THE DETERMINATION OF CARBON DIOXIDE IN FERMENTING MIXTURES.*

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INTRODUCTION.

In connection with a research on enzymic behavior it was necessary to develop a simple and expeditious method for the determination of the carbon dioxide formed during fermentations. The most suitable method of carbon dioxide analysis appeared to be the procedure evolved by Cain and Maxwell[1] for the determination of carbon in steel. They absorbed the carbon dioxide, formed by combustion, in known volumes of barium hydroxide solution and followed the precipitation of barium carbonate by measurements of the electrical conductivity of the solution. The idea has been applied by Spoehr and McGee[2,3] to the determination of carbon dioxide in their studies on plant respiration. This paper describes the development of this general method so as to render it applicable to studies on fermentation.

The technique which was finally adopted consisted in liberating the carbon dioxide from the fermentation mixtures by shaking, carrying it by means of a stream of air free from carbon dioxide to the absorption vessels containing barium hydroxide, and measuring the change in resistance of the barium hydroxide solutions.

1 Cain, J. R., and Maxwell, L. C., *J. Ind. and Eng. Chem.*, 1919, xi, 852.

2 Spoehr, H. A., and McGee, J. M., *Ind. and Eng. Chem.*, 1924, xvi, 128.

3 Spoehr, H. A., and McGee, J. M., *Carnegie Institution of Washington, Pub. No. 325*, 1923.

Conductivity of Barium Hydroxide Solutions.

Before undertaking the carbon dioxide determinations, it was necessary to determine the conductivities of pure barium hydroxide solutions. The conductivities found in the literature are almost exclusively at 25° and are not in very good agreement. As we wished to employ both 30° and 37°, we determined the conductivity at these temperatures over the concentration range of 0.065 to 0.12 N, and in addition repeated the observations at 25° for comparison with previously published data.

In making these determinations the usual precautions were observed. Conductivity water of specific conductance of from 0.8 to 1.2×10^{-6} mhos. was prepared by redistilling the laboratory supply of distilled water, first from acid permanganate and then from barium hydroxide, all in a current of air free from carbon dioxide. The potassium chloride employed as a conductivity standard was Baker's c.p. twice recrystallized. The barium hydroxide was Merck's c.p. similarly treated. Sodium carbonate was used as an acidimetric standard and was prepared by gentle ignition of pure sodium bicarbonate, which in turn was secured by saturating with carbon dioxide a cooled solution of Baker's c.p. sodium carbonate. Various samples of the final carbonate prepared as above gave reproducible analyses. Baker's c.p. hydrochloric acid was diluted and standardized against the sodium carbonate, using methyl orange as indicator. In all titrations the neutral solution was boiled to expel carbon dioxide, cooled, and the end-point redetermined. The final value chosen for the normality of the hydrochloric acid was the average of five determinations in which the greatest difference was 0.15 per cent. The barium hydroxide solutions were standardized against this hydrochloric acid using the average of three or four determinations, which in general showed differences of not more than 0.25 per cent.

The temperature of the thermostat was read to $\pm 0.01°$ by means of a standardized thermometer. Barium hydroxide solutions of various concentrations were immersed in the thermostat, and after allowing them to come to temperature their resistances were determined with a dip electrode. The thermostat temperature was then changed and the determinations repeated.

Measurements were made on each sample at 25°, 30°, and 37°. At each temperature the cell constant of the electrodes was determined by immersion in an accurately prepared potassium chloride solution both before and after measuring the conductivities. In no case did the cell constant undergo appreciable change during the measurements.

After several preliminary experiments the data given in Table I were secured in three runs, as indicated in the first column. Each sample was used at the three temperatures and the normali-

TABLE I.

