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ASSAY OF TRITIUM AND CARBON-14 IN ANIMAL TISSUES BY LIQUID SCINTILLATION*

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SUMMARY

1. Mouse organs are digested in hyamine hydroxide methanolic solution and the digest is diluted with dioxane before the addition of a toluene scintillating solution.

2. The vials ready for counting contain up to 155 mg of fresh tissue in a volume of 16 ml.

3. Without digest, with the Packard Tri-Carb spectrometer, the yields of counting of the final scintillating mixture are 50% for ^{14}C and 17.5% for ^{3}H. With 100 mg of stomach, intestine, brain or muscle, the yields are 40% for ^{14}C and 5% for ^{3}H. They are half these values with 100 mg of liver, lung or heart.

4. The chemiluminescence with the Tri-Carb spectrometer is negligible with ^{14}C, but a waiting period of 48 h is necessary for counting ^{3}H. During this time, the vials can stay at room temperature without particular screening from the light.

The total tritium and carbon-14 in animal tissues can be assayed by liquid scintillation counting after combustion of the sample in a current of oxygen. The carbon dioxide is absorbed in hyamine hydroxide in methanolic solution and subsequently diluted with toluene containing suitable fluorescent chemicals[1]. The water is absorbed in a dioxane scintillating solution[2]. The procedure is time-consuming and exacting; periodically the combustion train must be checked for the absence of contamination.

To simplify the procedure and permit many simultaneous determinations, digestion of the tissues into a form soluble in the scintillating solution has been suggested. Recently, BROWN AND BADMAN[3] published a method in which the tissues are treated with aqueous potassium hydroxide solution before addition of hyamine chloride and the scintillating solution; by this method, the ^{14}C of about 5 mg of fresh tissue could be counted with an efficiency of 60%. In our work with proteins, nucleic acids and whole tissues, we have employed digestion in a molar methanolic solution of hyamine hydroxide as described by STEINBERG *et al.*[4]. The present paper deals with some factors influencing the counting efficiency for both ^{14}C- and tritium-labelled substances in animal tissues; with the chemical nature of the solvents, and

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with quantities and origins of the organs. The importance and duration of chemiluminescence[5] have also been considered.

EXPERIMENTAL AND RESULTS

Preparation and counting of the samples

The hyamine hydroxide was prepared by the action of silver oxide on recrystallised hyamine chloride (di-isobutylcresoxyethoxyethyldimethylbenzyl ammonium chloride monohydrate = hyamine-10X, Rohm & Haas, Co.) following the procedure described by EISENBERG[6]; an approximately molar methanolic solution was obtained.

The experiments were performed with tissues from mice. In the first ones, intestines and stomachs were emptied, washed with physiological saline and dried with filter paper; 3.5 g of these mixed organs were digested with 15 ml of 1 *M* hyamine hydroxide in methanol for 12 h at 65° in the dark in a hermetically closed vessel. The final volume was 16 ml.

To the digest were added successively the *diluent* and the *scintillating solution*; the sample in the vial ready for counting is termed the *scintillating mixture*.

5 ml of the digest were diluted to 100 ml with one of the following diluents: methanol (Union Chimique Belge, 1120, *p.a.*): toluene (Merck, 8325, *p.a.*) (1:1 v/v), dioxane (Merck for chromatography, 9671): toluene (1:1 v/v) or pure dioxane.

For the preparation of the counting vial, to 5 ml of the diluted digest (corresponding to 55 mg of fresh tissues) were added 10 ml of a scintillating solution and 1 ml of toluene (or 1 ml of the ^{14}C- and tritium standard solution in toluene.) All the scintillating solutions contained 10 g of 2,5-diphenyloxazole, 250 mg of 1,4-bis-2(5-phenyloxazolyl)-benzene and 100 g of naphthalene per liter; they differed by the solvent which was either dioxane (Merck, 9671), toluene (Merck, 8325) or a mixture (1:1 v/v) of both. The standards were made from ^{3}H-naphthylacetamide or ^{14}C-naphthalene in toluene; 1 ml has an activity of 41,700 disintegrations per minute for ^{3}H, and 23,000 disintegrations per minute for ^{14}C.

