

A DIRECT TECHNIQUE FOR COUNTING ^{14}C AND ^3H IN TISSUES

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ABSTRACT - The technique to be described uses liquid scintillation counting to measure ^{14}C and ^3H present in tissues without the necessity of destroying the tissue by digestion or combustion. It is based on the property of cellular membranes to be permeated with liquid scintillators, and the extraction of certain cellular components. The use of the enzyme pronase makes it possible to measure the total activity in each sample. By using liquid scintillation which may or may not contain dioxane, it is possible to easily differentiate the fraction of radioactivity within the water soluble and lipid-soluble components. Preliminary calibration curves and the study of pulse height spectra make it possible to define in each case the best conditions for measurement. The calibration curves are reproducible and it is not necessary to estimate the counting efficiency in each case. This rapid technique gives results with high efficiency.

INTRODUCTION - The technique of direct counting, which we have perfected is based on the results obtained by one of us (1) in 1961 and which showed that: A) Microbial cells can be permeated with liquid scintillators; B) The radioactivity (^3H or ^{14}C) contained in micro-organisms can be correctly measured without the necessity of destroying them. When demonstrating that it was possible, without any preliminary treatment, to measure the radioactivity of body fluids (urine, whole blood, plasma), we noticed that liquid scintillators extracted various compounds from biological media (2). We then attempted to extend this direct technique to tissues.

MATERIALS

- I - Apparatus: a) For radioactivity measurements : automatic liquid scintillation spectrometer from Nuclear Enterprises (G.B.) with a single photomultiplier; b) ultrasonic generator from Ultrasons (Annemasse, France).
- II - Liquid scintillators: NE 220 (Nuclear Enterprises), containing dioxane, which can take up 10% water.
- III - Glass fibre papers: Whatman GF/A (referred to as fibre papers in the text).
- IV - Standard solutions of ^3H or ^{14}C
- V - Labelled experiments: Male or female mice of various strains fed with various labelled compounds; tritiated water; thymidine labelled with ^3H or ^{14}C ; pregnenolone labelled with ^3H or ^{14}C .
- VI - Enzyme: Pronase (Calbiochem activity 45,000 Proteolytic units/g) as a freshly prepared 1% solution in buffer TRIS-HCl 5.10^{-3}M , EDTA 2.10^{-2}M , pH 7.2.
- VII - Hyamine Hydroxide 10-X (Packard, USA)

DEVELOPMENT OF THE TECHNIQUE

At the time of dissection of the control or labelled mice, the samples were laid on fibre paper put into a stoppered vial and weighed. The radioactivity was then counted by the technique to be described or by a classical one (solubilization or combustion). We have worked only with a few organs: liver, mammary gland, muscle, brain, fat, ovaries, and adrenal gland.

I - Principle of the technique

The first attempts showed it was possible to measure the total radioactivity of some samples, by simply immersing them into the liquid scintillator NE 220. In these cases, the weighed samples were immersed in the liquid scintillator with their fibre paper (to avoid losses). When the whole radioactivity was measured in the untreated sample, we observed that the radioactivity was extracted by NE 220. Animal cells, similiary to bacterial ones, are therefore permeated with liquid scintillator.

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We assumed that the whole radioactivity might be measured in all cases if, by modifying the tissue and cell structures, the energy transfer became possible between the radioisotopes and the liquid scintillator. This assumption was proved correct by experiments in which the samples were treated with the enzyme, pronase.

Our first experiments showed that pronase did not act on an intact sample at room temperature. We therefore adopted the following procedure: after weighing, the sample and its fibre paper were laid on another fibre paper, covered with two others and crushed by rolling with the base of the cylinder and then with a glass stirrer. The four fibre papers surrounding the sample absorbed its fluids and the whole "packet", on which 0.15 ml of pronase solution was deposited was put into a vial. The vial was carefully closed and then left in an incubator at 37°C for three hr for liver, muscle, brain and fat or seven hr for other tissues or organs studied. The samples were then treated by ultrasound for 15 mins at room temperature before being immersed in the liquid scintillator. Working with tritiated water, we checked that losses were negligible, provided the vials were air-tight.

