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A Combustion Technique for the Assay of Tritium, ^{13}C and ^{14}C in a Single 10 mg. Sample of Biological Material

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Payne, Campbell & White (1952) have recently described their method for the quantitative combustion of 500–600 mg. samples of tritiated metabolites. This scale is dictated by the method they prescribe for the determination of tritium, namely the reaction of 100–200 mg. of water with aluminium carbide and the counting of the tritiomethane so produced in a proportional gas counter. As it is not always easy or even possible to isolate as much as 0·5 g. of a particular metabolite other workers may be interested to know that there was recently published (Glascock, 1951*a, b*) a method for determining tritium on the 5–10 mg. scale. Sufficient water for the determination of tritium by this method and sufficient carbon dioxide for the determination of both ^{13}C and ^{14}C are produced by the quantitative combustion of about 10 mg. of most organic substances. A method for doing this has been in routine use in this laboratory for the past two and a half years, and a description of it now seems appropriate in view of the communication by Payne *et al.* (1952). It is very much easier to burn 10 mg. quantitatively than 0·5 g. and thus obtain from 10 to 20 ml. of carbon dioxide. This is usually more than enough for the gas counting of several samples by the method of Brown & Miller (1947) and a few ml. can readily be spared for the mass spectrometer if ^{13}C is to be determined also. Then, if the compound is one which yields relatively little carbon dioxide, either a rather larger sample is burnt or the dose of ^{14}C is adjusted in advance so that smaller samples can be counted, thus leaving more for the spectrometer. The water produced usually amounts to about 10 mg. and it is treated with an excess of *n*-butyl magnesium bromide in a previously prepared 'Grignard tube' to yield *n*-tritiobutane almost quantitatively (Glascock, 1951*a, b*). If, for some reason, tritiomethane were preferred it could probably be prepared on this scale by using the appropriate Grignard reagent but butane has several important advantages. Firstly, it is an excellent counter gas and can be counted with high efficiency in the Geiger region of a suitable gas counter; hence the additional electronic equipment required for proportional counting as used by Payne *et al.* is unnecessary. Secondly, unlike methane, butane is fully condensed at the temperature of liquid oxygen and samples of gas can be easily measured out and quantitatively transferred. In fact it is possible to measure out and transfer to the counter between 10 μl. and 10 ml. of gas, thus permitting the counting of samples of widely differing specific activities. Tritium is available in very high activities, much higher, for example, than is ^{14}C, and it is therefore very easy to arrange the dose so that 10 mg. samples of metabolite have a suitable activity.

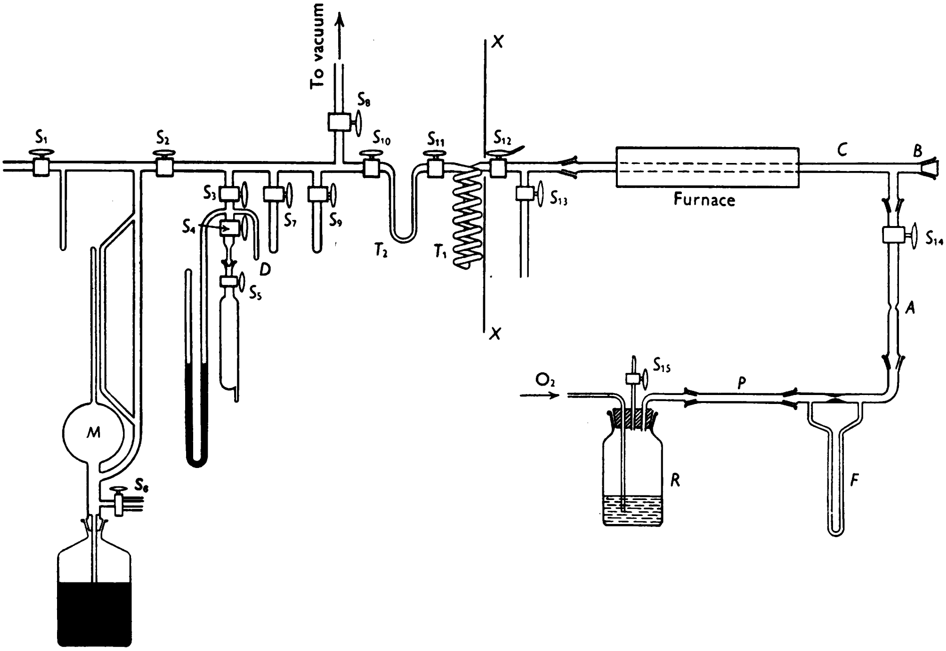

Fig. 1. Vacuum apparatus and combustion train. S_1 to S_{15} = stopcocks; M = McLeod gauge; C = part of combustion tube in which boat is placed; B = stoppered joint through which boat is introduced into combustion tube; T_1 = spiral trap; T_2 = U-trap; A = tube of Anhydrone and soda-asbestos; F = flowmeter; P = tube of platinized asbestos; R = oxygen reservoir.

