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ANALYSES FOR TOTAL AND ISOTOPIC CARBON IN INTERMEDIARY METABOLISM STUDIES

By

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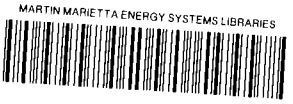

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ANALYSES FOR TOTAL AND ISOTOPIC CARBON IN INTERMEDIARY METABOLISM STUDIES

David S. Anthony* and Mary V. Long

A. INTRODUCTION

Interpretations of metabolic pathways in biological experiments with carbon isotopes usually rest on comparisons of concentration of isotope (specific activity) in the substrate with that in each carbon atom of suspected intermediates or end products. This requires measurement of specific activities of substrates, isolation of intermediates and end products in pure form, degradation of the resulting pure compounds a carbon atom at a time, and estimation of the specific activity of both the total pure compound and of each of the degradation products. The present paper is concerned only with the specific activity determinations. Such determinations actually require two kinds of analyses: total carbon estimations and measurements of tracer isotope abundance.

B. METHODS

1. For Determination of Total Carbon by Wet Combustion and Weighing as $BaCO_3$.

The most convenient method of analysis of the products of biological reactions and of degraded portions of these products for total and isotopic carbon required the oxidation of a variety of organic compounds to CO_2 and thence to $BaCO_3$ for weighing solid counting, or final conversion to dry CO_2 for gas counting or mass spectrographic analysis. Since the samples to be assayed were for the most part dilute aqueous solutions, a wet-oxidation scheme tolerant of large amounts of water was desirable. The system adopted was a slight modification of that of Osburn and Werkman[1] using potassium persulfate and silver nitrate. A simple representation of the reaction is given by the equation

$$2H^+ + S_2O_8 = + 2e \rightarrow 2HSO_4^-$$

The apparatus designed for the wet-oxidation is shown in Figure 1. The flask (A) in which the oxidation is carried out is equipped with one side arm

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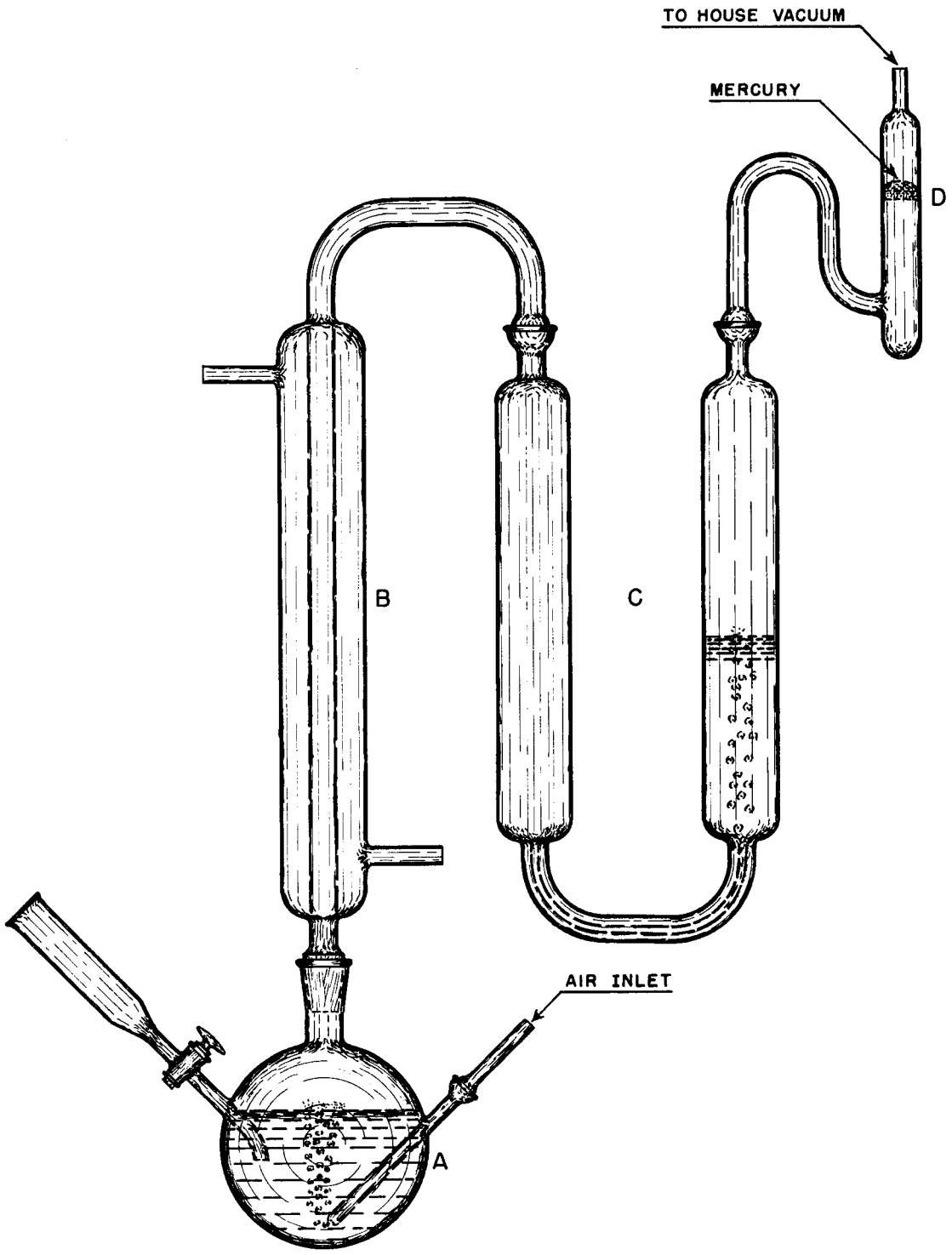


