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Study of the metabolism of Bacterium coscoroba on gluconic acid

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STUDY OF THE METABOLISM OF *BACTERIUM COSCOROBA* ON GLUCONIC ACID

by

Buford H. Butcher

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for the Degree of

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Approved

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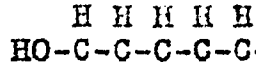
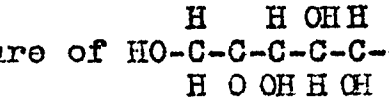
THE UTILIZATION OF CALCIUM GLUCONATE FOR THE DIFFERENTIATION OF MEMBERS OF THE COLON-AEROGENES GROUP

A perusal of the literature relating to the utilization of organic acids by bacteria gave no evidence that the glucose derivative, gluconic acid or its calcium salt had ever been used in a medium for the differentiation of the colon-aerogenes group. In fact, its use in the field of bacteriology is limited to three investigators. Boutroux (1898) reported that the action of a bacterium on calcium gluconate was one of oxidation. The gluconic acid which has a structural formula:



is oxidized to an hydroxy acid having the structure of



. In the same year Bertrand working along the same line found the same result as Bautroux. Recently Nagai (1924) has found that the action of Bacterium coli on calcium gluconate in presence of Na_2SO_3 produced acetaldehyde.

There was a possibility that by the addition of this gluconate molecule to a culture medium, it might be useful for the differentiation of the colon-aerogenes group. It was surmised that the gluconic acid or its calcium salt would be available for the growth of microörganisms because it so closely resembles glucose in structure. Later experiments showed this surmise to be true for the acid is readily available as a source of carbon for the members of the colon-aerogenes group investigated. The medium chosen for this investigatidn

was Koser's (1922) synthetic medium with the substitution of calcium gluconate for anyone of his list of organic acids. The following constituents were used:

Distilled water	1000.0 cc
NaCl	5.0 gr.
$MgSO_4.7H_2O$	0.2 gr.
$(NH_4)_2HPO_4$	1.0 gr.
K_2HPO_4	1.0 gr.
Calcium gluconate . . .	2.0 gr.

Andrades indicator was used for the detection of acidity. Durham tubes were used in order to detect the formation of gas in the culture. The medium was tubed, sterilized, inoculated with 19 different strains of the colon-aerogenes group kindly furnished by Dr. J. C. Weldin of the Bacteriology Department, Iowa State College. The temperature was maintained at 37-1/2°. Observations were made at the end of 2 days and again after 4 days. In all cases the organisms grew as is indicated in the table which follows:

TABLE A

The fermentative action of some members of the Colon-aerogenes group on calcium gluconate

Organisms	Aerobacter sub group	2 days acid	2 days gas	4 days acid	4 days gas
Bact. aerogenes	(isolated from soil)	+	+	-	+
" "	(" " manure)	+	+	-	+
" "	#730	+	+	-	+
" "	#731	+	+	-	+
" "	#117	+	+	-	+
" cloacae	#233	+	+	-	+
" oxytocum	#499	+	+	-	+
" "	#423	+	+	-	+
" "	#369	+	+	-	+
" "	#139	+	+	-	+
" "	#305	+	+	-	+
Organisms	**Escherichia sub group**				
Bact. coli variety	para-grunthali #13	+	+	-	+
" communior	# 41	+	+	-	+
" coscoroba	#100	+	-	+	-
" neapolitanum	#602	+	+	-	+
" "	#113	+	+	-	+
" schafferi	#131	+	+	-	+
" "	#152	+	+	-	+
" verkanda	#427	+	+	-	+

A study of the above table shows that the medium containing calcium gluconate has no value in the differentiation of the Escherichia from the aerobacter subgroups. On the other hand it clearly differentiates Bacterium coscoroba #100 from the other members of the colon-aerogenes group studied. In as much as this organism produces a permanent acidity and no gas while the other related organisms produce both acid and gas, this test proves to be specific for this particular organism.

Moreover it is noteworthy that the permanent acidity produced by Bacterium coscoroba is in direct contrast to the temporary acidity produced by the other organisms tried. In this series of tests no attempt was made to isolate and identify any of the fermentation products.

In conclusion it may be said that this series of tests has been the means of finding a specific differential character for Bacterium coscoroba #100. However, the use of calcium gluconate in a medium for the differentiation of the members of the colon-aerogenes group would be of no value.

Summary

1. Calcium gluconate has been found to be a satisfactory source of carbon for all organisms studied.
2. Calcium gluconate cannot be used to differentiate the Escherichia from the aerobacter subgroup.
3. It has been found that calcium gluconate may be used to differentiate Bacterium coscoroba #100 from all other members of the colon-aerogenes group studied since it produces no gas and a permanent acidity; all others tried generate gas and show temporary acidity.

UTILIZATION OF THE FORMATE ION BY BACTERIUM COSCOROBA

Certain detailed studies of the products of metabolism of Bacterium coscoroba, grown in a medium containing calcium gluconate, showed a marked increase followed by a decrease in concentration of formate ion. It was deemed desirable to determine the extent to which this organism would utilize the formate ion when this was present as the only source of carbon.

An examination of the literature showed that no studies had been made of the ability of Bacterium coscoroba to metabolize formate; however, several workers have reported concerning the ability of certain other organisms, more or less closely related, to utilize the formate ion.