Combinations of the different diluents and scintillating solutions afforded 9 kinds of counting vials differing in the proportions of methanol, dioxane and toluene.

The counts were made with the Packard Tri-Carb spectrometer, automatic model No. 314. This instrument is provided with two photomultipliers and a coincidence circuit. After maximum amplification, the pulses were selected between 10 and 60 V and the tensions on the photomultipliers chosen for maximum efficiency. In the present study, a high tension (H.T.) of 970 V (position 5 of the selector) for ^{14}C and of 1200 V (position 8) for tritium were used; these were very near the optimum for most of the cases analysed.

Each determination was done in duplicate. For the study of the chemiluminescence, one vial of the pair was left at room temperature without particular screening from the light, while the other was stored in the dark at —10° in the refrigerator of the Tri-Carb counter.

Chemiluminescence

After digestion for 12 h, the sample was allowed to cool for about 3 h; the digest was then diluted and, immediately after, the scintillating solution and 1 ml

TABLE I

CONDITIONS OF ^{14}C COUNTING

Chemiluminescence at H.T. = 5

Diluent	*Scintillating solution*	*Mode of storage*	*Counts/min at time t (h)*				
			t = 0	*t = 12*	*t = 24*	*t = 36*	*t = 48*
Methanol–toluene	Dioxane	Refrigerator	23	19	13	20	27
		room	18	16	16	27	15
	Dioxane–toluene	Refrigerator	27	16	20	24	19
		room	20	17	21	16	19
	Toluene	Refrigerator	20	14	22	17	21
		room	16	12	16	23	31
Dioxane–toluene	Dioxane	Refrigerator	18	19	26	18	19
		room	16	12	21	30	24
	Dioxane–toluene	Refrigerator	14	15	17	10	16
		room	15	18	14	15	14
	Toluene	Refrigerator	20	15	19	21	17
		room	17	12	17	13	19
Dioxane	Dioxane	Refrigerator	24	30	21	21	17
		room	29	21	23	17	15
	Dioxane–toluene	Refrigerator	21	18	25	21	16
		room	19	27	21	23	18
	Toluene	Refrigerator	29	20	22	15	20
		room	18	18	31	16	18

TABLE II

CONDITIONS OF ^{3}H COUNTING

Decrease of chemiluminescence with time at H.T. = 8

Diluent	*Scintillating solution*	*Mode of storage*	*Counts/min at time t (h)*				
			t = 0	*t = 12*	*t = 24*	*t = 36*	*t = 48*
Methanol–toluene	Dioxane	Refrigerator	5012	496	81	84	45
		room	2917	438	52	37	30
	Dioxane–toluene	Refrigerator	1121	239	65	63	48
		room	1043	280	46	34	34
	Toluene	Refrigerator	248	91	50	30	36
		room	183	76	34	31	36
Dioxane–toluene	Dioxane	Refrigerator	125	88	144	81	46
		room	713	40	37	40	43
	Dioxane–toluene	Refrigerator	25	65	25	49	35
		room	40	50	36	33	34
	Toluene	Refrigerator	41	43	46	37	35
		room	60	30	55	34	32
Dioxane	Dioxane	Refrigerator	1440	455	329	127	101
		room	1181	410	75	56	52
	Dioxane–toluene	Refrigerator	785	333	246	112	102
		room	803	396	62	59	43
	Toluene	Refrigerator	182	122	77	65	54
		room	175	117	60	46	47

of toluene were added. This precise moment is time o for the results recorded in Tables I and II.

The samples kept at room temperature were placed in the refrigerator of the Tri-Carb just before the counting which required only 1 min, and were removed immediately after. The statistical accuracy of the background values obtained during such a short time is not very great, but it sufficed for the present purpose.

At this stage, for obvious reasons, no radioactive standard was added to the

vials which were counted at the tensions used for the carbon-14 and tritium assays successively.

Table I shows that, with the Tri-Carb counter, under the conditions of ^{14}C assay (H.T. in position 5), no decrease of the recorded value with time was observed in any case.

