

CONCERNING DNA
LIQUID SCINTILLATION MEASUREMENTS
OF ^{14}C AND ^3H RADIOACTIVITY

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The liquid scintillation technique was utilized in the case of thymidine or DNA labelled with ^{14}C or ^3H . Measurements in the homogeneous phase have shown that the counting efficiencies depend on two variables : the β particles energy and the size of the molecule carrying the radioisotope. In the homogeneous phase, measurements are correct only if the liquid scintillation medium and the radioactive solution added give rise to a medium which is homogeneous, not only at the macroscopic level, but also at the molecular level. Experiments performed have shown that, for small sized molecules (like

thymidine), such homogeneity is maintained at both of these two levels ; however this is no longer the case for macromolecules such as DNA. In fact, for these molecules, only the homogeneity at the macroscopic level is stable, while the homogeneity at the molecular level is rapidly and irreversibly destroyed. Consequently, the measurement efficiency diminishes, and current techniques are not capable of correcting this phenomenon. Therefore, for a given radioisotope, preliminary calibrations performed with small sized molecules are not necessarily accurate for macromolecules, and vice versa.

I. INTRODUCTION

Measurement of the radioactivity of ^3H or ^{14}C is currently performed using the liquid scintillation technique. The compounds which are most frequently of interest to biologists or biochemists are hydrosoluble compounds, and the radioactivity of an aqueous solution can be measured either by the *homogeneous phase* method, when the radioactive solution is directly mixed with a liquid scintillator, or by the *heterogeneous phase* method, when a support (glass fiber, for example) is used as an intermediary. The choice between one or the other of these two techniques and that of the scintillator medium depends on the nature of the radioactive sample and on the experimenter, (The Current status of liquid scintillation counting, 1970 ; Liquid scintillation counting, 1974 ; Liquid scintillation, Science and Technology, 1976 ; and Fox, 1976).

The experimental difficulties encountered using the homogeneous phase method have shown that these measuring conditions are valid only if the homogeneity of the radioactive aqueous solution and the liquid scintillator selected is maintained, not only at the macroscopic level, *but especially* at the molecular level. Further, these difficulties have shown that the calibrations carried out with small sized molecules (tritiated water, ^3H -or ^{14}C - thymidine, for example) are not necessarily valid in the case of large sized molecules (DNA, for example), because of the physico-chemical properties of macromolecules. Finally, these difficulties have shown that the measurement artefacts encountered are not revealed by the standard methods of analysis and correction.

II. MATERIALS AND METHODS

1. *Measurement Apparatus.* Automatic spectrometer (Inter-technique, France).

2. *Liquid Scintillator.* a) Containing dioxanne : NE 220 (Nuclear Enterprises, G.B.). b) Emulsion cocktail type (New England Nuclear, U.S.A.).

3 ml of one or the other of these two liquid scintillators were used, the assays having shown that this is the optimum volume for the majority of the measurements described in this report.

3. *Counting Vials.* Either of glass, having a low ^{40}K content, or of polyethylene. For some of the trials, the inside wall of the vials was covered with a thin layer of paraffin.

4. *Radioactive Compounds.* a) Thymidine, labelled in position 2 with ^{14}C or in position 6 with ^3H (New England Nuclear, U.S.A.). b) DNA, labelled with ^{14}C or ^3H and prepared in the laboratory, according to the technique described by Marmur (1961), using a thymine-less *E. coli* strain. A single deproteinization was performed, and the radioactive DNA prepared in this manner was dissolved in the SSC medium (0.15 M sodium chloride-0.015 M sodium citrate), and stored at -75°C . Their radioactivity was determined using the combustion technique, and their DNA concentration was determined by measuring their optical density ($\lambda = 256 \text{ nm}$).

5. *Aqueous Solutions.* The concentrated solutions of radioactive DNA (solvent = SSC) were diluted, either in water or in an SSC solution having various concentrations (between 1 and 1/300), and in the presence of variable quantities of non-radioactive carrier DNA (calf thymus or salmon sperm - SIGMA).

