THE DETERMINATION OF CARBON DIOXIDE IN FERMENTING MIXTURES

Thesis by

Howard Merlin Winegarden

In Partial Fulfillment of the Requirements
for the Degree of Master of Science

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THE DETERMINATION OF CARBON DIOXIDE IN FERMENTING MIXTURES

By Albert L. Raymond* and Howard M. Winegarden

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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.

We wish to thank Professor A.A.Noyes for grants which made possible this investigation.

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°C. and are not in very good agreement. As we wished to employ both 30°C. and 37°C., we determined the conductivity at these temperatures over the concentration range of 0.12 to 0.065 N., and in addition repeated the observations at 25°C. 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 x 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 Merk'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%. 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%.

The temperature of the thermostat was read to ± 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 determination repeated. Measurements were made on a portion of each sample at 25°C., 30°C., and 37°C. 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.

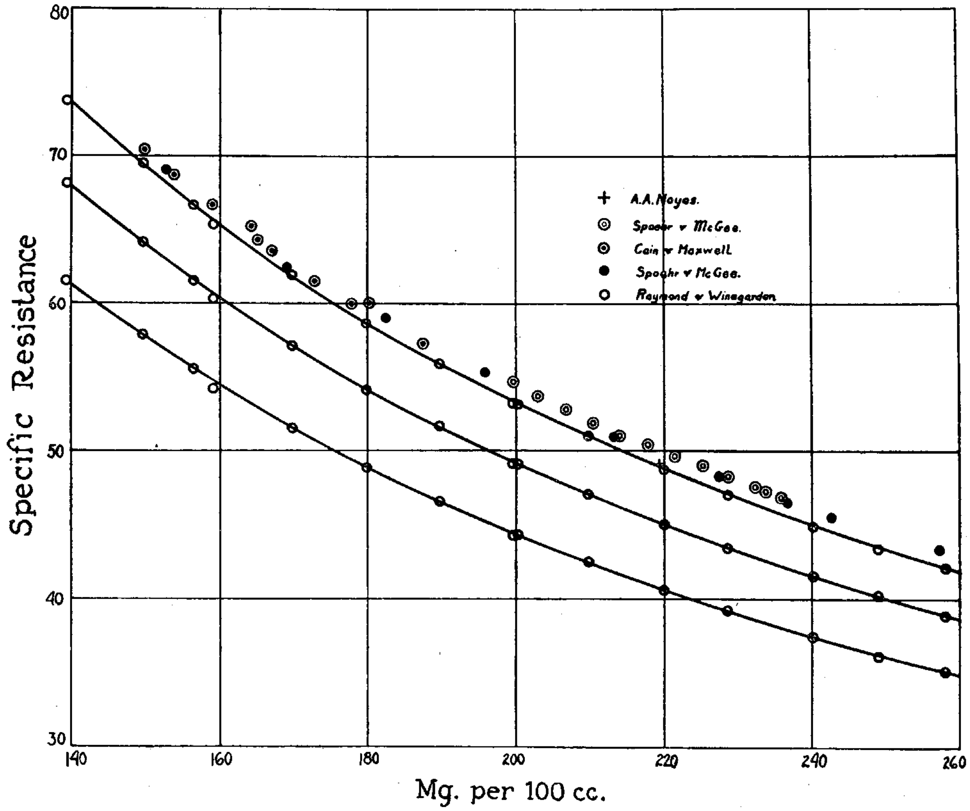
After several preliminary experiments, the data given in Table I were secured in three runs, as indicated in the first column. Each sample was used at the three temperatures, and the normalities given in column two are those which the solutions would have at 20°C. For convenience in our later work, the concentrations were recalculated and expressed in milligrams of carbon dioxide per 100 cc. of solution, by which is meant the milligrams of carbon dioxide stoichiometrically equivalent to the barium hydroxide in 100 cc. of solution. Thus 0.1 N. solution is expressed as 220 mg. CO_2/100 cc. These values appear in column three. The last three columns in the table give the specific resistances as calculated from the observed conductivities and cell constants.

Table I

Run	Norm. at 20°	MgCO$_3$ per 100 cc.	Specific Resistance 25°	30°	37°
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

For comparison with the data of Cain and Maxwell[1], and Spoehr and McGee[2,3] they are all plotted in Fig.I. A single point falling in this region determined by A.A.Noyes[4] is included.

Figure I



Apparatus and Method

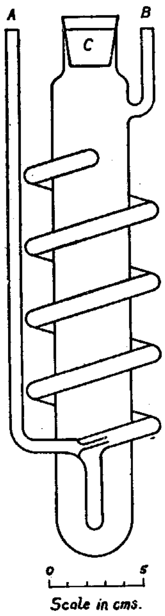
The electrical equipment for the determination of conductivities differed but slightly from that employed by Cain and Maxwell[1], A Weibel[5] type A.C. galvanometer operating on the 110 v. 50 cycle light circuit, supplied 6 v. A.C. for the bridge. The galvanometer was of low-resistance, and we therefore 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%, which was greater accuracy than we required. Carbon dioxide determinations were made with an accuracy of ± 0.0006 mg./cc. of absorbing solution, or ± 0.15 mg. for a 250cc. 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 ± 0.02 degrees 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 it through a calcium chloride tower and then through three soda-lime towers in series. By refilling the most used one of these every three to four 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 eight 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 stopcock omitted, as illustrated in figure II. 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.

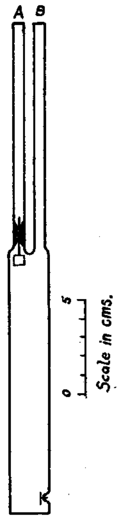
Fig. II.