On the contary, at the H.T. employed for tritium counting (position 8), the initial value was far above the usual background and decreased with time (Table II). In most cases, the stabilisation was incomplete even after 24 h and a waiting period of 48 h was necessary to ensure that the chemiluminescence was no longer visible. The presence of dioxane increased the chemiluminescence, but the mode of storage was without influence.

TABLE III

COMPARED EFFICIENCIES WITH ^{14}C AND BACKGROUNDS AT H. T. 5

(55 mg of tissues per vial. Assays performed after 48 h)

Diluent	*Scintillating solution*	*Mode of storage*	*^{14}C efficiencies (in %)*		*Backgrounds (counts/min)*	
			With tissues	*Without tissues*	*With tissues*	*Without tissues*
Methanol–toluene	Dioxane	Refrigerator	45	45	27	21
		room	45	48	26	23
	Dioxane–toluene	Refrigerator	46	48	20	21
		room	45	47	24	21
	Toluene	Refrigerator	46	47	19	24
		room	45	48	23	20
Dioxane–toluene	Dioxane	Refrigerator	48	47	23	22
		room	46	48	27	22
	Dioxane–toluene	Refrigerator	48	48	25	18
		room	46	47	25	20
	Toluene	Refrigerator	46	47	20	18
		room	46	47	17	20
Dioxane	Dioxane	Refrigerator	47	47	24	25
		room	47	48	25	22
	Dioxane–toluene	Refrigerator	48	48	24	24
		room	48	48	25	20
	Toluene	Refrigerator	47	46	22	19
		room	47	47	22	18

Relationship between counting efficiency and solvents

Various combinations of diluents and scintillating solutions were tried; all vials contained 55 mg of tissues from the same stock of digested stomachs and intestines. For each solvent combination, 2 vials were charged with 1 ml of toluene (background), 2 vials with 1 ml of the toluene solution of the ^{14}C standard (23,000 disintegrations/min) and 2 vials with 1 ml of the toluene solution of the tritium standard (41,700 disintegrations/min). The assays were performed after the disappearance of the chemiluminescence (one vial of each set being left at room temperature while the other was stored in the refrigerator of the Tri-Carb).

Table III shows that, in the case of ^{14}C, the 9 different mixtures gave approximately the same results. The unfavourable effect of methanol, very obvious with tritium, did not appear here.

From Table IV, it can be noted that for tritium the use of methanol–toluene as

TABLE IV

COMPARED EFFICIENCIES WITH TRITIUM AND BACKGROUNDS AT H.T. 8

(55 mg of tissues per vial. Assays performed after 48 h)

Diluent	*Scintillating solution*	*Mode of storage*	*Tritium efficiencies (in %)*		*Backgrounds (counts/min)*	
			With tissues	*Without tissues*	*With tissues*	*Without tissues*
Methanol–toluene	Dioxane	Refrigerator	7	8	44	37
		room	7	8	35	34
	Dioxane–toluene	Refrigerator	7	9	39	36
		room	7	9	34	36
	Toluene	Refrigerator	8	10	33	36
		room	7	10	30	34
Dioxane–toluene	Dioxane	Refrigerator	10	12	60	41
		room	10	12	36	43
	Dioxane–toluene	Refrigerator	11	12	34	38
		room	11	12	45	38
	Toluene	Refrigerator	9	14	35	34
		room	9	13	33	37
Dioxane	Dioxane	Refrigerator	11	13	50	50
		room	11	11	46	46
	Dioxane–toluene	Refrigerator	11	13	54	39
		room	11	12	40	38
	Toluene	Refrigerator	11	12	44	36
		room	12	13	40	34

TABLE V

EFFICIENCIES FOR ^{3}H AND ^{14}C AND BACKGROUNDS AS FUNCTIONS OF THE AMOUNTS OF TISSUE DIGEST

Amount of tissues per vial (mg)	*^{14}C efficiencies (in %) H.T. = 5*	*Backgrounds (counts/min) H.T. = 5*	*Tritium efficiencies (in %) H.T. = 8*	*Backgrounds (counts/min) H.T. = 8*
0	50.4	22	17.6	58
0	50.1	22.	17.1	61
15.5	53.9	24	15.1	61
15.5	51.2	24	15.6	61
31	52.2	19	11.4	61
31	53.5	21	11.4	62
62	48.2	19	8.3	63
62	49.1	20	8.3	57
93	44.3	19	6.5	61
93	43.8	18	6.8	58
124	38.3	20	5.0	66
124	38.7	20	5.1	61
155	28.4	17	3.3	63
155	27.1	17	3.5	66

diluent reduced the efficiency by about 25%, but the three scintillating solutions were about equally good.