6. *Measurement Techniques.* An aliquot portion of the radioactive solutions was directly added to the liquid scintillator, in the counting vials. As soon as prepared, these vials were shaken for 30 minutes (maximum speed of a rectilinear alternate shaker providing 280 shakes/minute), then counted. The measurements of each sample were repeated periodically.

7. *Decanting.* In order to estimate the quantity of DNA adsorbed on the wall of the counting vials, the following procedure was employed:

s_1 was the liquid scintillator containing, in vial p_1 , the measured sample (s_1/p_1); A_0 was the value of its activity,

measured as soon as prepared ($t = 0$), and A was the value measured at the time of decanting. At this time of decanting, medium s_1 was transferred to an empty counting vial, p_2 , with the aid of a pipette ; s_1/p_2 was this sample. 3 ml of new liquid scintillator, s_2 , were put into the initial counting vial, p_1 ; s_2/p_1 was this sample. The activity, V , of this sample, s_2/p_1 , corresponds to the DNA adsorbed on the wall of the vial, p_1 . The geometry of the DNA adsorbed on the vial walls was different from that of the DNA dissolved in the liquid scintillator, since a non-negligible portion of the β particles emitted from this adsorbed DNA was lost, due to the fact that they were directed toward the exterior of the liquid scintillator. Since the efficiency of this measurement is difficult to determine, the value, V , of this activity gives only an estimation, by default, of the DNA adsorbed. The activity, M , of the sample, s_1/p_1 , corresponds to the DNA which was still in the liquid scintillator. The difference, C , between the activity, A_0 , of the sample, s_1/p_1 , measured at time $t = 0$, and the activity, M , of the sample, s_1/p_2 , corresponds to the DNA adsorbed on the wall of the vial :

$$C = A_0 - M, \text{ with } C > V$$

8. *External Standard : Cs Ratio.* The automatic spectrometer used included an external ^{137}Cs standard, allowing the spectrum shift produced by quenching phenomena to be known. This shift was observed by measuring the ratio of the change in the counting rate in two contiguous channel settings. This ratio is described in this report as the *Cs ratio*. Its value diminishes in the presence of quenching.

III. RESULTS

Since DNA becomes denatured in water, it is usually handled in saline solutions such as SSC. The initial assays of this investigation dealt with DNA labelled with ^3H or ^{14}C and dissolved in the SSC medium. Such a solution is not miscible with the NE220 liquid scintillator, although it can be mixed with Biofluor.

When 0,1 ml of radioactive DNA in an SSC solution was added to glass vials containing 3 ml of Biofluor, it was noted that the measured activity, after shaking of the vials, diminished with time. This diminution began as soon as the samples were prepared ; it was more marked when the tracer was ^3H (Fig. 1). This diminution, in time, of the measured activity was always noted for all the samples prepared from different preparations of radioactive DNA, with the addition, or not,

of non-radioactive carrier DNA.

The various experiments, performed in order to understand this diminution, have revealed that it is independent of the shaking of the counting vials, but does depend on (Fig. 1 and 2) :

- . the radioactive isotope (^3H or ^{14}C)
- . the DNA concentration of the solutions
- . the SSC concentration of these same solutions.

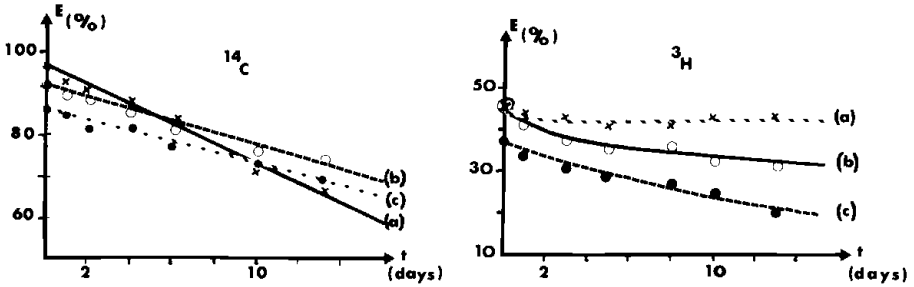


FIGURE 1. Course, with time, of the measurement efficiencies (E) for DNA in SSC solutions.

