

Chapter 16

Liquid Scintillation Counting of Biological Macromolecules: Extraction from Aqueous Solution and from Glass Fibre Filters

P. N. Paus

*Department of Microbiology, University of Oslo,
Blindern, Oslo 3, Norway*

INTRODUCTION

Modern liquid scintillation spectrometers enable one to measure simultaneously, with accuracy and great sensitivity, the presence of different isotopes, provided that samples have been prepared correctly. The difference between non-polar isotope standards and polar biological macromolecules introduces special difficulties. The measurement of the radioactivity in these macromolecules has been performed in several ways, most of which have disadvantages.

With filter precipitation techniques energy absorption is a great danger, and makes accurate double labelling work impossible as the weak β -emitters are affected more than the strong ones,¹ and the quench spectra are different from those obtained in solution. Combustion techniques have so far been too time-consuming to attain any great importance. Direct counting of aqueous solutions may necessitate the use of scintillation liquids with great water capacity. These have lower counting efficiencies than the pure toluene scintillation liquids, and most of them are quite expensive. When molecules with polar groups are counted in aqueous solution, the possibility of unstable count rates must be considered.²

Counting in aqueous solution

Figure 1 shows the decline in count rate with time obtained when ¹⁴C-labelled RNA, dissolved in 50 μ l buffer, was counted in 15 ml toluene–2-methoxyethanol (85:15 v/v) scintillation liquid in a spectrometer with discriminators set for counting of tritium and carbon-14 according to the simultaneous equation method.³ Similar results were obtained when proteins (cold TCA-precipitate from monkey serum) were counted, although the reduction in count rate was somewhat slower. The reduction was even greater when ³H-labelled RNA was counted, the final count rate being some 40% of the initial. After 5 to 36 h the rates became stable. Manual shaking of the vial restored the initial count rates, followed by a second decline. The more hydrophobic the scintillation liquid, the faster and more pronounced was the drop. External and internal count rates were constant, and no precipitation, phase separation or colour alteration could be detected by the naked eye. As the scintillation liquid was unaffected during the observation period, the reason

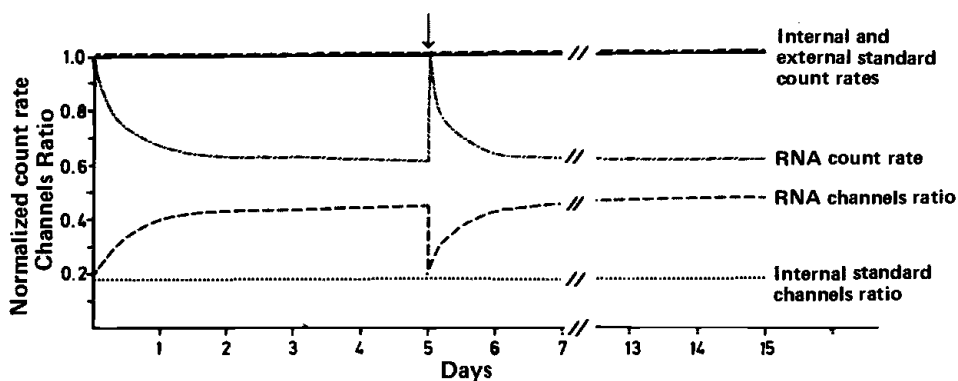


Fig. 1. Count rates and channels ratios as functions of time. $50 \mu\text{g}$ ^{14}C -labelled RNA in $50 \mu\text{l}$ 0.1 M sodium acetate, pH 6, dissolved in 15 ml toluene-2-methoxyethanol (85:15 v/v) scintillation liquid. Channels ratio: c.p.m. tritium channel/c.p.m. carbon-14 channel. Arrow indicates manual shaking.

