

LIQUID SCINTILLATION COUNTING
RECENT APPLICATIONS AND DEVELOPMENT
VOLUME II. SAMPLE PREPARATION AND APPLICATIONS

SOLVING THE PROBLEMS OF COUNTING $^{14}\text{CO}_2$
PRODUCED IN BIOLOGICAL EXPERIMENTS

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$^{14}\text{CO}_2$ can be trapped efficiently as $\text{Na}_2^{14}\text{CO}_3$ in aqueous solutions of NaOH. In this state it can be dispensed and counted with great accuracy and stability, provided the correct conditions are observed. In this paper the various separate causes of inaccuracy in handling and counting $^{14}\text{CO}_2$ are explained and demonstrated. These include the rapid loss of $^{14}\text{CO}_2$ from aqueous solutions of mid-range pH, the effects of varying the constitution of the mixture being counted, the use of high specific activity $\text{Na}_2^{14}\text{CO}_3$, adsorption on the surface of the vial, the misuse of the 'merit value' concept, and the effect of the dimensions of the counting vials on the rate of change of counting efficiency with volume of scintillant. The relevance of these conclusions to the work of other authors in this field is discussed. An account is given of some satisfactory procedures for counting $^{14}\text{CO}_2$ using both commercial liquid scintillation fluids and 'home made' mixtures. The applicability of the sample channels ratio and external standard channels ratio techniques to these mixtures is discussed. A method is described for the separation of $^{14}\text{CO}_2$ from larger amounts of ^{14}C labelled volatile fatty acids and alcohols, which may have been either evolved or used as substrates in biological experiments.

Many studies on the liquid scintillation counting of $^{14}\text{CO}_2$ have appeared over the last twenty years but an examination of

the more recent publications indicates that this measurement is still giving difficulty (Herbland, 1977; MacRae and Wilson, 1978). However it has been known for almost a decade (Murray, 1971) that emulsion counting of $^{14}\text{CO}_2$ trapped as $\text{Na}_2^{14}\text{CO}_3$ in aq. NaOH offers a solution to these problems. The reason that Murray's method has not been generally adopted appears to be that insufficient information about the capabilities and limitations of the technique is available. As a result the method can neither be used with confidence nor is it apparent that it is superior to the older methods which use organic bases or $\text{Ba}^{14}\text{CO}_3$.

We have examined a number of well known liquid scintillation counting mixtures with relation to their performance when counting $^{14}\text{CO}_2$. Although all were capable of giving good results, the conditions necessary to achieve this goal were different for each mixture. These differences were sufficiently large to make the choice of mixture for any particular application an important consideration. In addition we describe a number of other sources of inaccuracy which have come to light during the investigation.

MATERIALS

Liquid scintillation fluids: Unisolve 1 (Koch-Light Laboratories, Colnbrook, Bucks., England), Instagel and Packard Emulsifier Scintillator No.299 (Packard Instrument Co. Inc., 2200 Warrenville Rd., Downers Grove, Ill 60515).

Emulsifiers: Biosolve BBS-3 (Beckmann Instruments Inc., Fullerton, CA 92634), Fisons Emulsifier No.1, Triton X-100, Triton X-114 (Fisons Sci. App., Loughborough, Leics., England), and purified Triton X-100 (Koch-Light Laboratories, Colnbrook, Bucks., England).

Apparatus: Three scintillation counters were used: Nuclear Chicago Unilux II at 4°C , Analytic 92 at 9.5°C (G.D. Searle & Co., 2000 Nuclear Drive, Des Plaines, Ill. 60018). Packard Tricarb 3320 at 6°C . An Aminco Bowman spectrophotofluorometer (American Instrument Company, Silver Spring, Maryland 20910) was used in the light-scattering experiments. A 10 μl syringe (Scientific Glass Engineering Pty. Ltd., 111 Arden St., North Melbourne, Australia 3051) was used to measure out all radioactive samples. All samples were counted in standard 20 ml glass counting vials.

Chemicals: Radiochemicals were obtained from the Radiochemical Centre, Amersham, Bucks., U.K. All the home made scintillation fluid cocktails contained 5g/l PPO and 0.1g/l POPOP. Standardised n-[1- ^{14}C]hexadecane reference material (1.016 $\mu\text{Ci/g}$) was used to determine efficiencies.

METHODS

Many samples of different compositions were examined with the aim of discovering what effect the added constituents had on the $^{14}\text{CO}_2$ counting characteristics of the liquid scintillation fluids. In addition to the commercial scintillation fluids these included home made mixtures composed of toluene-Triton X-100, xylene-Triton X-114, toluene-Fisons Emulsifier No.1, and BBS-3 in toluene. All the samples prepared for examination contained 10 μl of the same solution of $\text{Na}_2^{14}\text{CO}_3$ in 5% (w/v) aq. NaOH. The channel settings used on the liquid scintillation counters were those recommended for single isotope counting of ^{14}C .

Phase Diagrams

The results were displayed for analysis on a number of phase diagrams and the characteristics examined were (i) appearance (ii) counting efficiency (iii) reproducibility of count rate. An average of one hundred samples were prepared and counted five times on the same counter over a two week period to obtain the information for each phase diagram. They were then stored to allow longer term examination. Three different types of phase diagram were used and the reason these particular ones were chosen is explained in the discussion.