EXPERIMENTAL

Apparatus. The combustion apparatus is shown in Fig. 1. It differs from the conventional micro-analytical apparatus in two important respects. Firstly, the products of combustion are not collected chemically, but are stripped from the O_2 stream in liquid-O_2-chilled traps (cf. Glascock, 1951*b*; Balmain, Folley & Glascock, 1951). Secondly, since a condensable component of a gas mixture is stripped out most efficiently at low pressures, the conditions are arranged so that the pressure in the traps is only a few mm. Hg.

The whole apparatus, with the exception of the quartz combustion tube, is made of Pyrex glass. The combustion tube itself is 50 cm. long with an internal diameter of 10 mm. and is fitted with B10 ground joints as shown in Fig. 1. The 30 cm. of its length within the furnace are packed with the standard CuO filling. It is maintained at a temperature of 600°, being switched on automatically in the morning, a few hours before work is due to start. The chilled traps, T_1 and T_2, consist of a spiral and a simple U-tube, respectively. The spiral comprises 50 cm. of 4 mm. bore tubing and condenses out the greater part of the combustion products, the rest being trapped in T_2.

The apparatus to the left of the line X–X is kept on open pumps and, by adjusting the stopcock S_{12}, O_2 is drawn through the combustion train from the reservoir R at about 50 ml./min. Thus the pressure in the chilled traps does not rise above about 2 mm. Hg. S_{12} is a 'control' stopcock, that is, one with a long handle to the plug and shallow file cuts on opposite sides of the bore. It is a convenient device for controlling gas streams accurately at low rates and has been described before (Arrol & Glascock, 1948).

The O_2 is fed into the reservoir R from a cylinder, is roughly dried by bubbling through conc. H_2SO_4 and then runs mostly to waste through the stopcock S_{15}. The O_2 drawn into the combustion tube passes first through hot platinized asbestos P (to oxidize any combustible gases), a simple flow meter F, and a tube packed half with soda-asbestos and half with Anhydrone A. Chemical rather than physical purification of the gas is preferable since several chilled traps would be needed to strip out contaminating water and CO_2 at atmospheric pressure. When a combustion is not being carried out the O_2 stream is diverted to waste through the stopcock S_{13} thus passing through the hot combustion tube.

The sample is weighed into a porcelain boat and placed in that part, *C*, of the combustion tube which is outside the furnace. O_2 is passed through the tube and the sample heated gently in a luminous flame until it starts to burn. It is then heated strongly for 5 min. in a Meker burner to complete the combustion. At the end of this time, which is ample for the quantitative combustion of 10 mg. samples, the stopcock S_{14} is closed and the control stopcock S_{12} opened wide, thus evacuating the combustion tube and drawing residual gas through the traps. The control stopcock is then closed and the traps evacuated to a very low pressure (mercury-vapour pump).

In checking this method (see Tables 1 and 2) the criterion of quantitative combustion has been the yield of CO_2, which can be measured manometrically with an error not exceeding 1%. It has been found that more than 25 mg. cannot always be burnt quantitatively owing to too vigorous firing; mixing the sample with about its own weight of CuO catalyst, however, permits a more controlled combustion of up to 100 mg. The contents of the traps T_1 and T_2 are combined in T_2 by allowing the first to warm to room temperature with the stopcock between them open and, by raising the temperature of trap T_2 from − 183° to − 78° (dry ice and acetone), the CO_2 is sublimed away from the water and is transferred to the calibrated space between stopcocks S_3 and S_4, measured and stored until required in a side arm of which two, attached to stopcocks S_7 and S_9, are shown. Any traces of contaminating tritium activity are eliminated

Table 1. *Quantitative combustion of substances of known composition*

(All weighings carried out on Oertling air-damped aperiodic balance. 1 div. = 0·2 mg.)