Run No.	Normality at 20°.	CO_2 per 100 cc.	Specific resistance.		
			25°	30°	37°
		mg.			
I	0.1131	248.9	43.43	40.13	36.16
	0.1000	220.0	48.78	45.04	40.60
	0.0907	199.6	53.21	49.12	44.30
	0.0817	179.9	58.63	54.12	48.85
	0.0723	159.1	65.40	60.33	54.48
	0.0633	139.3	73.68	68.13	61.48
II	0.1039	228.6	47.05	43.45	39.25
	0.0954	209.8	51.00	47.07	42.49
	0.0862	189.6	55.91	51.64	46.61
	0.0771	169.7	61.90	57.15	51.57
	0.0680	149.5	69.49	64.15	57.88
III	0.1172	257.9	42.12	38.94	35.15
	0.1092	240.2	44.89	41.50	37.41
	0:0910	200.3	53.15	49.11	44.32
	0.0711	156.4	66.70	61.52	55.58

ties given in the second column are those which the solutions would have at 20°. For convenience in our later work the concentrations were recalculated and expressed in mg. of carbon dioxide per 100 cc. of solution, by which is meant the mg. of carbon dioxide stoichiometrically equivalent to the barium hydroxide in 100 cc. of solution. Thus 0.1 N solution is expressed as 220 mg. of CO_2 per 100 cc. These values appear in the third column. The last three columns in the table give the specific resistances as calculated from the observed conductivities and cell constants.

For comparison with the data of Cain and Maxwell[1] and Spoehr

and McGee[2,3] they are all plotted in Fig. 1.   A single point falling
in this region determined by A. A. Noyes[4] is included.

Apparatus and Method.

The electrical equipment for the determination of conductivities
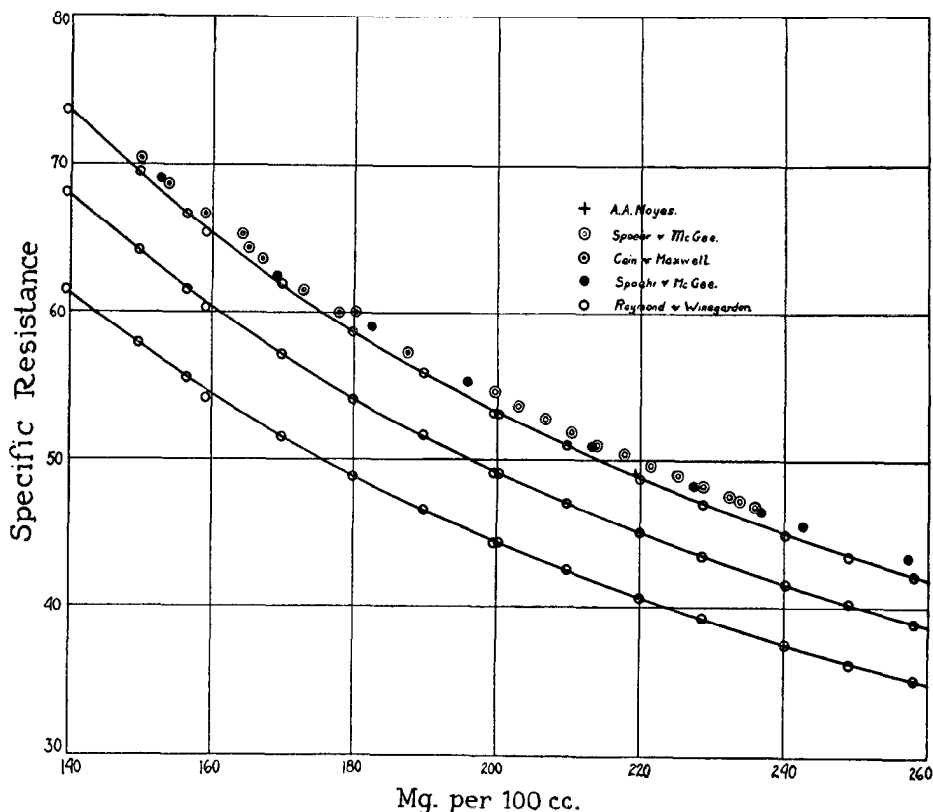differed but slightly from that employed by Cain and Maxwell.[1]

FIG. 1.

