ISOTOPIC GAS ANALYSIS FOR BIOCHEMISTS

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ACADEMIC PRESS INC., Publishers

New York, 1954

Library of Congress Catalog Card Number: 54-6348

PRINTED IN THE UNITED STATES OF AMERICA

PREFACE

Papers on the gas phase measurement of carbon and hydrogen isotopes have now become sufficiently numerous in the literature, and the methods themselves have aroused sufficient interest among biochemists, for a laboratory manual on the subject to appear worth-while. The particular advantages, especially in biochemical work, are discussed in the introduction. The author himself is responsible for a laboratory in which gas phase counting is the chief method of measuring these isotopes in the course of a fairly extensive program of biochemical and physiological research; he therefore hopes that the prominence given in this book to those methods of which he has first-hand experience will be thought justified. These are the ones described as carried out by means of *permanent vacuum-line technique.* Information on all others has been taken from the literature, augmented in a very few instances by private communications. It is hoped that any method described can be applied directly without its being necessary to refer back to the original paper. Full acknowledgment is given for all methods quoted but, unless the contrary is explicitly stated, it is to be understood that the author has not tested them himself but depends for criteria of reliability on the published data.

Acknowledgment is gladly made to all those whose permission to copy published diagrams has been obtained. The liberty of slightly simplifying diagrams has in some cases been taken, and it is hoped that the reason for so doing will be apparent from the context.

The author is also happy to acknowledge the patient and understanding cooperation of Mrs. D. E. Jones, who is responsible for all the drawings. The necessity of asking her to copy diagrams straight from the original papers has made it possible to use the same conventions in every drawing for commonly used items

such as stopcocks and standard joints. In the diagrams of the permanent vacuum line used by the author, however, the same conventions and notation have been used throughout the book, all stopcocks being shown in the open position for the sake of clarity. It is also to be noted that the numbering and notation of the master figure, Fig. 1, have been repeated in all other diagrams showing various sections of that apparatus.

While the author himself is alone responsible for any errors, thanks are gladly offered to all those whose valuable suggestions have helped him in compiling this book, and in particular to Dr. D. Taylor of the Atomic Energy Research Establishment, Harwell (Ionisation Chambers), Dr. D. H. Tomlin of the Physics Department, Reading University (Mass Spectrometry), and Mr. N. B. Balaam of 20th Century Electronics Ltd. (Counters); also to Dr. S. J. Folley, F.R.S., for his encouragement and advice; and to Mr. W. G. Duncombe and Mr. B. W. E. Peaple for help in the development of several of the methods described and for help in correcting the manuscript.

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June, 1954

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CHAPTER I

INTRODUCTION

Outline of Radioactive Assay Methods

End-Window Counters

The simplest and most commonly used method of measuring the activity of radioactive materials depends on the use of an end-window counter, beneath which the solid sample is placed. Even with samples emitting high-energy radiations, the geometry of this arrangement permits only a relatively small proportion of the radiations to enter the sensitive zone of the counter. If they are of low energy, such as those emitted by C^{14} (maximum energy 0.15 Mev) there is a further considerable loss of efficiency by absorption at the window of the counter and by self-absorption in the sample. Window absorption is reduced to a minimum by making it of very thin mica, usually less than 2 mg./cm.^{2}, or by the use of an internal gas-flow counter, which eliminates it altogether and much increases geometrical efficiency.

Self-absorption

The problem of self-absorption by weak emitters such as C^{14} can be dealt with in a number of ways. One method is to spread the sample in a thin layer so that self-absorption is negligible. This means that a relatively large area must be covered if reasonable counting rates are to be obtained. Entenman *et al.* (1) have described a method for counting very thin samples of fatty acids by deposition of 5–30-mg. samples from solution on a lens paper of total area 15 cm.^{2}. For such a method to be applicable, however, the substance used must be one that can be deposited from solution in a uniform layer (which is not possible for all substances),

and the counter must be designed so that most of the radiations from such a sample disk will enter the sensitive zone.

Another method of counting is to use a known thickness and correct the observed activity to zero thickness, i.e., to the value that would have been observed if no self-absorption had occurred. For this purpose, a self-absorption curve must be constructed by plotting counting rate against thickness for a sample of known specific activity. For accurate work, the self-absorption curve for every class of compound counted must be obtained because they will not all be identical, owing to backscattering (see below).