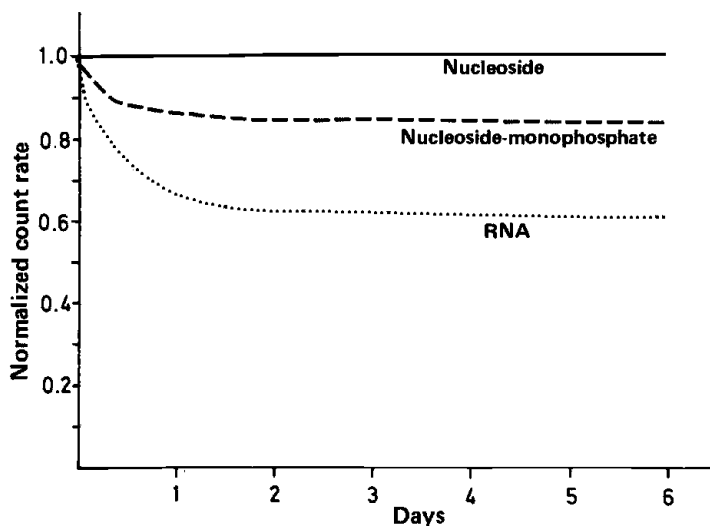


Fig. 2. Count rates of RNA, AMP and thymidine as functions of time. Each substance was dissolved in $50 \mu\text{l}$ 0.1 M sodium acetate, pH 6, before addition of 15 ml scintillation liquid.

for the instability had to be sought in the radioactive molecules themselves (see Fig. 2). A nucleoside-monophosphate (AMP) showed a less pronounced fall in count rate than RNA, and a nucleoside (thymidine) had a constant count rate.

The polar groups thus seem to be important for the instability of the count rates. With increasing amounts of water/ μg RNA, the drop became faster and more pronounced.

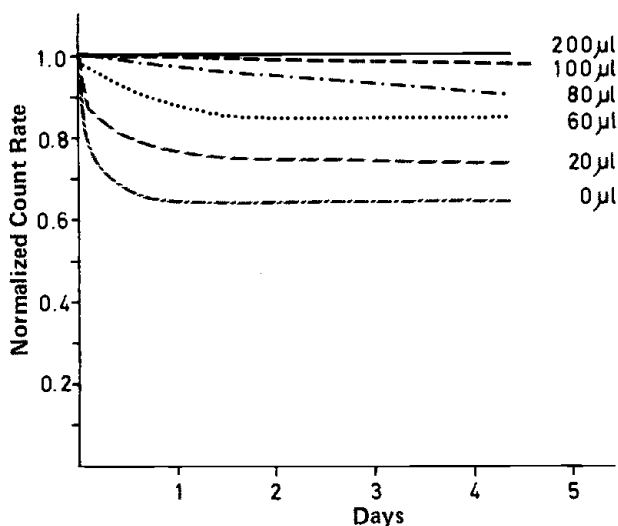


Fig. 5. Stabilization of count rates by addition of hyamine. 50 μ g RNA was dissolved in 50 μ l 0.1 M sodium acetate buffer, pH 6, and hyamine added in the indicated amounts before addition of scintillation liquid.

groups probably becoming associated with the acid groups of the nucleic acids, forming a hydrophobic coat around the molecules. However, stabilization depended upon the scintillation liquid and the buffer used. Some detergents, such as Triton X-100, tended to break the bonds and some buffer ions blocked the dissociable groups of the macromolecules or of hyamine.

Based on these experiments, the following methods were elaborated. Originally designed for counting of nucleic acids in aqueous solution,⁴ they probably have a wider field of application.

METHODS

Extraction from aqueous solution

By shaking the aqueous solution containing the radioactive molecules with a mixture of N.C.S. or Soluene-100 in n-pentanol, 1:4 v/v, or in n-hexanol, 4:6 v/v, the quaternary ammonium bases will form low quenching hydrophobic complexes with the molecules having sufficient dissociable acid groups. Upon phase separation, these complexes will be found in the organic, upper phase, which can easily be pipetted off and counted in a pure toluene scintillation liquid.

Using 1 ml extraction liquid, 1 ml of the Soluene-n-pentanol and 0.95 to 0.97 ml of the N.C.S.-n-pentanol mixtures could be recovered when 2 ml 0.04 M acetate buffer was extracted. N.C.S. count rates were higher than expected, compensating for the slightly reduced volume (see Table 1). Hyamine, being dissolved in methanol, did not give full phase separation. Increasing the buffer volume reduced the organic phase volume after phase separation. With 10 ml buffer, only about 0.7 ml Soluene-n-pentanol was recovered. Evidently, with 2 ml buffer, the volume of extraction liquid dissolved in the aqueous phase exactly balanced the amount of buffer dissolved in the organic phase. RNA was quantita-

Table 1. RNA extraction and counting with different complexing agents in pentanol (1 : 4 v/v).^a

