

## Chapter 20

# Factors influencing the Detection of Incorporated $^3\text{H}$ -Thymidine in Biological Material

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### INTRODUCTION

DNA synthesis is commonly measured by pulsing with labelled thymidine which is readily available labelled with either tritium or carbon-14. With the high counting efficiencies now found in modern liquid scintillation spectrometers, for convenience and economic reasons  $^3\text{H}$ -thymidine is becoming more widely used than  $^{14}\text{C}$ -thymidine.

It is often assumed that tritium can simply be substituted for carbon-14 in techniques originally developed for use with the latter. However we have found that this change in usage may give rise to problems. In particular, counting of incorporated tritium may be subject to considerable inaccuracy when methods of sample preparation are used that were developed in work with carbon-14.

### METHODS

Mouse ADJ PC6/A plasma cell tumour cells<sup>1</sup> were used as a convenient source of biological material in these experiments. The tumour was grown in BALB/c female mice. Labelled cells were prepared by pulsing a suspension of cells at a concentration of  $10^6$ /ml in a medium consisting of 70% medium 199 (British Drug Houses) and 30% horse serum (Burroughs Wellcome No. 2), for an hour with either  $^3\text{H}$ -thymidine (5 MeT), 5 Ci/mM at  $1.0 \mu\text{Ci/ml}$ , or with  $^{14}\text{C}$ -2-thymidine, 42 mCi/mM at  $0.05 \mu\text{Ci/ml}$ . (Labelled thymidine was obtained from the Radiochemical Centre, Amersham). Labelled DNA was isolated from solid tumours by the methods of Kirby,<sup>2</sup> tumours being taken one hour after the mice had been injected intraperitoneally with  $100 \mu\text{Ci/kg}$   $^3\text{H}$ -thymidine.

The radioactivity bound within the labelled cells or DNA was assayed by various methods:

1. **Assay on filter discs.** 0.5 ml aliquots of suspension containing  $0.5 \times 10^6$  cells were loaded onto Whatman glass fibre filter discs, (GF/A, 2.5 cm) contained in a Millipore 30-sample manifold. Each disc was then washed with approximately 10 ml aliquots of saline, 5% trichloroacetic acid (TCA), and ethanol in turn. The discs were then removed and placed in glass scintillation vials along with 10 ml scintillator.
2. **Perchloric acid extraction.** The total label incorporated into the cells was determined

by centrifuging a 5.0 ml aliquot containing  $5 \times 10^6$  cells, washing with saline and 5% TCA, and extracting the pellets twice with 5.0 ml of 0.5 N perchloric acid at 70°C for 30 min. 0.5 ml of the extracts was then assayed for radioactivity by mixing with 10 ml scintillator.

3. **Tetraethylammonium hydroxide (TEH) extractions.** TEH extractions of either TCA pellets or of cells on filter discs were carried out by using two 5.0 ml extractions at 50°C overnight. The radioactivity was then assayed in 0.5 ml aliquots of the extracts.

The radioactivity of the samples was measured in an Intertechnique ABAC SL 40 liquid scintillation spectrometer. The counter was set to accumulate 10 000 counts, i.e. with an inherent 1% counting error. The results were expressed as disintegrations/min (d.p.m.)/ $0.5 \times 10^6$  cells. Each point on the graphs is the mean of five samples; the standard deviations are too small to show on the scales used.