FIGURE 1

THE WET OXIDATION APPARATUS

for the admission of reagents (actually not required for this method but makes the same apparatus adaptable to other operations) and another for admitting dry, CO_2-free air. The purpose of the condenser (B) is to prevent the escape of volatile organic compounds from the pot and, also, as a precaution against "bumping" during heating of the solution.

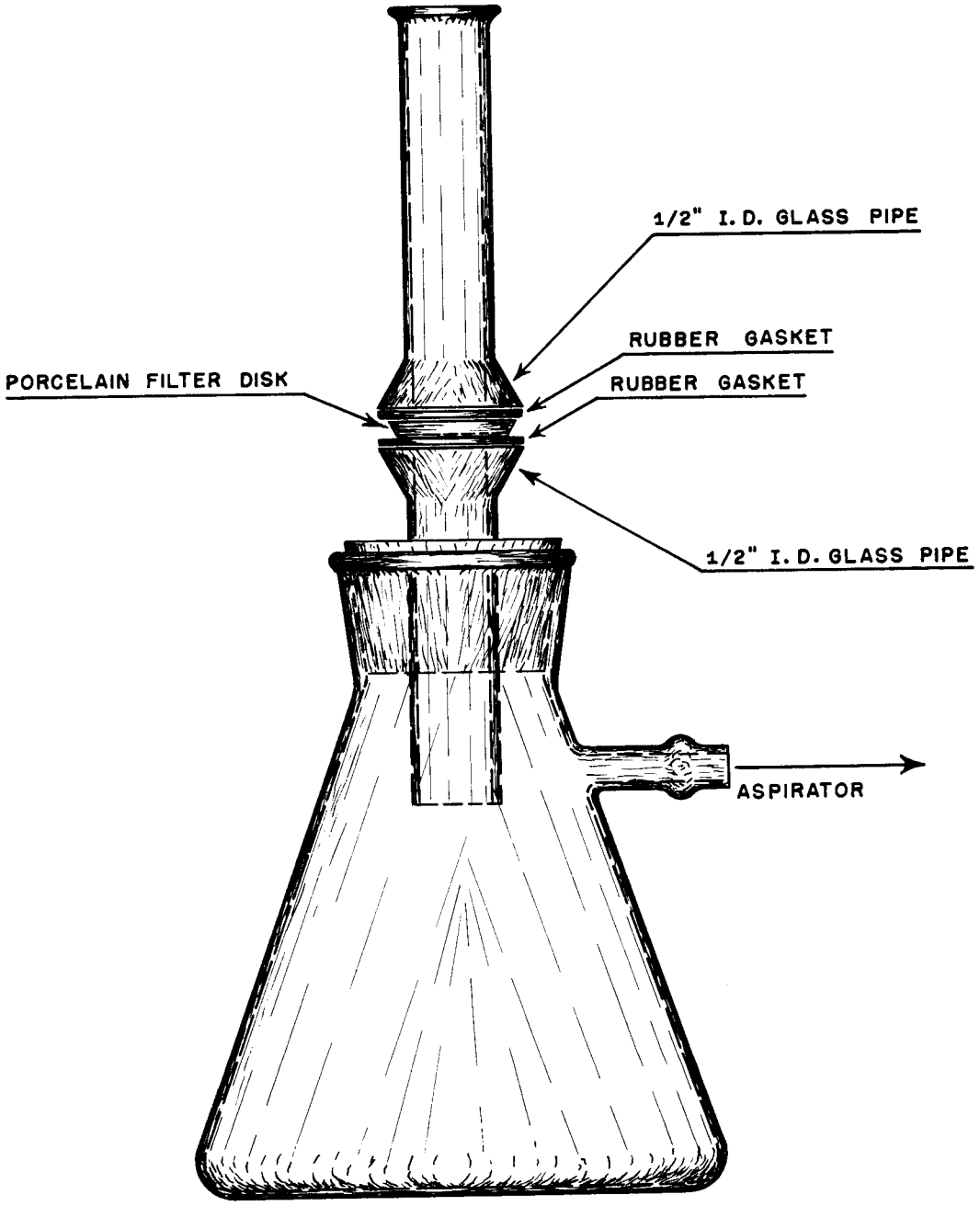
The third part of the apparatus is a two-armed trap (C) connected by capillary tubing to facilitate the bubbling of the CO_2 through alkali. The last section consists of a mercury check-valve (D) to prevent backup of the trap and entrance of room air from the vacuum line during pressure changes.