It was decided to use for the following experiments dioxane as diluent and toluene for the scintillating solution.

Quenching

In the above experiments with digests from intestines and stomachs, the efficiencies for ^{14}C and especially tritium were less than the maximum obtainable with the same scintillating solution (50% for ^{14}C, 17.5% for ^{3}H, see Table V). It seemed

worthwhile to investigate whether the quenching effect was due to the hyamine hydroxide solution, the digested tissues or both.

Therefore, 16 ml of 1 *M* hyamine hydroxide methanolic solution were warmed at 65° in the dark for 12 h before dilution and addition of the scintillating solution. The use of internal standards permitted determination of the efficiencies of counting under these conditions. The results are given in Tables III and IV where they can be compared with the results obtained when 55 mg of tissue was present in the vials. No significant difference can be noted with ^{14}C, but, for tritium, the yield was slightly decreased by the presence of the tissue digest.

It is thus evident that, for 55 mg of digested stomach and intestine, most of the quenching effect observed with tritium was due to the methanolic hyamine hydroxide solution and little to the tissue itself.

Influence of the amount of tissue

For this experiment, 4.2 g of mouse stomachs and intestines were digested in 16 ml of hyamine hydroxide for 12 h at 65° in the dark. The final volume was 17 ml.

Aliquots of 0, 0.5, 1, 2, 3, 4 and 5 ml were diluted to 40 ml with dioxane; 5 ml portions of these solutions were mixed with 10 ml of toluene scintillating solution and 1 ml of toluene or standard (^{14}C or ^{3}H) toluene solution in vials for counting. The vials thus contained respectively 0, 15.5, 31, 62, 93, 124 and 155 mg of mixed tissues and were in duplicate at each level. The assays were performed after the disappearance of the chemiluminescence. Backgrounds as well as yields for tritium and carbon-14 are recorded in Table V.

As expected, the efficiency for tritium decreased with the amount of digest added (tissue + hyamine solution). It can be seen that, without the quenching effect of these components, the yield was 17.5% and decreased to 3.5% when the vial contained the digest of 155 mg of fresh tissue.

TABLE VI

DIGESTION OF DIFFERENT TISSUES AND USED DILUTIONS

Kind of tissues	*Weight tissue digest (mg)*	*Amount of hyamine hydroxide solution (ml)*	*Volume after dilution*	*Amount of tissue per vial (mg)*
Stomach–intestine	1500	6	75	100
Lung	600	2.5	30	100
Heart	600	2.5	30	100
Brain	1500	6	75	100
Liver	1500	6	75	100
Muscle	1500	6	75	100

For ^{14}C, the result was nearly unaffected until the amount of digest reached 3 ml (around 100 mg of fresh tissue); at this level, the tritium counting efficiency had already decreased by a factor of nearly 3.

Influence of the kind of tissue

The various mouse organs tested were: digestive tract, brain, muscle, heart, liver and lung. They were digested with a number of ml of 1 *M* hyamine hydroxide in methanol equal to four times their weight in g (Table VI). The digest was diluted

with dioxane and the toluene scintillating solution was used for the preparation of the counting vial which contained 100 mg of digested tissue; 1 ml of toluene or toluene standard solution was added. Table VII gives the results of this experiment.

The organs can be divided into two main groups.

Stomach, intestine, brain and muscle form the first one. The efficiencies were around 40% for carbon-14 and 5% for tritium. The second group contains heart, lung and liver. Here the digest was deeply brown and the counting vials were highly coloured. The efficiencies were only around 25% for carbon-14 and 2–3% for tritium, about half of those obtained with the organs of the first group. Blood gave still lower results.