A Weibel[5] type A.C. galvanometer operating on the 110 volt 50
cycle light circuit supplied 6 volts A.C. for the bridge.   The

[4] Noyes, A. A., *Carnegie Institution of Washington*, Pub. No. 63, 1907, 254.
[5] Weibel, E. E., *Bureau of Standards, Scientific and Technical Papers, No. 297*, 1917.

galvanometer was of low resistance, and we therefore substituted a low resistance Kohlrausch bridge and a four dial Curtis box for their self-contained bridge.   An advantage of this change will be discussed later.   The instruments were manufactured by Leeds and Northrup and gave complete satisfaction.   Resistances up to 1000 ohms could be measured to within 0.05 per cent, which was greater accuracy than we required.   Carbon dioxide determinations were made with an accuracy of $\pm$ 0.0006 mg. per cc. of absorbing solution or $\pm$ 0.15 mg. for a 250 cc. cell.

For convenience as well as for reproducibility and accuracy in the carbon dioxide determinations, it was decided at the outset to immerse both the fermentation vessels and barium hydroxide solutions in a single large thermostat.   One equipped with the usual stirrer and regulator and capable of maintaining a desired temperature to within $\pm$ 0.02° was employed throughout.

For transferring the carbon dioxide to the absorption vessels a stream of air free from carbon dioxide was used.   The air was purified by passing through a calcium chloride tower and then through three soda-lime towers in series.   By refilling the most used one of these every 3 to 4 weeks and moving the towers in a counter-current fashion, no trouble was experienced from carbon dioxide in the gas stream.   A pressure-regulating device consisting of an 8 inch U-tube about one-fourth filled with mercury was connected to the air line by means of a large T-tube.   If the line pressure exceeded the desired value, the gas stream escaped through the mercury.   This was found to give as close regulation as was necessary.   To maintain constant rates of gas flow a piece of glass capillary tube was placed in the line following the pressure regulator.   By suitably choosing the size of the capillary.and the amount of mercury in the U-tube, the gas flow was easily adjusted to any desired value.   For our work we found 200 cc. of air per minute to be satisfactory.   An exactly constant rate of gas flow is, however, probably unnecessary, as will be indicated later.

After trying several types of gas absorbers we decided that the design of Weaver and Edwards[6] was best suited to our purpose. It is a compact and very efficient absorber, even at high rates of gas flow.   Their original design was simplified and the lower

[6] Weaver, E. R., and Edwards, J. D., J. Ind. and Eng. Chem., 1915, vii. 534.

stop-cock omitted, as illustrated in Fig. 2. The absorber is for 250 cc. of solution but may be altered to permit the use of any quantity. The gas stream enters through the tube A, carries the absorbing liquid up the spiral, and finally escapes through the outlet B. The spirals were built of tubing which was 8 mm. inside diameter and although considerable precipitation of barium carbonate occurred in them, the absorbers could be used for several runs before cleaning and refilling. A difficulty which has been
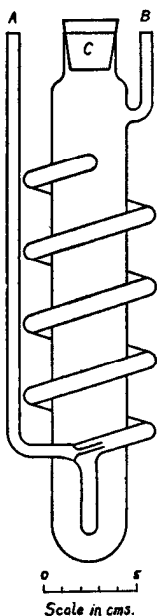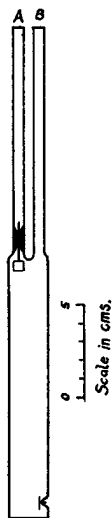

FIG. 2.   FIG. 3.

experienced, however, is that the injector may become clogged during a run. To avoid this the constriction can be omitted as the bubbles are still small enough to produce efficient absorption at low rates of gas flow. A soda-lime tube was attached to the small upper outlet of the absorber to prevent carbon dioxide entering from the air.