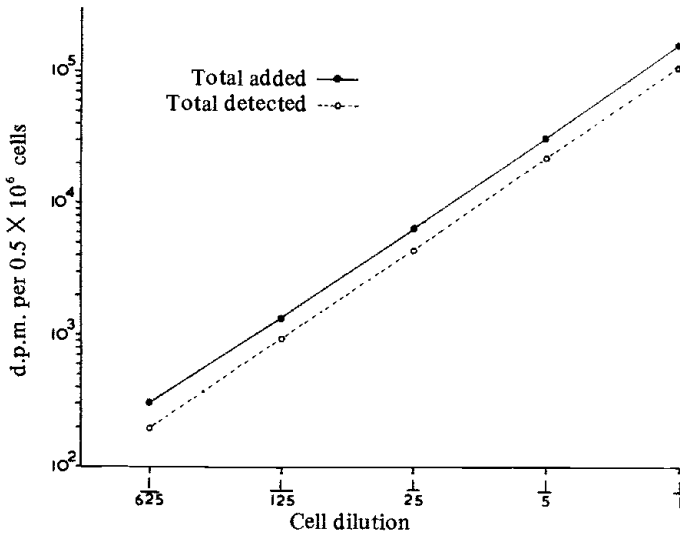


Fig. 1. Radioactivity on filter discs. Cells were labelled with  $^3\text{H}$ -thymidine and serially diluted with unlabelled cells. The radioactivity incorporated was estimated by perchloric acid hydrolysis of a 5% TCA pellet ('Total added') and by the filter disc technique by direct measurement ('Total detected'). Each point in this and subsequent figures represents the mean of five samples. The standard errors are too small to plot.

## RESULTS

### Effect of sample preparation on amount of activity detected

In a series of experiments cells were labelled with  $^3\text{H}$ -thymidine and serially diluted with unlabelled cells. Aliquots were assayed for tritium incorporated into DNA using different methods of sample preparation. Figure 1 shows the results of experiments in which the incorporated radioactivity was measured either on filter discs or after 0.5 N perchloric acid hydrolysis of a 5% TCA pellet. It is clear that there is a considerable discrepancy between the amounts of radioactivity detected by the two methods, activity after perchloric extraction being about 50% higher than that detected on the discs. This

Table 1. Labelled cells on filter discs. Aliquots of cells, labelled either with  $^3\text{H}$ -thymidine or  $^{14}\text{C}$ -thymidine were divided into three groups. The first was precipitated with 5% TCA and the second two loaded onto filter discs. The 5% TCA pellets were extracted with 0.5 N perchloric acid and were considered to be equivalent to the material added ('Amount added') to the discs. One group of discs was counted directly ('Amount detected'), the others were extracted with 0.5 N perchloric acid ('Amount extracted').

Isotope	Amount added	Amount detected	Amount extracted
Tritium	30716	20125	29172
Carbon-14	5072	4998	5017

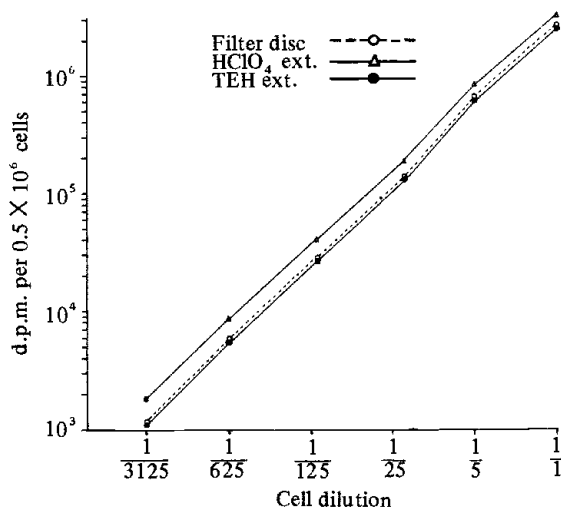


Fig. 2. Radioactivity on filter discs. Cells were labelled with  $^3\text{H}$ -thymidine and serially diluted with unlabelled cells. The radioactivity incorporated was measured by the filter disc technique, either by direct measurement ('Filter disc'), or after perchloric acid extraction of the disc ('HClO<sub>4</sub> extraction') 2 × 5.0 ml 0.5 N at 70°C for 30 min or after TEH extraction of the material on the disc ('TEH extraction') 2 × 5.0 ml at 50°C overnight.

discrepancy could be the result of many factors, loss through the disc being the most obvious. However, Table 1 shows the results of an experiment in which aliquots of labelled cells were either loaded onto a disc or precipitated with TCA and the discs were then either counted directly or extracted with perchloric acid. It is clearly seen that the amount extracted agrees with the amount loaded onto the disc showing that little activity is lost through the filter.