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 experienced, however, is that the injector may become clogged during a run. To avoid this the constriction could 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 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 figure III.

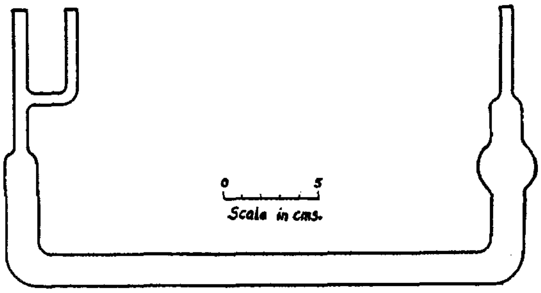
Fig. III.



The upper electrode 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 two-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 be only 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 three-way stopcock was connected to a source of air, free from carbon dioxide in such a way that the electrode might be alternately blown out and allowed to refill by merely turning the stopcock.

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 figure IV was employed, the horizontal part of the tube being half filled by 20-25 cc. of solution.

Fig. IV.



Solutions could be added or withdrawn through the straight tube with a pipette without disconnecting 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 degrees from the horizontal and a frequency of about 120/min. were adopted.

In our final apparatus we mounted six identical sets side by side in the thermostat, the shaking table being built to accomodate 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 were 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 the 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, but was not of sufficient magnitude to introduce appreciable error. The cause of this phenomenom was not investigated.

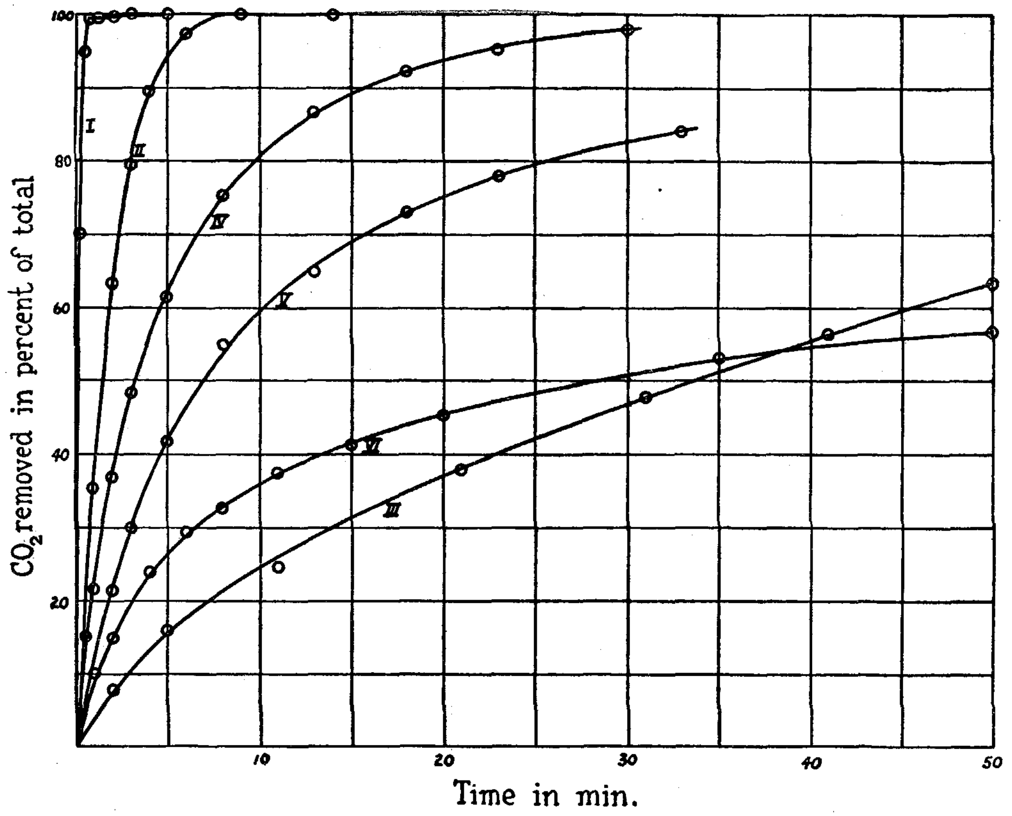
An additional difficulty which we had anticipated was that the precipitation would be so slow as to require a correcting factor. To examine this possibility, resistance determinations were made one-half, one, and five minutes after introducing carbon dioxide and again after ten to twenty-four hours. Changes after one 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 sulphuric acid. A stream of air, free from carbon dioxide, was bubbled thru 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 one percent, and were systematically in the direction which would indicate that not all of the carbon dioxide had been evolved from the acid solution.

Rate of Evolution of Carbon Dioxide from Solution

Using the apparatus described above the rate of removal of carbon dioxide from solutions was investigated. Figure V illustrates the results obtained. The graphs represent the carbon dioxide removed in different periods of time, the removal being expressed in percent of the total amount originally present.

Fig. V.



In the first experiment 20 cc. of mercury were placed in the fermentation tube, and the carbon dioxide introduced in the space above. The shaker was started and the air stream turned on. In one half minute 95% 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.

