric acid solutions (see Fig. 7), but these are different. However, one can superimpose the merit diagrams and locate a counting area where a constant merit value can be obtained (see Fig. 8) so that no quench correction will be necessary, although the merit values are slightly less than optimal. A similar estimate may be made from the ammonium formate system for separating nucleic acid bases (see Table 2). A further advantage of the colloid system is, of course, in the counting of complex biological solutions such as cell media, urine and plasma. The diagram for Fischer's medium containing 20% serum is shown in Fig. 9, and for urine, Fig. 10 demonstrates the kind of stability profile obtainable. These are summarized in Table 3.

### Quench correction

The colloid scintillation system should be regarded in the same way as any heterogeneous system. The only satisfactory way in which quench correction can be undertaken is by spiking with a standard of comparable polarity to that of the  $\beta$ -emitter. If there is any doubt, standards with two extremes of solubility, namely tritiated water and tritiated toluene can be compared and a region where a common merit value occurs is selected. Channels ratio measurements follow very closely those of internal standards in those systems which have been studied but it is reasonable to expect that divergences may occur. Indeed, Turner<sup>28</sup> has pointed out some small divergences in the less stable parts of the diagram. External standard ratios have very wide divergences in this system and should not

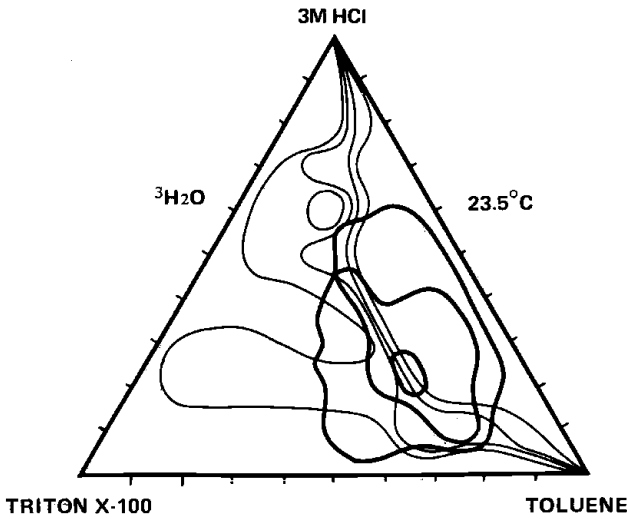


Fig. 7. A combined phase/merit value diagram for the system 3 M HCl: Triton X-100: toluene, using PPO as fluor. (See legends to Figs. 2 and 3 for interpretation).

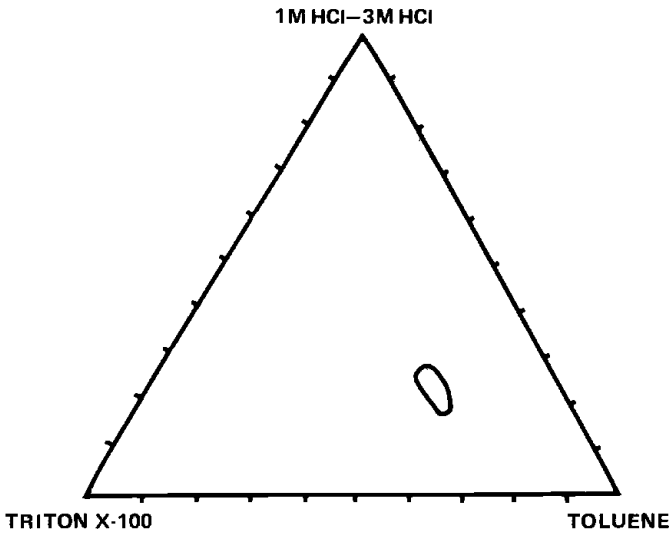


Fig. 8. The result of combining the merit values for 1 M and 3 M HCl: Triton X-100: toluene systems. The contour encloses that area where the merit value remains constant.

be used. It is possible that important information on colloid structure can be obtained from these measurements but they are useless for quench correction. I cannot stress too highly the importance of knowing whether one is dealing with a homogeneous or liquid-liquid two-phase system when one employs the newer gelling or solubilizing agents.