Figure 2 shows the results of an experiment in which serial dilutions of labelled cells were processed on filter discs which were then divided into three groups. The first was counted directly, the second extracted with tetraethylammonium hydroxide (TEH) and the third with 0.5 N perchloric acid. The radioactivity measured in the TEH extract was almost the same as that measured on the discs. However with 0.5 N perchloric acid extraction there was a 50% increase in the amount of radioactivity measured.

Table 2. Labelled DNA on filter discs. Aliquots of  $^3\text{H}$ -labelled DNA or  $^{14}\text{C}$ -labelled DNA in 2% NaCl were loaded onto filter discs and, after precipitation and washing, the radioactivity on the discs was measured ('Amount detected'). Further aliquots were hydrolysed in 0.5 N perchloric acid and assayed for radioactivity to determine the total amount of activity added to the disc ('Amount added'). Other discs were extracted with perchloric acid and the radioactivity of the extracts measured ('Amount extracted').

Isotope	Amount added	Amount detected	Amount extracted
Tritium	147974	72589	138648
Carbon-14	5277	5090	5171

Table 3. Isotope ratios of labelled DNA on filter discs. Samples of double labelled DNA and single labelled DNA were dissolved in 2% NaCl. Aliquots of double labelled DNA and mixtures of single labelled DNA were assayed for radioactivity after perchloric acid hydrolysis ('Amount added'), or after precipitation and washing on filter discs ('Amount detected'). Samples of DNA precipitated on the discs were also extracted with perchloric acid and the radioactivity of the extracts measured ('Amount extracted'). The isotope ratios tritium:carbon-14 of the samples were then calculated.

Sample		Amount added	Amount detected	Amount extracted
$^3\text{H}/^{14}\text{C}$ dual label	$^3\text{H}$	34820	20492	33539
	$^{14}\text{C}$	3972	3945	3929
	Ratio	8.76	5.19	8.54
$^3\text{H}$ plus $^{14}\text{C}$	$^3\text{H}$	70777	52784	63510
	$^{14}\text{C}$	2474	2564	2371
	Ratio	28.6	21.0	26.8

In further experiments DNA that had been labelled with either  $^3\text{H}$ -thymidine or  $^{14}\text{C}$ -thymidine was assayed for radioactivity either directly on filter discs, in perchloric acid extracts of filter discs or after perchloric acid hydrolysis of the native material. Results are shown in Table 2. It is clear that with tritium labelling there is considerable loss of activity when DNA is measured on the filter discs but 94 to 98% of the total activity is recoverable by perchloric extraction of the discs, again showing that very little of the loss is due to passage of material through the disc. With the  $^{14}\text{C}$ -labelled DNA there is no significant variation with sample preparation in the amounts of radioactivity detected.

These results suggest that in experiments using both tritium and carbon-14 the method of sample preparation could influence measurements of isotope ratio if tritium were used to label DNA. Table 3 shows the results of such an experiment using doubly labelled DNA and mixtures of  $^3\text{H}$ - and  $^{14}\text{C}$ -labelled DNA. Aliquots were assayed for radioactivity of filter discs and after perchloric extraction of the precipitated material on the discs. The results show that the ratio of tritium to carbon-14 appears to be much greater after perchloric acid hydrolysis.

The influence of sample preparation on the apparent activity of labelled DNA is further illustrated in Table 4. Radioactivity of equal aliquots of labelled DNA was measured after different methods of sample preparation. It is seen that with  $^3\text{H}$ -labelled DNA

Table 4. Effect of sample preparation on activity detected. Samples of <sup>3</sup>H-labelled and <sup>14</sup>C-labelled DNA in 2% NaCl were assayed for radioactivity using different methods of sample preparation. Perchloric acid hydrolysis was carried out using 0.5 N acid at 70°C for 2 h. TEH solubilization was effected by using 25% TEH at 50°C overnight. Biosolv 111, Hyamine and Soluene were used according to the manufacturers' instructions.