Liquid scintillator = Biofluor

^{14}C -DNA : a) 95 $\mu\text{g/ml}$ (x) ; b) 190 $\mu\text{g/ml}$ (O) ; c) 395 $\mu\text{g/ml}$ (●) ;

^3H -DNA : a) 2 $\mu\text{g/ml}$ (x) ; b) 17 $\mu\text{g/ml}$ (O) ; c) 60 to 200 $\mu\text{g/ml}$ (●).

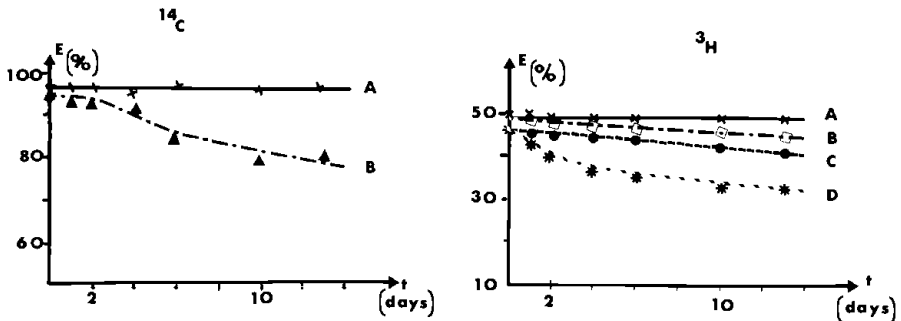


FIGURE 2. Course, with time, of the measurement efficiencies (E) for DNA in SSC solutions : role of the concentrations. (Liquid scintillator = Biofluor).

^{14}C DNA : A) 95 to 395 $\mu\text{g/ml}$ in SSC 1/100 ; 95 to 190 $\mu\text{g/ml}$ in SSC 1/10 ; B) 395 $\mu\text{g/ml}$ in SSC 1/10.

^3H -DNA : A) 0.2 to 10 $\mu\text{g/ml}$ in SSC 1/300 to 1/10 ; B) 50 to 100 $\mu\text{g/ml}$ in SSC 1/300 to 1/10, and 200 $\mu\text{g/ml}$ in SSC 1/300 ; C) 100 to 200 $\mu\text{g/ml}$ in SSC 1/10 ; D) 425 $\mu\text{g/ml}$ in SSC 1/10.

For each DNA and SSC concentration, three samples were prepared and measured. Each point represents the average of all the trials which gave the same order of results.

Since the SSC diluted solutions are miscible with the NE 220 liquid scintillator, some assays were carried out. They revealed a low measurement efficiency as soon as the SSC concentration reached 10%, and, in all the cases, a diminution of the measured activity, with time, which was greater and more rapid than that observed with Biofluor (Fig. 3).

Neither the low efficiencies of the initial measurements, nor their diminution with time, were ever reflected in the value of the Cs ratio. In effect, the values of this ratio remained independent of the counting efficiencies of the samples, and, most frequently, remained constant during the course of time. In some cases, these values increased slightly, but they never decreased (Fig. 4).

In the case of a small sized molecule, such as thymidine labelled with ^{14}C or ^3H , the measurement efficiencies and the value of the cesium ratio always remained constant with time ; the SSC concentration of the solutions never diminished the measurement efficiencies (Fig. 5).

The absence of any modification, with time, in the measured activities when ^3H or ^{14}C was carried by thymidine, as well as the absence of a decrease in the value of the Cs ratio when the ^3H or the ^{14}C was carried by DNA, demonstrate that neither the SSC medium nor the DNA modify the liquid scintillator (Biofluor or NE 220). The diminutions observed in the measured activity cannot, therefore, be linked to a quenching phenomenon. In order to understand them, the counting vials were measured until their activity became constant.