Substance	Wt. combusted (mg.)	CO_2 at N.T.P. (ml.) Found	Calc.
Palmitic acid, $C_{16}H_{32}O_2$	10·4	14·50	14·50
Palmitic acid, $C_{16}H_{32}O_2$	8·6	11·80	12·00
Stearic acid, $C_{18}H_{36}O_2$	5·8	8·10	8·20
Stearic acid, $C_{18}H_{36}O_2$	8·0	11·05	11·30
Glucose, $C_6H_{12}O_6$	12·3	9·20	9·20
Glucose, $C_6H_{12}O_6$	13·4	10·00	10·00

Table 2. *Reproducibility of results*

(Sample combusted and assayed was calcium salts of mixed total fatty acids isolated from mammary-gland slices after incubation *in vitro* in bicarbonate-Ringer containing [^{14}C]glucose and doubly labelled acetate ($CH_2{}^3H.{}^{13}COONa$) as substrate. (Unpublished results of Balmain, Folley & Glascock.))

A. Triplicate combustions: yield of CO_2

Combustion no.	Weight combusted (mg.)	CO_2 obtained at N.T.P. Total (ml.)	(ml./mg. salt)
1	13·1	13·60	1·037
2	8·8	9·05	1·029
3	11·1	11·40	1·029

B. Assay of products (tritiobutane first being prepared from combustion water)

Gas assayed	Combustion no.	Vol. counted (ml. at N.T.P.)	Activity (counts/min.)	Specific activity (counts/min./ml.)
Butane	1	2·60	429	165·0
		3·10	500	161·0
	2	2·20	358	162·5
		3·15	504	160·0
	3	2·80	447	159·5
		2·50	399	159·5
Carbon dioxide	1	4·35	1883	433·0
		2·95	1297	440·0
	2	2·80	1187	424·0
		1·90	828	436·0
	3	0·88*	387	440·0
		3·70	1576	426·0
			Total ^{13}C abundance (atom %)	Excess ^{13}C (atom %)†
Carbon dioxide	1	—	1·230	0·140
			1·229	0·139
	2	—	1·232	0·142
			1·231	0·141
	3	—	1·233	0·143
			1·228	0·138

* Measured in McLeod gauge.

† Experimental value for natural abundance of ^{13}C found to be 1·09 atoms %.

from the CO_2 before counting by adsorption on active charcoal at − 78° and desorption at 0°. Both processes take place very rapidly and cause negligible delay in the course of an assay. The water, which remains in trap T_2, is distilled *in vacuo* into a previously prepared 'Grignard tube' for the generation of butane and assay of tritium.

As the actual combustion takes only 5 or 6 min. the time taken for the assay of each sample depends chiefly on the speed of counting and the time needed to clear one batch of isotopic gases from the apparatus to make room for the next.

The presence of combined nitrogen in the sample interferes with the assay of none of the isotopes. Thus, separate samples of glyceryl tri-*p*-nitrobenzoate labelled with ^{14}C and tritium, respectively, have been successfully assayed.

Measurement of gas samples. In the calibrated space, S_3–S_4, volumes of gas between 1 and 15 ml. can be measured manometrically, with an error of less than 1%. In the McLeod gauge, M, volumes between 10 µl. and 1 ml. can be measured with about the same accuracy. The reservoirs of inactive butane and CO_2 (2 l. bulbs), not shown in the diagram, communicate with the space S_3–S_4 and are used to adjust the pressure of the gas in the counter to its working value after condensing in a measured sample of radioactive gas. In routine use it has been found that two samples usually give closely agreeing specific activities (see, for example, Glascock, 1951 *a*, *b*; and Table 2). If the sample to be counted is less than 1 ml. it is convenient to fill the counter with inactive gas, count the background, and then, after re-attaching to the vacuum apparatus, add the sample to be counted. Counters filled with either CO_2 or butane have such good characteristics that the slight increase in pressure introduces no measurable error.

Elimination of contamination. There has been much reference in the literature to the errors arising from contamination of silica and glass apparatus by hydrogen isotopes. No such contamination has been detected after combustion of radioactive samples by the method and on the scale described in this paper. Thus no tritium or ^{14}C activity was found when a sample of inactive glucose was combusted and assayed immediately after a 12 mg. sample of fatty acid containing a total of 1150 counts/min. of tritium and 3300 of ^{14}C. This may be due partly to the fact that a stream of O_2 is continuously passed through the hot combustion tube whether it is in use or not. As an additional precaution the two traps are flamed out under high vacuum with the luminous flame of a hand torch between combustions.