	Tritium (d.p.m.)	Carbon-14 (d.p.m.)
Perchloric acid	34050	11050
TEH	20500	10311
Biosolv 111	20470	10165
Hyamine	31810	11248
Soluene	20710	11010

Table 5. Effect of sample preparation on DNA specific activity. Aliquots of labelled DNA at 0.1 mg/ml in 2% NaCl were made up and the specific activities in terms of d.p.m./μg DNA measured after different methods of a sample preparation. Perchloric acid hydrolysis was carried out using 0.5 N acid at 70°C for 2 h. TEH solubilization was effected using 25% solution at 50°C overnight. N.C.S., Soluene and Hyamine were used according to the manufacturers' instructions.

Agent	Specific activity	Percentage perchloric acid
0.5 N Perchloric acid	929	100
TEH	734	79
N.C.S.	723	78
Soluene	718	77
Hyamine	845	91

activity detected depends greatly on the method of extraction and that maximum activity is only found after perchloric acid hydrolysis. With the carbon-14 label, measurement of activity is unaffected by sample preparation. Table 5 shows the results of a similar experiment in which the radioactivity of <sup>3</sup>H-DNA is expressed in terms of specific activity (d.p.m./μg). Again this depends on the method of sample preparation and was maximal after perchloric acid hydrolysis.

From the previous experiments it is clear that the maximum count rate from <sup>3</sup>H-labelled DNA is only found after perchloric acid hydrolysis. A similar result is found after hydrolysis with DNase. It is well known that both procedures result in the production of small molecular weight fragments, bases, phosphates and sugar degradation products with perchloric acid and mononucleotides with DNase. Figure 3 shows results of an experiment in which equal amounts of DNA were either hydrolysed in perchloric acid or solubilized in TEH and then fractionated on a G50 Sephadex column using 2% sodium chloride as the eluent. Optical density measurement of the fractions shows a considerable difference in distribution of molecular weight of the fragments produced. Perchloric acid produces small molecular weight fragments, the peak corresponding to that of the thymidine marker, whereas the TEH solubilization produces a large range of molecular weight fragments,

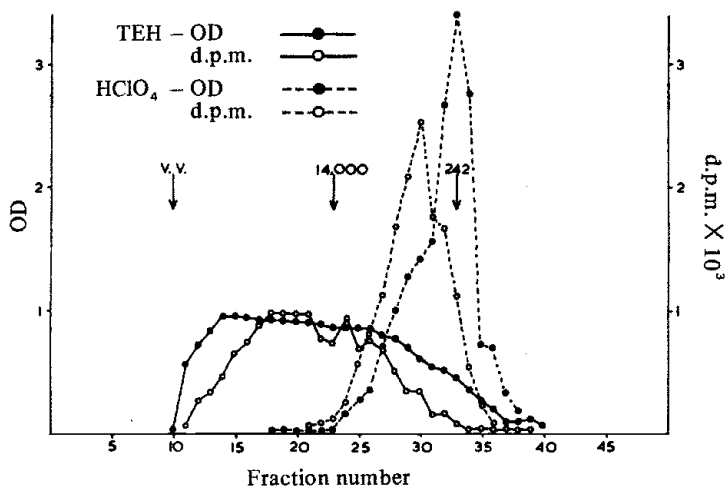


Fig. 3. Sephadex G50 fractionation of  $^3\text{H}$ -DNA after solubilization in TEH or hydrolysis in 0.5 N perchloric acid.  $^3\text{H}$ -DNA in 2% NaCl at 10 mg/ml was either solubilized in TEH or hydrolysed in 0.5 N perchloric acid. 1.0 ml of the resulting solutions was loaded on a  $30 \times 2.5$  cm column of G50 Sephadex. 2% NaCl was used as the eluent, and 100-drop fractions were collected (approximately 5.0 ml). The optical density and radioactivity of these fractions were then measured and plotted as shown. Native DNA was used as a marker to find the void volume. Lysosyme (molecular weight 14 000) and thymidine (molecular weight 242) were also used as markers.