Phase Diagrams Using Rectangular Co-ordinates (Findlay, 1904). Volume of water was the abscissa and weight of NaOH was the ordinate in these diagrams. In the preparation of the samples aliquots of water from 0 to 3.0 ml in steps of 0.25 were added to series of vials containing 10 ml scintillation fluid. Ten such series of vials were prepared from each scintillation fluid and to each series was added a fixed amount of 5% (w/v) aq. NaOH. The amounts used were 0, 20 μl , 50 μl , 0.1 ml, 0.2 ml, 0.3 ml, 0.4 ml, 0.5 ml, 0.7 ml, 1.0 ml. All the scintillation mixtures were first examined by this means. In the 'home made' mixtures the solvent:emulsifier ratio was 2:1 except with BBS-3 when a 16% solution in toluene was used. The reason for choosing these ratios for the initial investigation was that they are the recommended proportions for counting aqueous samples and there was no evidence to suggest that aqueous solutions of NaOH and Na_2CO_3 should require different conditions. Also it was felt that the potential convenience of being able to use general purpose scintillation mixtures for counting $^{14}\text{CO}_2$ and alkaline solutions justified their investigation before others.

Triangular Phase Diagrams (Gibbs, 1876). The solvent-emulsifier mixtures were then examined using triangular phase

diagrams in the conventional manner (Fox, 1976) but using the information derived from the rectangular phase diagrams to choose suitable concentrations of aqueous solutions.

Triangular Phase Diagrams Using Ethanol. Triangular phase diagrams were constructed in which ethanol, water and a scintillation mixture were the three components. Unisolve 1 and three different toluene-Triton X-100 counting mixtures were examined in this way. The toluene-Triton X-100 ratios used were 2:1, 1:1 and 1:2. The samples prepared for this type of phase diagram all contained 22 mg Na_2CO_3 and 0.1 ml 5% NaOH in addition to the other components.

Low Specific Activity $\text{Na}_2^{14}\text{CO}_3$

The effect of Na_2CO_3 'carrier' on the counting mixtures was examined in a less complete way for the reasons explained in the discussion. The procedure used was to add increasing amounts to mixtures which showed good counting characteristics for high specific activity $\text{Na}_2^{14}\text{CO}_3$ to determine the amount required to affect their performance adversely.

Efficiency measurements

The efficiency of counting of the emulsion mixtures was measured using standardised n-[1- ^{14}C]hexadecane reference material (1.016 $\mu\text{Ci/g}$) and [U- ^{14}C]oxalic acid. An aqueous solution of the oxalic acid was standardised against the n-hexadecane by counting both in an ethanol-toluene scintillation mixture (Hall and Cocking, 1965) in which both are soluble.

The Tyndall Effect

Light-scattering experiments in the spectrophotofluorometer were done using a mixture of toluene and purified Triton X-100 (ratio 2:1) combined with various amounts of water. The light scattered at 90° to the incident beam was measured between wavelengths of 350 and 380 nanometres.

Preparation of Samples

The following procedure was used for preparing the counting samples. The reason this is described is explained in the discussion.

- (i) Add 10 ml scintillation fluid to vial and replace cap loosely.
- (ii) Add required volume of water and replace cap loosely.

- (iii) Add required volume of 5% NaOH aq. and replace loosely.
- (iv) Add required volume of Na_2CO_3 solution and replace cap loosely.
- (v) Allow to stand for approx. 30 min for vapour to equilibrate.
- (vi) Screw cap tight and shake vigorously.
- (vii) Allow to stand for approx. 30 min to drain cap.
- (viii) Draw up radioactive solution in 10 μl syringe excluding air.
- (ix) Wipe syringe needle with clean dry tissue.
- (x) Dispense radioactive solution beneath surface of scintillation mixture.
- (xi) Move needle quickly from side to side to rinse tip.
- (xii) Remove needle from scintillation mixture and wipe.
- (xiii) Replace cap loosely and allow to stand for approx. 30 min.
- (xiv) Screw cap tight and shake vigorously.
- (xv) Allow to stand for 30 min to drain before counting.

Quench Correction

The applicability of the sample channels ratio (SCR) and external standard channels ratio (ESCR) for determining the efficiency of counting of quenched samples was studied using phenolphthalein, chloroform, and alkaline extracts of sheep's liver as quenching agents when counting $^{14}\text{CO}_2$.

RESULTS

All the mixtures examined were capable of successfully counting high specific activity $\text{Na}_2^{14}\text{CO}_3$ (59.5 mCi/mmol) in the absence of any carrier provided that the correct quantities of NaOH and water were present in the counting mixtures.

For each mixture the range of compositions that gave the highest count rate coincided with those which gave the lowest standard deviation of repeated counts and these samples always had the appearance of crystal clear liquids at temperatures from 3°C to 21°C. Other compositions did not appear to be of any value for accurate counting. The compositions of the best mixtures are given in Table 1.

The Effect of Water

When known small amounts of water were added to the mixtures a macroemulsion was formed which separated and settled causing a change in count rate. As the amount of water increased a

TABLE 1. The relative amounts of constituents recommended for use when counting $\text{Na}_2^{14}\text{CO}_3$ or biological samples digested in aqueous NaOH^b

Scintillation fluid	Volume of scintillation fluid (ml)	Volume of water (ml)	Weight of NaOH (mg)	Weight of Na_2CO_3 (mg)
Instagel	10	1.10 ± 0.2	7.0 ± 4.0	0-9
Packard Emulsifier Scintillator No.299	10	1.00 ± 0.2	4.0 ± 1.0	0-7
Unisolve 1	10	0.90 ± 0.1	4.0 ± 1.0	0-3
Unisolve 1-ethanol (8:1) ^a	11.25	2.25	10	0-30
Toluene-Triton X-100 (2:1)	10	1.20 ± 0.2	3.5 ± 2.0	0-9
Toluene-BBS-3 (21:4)	10	1.30 ± 0.3	5.0 ± 2.0	0-10
Toluene-Fisons Emulsifier No.1 (2:1)	10	1.40 ± 0.2	4.0 ± 1.0	0-18
Xylene-Triton X-114 (2:1)	10	0.90 ± 0.1	4.0 ± 1.0	0-6

Note: To avoid problems with alkaline chemiluminescence in freshly prepared counting samples it is advisable to prepare a stock solution of alkaline scintillation fluid (see conclusions)

^aFor further information on this mixture see Huskisson and Ward, 1978.