Loew (1892) reported that Bacillus methylicus assimilated formic acid and could change it into formaldehyde and CO_2 according to the reaction:

$$\begin{matrix} HCOOH \\ HCOOH \end{matrix} \longrightarrow \begin{matrix} CHO \\ COOH \end{matrix} + H_2O$$

$$\begin{matrix} CHO \\ COOH \end{matrix} \longrightarrow CO_2 + CH_2O$$

Harden (1901) stated that a culture of Bacterium coli commune produced comparatively little change in a 2% sodium formate solution in a 1% peptone medium. However, Pakes and Jollyman (1901) who studied some 80 strains of Bacterium coli commune and related intestinal bacteria, found that the organism when grown under anaerobic conditions were able to utilize sodium formate. They found H_2 and CO_2 to be produced in equimolecular proportions and concluded that formic acid was decomposed according to the reaction: $HCOOH \longrightarrow H_2 + CO_2$ or inasmuch

as the sodium salt was used, the reaction might be written: $NaOOCH + H_2O \longrightarrow NaHCO_3 + H_2O$. Omeliansky (1903) isolated Bacterium formicum from horse manure and found that it decomposed considerable quantities of calcium formate under aerobic conditions. Franzen and Braun (1908) investigated the fermentation of formic acid by Proteus vulgaris and stated that the same amount of acid is utilized within a given time and at a given concentration whether the acid is present as a potassium or a sodium salt. They found that the reaction increased in velocity as the concentration of the formate was increased. Grey (1914) published an account of the decomposition of formates by Bacterium coli commune . He reported that the power of this organism to utilize formic acid varies considerably after the organism has been kept for some time on artificial media. Furthermore he stated that the decomposition of formates is inhibited by a very small excess of either acid or alkali.

Literature references found, failed to solve the problem without further experiment. A medium was prepared in which the following constituents were used:

H_2O (distilled)	500.0 cc
$(NH_4)_2HPO_4$	0.5 gr.
NaCl	1.87gr.
K_2HPO_4	0.5 gr.
$MgSO_4$	0.1 gr.
HCOOH	5.0 gr.
$CaCO_3$	15.0 gr.

This is a modified Koser medium: only three-fourths as much sodium chloride was used as Koser's formula calls for; formic acid and calcium carbonate were also added, neither of which Koser employed.

The medium was placed in a 1-1/2 liter Erlenmeyer flask, sterilized in a steam autoclave for 20 minutes at 15 pounds pressure, cooled and inoculated with 2.0 cc of a broth culture of Bacterium coscoroba 24 hours old. The culture was incubated for seven days at 37-1/2° and was shaken twice each day so as to introduce air into the substrate. After 18 hours of incubation a strong fermentation was indicated by the amount of gas evolved from the culture flask. This strong evolution of gas continued for about four days, then it decreased sharply and at the end of six days it had entirely ceased.

In order to discover whether there was a simple decomposition of the formate or whether there was a synthetic action occuring, the culture was treated according to the following method: the contents of the culture flask were filtered after first reserving 2.0 cc for an aldehyde test with Fehling's solution which proved negative. The residue containing $CaCO_3$ and possibly other insoluble calcium salts was treated with a slight excess of sulphuric acid; the whole was warmed, filtered, washed and the residue discarded. This was done to recover any calcium oxalate that might be present for oxalic acid could be synthesized by removal of one hydrogen atom from each of two molecules of formic acid: $\begin{matrix} HCOOH \\ HCOOH \end{matrix} \longrightarrow \begin{matrix} COOH \\ COOH \end{matrix} + H_2$

The filtrate of 250 cc was added to the original filtrate, the whole made acid with H_2SO_4 until a blue coloration was obtained with congo red paper. It was then steam distilled from a 500 cc Kjeldahl flask. Distillation was continued until 10 cc of distillate would give a distinct pink coloration with phenolphthalein when one-half drop of 0.1 N NaOH was added. This stage was not reached until about 10 liters of distillate had been collected. The entire distillate was titrated in a 12 liter balloon flask, using phenolphthalein as an indicator. It was evaporated to small volume then made up to 500 cc, an aliquot part of 25 cc taken and titrated for formic acid with $KMnO_4$ according to the method of Klein (1906). The method in brief is to add an excess of $KMnO_4$ to the hot sodium salt; then add oxalic acid in excess, then sufficient dilute H_2SO_4 and the mixture back titrated with $KMnO_4$. In this way it was found that the formic acid present checked well with the total volatile acids present in the distillate. This eliminates the presence of all other volatile acids which might have been produced by the fermentation of the formate ion by Bacterium coscoroba. The formate found by $KMnO_4$ titration is that which was not utilized by the growing culture and amounted to about one-half of the 5 grams originally added. The formic acid used for this work was Merck's 90%, the impurities being traces of other volatile acids and water.

The non volatile residue from the distillation flask, which now had a volume of about 125 cc was poured into a continuous

extractor (Laquer 1921) and extracted for 60 hours with ether, the ether evaporated, 100 cc of water added and the extract titrated with .1 N $Ba(OH)_2$ phenolphthalein being used as an indicator. Only about 4 cc of 0.1 N $Ba(OH)_2$ was necessary for neutralization and the small amount of acid in the extract proved to be sulphuric which had gone over in small amount with the ether during extraction. It was now proven that during the fermentation of calcium formate by Bacterium coscoroba, no volatile or nonvolatile organic acids were formed.