DISCUSSION

Solid and gas counting: sample size. When the stable isotope ^{13}C is to be determined, combustion to carbon dioxide cannot be avoided since only gaseous substances can be assayed in the mass spectrometer. Compounds containing ^{14}C, however, can be counted directly either by means of a thin end-window counter or in a flow-type counter. Although the direct counting of organic substances is very convenient and widely used, the method suffers from certain disadvantages: firstly, not all compounds can be spread on a disk to form an even layer giving reproducible counting rates; secondly, some loss of accuracy is involved in comparing activities when the radioactive isotope is combined in compounds of markedly different chemical composition. Many workers therefore prefer to reduce all their organic samples to a common chemical form, usually barium carbonate. This is accomplished by combustion and absorption of the carbon dioxide in barium hydroxide. Its activity as measured with an end-window counter is compared with that of a standard barium carbonate sample of known activity which is counted at the same time.

An elegant method for counting very thin samples of fatty acids spread over a large area (from 5 to 30 mg. on 15 sq.cm.) by deposition from solution on a lens paper has been described by Entenman, Lerner, Chaikoff & Dauben (1949). This is applicable, however, only to certain substances, and for general work most workers prefer to count samples at infinite thickness, which for ^{14}C is about 20 mg./sq.cm. (Kamen, 1947), since this eliminates the correction for self-absorption and consequent errors. Disks of 2 or even 3 sq.cm. area are commonly used since the larger the disk the relatively smaller the 'edge effect'. Thus, allowing for transfer losses, it is inconvenient if less than 75–100 mg. of sample are available for solid counting at infinite thickness. This is to be contrasted with the 10 mg. which are sufficient for the combustion method described in this paper; for although 100 mg. of metabolite are easier to obtain than 0·5 g. it is often still more convenient if much less can be accurately assayed. For example, not more than about 80 mg. of mixed fatty acids may be obtainable from the liver of a single rat and it is sometimes necessary to fractionate this quantity into several components. Similarly, the quantity of fatty acids obtainable from 1 g. of mammary tissue used in a tissue-slice experiment is only 30–40 mg. and is too little to count conveniently in the solid state.

Counter efficiency: dose levels. For the counting of β-particles from ^{14}C the efficiency of a gas counter is at least 80%. The efficiency of an end-window counter mounted in the conventional lead castle (i.e. about 5 mm. from the source) is reduced by more than a factor of 10 as a result of geometry and absorption by the window and there is a further loss due to self-absorption by the sample. For example, it was found that a sample of fatty acid spread at infinite thickness on a disk of area 1 sq.cm. gave 100 counts/min. when measured with an end-window counter (EHM 2). The same sample combusted and the whole of the carbon dioxide introduced into a gas counter had an activity of 4200 counts/min. There is thus a gain in efficiency by a factor of 42. The corresponding gain for glucose is 56. The experimental results from which these values are calculated are given in Table 3.

If an organic compound labelled with ^{14}C has an activity at infinite thickness of A counts/min. it can be shown that if the carbon in it were isolated and counted at infinite thickness it would have an activity of $100A/BC$ counts/min., where C is the percentage of carbon in the compound and B is the backscattering factor. For compounds containing only light elements B has the value of 1·04 (Calvin, Heidelberger, Reid, Tolbert & Yankwich, 1949), and for purposes of comparison of activities it can therefore be neglected. For compounds containing heavy elements, however, B is larger and must be taken into consideration. Thus the value for $BaCO_3$ is 1·30.

Workers who prefer to count uncombusted compounds at infinite thickness claim that this formula gives a measure of the specific activity of the carbon to within 5 or 6 % providing care is taken to spread the sample so that a uniform surface is obtained. Other workers find that the counting of uncombusted samples is subject to inaccuracies greater than this. For example, Abrams & Clark (1951) say, 'We have been able to get ^{14}C counts accurate to within 5 % only by completely combusting the sample and counting it as an infinitely thick layer of $BaCO_3$. In attempting to count thin layers of uncombusted compounds we have observed inaccuracies as high as 30 %.'

Table 3. *Comparison of ^{14}C solid counting at infinite thickness and gas counting of $^{14}CO_2$*

(For details of calculation see text.)