The following procedure was found most efficient in oxidizing such substances as sodium acetate, sodium succinate, sodium propionate, lactic acid, ethanol, acetone, etc. A sample of sufficient size to yield $\sim$50 mgs of $BaCO_3$, water to about 20 ml total volume, 0.20 g of potassium persulfate, and 1 ml of 5% silver nitrate solution were introduced into the pot. The trap was loaded with 10 ml of $\sim$1N carbonate-low alkali (NaOH), and the system connected and checked (very important) to be sure that all joints were air tight. The pot was then placed in a water bath which was brought to 70°C and held at this temperature for 20 minutes. At the end of this time the temperature of the water bath was increased slowly to boiling and maintained until the solution in the pot became clear or until the persulfate was entirely dissolved. Following this the water bath was removed, the solution in the pot was boiled, gently, with a free flame, and the system was swept for 10 minutes by applying a slight vacuum at the end of the check-valve while admitting CO_2-free air through the side arm of the pot. The CO_2 trapped in the alkali was then ready to be precipitated as $BaCO_3$ for weighing and solid counting. The alkali with its load of carbonate was transferred from the traps to large test tubes by direct dumping and rinsing with boiled, distilled water in the open air. Then, approximately 5 ml of 0.2 M $BaCl_2$ was tipped in at once from a graduated cylinder. The tube was swirled, very lightly stoppered, and then was immersed in boiling water for 10 to 20 minutes. At the end of this digestion period, the tube was tightly stoppered, removed from the hot water, and placed in cold water. The cooled, digested $BaCO_3$ was filtered through a porous porcelain disk, rinsed with freshly boiled, cooled water, and rinsed with acetone in the apparatus shown in Figure 2. The disk bearing the cake of $BaCO_3$ is dried in a 110° oven for longer than 20 minutes.

2. Procedure For Estimation of Carbon^{14}

a. Solid counting

After total carbon had been determined by weighing the $BaCO_3$ on the porcelain filter disk, the C^{14} content was determined by counting the disk directly with a mica end-window Geiger-Mueller tube. The sample disks



FILTER FOR BARIUM CARBONATE
(EXPLODED PARTS DIAGRAM)

FIGURE 2

were mounted for counting on a metal plate which was designed so that a pedestal engaged the recessed bottom of the disks while the edges of the plate were firmly held by slots in the inside of the lead shield surrounding the counter tube. In this manner the disks could be centered under the counter tube and could safely be placed within 2 mm of the mica window.

All counts were corrected for self-absorption from a knowledge of the sample weight and area of the disks. The area had been determined previously by the method described below.

b. Method of standardization of porcelain counting disks

A fixed aliquot of a standard Na_2CO_3 solution which would give a barium carbonate sample of about 25 mg/cm^2 yielding about 50 counts per second was used for disk standardization. Replicate samples were precipitated with barium chloride and were filtered onto the disks. Since the total weight of barium carbonate on each disk and the total amount of C^{14} was the same, the gross count observed was a function of the effective area of the disk. With the counts per second and the disk loading in milligrams both known, the effective area (in cm^2) could then be read off of the self-absorption curve (which plots counts/sec vs mg/cm^2).

In practice, the inside diameters of the first few disks received in the laboratory were carefully measured with a caliper and their area was calculated; these disks were used to establish the self-absorption curves. In standardization of any subsequent batch of disks, these measured disks were used to fix the counting yield of the particular amount of $Na_2C^{14}O_3$ used. Then the relative effective areas of the new disks were read off the curve. Area measurement by direct determination of radius with a caliper is satisfactory only with a few disks selected for obvious uniformity from a given shipment. Other disks in the same batch, and particularly, disks from separate shipments show enough variation in height and pitch of lip, thickness of peripheral cement ring joining frit and lip, etc., to have different counting yields even though they may measure the same in inside diameter.

c. Quick survey for radioactivity without combustion

Frequently it was important to know which fraction or compound was highly radioactive, which was moderately so, and which was inactive as soon as a separation procedure was under way. Without waiting for completion of the procedure or for combustions, etc., a quick answer would often save much valuable time in initiating further steps.

