

Solubilisation of Mammalian Tissue for Scintillation Counting

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INTRODUCTION

The problems facing the biologist in the preparation of biological samples for liquid scintillation counting are echoed in Hamlet's words: 'Oh that this too, too solid flesh would melt'.

There exists a plethora of commercially available solubilising agents, the main characteristic of many of them being their high cost. The need arose in our department to analyse large numbers of samples for Carbon-14-labelled material. The use of any of the commercial solubiliser systems would have led to an economic disaster in the department and the use of combustion techniques was impractical due to the large numbers of samples involved. We therefore decided to re-investigate some of the older published methods, taking as a basis sodium hydroxide digestion and the perchloric acid digestion method of Mahin and Lofberg.¹

PRELIMINARY STUDIES

It was intended to develop a method whereby large pieces of tissue could be digested and from the final digestion mixture portions could be taken for counting. If aqueous sodium hydroxide was used for this purpose and the tissue involved contained large amounts of fat, sodium soaps resulted and these were, as a rule, insoluble in the final digest. This problem was overcome by the addition of Triton and methanol to the digestion mixture resulting in a solubiliser (SHT) with the composition shown in Table 1.

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Table 1. SHT solubiliser.

Sodium hydroxide	80 g
Distilled water	600 ml
Methanol	300 ml
Triton X405 (75% aqueous solution)	100 ml

SHT may be counted, after preliminary neutralisation, in a toluene/triton scintillator of the type described by Patterson and Greene.² The composition of this scintillator is shown at the top of Table 2 and is referred to as tT21. We also devised a scintillator (S.20),^a the composition of which is shown at the bottom of Table 2, 10 ml of which would accept and neutralise 1 ml of a 2M aqueous alkali solution. Nonanoic and lauric acids are present in quantities just sufficient to neutralise the alkali. Dimethyl POPOP and PPO are used as the fluors rather than butyl PBD which shows a tendency to decompose in the presence of alkali. It should be noted that when SHT is used with scintillator S.20 the resulting solution does tend to chemiluminesce for 2 to 4 h.

Table 2. Composition of scintillators S.20 and tT21.

Scintillator tT21	
Toluene	1000 ml
Triton X100	500 ml
Butyl PBD	10.25 g
Scintillator S.20	
Toluene	600 ml
Triton X100	300 ml
Oxitol	30 ml
Nonanoic acid	20 ml
Lauric acid	12 g
Dimethyl POPOP	0.5 g
PPO	6.0 g

As mentioned previously, SHT when used with tT21 needs preliminary neutralisation with an acid. We tried several acids for this purpose and found that nitric acid gave the least quenching and had less tendency to cause precipitation. In the normal laboratory procedure we neutralised 1 ml of an SHT digest with 0.5 ml of 4.4M nitric acid. For the purposes of investigating the effect of excess acid on the counting efficiency of the SHT/tT21 system, we used 8M nitric acid.

It may be observed in Table 3 that the counting efficiency of SHT/tT21 is little affected by excess acid. The same is true of SHT tissue digests. Even if a sample received two additions of acid in error, the resulting counting efficiency would not be seriously affected. Table 4 shows the solubilising ability of SHT, in conjunction with the two scintillators already described. It also shows the stability of the tissue digest/scintillator cocktail combination. tT21 will accept up to 500 mg of homogenate, i.e. 250 mg wet weight of tissue, and give a stable counting solution. Scintillator S.20, however, has a lower capacity, 300 mg of homogenate, i.e. 150 mg of tissue. As would be expected, efficiencies decrease with increasing tissue concentration. However, 500 mg of

^a Composition based on unpublished results of Dr. C.G. Raison.

Table 3. Effects of excess nitric acid on counting efficiency of SHT/tT21.

Acid (ml)	Excess acid (%)	Water (ml)	Efficiency (%)	
			³ H	¹⁴ C
0.25	0	0.25	19.5	85.0
0.3	20	0.20	19.5	85.0
0.4	60	0.10	15.5	82.0
0.5	100	0.00	12.5	78.0

1 ml SHT + 8M nitric acid, water and 10 ml tT21. Spiked with ¹⁴C hexadecane or ³H hexadecane.