Table 2. Column chromatography.

Aqueous solution	Solvent composition		Phosphor mix		Tritium counts	
	Triton X-100	Toluene	Phosphor	Aqueous	Merit value	Efficiency
Ammonium formate 1 M	17	23	7	3	502	16.7
Ammonium formate 0.03 M	2	3	5	5	504	10.1
Gradient 0.03M→1.0M	1	1	7	3	392→377	13.1→12.6
Hydrochloric acid 1 M	2	5	7	3	461	15.3
Hydrochloric acid 3 M	2.3	5	8	2	339	16.9
Gradient 1M→3M	1	2	7.5	2.5	321→326	12.9
Formic acid 0.1 N	6	11	8.5	1.5	320	21.3

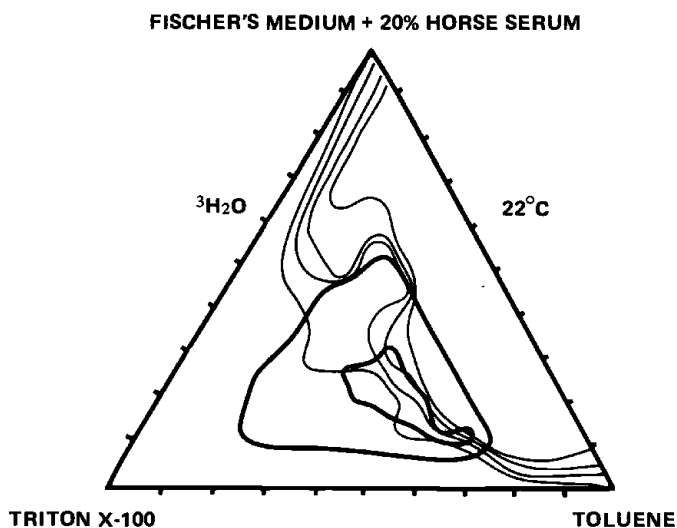


Fig. 9. A combined phase/merit value diagram for the Fischer's Medium containing 20% horse serum, Triton X-100 and toluene. (See legends to Figs. 2 and 3 for interpretation).

## CONCLUSION

In conclusion, I hope I have shown that heterogeneous systems play an extremely important part in modern biochemical procedure and the scope for their further exploitation is considerable. I am sure that the potentialities of the colloid system have only just been realized and that with a more sophisticated appreciation of the physical properties

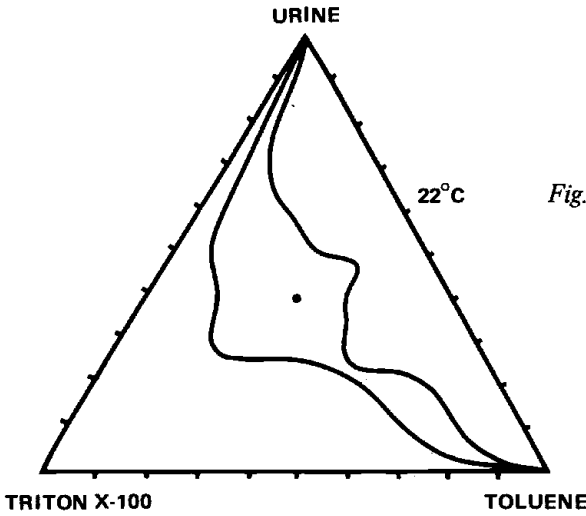


Fig. 10(a). A simplified 'phase' diagram of human urine.

Fig. 10(b). The merit diagram using PPO as fluor.