A quick, qualitative or very rough quantitative survey method based on the activity of a dried sample on watch glasses was developed. An aliquot of a solution carrying suspected (nonvolatile) radioactive components was

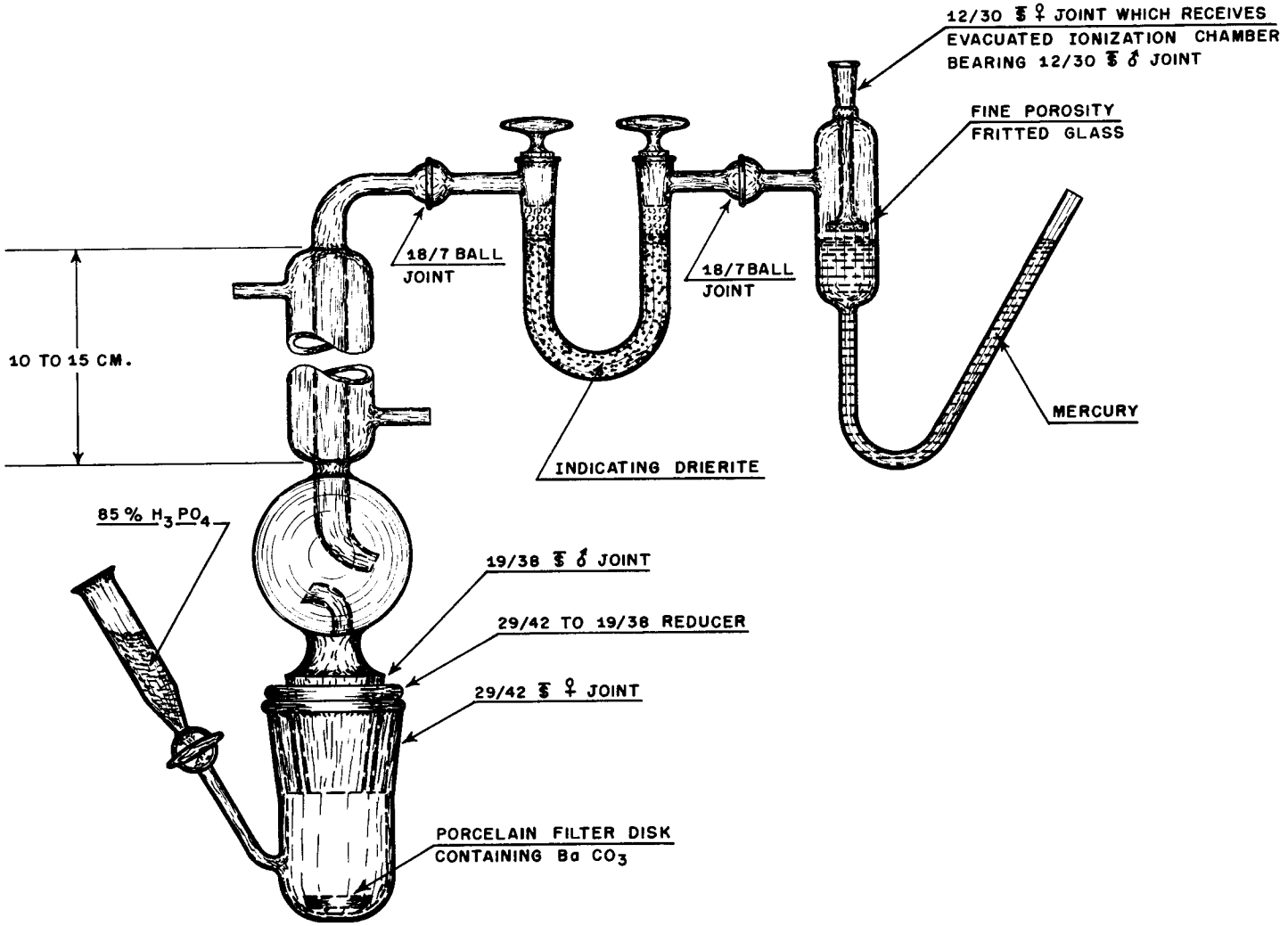
evaporated to dryness on a watch glass about 3 cm in diameter and the watch glass was counted exactly as though it was the usual disk containing $BaCO_3$. When the total solids on the disk did not exceed a few milligrams per cm^2, the counts ran about 8 to 10 times higher than the same amount of activity counted as an infinitely thick sample of $BaCO_3$. Of course such a method is subject to large errors due to irregular deposition of solids on the watch glass, to deliquesence of many substances under investigation, to spattering, and to chemical destruction of labile compounds during the drying procedure.