Table 4. Solubilising ability of SHT

Weight homogenate (mg)	Stability 3 days		Counting efficiency (%)			
			³ H		¹⁴ C	
	tT21	S.20	tT21	S.20	tT21	S.20
100	yes	yes	14	23	80	85
200	yes	yes	10	19	74	84
300	yes	yes	9	16	71	82
350	yes	no	9	14	71	81
400	yes	no	7	13	68	80
500	yes	no	4	11	61	75

Aqueous rat liver homogenate (1:1) was solubilised in 1 ml SHT and counted in 10 ml of scintillator.

liver may be counted in 20 ml of tT21 at a Carbon-14 efficiency of 60%. A more extensive description of the practical problems with and limitation of SHT, when used as a routine laboratory method, will be described elsewhere.³

The general performances of SHT solubilisation and perchloric acid digestion followed by treatment with hydrogen peroxide were compared with four commercial products: NCS (Searle), Protosol (New England Nuclear), Soluene 100 (Packard) and Biosolve (Beckman). At the time this study was undertaken Soluene 350 (Packard) and TS1 and TS2 solubilisers (Koch Light) were not available.

METHODS

The initial comparisons were carried out as described below.

Tissues were removed from rats and prepared for solubilisation in triplicate both as tissue snips of 100 ± 5 mg and homogenates (1:1 aqueous) of 200 ± 10 mg. Where commercial solubilisers were used the manufacturer's recommended methods were followed without modification. Solubilisation was brought about in an ultrasonic cleansing bath (Kerry Pulsatronic K901), the vials being held in a wire cradle in strong detergent solution (5% Lab Brite). The samples were visually assessed at regular intervals and when they had been completely solubilised, scintillator was added. The samples were spiked and counted in an Intertechnique SL40 ABAC 4K scintillation counter.

Details of the methods and of the solubiliser/scintillator combinations used are shown in Table 5. In all cases except SHT/S.20 a digestion temperature of 50°C was used. With SHT/S.20 it was found that a digestion temperature of 70°C was necessary to ensure that the sample did not precipitate out in the scintillator

Table 5.

Solubiliser	Volume of solubiliser	Scintillator	Extra steps	Time for satisfactory solubilisation (h)
NCS	1 ml	10 ml toluene butyl PBD (7.5 g/litre)	Add 30 µlitres glacial acetic acid after addition of scintillator	2
Solvent	2 ml	10 ml toluene, Me ₂ POPOP 100 mg/litre PPO 6.0 g/litre		7
BBS	1 ml 2M NaOH	12 ml toluene/BBS3 (5:1) butyl PBD 6.25 g/litre	Add 2.5 ml BBS2 to NaOH digest prior to addition of scintillator	0.5
Protosol	1 ml	10 ml toluene Me ₂ POPOP 100 mg/litre PPO 6 g/litre 16 ml tT21		22
Perchloric acid	0.4 ml HClO ₄ 0.8 ml 60% H ₂ O ₂			0.5
SHT	1 ml	10 ml tT21		0.5
SHT	1 ml	10 ml S.20	Neutralise with 0.5 ml 4M HNO ₃	0.5

Table 6. Mean counting efficiency (%) of Carbon-14 for solubilised rat tissues.

Solubilised tissue	NCS	Soluene 100	BBS	HClO ₄	Protosol	SHT	SHT/S.20
Brain	86.9 [±] 0.6	70.7 [±] 18.7	67.4 [±] 1.3	78.3 [±] 1.0	87.4 [±] 1.5	79.7 [±] 4.3	82.4 [±] 2.3
Skin	84.1 [±] 1.7	87.7 [±] 2.4	72.4 [±] 6.4	80.1 [±] 6.1	77.3 [±] 2.1	76.1 [±] 1.6	82.3 [±] 2.2
Lung	61.9 [±] 5.6	45.9 [±] 14.0	63.1 [±] 2.4	82.5 [±] 3.7	51.6 [±] 13.2	57.3 [±] 4.1	76.1 [±] 3.3
Heart	81.5 [±] 3.9	66.7 [±] 9.7	66.2 [±] 4.0	77.7 [±] 1.4	66.1 [±] 12.8	70.5 [±] 1.7	79.9 [±] 2.8
Liver	70.1 [±] 3.7	47.0 [±] 14.9	60.6 [±] 1.6	74.9 [±] 0.7	41.0 [±] 20.5	65.5 [±] 3.6	80.3 [±] 3.8
Spleen	17.7 [±] 1.3	15.8 [±] 5.0	46.6 [±] 2.2	75.6 [±] 2.2	15.7 [±] 6.6	29.7 [±] 11.5	63.9 [±] 2.6
Kidney	72.2 [±] 0.9	46.3 [±] 10.6	53.8 [±] 16.3	73.9 [±] 1.2	58.9 [±] 12.1	60.7 [±] 2.9	77.8 [±] 2.0
Intestine	87.0 [±] 1.5	74.9 [±] 11.3	67.2 [±] 7.4	78.5 [±] 2.0	80.2 [±] 1.5	79.4 [±] 3.6	81.5 [±] 1.3
Muscle	87.0 [±] 3.0	67.1 [±] 14.6	65.9 [±] 3.9	78.1 [±] 1.3	79.1 [±] 4.2	78.8 [±] 2.0	81.5 [±] 0.8
Adipose	90.3 [±] 1.8	77.2 [±] 19.6	78.4 [±] 1.3	81.3 [±] 1.5	68.7 [±] 22.2	82.1 [±] 2.9	83.7 [±] 2.8
Blood	8.0 [±] 0.4	5.1 [±] 4.4	4.7 [±] 1.6	77.2 [±] 1.1	4.7 [±] 2.7	2.5 [±] 0.3	12.6 [±] 1.0