many of which are larger than the lysosyme marker (molecular weight 14 000). (This and the previous results suggest that molecular weight may influence resultant count rate of  $^3\text{H}$ -labelled DNA). A possible explanation of this phenomenon is self absorption of the low energy  $\beta$ -particles within the larger DNA polymer. The total d.p.m. eluted from the column with the TEH solubilized DNA was approximately 55% of the total after perchloric acid hydrolysis which is in good agreement with the previous experiments.

### Effect of quenching

It could be argued that the discrepancies shown when  $^3\text{H}$ -labelled DNA is counted under different conditions were due to errors in quench correction in the systems used. The original quench curve had been made up using chloroform but the experiment summarized in Table 6 shows that all the other agents fall well within the limits of experimental error and could not possibly account for discrepancies of the magnitude found. In this experiment a series of  $^3\text{H}$ -hexadecane standards in scintillation counting fluid were assayed and then reassayed after the addition of various quenching agents. Some deviation is observed, but some must be expected since the counter was set to accumulate 10 000 counts, i.e. with an inherent 1% error, and since samples are counted at about 25% efficiency multiplication of counts/min (c.p.m.) to d.p.m. would increase this error fourfold.

Figure 4 shows the chloroform quench curve used for the work. The curve was unaltered by the presence or absence of glass fibre filter discs, nor did the discs influence the d.p.m. output. The results of the external standard quench corrections were shown to be accurate by internal standardization. We do not suggest that external or internal stan-

Table 6. Accuracy of quench correction. <sup>3</sup>H-hexadecane was made up in scintillation counting fluid and aliquots were assayed for radioactivity. Various quenching agents were then added to the samples at a final concentration of 2%, and the samples were then reassayed for radioactivity. The error in d.p.m. measurement before and after quenching was then calculated.

Quencher (2%)	d.p.m. before quench	d.p.m. after quench	% Error
0.5 N Perchloric acid	9492	9767	+2.90
TEH	9281	9466	+1.78
5% TCA	9110	9167	+0.63
N.C.S.	9526	9492	-0.36
Water	9301	9503	+2.17
0.1 N NaOH	9401	9089	-3.41

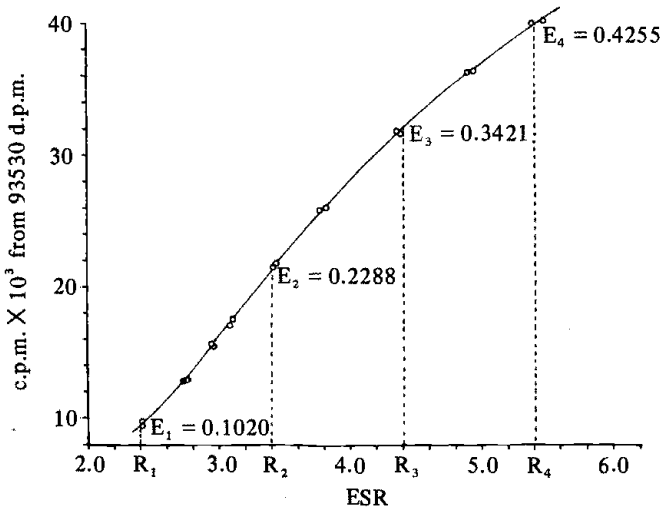


Fig. 4. Tritium quench curve – external standard ratio versus efficiency (chloroform). <sup>3</sup>H-hexadecane (Amersham) was used as a standard, diluted with scintillator to give 93530 d.p.m./vial. Chloroform was used as a quenching agent, in final concentrations ranging from 0.1 to 10% (v/v). Duplicate vials were counted and the mean c.p.m. plotted against the mean external standard ratio (ESR). The curve follows a third order polynomial function and the coefficients to the equation  $y = ax^3 + bx^2 + cx + d$  (where  $y$  = efficiency and  $x$  = ESR) were calculated from the coordinates  $R_1E_1$ ,  $R_2E_2$ ,  $R_3E_3$  and  $R_4E_4$ . These coefficients were then fed into the computer of the Inter technique ABAC SL 40 where they were used in the conversion of c.p.m. to d.p.m.