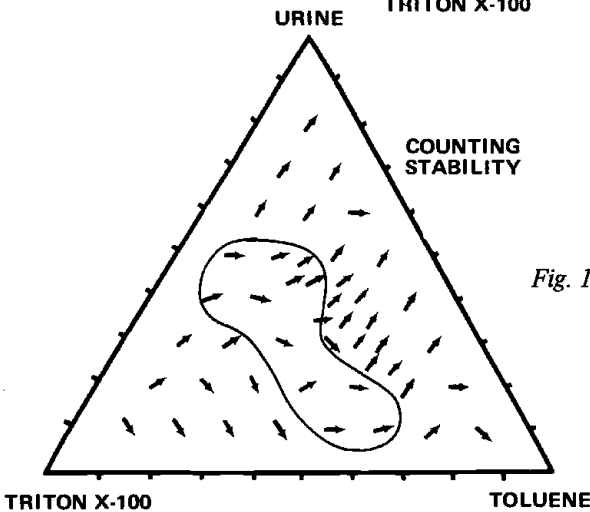
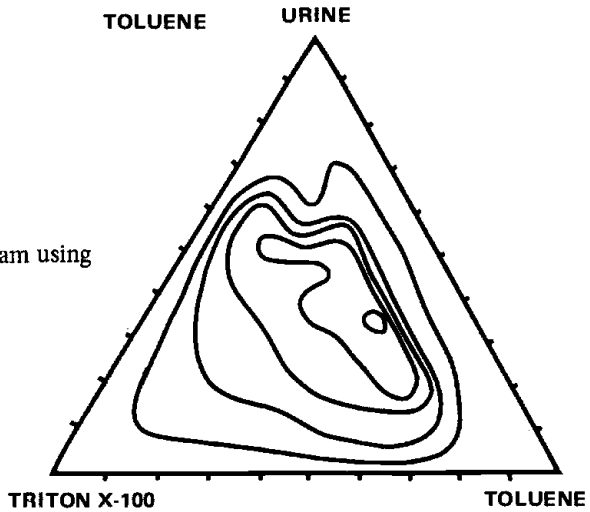


Fig. 10(c). The relative stability of the regions. Direction of arrows symbolizes the direction of counts during a recycling period of 24 h. Contour encloses area of relative stability.

Table 3. Media and biological samples.

Solution	Solvent composition		Phosphor mix		Tritium counts	
	Triton X-100	Toluene	Phosphor	Aqueous	Merit value	Efficiency
Fischer's medium + 20% serum	7	9	8	2	217	10.9
TYG micro-biological medium	1	1	8	2	480	24.2
Human urine	1	1	6	4	439	11.0
Human plasma	4	7	8	2	177	8.9

of the colloidal particles in relation to the very wide range of surfactants available, new techniques of assay of soft  $\beta$ -emitters in complex media could be developed.

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## DISCUSSION

**B. Scales:** I would like to re-enforce the warnings given by Dr. Fox about the uncritical use of Triton X-100. I have two slides which illustrate the peculiarities of the toluene: Triton X-100: water system, and show that gross errors can be incurred in the calculation of d.p.m. if these emulsion systems are used without due care and attention.

Figure 11 shows two identical series of 14 vials made up by adding increasing volumes of water to a fixed volume (10.0 ml) of a 2:1 toluene: Triton X-100 system containing 0.6% butyl PBD. Two important points to note are a) the general decrease in opacity (and increase in viscosity) as the volume of water increases, and b) the presence of two regions of instability; the first, which occurs in this example with the addition of about 0.25 ml water probably corresponds to the small pool of instability noted in Fig. 4 of Dr. Fox's paper, the water droplets visibly setting out on the base of the vial. The second occurs with the addition of from 2 to 3 ml water and corresponds to the larger central area of instability in that figure.