A further advantage of this type of absorber is that it is admirably suited to the use of pipette conductivity cells, which were inserted through the rubber stopper C and left in place. The

cell which we employed is illustrated in Fig. 3.   The upper elec-
trode is sealed into the tube A, which also serves to insulate the
lead wire.   The connecting wire to the lower electrode is stretched
along the side of the cell and held in place by the 2-hole stopper
into which the two tubes are inserted.   The open tube B serves
to admit air free from carbon dioxide, in order to blow out the
contents of the electrode as well as to mix the solution in the
absorber.   The cell is immersed so that the upper electrode is
below the liquid level and the pipette need therefore only be blown
out, as it refills of itself.   It was found by blowing out the contents
three or four times that the solution was uniform and further
mixing produced no changes in resistance.   A 3-way stop-cock was
connected to a source of air free from carbon dioxide in such a
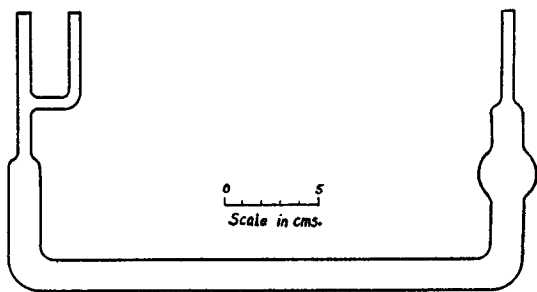

FIG. 4.

way that the electrode might be alternately blown out and allowed
to refill by merely turning the stop-cock.

   In order to liberate the carbon dioxide from the fermenting
mixtures it was desirable to expose as large a liquid surface as
possible to the gas stream.   For this reason the fermentation tube
illustrated in Fig. 4 was employed, the horizontal part of the tube
being about half filled by 20 to 25 cc. of solution.   Solutions
could be added or withdrawn through the straight tube with a
pipette without discontinuing the current of air free from carbon
dioxide which entered through the branch tube.   The straight
tube was capped, except during such removals or additions.   In
order to accelerate the evolution of carbon dioxide, the tube was
fastened with small clamps to a rocking table immersed in the

thermostat.   The table was oscillated by means of a small electric motor fitted with a worm drive and so arranged that both the frequency and the amplitude of the oscillation could be changed. After some preliminary experiments an amplitude of ± 6° from the horizontal and a frequency of about 120 per minute were adopted.

In our final apparatus we mounted six identical sets side by side in the thermostat, the shaking table being built to accommodate six tubes.   The air lines for transferring the carbon dioxide and for blowing out the pipette electrodes were manifolded.   Gas-washing towers filled with water were placed in the air lines and immersed in the thermostat to saturate the incoming air and prevent evaporation.

For convenience an automatic pipette delivering exactly 250 cc. was used to fill the absorption vessels.   A further time-saving device was made possible by our electrical hook up.   The cell constants of our different electrodes were in general not the same. However, by setting the four dial resistance box to correspond to the constant of the cell employed (the setting takes but a few seconds) it was possible to compensate for these differences.   The strength of the barium hydroxide solutions was then calculated from the data of Table I and plotted as a function of the bridge reading.   The graph was found to be very useful, as the calculations were otherwise quite tedious.

Reliability of the Analytical Method.

The observation of Spoehr[4] that the constant of an immersed cell is subject to change due to precipitation of barium carbonate led us to examine this question.   We found that removing the cell from solution and allowing carbon dioxide from the air to form barium carbonate, as suggested by Spoehr, did produce a change in cell constant.   However, as long as the electrodes were kept completely immersed no appreciable change in cell constant was observed during a run.   This is presumably due to the fact that the barium carbonate is largely precipitated in the spiral, before coming into contact with the electrodes, changes in cell constant being due to the precipitation of barium carbonate upon the platinized surface and not to adsorption of previously precipitated barium carbonate.