Standardization corrects for quenching occurring in the filter discs but only for contamination of the scintillator by water etc.

Figure 5 shows an absolute activity quench curve. This assumes that the effects, if any, produced by the presence of the filter disc are constant in all samples. The absolute activity of aliquots of <sup>3</sup>H-labelled cells was determined by perchloric acid hydrolysis of an equivalent 5% TCA pellet. The apparent activity in terms of c.p.m. detected in the

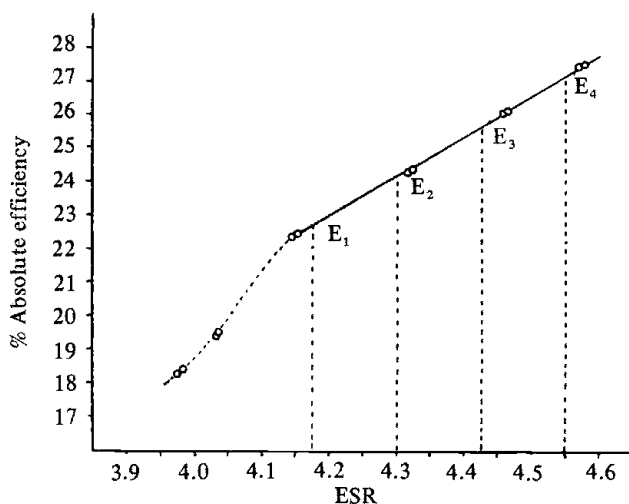


Fig. 5. Absolute activity quench curve — water quenching. The absolute activity present in aliquots of  $^3\text{H}$ -labelled cells was determined by perchloric acid hydrolysis of a 5% TCA pellet. The apparent activity, in terms of c.p.m., detected in corresponding aliquots of cells on filter discs was also measured and, from the two measurements, an efficiency figure was calculated that would allow for the loss of energy due to self absorption and any loss through the disc. Water was used as a quenching agent since it was the agent most likely to be left in the filter disc technique. Water was added in final concentrations ranging from 0 to 5% (v/v) as shown by the six pairs of points on the graph, the 0% concentration being represented by the top, right hand pair of points and the 5% concentration by the bottom, left hand pair. Over the working range, i.e. 0 to 3% the curve is linear. The coefficients to the equation  $y = ax^3 + bx^2 + cx + d$  (where  $y$  = absolute efficiency and  $x$  = ESR) were calculated from the coordinates,  $R_1 E_1$ ,  $R_2 E_2$ ,  $R_3 E_3$  and  $R_4 E_4$ . As the curve is linear,  $a$  and  $b = 0$ .

corresponding aliquots of cells in filter discs was also measured and from the two measurements an efficiency figure was calculated that would allow for loss of energy due to self absorption and any loss through the disc. Water was used as a quenching agent since it was the agent most likely to be left in the filter disc technique.

## CONCLUSIONS

These findings show that sample preparation can influence the activity detected when measuring  $^3\text{H}$ -thymidine labelled DNA. This does not invalidate techniques using  $^3\text{H}$ -thymidine providing they are internally controlled and absolute values are not required. In experiments using different methods of sample preparation caution should be applied in determining absolute values. The same caution must also be applied in systems using tritium and carbon-14 together, where DNA is labelled with tritium and carbon-14 used to determine another parameter.