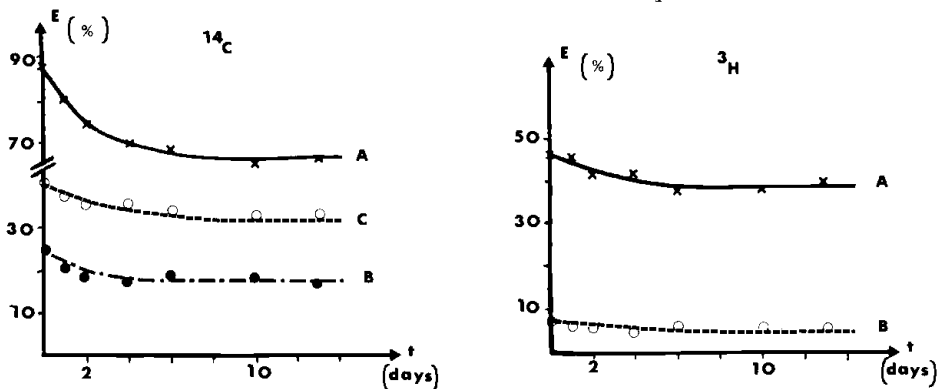


FIGURE 3. Course, with time, of the measurement efficiencies (E) for DNA in NE 220.

^{14}C -DNA : A) 85 to 390 $\mu\text{g/ml}$ in SSC 1/100 ; B) 85 to 190 $\mu\text{g/ml}$ in SSC 1/10 ; C) 390 $\mu\text{g/ml}$ in SSC 1/10.

^3H -DNA : 0.02 to 200 $\mu\text{g/ml}$ in SSC at A) 1/300 ; B) 1/10.

For each DNA and SSC concentration, three samples were prepared and measured. Each point represents the average of all the trials which gave the same order of results.

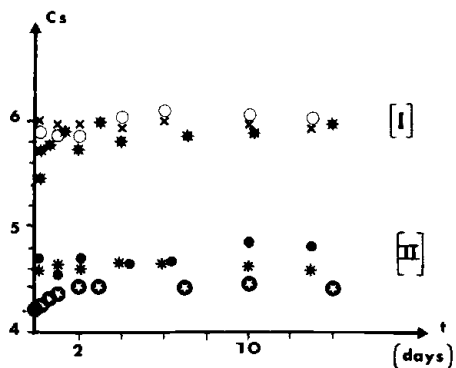


FIGURE 4. Course, with time, of the cesium ratio (Cs) (^{14}C -DNA).

Curve I. Liquid scintillator = NE 220. 3 ml of this medium corresponds to a Cs ratio between 6.100 and 6.300.

(*) : 31 $\mu\text{g/ml}$ in SSC 1/100 (curve gl, Fig. 8)

(x) : 190 $\mu\text{g/ml}$ in SSC 1/10 (curve B, Fig. 3)

(O) : 390 $\mu\text{g/ml}$ in SSC 1/100 (curve A, Fig. 3).

Curve II. Liquid scintillator = Biofluor. 3 ml of this medium corresponds to a Cs ratio between 4.800 and 5.000.

(*) : 31 $\mu\text{g/ml}$ in SSC 1/100 (curve gl, Fig. 8)

(*) : 95 $\mu\text{g/ml}$ in SSC (curve a, Fig. 1)

(O) : 395 $\mu\text{g/ml}$ in SSC (curve c, Fig. 1).

With ^3H -DNA, the results are of the same order, since the Cs ratio is not dependent on the radioelement.

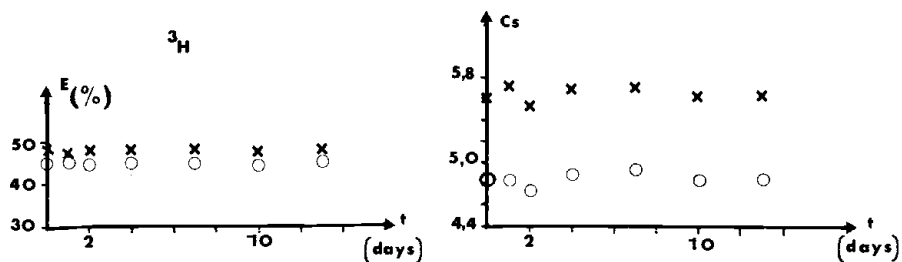


FIGURE 5. Case of Thymidine. Liquid scintillator = NE 220 (x) or Biofluor (O).