No attempt was made to measure or identify the gas or gases which were evolved during the growth of the organism in the formate medium.

These results show that when calcium formate is fermented by Bacterium coscoroba, the action is one of simple decomposition in which the formate radicle is converted into simpler compounds. However, a small part of the carbon from the formate is used in synthesizing the compounds for new bacterial cells.

From the results obtained, it may be concluded that the formate ion is readily available for Bacterium coscoroba, when partial aeration is obtained and when grown in the presence of an excess of Calcium carbonate.

Summary

1. It was found that the formate ion was utilized by Bacterium coscoroba.
2. The organisms did not synthesize aldehydes or higher organic acids.
3. The action of the organism on the formate ion is a destructive one with the exception of the synthetic action occuring within the cells.

THE UTILIZATION OF THE ACETATE ION BY BACTERIUM COSCOROBA

Previous and more extensive work done, along the same general line of this investigation, gave rise to the necessity of determining the power of Bacterium coscoroba to utilize calcium acetate. In determining the action of this organism on the acetate ion, some specific data has been collected and the chain of evidence bearing on a larger problem has been considerably strengthened.

Literature references concerning the action of microorganisms on the acetate ion are not numerous and there are none bearing directly on the utilization of the acetate ion by Bacterium coscoroba. Certain investigators have secured data which are related to the problem under discussion. Pasteur (1864), reported that a culture of Bacterium aceti completely oxidized acetic acid to carbon dioxide and water. His work was later confirmed by Brown (1886). Hoppe-Seyler (1887) inoculated a flask containing a solution of calcium acetate with river mud. The mixture stood exposed to the atmosphere for seven months and occasionally the gases were analyzed. During the first few weeks nitrogen was evolved with other gases then only carbon dioxide and methane according to the equation $Ca(CH_3COO)_2 + HOH \longrightarrow CaCO_3 + CO_2 + CH_4$. If acetic acid were decomposed instead of the calcium salt the reaction would be $CH_3COOH \longrightarrow CH_4 + CO_2$.

Kruse (1910) stated that acetic acid made a poor source of carbon for most microörganisms. Since literature references

contribute nothing directly towards the solution of the problem, a medium was prepared using the following constituents:

Distilled water	500.0 cc
$(NH_4)_2HPO_4$	0.5 gr.
NaCl	1.25gr.
K_2HPO_4	0.5 gr.
Acetic acid	5.0 gr.
$CaCO_3$	15.0 gr.

The glacial acetic acid used was especially purified according to the method of Blousefield and Lowry (1911). The treatment removes the small quantities of formic and other volatile acids which are readily found present; some water is also removed, thus leaving a high purity.

The 500 cc of medium was placed in a 500 cc Erlenmeyer flask, inoculated with 2 cc of a broth culture 24 hours old. The culture was grown for 7 days under the same conditions and analyzed as has already been described (see page 10). No gas was being evolved from the culture after 6 days so it was assumed that growth had ceased.

Results of the analysis show:

(1) No aldehydes were present.

(2) No formic had been produced by bacterial growth.

(3) The bacteria had utilized 1.23 grams of acetic acid out of the 5 grams used.

(4) A para toluidine derivative made with the volatile acids melted at 148° thus showing that no other volatile acids were present along with acetic.

(5) Ether extract of liquid residue remaining after volatile acids had been distilled contained no acids or any other organic compound in sufficient quantity to identify.

During the first two or three days of growth there was a considerable evolution of gas from the culture flask but the gas was not determined.

.The results obtained from this experiment confirm earlier observations that the acetate ion is readily available for Bacterium coscoroba but that toxic products resulting from bacterial growth soon prevent the further destruction of the acetate radicle.

Summary

1. The acetate ion is readily available for Bacterium coscoroba. 1.23 grams of a total 5 grams used were decomposed in 7 days.
2. Toxic products resulting from growth of organism stop bacterial action before much of the acetic acid is used.

THE UTILIZATION OF THE LACTATE ION BY BACTERIUM COSCOROBA

The specific problem concerning the utilization of lactic acid by Bacterium coscoroba arose during the progress of some further work which was being carried out at this laboratory.

The action of various kinds of bacteria on lactic acid and its salts has long been known. Various investigators have contributed data bearing on the decomposition of this acid by bacteria grown under widely varying conditions and consequently different results have been obtained.

Fitz (1878, 1880, 1882) was one of the earlier workers who used the lactate ion as a source of carbon. He found that the butyl bacillus, isolated from cow dung utilized calcium lactate rapidly and easily. No doubt the greater part of the bi-product was butyric acid with some caproic and acetic acids. In another fermentation test with calcium lactate he discovered a small amount of alcohol, propionic acid and a minute quantity of succinic acid:

$$3C_3H_6O_3 \longrightarrow 2CH_3CH_2COOH + C_2H_5OH + CO_2 + H_2$$

Again he used the Bacillus butylicum on a medium containing the calcium salt of lactic acid and an excess of calcium carbonate; as a result of this experiment he found the lactate utilized by the bacteria. Hoppe-Seyler (1887) reported that when calcium lactate was inoculated with river mud, the fermentation products were calcium acetate, carbon dioxide and methane. However, not all kinds of organisms are able to