Extraction liquid	Extracted from	No. of parallels	Observed count rates in per cent of calculated (\pm SD)	
			After 1 h	After 14 days
Soluene/pentanol	0.04 M Na acetate pH 6.0	5	96 \pm 3%	94 \pm 4%
Soluene/pentanol	0.1 M Na acetate pH 6.0	5	101 \pm 2%	98 \pm 3%
N.C.S./pentanol	0.04 M Na acetate pH 6.0	5	91 \pm 9%	88 \pm 9%
N.C.S./pentanol	0.1 M Na acetate pH 6.0	5	105 \pm 1%	102 \pm 1%
Hyamine/pentanol	0.04 M Na acetate pH 6.0	5	80 \pm 1%	65 \pm 9%
Tetraethylammonium hydroxide/pentanol	0.04 M Na acetate pH 6.0	3	0	0
Diethyldihexylamine/pentanol	0.04 M Na acetate pH 6.0	3	0	0
Diethyldihexylamine/pentanol	0.1 M Na acetate pH 6.0	3	0	0

^a A toluene-2-methoxyethanol (85 : 15 v/v) scintillation liquid was used.

Table 2. Effect of Soluene in different solvents (1:4 v/v).^a

Extraction liquid	No. of parallels	Observed count rates in per cent of calculated (\pm SD)	
		After 1 h	After 14 days
Soluene/pentanol	5	101 \pm 2%	98 \pm 3%
Soluene/hexanol	5	96 \pm 1%	96 \pm 1%
Soluene/heptanol	3	7 \pm 1%	—
Soluene/benzyl alcohol	3	11 \pm 3%	—
Soluene/cyclohexane	3	12 \pm 1.5%	—
Soluene/heptane	3	16 \pm 1.5%	—
Soluene/benzene	3	0	—
Soluene/toluene	3	0	—

^a RNA was extracted from 0.1 M sodium acetate pH 6.0 and counted in a toluene-2-methoxyethanol (85:15 v/v) scintillation liquid.

tively extracted from buffer volumes up to 7 to 8 ml, rising count rates compensating for the decreasing volume of the organic phase. Soluene-n-hexanol permitted nearly complete phase separation with complete extraction of the same volumes. The other solvents tried were too hydrophobic to allow quantitative extraction (see Table 2).

The ratio of the quaternary ammonium base to the aliphatic alcohol was adjusted according to the ionic strength of the solution. A high ratio permitted extraction from solutions of high ionic strength. When the ratio was too high, however, phase separation did not take place. RNA was quantitatively extracted from solutions with ionic strengths between 0.02 and 0.12 by a 20% v/v solution of Soluene in pentanol, and a 80% v/v solution extracted RNA quantitatively from solutions of ionic strengths as high as 0.6.

Complete extraction of RNA was obtained over the entire pH range tested (3.25 to 9.5), as expected from the dissociation constants of RNA and Soluene.

Ions that blocked the complexing groups depressed the extraction (e.g. RNA extraction was depressed by magnesium, and a high chloride content unbalanced by strong cations depressed extraction generally). Sucrose, EDTA and SDS in the usual concentrations did not affect the extraction beyond their effect on the ionic strength of the solution.

To avoid photo- and chemiluminescence, sufficient acid should be added to the scintillation liquid to give a final acidic pH in the counting vial, causing the spurious counts to disappear during the time needed for sample temperature equilibration in the spectrometer. Pure toluene scintillation liquids became milky white on addition of the extraction liquid. This cleared in a few hours as water collected in small droplets at the bottom of the vial. Tritium count rates rose from 98% to 100% of the expected rate during this time. Carbon-14 count rates were unaffected. Scintillation liquids containing Triton X-100 gave unstable count rates.

Some buffer ions interfered with the complexes, causing unstable count rates. In these cases, extremely hydrophobic or hydrophilic scintillation liquids should be used. In the former, the buffer is not incorporated into the scintillation liquid. In the latter, the thermodynamic equilibrium favours stability. We find that 0.8 to 0.9 ml can routinely be pipetted off with a constriction pipette from 1 ml upper phase. When 0.9 ml was pipetted

off, 53% tritium efficiency and 93% carbon-14 efficiency were obtained by balance point counting in a closed channel system. Appropriate corrections should be made for the size of the aliquot. The efficiencies of the pure scintillation liquid were 55% and 94% respectively.

The method is fast. A hundred fractions may be extracted and transferred to the scintillation vials in 1 to 1½ h, excluding the time needed for phase separation.