^bThe efficiencies of counting of these mixtures are not given because they vary according to the scintillation counter and the channel settings used. However under any particular set of conditions they did not differ greatly from one another. Under each set of conditions we used, the counting efficiency of the least efficient mixture (Unisolve 1-ethanol, 8:1) was only 4% less than that of the most efficient mixture (toluene-BBS-3, 21:4). The efficiencies observed were always in the region of 85-90%.

clear microemulsion was formed which had good counting characteristics for n-[1- ^{14}C]hexadecane and [U- ^{14}C]oxalic acid, but not for $\text{Na}_2^{14}\text{CO}_3$ unless some NaOH was present. When the amount of water was increased further the microemulsion was destroyed and mixtures with inferior counting characteristics resulted.

The Effect of NaOH

When counting $\text{Na}_2^{14}\text{CO}_3$ all the mixtures displayed falling count rates when the NaOH concentration was below a certain amount (3 mg NaOH/10 ml scintillation fluid in most cases). It has been established that this is due to loss of gaseous $^{14}\text{CO}_2$ (Herbland, 1977). On the other hand unstable and dropping count rates resulted when the NaOH concentration exceeded a certain amount (5 mg NaOH/10 ml scintillation fluid in most cases). This was evidently due to the NaOH exhibiting the well known property of electrolytes in destroying, or "breaking", emulsions.

The Effect of Na_2CO_3

Marked differences were found in the amount of Na_2CO_3 that could be present without adversely affecting the performance of the mixtures. These results are shown in Table 1.

The Effect of Altering Solvent-Emulsifier Ratios

The triangular phase diagram of the toluene-Triton X-100 0.5% aq. NaOH system showed that compositions of solvent-emulsifier ratio between 1:1 and 4:1 are capable of forming clear microemulsions with good counting characteristics. The normally used ratio of (2:1) is therefore in the middle of the usable range.

The Effect of Ethanol on the Counting Mixture

In the examination of the effect of various combinations of ethanol and water on the toluene-Triton X-100 counting mixtures the toluene-Triton X-100 ratio 1:1 yielded the widest range of compositions with improved Na_2CO_3 capacity. However this capacity was not greatly increased and the counting efficiency was reduced considerably by the large water content required. Possibly these mixtures could be useful for some applications but they were of no immediate interest in the present study.

A similar phase diagram was drawn using the system Unisolve 1-ethanol-water. This behaved quite differently as would be expected from a previous study (Huskisson and Ward, 1978).

The results showed that only the relatively restricted range of compositions that we reported had good counting characteristics.

Counting Efficiencies

The counting efficiencies of the mixtures were measured using standardised n-[1-¹⁴C]hexadecane reference material and [U-¹⁴C]oxalic acid to see if there was any difference in the efficiency of counting of substances in the dispersed aqueous phase and the organic phase. Both were counted with the same efficiency. The addition of NaOH and Na₂CO₃ made a negligible difference to the efficiency of counting until the amounts present were large enough to adversely affect the physical stability of the counting mixtures.

When the aqueous solution of [U-¹⁴C]oxalic acid that was used for the efficiency determinations was counted in the toluene-ethanol mixture (Hall and Cocking, 1965) a diminished and dropping count rate was noted. This was presumably due to adsorption and therefore insufficient carrier oxalic acid was present. The aqueous solution used contained 2% oxalic acid in addition to the [U-¹⁴C]oxalic acid (74 mCi/mmol) and 10 μl was counted in 10 ml scintillation mixture, so each vial contained 200 μg oxalic acid. Therefore fresh samples were prepared in which 10 mg oxalic acid was dissolved in each vial before the [U-¹⁴C]oxalic acid solution was added. (The mixture was found to be capable of dissolving at least 250 mg oxalic acid per 10 ml at 3°C). Even with this composition a drop in the count rate from these samples was detectable after three or four days. To date this has not been investigated further.

10 mg oxalic acid and 10 μl water was added to the n-[1-¹⁴C]hexadecane standards and 10 μl n-hexadecane added to the [U-¹⁴C]oxalic standards, so that their chemical composition was the same. This was to ensure identical efficiency of counting.

No drop in count rate was seen when counting [U-¹⁴C]oxalic acid using the compositions of emulsion counting mixtures that were suitable for counting ¹⁴CO₂.

Light-Scattering Experiments

In the light-scattering experiments the scattered light was observed to be consistently of the same wavelength as the illuminating source. The clear transparent samples scattered light as effectively, and sometimes more effectively than the cloudy samples which were obviously macroemulsions. This suggested the presence of large numbers of very small particles in the clear fluids, indicating that they are microemulsions and not solutions. Unpurified Triton X-100 exhibited a

fluorescence spectrum in the region 350-380 nm. That is why it was necessary to use purified Triton X-100. At other wavelengths the light is absorbed.

Quench Correction

A family of curves was obtained when efficiency was calibrated against either SCR or ESCR using the three different quenching agents in the counting mixtures recommended in Table 1 for counting $^{14}\text{CO}_2$. The curves diverged with increased quenching to such an extent that when the count rate was reduced by 15% due to quenching, the estimation of the radioactivity present calculated from the ESCR or SCR using the chloroform calibration curve would be higher than that indicated by the phenolphthalein curve to the extent of 2.5% when using ESCR and 5% using SCR. The calibration curve using the alkaline liver extract as quenching agent lay between the other two.