d. Conversion of $BaCO_3$ to CO_2 for gas ionization measurements

If for some reason the sample of $BaCO_3$ on the porcelain disk could not be properly counted for C^{14} (specific activity too low) it was converted into CO_2 and introduced into a previously evacuated ionization chamber in the apparatus sketched in Figure 3. The apparatus was originally developed by W. B. Leslie and is reported by O. K. Neville elsewhere.[2] When the stopcock on the evacuated ionization chamber was opened to this apparatus, the mercury quickly rose to the level of the fritted disk and stopped the gas flow. When H_3PO_4 was introduced into the other end of the apparatus, CO_2 was produced from the $BaCO_3$, raising the pressure in the apparatus, forcing the mercury away from the fritted disk and admitting a "burp" of CO_2 until the mercury rose and again sealed the disk. Acid addition while heating was continued until no more CO_2 was evolved. Then the stopcock in the H_3PO_4 reservoir was opened wide, permitting room air to enter, sweeping the last CO_2 with it into the chamber. Since the volume of the chamber (250 ml) was about 10 times that of the apparatus, very efficient transfer of CO_2 would be expected. The chamber was then connected to a vibrating reed electrometer and the amperes of ion current were recorded.

e. Sampling for C^{13} measurement

The apparatus shown in Figure 4 was evacuated to a pressure of about 10 microns, the stopcock on the vacuum line closed, and the $BaCO_3$ to be assayed was converted to CO_2 by the cautious, slow addition of H_3PO_4. The gas was dried by passage through Drierite and a cold finger. The CO_2 was frozen out in liquid nitrogen and after allowing about 5 minutes to complete the distillation, the break-off ampoule was slowly melted in and pulled off at the previously prepared heavy-walled constriction. At any convenient time in the future these ampoules, bearing identifying numbers of burned-in black laboratory ink, could be sealed to the mass spectrometer for analysis. The over-all precision including mass spectrography, wet oxidation, trapping, precipitation, and the above distillation with samples of near natural C^{13} content was about ± 0.01 atom per cent carbon[13].[3]



APPARATUS FOR CONVERSION OF Ba CO_3 TO CO_2 FOR FILLING AN IONIZATION CHAMBER.