Figure 12 illustrates the count rates of toluene-soluble and a toluene-insoluble material in these systems at 18°C and 2 to 4°C. Most samples were made up in triplicate and counted for 10 min intervals over a 3-day period. The points show the average of these counts, and the bar lines illustrate the maximum drift in observed count rate (or calculated disintegration rate) over the full counting period. With <sup>14</sup>C-hexadecane at 18°C, the pattern is similar to that reported by Turner (*Intern. J. Appl. Radiation Isotopes* **19**, 557 (1968)). A marked decrease in count rate occurs with the addition of 2.5 to 3.0 ml water, and this only returns to normal when the system is stabilized by the further addition of water. Under refrigerated conditions, the same sample showed gross instability of count rate with 4.0 ml water. With higher water concentrations, the count rate was more stable but was drastically reduced. When the patterns are examined using a water-soluble material (<sup>14</sup>C-sodium succinate), a marked drop in count rate occurs with the addition of 0.25 ml water. Thereafter, a steady count rate is observed at 18°C, but at 2 to 4°C it falls rapidly when more than 4.0 ml water is present.

Any attempt to calculate d.p.m. of the <sup>14</sup>C-sodium succinate using the counting efficiency derived from a <sup>14</sup>C-hexadecane spike of the same samples results in the graphs within the lower axes. Instead of obtaining a constant d.p.m. value (4800 d.p.m. expected) at all water concentrations, the results can range from 2200 up to 5700 d.p.m. depending on the system used.

In metabolism studies, labelled materials of unknown structure present in biological samples are frequently quantitated using Triton X-100 systems. The lipophilic characters of the various labelled chemical entities present, will, by virtue of the normal detoxification mechanisms, often be very different from each other, and from that of the parent compound. For these reasons, the incautious use of internal standardization procedures in emulsion counting, whether carried out with lipophilic or hydrophilic substances, can give very misleading results.