Both chloroform and phenolphthalein in dilute aq. NaOH could be used to produce smooth calibration curves from minimum quench to almost total quenching of the count rate, but much smaller amounts of phenolphthalein were required to produce a given degree of quenching. In the phenolphthalein quenched samples 2 moles of NaOH per mole of phenolphthalein were added in addition to the amount of NaOH recommended in Table 1.

DISCUSSION

One of the objectives of this work was to see if the results of a previous study (Huskisson and Ward, 1978) were applicable to other commonly used emulsion counting mixtures, and if so to discover the best possible compositions for $^{14}\text{CO}_2$ counting mixtures using each of the scintillation fluids examined. The results show that all the mixtures examined can be used for accurately counting both $^{14}\text{CO}_2$ and alkaline extracts of biological materials. The question of whether the best possible mixtures for these purposes have been discovered is a matter requiring considerable discussion and the following description explains the rationale behind our investigation.

Description of the Phase Diagrams

Phase diagrams have been used to investigate the properties of emulsion counting systems before, but the ones we used are somewhat different from those that have been published. The choice of phase diagram is a matter of some importance because the systems being examined have many components and to investi-

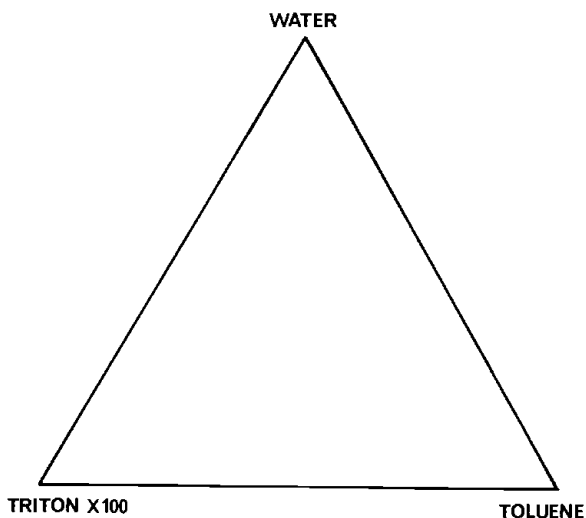


FIGURE 1

gate them fully would be a very large undertaking. Consequently it is necessary to choose carefully if the required information is to be obtained from the minimum number of samples.

Previously published phase diagrams of the three component system toluene, Triton X-100, water (Van der Laarse, 1967; Fox, 1968) used the triangular phase diagrams of J. Willard Gibbs (Gibbs, 1876). They were laid out as in Fig. 1. In these diagrams each apex represents 100% of the component with which it is labelled. The side of the triangle opposite the apex represents 0% of that component, and lines across the triangle parallel to this side represent a linear progression from 0% to 100% of that component as the apex is approached.

When it is desired to investigate the effect of an aqueous solution on the counting mixtures another variable, the solute, is introduced. The most thorough method of investigating the system is to prepare a whole series of phase diagrams of the type already described but for each of which the samples used to investigate the phase relationships contain a fixed amount of solute. The solute is then not a variable for any individual phase diagram but is increased in steps from one phase diagram to the next. If the phase diagrams so derived are then stacked one above the other a solid phase diagram is constructed in the form of a prism (Fig. 2), the long axis of which represents the amount of solute present (Findlay, 1904). (In fact the water apex represents less than 100% water in the triangular phase diagrams containing solute by an amount depending on the increase in the volume of the solution on addition of the solute, but in practice the amount of solute present is small so this effect can be ignored).

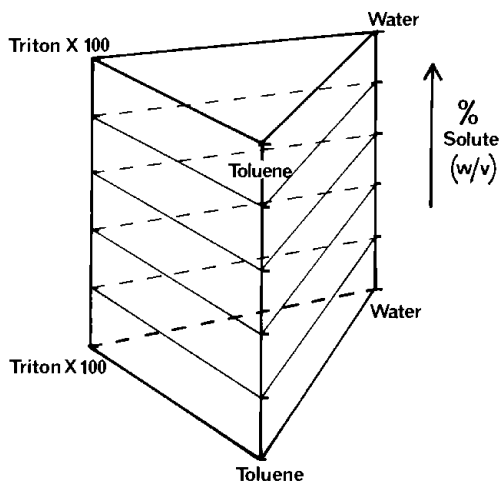


FIGURE 2

The advantage of this method is that the whole system can be examined. The disadvantage is that the amount of work and materials involved is large. An abbreviated method which will yield the required information is therefore desirable. The usual abbreviation is to use an aqueous solution of fixed concentration in place of water. We used 0.5% (w/v) aq. NaOH. This is equivalent to examining the plane section through the prismatic phase diagram indicated in Fig. 3(a) by the diagonal heavy lined triangle. Examination of this figure shows that if the region of good counting characteristics is small it may be easily missed by using the wrong concentration of aqueous solution. In the case of the toluene-Triton X-100-aq. NaOH system our results showed that it was necessary to choose a concentration of 0.5% NaOH to intersect the centre of the region of best counting characteristics. If a concentration greater than 2% NaOH was used, the region of microemulsion formation at 3°C would be completely missed. In previously published investigations of the effect of various aqueous solutions, concentrations of 5% and 10% have been used.