The commonest method of dealing with the problem of self-absorption, however, is to count at infinite thickness at which, for any particular chemical compound, the observed activity is dependent only on the specific activity of the sample. At infinite thickness, the radiations from the lowest layers are completely absorbed before reaching the surface and therefore never enter the counter; it corresponds with the horizontal portion of the self-absorption curve where the counting rate is independent of thickness. Its actual minimum value depends on the energy of the radiations emitted and is 20 mg./cm.^{2} for C^{14} (and less than 1 mg./cm.^{2} for tritium).

Determination of C^{14}

The specific activity of the carbon contained in the sample is the quantity usually sought, and, for a given chemical compound, this may be found by calculating the activity that the carbon would have if isolated and counted at infinite thickness; this activity is then multiplied by an empirical factor to give specific activity. Many workers omit the second part of the calculation since a valid comparison of carbon specific activities may be made by comparing activities at infinite thickness. This is given by

$$A_c = \frac{100 A_s}{C}$$

where A_c is the activity of the carbon, A_s is the observed activity of the sample, and C is the percentage of carbon contained in it.

For a comparison of the specific activity of carbon in com-

pounds of markedly different chemical composition, however, the effect of backscattering must be taken into consideration. More radiations than would be expected from a consideration only of the specific activity of the carbon and absorption in the sample are emitted from thick samples, particularly those containing heavy elements. This is due to reflection back to the surface of radiations originally directed away from the counter. The increase in counting rate due to this backscattering may be by as much as a factor of 1.3 for barium carbonate or by as little as a factor of 1.04 for substances containing only light elements (2). The formula for calculating the activity of the carbon in the sample must therefore be modified to

$$A_c = \frac{100A_s}{BC}$$

where B is the backscattering factor. Unfortunately there is not universal agreement as to the value of backscattering factors, and some workers think it necessary to evaluate them for themselves for their own counters and on their own equipment.

Nature of the Sample

An additional difficulty inherent in solid counting of organic samples is nonuniformity of surface. Some substances are much more easily spread in uniform layers than others, and truly comparable results obviously cannot be obtained unless each sample counted has the same effective surface area. An additional difficulty may arise from the necessity of counting substances which are not chemically pure and whose carbon content is not, therefore, accurately known. An example of such a class of compounds is the mixture of fatty acids extracted from tissue slices in studies on fat synthesis. Many workers therefore prefer to convert all their samples to a common chemical form; this obviates the necessity of calculating the specific activity of the carbon and of applying the backscattering correction with its possible attendant errors. At the same time it ensures a reasonably uniform surface which is the same for each sample. The compound usually chosen is barium carbonate obtained by combustion, absorption of the

carbon dioxide in barium hydroxide solution, and filtration, drying, and plating of the precipitate.

Gas Phase Measurement of Radioactivity

It is particularly to be noted that organic samples are also reduced to a common chemical form by conversion to carbon dioxide; and the introduction of it into instruments designed for gas phase assay provides a reliable means of directly comparing carbon specific activities. Once the apparatus has been built, it is considerably easier to introduce the carbon dioxide into a counter or ionization chamber than to convert to barium carbonate and plate it ready for solid counting. Furthermore, this procedure results in an increased instead of a diminished efficiency.

Relative Sample Size for Solid and Gas Counting

Apart from those substances which are suitable for spreading in very thin layers over large surfaces, it is usually inconvenient, especially for counting at infinite thickness, if less than about 75 mg. sample are available. It is true that less than this can be spread on small disks 1 cm.^{2} in area but, for the purpose of avoiding inaccuracies due to edge effects, most workers prefer disks of 2 or even 3 cm.^{2} area which require 40 and 60 mg., respectively, of C^{14} compounds for counting at infinite thickness. Thus, allowing for transfer losses, the 75 mg. quoted is not usually excessive. This is to be compared with the 5–10 mg. which is sufficient for many methods of gas phase assay. Thus, in a method described by the present author (Chapter VI), three isotopes (C^{13}, C^{14}, and tritium) can be determined in the products of combustion of a single 10-mg. sample. This is an important advantage since it may often be difficult to isolate more than this in biochemical experiments, and the addition of carrier, even if possible, may reduce the observed activity to an inconveniently low level. It is true that combustion and conversion to barium carbonate, already referred to as a procedure designed to eliminate the errors arising from the counting of chemically different compounds, brings about an effective increase in sample size; thus glucose yields 6.6 times its weight of barium carbonate. But this increase in weight

is, of course, accompanied by a proportional drop in specific activity.