Course, with time, of the measurement efficiencies (E) and of the cesium ratio (Cs) for ^3H -6-thymidine in an SSC (0.8) solution (300 $\mu\text{g/ml}$). The results are of the same order for ^3H -6-thymidine in water and for ^{14}C -2-thymidine (300 $\mu\text{g/ml}$) in water or SSC (0.8); for the latter, the measurement efficiency, E, is equal to 97% for NE 220 or for Biofluor. (Each point is the average of 3 trials).

TABLE 1. Adsorption of DNA on the Walls of the Counting Vials

Isotope	DNA solutions		Liquid scintillator	Curve	A ₀ (cpm)	E ₀ (%)	Equilibrium		After decanting (cpm)	
	μg/ml	SSC					t (days)	A (cpm)	M (s ₁ /p ₂)	V (s ₂ /p ₁)
¹⁴ C	95	1	Biofluor	Fig. 1a	16900	96	18	11000	8200	3980
	395	1	"	Fig. 1c	15500	88.5	20	11500	3700	8940
	390	1/10	"	Fig. 2B	16630	94.5	60	12880	4705	7950
	85	1/100	NE 220	Fig. 3A	15490	88	8	11265	385	12035
³ H	2	1	Biofluor	Fig. 1a	72800	45	2	66600	51480	6000
	80	1	"	Fig. 1c	62190	37	60	13180	1180	20800
	70	1/300	"	Fig. 2B	8225	50	32	2631	55	3275
	0.2	1/300	NE 220	Fig. 3A	7570	46	6	7310	6070	685
	0.2	1/10	NE 220	Fig. 3B	1150	7	6	655	270	2950

These results are related to the experiments shown in Figures 1 to 3.

E₀ = efficiency of the initial measurement performed just after the vials were shaken (this time is taken as t = 0).

s₁/p₂ : liquid scintillator of the sample decanted into a new, empty vial : this is activity M.

s₂/p₁ : fresh liquid scintillator put in the initial vial : this is activity V.

(A₀, A, M and V are defined in Materials and Methods, § 7).

At that time, they were decanted according to the procedure described in *Materials and Methods*. The counting results of the new samples, obtained after decanting procedures, revealed that the radioactivity is also adsorbed on the walls of these vials (Table 1). Such an adsorption phenomenon has already been mentioned by Kobayashi and Maudsley (*in Liquid scintillation counting, Recent developments, 1974*).

The overall results obtained in this investigation show that there is a relationship between the measured activity and the activity fixed on the walls of the vials (Fig. 6). This relationship depends only on the liquid scintillator and is independent of the concentration of the DNA solutions (from 0.2 to 500 $\mu\text{g/ml}$), and the SSC solutions (from 1/300 to 1).

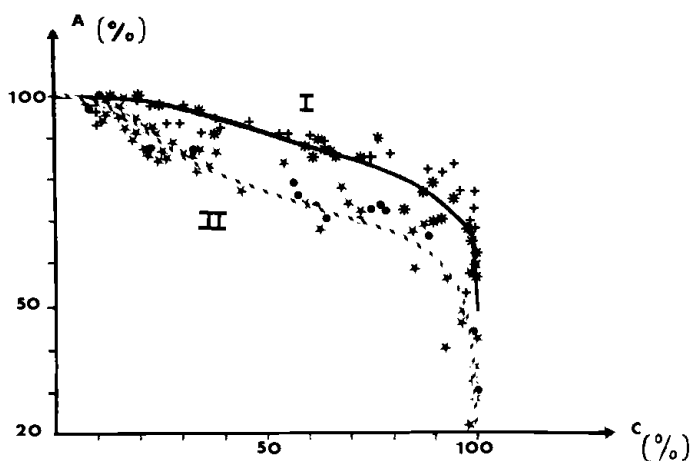


FIGURE 6. Relationship between the measured activity (A) and the activity (C) adsorbed on the vial walls.