Results are the means of six determinations [±] S.D.

Table 7. Mean counting efficiency (%) of Hydrogen-3 for solubilised rat tissues.

Solubilised tissue	NCS	Soluene 100	BBS	HClO ₄	Protosol	SHT/tt21	SHT/S.20
Brain	32.1 [±] 2.9	6.1 [±] 0.8	13.9 [±] 0.8	11.4 [±] 0.6	23.2 [±] 0.8	15.9 [±] 0.9	18.5 [±] 0.9
Lung	14.6 [±] 3.4	9.6 [±] 6.8	6.3 [±] 2.6	6.4 [±] 0.8	13.7 [±] 1.6	7.8 [±] 1.2	9.1 [±] 1.7
Liver	13.2 [±] 5.7	4.8 [±] 0.4	10.3 [±] 0.9	8.1 [±] 0.4	15.9 [±] 1.4	6.4 [±] 0.3	11.1 [±] 0.2
Spleen	2.4 [±] 0.5	2.0 [±] 0.3	2.2 [±] 0.9	7.6 [±] 0.9	4.2 [±] 0.1	3.1 [±] 1.9	4.5 [±] 1.4
Blood	0.4 [±] 0.1	1.6 [±] 0.1	0.1 [±] 0.1	9.1 [±] 0.4	0.5 [±] 0.1	0.1 [±] 0.1	0.2 [±] 0.1
Stomach and contents	1.1 [±] 0.1	2.6 [±] 0.5	6.1 [±] 0.9	8.8 [±] 0.9	8.7 [±] 1.4	2.3 [±] 0.1	7.3 [±] 0.9
Caecum and contents	1.2 [±] 0.1	2.4 [±] 0.1	4.6 [±] 0.6	9.6 [±] 0.5	8.4 [±] 0.1	2.8 [±] 0.4	8.3 [±] 1.2

Results are the means of six determinations [±] S.D.

cocktail. It can be seen that those methods involving NaOH, namely SHT and BBS, needed only 30 min for complete solubilisation as did perchloric acid. Whereas NCS needed 2 h, Soluene required 7 h and Protosol 22 h. The Protosol digestions were not carried out entirely in the ultrasonic bath. The first 7 h were in the bath and the rest of the time in an oven at 50°C.

CARBON-14

Table 6 shows the mean Carbon-14 counting efficiencies for solubilised rat tissues. In each case the results are the means of six determinations, three for homogenates and three for tissue snips. We did not find any significant difference between the counting efficiencies of tissue snips as compared with homogenates. However, in those cases where long digestion periods were necessary, the homogenates tended to solubilise more rapidly than the tissue snips. No appreciable differences were observed in the efficiencies with the various methods. In general, as would be expected, white tissues such as brain gave higher counting efficiencies than red tissues. The range for white tissues was 67-87% and the range for red tissues, such as liver, was 41-80%. BBS and perchloric acid gave the most consistent counting efficiencies and Soluene 100 exhibited the highest variations in counting efficiencies of individual tissues, as shown by the standard deviation.

HYDROGEN-3

Table 7 shows the mean Hydrogen-3 counting efficiencies of tissue digests. The results are the means of six determinations, three for homogenates and three for tissue snips. None of the solubilisers completely digested the fibrous material which is found in the rat stomach and caecum, as a result of digested food. As with Carbon-14, red tissues quenched most, white tissues quenched least but there were no general trends in the results. NCS and Protosol, however, gave the highest counting efficiencies, but perchloric acid gave the most consistent counting efficiency over the range of tissues.