The samples with and without pronase treatment * were then measured. In order to determine in each case the counting efficiency, calibration experiments were carried out.

II - Calibrations

20 μ l of standard solutions (^{14}C or ^3H) were put into vials containing the liquid scintillator. After counting the activity, we added the samples of organs taken from non-treated mice: these were of various weights and with or without pronase treatment. Preliminary tests enabled us to choose the optimum volume of NE 220 in each case. One ml was used for samples counted intact, and 3 ml for those treated with pronase.

*) experiments showed that, when the total radioactivity could not be measured on intact samples the action of pronase was indispensable; in fact, the measurement of samples only crushed, never gave the total activity (3).

The study of the pulse height spectra of NE 220 containing organ pieces either labelled in-vivo, or simply immersed in the presence of ^3H or ^{14}C , showed that calibrations had to be made under the conditions we adopted. The simple presence of organs alters the pulse height spectra; this alteration depends only slightly on the nature and weight of the organ and has the same order of magnitude with all samples, whether labelled in-vivo or measured in the presence of radioactivity. The spectra are displaced towards low energies, principally in the case of ^{14}C . Similar results had been obtained with whole blood (2). This led us to measure ^{14}C in the presence of organs with the P setting previously established for the counting of ^{14}C in whole blood; this setting corresponds to the same gain and threshold which was used for ^3H , but without an upper discriminator, for high energy pulses remain in such ^{14}C spectra. As observed in the case of whole blood, the modifications of spectra in the presence of organs remain unchanged when the latter are removed from the liquid scintillator (Fig.1). Therefore, the modifications in spectra are due to organic substances extracted by the liquid scintillator. The efficiencies of measurement with this calibration technique should give satisfactory results in the case of organs labelled in-vivo.

It was experimentally shown that, when a piece of organ, is immersed into NE 220 in the presence of ^3H or ^{14}C , the counted activity decreases with time. It reaches a steady value in a few hours and then remains stable for several days. This drop of activity depends on the nature and weight of the organ and on the added isotope. We think that the decrease of the counted activity is associated with the extraction of various compounds by NE 220 which modify the pulse height spectra. Therefore, the activity measurement must be made at equilibrium, that is 3 to 4 hr after putting the sample into the liquid scintillation solution.

On the contrary, when the sample is treated with pronase, the counted activity most often increases before reaching a constant value 4 to 6 hr later. It was shown experimentally that pronase diminishes the transparency of fibre paper to photons; it recovers slowly, hence, the increase in the counted activity, as time passes. If the sample