Our first investigations were done on commercial scintillation fluids in which the emulsifier solvent ratio is fixed. It has been pointed out (Fox, 1968), that in a triangular phase diagram of the type shown in Fig. 1, all possible compositions of a toluene-Triton X-100 (2:1) mixture with the third component fall on a straight line drawn from the apex of the third component to a point on the opposite side that is nearer the toluene apex than the Triton X-100 apex and divides the side in the ratio 2:1 (Fig. 4). Therefore taking a fixed emulsifier solvent ratio and testing it with various amounts of water and NaOH is equivalent to taking a longitudinal section through

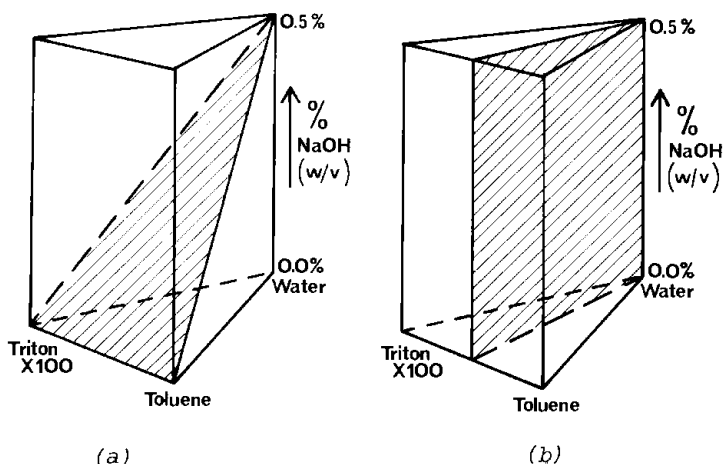


FIGURE 3

the prismatic phase diagram as shown by the dotted rectangle in Fig. 3(b). This represents the phase diagram using rectangular co-ordinates by means of which we examined the commercial scintillation fluids. In a previous study (Huskinson and Ward, 1978) we used a further simplification. Instead of examining the whole of the rectangular section by making up samples containing all possible combinations of NaOH and water, we simply used 1 ml water to 10 ml Unisolve 1 with differing amounts of NaOH. From this we obtained the amount of NaOH that would keep the $^{14}\text{CO}_2$ in solution without having any adverse effect on the counting properties of the scintillation fluid. Then we used this amount of NaOH with 10 ml Unisolve 1 and varied the water content to discover the correct amount of water to use. This gave us a suitable mixture for counting $^{14}\text{CO}_2$ which was our objective, but it didn't examine all the possibilities. The current investigations had the wider objective of finding the best possible mixtures and therefore the more complete approach was adopted. The investigations showed that in fact no better mixture existed.

In examining the emulsifier-solvent mixtures different approaches are possible but in order that our results should be directly comparable with the results obtained with the commercial scintillation fluids we first adopted the same method of rectangular co-ordinates, using the emulsifier-solvent ratio recommended for general use. The information obtained in this way showed that a concentration of 0.5% NaOH in water was likely to yield useful information when a triangular phase diagram of the toluene-Triton X-100-aq. NaOH system was examined. By this means the prismatic phase diagram for this system was examined by means of two mutually perpendicular plane sections that both yielded useful information.

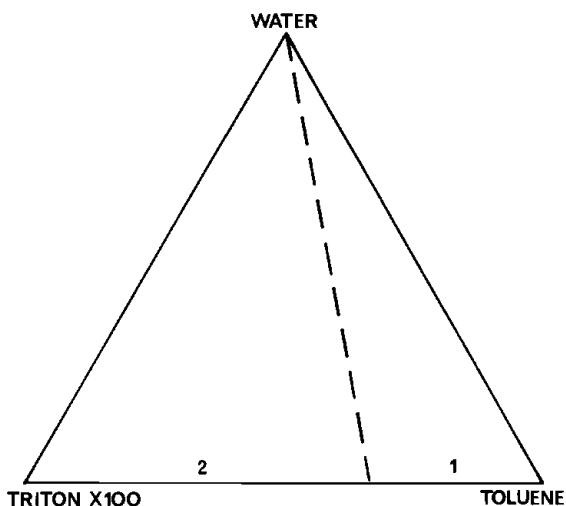


FIGURE 4

The Na_2CO_3 capacity of Unisolve 1 can be increased by using ethanol (Huskisson and Ward, 1978) and the use of other alcohols in this way has been suggested (Fox, 1977). We have also examined the effect of ethanol on the toluene-Triton X-100 system. Triangular phase diagrams were constructed with ethanol and water as two of the components and a fixed ratio of toluene-Triton X-100 as the third component. The results can be related to the ordinary phase diagrams (Fig. 1) by imagining the ethanol (or other alcohol) to be another dimension in space. In this way a solid phase diagram in the form of a tetrahedron (Fig. 5) is formed, each apex of which represents 100% of one of the four components (Friberg, 1977). The triangular phase diagram of toluene-Triton X-100 (2:1) with water and ethanol is then the plane section of this tetrahedral phase diagram that passes through the straight line on the toluene-Triton X-100-water surface shown in Fig. 4 and the ethanol apex. In addition to toluene-Triton X-100 (2:1) the ratios (1:1) and (1:2) were also examined in this way, so the actual sections of the tetrahedral phase diagram that were examined were as shown in Fig. 6. When examining a commercial liquid scintillation fluid it is of course only possible to examine one section of this tetrahedral phase diagram because the emulsifier-solvent ratio is fixed.

The effect of NaOH on each of these sections should strictly be determined in the same way that was explained for the phase diagram shown in Fig. 1 i.e. by constructing a prismatic phase diagram. Furthermore, to examine the effect of Na_2CO_3 on all the mixtures of emulsifier, solvent, water and NaOH so far discussed the whole process has to start again. Each possible plane section of the prismatic phase diagrams has to be itself