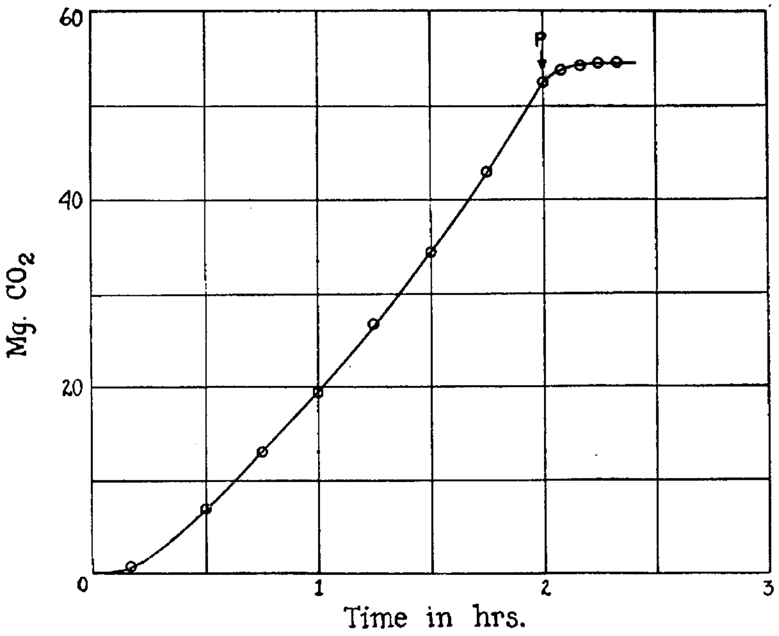
The experiment was then repeated with the exception that 20 cc. of water saturated with carbon dioxide were substituted for the mercury. In this way Curve II was obtained. For comparison, this second run was repeated, except that the shaker was not operated. The readings were continued and the value at 24 hours was taken as representing the total amount present. The data for the first 30 minutes are given in Curve III.

From curves I and II may be seen that removal of the gas from the space above the liquid is, for all practical purposes complete in one 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 minute at the start, dropping rapidly to 2% per minute. From the agitated liquid, the rate of escape was about 60% 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 to still further 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% of the residual carbon dioxide removed per minute.

If we assume 50% per minute to be certainly 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 first minute after the beginning of such production will amount to 50% of the production. The second minute the error will be only 25%, and the third minute only 12%, 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% of the increment. For small uniform acceleration, 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 five minutes. Observations may, therefore, not be made less than five minutes after some sudden change in rate. As an example, there may be included the results (Fig. VI) on a fermentation employing live yeast.

Fig. VI



At the point P saturated mercuric chloride solution was introduced. This reagent causes an almost immediate cessation of fermentation, but the observations do not report this fact for several minutes, as may be seen from the curve.

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 CO_2 (or H_2CO_3). If, however, the solution is made more alkaline, the proportion of carbon dioxide as $CO_3^=$ and HCO_3^- increases, as shown in Table II. Since 50% of the total carbon dioxide is removed per minute in the acid solutions, it might be expected that 50% of the free carbon dioxide (CO_2 not present as HCO_3^- or $CO_3^=$) would be in general 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 until equilibrium was reached. Curves IV, V, and VI in Fig. V represent the results at pH 7, 8, and 9, respectively. It may be seen that the rate of evolution is much less in these more alkaline solutions.

Table II

Percent 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

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 minute is evolved at pH 5, (Table III).

Table III

pH	Calc. rate % per min.	Observed rate 10 min.	20 min.	30 min.
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

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 of securing sterility. The tubes may be easily sterilized, but the air stream and connections may 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 0.12 to 0.065 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.

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[1] Cain and Maxwell, Jour. Ind. Eng. Chem., 11, 852 (1919).

[2] Spoehr and McGee, Ibid, 16, 128 (1924).

[3] Spoehr and McGee, Carnegie Inst. Publ, 325 (1923).

[4] A.A.Noyes, Carnegie Inst. Publ., 63, 254 (1907).

[5] E.E.Weibel, Bur. of Stand. Sci. Paper, 297 (1917).

[6] Weaver and Edwards, Jour. Ind. Eng. Chem., 7, 534 (1915).

CO-ZYMASE - A STUDY OF PURIFICATION METHODS

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CO-ZYMASE - A STUDY OF PURIFICATION METHODS

By Albert L. Raymond[1] and Howard M. Winegarden.

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[1] National Research Fellow in Chemistry.

Introduction

Co-zymase is one of the essential components of the complex enzyme mixture which effects alcoholic fermentation in the absence of living cells. The separation of the mixture into "zymase" and "co-zymase" was first accomplished by Harden and Young[2] by means of ultrafiltration through a gelatin impregnated Chamberland filter candle. The residue and filtrate as thus prepared possessed, separately, no fermentative action, but when mixed were found to produce a rapid fermentation. The active constituent of the residue was named zymase, while that constituent of the filtrate responsible for the reactivation of the residue was named co-zymase.

The mechanism of the activation of the zymase was investigated by Euler and Myrbach[3], who came to the conclusion that the co-zymase was involved in that stage of the process in which inorganic phosphates are converted to carbohydrate esters. The same authors[4] have described a comprehensive and successful series of experiments on the purification of co-zymase.

A complete knowledge of the properties and function of co-zymase would be of extreme importance, as it should throw considerable light upon the mechanism of enzyme action as a whole, and should in addition have important bearing upon the entire question of carbohydrate utilization. It has been shown, for example, by Meyerhof[5] that a substance, either identical with, or very similar to co-zynase exists in most animal tissue, and the demonstration of a complete identity of the two would have important metabolic connotations.