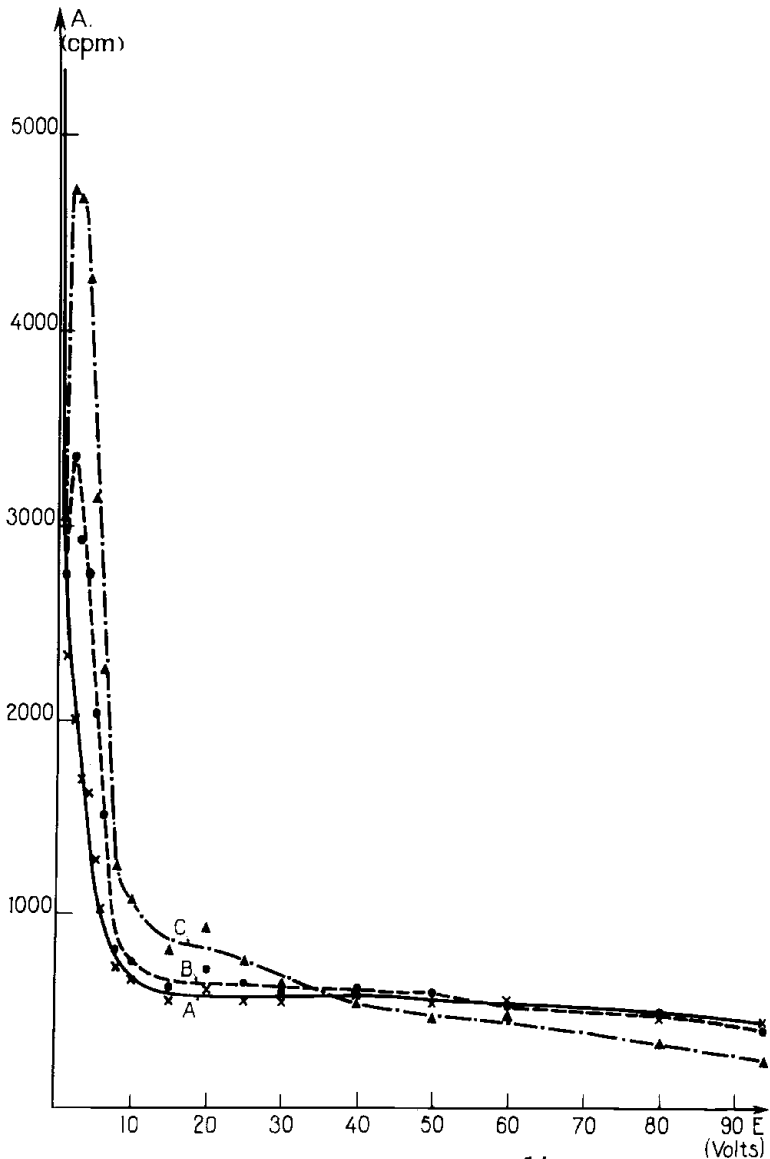


Fig.1 Pulse height spectrum in NE 220, ^{14}C samples (calibrations; P.setting).

The setting of the gain corresponded to that of ^3H . ^{14}C standard (48.800 dpm) was added to the following samples: Curve A) Four fibre papers were immersed for 19 hours in 2ml of NE 220, then removed; ^{14}C standard was then added; Curve B) 19,8 mg of spleen crushed between four fibre papers were immersed for 19 hours in scintillator liquid and removed; ^{14}C standard was then added; Curve C) 19,5 mg of spleen crushed between four fibre papers were immersed and left in NE220; ^{14}C standard was then added.

A : counted activity in cpm.

are not treated by ultrasound when taken out of the incubator at 37°C, equilibrium is reached much more slowly. Ultrasound therefore speeds the recovery of the fibre paper transparency.

In this study, we expressed the counted activities, not as counting efficiencies E, but as relative efficiencies (or counting yields) R, calculated with respect to measurements made on vials containing the standard radioactive solution, before introducing the organ samples. In this way, the characteristics of the scintillation counter are eliminated and the part played by the samples, is made evident. This study showed (Fig. 2 and 3) that the relative efficiency depends on the state of the sample (taken intact or treated with pronase); it decreases when sample weight, ^3H increases. This drop is more important in the case of ^3H than ^{14}C measured with the P setting. The relative efficiency also depends on the volume of NE 220.

The reproducibility of the calibration curves was shown experimentally, first in the course of time, second with various samples of the same organ taken from various strains of mice. The fluctuation of the results was within 10%. We did not notice any modification of results with various stocks of NE 220. Therefore, the calibration curves (Fig. 2 and 3) can be used systematically.

III - Counting of labelled samples

In the case of mice treated with ^{14}C or ^3H -labelled compounds, we have used the experimental conditions defined above. The samples, weighed on fibre paper in weighing flasks were placed in the NE 220, either intact with the weighing paper or treated with pronase. In the case of ^{14}C the P setting was always used. The relative efficiency, R, was determined from previous calibration curves (Fig. 2 and 3); knowing the efficiency (E) of the scintillation counter, we calculated the efficiency (e) of the measurement ($e = E \times R$) which permitted us to express the activity of the sample as dpm/mg.