## ACKNOWLEDGEMENTS

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## DISCUSSION

**P. Stanley:** I would like to raise three points.

Firstly, I believe the problem you have encountered is due to the very low  $\beta$ -energy associated with tritium. Both  $\beta$ -absorption and quenching of this isotope diminishes its counting efficiency. In addition biological samples on filters tend to absorb water thus aggravating the situation. It is also difficult to obtain precipitates on filters in a reproducible manner and to ensure that those precipitates are thinly and evenly spread.

<sup>3</sup>H-thymidine has been shown in many systems to label macromolecules other than DNA, for instance RNA and lipid. Is the DNA you purify free from these components?

Is your <sup>3</sup>H-thymidine free from contaminating breakdown products of the compound?

Was it rechromatographed to check this before use?

**J. A. Double:** We have suggested in the text that the problem we encountered was due to the very low  $\beta$ -energy associated with tritium, that energy losses occurred within the DNA polymer, and that external counting conditions, i.e. within the scintillation system have little effect on the resulting observed efficiency. The effect of water quenching was shown in Fig. 5. It is clearly seen that only when unrealistic amounts of water are added is there a significant reduction in efficiency. From our results, where in all instances count rates from five identical samples are within 5% of one another, it is unlikely that the spreading of the precipitates has any great influence on the resultant efficiency.

The DNA used was highly purified and definitely free from RNA and lipids. However, it is possible that if these were present and labelled, the same phenomenon of self absorption of the low energy  $\beta$ -particles might still be observed.

The <sup>3</sup>H-thymidine used was free from contaminating breakdown products as shown by TLC. However, such products would not label DNA and any unbound label would be removed by the extraction and purification procedures.

**M. Grant:** Are you sure that your DNA preparations are free from other materials such as lipids? Since you are working with mouse tumour cells which unlike many of the bacterial systems probably contain the enzymes necessary to degrade thymidine, one might expect to find the label from thymidine in a variety of cellular components.

**J. A. Double:** The DNA samples in these experiments were very clean.

**D. E. Bowyer:** High temperature digestion of samples may lead to a loss of isotope as volatile materials such as <sup>3</sup>H<sub>2</sub>O and <sup>14</sup>CO<sub>2</sub>.

**J. A. Double:** The digestions were carried out in sealed tubes, and it was highly unlikely that any such losses occurred.

**P. Johnson:** I would like to follow up Dr. Bowyer's point about possible loss of volatile materials with high temperature digestion procedures. A very simple answer to this is to modify a standard scintillation vial as shown in Fig. 6, in which a snug fitting polythene shive has a central well to accommodate a gas chromatograph septum. By drilling a central hole in the vial cap and in the polythene shive, samples and reagent can conveniently be withdrawn or added, but the system is sufficiently gas tight to allow high tem-

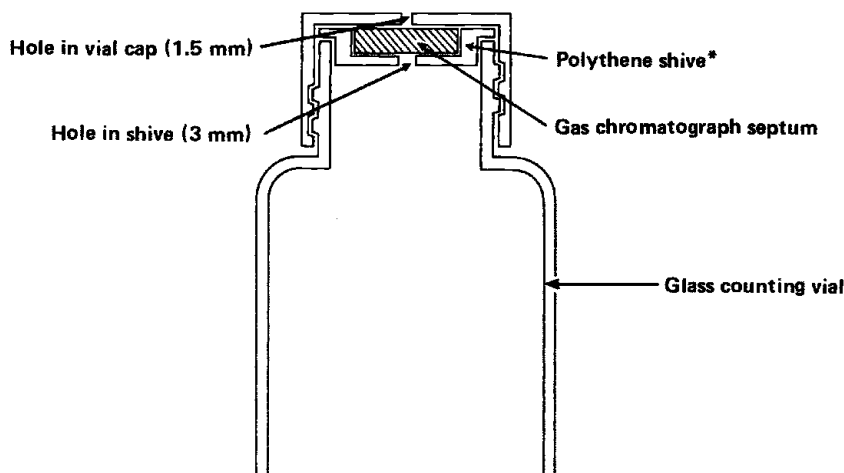


Fig. 6. Modified scintillation vial.

perature digestion without losses of volatile material such as  $^{14}\text{CO}_2$ .