In view of the importance of the field, as well as of the interest of one of us[6] in the problem of carbohydrate utilization, we decided to attempt an extension of the work of Euler and Myrbach on the purification of co-zymase. At the beginning, however, we found that our yeast produced much less active enzyme preparations than those of Euler and Myrbach, as regards both the zymase and co-zymase content, and also that the technic of the above authors with lead precipitation, which enabled them to secure an initial purification of from ten to thirty fold, was in our case practically useless.

We therefore abandoned our original intention and have instead studied the purification produced in our material by a variety of reagents. In the investigation we have repeated much of the work done by Euler and Myrbach, and several differences have been found, which appear difficult to explain solely upon the basis of the lower initial purity of our material. As certain of the experiments show distinct promise, we hope to be able to extend the work upon a material of considerably higher original purity, such as was employed by Euler and Myrbach.

We desire to thank Professor A.A. Noyes for grants and facilities which allowed us to pursue these studies, and Professor R.C. Tolman for his constant interest and encouragement.

Materials and Technic of Assay.

The yeast which we employed throughout was a bottom yeast supplied by the Eastside Brewery[7] in Los Angeles. It was obtained fresh, transported immediately to Pasadena, and used the same day.

For analyzing extracts for co-zymase activity, the analytical procedure adopted was similar to that employed by Euler and Myrbach. When zymin (acetone-dried yeast) is washed with water, the co-zymase is largely removed. The washed material is therefore a source of zymase practically free from co-zymase, and on addition of the latter is again capable of producing fermentation. Euler and Myrbach found on adding co-zymase extracts to such washed zymin that within reasonable limits of concentration the maximum rate of carbon dioxide production was proportional to the amount of extract added. Our experience in general confirmed this, as well as the fact that it is necessary to keep the phosphate concentration constant throughout. All solutions were adjusted to pH 6.3 - 6.5 before testing, but even in those cases in which all precautions were observed, we found variations of 5 to 10 per cent in certain analyses and our assays are therefore only reliable within these limits.

In order to prepare our zymin the prodecure of Albert[8] was used. Fresh yeast was filtered through canvas in a small plate and frame press under five to fifteen pounds pressure. The press was then blown out with compressed air to secure partial drying, and the yeast was pressed in a hand press to remove as much water as possible. This drying was facilitated by making cakes of the washed yeast, wrapping them in sheets of filter paper, and placing them between pads of blotting paper. The bundle was wrapped securely in canvas, and pressed at about 300 lbs. per sq. in., producing a friable, light-colored product. This was converted to zymin in 500 gram lots by passing it through a ten mesh sieve into three liters of c.p. acetone. After stirring for ten minutes, the acetone was removed by suction filtration. The partly dried yeast was next introduced into one liter of fresh acetone for two minutes, filtered, ground with 500 cc. of ether for three minutes, and again filtered. The almost white powder which resulted was air-dried.

Although the procedure appears quite simple, we found it difficult to prepare an actively fermenting zymin from our yeast. On substituting technical acetone for the c.p. material, a completely inactive preparation resulted, as was the case when the acetone was added with stirring to the yeast instead of the reverse as described above. Even those lots which were prepared by apparently identical procedures showed considerable differences in activity. The experimental work to be reported was, therefore, performed with two very similar lots which were, incidentally, the best which we secured.

For assay purposes, the zymin was freed of co-zymase by mixing with eight parts of water and centrifuging. This washing was repeated twice more, and the zymin then produced almost no fermentation in the absence of added co-zymase. The zymin was freshly washed as needed.

The solution used for making the assays was prepared as follows. Eleven grams of zymin were washed as above, and diluted to a total weight of 48.4 gms. (44 cc.) by adding distilled water and a small amount of "zymophosphate" solution. There were then added 18.9 cc. of a solution of the following composition:

$Na_2HPO_4 \cdot 6H_2O$	18.8 grams
KH_2PO_4	15.6 "
Glucose	583.0 "
Gentian violet	0.73 "

Distilled water to 1000 cc.

The mixture was made homogeneous by stirring and shaking, and 10.0 cc.

were removed and added to 9.0 cc. of the co-zymase solution to be tested (or a lesser amount diluted to 9.0 cc.). The final concentration in this fermenting mixture were:

Na_2HPO_4	0.013 molal
KH_2PO_4	0.020 "
Glucose	10.0%
Gentian violet	0.125%
Zymin	10.0%

The "zymophosphate solution" was added because of its known property of reducing the incubation period of fermentation. The "zymophosphate" was prepared from the first zymin washes by precipitation first as a barium and finally as a lead salt. Lead was precipitated with hydrogen sulfide, the latter removed in vacuo, and the resulting solution adjusted to pH 6.4. It had no co-zymase activity, and enough was added to diminish the incubation period to from o to $\frac{3}{4}$ hours.

The gentian violet was added as an antiseptic, and at the above concentration we observed no growths for the periods over which we worked (two to twelve hours), and there was no inhibition of the enzyme action.

The pH of the mixture was 6.4, and the total phosphate concentration was optimum for our yeast.

For determining the carbon dioxide formed an electrometric[9] method devised for the purpose was employed. Readings were made at 15 minute intervals, and the maximum rate was taken as a measure of the co-zymase activity of the sample.

Typical fermentation curves are given in Fig. I. In these the rates of carbon dioxide production are plotted as a function of the elapsed time. Curves I and II were obtained with different quantities of co-zymase, using the glucose-phosphate mixture described above, while Curves III and IV are the corresponding ones for runs employing twice the concentration of phosphate, other factors being unchanged. The longer periods before attainment of the maximum rate in the latter case are characteristic of high phosphate concentrations, as are the larger maximum rates for the same co-zymase concentration.

