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Note

Liquid scintillation counting of ^{32}P-labelled compounds after micro-thin-layer chromatography

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^{32}P is a radioactive isotope that has had extensive applications in biochemistry for many years[1] and, despite some disadvantages, it is still widely used today. In recent years some papers have appeared on liquid scintillation counting (LSC) of this isotope[2–6].

Our laboratory used micro-thin-layer chromatography (TLC), Belenky *et al.* variant[7] of high-performance TLC, to separate phospholipids[8] and polar phosphorus products[9]. In this paper the influence of various factors on the LSC of ^{32}P-labelled compounds separated by micro-TLC is described. It is shown that the LSC procedures may be simplified, thus reducing the cost of counting.

EXPERIMENTAL

PPO, POPOP and reagent-grade and highly purified toluene, benzylamine, ethanolamine (Reachim, Moscow, U.S.S.R.), Triton X-100 (Serva, Heidelberg, G.F.R.), putrescine (Fluka, Buchs, Switzerland) and $Na_2H^{32}PO_4$ (Isotope, Moscow, U.S.S.R.) were used.

The ^{32}P-labelled phospholipid fraction was isolated by the Folch *et al.* extraction procedure[10] and column chromatography from *Saccharomyces cerevisiae*, which had been cultivated with [^{32}P]phosphate[11]. The fraction was vacuum re-evaporated with toluene to remove the remains of chloroform. Working phospholipid solutions prepared in toluene had radioactivity levels of 3000–4000 cpm/μl. Micro-TLC plates were prepared as described previously[8]. The solution was placed in 20-ml standard glass counting vials or spotted on to micro-TLC plates with volumetric micropipettes. The spots were rendered visible with iodine vapour or malachite green reagent[12]. The zones of silica gel were transferred from the TLC plates into the vials with a microspatula.

Radioactivity was measured with an SL-30 liquid scintillation spectrometer (Intertechnique, Plaisir, France). The results of counting were registered in two channels: (1) fully opened window; (2) standard ^{32}P channel of the instrument. The backgrounds of the first and second channels were 40–57 and 20–35 cpm, respectively.

RESULTS AND DISCUSSION

In recent years, much attention has been paid to measurements of the ^{32}P radioactivity in solutions without scintillator by the Cerenkov effect[2,3,5]. As our preliminary results demonstrated, Cerenkov counting of the ^{32}P-labelled phospholipids on silica gel in toluene, the most effective solvent for LSC without scintillator[2], yielded results that were not more than 75% as high as those obtained with PPO solutions. Therefore, we studied in detail ^{32}P counting in mixtures with fluorescent substances. A 0.5% solution of PPO in toluene was taken as the initial counting solution. This solution had been recorded for ^{32}P counting[4] and is often used in modern spectrometry[13,14].

Comparison of counting efficiencies in different volumes of solution

The volume of scintillation solution commonly used for a standard vial is 10–15ml[13,14]. One of the developments in modern LSC is counting in small volumes of fluorescent fluids[5,15–19], and special minivials or plastic bags are used for such volumes. There are indications (for example, ref. 2 and Fig. 4.4 in ref. 13) that small volumes of scintillation solutions in standard vials provided high efficiencies of counting for isotopes of higher radioactivity.

As the decrease in solution volumes and the adsorption of compounds on silica gel causes the shift of the ^{32}P spectrum, we counted the probes in two channels, one of which had a fully opened window.

Table I shows that a decrease in the volume of the scintillation solution from 15 to 0.25 ml reduced the counting efficiency of ^{32}P-labelled phospholipids in toluene solution by less than 1.5% in channel 1 and about 11% in the standard ^{32}P channel. Samples on silica gel gave the same results in channel 1, while the efficiency in the standard channel decreased sharply with diminishing volume.

TABLE I

EFFECT OF VOLUME OF SCINTILLATION SOLUTION ON COUNTING EFFICIENCY

Volume of solution (ml)	*[^{32}P]Phospholipids in toluene solution (cpm ± s.d.)**		*[^{32}P]Phospholipids on silica gel (cpm ± s.d.)**	
	Channel 1	*Channel 2*	*Channel 1*	*Channel 2*
15	18555 ± 284	18060 ± 269	18679 ± 336	16112 ± 396
10	18616 ± 228	18144 ± 227	18836 ± 298	16713 ± 419
5	18594 ± 251	18020 ± 223	18772 ± 237	16226 ± 296
2.0	18655 ± 157	17973 ± 142	18550 ± 270	16000 ± 352
1.0	18529 ± 127	17488 ± 146	18524 ± 112	14881 ± 121
0.7	18475 ± 317	17278 ± 308	18441 ± 237	14421 ± 199
0.5	18461 ± 278	16935 ± 268	18451 ± 252	13983 ± 218
0.2	18345 ± 387	16108 ± 370	18139 ± 358	11877 ± 795

* s.d. = standard deviation ($n = 5$).

All of the ensuing experiments were carried out with 1-ml portions of scintillation fluids.

Effect of amount of silica gel

The introduction of large amounts of silica gel into a vial is known to reduce

the efficiency of isotope counting[2,20,21]. Therefore, we measured the activity of phospholipid probes spotted on plates in zones of three different sizes (4 × 4, 7 × 7 and 10 × 10 mm). The spots of the phospholipid solution covered an area less than the whole zone. The middle zone is usually preferred for lipid analysis by micro-TLC. The following values were obtained for probes counted in channel 1:15,019 ± 179, 15,316 ± 188 and 15,698 ± 165.

The results were unexpected, in that with an increase in the amount of silica gel the efficiency of counting was increased. This effect may be due to inadequate binding of the various portions of the microsilica gel with equal amounts of phospholipids; as the solution is distributed over a more extensive area, the aggregation decreased, resulting in a reduction in quenching. However, a 2- or 3-fold change in area did change the efficiency of counting by more than 2%.