It was observed, however, that the resistance of the barium hydroxide solutions increased slightly when kept 24 to 48 hours in our cells. This occurred, nevertheless, even in the absence of carbonate, and was not of sufficient magnitude to introduce appreciable error. The cause of this phenomenon was not investigated.

An additional difficulty which we had anticipated was that the precipitation would be so slow as to require a correction factor. To examine this possibility, resistance determinations were made $\frac{1}{2}$, 1, and 5 minutes after introducing carbon dioxide and again after 10 to 24 hours. Changes after 1 minute were found to be negligible as long as the solutions always contained a relatively large excess of barium hydroxide, as was the case in the range of concentrations which we employed.

In order to show that the method actually gave correct results on carbon dioxide, we prepared samples of gas by adding measured volumes of standard sodium carbonate solution to an excess of dilute sulfuric acid. A stream of air free from carbon dioxide was bubbled through the mixture and then through the absorber. After 15 to 20 minutes the amount of carbon dioxide as determined by the change in resistance was compared with that from the amount of carbonate used. The differences were always less than 1 per cent, and were systematically in the direction which would indicate that not all the carbon dioxide had been evolved from the acid solution.

Rate of Evolution of Carbon Dioxide from Solution.

Using the apparatus described above we investigated the rate of removal of carbon dioxide from solutions. Fig. 5 illustrates the results obtained. The graphs represent the carbon dioxide removed in different periods of time, the removal being expressed in per cent of the total amount originally present.

In the first experiment 20 cc. of mercury were placed in the fermentation tube and carbon dioxide introduced in the space above. The shaker was started and the air stream turned on. In $\frac{1}{2}$ minute 95 per cent of the carbon dioxide was precipitated as carbonate, as shown by Curve I. The total amount present was taken as being the value after 10 minutes, as this was identical with the results at 3 and 5 minutes.

The experiment was then repeated with the exception that 20 cc.
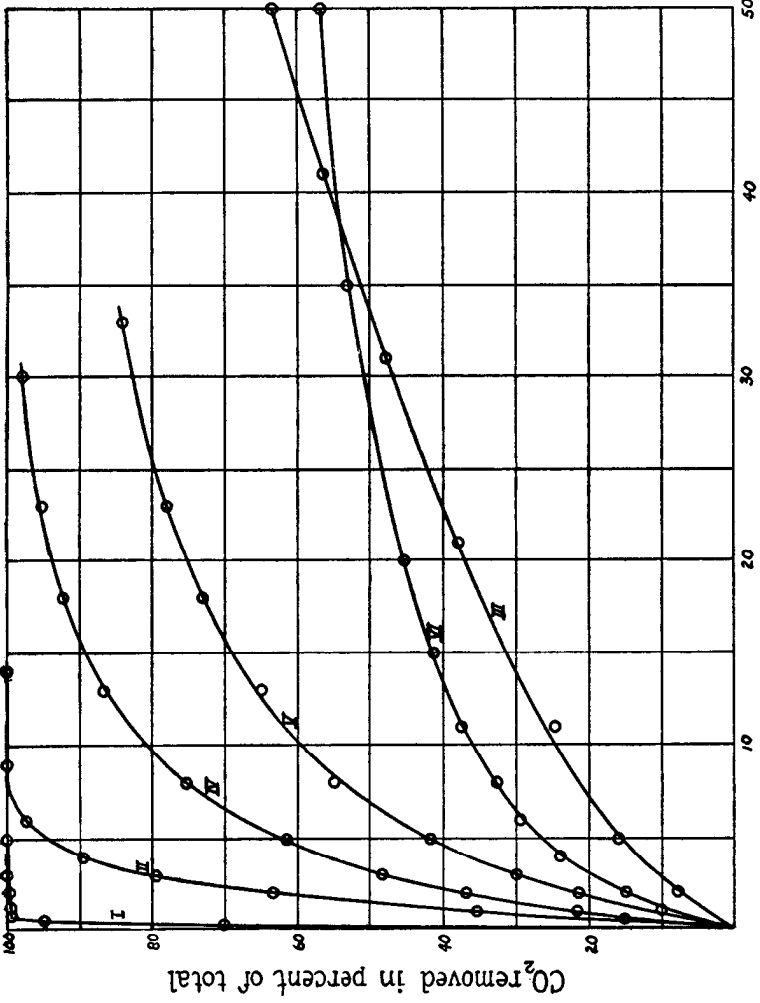
Fig. 5.