utilize lactic acid as a source of carbon for Harden (1901) in working with Bacterium coli commune stated that the lactate ion was practically unchanged when used as an ammonium, sodium, or calcium salt. Most of the propionic acid in Emmenthaler cheese is produced from lactates by specific propionic bacteria; acetic acid and carbon dioxide are also produced. There is also a pure acetic acid fermentation of calcium lactate induced by propionic and lactic acid bacteria. Such a fermentation has been reported by Freudenreich and Jensen (1906). Máze (1918) selected six species of bacteria and grew them in a medium containing calcium lactate. Pyruvic acid and ketone compounds were formed; the rate of formation and destruction of pyruvic acid varied according to the species concerned. The amount of lactic acid destroyed also varied with the particular bacteria used. No formic acid was encountered as a biproduct and the acetic acid formed was variable. Lemoigne (1923) found that lactic acid like sugar was decomposed by Bacillus subtilis with formation of butylene glycol and acetyl methyl carbinol. Owing to the great stability of lactic acid, these products are formed slowly and are detectable only in old cultures. So far as could be determined no one had ever used lactic acid as a source of carbon for Bacterium coscoroba. For this work the following medium was selected:

H_2O	500.0 cc
$(NH_4)_2HPO_4$	0.5 gr.
NaCl	1.87gr.

K_2HPO_4	0.5 gr.
$MgSO_4$	0.01gr.
Lactic acid	5.0 gr.
$CaCO_3$	15.0 gr.

This was placed in a 1-1/2 liter Erlenmeyer flask, sterilized at 15 pounds for 15 minutes, cooled and inoculated with 2 cc of a 24 hour broth culture of Bacterium coscoroba. The flask was shaken twice each day and kept at 37-1/2° for seven days. Judging from the amount of gas evolved, vigorous growth began after about 18 hours and continued until about the fifth day when it ceased to be evolved. At the end of the seventh day the culture was treated according to a method already given (page 10). A total of 62 cc NaOH N = 0.1036 were necessary to titrate all the volatile acids which come over with steam distillation. No formic acid was present as was shown by adding $KMnO_4$. Upon evaporation of the sodium salt of the volatile acid or acids, a derivative was made using pure toluidine and this acid-para—toluidine compound melted sharply at 147° which indicated that only acetic acid was present as a volatile acid. There were no non-volatile acids present except a small amount of unused lactic acid. In this work the interest lay only with the acid biproducts formed from the lactate ion so there was no attempt to identify any alcohols or ketones that might have been present in small quantities. Neither were the gases evolved from the culture examined.

The results secured from the experiment clearly indicate that the lactate ion is readily available as a source of carbon when Bacterium coscoroba is grown in a medium containing calcium lactate.

Summary

1. Calcium lactate is readily utilized by Bacterium coscoroba.
2. Acetic acid is the only volatile organic metabolic acid produced.
3. There are no more volatile acids formed by the action of Bacterium coscoroba on calcium lactate.
4. No attempt was made to identify any metabolic products other than the acids mentioned.
5. The gases evolved were not examined.

THE UTILIZATION OF THE SUCCINATE ION BY BACTERIUM COSCOROBA

The problem of whether or not calcium succinate was utilized by Bacterium coscoroba, arose in connection with the metabolic studies of this same organism grown in another medium which contained calcium gluconate. Succinic acid was found to be one of the several decomposition products. Did the amount of succinic acid found in the culture represent only a small portion of that produced and later used by the organism or was the acid molecule completely or relatively unavailable to Bacterium coscoroba?

It was soon discovered that no investigator had determined the extent to which Bacterium coscoroba can utilize the succinate ion. What little experimental work has been done along this line is widely scattered in the matter of years; moreover, it does not relate directly to the problem discussed in this paper because the organism used and conditions of growth do not in any case correspond to these of earlier investigators. Béchamp (1870) observed that a ferment transformed calcium succinate into butyric acid, carbon dioxide and hydrogen but he does not state how much of the succinate ion was so used. Aubel (1921) working with Bacterium pyocyaneus and its action on ammonium succinate, obtained propionic acid and formic acid or fermentation products. Koser (1922) obtained growth of some members of the colon-aerogenes group when he used a sodium salt of succinic acid as a source of carbon. He was not interested

in the decomposition products so made no attempt to identify them. A fermentation of interest is that reported by Grey (1924). He studied the actions of Bacterium coli commune on succinic acid in presence of calcium formate. The calcium salt of formic acid presumably accelerates the decomposition of succinic acid because the formic salt is itself broken down leaving the metallic ion free to combine with any biproduct acid formed so the acidity is partially controlled. Grey reports that about 50% of the 5.3g. of succinic used per 1000 cc of medium was used by Bacterium coli commune in 72 hours. The succinic acid is decomposed chiefly into hydrogen, formic and acetic acids:

$$\begin{matrix} COOH \\ | \\ CH_2 \\ | \\ CH_2 \\ | \\ COOH \end{matrix} + 2H_2O \longrightarrow CH_3COOH + 2HCOOH + H_2$$

These references indicate that the extremely stable succinic acid molecule can be broken down by enzymatic action under the specific conditions prevailing for each individual experiment cited.