Beside nucleic acids, quantitative extractions of monkey salivary α -amylase (pI about 6.0 and 8.0), of cytochrome-C (pI 10.6), of a crude total TCA precipitate from serum in monkeys labelled with radioactive amino acids, and of soluble HeLa cell protein were obtained from 0.05 M sodium chloride in 0.01 M sodium acetate buffer pH 6. Smaller molecules, like adenosine monophosphate and orotic acid, were only partially extracted.

Obviously, the method outlined has its limitations. The need for unblocked complexing groups is absolute. Conditions found for one kind of biological macromolecule may therefore be invalid for another. Consequently, in every case, the recovery of isotope should be checked under the actual conditions used. On the other hand, extraction is insensitive to volume changes over quite a wide range. Dilution with water or salt solution may extend the limits for complete extraction. In the case of complete extraction, the method provides high counting efficiencies and the possibility of performing multiple labelling experiments.

Extraction from glass fibre filters

When complete extraction is not possible, precipitation with collection of the precipitates on glass fibre discs may be preferable. Precipitated macromolecules with sufficient acid groups may then be brought into solution by quaternary ammonium bases. The procedure should not be used with nitro-cellulose filters, as these give a severely quenching yellow colour upon incubation.

Routinely, the dried filters are incubated in closed scintillation vials with 1 ml 20% v/v Soluene in toluene at 50°C. Then 15 ml pure toluene scintillation liquid is added, the vial shaken until the filter floats freely, and counted as soon as sample temperature equilibrium is obtained in the spectrometer. To avoid photo- and chemiluminescence enough concentrated acetic acid is added to the scintillation liquid to acidify the samples.

Using this method, 98 to 99% of rat liver total RNA and DNA was brought into solution after incubation for 1 to 2 h. Elution was checked by removal of the filters from the vials after counting, and recounting of the vials and filters separately. 3 to 5 h were needed for an equal elution of soluble HeLa cell protein (labelled with radioactive amino acids for 48 h). Recently, a similar method has been published by Birnboim⁵ using 25% v/v N.C.S. in toluene for counting ³H-labelled RNA.

Increase of the Soluene content beyond 20% reduced the counting efficiency, as Soluene had a marked quenching effect compared with toluene. Less than 20% Soluene reduced the elution. Increase in incubation time of up to 24 h did not increase the count rates. The glass fibre paper disintegrated when incubated in 50% Soluene for more than 12 h.

High counting efficiencies with stable count rates were obtained. Direct balance point counting gave 52% tritium efficiency and 93% carbon-14 efficiency, while the eluted molecules were counted with 53% tritium efficiency and 93% carbon-14 efficiency as judged by the channels ratio method.

If quantitative direct extraction is possible, it is probably the method of choice, because it is faster, and because errors due to incomplete precipitation or absorption to

the filter during filtration are avoided. With both methods, however, a complete account of disintegrations should be made. Even a slightly reduced recovery conveys the danger of selection because of different polarity of the molecules.

CONCLUSIONS

Molecules with polar groups may show unstable count rates when counted in aqueous solution. RNA count rates may be stabilized by addition of hyamine, presumably because of the formation of RNA—quaternary ammonium base complexes.

RNA, DNA and many proteins in aqueous solution may be extracted into an organic phase, suitable for liquid scintillation counting, by shaking with a mixture of Soluene-100 or N.C.S. in n-pentanol or n-hexanol.

RNA, DNA and many proteins may be eluted from glass fibre filters by incubation with quaternary ammonium base solubilizers.

The two latter methods abolish energy absorption by filters or precipitates, give high counting efficiencies, and allow double labelling experiments to be performed with greater accuracy.

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DISCUSSION

B. W. Fox: Have you tried separating double and single stranded DNA by this procedure?

P. N. Paus: No. There might be differences in extraction under conditions when extraction is not complete (*cf.* RNA).

B. W. Fox: Have you noticed any differential extraction of RNA species since the use of quaternary bases is well known in counter current separation procedures?

P. N. Paus: Differential extraction has only been obtained with magnesium present in the aqueous phase. Ribosomal RNA extraction was then more depressed than rapidly labelled RNA.

B. W. Fox: Comment: Lithium is normally used in the aqueous phase for RNA separation; it would be interesting to try this ion in your system.

H. E. Dobbs: Dr. Paus has described a method, using liquid reagents, for preparing biological macromolecules for liquid scintillation counting. When selecting the papers for this session, we included a paper in which the preparation of biological samples for liquid scintillation counting by combustion techniques was reviewed. Unfortunately that paper was withdrawn. Suitable combustion techniques can be applied to all biological samples which are not easily soluble in liquid chemical reagents.