Curve I. Liquid scintillator = NE 220. ^{14}C -DNA (*) and ^3H -DNA (+).

Curve II. Liquid scintillator = Biofluor. ^{14}C -DNA (●) and ^3H -DNA (★).

C = the activity of the DNA adsorbed on the walls and calculated.

A and C are described in *Materials and Methods* § 7 (decanting); they are expressed here in % of the initial activity, A_0 , measured once the shaking of the vials has occurred.

The courses, with time, of the measured and adsorbed activities are complementary (Fig. 7). The kinetics of these courses depends on the concentration of the DNA and the SSC solutions.

These diminution phenomena of the measured activity, during the course of time, which are not accompanied by a decrease in the value of the Cs ratio, and those of the DNA adsorption on the walls of the counting vials, were found to exist by using either polyethylene vials or glass vials, their internal wall being covered with a thin layer of paraffin. For a given liquid scintillator and a DNA solution, only the speed and the scope of these phenomena depend on the nature of the vial wall (Fig. 8).

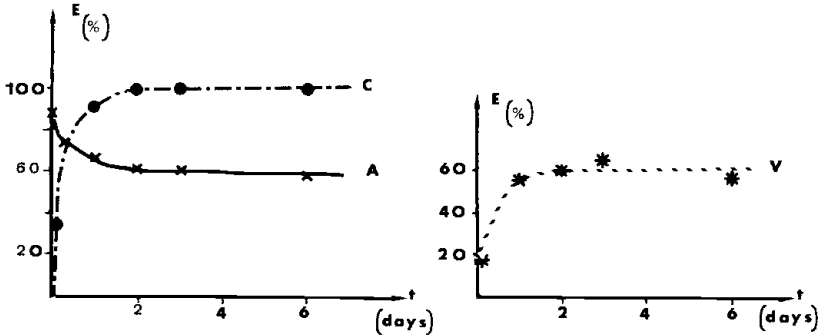


FIGURE 7. Kinetics of the variation in measured (A) and adsorbed (V or C) activities.

Aliquot parts of 0.1 ml of a 95 $\mu\text{g/ml}$, ^{14}C -DNA solution (SSC-1/20) were added to 3 ml of NE 220 (s_1/p_1). Ten s_1/p_1 samples were prepared at the same time, were measured at the same time, but were decanted, during the course of time, two by two, as described in Materials and Methods, § 7 (decanting).

Curve A : measured activity in the vials - s_1/p_1

Curve V : measured activity in the p_1 vials, after the initial NE 220 s_1 had been replaced with fresh NE 220 - s_2 (s_2/p_1).

Curve C : activity adsorbed on the vial walls, and calculated.

The principle of the activities A, C and V is defined in Materials and Methods, § 7 (decanting).

The activities are expressed in relation to the real activity (dpm) of the DNA sample used. The difference between curves C and V is due to the difference between the geometry which characterizes, respectively, the adsorbed DNA and the DNA dissolved in the liquid scintillator.

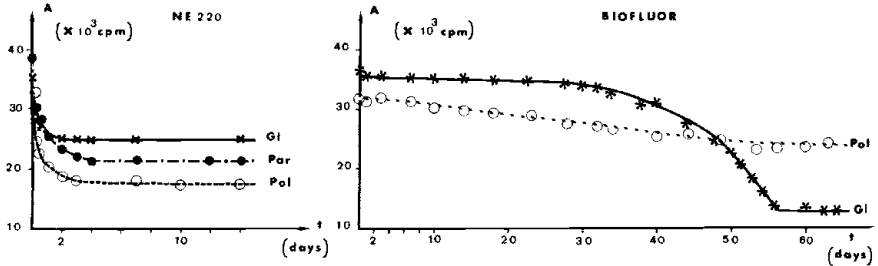


FIGURE 8. Role of the counting vial wall.