Substance	Activity of 20 mg. on 1 cm. disk (X) (counts/min.)	Vol. CO_2 from combustion of 20 mg. (V) (ml. at N.T.P.)	Specific activity of CO_2 (Y) (counts/min./ml.)	Total activity of V ml. (Z) (counts/min.)	Ratio Z/X = gain in efficiency	(Calc.) activity of C at infinite thickness ($=100A/BC$) (N) (counts/min.)	Ratio N/Y
Ca salts of mixed fatty acids containing ^{14}C	2439	24·6	4150	102 000	42	3620	0·87
[^{14}C]Glucose	120	15·0	450	6760	56	288	0·64
$Ba^{14}CO_3$	26	2·28*	320	730	28	329	1·03
$Ba^{14}CO_3$	2204	2·28*	29 300	66 700	30	29 000	0·99

* Liberated with acid.

The results in Table 3 are presented chiefly to demonstrate the much greater efficiency of gas counting compared with solid counting, and are not represented as the result of a rigorous comparative study of the two methods. It will, however, be noted that the ratios of the observed activity of the gas samples to the calculated activities of the carbon at infinite thickness are not the same for the few compounds listed. This suggests that, for these compounds in the particular equipment used, solid counting suffers from inaccuracies of the same order as those observed by Abrams & Clark (1951) and that a more detailed comparison of the two methods might be profitable. It is to be noted, moreover, that if direct counting of organic samples is rejected as insufficiently accurate for a particular purpose then it is considerably easier to count carbon dioxide in a gas counter than to prepare barium carbonate samples for solid counting.

The high efficiency of gas counting shown in these results means that if metabolites are to be assayed by this method instead of by solid counting the dose of isotope used can be reduced by a factor of 10 or more. This represents a considerable saving of money as well as a tenfold reduction in health hazards and the precautions needed to combat them. This reduction in dose is also of considerable advantage in experiments where a physiologically excessive amount of labelled substrate would be required to produce metabolites sufficiently active for solid counting.

Flow counters: assay of tritium. Windowless flow-type counters have been described for the more efficient counting of solid samples. The sample is put inside the counter which is operated with a gas continually passing through it at atmospheric pressure. If helium containing a suitable quenching vapour is used the counter can be operated in the Geiger region (Kelsey, 1949; Damon, 1951). If methane is used the counter is operated in the proportional region (Eidinoff & Knoll, 1950; Sharpe & Taylor, 1951).

There is obviously a considerable gain in efficiency by this technique since window-absorption is eliminated and there is a more favourable geometry. It is particularly significant if solid tritiated compounds are to be counted since it is virtually impossible to make a window transparent to such low energy β-particles. Eidinoff & Knoll (1950) describe the counting of a tritiated steroid in their methane-flow type proportional counter. By spreading their sample in a thickness of 1 mg./sq.cm. over an area of 10 sq.cm. they were able to count it with an efficiency of as much as 2·6 %. This is the infinite thickness value for tritium (0·8 mg./sq.cm.) and was obtained by evaporating a solution of the steroid. The authors observe that their technique will probably be applicable only to labelled substrates and that the metabolites obtained from biochemical systems will usually have such a low activity that they will have to be assayed by gas-counting methods. It is to be noted that if ^{14}C were present in the same material, solid counting would involve a rather complex procedure for the determination of both isotopes. On the other hand, by the combustion method described in this paper three isotopes can be determined with high efficiency and satisfactory accuracy as a routine procedure.

SUMMARY

1. A rapid method is described for the quantitative combustion of 10 mg. samples of biological materials.

2. Methods for the assay of tritium as tritiobutane and ^{14}C as $^{14}CO_2$ on this scale are briefly summarized, and methods of measuring and counting 10 μl.–10 ml. samples of radioactive gases are described.

3. Results are presented which demonstrate the quantitative combustion of some standard substances. The closely agreeing results of combusting and assaying in triplicate a sample containing the three isotopes are also presented.

4. The various methods of assaying the three isotopes are discussed with special reference to ^{14}C. The conclusions are drawn: (i) that gas counting is preferable for tritium especially at low activities and in the presence of ^{14}C; (ii) that the counting of solid samples containing ^{14}C is most reliable if first reduced to a single standard compound (usually $BaCO_3$); (iii) that gas counting is simpler than this and more efficient by at least a factor of 10, thus allowing a tenfold reduction in the dose of isotope used in biological experiments.

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