TABLE VII

EFFICIENCIES FOR ^{3}H AND ^{14}C WITH 100 mg OF DIFFERENT TISSUES PER VIAL

Kind of tissues	*Counting efficiencies (in %)*		*Backgrounds (counts/min)*	
	^{3}H (*H.T.* = 8)	^{14}C (*H.T.* = 5)	*H.T.* = 8	*H.T.* = 5
Stomach–intestine	5.6	40.8	58	20
	5.6	40.7		
Lung	2.1	22.1	68	61
	2.1	21.4		
Heart	3.2	28.9	79	58
	3.2	29.1		
Brain	5.2	40.7	70	82
	5.1	40.6		
Liver	2.1	21.8	81	70
	2.2	21.6		
Muscle	4.6	41.2	77	79
	4.7	41.5		

DISCUSSION

The present paper deals with the determination of total carbon-14 or tritium in animal tissues by liquid scintillation counting after digestion in hyamine base. To obtain complete digestion after 12 h warming at 65°, it is necessary to use an amount of methanolic 1 *M* hyamine hydroxide in ml equal to 4 times the wet weight of the organ in g; the fresh tissues are used without preliminary desiccation. To the digest are added successively the diluent and the scintillating solution; the sample in the vial ready for counting is termed the scintillating mixture.

In this method, not only the digestion of the tissues must be complete but also no precipitation must be allowed to occur when the diluent and the scintillating solution are added. Only a final clear solution in the counting vial can give a reliable result.

The diluents tried were methanol–toluene (1:1, v/v), dioxane–toluene (1:1, v/v) and pure dioxane. The scintillating solutions contain 10 g of 2,5-diphenyloxazole, 250 mg of 1,4-bis-2(5-phenyloxazolyl)-benzene and 100 g of naphthalene per liter of toluene, dioxane or a mixture of both (1:1, v/v). The counting was performed in a Tri-Carb automatic spectrometer provided with two photomultipliers and a coincidence circuit.

The chemical nature of the diluent and the solvent of the scintillating solution

has no effect on the yield of counting for carbon-14, but for tritium, methanol has a strong quenching effect and must be avoided. Pure dioxane is the best diluent, in spite of its unfavourable effect on chemiluminescence, because the solubility of polar components of the digest is increased and unwanted precipitation is avoided.

For the scintillating solution, toluene is the best solvent. The introduction of naphthalene is justified because it reduces the quenching effect [7] of the digest. The increased amounts of 2,5-diphenyloxazole and 1,4-bis-2(5-phenyloxazolyl)-benzene (respectively 6.25 g and 156 mg per liter of the final mixture instead of the usual 4 g and 100 mg) were suggested by the work of WERBIN *et al.* [2] for the determination of tritiated water.

For carbon-14, the problem of chemiluminescence can be practically ignored when a good hyamine hydroxide preparation is used even in the presence of dioxane (Merck, 9671, for chromatography). But, for tritium, chemiluminescence is a serious problem especially when dioxane is used. A 48-h storage period before the assay in the Tri-Carb counter is to be recommended; the storage can be done at room temperature without particular screening from light. It is worthwhile noting that the nuisance of chemiluminescence is considerably aggravated with a counter having a longer time constant in the coincidence circuit; it would be overwhelming with a counter utilizing a single photomultiplier without the security of such a circuit.

Different organs from mice have been tested. They may be divided into two groups: (1) stomach, intestine, brain and muscle; (2) liver, lung and heart.

For carbon-14, the introduction of the hyamine hydroxide tissue digest has no quenching effect for the organs of the first group until the amount per vial reaches 100 mg of fresh tissue. At this level, however, the quenching is by a factor of two for the organs belonging to the second group.

With tritium, we have already seen that methanol must be excluded from the diluent for optimum efficiency of counting. The addition of the digest causes an important quenching. For the organs of the first group, most of the quenching seems to be due to the hyamine hydroxide methanolic solution and little to the tissue itself. However, the tissues of the second category seem to quench strongly; the final solution is deeply coloured (brown), probably because the organs belonging to this group contain much blood.

The efficiencies given in the tables relate to assays performed in the Packard Tri-Carb spectrometer; the maximum yields observed with the scintillating mixture used in these experiments were 50% for carbon-14 and 17.5% for tritium.

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