The medium used for this work was made as follows:

H_2O	500.0 cc
$(NH_4)_2HPO_4$	0.5 gr.
NaCl	1.87gr.
K_2HPO_4	0.5 gr.
$MgSO_4$	0.1 gr.
Succinic acid	5.0 gr.
$CaCO_3$	15.0 gr.

After sterilization in 1.5 liter flask for 20 minutes at 15 pounds live steam the medium was cooled, inoculated with 2.0 cc broth cultures of Bacterium coscoroba and incubated at 37°C. Within 24 hours growth was indicated by the evolution of a moderate amount of gas which continued for about two days. In order to admit air into the culture the flask was agitated twice each day for seven days and the contents of the flask were analyzed according to the procedure already discussed (page 10). All volatile acids had passed over when only about four liters of distillate were collected.

Results

Total volatile acids required only 1.84 cc of NaOH, the normality of which was 0.9916. Potassium permanganate titration showed only a trace of formic acid amounting to less than 0.01 gram. The small amount of acid not oxidized by permanganate was assumed to be acetic since the sodium salt when evaporated to small volume gave the characteristic ethyl acetate odor when an excess of H_2SO_4 then some ethyl alcohol were added and the contents of the test tube warmed. Approximately only 0.1 gram of acetic acid was present and it was not convenient to prepare a derivative with such a small amount. The exact identity of such a minute quantity of acid was not considered essentially important.

The ether extract of the non volatile residue was made up

to a volume of 250 cc, an aliquot of 25 cc taken and titrated with standard NaOH. This aliquot gave a value of 0.46 gram when calculated as succinic acid. The ether from another aliquot was evaporated, the residue recrystallized twice; a mixed melting point was taken by adding some known succinic acid to the unknown residue which proved to be succinic acid as the melting point of the mixture was 184°. It is now established that the total amount of succinic acid utilized by Bacterium coscoroba was 0.40 gram out of a total of 5.0 grams added to the medium.

Thus under the conditions succinic acid is not available to a great extent. It was found to support the growth of Bacterium coscoroba only about two days while under similar conditions formic, acetic and lactic acids sustained the growth of the organism for six days.

Summary

1. Calcium succinate will support the growth of Bacterium coscoroba only for about two days during which time only about 8% of the succinic acid is used.
2. The total volatile acids present amount to a little more than 0.1 gram. Formic was found only in traces (less than 0.01 gram). The remainder was qualitatively identified as acetic which amounted to about 0.10 gram.
3. The non volatile acid was identified as succinic and only a small portion of it had been used.

THE METABOLISM OF *BACTERIUM COSCOROBA* GROWN IN AN AERATED MEDIUM CONTAINING CALCIUM GLUCONATE

The calcium salt of gluconic acid was one of a number of pure hexose derivatives used in a culture medium as the sole source of carbon for microörganisms. The availability of the gluconate molecule and the unusual fermentative reaction caused by *Bacterium coscoroba* have been referred to (page 6). These observations led to the selection of the gluconate molecule and this particular organism for more extended study. In that gluconic acid and the glucose molecule are closely related in structure it was thought that the study of the metabolic products of this acid might be of value in interpreting the action of microörganisms on glucose, a problem which is by no means completely solved.

At the outset it was decided to study the bacterial decomposition products of calcium gluconate, at the period of maximum growth and at certain periods thereafter; the maximum growth has been found to occur at about 24 hours after inoculation. Various time intervals in addition to the 24 hour period such as 36 hours and seven days were chosen and the metabolic products determined in each case.

Historical Survey

Although gluconic acid has been known for half a century (Hlasiwetz and Habermann, 1870) and during all these years few

investigators have determined the action of organisms on the molecule.

The action of the animal organisms on gluconic acid was observed by Mayer (1901). He injected rabbits with a salt of gluconic acid and reported saccharic acid occuring in the urine as an oxidation product. Later when Schott (1911) carried out the same experiment he observed mainly gluconic acid in the urine together with a small amount of saccharic acid. Liver infusion decomposes gluconic acid only partially; the oxidation products such as saccharic, oxalic and glycollic acids were not found. Paderi (1915) who carried out this liver infusion work reports that gluconic acid is normally not found in the animal even after a diet rich in carbohydrates. The investigations made by these men seem to indicate that, in the animal organism, gluconic acid is a stable molecule. References already alluded to (page 6) make it quite clear that when the same acid is attacked by microörganisms it becomes readily available as a source of carbon.

No investigator has published results of any metabolic work which in any way is closely related to this present problem. Numerous metabolic experiments have been run by growing organisms related to Bacterium coscoroba on monosaccharides and disaccharides and even polysaccharides but not under the same conditions as this work was done. Earlier problems solved by Harden (1901 and 1905), Harden and Penfield (1912) are of the same general type and scope as the one at hand. Such references were helpful since they outlined certain methods which

could be modified and used in this work.