**B. W. Fox:** I agree with these comments. The only really satisfactory standard would be

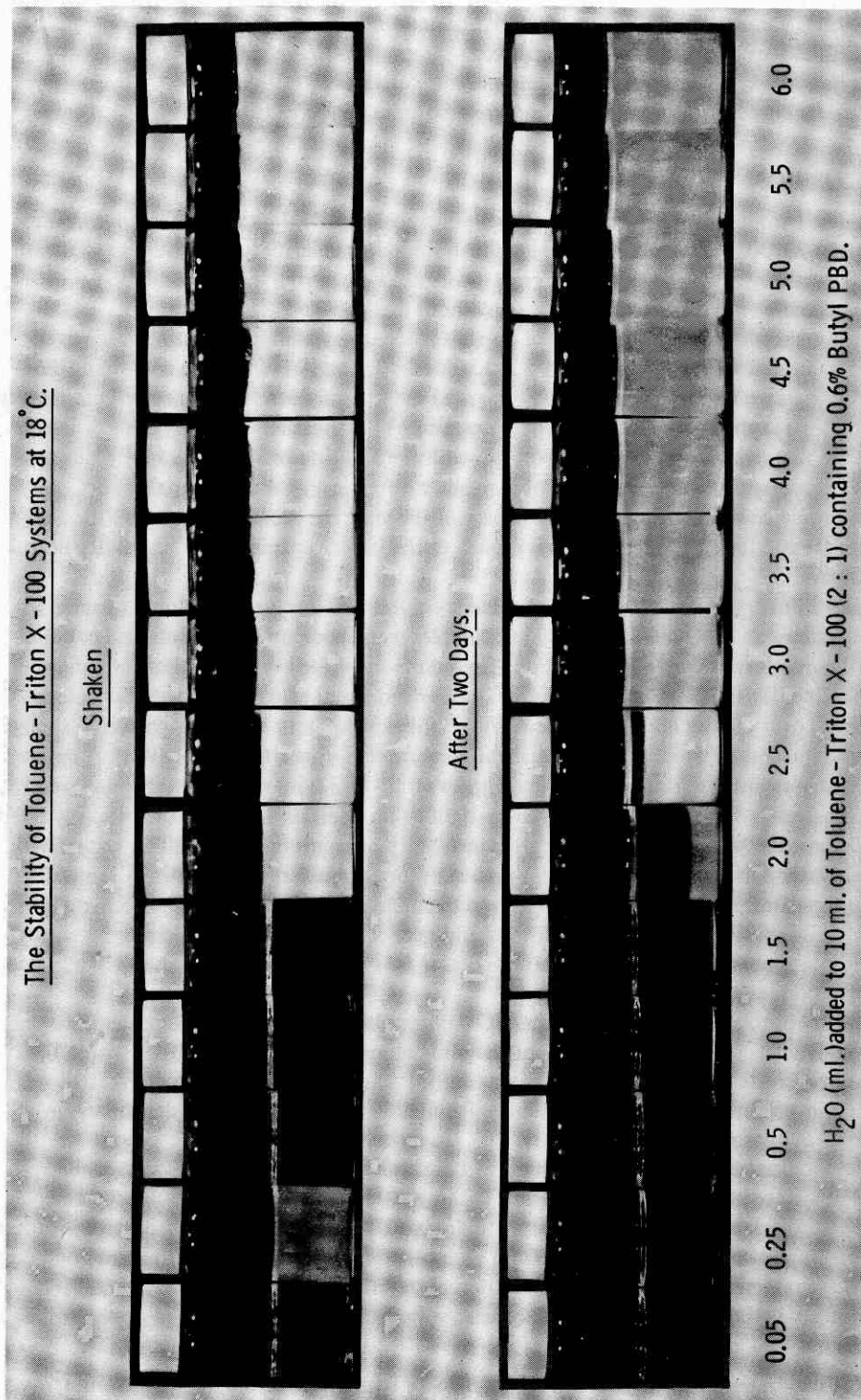


Fig. 11. The stability of toluene: Triton X-100 systems at 18° C.