FIGURE 3

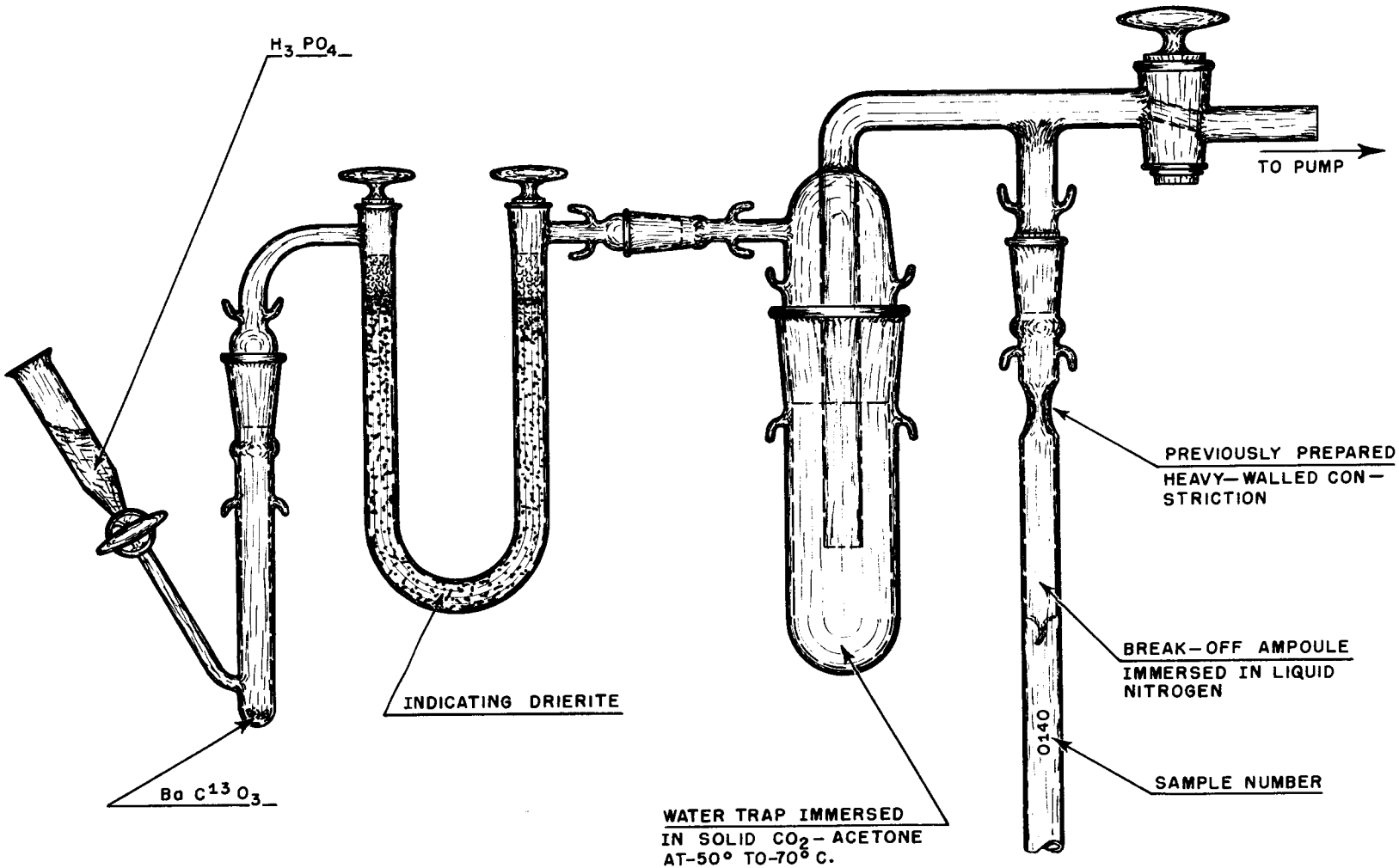


FIGURE 4

APPARATUS FOR CONVERSION OF Ba CO_3 TO CO_2 FOR MASS SPECTROGRAPHIC ANALYSIS

C. RESULTS

1. Accuracy and Precision of Total Carbon Analysis by Wet Oxidation.

Results of carbon analyses by the persulfate wet oxidation procedure are given in Table 1. A total of 41 assays of eight different substances are recorded. The average yield of $BaCO_3$ ranged from 97.0% of theory (on zinc lactate) to 101.6% (on glucose) while the highest individual determination was 103.9% on one sample of glucose and the lowest was 94.8% on a sample of zinc lactate. Very likely the true accuracy of the method is even slightly better than indicated above since no great effort was made to dry any samples before combustion. Hence some of the values (especially that for acetone) might be slightly low due to presence of water. It should be pointed out that the method has limitations. It gives satisfactory yields only for materials in aqueous solution and not for solids in suspension. A few compounds in true solution will not be satisfactorily oxidized by the persulfate procedure. For example, ethyl and methylamines give only about 75% yield of $BaCO_3$ with this method.

A study of the precision of the method shows the average deviation from the observed mean usually was ± 1 to 2%. The range in deviations of individual determinations from the mean was +3.0% to -3.7%.

The chief sources of error included small hold-up of volatile compounds in the reflux condenser, contamination of neutral or alkaline samples with carbonate, 0.1% to 0.5% changes in tare weight of the disks, and perhaps occasional inefficiency in trapping of CO_2 in the single alkali trap used for all of the above determinations. The latter might occur if the operator was careless when he first applied vacuum for sweeping purposes, permitting a burst of gas to pass before control was achieved.

A blank determination of carbon must be frequently run when the operator takes as few precautions against contamination with atmospheric CO_2 as was the case here. For trapping purposes, 1N NaOH was made up frequently in small amounts by simple dilution of saturated NaOH with boiled distilled water. In the interests of speed and convenience, the 1N alkali was stored in an ordinary volumetric flask closed by a rubber stopper and not protected against atmospheric CO_2 by any of the elaborate arrangements often used. Transfer of this alkali to the traps by direct pipetting (vacuum supplied by aspirator rather than by mouth) rinsing of the traps (with boiled, distilled water), addition of $BaCl_2$, and filtering were all done as rapidly as possible in the open room. The carbon picked up by this procedure plus the small amount from oxidation of impurities in the water, $K_2S_2O_8$, and $AgNO_3$ resulted in a quite consistent blank 1.3 to 1.8 mg.