IN VIVO STUDIES

The use of some of the solubilisers with labelled tissues obtained after *in vivo* dosing was investigated. The methods chosen for this investigation were SHT/tT21, SHT/S.20 and perchloric acid, the three cheapest methods, as may be seen from Table 10. NCS was also included as the cheapest commercial solubiliser and the one with which we had the most experience.

To obtain labelled tissues intraperitoneal injections of labelled glucose were administered to four rats, using Carbon-14 or Hydrogen-3. The rats were killed after one hour and tissues were removed and pooled. Homogenates of the tissues (1:1 aqueous) were prepared and then 200 mg homogenate were solubilised in triplicate by each method. We also combusted portions of each tissue. For Carbon-14-labelled samples a tube furnace was used and in the case of Hydrogen-3 a Model 305 Packard oxidiser was used. The radioactivity content of each sample in d.p.m. was computed by the external standard channels ratio method using quench curves prepared from biological samples.

The levels of Carbon-14 in tissues from rats dosed with D-(U-¹⁴C) glucose as measured by the different methods are shown in Table 8. The levels of Hydrogen-3 from rats dosed with D-(6-³H) glucose are shown in Table 9.

The solubilisation methods generally give higher results than combustion for Carbon-14 and lower results than combustion for Hydrogen-3. It would appear that the more quenched the samples, i.e. the more coloured the tissue, the greater

Table 8. Assay of Carbon-14 in tissues from rats dosed with D-(U-Carbon-14) glucose.

Tissue	Solubiliser				Furnace combustion
	SHT/tT21	SHT/S.20	HClO ₄	NCS	
Liver	27600 ±310	26300 ±230	26900 ±550	25300 ±700	24500 ±650
Heart	2900 ±50	3000 ±30	2600 ±40	2700 ±40	2700 ±130
Brain	5000 ±190	4900 ±80	4700 ±230	4800 ±10	4900 ±210
Lung	2700 ±50	2800 ±50	2600 ±10	2600 ±30	2000 ±10
Muscle	5700 ±160	5800 ±140	4700 ±260	6100 ±290	5500 ±510
Blood	2400 ±100	2300 ±160	2700 ±160	2000 ±90	2800 ±170
0.1 ml Plasma	13500	13500	12700	13400	13300
0.5 ml	±30	±90	±10	±240	±520

d.p.m./100 mg tissue ± S.D. of mean.

Table 9. Assay of Hydrogen-3 in tissues from rats dosed with D-(6-Hydrogen-3) glucose.

Tissue	Solubiliser				Packard oxidiser
	SHT/tT21	SHT/S.20	HClO ₄	NCS	
Liver	129700 1400	128900 2300	141800 2800	134900 8500	152900 2600
Heart	51300 500	45500 900	59200 2600	34000 1500	55600 900
Brain	70600 420	66300 1000	72600 1500	61300 3100	68800 2800
Lung	50100 1800	44800 1500	57200 1600	39600 900	55600 900
Muscle	58000 2000	55000 350	58700 1500	50000 1600	59000 900
Blood	55300	58300	69200	—	70700
0.1 ml Plasma	3600	1600	3000		1400
0.5 ml	7700	5600	4100	6700	27300

d.p.m./100 mg wet weight tissue ± S.D. of mean.

the difference between the results obtained by solubilisation as compared with combustion. This was initially taken to indicate a possible inaccuracy in the quench curve.

However, we digested samples of the *in vivo*-labelled liver with all the solubilisers and counted them, using the internal standard technique to calculate d.p.m. The results obtained in this way were identical to those obtained with a quench curve.

We also incubated a liver homogenate with D-(1-¹⁴C) glucose and subsequently

assayed portions of this for Carbon-14 activity by each of the five methods; no significant differences were observed in the results obtained by each method. The present observations do not allow a satisfactory explanation of the discrepancies observed with in vivo-labelled tissues.

COST OF METHODS

Perchloric acid and SHT in conjunction with either tT21 or scintillator S.20 are much cheaper than the other methods. The most expensive of these three methods, perchloric acid, costs one-third of the price of NCS, the cheapest commercial product.

Table 10. Cost comparison of solubilisers (costs in £ for 100 samples (100 mg tissue)).