Experimental Work

The calcium gluconate used in all this work could not be purchased as it was not listed by any of the standard chemical supply houses. The same applied to the acid as well as to any of its salts. Most of the calcium salt used was made according to the method of Kiliani and Kleemann (1884). This method consists in the oxidation of glucose with bromine water which gives the free acid together with some HBr; this HBr is removed by treatment with lead carbonate; the last traces of lead are removed by H_2S; the solution is then boiled and the calcium salt precipitated; this salt is easily purified as it can be recrystallized from hot water. The addition of ethyl alcohol to the mother liquor assists in the crystallization of the compound. This method gives a yield which is about 70% of the theoretical. Brown (1886) found that Bacterium aceti grown on glucose in presence of calcium carbonate would produce considerable quantities of calcium gluconate. Somewhat later Seifert (1897) in growing Bacterium aceti on glucose confirmed the work of Brown. An attempt was made in this laboratory to produce calcium gluconate by seeding a glucose medium with Bacterium aceti but the culture was not viable and all attempts to revive it failed. The most successful attempt to produce calcium gluconate by bio-chemical means was reported by

Alsberg (1911). He succeeded in getting nearly theoretical yields by growing Bacterium savastanoi in a medium containing glucose and calcium carbonate. The most convenient chemical method of preparation and the one representing theoretical yields is that of Ling and Nanji (1922). In this method as well as all others, glucose is oxidized. Bromine is the oxidizing agent and the source is the calcium salt of hydrobromic acid.

As might be supposed the calcium gluconate molecule is much more stable toward chemical reagents and heat than glucose. It shows no signs of caramalization when sterilized in live steam at 15 pounds pressure for 20 minutes. In this connection it shows a decided advantage over glucose as well as other sugars. One hundred parts of water at room temperature will dissolve about four and one-half parts of calcium salt; it is easily soluble in hot water and insoluble in alcohol. Since the salt is only slightly soluble in water at room temperature one could never use a medium containing more than 4% of calcium gluconate in solution. As a matter of arbitrary choice it was decided to work with a 1% solution and all subsequent determinations were made by growing Bacterium coscoroba in a medium containing 1% calcium gluconate. Koser's inorganic medium (page 5) with some modification and additions was used for this work. The amount of NaCl was decreased from 5 parts per 1000 to 3.75 parts per 1000. This was the least soluble of the inorganic salts used and it was found that it interfered with certain later operations in the analysis of the culture. Perhaps the

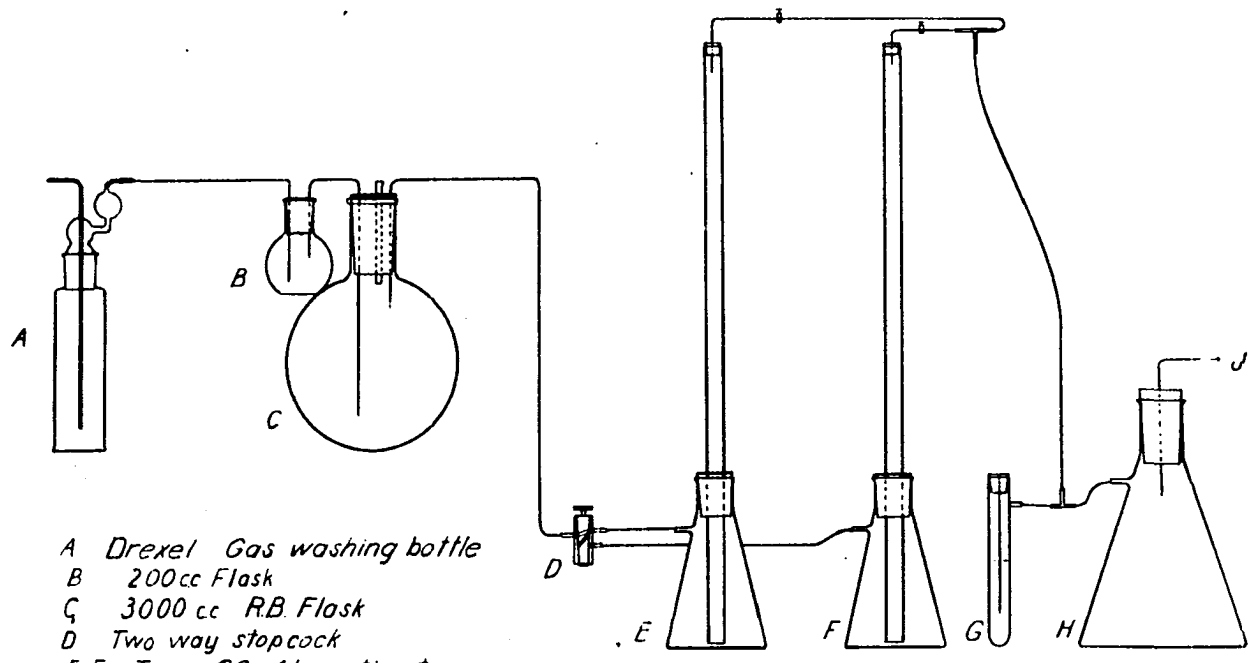
NaCl could have been reduced to a much smaller quantity without imparing the growth of the organism. The complete formula for the medium is as follows:

H_2O, distilled	2000.0 cc
$(NH_4)_2HPO_4$	2.0 gr.
NaCl	7.5 gr.
K_2HPO_4	2.0 gr.
$MgSO_4$	0.3 gr.
Calcium gluconate . . .	20.0 gr.
Calcium carbonate . . .	30.0 gr.

The calcium carbonate used is an addition to the inorganic constituents of Koser's medium. It was known that a considerable quantity of acids was produced by the fermentation of the gluconate molecule and the calcium carbonate was added in excess in order to neutralize the acids formed and thus prevent their accumulation which would inhibit the growth of the organisms.