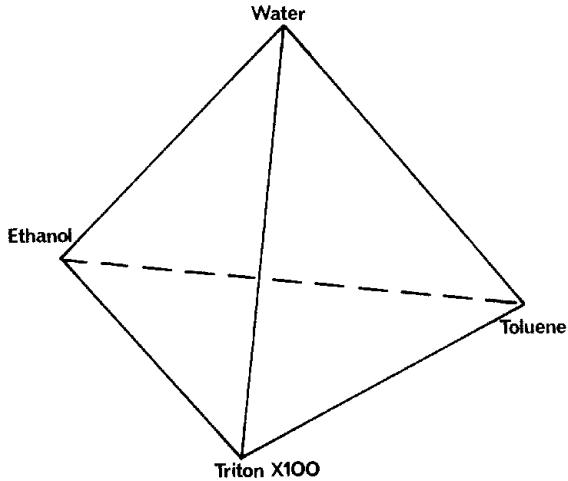


FIGURE 5

expanded into another solid phase diagram with the amount of Na_2CO_3 forming the new dimension. This sort of procedure is only practicable if we want to define the limitations of a counting mixture that looks promising for our purposes. In the present study we have simply determined the maximum amount of Na_2CO_3 that can be added without affecting the efficiency of counting of the recommended mixtures (Table 1). It will be appreciated that we cannot pretend to have fully investigated the systems we have examined but we hope we have shown that we have picked our way through the maze with a good deal of care.

The Stability of Samples

The difficulties of constructing phase diagrams for the non-ionic systems that are used in emulsion counting have been described (Prince, 1977). This author recommended that the samples used should be stored for at least three months due to the long period of time required to attain true equilibrium. It does not seem likely that mixtures which are subject to phase changes over such long periods would be suitable for accurate liquid scintillation counting. However in the range of compositions which we found best for counting $^{14}\text{CO}_2$, equilibrium appeared to be reached rapidly and this was reflected in the stability of the count rates derived from them. These remained constant for at least a year. The durability of Unisolve 1-ethanol mixture previously published (Huskisson and Ward, 1978) was even greater than this. Samples of this mixture containing 0.5 ml of 5% aq. NaOH (various amounts of which had been converted to $\text{Na}_2^{14}\text{CO}_3$ by trapping $^{14}\text{CO}_2$), have

been stored at room temperature for 2½ years and still show no sign of a drop in count rate. These counting mixtures are water in oil microemulsions in which the microemulsion is the stable state. This explains their long term stability. The eventual breakdown which occurred in some of the samples appeared to be due to reaction of the NaOH with the aluminium liners of the vial caps and with the glass walls of the vials which showed considerable etching.

Of course it is most unlikely that one would want to count samples after such a long period but the long term stability that is demonstrated by these samples does have an important practical aspect. It offers a means by which the notorious problem of chemiluminescence in freshly prepared alkaline counting samples can be circumvented. Chemiluminescence produced by basic samples has been shown to be due to at least two reactions of half-life about 0.45 h and 12.2 h (Benson, 1976). If a counting mixture containing suitable quantities of aqueous NaOH is made up well in advance of counting requirements these reactions will have proceeded to completion by the time samples are prepared for counting. Further addition of moderate amounts of alkaline solutions does not produce further chemiluminescence. This procedure also has the advantage that the sample that contains the $\text{Na}_2^{14}\text{CO}_3$ to be counted is added to a mixture that is uniformly alkaline and so there is no chance of any loss of $^{14}\text{CO}_2$ due to poor mixing. In addition to long term stability the count rates from the samples of best composition showed little statistical variation (Huskisson and Ward, 1978). This reproducibility of count rate is the reason that it is so desirable to count all emulsion samples within the range of compositions in which they form true water in oil

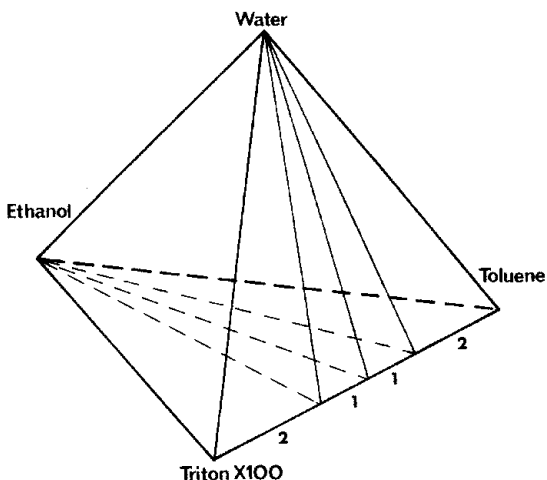


FIGURE 6

microemulsions. It is important to bear this in mind when trying to achieve improved accuracy of counting by adding more aqueous phase and so increasing the count rate. If, as a result, the system ceases to be a true microemulsion, a large decrease in the reproducibility of the count rate can occur. This will introduce a statistical error that may far outweigh any improvement in the statistical random error of decay due to an increased count rate. Many publications give details of merit values of various cocktails, but we are not aware of any that give details of the variability of the count rate at the different compositions.

The role of ethanol in increasing the Na_2CO_3 and NaOH capacity of emulsion counting mixtures is presumably due to its action as an amphiphile i.e. a substance that stabilises emulsions.

Efficiency of Counting

Differences in the efficiency of counting of ionic and non-ionic compounds have been noted using emulsion counting (Van der Laarse, 1967). Therefore it is necessary to use both an ionic and non-ionic standard if the efficiencies of a mixture are to be properly investigated. Only non-ionic ^{14}C reference materials are available commercially so an ionic solution has to be prepared and standardised against one of the non-ionic reference materials. Oxalic acid has been suggested as a suitable ionic standard (Fox, 1976). This compound has the added advantage that it is soluble in mixtures of toluene and ethanol and therefore can be standardised against non-ionic reference materials such as $n\text{-}[1\text{-}^{14}\text{C}]\text{hexadecane}$ in a medium in which both are in true solution and so can be expected to be counted with the same efficiencies. $[\text{U-}^{14}\text{C}]\text{oxalic acid}$ was therefore used in these efficiency determinations (Table 1). The adsorption problems we noted with oxalic acid are likely to occur with any strongly polar compound.