The experiments showed that for samples taken from animals having received ^3H or ^{14}C -labelled compounds, the measured activity, most often increases with time, reaching a limit

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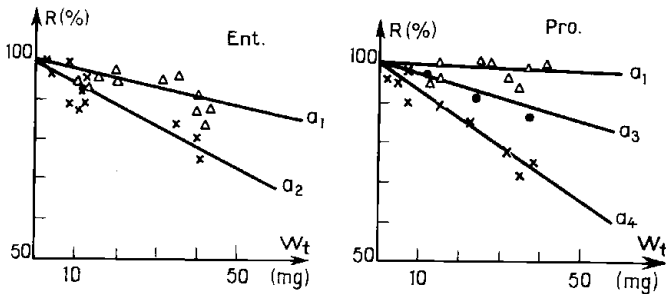


Fig.2 The effect of organs on the activity measurement with ^{14}C (P Setting)

R represented the ratio of the activity counted at equilibrium in the presence of sample, to that counted before the sample was immersed into the scintillator liquid. Measurements made with the samples intact (1 ml NE 220) or treated with pronase (3 ml NE 220), respectively, the curves Ent. and Pro. Curves index 1 : brain, fat, mammary glands and muscle; index 2 : liver spleen, ovaries and adrenal glands; index 3 : liver; index 4 : spleen, ovaries and adrenal glands. Each point represented the mean value of 2 to 4 assays having about the same weight. In the case of ^{14}C , the effect of the fibre papers and that of pronase on the relative efficiency R is negligible : the curves are extrapolated to $R = 100\%$.

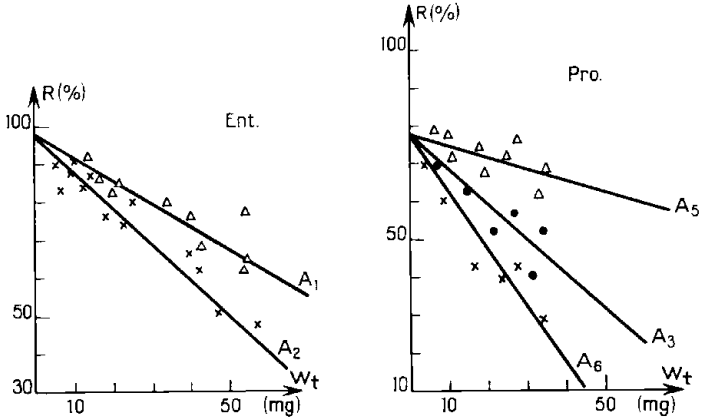


Fig.3 The effects of organs on activity measurements with ^3H

R has the same meaning as figure 2.

The measurements made with samples intact (1 ml NE 220) or treated with pronase (3 ml NE 220) respectively, the curves Ent. and Pro. Curves index 1 : brain, fat, mammary glands and muscle; index 2 : spleen, liver, ovaries and adrenal glands; index 3 : liver; index 5 : brain, fat, mammary glands, muscle and ovaries; index 6 : spleen and adrenal glands. In the case of ^3H , the effect on the relative efficiency R of the fibre papers and pronase is no longer negligible and corresponds to the extrapolation to the origin of the curves.

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which remains stable for a few days. The increase in activity and the time necessary to reach this plateau depend on the nature of the radioactive compound and on the state of sample (intact, or treated with pronase); this increase can be quite significant (up to five times the initial activity). Equilibrium is reached five to seven hr after the sample was placed in the liquid scintillator. This kinetics corresponds to the extraction of organic substances by the liquid scintillator, some of which are labelled and alter the pulse height spectra. The extraction of these compounds involves both the increase of counted activity and the decrease in relative efficiency. The experiments also show the preponderance of the former phenomenon, since the measured activity increases. This extraction is confirmed by the following facts: if, after measurement, we remove the radioactive sample with the fibre paper, we find that : a) the pulse height spectra remains modified (Fig.1); b) the radioactivity initially counted, is found, in a significant fraction, in the liquid scintillator (measurement "fibre papers out").

$$A - \frac{14}{C}$$

The experiments show that the relationship (activity (dpm) versus weight) is linear for pronase-treated samples, but occurs only rarely for intact samples (Fig. 4). In the latter case, we believe that we have counted all of the "x" compounds which are extracted by the liquid scintillator, and only a fraction of the "y" compounds, which are not extracted (the fraction located near the surface of the sample). The linear relation between activity (dpm) and the weight of the assay, obtained with the pronase-treated samples (Fig. 4)) seemed to indicate that all of the radioactivity was measured in these conditions. This was demonstrated by comparing the results obtained by this technique with those obtained by a classical method (Table 1).