By preliminary experiment it was found that 20 grams of calcium gluconate would furnish metabolic products in such quantity sufficient to admit of identification and quantitative estimation. It was also learned that when the medium was inoculated with Bacterium coscoroba there was an inhibition of growth for the first two or three days judging from the fact that no carbon dioxide was evolved; these culture flasks were not aerated as in later work. The assumption was reached that sterilization might leave the contents of the flasks deficient in carbon dioxide or in other words that the medium might be too

Plate I



A Drexel Gas washing bottle
B 200cc Flask
C 3000 cc R.B. Flask
D Two way stopcock
E&F Truog CO_2 Absorption tower
G Safety vacuum control
H Safety Flask
J To suction

alkaline for the immediate growth of the organism. Acting on this assumption it was decided to add 1.0 cc of 85% H_3PO_4 which would be correct for this condition, if it existed. Later observations showed that it did not exist. The acid would react on the excess $CaCO_3$ with the evolution of carbon dioxide and thus the medium would be saturated with this gas and any alkalinity would be corrected before inoculation which immediately followed the addition of H_3PO_4. Since it was desired to estimate the carbon dioxide evolved from the growing culture, the total quantity of this gas collected must be corrected for the amount of carbon dioxide produced by the action of 1 cc of H_3PO_4 on the calcium carbonate in the medium.

A brief description of the aeration - carbon dioxide absorption apparatus follows. For a schematic view of the apparatus see Plate I. Flask C in operation contains 2 liters of medium; flask B is permanently secured to the large one by means of a wire support about the necks of the two containers. Flask B contains about 150 cc water. Cotton plugs are fitted into the necks of B and C; a short piece of bent tubing leading from A to B, the tubing from B to C and from C towards D and a larger piece of straight tubing inserted through the cotton plug in C are fixed in place and sterilized along with the two flasks; the exterior terminals of the tube from A to B and from C toward D are protected by cotton plugs which are flamed and burned when it is desired to connect to the remainder of the set-up. The bent tubing leading from B to the bottom of C may

be raised above the surface of the medium in C to prevent syphon action. If so, its exposed end above the stopper in B is protected by a cotton plug; it is convenient to insure its insertion through the plug in B by previously fitting in position a short section of a glass tube about 12 mm in diameter which may be flamed and removed after sterilization when it is desired to insert the flamed end of the bent tube through the plug in B. With such an arrangement no contamination has ever occurred. Flask A with its contents of 50% KOH does not need to be sterilized for the sterile water in B acts as a safeguard against contamination of C. The stopcock D is employed as a matter of convenience in directing the continuous current of gases alternately through the absorption towers E and F. These carbon dioxide towers are a slight modification of those designed and described by Truog (1915). Before use they are assembled and by a separate operation they are swept out by carbon dioxide-free air and the standard $Ba(OH)_2$ measured from a burett directly into the top of the tower; if necessary, carbon dioxide-free water may be added to make up to the proper volume.

The mercury trap G is inserted in order to maintain or regulate a steady stream of gases pulled through the apparatus by means of a water suction pump attached to H. The trap permits the pump to draw an amount of air in excess of that desired through the apparatus, the extra amount of air being drawn through the glass tubing which extends through the rub-

ber stopper and down into G. It may be mentioned that G is a side arm test tube 30 cm x 2.5 cm, mercury being added to a determined height. This regulator will take care of any variation in the water pressure.

The flask H is a two liter suction filter which is inserted to guard against any possible back flow of water from the pump.

Preparatory to operation the sterilized flasks B and C together with their contents are placed in a water bath at 37° and immersed almost to the neck of the larger. All necessary connections made with sections of pressure tubing, the joints protected with beeswax to prevent leakage. The cotton plugs are depressed slightly into the neck of each flask and melted sealing wax poured over the plugs to a depth of one quarter inch. If any doubt exists about these built-up stoppers being air tight, they may be coated over with a layer of melted beeswax. The third and larger glass tube opening into C is for the purpose of inoculation and also serves as a mode of entrance when it is necessary to withdraw samples of the culture from time to time. During aeration this opening has its cotton plug pushed far enough within to allow a rubber stopper to be inserted and if necessary sealed with wax. The apparatus should always be tested for any possible leaks by closing of the inlet tube of A while the pump is running; if the apparatus is air tight throughout, bubbles will soon cease to rise in the particular absorption tower connected.

