A LIQUID SCINTILLATION METHOD FOR MEASUREMENT OF RADIOACTIVITY IN ANIMAL TISSUE AND TISSUE FRACTIONS

D. S. KINNORY, E. L. KANABROCKI, J. GRECO, R. L. VEATCH, E. KAPLAN and Y. T. OESTER

Radioisotope Service, V.A. Hospital, Hines, Illinois and Loyola University, Chicago, Illinois

THE high counting efficiency of beta-emitting isotopes and the relatively low loss of activity through self-absorption render the internal liquid scintillation technique useful for the counting of relatively large samples with even low levels of activity. This consideration makes it valuable for many metabolic investigations, particularly on humans. As compared with the Geiger–Mueller counter, any one or combination of the following desired advantages can be obtained: the amount of the radioactive material administered may be reduced with consequent decreased exposure of the subject to radiation or lessened cost of material; low-activity samples can be counted with increased precision; or the counting time can be reduced with no loss in accuracy.

One of the current limitations hindering the more extensive utilization of internal liquid scintillation counting is the problem of availability of solvent systems which, near freezing temperatures, would dissolve both the material to be counted and the scintillation phosphor, without having an appreciable quenching effect.

This paper describes a solvent system, developed in response to our research needs, which is suitable for the liquid scintillation measurement of radioactivity in tissue homogenates, lipids, proteins, pentose and deoxypentose nucleic acids isolated from animals following administration of C^{14}-labeled precursor. The procedure makes use of the observation by LAHR, *et al.*[1] and by PEARCE, *et al.*[2] that formamide can be used as solvent for tissues. We have also investigated the use of the hyamine solvent described by PASSMAN, *et al.*[3] for the liquid scintillation counting of $C^{14}O_2$ and compared it with the formamide–ethanol–toluene solvent.

METHOD

In the particular investigation for which the liquid scintillation method described below was developed, a means for fractionating tissue into various components was employed.

(1) *Procedure for isolating tissue components*

The isolation procedure, (Fig. 1), for tissue nucleic acids, proteins, and lipids was accomplished by a modification of the procedure of DAVIDSON and SMELLIE[4] and of TYNER *et al.*[5] The experimental animals were injected with formate-C^{14} and after a time-interval the livers or small intestines were pooled, minced and homogenized in ice-cold 0.9% NaCl.

The acid-soluble compounds, among the inorganic phosphorus, phosphorylated monosaccharides and nucleotides, were removed by repeated extraction with ice-cold 10% trichloroacetic acid, which precipitates proteins, lipids and nucleic acids.

The lipids were next extracted with 3:1 ethanol–ether solution and the residue boiled with 10% NaCl at neutral or slightly alkaline pH to dissociate the nucleic acids from the proteins, which are denatured. The sodium nucleates go into solution and are precipitated with 3 volumes of cold 95% ethanol.

Separation of PNA from DNA is achieved by taking advantage of the ease of alkaline hydrolysis of PNA to its mononucleotides and the resistance of DNA to basic solution. The DNA is then separated from the PNA mononucleotides by acid precipitation, and redissolved in alkali.

(2) *Internal standardization*

A correction factor to compensate for quenching and for the effect of color, in the case of some tissue fractions, was obtained by dividing the radioactivity measurements of the vial containing only the C^{14}-labeled stearic acid standard by the difference between the count in the vial containing the C^{14}-labeled stearic acid plus the tissue fraction and the count of the tissue fraction alone.

Correction factor = Stearic acid count/(stearic acid + tissue fraction count) − (tissue fraction count).

This correction factor expression compares the C^{14}-stearic acid standard count in the pure solvent system used with that containing the tissue fraction, and compensates for any characteristics due to the presence of the tissue fraction, such as color production.