of water saturated with carbon dioxide were substituted for the mercury. In this way Curve II was obtained. For comparison this second run was repeated, except that the shaker was not operated. The readings were continued and the value at 24 hours was taken as representing the total amount present. The data for the first 50 minutes are given in Curve III.

From Curves I and II it may be seen that removal of the gas from the space above the liquid is, for all practical purposes complete in 1 minute, but that the rate at which carbon dioxide escapes from the liquid into the gas space is in comparison very slow. From the data for Curve III the rate of escape from an unagitated liquid was found to be 4 per cent per minute at the start, dropping rapidly to 2 per cent per minute. From the agitated liquid, however, the rate of escape was about 60 per cent of the residual carbon dioxide per minute. This illustrates the extreme importance of the shaking. Increasing the shaking, placing beads in the tube, and using a corrugated tube that broke up the liquid surface more thoroughly, each produced slightly better results, but not enough to compensate for certain mechanical disadvantages. With different mechanical arrangement, which would allow very violent shaking, it should be possible still further to increase the rate of removal.

The rate of gas flow was found, as mentioned above, to be of but slight consequence. By shaking the mixture with the air stream off, stopping the shaking, and turning on the air, a curve almost identical with Curve III was obtained. The average rate in this case was about 55 per cent of the residual carbon dioxide removed per minute.

If we assume 50 per cent per minute to be obtainable it is possible to calculate the limitations of the method. If carbon dioxide is being produced at a constant rate, then the error in observation the 1st minute after the beginning of such production will amount to 50 per cent of the production. The 2nd minute the error will be only 25 per cent, and the 3rd minute only 12 per cent, etc. Similarly, if some acceleration (either positive or negative) in the rate of production suddenly occurs, then after 5 minutes this will be reported to within about 3 per cent of the increment. For small uniform accelerations the error is not appreciable. It may therefore be stated that the method may be used with negligible

errors for uniformly accelerated changes, while for instantaneous changes the error is negligible after 5 minutes.   Observations may, therefore, not be made less than 5 minutes after some sudden change in rate.   As an example, there may be included the results (Fig. 6) on a fermentation employing live yeast.   At the point P saturated mercuric chloride solution was introduced.   Although carbon dioxide production probably ceased almost immediately, the observations do not report this fact for several minutes, as may be seen from the curve.
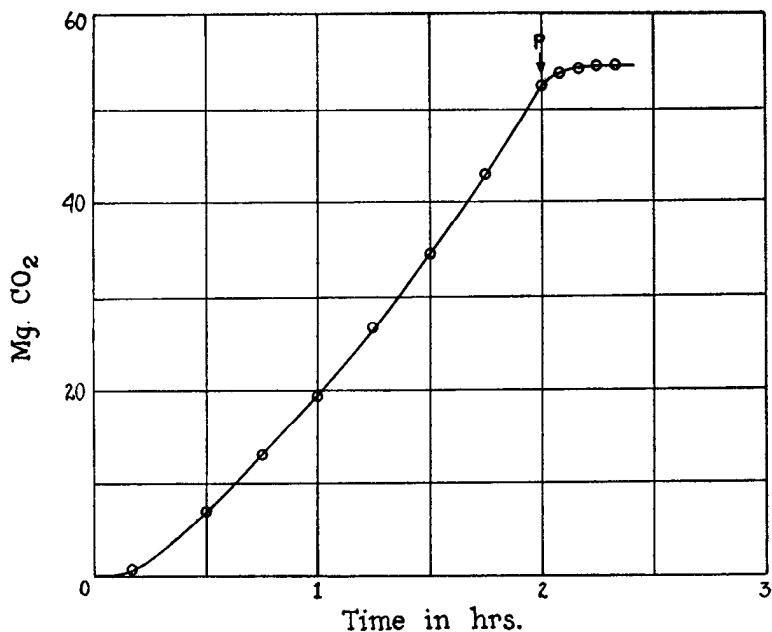