The error caused by the use of different amounts of silica gel will be decreased if zones of standard dimensions are selected for counting.

Effect of composition of scintillation mixtures

Although the PPO solution in toluene, the simplest scintillation mixture, yielded good results, we checked the effect of the addition of POPOP to the mixture and tested the scintillation liquids in the presence of Triton X-100. Sometimes POPOP is added to scintillation solutions used in modern spectrometers for heterogeneous counting. Counting mixtures with Triton X-100 are widely utilized[14,22], including also the counting of radioactivity in the presence of silic agel[23,24].

Table II demonstrates that polar mixture D, containing water, produces a higher result in the ^{32}P channel. This may explain the elution of phospholipids from silica gel by this scintillation cocktail. The low efficiency with mixture C is probably due to its higher viscosity, which contributes to formation of large aggregates of silica gel.

TABLE II

EFFECT OF COMPOSITION OF SCINTILLATION MIXTURE ON COUNTING EFFICIENCY

Scintillation mixture	*Channel 1 (cpm ± s.d.)**	*Channel 2 (cpm ± s.d.)**
A (0.5% PPO in toluene)	16524 ± 153	13276 ± 271
B (0.5% PPO + 0.025% POPOP in toluene)	16553 ± 636	13033 ± 562
C [0.5% PPO in toluene–Triton X-100 (2:1, v/v)]	15718 ± 168	12176 ± 220
D [0.5% PPO in toluene–Triton X-100–water (2:1:0.2, v/v)]	16775 ± 352	15357 ± 566

* s.d. = standard deviation (n = 5).

The results obtained in channel 1, when using mixtures A, B and D, are almost adequate, so in our further investigations we used the simple and cheaper mixture A.

Effect of PPO concentration and quality of toluene

Usually, 4–6 g of PPO were dissolved per litre of scintillation solution[13,14]. We found that when the PPO concentration was decreased from 0.5% to 0.1%, the efficiency remained virtually unchanged. The conclusions drawn from our experiments

also confirmed (ref. 13, p. 68) that reagent-grade toluene has the same effects as specially purified toluene in scintillation counting. In this respect, the counting of probes with ^{32}P may be less expensive when using 1 ml of 0.1% PPO solution in reagent-grade toluene.

^{32}P counting after spot detection with malachite green reagent

The detection of phospholipids on thin-layer chromatograms is a simple procedure, as 1 μg of most of them can be readily revealed with iodine vapours, the residue of which does not interfere with ^{32}P counting. The location of polar products of phospholipid metabolism is complicated, however. The amounts of products found in natural mixtures are not revealed with iodine vapours. Autoradiography, used for detection of labelled phosphates, requires prolonged exposures of hours and even days[25,26]. Processing of photomaterials following the exposure makes the detection both complicated and prolonged.

Recently, we suggested a relatively simple and very sensitive method that permits the detection of organic phosphates separated by micro-TLC[12]. As the phosphates must not be lost during the procedure, we tried to verify its possible utilization for ^{32}P counting.

As shown in Table III, the treatment of micro-TLC plates with perchloric acid and malachite green induced a noticeable decrease in the ^{32}P count. The scintillation mixture and silica gel, including the remains of the dye, were coloured green-blue. As the most severe quenching was due to the red colour of the scintillation solution[13], this decrease may be accounted for by the property of strong acids to cause chemical quenching of ^{32}P counting[6]. Amines, which are able to decolorize the probes, were added to the scintillation mixture to neutralize the acids. Benzylamine and ethanolamine are widely used in scintillation counting to trap $^{14}CO_2$ (refs. 13 and 14). However, benzylamine did not have the appropriate effect here. Ethanolamine, although it elevated the counting efficiency, is poorly soluble in toluene. Therefore, we used a more soluble aliphatic amine, putrescine. A 0.2% concentration was optimal, as even 0.5–1.0 ml of this mixture decolorized the probe and the efficiency of counting was noticeably higher than in the absence of the amine. When the concentration of putrescine was increased to 0.5%, the counting decreased in comparison with that with a 0.2% solution. The addition of amines does not increase the counting efficiency to values obtained by measuring the activity of the initial phospholipids before combustion, but amines favour more efficient and

TABLE III

EFFECT OF PROCEDURE FOR SPOT DETECTION AND COMPOSITION OF SCINTILLATION MIXTURE ON COUNTING EFFICIENCY

*Procedure for spot detection**	*Scintillation mixture*	*Channel 1 (cpm ± s.d.)***	*Channel 2 (cpm ± s.d.)***
a	0.5% PPO in toluene	23744 ± 1317	19614 ± 1469
b	0.5% PPO in toluene	19703 ± 911	14627 ± 730
b	0.5% PPO and 0.1% ethanolamine in toluene	20917 ± 462	16857 ± 459
b	0.5% PPO and 0.2% putrescine in toluene	21244 ± 691	17280 ± 816

* a, Iodine vapour; b, malachite green reagent.

** s.d. = standard deviation (n = 5).

stable counting. To obtain reproducible results, one should standardize the drying of chromotograms after treatment with the reagent, as water in the silica gel reduces the efficiency of counting.

CONCLUSION

The results obtained are not considered to be sufficiently satisfactory for the procedures to be recommended in all applications of ^{32}P counting. In order to avoid a decrease in the efficiency of counting, one should be accurate in using the minimal amount of scintillation mixture suitable for counting in standard vials, as "liquid scintillation counters even of the same design may differ greatly, each instrument having its own personal characteristics" (ref. 13, p. viii).

However, the results indicate that even a small number of preliminary experiments enable one to apply less expensive and more convenient methods of LSC than the standard procedures used in many laboratories.

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