(3) *Procedure for preparing specimens for liquid scintillation counting*

(*a*) *Tissue homogenate extracts*—Two ml aliquots of the various tissue extracts were placed into each of the two 25 ml vials and evaporated by means of forced air and gentle heating, (Fig. 2). One hundred λ of C^{14}-labeled stearic acid dissolved in toluene and having about 30,000 counts/min were then added to one of the dried vials and to an additional empty vial. Into all three vials, 1.0 ml of formamide was introduced and the dried residue dissolved by swirling. This was followed by the addition of 8.6 ml of absolute ethanol and 10.4 ml of 0.58% of the phosphor diphenyloxazole dissolved in

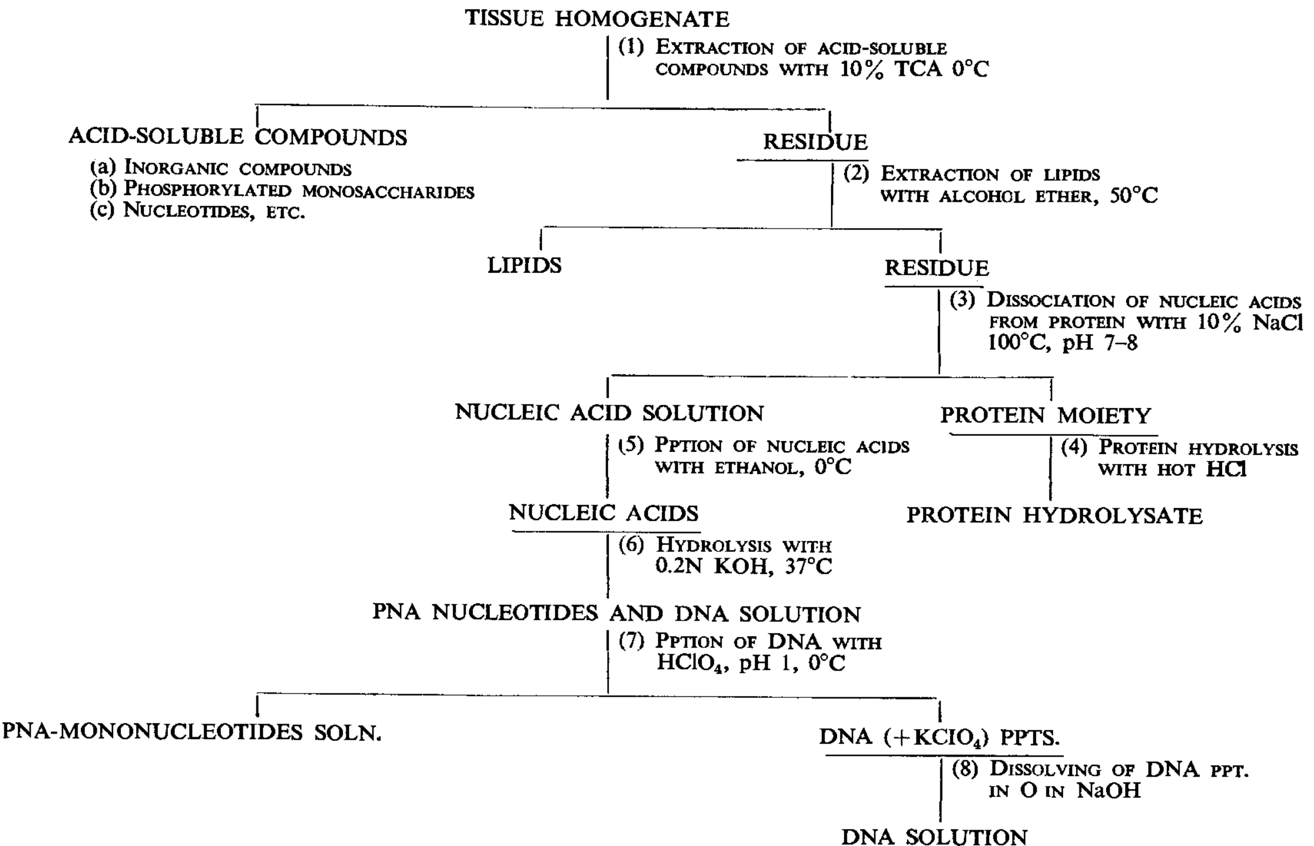

Fig. 1. Flowsheet for the isolation and quantitative determination of lipids, proteins, PNA, and DNA from tissue homogenate.

toluene. Each vial was closed with a tin-foil lined cap and mixed by inversion. A blank for background counting contained only the solvent system composed fo 1.0 ml formamide, 8.6 ml of absolute ethanol and 10.4 ml of the diphenyloxazole phosphor solution. The vials were kept in the Tri-Carb refrigerator overnight, before counting. Since a slowly-settling, fine, flocculent precipitate may form, the contents of each vial should be mixed by inversion before counting. Repeated checks have shown that this precipitate, which may form, does not interfere with the reproducibility of the results. The Tri-Carb liquid scintillation counter was used at high-voltage tap 8 with a window opening of 10–100.