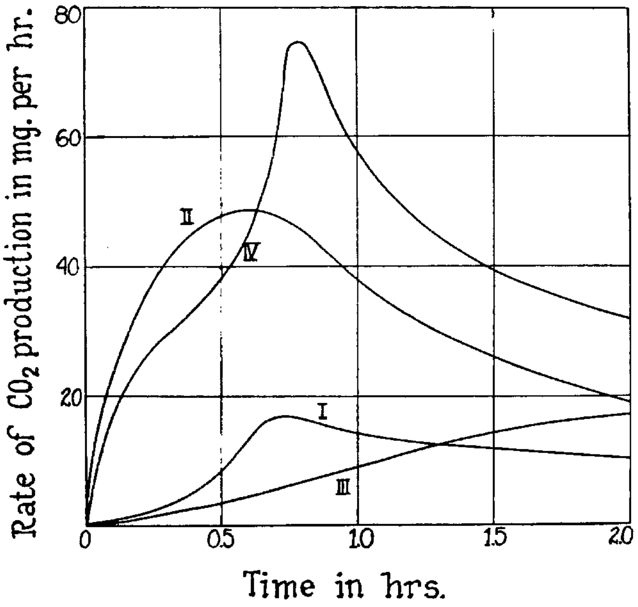

Fig. 1.

It is of interest that at no time did we find co-zymase preparations which gave curves markedly different from those of Fig. I. With many of the purified preparations, however, an incubation period of from one to three hours was observed, and as "zymophosphates" have been stated to remove these lag periods, we added such preparations, as mentioned before, to our fermentation mixtures.

Hydrogen ion concentrations were determined throughout by means of indicators, the colors being checked with electrometrically standardized buffers. In addition to Clark and Lub's standard set of indicators, we employed phenolphthalein, thymophthalein, and in a few cases, methyl blue.

For convenience in reporting our results, we have defined a "unit" as being that quantity of co-zymase required to produce a maximum rate of fermentation of one mg. of carbon dioxide per hour. We have also used Euler's activity coefficient, which is defined as the maximum rate of carbon dioxide production in cc. per hour, divided by the solid content of the sample expressed in grams. The symbol Aco was suggested by Euler to designate this unit and has been employed throughout this paper. The Aco, except where otherwise noted, refers to the ash-free material. Solids were determined by drying to constant weight at 105-110°C and ash by igniting at dull red until the residue was gray or white in color.

Extraction of Co-zymase from Yeast

Our co-zymase solutions were prepared by extracting yeast with hot water, a procedure which has been shown by others to destroy the zymase activity without appreciably impairing the co-zymase. Fresh and washed yeasts were both investigated, but as the only difference between the two extracts lay in the fact that those from the washed yeast contained slightly less solids, the latter were not extensively employed.

In order to obtain an efficient extraction, various factors were examined with a view to determining the optimum yield of co-zymase, without regard to the purity of the resultant product. Batches of 10 to 20 liters were prepared by adding yeast to water at 90-92^{0}, stirring two to three minutes, and cooling as rapidly as possible by immersing the metal container in running water.

The effect of concentration of the yeast upon the yield was studied, and it was found that the efficiency of the extraction increased largely with decreasing concentration down to 0.1 gm. fresh yeast per cc. solution. More dilute extracts were not examined, as solutions of much lower activity required too extensive evaporation before they could be used.

Acidity was also examined with regard to its effect on the yield, glacial acetic acid and sodium carbonate being added to the hot water to vary the acidity. The pH was determined and the solution neutralized to pH 6.4, and tested. The results are included in Table I.

Table I.

Reagent	HOAc		Control	Na_2CO_3	
Concentration (g/L)	5	1		1	5
pH of extract	3.8	4.6	6.0	7.0	10.0
Activity (Units/cc.)	3.6	3.6	4.7	2.9	2.5

Electro Dialysis

No attempts appear to have been made toward purifying co-zymase by means of electro-dialysis. As the method offers great possibilities with those substances which migrate under the influence of an electric field, we decided to investigate the results with our material.

A cell was built consisting of seven 50 cc. compartments, in a row, and separated by parchment paper partitions. There were placed in the compartments in order: I, copper sulfate solution (1 molal) and a copper electrode; II, potassium chloride (0.1 m.); III, potassium chloride (0.1m.) adjusted to the desired pH; IV, co-zymase solution similarly adjusted; V, VI, and VII were duplicates of III, II, and I respectively. The potassium chloride solution in II and VI prevented contamination of that in III and V.

To see if the pH of the solution affected either the magnitude or direction of migration of the activity, a set of four experiments was performed covering pH range of 2 to 10. A potential drop of 4 volts/cm. was maintained in the co-zymase solution and 1000 to 1800 coulombs passed in 40 to 60 minutes. In the case of experiment III, the apparatus was kept in the ice-box to decrease the destruction due to the alkalinity. The pH did not change appreciably during the run. The activities of the solutions are given in Table II.

Table II.

		Activity (Units/cc.)		
Expt.	pH	Anode	Center	Cathode
IV	2.0	0.2	1.4	0.3
II	4.4	0	2.7	0
I	6.4	0.2	3.2	0.2
III	9.7	0	2.3	0

In no case, therefore, was an appreciable amount of co-zymase transferred to the potassium chloride compartments, although tests showed the parchment paper was permeable to the co-zymase over the entire pH range employed.

In view of the fact that the paper was permeable to the co-zymase and yet practically no transference occurred in an hour, it seems justifiable to conclude that the co-zymase carries no appreciable fraction of the current over the pH range examined. Whether this is due to its being present at low concentration, or to its having a low degree of ionization, or to both, can not be determined from the above data. However, the possibility of purifying crude solutions by this means seems to be definitely excluded.