TABLE 1

ACCURACY AND PRECISION OF TOTAL CARBON DETERMINATION BY WET OXIDATION

Substance	Theoretical yield mg $BaCO_3$	Observed yield mg $BaCO_3$	Observed yield % of theory	Range & Av. Deviation from observed mean %
Sodium oxalate				
1	39.4	40.0	102.1	+ 0.9
2		40.0	102.1	+ 0.9
3		39.5	100.8	- 0.4
4		39.7	101.3	+ 0.1
5		38.4	97.5	- 3.7
6		40.2	102.5	+ 1.3
7		40.7	103.8	+ 2.6
8		40.6	103.6	+ 2.4
9		39.4	100.5	- 0.7
10		39.8	101.6	+ 0.4
11		40.3	102.8	+ 1.6
	Average	39.9	101.2	± 1.4
Zinc lactate				
1	39.0	37.9	97.2	+ 0.2
2		38.0	97.5	+ 0.5
3		38.4	98.6	+ 1.6
4		38.4	98.6	+ 1.6
5		37.2	95.6	- 1.4
6		36.9	94.8	- 2.2
	Average	37.8	97.0	± 1.3
Fumaric acid				
1	34.0	33.7	99.2	0
2		33.7	99.2	0
	Average	33.7	99.2	+ 0
1	36.0	34.9	96.9	+ 0.2
2		34.7	96.4	- 0.3
	Average	34.8	96.7	+ 0.25
1	45.1	43.8	97.1	- 0.9
2		44.6	98.9	+ 0.9
	Average	44.2	98.0	± 0.9
Sodium acetate				
1	19.4	18.8	96.9	- 3.7
2		20.1	103.6	+ 3.0
3		19.4	100.0	- 0.6
4		19.5	100.6	0.0
	Average	19.5	100.6	± 1.8
1	39.1	38.9	99.5	+ 2.1
2		37.2	95.2	- 2.2
	Average	38.1	97.4	± 2.2

Table 1 cont.

Succinic acid				
1	40.3	41.1	102.0	+ 0.5
2	↓	41.2	102.3	+ 0.8
3		41.6	103.3	+ 1.8
4	↓	39.7	98.3	- 3.2
	Average	40.9	101.5	± 1.6
1	41.0	40.2	98.0	
Glucose				
1	39.4	39.9	101.4	- 0.2
2	↓	40.6	103.2	+ 1.6
3		40.9	103.9	+ 2.3
4	↓	38.6	98.0	- 3.6
	Average	40.0	101.6	± 1.9
Acetone				
1	60.4	58.9	97.6	± 0.1
2	↓	58.8	97.4	- 0.1
	Average	58.9	97.5	± 0.1
Sodium lactate				
1	42.6	41.3	97.0	

A subtle error once arose when the source of alkali was changed to a commercial, standard 1N NaOH preparation. This gave the usual blank, but gave apparent $BaCO_3$ yields that were about 10 mg high when a sample was present. Only when considerable $BaCO_3$ was precipitating would this error appear, and treatment with dilute acid indicated the substance was not $BaCO_3$. It has been postulated that this was barium silicate co-precipitating with $BaCO_3$. This type of error can be detected by oxidizing a standard test organic substance at regular intervals.

2. Measurement of Carbon^{14}

a. General

All counting was done on the porcelain disks of about 1.8 cm^2 area with samples as nearly equal to infinite thickness as possible. Mica end-window counters of 1.3 to 2.4 mg/cm^2 thickness were used with the sample centered within about 2 mm of the tube face. The counting efficiency of this arrangement, as determined by counting a sample of Bureau of Standards $BaCO_3$, was about 1.5%.

Background of the shielded counter with disk and inactive $BaCO_3$ in place was about 0.4 counts/second and no counts in the biological studies were considered significant unless they were at least three times the background. Nearly all counts were run in triplicate and each count was allowed to continue until it had accumulated at least 1000 events.

Occasionally, samples were too active to be counted on the first shelf of the rack in the counter housing. By dropping to the second shelf, the counting yield could be dropped by a factor of about 3.3 and a moderately accurate measure of activity could be obtained.

b. Precision of specific activity determinations

Typical data showing the precision of specific activity measurements are shown in Table 2. These values do not represent special test samples

TABLE 2

TYPICAL DATA SHOWING THE PRECISION OF SPECIFIC ACTIVITY DETERMINATIONS*

Sample #	Specific activity c/s/mg $BaCO_3$	Deviation from mean %
8-112-3	1.46	+ 0.3
8-112-4	1.45	- 0.3
mean	1.45 (5)	
8-112-5	1.20	- 2.4
8-112-6	1.26	+ 2.4
mean	1.23	
7-27-1	1.31	- 1.1
7-27-2	1.36	+ 1.1
mean	1.33 (5)	
7-21-3	2.97	- 1.8
7-21-4	3.08	+ 1.8
mean	3.02 (5)	
30-91-1	1.12	+ 3.2
30-91-2	1.05	- 3.2
mean	1.08 (5)	
30-91-3A	0.63	+ 5.0)
		) total
30-91-3B	0.58	- 3.3) deviation =
		) 10.5
30-91-3C	0.60	+ 0.0) 10.5/4 =
		) 2.6% Av
30-91-3D	0.59	- 1.7) deviation
mean	0.60	