**J. A. Double:** From the answer to the previous question, I do not think that the complexities you suggest are necessary.

**P. Johnson:** Comment: Except that sealed tubes do not allow convenient repetitive withdrawal of samples or addition of reagents; neither are tubes compatible with direct scintillation counting, so that further transfers are necessary.

**B. E. Gordon:** Special comment to all research workers: It must be pointed out to those doing biological and biochemical research that there is a major problem associated with liquid scintillation counting of materials not truly soluble in the counting solvent. The problem is that losses due to absorption or self absorption cannot be corrected for. Thus when counting a protein, a degraded protein, or any metabolite of unknown solubility, one may achieve highly reproducible but severely inaccurate results. This arises from the adsorption of a fixed amount of the labelled materials on any available surface. The use of auxiliary reagents which raise the counting rate (e.g. solubilizers) is also suspect since there is no test to determine when all the material has been desorbed. Furthermore, the argument that after the count rate has stabilized one has useful data is invalid for the same reasons.

The situation is usually worse with  $^3\text{H}$ -labelled materials than with carbon-14, but carbon-14 is subject to the same uncertainties. Therefore, it is essential that one establish, beyond doubt, that the counting system employed in work reported is free of such errors. This can only be done by calibrating the method against a series of combustion analyses and counting the  $^{14}\text{CO}_2$  and/or  $^3\text{H}_2\text{O}$ .

The comment that one must calibrate the combustion method is easily answered by combusting standards. There is no merit whatsoever in the argument that samples high in salts, metals, etc. cannot be combusted to yield quantitative recovery of carbon and hydrogen. There are a number of variations in the combustion method in the literature which can take care of refractory materials such as inorganics.

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\* Amplex Appliances Ltd., Tylney Road, Bromley, Kent.

Finally, it should be stressed that, because of the great tendency of such high polymers to adsorb, absorb, or coagulate small changes in the composition of the samples (e.g. molecular weight of a protein) or in the counting solution can change a quantitative system to a non-quantitative one. Thus one must be exceedingly careful to preserve the conditions of a proven (i.e. by combustion) counting technique.

I believe that referees of papers submitted to journals should view critically the point of accuracy of counting data and should in the future reject those which do not demonstrate, unequivocally, this accuracy.

**P. Johnson:** Comment: The question of precision versus accuracy is an old one and the two are sometimes confused. With regard to our own work, I am inclined to agree with the comments of Dr. Dobbs earlier in this meeting (pp. 215, 231) about burning a sample if other methods are difficult. We also routinely attempt some cross check on our other methods by using a combustion technique on random samples but no method is foolproof.

**B. Scales:** I agree wholeheartedly with Dr. Gordon's comments, but how do you check the efficiency of your combustions? Some samples are undoubtedly difficult to combust; whilst it is not advisable to simply place an aliquot of labelled materials on the surface of the sample prior to combustion, it is impossible to go to the other extreme and prepare, as a standard, a sample of animal tissue which contains the various radioactive materials dispersed in the same manner as would occur after dosing the animal with labelled compound. So very often a compromise has to be reached. Whether you use a commercially available automatic combustion system, a semi-automatic oxygen flask technique, or a combustion furnace, the efficiency of combustion of some types of samples leaves much to be desired.

**B. E. Gordon:** I am still at a loss to understand the difficulty of quantitatively combusting an organic material (particularly a biological sample) to carbon dioxide and water. A quartz combustion tube with appropriate packing has worked for decades on the most refractory samples (polynuclear aromatics, carbon on silica or alumina particles, and metallic carbides). The question of dispersion of the labelled materials in a tissue sample is irrelevant — it all goes to carbon dioxide and water.