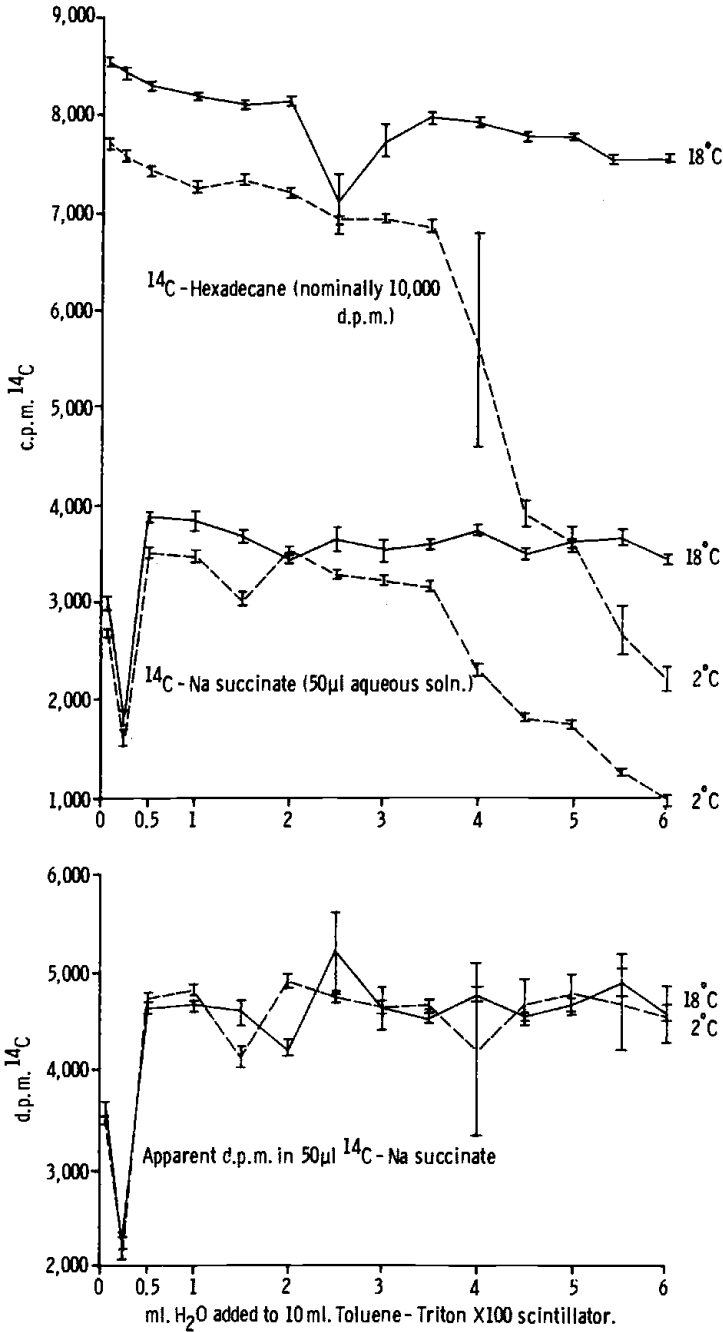


Fig. 12. Variations in carbon-14 counting efficiency of lipophilic or hydrophilic substances in toluene: Triton X-100 systems.

one which is made up of the species being studied. In biochemical work, most of the species of interest are water-soluble and hence tritiated water and  $^{14}\text{C}$ -sodium succinate would be a reasonable compromise to be used in colloid counting. I published merit diagrams comparing tritiated water and tritiated toluene in *Intern. J. Appl. Radiation Isotopes* 19, 717 (1968), and it can be seen from these curves that the major discrepancies occur in low surfactant and hence unstable regions, and not the regions recommended for counting. Your comments underline the necessity to adhere to the recommended composition.

**D. Bowyer:** In view of the apparent ease of determination of counting efficiency by means of the external standard method it would seem important to try to find external standard counting conditions suitable for use with heterogeneous systems; in this respect the energy of the external standard is important.

Which external standard sources were used in the experiments which demonstrated that the method was unreliable for heterogeneous systems?

**B. W. Fox:** In the initial studies, the relatively weak caesium-137 ( $20\ \mu\text{Ci}$ ) was used as external standard, and this gave meaningless ratios. In other machines with stronger sources a figure is obtained, but I feel that this is in general unreliable due to the unknown relationship of droplet to environment producing conditions not unlike those in any other form of heterogeneous system. What is the external standard measuring? The efficiency of the droplet or the efficiency of the environment? The net result is some complex function of both. It is possible that providing the micelle is very small in size, the system may approach that of a homogeneous solution, but at this moment I would prefer to avoid using the external standard as a means of quench correction in this system.

**F. Battig:** Contrary to your observations, we find excellent agreement of external standard ratios with tritium efficiency if using soluble regions of mixtures of biological fluids like urine, bile, plasma and serum in Instagel.

**B. W. Fox:** I think you have been fortunate. In our experience the external standard ratio is too low to be meaningful. In the case of high activity external standard sources, it is possible that data from a single composition within the phase diagram may be meaningful, say for colour quenching, but it is dangerous to apply it when crossing the phase diagram.

**H. Dobbs:** I would like to thank Dr. Fox for presenting the plenary lecture in this session. I would like to comment on the high quality of his slides. I found them aesthetically pleasing and am sure they reflect the excellence of his work. As always, his presentation was both eloquent and informative. I am sure that the systems he has described give accurate results when used correctly. However, I would like to sound a word of warning. Many of the people who use liquid scintillation counting techniques have little knowledge of the fundamental processes. Once they have been introduced to a method of sample preparation they stick to it slavishly regardless of the possible pitfalls. I am concerned that the methods used with great success in Dr. Fox's laboratories may be misused in other laboratories. Careless or ignorant application of Dr. Fox's elegant techniques to unknown samples could lead to gross errors in the measured counting efficiency.