It should be noted that we attempted to define the optimum reaction conditions of pronase by counting the activities of different fractions of the same organ (taken from a mouse fed with [2-¹⁴C] thymidine) treated during different incubation times at 37°C and with different concentrations of the enzyme.

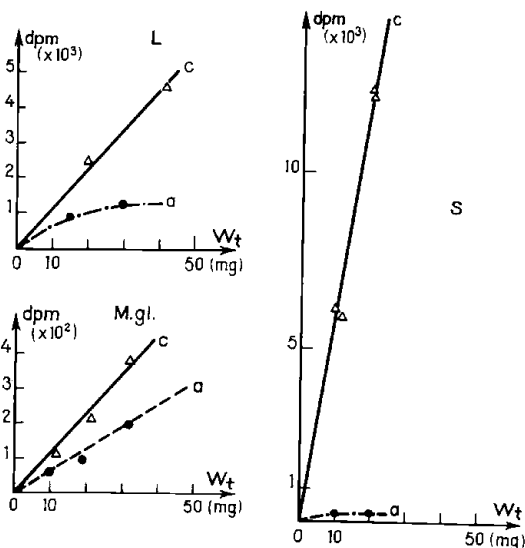


Fig.4 The dependence of activity (dpm) on weight: for ¹⁴C
 L : liver of a mouse killed two hours after being fed with 200 µg (20 µCi) of [2-¹⁴C] thymidine. S and M gl.: respectively, spleen and mammary glands of a mouse killed two hours after being fed with 121 µg (8 µCi) of [2-¹⁴C] thymidine.

Curves a (●) : samples counted intact; curves c (Δ) : samples counted after treatment with pronase.

The "a" curve for mammary gland gives an example of a linear relationship when the total activity is not measured since the "a" curve represented only about half of the "c" curve, in this case.

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B - ^3H

When we applied the technique established for ^{14}C to ^3H , we found that the activity-weight relationships were linear in all cases. However, for the same tissue, the activity measured with untreated samples was sometimes lower than that found with pronase - treated samples (Fig. 5). When the untreated sample is placed in NE 220, we count the total amount of the "X" compounds extracted by the liquid scintillator, but in no case, the non-extractible "Y" compounds, this being due to the weak average β path length of ^3H (1 μl in water). A systematic comparison was made between counting results obtained with untreated samples and either those treated with pronase or those treated by a classical method of digestion or combustion. They showed that the results for the pronase-treated samples are always in agreement with those obtained by classical methods, whereas the results from untreated samples are only sometimes in agreement (Table II). These results demonstrated that with ^3H , the linearity of the activity-weight relationship is not a sufficient test of the method's validity. However, we must try to interpret why in many cases, it was possible to measure the total ^3H on untreated samples. This last result was surprising as it did not occur with ^{14}C (Table I). These results can be explained by the fact that in-vivo the ^3H label of organic compounds quickly ends up in a water molecule. Wade and Shaw demonstrated that this transfer exists even when the ^3H label of thymidine is in the 2 or methyl position (4). After ingestion of 1 to 10 μCi of this compound, 85-90% of ^3H is found in the form of water after 24 hours. A similar result was found in the case of [^3H] chlormadinone (5). Our experiments show that water is easily extractable from tissues by NE 220, and this is why, for many types of tissues, the total ^3H is counted by simple immersion in liquid scintillator. In the [^3H] thymidine experiments (Table II), these are tissues with little mitotic activity (e.g., muscle, brain). In tissues with a greater mitotic activity e.g., spleen, ovaries, adrenal glands, pronase treatment must be used to obtain a measurement of total activity (Table II). However, in order that this non-extractable activity would remain significant compared to the extractable activity we killed the mice after a short period of time ($\frac{1}{4}$ hour). In the case of compounds such as