Alcohol Precipitation

As alcohol has been suggested for purifying co-zymase, we performed a number of experiments with this reagent. We found that an alcohol concentration of 40% produced a fine flocculent precipitate and left the majority of the activity in the solution, but that 80% alcohol, on the other hand, produced a less satisfactory separation and frequently occasioned considerable losses of activity.

It seemed well to learn, therefore, how much purification was being produced, and a set of experiments was performed with this in mind. A co-zymase solution was concentrated five fold in vacuo and divided into two portions. The first was brought up to 80% by the addition of 95% alcohol, and the fractions separated. (Table III)

Table III

	Yield (%)	Aco	
		Dry	Ash-free
Filtrate	59	28	38
Precipitate	48	30	41
Original		26	36

To the remaining portion of the concentrated solution alcohol was added to 40% and the filtrate was then brought up to 80% as before. (Table IV) The Aco was determined on each fraction and the yields calculated. The yields are expressed as the percentage of the original co-zymase, present in the different fractions. The failure to add up to 100% is presumably due either to varying phosphate concentrations in the different fractions, or else to the removal of some inhibiting material. This phenomenon was frequently observed.

Table IV

	Yield (%)	Aco	
		Dry	Ash-free
40% precipitate	11	15	29
80% "	51	36	42
80% filtrate	61	31	44
Original		26	36

It will be seen that even though there were apparently no losses of activity, the purification was not appreciable in any case. It must be noted that these experiments were performed only upon crude solutions and the possibility, therefore, remains that different results might be obtained on a purer material.

Lead Precipitation

Due to the great success of Euler and Myrbach using lead precipitation as a means of purification, we attempted to repeat their experiments with this reagent. The procedure evolved by them consisted in a precipitation at pH 6, which left the active material in the filtrate, followed by precipitation of the activity at pH 10.

We employed both washed and unwashed yeast and prepared an extract exactly as they described. Precipitation at pH 6 gave a mixture which was very difficult to filter and which was turbid after centrifugation. Practically none of the activity passed into the precipitate. Solid determinations indicated only very slight increase in the Aco. Euler and Myrbach, on the other hand, obtained three fold purification and reported no mechanical difficulties.

At pH 10 we found the activity to be again almost entirely in the filtrate. This is contrary to the results of the above authors, who found precipitation to begin at pH 8.5 and be complete at pH 10. We also duplicated our own results with pH 6 filtrates to which additional lead acetate had been added, indicating that the difference in the alkaline precipitation was not due to insufficient lead. To ascertain whether we were dealing solely with a concentration effect, we evaporated a sample of our material to one sixth volume in vacuo and repeated the experiment. Again the activity remained almost completely in the filtrates, both at pH 6 and 10.

In view of these facts, as well as the one mentioned above that the precipitation is not at all satisfactory at pH 6, we decided to modify Euler's first procedure as follows. Batches of co-zymase solution were prepared by adding 2½ kg. of fresh yeast to 15 L of water at 90-92°. The solutions were filtered immediately and cooled. Centrifugation probably would have been preferable but no continuous centrifuge was available. The cooled, filtered solutions were placed in a large glass container capable of holding 25-30 L and a hot concentrated lead acetate solution was added to the extent of 7.5 g. of lead acetate per liter. The pH was then adjusted to 9.0 by the addition of 6 N. NaOH with constant stirring, and the mixture was allowed to settle for one or two minutes. The supernatant portion was occasionally perfectly clear and filtered rapidly giving a light yellow, crystal clear solution. When this was not the case, additional portions of 0.5 g/L of lead acetate were added, and the pH readjusted after each addition to 9.0. In no case did we need to add more than 8½ g/L total lead acetate to produce perfect precipitation. The phosphate content[10] of the solution dropped from an average of 0.35 mg/cc. to about 0.007 mg/cc. which accounts for a large portion of the added lead.

These final solutions, after neutralization and removal of lead, contained from 90-100% of the activity of the original extract, but solid determinations showed only slight changes in Aco. A few experiments chosen at random are given to illustrate these points. (Table V)

Table V

Expt.	Remarks	Yield (%)	Aco	
			Dry	Corr.
19	Orig. unwashed		36	
	Precipitated	109	39	73
40	Orig. unwashed		50	
	Precipitated	96	45	73
41	Orig. washed		73	
	Precipitated	97	62	130
66	Orig. unwashed		21	
	Precipitated	90	20	32

The solid content of the final solution may be corrected for those ions added during the precipitation which would be carried through into the filtrate. These include Na^+ and NO_3^- from the base and acid used and Ac^- from the lead acetate. The final column of the table gives the activities corrected by these factors, from which it may be seen that an actual slight purification did result in each case.

Solutions prepared as above, still containing a small amount of lead, were quite stable. Unlike the original boiled yeast extracts, they exhibited little tendency to develop mold or bacterial growths, and lost less than 50% of their activity on being kept in the ice-box for two or three weeks. As mentioned above, inorganic phosphates were almost completely removed by the lead precipitation, and therefore did not interfere with additional reagents. For these reasons, we adopted this precipitation as standard, and the further work described in this paper was largely done on such lead treated solutions, from which excess lead was not removed. No distinction will be made between such solutions prepared from washed and unwashed yeast, as no differences in their behavior were observed.