*All values in this table were obtained by combustion of C^{14}-ethanol samples. Precision on other, usually less volatile substances, was at least as good.

handled with extreme care, but rather, were obtained from time to time over a two year span and in routine determinations of specific activities of feed materials for biological experiments. Only ethanol samples were included in this table since many more ethanol samples were assayed than any other substrate. It was felt also that the radioactive and chemical purity of the ethanol might well be the highest of any of the materials examined.

In 13 out of 14 analyses, the deviation from the mean specific activity did not exceed ± 3.3% and in the other case, it was only 5%. Thus, it could be conservatively stated that the over-all precision of our analytical methods was ± 5%. This includes all errors of pipetting, oxidation, transfer, precipitation, weighing, self absorption correction, calculation, and statistical error of the count. Since the minimum number of counts recorded for any value in this table (samples 30-91A to D inclusive) was a total of 4500 events, the maximum "95/100" statistical error was ± 2.2%. The minimum "95/100" statistical error in Table 2 was ± 1.7%. (Samples 7-21-3 and 4.)

c. Gas ionization measurements

At times, samples from the biological experiments were obtained which were imporper for solid counting with the G-M counter for one or more of the following reasons:

1. Insufficient total activity.
2. Insufficient weight of sample.
3. Too large an amount of $BaCO_3$.

Such samples were converted to CO_2 and run into the gas ionization chamber (as described in Sec. B-2d) which had an absolute sensitivity 15 to 30 times that of the G-M counter. Furthermore, within wide limits the specific activity of the sample did not affect the count. This was true because the 250 ml volume of the ionization chamber permitted the introduction of samples ranging from vanishingly small amounts to a millimole of CO_2 without making the CO_2 content of the chamber as much as 10%. Essentially, then, the composition of the filling gas and thus the ion pairs per cm of path length remained the same.

Since the bulk of the radioactivity measurements in any experiment were carried out with the mica end-window counter, it was often desirable to calculate the few gas ionization measurements in terms of counts per second as obtained with the G-M counter. Table 3 shows the data obtained for such an interconversion with a 1.4 mg/cm^2 counter tube. It will be noted that the mean ion current is 2.02 x 10^{-14} amperes per 1.0 count per second. With this particular ion chamber and counter tube on this day, the former was 67 x background and the latter was 2.7 x background, giving a ratio of sensitivities of 25 to 1.

TABLE 3

CONVERSION OF GAS IONIZATION MEASUREMENTS TO COUNTS PER SECOND
(Samples 30-91-3A to 30-91-3D)

Amperes ion current	Counts/sec	Counts/sec/10^{-14} ampere	Deviation from mean
A 9.3×10^{-14}	19.1	2.06	+ .04
B 9.0×10^{-14}	17.3	1.92	- .10
C 8.9×10^{-14}	18.2	2.04	+ .02
D 9.0×10^{-14}	18.6	2.07	+ .05
	mean	2.02 average	± .05

$\frac{0.05}{2.02} \times 100 = \pm 2.5\%$ av deviation from the mean ion current

Specific activities calculated from above ion currents:

	% deviation from mean
$\frac{2.06 \times 10^{-14}}{33.6 \text{ mg}} = 6.14 \text{ amp} \times 10^{-16}/\text{mg}$	+ 2.0
$\frac{11.92 \times 10^{-14}}{33.1 \text{ mg}} = 5.82 \text{ amp} \times 10^{-16}/\text{mg}$	- 3.3
$\frac{2.04 \times 10^{-14}}{33.2 \text{ mg}} = 6.15 \text{ amp} \times 10^{-16}/\text{mg}$	+ 2.2
$\frac{2.07 \times 10^{-14}}{34.6 \text{ mg}} = 5.99 \text{ amp} \times 10^{-16}/\text{mg}$	- 0.5
mean $= 6.02 \text{ amp} \times 10^{-16}/\text{mg}$	± 2.0

Bkgd = 0.3×10^{-14} amps.

The specific activities in terms of ion current per mg $BaCO_3$ for the four samples showed an average deviation from the mean of ± 2.0% and a maximum deviation of 3.3%. This should be compared with the same four samples as measured by solid counting with the counter (last 4 entries, Table 2). Where the average deviation was 2.6% and the maximum was 5.0%. As indicated previously, the spread of these latter values was in considerable measure due to the statistical uncertainty of the counts.

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