FIG. **6.**

A further factor which must be considered in this connection is the pH of the solutions.   In the experiments just reported, since the solutions were unbuffered, the pH was between 4 and 7. In this region the carbon dioxide is largely present as carbon dioxide (or H_2CO_3).   If, however, the solutions be made more alkaline, the proportion of carbon dioxide as $CO_3^=$ and HCO_3^- increases, as shown in Table 1I.   Since 50 per cent of the total

carbon dioxide is removed per minute in the acid solutions, it might be expected that 50 per cent of the free carbon dioxide (CO_2 not present as HCO_3^- or $CO_3^=$) would in general be removed per minute. This was investigated experimentally. Buffered solutions were substituted for the water and the shaker was operated throughout. To obtain the total amount originally present, the solutions were acidified after 30 to 50 minutes, and shaken till equilibrium was reached. Curves IV, V, and VI in Fig. 5 represent the results at pH 7, 8, and 9, respectively. It may be

TABLE II.

Per cent of total present as:	pH					
	4	5	6	7	8	9
HCO_3^-....................	0.3	2.9	23.1	75.0	96.8	93.9
$CO_3^=$......................			0.001	0.05	0.6	5.6
$CO_2 + H_2CO_3$.............	99.7	97.1	76.9	25.0	2.6	0.5

TABLE III.

pH	Calculated rate per min.	Observed rate.		
		10 min.	20 min.	30 min.
	per cent			
4	57			
5	55	57		
6	43	42	35	
7	14	12	11	10
8	1.5	6	4	3
9	0.3	2.2	1.2	0.9

seen that the rate of evolution is much less in these more alkaline solutions.

The rates which would be predicted from the fraction present as $CO_2 + H_2CO_3$ at the different hydrogen ion concentrations were compared with rates actually observed at 10, 20, and 30 minutes. In the calculations it was assumed that 55 per cent per minute is evolved at pH 5 (Table III).

We may therefore conclude that without employing correction factors the method is not easily applicable in its present form to investigations in alkaline media.

Applications.

A particular advantage of the method is that it is ideally suited to investigations on aerobic and anaerobic fermentations, as a change from compressed air to compressed nitrogen is all that is required. A disadvantage involved in the application of the procedure to biological studies, however, lies in the difficulty in securing sterility. The tubes may be easily sterilized but the air streams and connections are apt to present difficulties. Our own work has been confined to enzyme systems and we have therefore used an inhibiting agent in the mixture. Toluene, which is most often used for this purpose, is too volatile and we have employed instead a 1:5000 concentration of gentian violet. At this concentration there is no appreciable inhibition of zymase, but no live yeasts have been observed even after 3 or 4 days.

SUMMARY.

The specific resistances of barium hydroxide solutions from 0.065 to 0.12 N have been determined at 25°, 30°, and 37°.

The rate of the evolution of carbon dioxide from aqueous solutions under various conditions has been examined.

A method has been developed for the rapid and accurate determination of the carbon dioxide formed in fermenting mixtures. The method is applicable to either aerobic or anaerobic investigations. The advantages and limitations of the method are pointed out.