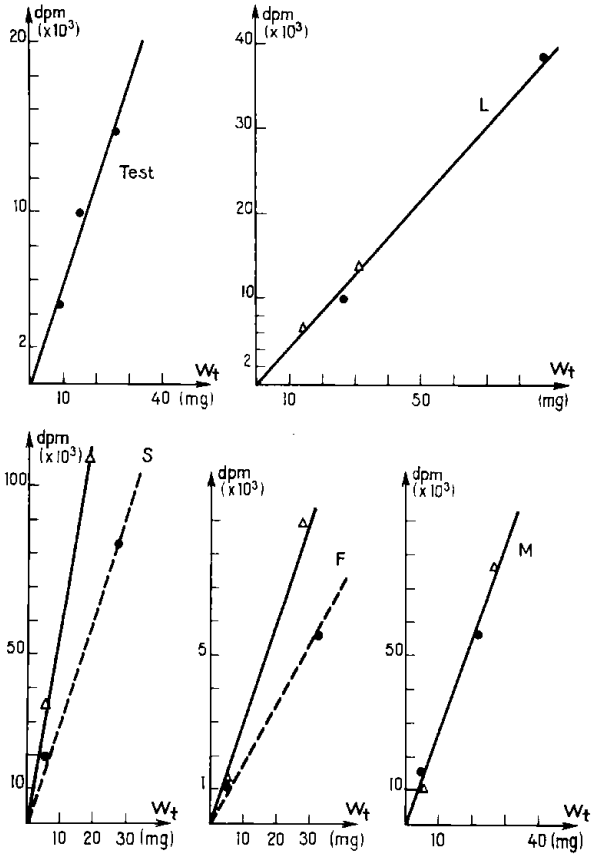


Fig.5 The dependence of activity (dpm) on weight : for ³H
 L and Test: respectively liver and testis of a male
 mouse killed two hours after being fed with 1,7 μg =
 100 μCi of [6-³H] thymidine. S, M and F. : respectively
 spleen, muscle and fat of a female mouse killed two hours
 after being fed with 1,7 μg = 100 μCi of [6-³H] thymidine.
 Points (●) : samples counted intact; points (Δ) samples
 counted after treatment with pronase.

TABLE I

Comparison of results of measurements obtained by different techniques for ^3H and ^{14}C Results expressed as dpm/mg tissue

	[methyl- ^{14}C] Thymidine			[2- ^{14}C] Thymidine		
	Intact	Direct Pronase	Combustion	Intact	Direct Pronase	Combustion
L.	760 ± 95	1.477 ± 230	1.310 ± 81	40 ± 6	71 ± 12	78 ± 5
S.	129 ± 17	998 ± 156	1.025 ± 64	57 ± 8	417 ± 70	400 ± 27
M.	120 ± 15	146 ± 25	144 ± 10	20 ± 3	23 ± 4	25 ± 1,5
B.	441 ± 55	512 ± 84	559 ± 35	21 ± 3,5	20 ± 3,5	21 ± 1,5
M.Gl	153 ± 20	249 ± 41	285 ± 19	28 ± 4	32 ± 5,5	33 ± 2
F.	39 ± 5,5	52 ± 9	42 ± 3	19 ± 3	20 ± 3,5	18 ± 1,5
Ov.		452 ± 84	433 ± 43		109 ± 23	89 ± 9
Adr.		566 ± 136	584 ± 70		79 ± 20	85 ± 6

B: Brain; Ov.: ovaries; Adr.: adrenal glands

The other letters and abbreviations have the same meaning as in Fig.2,4 and 5.

Mouse killed one hour after being fed with 35 µg (8 µCi) of [methyl- ^{14}C] thymidine or 38.7 µg (8 µCi) of [2- ^{14}C] thymidine

TABLE II

Comparison of results of measurements obtained by different techniques for ^3H
Results expressed as dpm/mg tissue

ORGANS	^3H Pregnenolone		^3H Thymidine			
	Intact	Direct Pronase	Hyamine	Intact	Direct Pronase	Combustion
L.	12.500±2000	14.820±3000	15.490±2560	3.144±490	10.970±1725	11.350±850
S.	1.555± 250	2.065± 435	1.388± 270	961±170	1.695± 281	2.090±172
M.	1.100± 175	1.050± 190	1.050± 204	1.004±164	864± 141	926± 70
B.	7.005±1050	7.262±1190	6.350± 920	817±133	735± 124	843± 67
M.GI	5.720± 860	5.183± 855	5.110± 690	486± 82	656± 111	589± 47
F.	--	--	--	148± 26	250± 44	190± 15
Ov.	--	--	--	--	1.052± 205	834± 92
Adr.	--	--	--	--	1.312± 298	564± 62

The letters and abbreviations have the same meaning as in Table I.