TABLE 1

COMPARISON OF C^{14} SOLID COUNTING AT INFINITE THICKNESS AND GAS COUNTING OF $C^{14}O_2$

	I	II	III	IV	V	VI	VII
Substance	*Activity of 20 mg. on 1-cm.² disk (counts/min.) (X)*	*Volume CO_2 from combustion of 20 mg. (std. ml.) (V)*	*Specific activity of CO_2 (counts/min./ml.) (Y)*	*Total activity of V ml. (counts/min.) (Z)*	*Ratio Z/X = gain in efficiency*	*Activity at ∞ thickness = 100A./BC (counts/min.) (N)*	*Ratio N/Y*
Ca salts of mixed fatty acids containing C^{14}	2,439	24.6	4,150	102,000	42	3,620	0.87
C^{14}-glucose	120	15.0	450	6,750	56	288	0.64
$BaC^{14}O_3$	26	2.28	320	730	28	329	1.03
$BaC^{14}O_3$	2,204	2.28	29,300	66,800	30	28,000	0.96

Relative Efficiency of End-Window and Gas Counting

Gas counting is about 50 times more efficient than solid counting at infinite thickness using a counter fitted with a window of about 2 mg./cm.² Some figures accumulated in the author's laboratory (3), illustrating the differences between end-window and gas phase counting of some organic samples are given in Table 1. In column I is given the observed activity of each sample when counted on a 1 cm.² disk at infinite thickness (20 mg./cm.²). In column II is given the volume of carbon dioxide obtained from that 20 mg. by combustion or decomposition, and, in column III, its specific activity. Thus the product of the figures in these two columns gives the total observable activity in the CO_2 obtainable from the 20 mg. combusted (the gas counter used held 25 standard ml. at its operating pressure), and is shown in column IV. This activity

is, in all cases, very much greater than the activity at infinite thickness, and the factor by which it is greater is shown in column V. It will be seen that the gain in efficiency is as much as 56 (for glucose).

Although this table is not the result of a rigorous comparative study of the two methods, it gives some indication of the reliability of the calculation of carbon specific activity from known carbon content, published backscattering factor, and observed activity at infinite thickness. The calculated infinite-thickness activity of the carbon in the samples is shown in column VI (the carbon content of the fatty acid calcium salts being obtained from a microanalysis). If it is a true measure of the specific activity of the carbon, then for all samples it should bear the same ratio to the specific activity of the carbon dioxide shown in column III. This ratio is shown in the last column and will be seen to vary between 0.64 for glucose and 1.03 for barium carbonate. The agreement for the ratio for the two barium carbonate samples is quite good and it would thus appear, apart from considerations of relative efficiency, that more reliable results are obtained if the compounds to be assayed are always reduced to the same chemical form. This is in agreement with the finding of other workers; for example, Abrams and Clark (4) who say they have been able to get C^{14} counts to agree within 5 per cent only by completely combusting the sample and counting as an infinitely thick layer of barium carbonate. In attempting to count thin samples of uncombusted compounds, they observed inaccuracies as high as 30 per cent. As already noted, however, it is easier to introduce carbon dioxide into a gas phase counter than to convert it to barium carbonate for solid counting.

Gas-flow Counters

These counters are not factory-filled but are constructed so that the filling gas continually passes through them at a pressure slightly in excess of atmospheric, thus preventing the entry of air. They may be operated in the Geiger region by using helium containing a suitable quenching vapor (5, 6), or in the proportional region using methane (7, 8). The sample to be counted is intro-

duced into the body of the counter by means of a suitable mechanical device, the radiations from it then passing into the sensitive zone without interference by a window. There is also a very favorable geometry, and the resultant efficiency may be as high as 50 per cent under conditions of zero self-absorption. This is about ten times higher than is possible with an end-window counter. The elimination of the window also makes possible the counting of tritiated compounds in the solid state (7).

Determination of Tritium

For the counting of tritium, gas phase assay is undoubtedly the most sensitive and accurate, notwithstanding the development of the internal gas-flow counter and scintillation methods referred to above and in Chapter VIII. Self-absorption reduces the efficiency of the internal gas-flow method from 50 per cent, which is expected from geometry alone, to 2.6 per cent. Eidinoff and Knoll (7) observe that their gas-flow counter method will normally be applicable only to highly active compounds and that labeled metabolites isolated from biochemical systems will usually have to be assayed by gas phase methods.