In order to determine the effect of higher pH on the precipitation of the active material, we added lead acetate in different concentrations and tried pH values of 10.5 and 11. The behavior was quite variable and appeared to depend upon the particular batch of material employed. For example, lot no. 41 made 0.005 M. with lead acetate, gave a precipitate containing 79% of the activity at pH 10.5 and 72% at pH 11.0, while another, lot no. 66, using lead ion concentrations of 0.005, 0.01, and 0.015 M., at pH's 10, 10.5, and 11 gave a maximum precipitation of 27%. It is possible that the differences between Euler's results and our own regarding the precipitation at pH 10 are dependent upon the same factors that must be operating in the cases cited. In any event, the lead is not a satisfactory precipitant until these apparent anomalies are understood.

In those cases in which the lead did precipitate the activity, the purity was considerably increased. The results obtained for lot no. 41 are given in Table VI.

Table VI

Ppt. pH	Yield (%)	Aco	
		Dry	Ash-free
Orig.		73	
10.5	79	358	524
11.0	72	291	413

Although this lot gave the most effective purification which we secured with lead, it was but seven fold increase over the original material. In view of the fact that similar precipitations in the hands of Euler and Myrbach produced 10 to 30 fold increase over their original, the above results do not appear particularly encouraging.

There is one further point to be noted in connection with the experiments on lead, regarding the poisoning of zymase by that substance. A duplicate experiment was performed, using in one case the lead treated co-zymase solution directly, and in the other an identical quantity from which the lead had been removed with hydrogen sulfide. The two runs were identical in fermentative activity within the limits of experimental error. An analytical determination demonstrated a lead concentration of 0.00048 M. in the fermentation mixture, which would seem to exceed the "trace" which Euler and Myrbach found produced poisoning.

Mercury Precipitation

The results with mercury as a precipitant for co-zymase were particularly interesting, as they showed certain peculiarities that do not appear to have been previously noted, in biological purifications.

Mercuric chloride is by far the most common mercury salt employed in biological work. Due, however, to its low degree of ionization, it might be predicted that less precipitation would occur with this salt than with mercuric nitrate, for example, at the same molalities. This was verified experimentally. On adding $Hg(CN)_2$, $HgCl_2$, and $Hg(NO_3)_2$ of the same concentration to the lead-treated solution, there were observed, respectively, no, slight, and heavy precipitation. This is in exact correspondence with the degree of ionization of these salts.

More quantitative experiments were therefore performed with $HgCl_2$ and $Hg(NO_3)_2$. To samples of co-zymase solution, $HgCl_2$ solution was added to concentrations of 0.0025, 0.005, and 0.0075 M., the pH was adjusted to 7.2 - 8.0, and the precipitate centrifuged off. The addition of a few drops more of $HgCl_2$ solution produced no precipitation or opalescence in the filtrates, indicating "complete precipitation" in each case. It was therefore of great interest that not only did the yield of active material carried down by the precipitate increase with increasing concentration of $HgCl_2$, but the Aco's likewise progressively increased. The data are given in Table VII.

Table VII

$HgCl_2$ conc.	Yield (%)	Aco Dry	Aco Ash-free
Orig.		20	31
0.0025	25	75	139
0.005	42	125	185
0.0075	65	117	192

The above experiment was then repeated with $Hg(NO_3)_2$ at the same concentrations. The clear solutions, after centrifuging off the precipitate, were tested with more of the reagent and further precipitation was found to occur with the 0.0025 and 0.005 M. concentrations, but not with the 0.0075. In this case, the yields increased as before, but the purity underwent a constant decrease. (Table VIII)

Table VIII

Molal	Yield (%)	Aco Dry	Aco Ash-free
Orig.		20	31
0.0025	45	135	214
0.005	57	106	166
0.0075	75	106	140

In order to elaborate upon these observations, the experiments were repeated in a more acid medium. A pH of 6.5 - 6.7 was employed, and the same concentrations of mercury as above. Precipitation was practically complete with the $HgCl_2$ at 0.0025 M. and with the $Hg(NO_3)_2$ at 0.005 M. In this experiment the results were similar to those at the higher pH, but the yield was in each case lower, as illustrated in Table IX.

Table IX

Precipitate	Orig.	$HgCl_2$			$Hg(NO_3)_2$		
Conc. molal		0.0025	0.005	0.0075	0.0025	0.005	0.0075
Yield percent		12	37	38	42	46	59
Aco (ash-free)	31	47		83	121	93	93

The results confirm the experiment of Euler to the extent that $HgCl_2$ to "complete precipitation" in neutral or acid solution is a poor precipitant. They are, however, quite different in their final interpretation, for they indicate $HgCl_2$ to have considerable possibilities in the purification, if used in larger quantities than necessary for "complete precipitation".

The experiments are also of importance in connection with biological purifications employing mercury, as they indicate important differences resulting from different salts of this element, and serve as well to invalidate "complete precipitation" as a reliable guide.

Silver Precipitation

Silver, like mercury, was found to be a fairly satisfactory precipitant for co-zymase. Our preliminary experiments indicated approximately neutral solutions to be satisfactory, and the effect of concentration was therefore investigated at pH 7.2 - 7.3. The results are listed in Table X.

Table X

Conc. (molal)	0.0	0.0025	0.005	0.0075
Yield (%)		48	61	63
Aco (ash-free)	30	173	206	203

Attempts to increase the above yields by altering the acidity met with failure. Occasional batches, however, gave yields of 75%

to 85% at the above pH. As the Aco was likewise fairly high, as will be observed above, it would appear that silver might perhaps be profitably employed.