When using emulsion counting systems of compositions suitable for counting $^{14}\text{CO}_2$ no adsorption of $[\text{U-}^{14}\text{C}]\text{oxalate}$ was noticed. This may be because these solutions all contain sodium hydroxide and so the oxalic acid is present as the sodium salt.

Solutions of Sodium ^{14}C bicarbonate have also been suggested as an ionic standard. In our experience solutions of this salt lose $^{14}\text{CO}_2$ unless they contain NaOH . In addition alkaline solutions absorb CO_2 from the atmosphere and this results in a drop in pH unless excess NaOH is present. When we used a stock solution of $\text{Na}_2^{14}\text{CO}_3$ in 0.02 M NaOH for the preparation of counting samples over a period of time, a drop in count rate due to this effect was noted after a few weeks. However we have found that a solution of sodium ^{14}C carbonate (or bicar-

bonate) in 5% NaOH (1.25 M) forms a very reliable and stable internal standard when used with solutions suitable for counting $^{14}\text{CO}_2$. A solution of this composition which had been stored at room temperature and periodically used as a standard still gave the same count rate when fresh counting vials were prepared one year later.

Preparation of Counting Samples

Experience showed that a great deal of care was necessary in preparing the samples for counting if reproducible results were to be obtained. This apparently simple operation is full of unsuspected sources of error which only became apparent when large numbers of samples were being examined for reproducibility. There seems little point in describing scintillation fluids which give highly accurate count rates if the accuracy will be lost in use. That is why a detailed account of the routine we eventually adopted is given in the methods section. The procedure may seem rather tedious and the temptation to stray from it is great. In fact we have ourselves succumbed to this temptation many times, but we have always regretted doing so. Therefore it will perhaps be useful to comment on it.

The reason for allowing the vapour to equilibrate before shaking is that the solvent is a volatile liquid with an appreciable vapour pressure (40 mm Hg at 31.8°C for toluene). Therefore if a vial is capped and shaken immediately, a pressure in the region of half a pound per square inch can be produced inside the vial (Findlay, 1904) and this will force out some of the contents if the seal is not perfect. In practice this is a monotonously frequent occurrence. However if the vapour is properly equilibrated this type of leak doesn't occur because any fluid that leaves the vial must be replaced by air; otherwise a slight vacuum is created. The effect therefore is to hold the liquid in the vial. The volatility of the solvent is also the reason for keeping caps on the vials between additions. The constitution of these mixtures is critical and if some of the solvent is lost by evaporation the composition will be altered.

The addition of the radioactive samples was an operation that provided a great many opportunities for error. In these samples 10 μl aliquots of $\text{Na}_2^{14}\text{CO}_3$ in 5% aq. NaOH were used. To obtain reproducible results, it was necessary always to use the same syringe, because although any particular syringe can deliver a reproducible amount, apparently similar syringes deliver considerably different amounts. It was also found necessary to use a syringe with a needle cemented in place with epoxy resin (Araldite) because syringes with detachable needles delivered different amounts at different times for no obvious

reason. We find syringes with 11 cm needles most convenient but the syringes supplied commercially always have very fine bore needles which are undesirable for two reasons. First it is difficult to draw up solutions through them without including air bubbles. This is due to the viscosity of the liquid causing a pressure drop along the needle resulting in air entering the barrel of the syringe past the plunger. Secondly the same pressure difference in reverse occurs when the sample is delivered from the syringe and a variable amount of the sample is forced past the plunger instead of out of the needle. Therefore when we purchase a new syringe we always replace the needle with a new one of wider bore stainless steel hypodermic tubing. If the needle supplied is cemented in place, it can easily be removed by placing the syringe, needle-downwards, in a measuring cylinder full of concentrated H_2SO_4 for several weeks. An additional advantage of the wide bore needles is that any air bubble which is in the barrel of the syringe can be ejected by rapid depression of the plunger whilst the tip of the needle is in the liquid. This is much more difficult with narrow bore needles.

Having filled a suitable syringe with the radioactive solution it is necessary to wipe the needle with a tissue because at such small volumes the solution wetting the needle represents a considerable and variable quantity. Then the needle is dipped into the scintillation fluid and the plunger depressed. The tip of the needle is then moved quickly from side to side to rinse it and removed from the vial. It is then wiped clean to avoid transferring scintillation fluid back to the radioactive solution. The radioactive solution should be dispensed under the surface of the scintillation fluid and not into an empty vial, otherwise approximately 1% of the sample is retained on the outside of the needle by wetting.

If the above procedure is followed the reproducibility between samples is within 0.1% which is better accuracy than can be attained by weighing such small amounts and the procedure is very much less tedious. The exact amount of activity delivered by the syringe was determined by weighing larger amounts of n-[1- ^{14}C]hexadecane reference material (1.016 $\mu Ci/g$) into empty vials and then adding scintillation fluid and counting to compare the count rates with the count rate obtained from the nominal 10 μl delivered by the syringe.

Quench Correction

It has been established that SCR and ESCR cannot be used to compensate for quenching due to variations in water content in emulsion counting (Noujam *et al.*, 1976). However the results discussed below show that these channels ratios can be used

with great advantage provided that the proper constitution of the samples is observed.

Chloroform causes chemical quenching whereas alkaline phenolphthalein, which is insoluble in toluene, causes colour quenching of the scintillation process. Liver extract, due to its complex nature would be expected to produce both colour and chemical quenching. Colour quenching behaves differently from chemical quenching because of the path length through the scintillation medium which alters the pulse height distribution (Neary and Budd, 1970). This effect increases with increased quenching, and therefore the divergence of the calibration curves using respectively, phenolphthalein and chloroform, was to be expected. A biological sample which causes both colour quenching and chemical quenching would be likely to show this divergence to a lesser degree because for each drop in efficiency there is relatively less colour quenching present to alter the pulse height distribution. This was confirmed in our experiments because the calibration curve using alkaline extract of liver as a quenching agent lay between the other two when either SCR or ESCR was used.