Assay of Compounds Containing Several Isotopes

There are a number of elegant methods reported in the literature for the separate assay of two radioactive isotopes contained in the same compound. These usually take advantage of markedly different properties possessed by the radiations emitted by the two isotopes. For example, a thin-window counter filled with helium is 30 times as sensitive to the radiations from Fe^{59} as to those from Fe^{55}, whereas one with a thick beryllium window and filled with argon is sensitive only to the radiations from Fe^{55}. This is because the two radiations differ both in character and in energy. Those from C^{14} and tritium also differ markedly, but only in maximum energy; hence although C^{14} may be counted in the presence of tritium by means of a thin-window counter, the separate counting of tritium in the presence of C^{14} is not possible, as only the total radiation can be measured by means of a flow-type counter. Even then, owing to the difference in efficiency of

counting of the two isotopes in the flow counter and the difference in efficiency between the two kinds of counter, the calculation would be somewhat laborious and inaccurate. Combustion and separate assay of the carbon dioxide and water by gas phase methods is much more reliable. If the stable isotopes C^{13}, N^{15}, or deuterium are also to be determined, combustion and gas phase manipulation cannot of course be avoided; C^{13} and N^{15} are determined in the mass spectrometer as carbon dioxide and elemental nitrogen, respectively, and deuterium is determined either in the form of water or as hydrogen gas.

Economy of Labeled Substrates

In addition to the advantages already enumerated, gas phase assay makes possible a 10- to 100-fold reduction in the amount of labeled material used in an experiment. This may not alone always justify the use of a method that would not otherwise be used, but it is still a relevant consideration in many circumstances. Firstly, even a 10-fold reduction in the cost of labeled materials is not to be neglected when substrates cost as much as £200 ($560) per millicurie (e.g. 1-C^{14} glycerol). A large reduction in the amount of activity used also brings about a corresponding reduction in health hazards to the workers and may even permit of experiments on human subjects. Furthermore, in some experiments, for example, with labeled hormones, the specific activity of the available material may be such that the dose used contains an activity so low that the most sensitive methods of detection are essential if the experiment is to succeed.

Technical Difficulties

Although the arguments in favor of the superior accuracy and sensitivity of gas counting methods over all others seem incontrovertible, it must also be noted that more skill is needed for the successful running of a gas analysis apparatus and, at least compared with the solid counting of uncombusted samples containing only C^{14}, the method is more lengthy. A considerable knowledge of high-vacuum technique is desirable, and some workers, particularly those trained only in the biological sciences, may

consider the learning of what almost amounts to a new discipline more trouble than it is worth, or, having mastered the technique themselves, may find it difficult to secure the services of assistant staff with the necessary capacity for using and maintaining relatively fragile apparatus. There is also a great investment of time and money in gas phase assay equipment—an investment which is justified only if the equipment is to be kept in continuous use. In the author's laboratory, this condition is satisfied and in fact it has been found necessary to arrive at a compromise whereby the burden of work on the gas analysis apparatus is somewhat diminished. This is achieved by carrying out small scale pilot experiments using end-window solid counting of uncombusted samples, followed, if preliminary results justify it, by more extensive experiments using doubly or triply labeled substrates (C^{13}, C^{14}, and tritium) to which gas analysis technique is applied.

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CHAPTER II

HIGH-VACUUM APPARATUS

General Requirements

Gas phase assay of the radioactive isotopes of carbon and hydrogen necessitates the quantitative manipulation of gas samples, ranging from as little as 10 μl. to as much as 100 ml., and also the evacuation to very low pressures of the counters and ionization chambers used for radioactive measurements. This is not only to permit the introduction of the measured sample to be assayed but because counters, in particular, are very sensitive to the presence of the air gases which interfere with their counting properties. A source of high vacuum and the application of high-vacuum techniques is therefore inevitable.

One of the chief advantages of gas phase assay over solid counting methods lies in its high efficiency and the consequent possibility of using activities smaller by a factor of at least ten; elaborate health precautions are therefore unnecessary. Nevertheless, the equipment should be housed in a large, well-ventilated laboratory provided with an extraction fan capable of changing the air several times an hour. Alternatively, if desired, the vacuum apparatus can be housed in a large cabinet with extraction fan and sliding glass doors. The temperature of the laboratory should be maintained at about 20°, which is not only comfortable for the workers but is a temperature at which vacuum grease is neither too stiff nor too fluid.

If it is possible to design the laboratory right from the start, it is an advantage, when a permanent installation is planned, to provide a bench lower than the usual laboratory bench by about 6 in. and accessible from three sides. This makes it possible for workers of average height to reach all stopcocks on the apparatus without uncomfortable stretching, with consequent risk of break-