Solubiliser	Cost		
	Solubiliser	Scintillator	Total
BBS	7.5	6.93	14.43
Protosol	9.60	1.15	10.75
Soluene 100	5.16	1.15	6.31
NCS	4.80	0.93	5.73
HClO ₄	0.20	1.66	1.86
SHT/S.20	0.20	1.54	1.74
SHT/tT21	0.20	1.04	1.24

SUMMARY

Experiments using different solubilisers indicated that different methods gave different counting efficiencies. It is apparent that none of the methods investigated are generally superior to any other except in terms of time and cost. The assays of in vivo-labelled tissue indicate that the results obtained by solubilisation differ from those obtained by combustion, in some cases by up to 12% for Carbon-14 and by up to 40% but more generally about 15% for Hydrogen-3. These discrepancies may be due to different interactions between the various solubilisers and metabolites of glucose.

Table 11 shows the ideal qualities of a solubiliser. We believe the three inexpensive systems described conform to these requirements as well as any of the commercial systems and a good deal better than some. They are certainly cheaper than all the commercially available solubilisers. Dr. Gordon has commented that only combustion is really reliable but he also agrees that with large

Table 11. Requirement for solubiliser.

1.	Inexpensive in large quantities
2.	May be used with an inexpensive scintillator
3.	Will digest all small animal tissues
4.	Gives a stable solution with scintillator cocktail
5.	Gives 'good' and reproducible counting efficiency
6.	Ideally should be suitable for Carbon-14 and Hydrogen-3
7.	Solubilises rapidly
8.	Minimal number of steps involved in sample preparation
9.	Sample preparation should not require great precision or accuracy

numbers of samples combustion is impractical. Certainly we have shown that with some types of samples and some solubilisers agreement with combustion methods is very good but with other samples there can be large discrepancies, for reasons as yet unknown.

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REFERENCES

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- 2 M.S. Patterson and R.C. Greene, *Analyt. Chem.* **37**, 854-857 (1965).
- 3 J.G. Dent and P. Johnson, in preparation.

DISCUSSION

T.J. Rising: I noticed that your d.p.m. values for Carbon-14 for HClO_4 digestions were low when compared to other solubilisation and combustion methods. Could this possibly be due to the evolution of $^{14}\text{CO}_2$, since the tritium values from HClO_4 digests were comparable with those from other techniques?

J.G. Dent: Yes it could be loss of CO_2 . We investigated this possibility by digesting liver homogenates (after dosing ^{14}C -glucose) under conditions where we were able to collect any $^{14}\text{CO}_2$ in phenylethylamine. With perchloric acid digestion, 2-4% of the estimated total activity in the sample appeared in the phenylethylamine. This could explain the lower Carbon-14 results obtained with perchloric acid.

P. Johnson: I would add that we do not claim to have solved other peoples' chemical problems. The onus is on the worker concerned to prove that with his given material in a given solubiliser system he is not getting loss of carbon dioxide or other volatile components. Also the hazard exists not only with perchloric acid, and we have experienced one compound which underwent a small loss of labelled carbon dioxide when treated with sodium hydroxide. If volatility problems are experienced, they can be overcome by use of a 'sealed' vial as described at the last Brighton Symposium {P. Johnson, in *Liquid Scintillation Counting*, Vol. 2, (Eds. M.A. Crook, P. Johnson and B. Scales) Heyden & Son, London, 1972, p.241}.

K. Painter: Neutralisation with glacial acetic acid is preferable to either nitric acid or hydrochloric acid as it buffers the cocktail; titration of each sample is not necessary. Workers should be cautioned against perchloric acid wet digestion techniques as there are many reports of serious explosions in the literature.

J.G. Dent: Concerning Dr. Painter's point about the choice of acid for neutralisation, we have tried a whole range of acids including glacial acetic and in our experience nitric acid is preferable. It does not tend to cause precipitation and gives the most stable samples in the T21 system.

B.W. Fox: If the Mahin Hofberg procedure is carried out on wet or dry samples, i.e. 20 mg sample + 0.2 ml perchloric acid (70%) and 0.4 ml H_2O_2 heated to not more than 80°C (usually 60°C), then we have not obtained any explosions in many

such measurements. If one exceeds 100°C , chlorine peroxide is formed and explosions occur, usually on handling the vial.

P. Johnson: Our experience agrees with that of Dr Fox, although one should always treat perchloric acid with respect.

B. E. Gordon: Wet oxidation with HClO_4 is safe if preceded by complete pre-oxidation with $\text{H}_2\text{SO}_4/\text{HNO}_3$ until a clear solution is obtained. Then 0.1 ml 72% HClO_4 added to the cooled solution followed by heating to boiling H_2SO_4 will complete the oxidation.