These results show that the counting mixture described in this paper can be used to count heavily quenching alkaline biological extracts with good accuracy using SCR or ESCR as a measure of counting efficiency. This is useful because alkaline digestion of biological materials is widely used for preparing samples for counting. However the methods currently used (Fox, 1976) involve acidification of the extract and this results in the loss of any activity present as $^{14}\text{CO}_2$.

Adsorption

There is some controversy as to whether adsorption occurs in emulsion counting of $\text{Na}_2^{14}\text{CO}_3$ (Wigfield, 1976). Our results agree with those of that author in that we have not seen effects that could be attributed to adsorption. Furthermore we have never had any problems with low count rates when using counting mixtures of the recommended compositions even with the highest specific activity $\text{Na}_2^{14}\text{CO}_3$ we used (59.5 mCi/mmol). The maximum specific activity theoretically possible for $\text{Na}_2^{14}\text{CO}_3$ at 100% isotopic abundance is 64 mCi/mmol (Wilson, 1966).

Variations with Volume of Vial Contents

The variation of channels ratios with volume in the vial has recently been reported (Knoche et al., 1979). It is essential when accurate counts are required to keep both the volume of counting mixture and vial type constant. Otherwise it is necessary to calibrate the liquid scintillation counter being used.

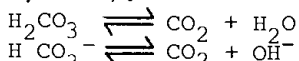
Loss of $^{14}\text{CO}_2$ from Aqueous Solutions of $\text{NaH}^{14}\text{CO}_3$ and $\text{Na}_2^{14}\text{CO}_3$

The problems that are encountered in handling and counting $^{14}\text{CO}_2$ originate in the way it escapes from aqueous solution. The extent to which this occurs appears to be frequently underestimated. A method for the separation of $^{14}\text{CO}_2$ from other labelled metabolic products has been described elsewhere (Ward and Huskisson, 1979). In this method $^{14}\text{CO}_2$ is flushed by nitrogen from an aqueous solution buffered at pH 7.8. This value was chosen because all traces of $^{14}\text{CO}_2$ can be flushed from an aqueous solution of this pH within one hour. This illustrates the high volatility of $^{14}\text{CO}_2$ in slightly alkaline aqueous samples.

By comparison with similar acids the pK_1 of the reaction

$$\text{H}_2\text{CO}_3 \rightleftharpoons \text{H}^+ + \text{HCO}_3^-$$

should lie in the range $2.8 + 0.9$, but the directly measured pK_1 is 6.38. This is because carbon dioxide in aqueous solution is only partly in the form of H_2CO_3 . It is largely present in more loosely hydrated forms. In the pH range 8-10 both of the following reactions are recognised to be important (Cotton and Wilkinson, 1966).



However we have found that $^{14}\text{CO}_2$ escapes from aqueous solutions of higher pH than this. It was found that 0.02 M NaOH (pH > 12) was the *minimum* concentration that was capable of preventing loss of $^{14}\text{CO}_2$ from a solution of $\text{Na}_2^{14}\text{CO}_3$ when evaporated to dryness *in vacuo* at 40°C on a rotary evaporator. It can be seen from Table 1 that the lowest concentration of NaOH in the aqueous phase that is compatible with the accurate counting of $^{14}\text{CO}_2$ is 1.25 mg in 1.40 ml water (0.025 M) for the toluene-Triton X-100 (2:1) mixture. This shows that the minimum concentration of NaOH in the aqueous phase that is effective in preventing loss of $^{14}\text{CO}_2$ from the mixtures is about the same as the minimum required to prevent loss from an aqueous solution if it is evaporated to dryness on a rotary evaporator at 40°C *in vacuo*. This is not too surprising since under the conditions of enormous surface area encountered when the aqueous phase is dispersed as a microemulsion, the tendency to lose $^{14}\text{CO}_2$ must be much the same as under evaporation.

The fact that this minimum alkali concentration is less than that required to break the microemulsion is the happy coincidence that makes the accurate emulsion counting of $^{14}\text{CO}_2$ possible.

CONCLUSIONS

The use of emulsion liquid scintillation counting fluids to count $\text{Na}_2^{14}\text{CO}_3$ in the absence of NaOH or any other base is still current practice (Major, 1979; Mayobre *et al*, 1979), despite the fact that several studies have shown that this method is subject to serious errors (MacRae and Wilson, 1978; Huskisson and Ward, 1978; Herbland, 1977; Murray, 1971).

The work described here shows that the accurate emulsion counting of $^{14}\text{CO}_2$ trapped in aq.NaOH is not subject to any special difficulty provided the relevant information is available.

Alkaline chemiluminescence in freshly prepared samples can be avoided by preparing a stock solution of alkaline scintillation fluid. This is done by incorporating into the scintillation fluid the *minimum* amounts of water and NaOH that will give good counting characteristics for $^{14}\text{CO}_2$ (Table 1). Any ^{14}C sample can then be counted by simply adding it to a portion of this stock solution. Provided that the sums of the water and NaOH already present in the scintillation fluid, and the water and NaOH (if any) added with the ^{14}C sample, do not exceed the maximum amount shown in Table 1, the sample will be accurately counted. For the most precise results, the total amount of water and NaOH present in the counting vial should be the mean values shown in Table 1.

If such an alkaline stock solution is prepared, then $\text{Na}_2^{14}\text{CO}_3$ in 5% aq.NaOH can be used as a reliable and inexpensive internal standard.

We have used this system for all ^{14}C counting requirements for several years and it has proved to be very satisfactory.

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