0.1 ml of a ^3H -DNA solution (31 $\mu\text{g}/\text{ml}$) in SSC-1/100 was added to 3 ml of NE 220 or Biofluor. A = measured activity ; gl = glass vials ; POL = polyethylene vials ; PAR = glass vials, the inside surfaces of which had been coated with a thin layer of paraffin.

For Biofluor, the form of the gl curve is characteristic of the course of the measured activity in this liquid scintillator, when the vials are made of glass.

For all the samples, the value of the Cs ratio remained constant, or increased slightly with time, identical to the curves in Figure 4.

IV. DISCUSSION

The overall results obtained reveal that although the measured activity remains very constant during the course of time, when the radioactive compound is a small sized molecule, such as thymidine, (Fig. 5), this is no longer the case when the radioactive compound is a macromolecule such as DNA (Fig. 1 to 3).

In effect, for radioactive DNA, the diminution of the measured activity, during the course of time (Fig. 1 to 3 and 8), is never accompanied by a decrease in the value of the Cs ratio (Fig. 4). The course of these two given phenomena is true for DNA labelled with ^{14}C , as well as with ^3H . These two courses depend therefore on the macromolecule, and not on the radioisotope. It is known that the addition, to any liquid scintillator, of an aliquot portion of aqueous solution has as a consequence the diminution of the value of the Cs ratio which characterises the pure liquid scintillator. When the added aqueous solution contains small sized molecules, the value of this ratio and the measured activity remain stable with time. Conversely, when the added solution contains a macromolecule, such as DNA, the measured activity decreases with time, while the value of the Cs ratio remains constant, or increases. That means that, in these cases, it became closer

to the value of the Cs ratio which characterizes the pure liquid scintillator (Fig. 4). This tendency of the Cs ratio value to return to the value of the pure liquid scintillator might indicate a phase separation of the liquid scintillator-DNA aqueous solution mixture. This phase separation was demonstrated using dielectric measurements in the microwave domain (the C band), via a method already described by one of the authors (David *et al.*, 1975). This method provides information on the *bound water/free water* phenomenon.

It should be pointed out that the appearance of these solutions remains that of a homogeneous solution, which is not modified during the course of time. The phase separation is thus produced only at the molecular level and not at all at the macroscopic level. If such a phase separation had occurred at the macroscopic level, a visible modification - a cloudy appearance, for example - would have been noted, but this was not the case.

It may be supposed that, during the course of this phase separation, the migration of the DNA molecules takes them close to the vial walls, where they are strongly adsorbed, and from which they are disadsorbed only with difficulty. In effect, they cannot be disadsorbed by any amount of shaking; they remain adsorbed when the vials are decanted (Fig. 6 and 7). Finally, it is noted that the glass vials are particularly difficult to decontaminate. This adsorption on the glass surfaces was also seen when a glass chip, having the same composition, (a fresh vial was broken for this purpose), was added to the two scintillators employed (NE 220 and Biofluor). When the vials are of polyethylene or of glass, covered inside with a thin layer of paraffin, the DNA does not remain adsorbed only on the surface of the wall, but penetrates slightly into the thickness of the polyethylene or the paraffin. In the case of ^{14}C -DNA, it is possible to follow this penetration by means of the deformation of the scintillator spectra during the course of time.

By trying to solubilize radioactive DNA in a 90% DMSO solution, such an adsorption of DNA on the walls of the recipients used was noted once again (Table 2). This adsorption seems to exist when the DNA is not very soluble in the medium employed, as is the case even for 90% DMSO, and also, doubtless, for the NE 220 and Biofluor liquid scintillators. This insolubility must also explain why the measurements efficiencies and their stability, with time, are always better when the concentration of DNA solutions is lower (Fig. 1 to 3).

It seems that the capacity of water molecules and ions to be fixed to DNA is concomitant with the property of adsorption of the DNA on the vial walls. It has been noted, in effect, that the measured activity in the initial counting vial, p_1 , containing, after decanting, the fresh liquid scintillator, s_2 , (activity $V = s_2/p_1$) was sometimes greater than the activity measured just before decanting (Table 1).