Removal of Metals as Sulfides

In the case of the three metals described above, lead, mercury, and silver, it was frequently observed that the loss in total co-zymase was very great. This was assumed to be due to the fact that when the metals were removed as sulfides, as a preliminary to the tests of activity, some of the co-zymase was adsorbed on the precipitated sulfides. An experiment was undertaken to examine this possibility.

To pairs of samples of co-zymase solution, the above three ions were added to a concentration of 0.0075 M., and the pH was adjusted in one of each pair to 10 and in the other to 5. Without removing any precipitate, the solutions were saturated with hydrogen sulfide, filtered, and the hydrogen sulfide removed in vacuo. The solutions were then tested and the losses in activity thus determined. They are listed below in Table XI in percent of the original amount present.

Table XI

Percent Loss in Activity by the Precipitation of Sulfides

pH	Lead	Mercury	Silver
5	6	16	22
10	0	9	2

In purer samples the above phenomenon was of even greater magnitude. With silver, in particular, we had great difficulty, and in a few cases losses of as high as 60% and 80% occurred. As the precipitation of the sulfides is best accomplished in acid solutions and as it is here that the greatest losses occur, this factor must be given close attention. We avoided the difficulty as best we could by precipitating the sulfides from dilute solutions, in which case the losses were much lower. The matter is of interest because of its general bearing on the problem of biological purifications by means of the heavy metals.

Cadmium

Turning from the heavy metals, we examined the behavior of cadmium, iron, and aluminum hydroxides. Cadmium was not particularly effective in carrying down the activity, as indicated by the table below (XII). The experiments were performed by adding a solution of $CdCl_2$ to the desired molality, adjusting the solution to pH 10.5 - 10.7, centrifuging off the precipitate, neutralizing both portions, and removing the cadmium as a sulfide. Preliminary experiments had shown practically no losses from precipitation of cadmium sulfide.

Table XII

Conc. (molal)	0.005	0.0075	0.01	Orig.
Yield in precipitate (%)	9	24	37	
Aco (ash-free)	39	42	42	30

It will be observed that not only are the yields low, but that almost no purification results.

Iron

As the precipitation of iron hydroxide in the co-zymase solutions was found to be difficult, the hydroxide was freshly prepared, washed, and added to the co-zymase solution to 0.05 molal. The solution was made alkaline to pH 10.1 - 10.2, and shaken for ten to twenty minutes. On centrifuging off the precipitate, neutralizing, and testing, 88% of the original activity was found to remain in the filtrate, while the precipitate was inactive. The 12% loss might well have been a result of the alkalinity of the solution. In any event, iron would not appear to be a useful reagent.

Aluminum.

The use of aluminum hydroxide as an adsorbent for the co-zymase activity has been discussed by Euler. He found that the hydroxide was quite an effective adsorbent at pH 10, and deduced the Aco of the material to be rather high. He did not, however, utilize the method.

On original extracts, untreated with lead, we found aluminum hydroxide to be quite ineffective. Aluminum sulfate was added to give aluminum ion concentrations of 0.005, 0.01, and 0.015 molal, and then NaOH to bring the pH to 10. The precipitates were centrifuged off and both portions neutralized. Table XIII gives the distribution of the co-zymase in per cent of the original.

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Table XIII

Conc. (molal)	0.005	0.01	0.015
Precipitate	3	7	25
Filtrate	90	61	59

However, in repeating the experiment, with the exception that a lead precipitated solution was employed, more promising results were secured, as indicated in Table XIV.

Table XIV

Conc. (molal)	0.005	0.01	0.015
Precipitate	77	103	75
Filtrate	33	4	0

The difference in the behavior with the two solutions may perhaps be accounted for by the absence of phosphate in the lead precipitated material, as aluminum phosphate, which is surely formed in the original material, may interfere.

The problem of recovering the activity from the hydroxide still remained. Two methods were examined and both found to be useful. The first consisted in dissolving the precipitate in a minimal quantity of 6 N. hydrochloric acid, diluting, and neutralizing. In the second method the precipitate was suspended in water and adjusted to pH 5.9 - 6.1 and kept in the ice-box for 12 to 24 hours. As the mixture became more alkaline on standing, it was readjusted once or twice during the period.

Both methods were found to give filtrates containing the major portion of the activity. The second procedure, moreover, resulted in a fairly pure product, the Aco being increased, for example, in one experiment from 30 to 275 (ash-free) by precipitating at pH 10 and reversing the adsorption at pH 6.0. Repeating the adsorption and reversal once more caused an increase to only 335.

Miscellaneous.

The remaining reagents which we employed were examined only casually and will, for this reason, be only briefly mentioned.

Barium, in accordance with the statements of Euler, was found to produce no precipitation of the activity, even in solution as alkaline as pH 10.5.

Tannic acid was likewise found to precipitate none of the activity from dilute solutions, and concentrated solutions were not investigated. The filtrate from the tannic acid precipitation was noted to develop only faint precipitates with either phosphotungstic or silicotungstic acids.

Silicotungstic and phosphotungstic acids were both tried, but without particular success. Considerable loss of activity in general resulted with no very great increase in the purity of the co-zymase preparation.

Summary

Electro dialysis and a number of reagents have been examined with regard to their applicability to purifying co-zymase extracts.

Lead salts were found to give variable results. The experiments of Euler and Myrbach could not be duplicated on the available co-zymase extracts.

Mercuric chloride and nitrate were found to effect different results. The experiments appear to have considerable bearing on biological purifications with this element.

Mercury, silver, and aluminum were found to be most useful.

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FOOTNOTES.

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