(*b*) *Tissue homogenate*—Two to five grams of whole tissue homogenate were placed into a 200 ml round-bottom flask containing 75 ml of formamide. With an electrically heated jacket, the mixture was refluxed for 2 hr at 145°C using a water-cooled condenser. This was cooled and transferred to a 100 ml volumetric flask. The round-bottom flask was rinsed with three 5 ml portions and then one 10 ml portion of formamide. Each time the refluxed solution was transferred and finally made up to the 100 ml mark with formamide. At first the solution was clear and slightly brownish in color. On standing, some fine floccuous matter settled to the bottom. It was well dispersed by shaking before an aliquot was taken out for counting. To 1.0 ml of this formamide tissue system 8.6 ml of ethanol and 10.4 ml of toluene containing PPO were added.

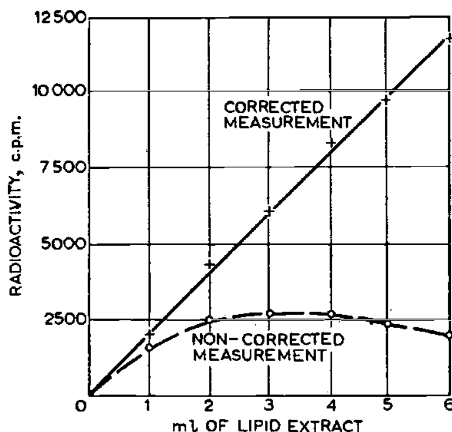

Fig. 3. Relation of corrected to non-corrected values in counts/min of lipid fraction using the C^{14}-stearic acid internal standard method.

Since some $C^{14}O_2$ may be liberated from certain tissue during the reflux described above, it is necessary to set up a hyamine-containing trap with each new tissue homogenate under study. One 25 ml hyamine aliquot is sufficient for such a purpose. $C^{14}O_2$ recovered in that manner accounted for less than 0.1 % of the total radioactivity in the case of small intestine and the thymus

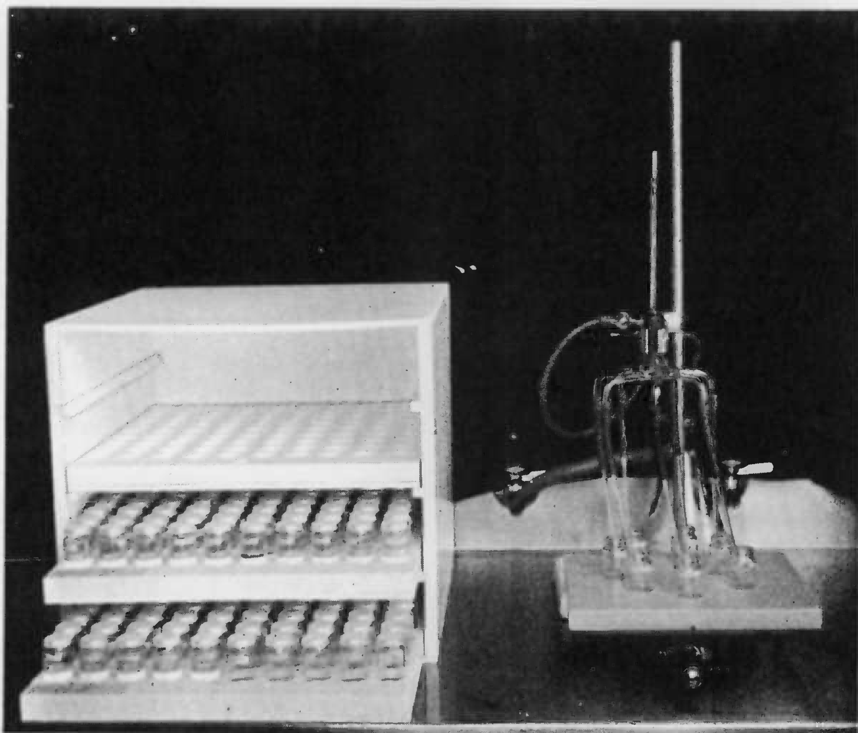

Fig. 2. Set-up for evaporating and storing tissue extract specimens.

gland. Considerably greater quantity of C^{14} material was trapped in hyamine during dissolving of liver homogenate.