TABLE 2. Counted Activity of DNA Dissolved in DMSO (90%)

t (h)	(cpm)	
	³ H-DNA	¹⁴ C-DNA
0	2390	9435
2	1945	8020
4	1865	7550
6	1700	7100
19	1520	7025

The DNA solutions in 90% DMSO had concentrations of 0.85 μg DNA/ml for ³H-DNA, and 190 μg /ml for ¹⁴C-DNA.

The measurements were performed in NE 220 with aliquot portions of 0.1 ml of these DNA solutions, sampled at different time intervals, and placed on glass fiber.

At time $t = 19$ h, the vials containing the solutions of DNA in DMSO were emptied, washed with 90% DMSO and refilled with 3 ml of NE 220. The lost activity was found in this final measurement.

Each value is the average of 3 trials.

A result as illogical in appearance as this may be understood if it is supposed that the salt molecules, (which are more or less disassociated), and the water molecules can be adsorbed in the form of microstructures on the surface of the DNA attached to the wall. These microstructures bring about a local self-adsorption of the β particles emitted by the ³H or the ¹⁴C of the DNA. This is seen in the low measured activity. The decanting replaces the initial liquid scintillator, s_1 , with a fresh liquid scintillator, s_2 . This seems to destroy these microstructures on the DNA surface, at the same time that the macromolecule stays adsorbed on the wall. The measurement of this new sample $V = s_2/p_1$ gives a measured activity which is greater than that of sample s_1/p_1 , following the elimination of these microstructures. This hypothesis explains, therefore, why the increase in the measured activity s_2/p_1 , in relation to that of s_1/p_1 , is always higher in the case of ³H-DNA, whose β particle energy is lower than that of the ¹⁴C β particle.

These two phenomena, adsorption on the vial walls, and the attachment of microstructures (salt molecules, H₂O or ions hydrated at varying degrees) to the surface of the DNA itself, have been found for DNA solutions placed on glass fibers (Rebeyrotte and Apelgot -unpublished results). For the measurement of radioactive DNA in a solution, the best technique

appears to be that described by Carrier and Setlow (1971). This technique consists of washing the fibers in a 5% trichloroacetic acid solution. The adsorption forces of DNA onto the glass fibers are such that this treatment frees the DNA from its salts, without disadsorbing it itself. Of course, the combustion technique also gives correct results, independently of the solution concentrations of DNA or salts.

V. CONCLUSION

The same radioactive atom can, therefore, give two different results, depending on the molecule which carries it. In liquid scintillation, the results of the homogeneous phase measurements do not, therefore, depend only on the energy of the β particles emitted by the radioactive isotope, but also on the size of the radioactive molecule. The difficulties encountered in the case of DNA appear to be a consequence of the physico-chemical properties of the macromolecules in general, and should be evidenced as well in the case of RNA or of proteins.

The role played by the size of the molecules is already known in the case of paper supports (S. Apelgot and M. Duquesne, *in* Liquid scintillation, Science and Technology, 1976). It is seen again in this investigation, for homogeneous phase measurements. The difficulties associated with the size of the molecules, using these two techniques, have different origins. Nonetheless, in the two cases, the measurement of the β particles corresponds to an *all or nothing* phenomenon, and in both cases renders the classical efficiency controls, using an external standard method, the standard ratio method, or even the internal method quite useless, when the radioactive compound standard is a molecule whose physico-chemical properties are different from those of the experimental compound.

Since the measurement results depend on two variables, the energy of the β particles of the radioelement and the physico-chemical properties of the molecules labelled with this radioelement (macromolecules or other molecules), for any preliminary calibrations this factor must be borne in mind. Consequently, for each radioelement, calibrations with a macromolecule and with a small sized molecule, both labelled with the radioelement under investigation, are necessary. The results obtained with these two families of molecules can be different.

AKNOWLEDGMENTS

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