Mice fed with: [^3H] pregnenolone: 18,2 μg (560 μCi) and sacrificed two hours later;
[^3H] Thymidine: 0,92 μg (53,5 μCi) " " $\frac{1}{2}$ hour "

The contradictory result obtained for adrenal glands is probably due to the fact that the two glands were not identical.

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tritiated water, [^3H] chlormadinone or [^3H] pregnenolone, the total activity is recovered for samples counted untreated (Table II).

To avoid loss of ^3H corresponding to autolysis of tissues, it is necessary to keep the samples, in liquid nitrogen (-196°C), until pronase is added (3).

DISCUSSION - This study shows that it is possible to measure ^{14}C or ^3H which has been incorporated in organs, without using the classical methods of combustion or digestion. The combustion method is precise but requires a special apparatus and presents certain difficulties well known to those who use it. The digestion method gives variable results due to artifacts, of which the two principal ones are, a) the difficulty in obtaining a perfect solubilization of the samples and b) the occurrence of spurious luminescence present in liquid scintillators which never completely disappears (6 - 8).

A comparison of the results obtained by the technique described here with those obtained by one of the classical techniques demonstrates that it is possible to directly count the ^{14}C or ^3H incorporated in the organs. It also proves the validity of the calibration curves which were established when the organs (or fragments) were added to the liquid scintillator containing one or the other of the radioisotopes. The validity of the curves can be seen following the studies of the pulse height spectra, which have shown that the alterations were of the same order when the sample was either itself radioactive or simply immersed into the liquid scintillator containing radioactivity. We have seen that this result is explained by the fact that the cell membranes are permeated with the liquid scintillator which extracts different substances responsible for the modification of the spectra. These modifications are of the same order as those obtained with whole blood. The reproducibility of the calibration curves shows that it is unnecessary to determine, for each test, the counting efficiency by the internal-standard method, as it is possible to use previously established calibration curves.

The only special equipment necessary for the described

technique is an ultrasonic generator which is used to reduce the time required for the equilibration of the pronase-treated sample in the liquid scintillator. A counter with a single photomultiplier was used in this study. We have shown that the results are similar if one uses a counter with two photomultipliers; thus, with a different geometry the volume of scintillation fluid does not need to be changed. Nuclear Enterprises liquid scintillator NE 220 containing dioxane was also used. It is possible, however, to use liquid scintillators which do not contain this compound. The experiments show that the cell membranes are permeated with the solvent (toluene or xylene) which is common to all liquid scintillators, and that these extract the soluble component from the cells. These two properties are general, and the experiments, show that the activity counted in a labelled sample increases with time, reaching a plateau, whereas that of a control sample, in the presence of a radioactive solution, decreases before reaching a limit; the pulse height spectra are altered, as with NE 220. However, with the liquid scintillator not containing dioxane, it is not possible to treat the samples with pronase, since such media are not miscible with water. The comparison of the activity obtained with two liquid scintillators, of which only one contains dioxane, can give a preliminary indication of the nature (water-soluble and lipid-soluble) of the radioactive components existing in the sample.

In order to count the radioactivity of heavier samples it is necessary to treat several fragments of the same organ. The experiments show that such samplings always give results of the same order when the organ is homogeneous. For precise measurements, the samples must be at equilibrium in the liquid scintillator fluid, that is, 5 to 7 hours after their initial immersion. However, measurements made immediately after immersion give significant approximate pre-results. In the case of ^{14}C , measurements should be made with the "P" setting. Finally, the study shows that samples prepared by this technique remain stable several days in the liquid scintillator at ambient temperature.

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