RESULTS

The color and quenching ability of the specimens counted made obligatory the use of the internal standard technique described.

The non-corrected measurement can be so strongly affected as to show no increase in radioactivity counts for larger quantities of the tissue fraction. In contrast, the use of the correction factor brings about a satisfactory proportionality relationship between radioactivity counts and the amount of the tissue used. The true count of the tissue fraction can be satisfactorily obtained by multiplying the observed tissue fraction count by the correction factor.

TABLE 1

Balance Study of Formate-C^{14} Incorporation into Various Tissue Fractions and Whole Homogenate

Tissue homogenate fractions	Liver		Small intestine	
	counts/min	Average counts/min	counts/min	Average counts/min
TCA-soluble substances	28,812 28,523	28,667	70,153 67,815	68,984
Protein hydrolysate	100,627 96,873	98,750	238,333 239,612	238,972
Lipids	60,704 62,607	61,655	47,856 46,384	47,120
PNA	7810 8121	7965	80,518 77,100	78,809
DNA	2789 2967	2878	30,231 29,986	30,109
Total		199,915		463,994
Total tissue homogenate	197,779 202,258	200,019	458,355 460,548	459,451

An application of the use of the described solvent and correction factor calculation is illustrated in the balance study shown in Table 1. The sum totals of the radioactivities of all fractions of the liver aliquot counted is about 200,000 counts/min and agrees well with the total count of the whole tissue

homogenate dissolved directly in the solvent system used. An equally satisfactory agreement was obtained in the case of the small intestine, when the sum total of the radioactivities in the isolated fractions was compared with the count of the whole tissue homogenate. In both instances, the results were close to 460,000 counts/min for the aliquot measured.

TABLE 2

Comparison of Counting Efficiencies of Various Formamide and Hyamime Concentrations in Solvent

Formamide			Hyamine		
Grams	% Efficiency	H.V. tap 10–100	Grams	% Efficiency	H.V. tap 10–100
0.12	59.1	7	0.12	59.0	7
0.25	56.4	7	0.25	54.7	7
0.30	55.4	7	0.30	51.8	8
0.40	53.8	7	0.40	48.8	8
0.50	51.5	8	0.50	45.6	8
0.75	47.7	8	0.75	36.9	9
1.00	38.5	9	1.00	29.8	9

Final volume of above preparations: Formamide 10.0 ml and Hyamine 5.25 ml. All solutions contained 0.3% PPO.

Formamide dissolved the various *tissue extracts* with a greater ease than hyamine solvent. This was shown by the measurement of radioactivity in samples dissolved in formamide and in hyamine. Measurements in formamide could be duplicated within 3% from day to day. Measurements of similar aliquots dissolved in hyamine increased daily in magnitude until they reached a constant after several days. This constant agreed well with the readings obtained with the formamide.

SUMMARY

(1) A method has been developed for the estimation of radioactivity by liquid scintillation counting of tissue homogenates, lipids, proteins, pentose and deoxypentose nucleic acids. These fractions were isolated from animals following administration of C^{14}-labeled precursor. The tissue homogenates and the isolated tissue fractions were all dissolved in formamide and counted in the toluene–ethanol medium containing diphenyloxazole as phosphor. Hyamine can be used as solvent for the above tissue fractions in place of formamide.

(2) An internal standard technique is described.

(3) Combined with the existing methods for tissue fractionation, the described procedure should be applicable to the determination of incorporation of C^{14}, P^{32} and S^{35}-labeled precursors into a variety of cell components.

REFERENCES

[1] T. N. LAHR, R. OLSEN, G. I. GLEASON and D. L. TABERN. *J. Lab. Clin. Med.* **45**, 66, (1955).
[2] E. M. PEARCE, F. DEVENUTO, W. M. FITCH, H. E. FIRSCHEN and U. WESTPHAL. *Anal. Chem.* **28.** 1762 (1956).
[3] J. M. PASSMANN, N. S. RADIN and J. A. D. COOPER. *Anal. Chem.* **28**, 484 (1956).
[4] J. N. DAVIDSON and R. M. S. SMELLIE. *Biochem. J.* **52,** 599 (1952).
[5] E. P. TYNER, C. HEIDELBERGER and G. A. LEPAGE. *Cancer Res.* **13**, 186 (1953).