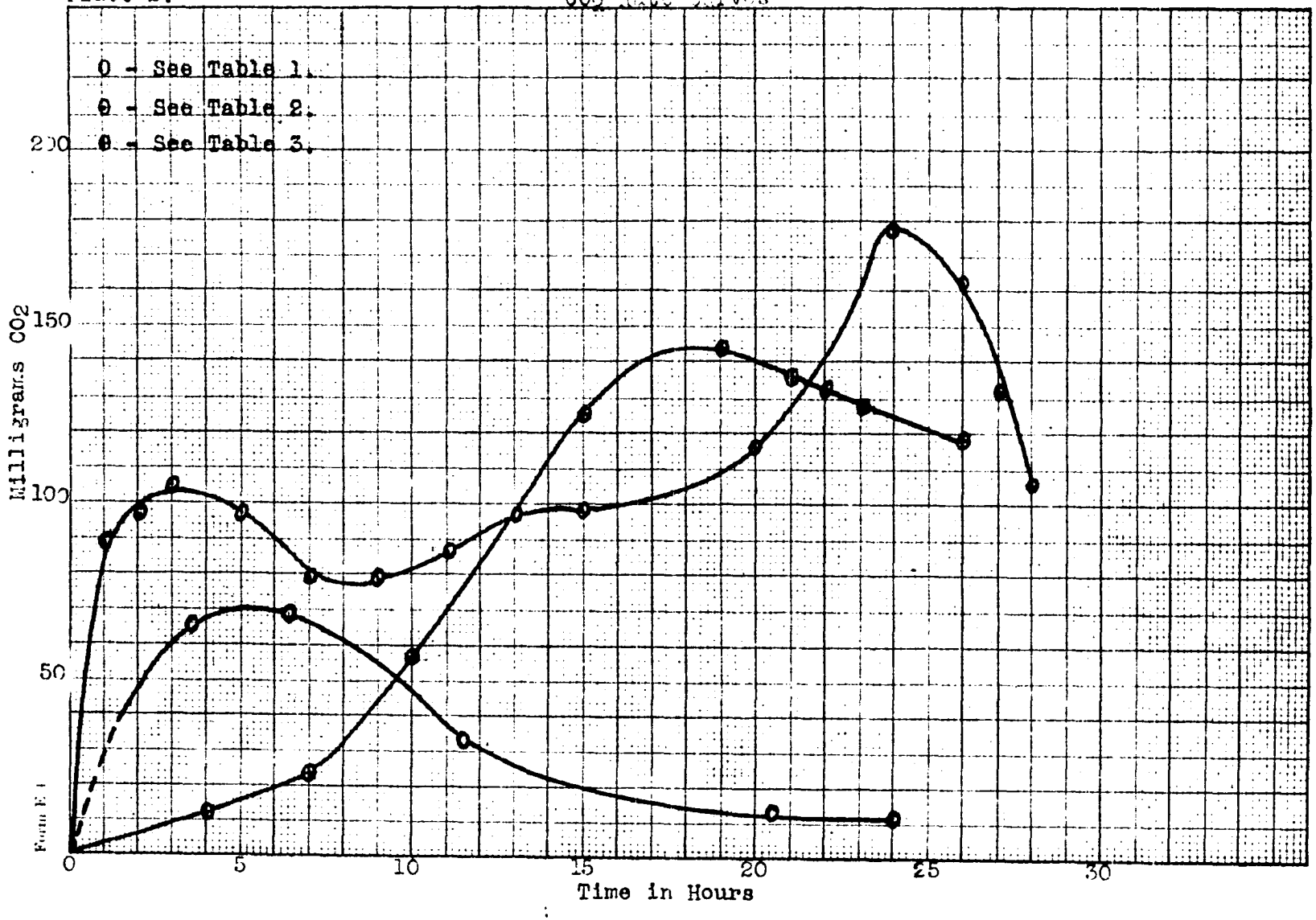
By means of screw clamps, one of which may be in position between G and the top of the absorption tower E or F, the other between A and B, it is an easy matter to maintain a constant flow of air through the apparatus; in most of this work the apparatus was adjusted so that one bubble of air per second would enter through the KOH solution in container A. The 50% solution of alkali was used to remove all carbon dioxide from air entering the system.

A brief description of a typical carbon dioxide determination follows: In this particular instance the medium was sterilized, the flasks B and C then attached to the aeration system as has been described above; 1.0 cc H_3PO_4 added by means of a sterile pipette but no inoculation was made since these carbon dioxide data were to be used to correct the values obtained in later work where both the H_3PO_4 and the inoculum were added. In this correction it may be recalled that interest lays only in the carbon dioxide produced by the growing organisms. The carbon dioxide evolved as a result of chemical action of H_3PO_4 on $CaCO_3$ was collected continuously for a period of 24 hours with the exception of the first one and one-half hour and this can easily and rather accurately be interpolated from the curve plotted from the data collected. The carbon dioxide was swept over into the absorption towers by air entering at the constant rate of one bubble per second. The gas absorbed in standard $Ba(OH)_2$ was determined by direct titration with standard HCl, phenolphthalein being the indicator

EXPLANATION OF SYMBOLS USED IN PLATE 2.

O — CO_2 curve — 1 cc H_3PO_4; no inoculation.
◉ — CO_2 curve — 1 cc H_3PO_4; inoculation.
⊕ — CO_2 curve — no H_3PO_4; inoculation.

Plate 2. CO_2 Rate Curves



used. The rate curve for the evolution of carbon dioxide has been constructed from the data obtained and will be found by referring to Plate 2. Each small circle on the curve indicates a point actually determined by data. The part of the curve indicated by the broken line represents the first one and one-half hours during which no gas was collected. For actual and estimated carbon dioxide data see Table I. Hourly estimates were taken from curve in order to complete the data. It is true that determinations could have been easily made each hour but experience has shown that estimates can be made which are within the limits of error allowable in such biological determinations.

Table I.

CO_2 rate curve.
Determinations made on sterile medium.
1 cc H_3PO_4 added to medium at 6:30 A.M.
Collection of CO_2 began at 8:00 A.M.

Time interval	Milligrams CO_2 collected per hour	Milligrams CO_2 collected and estimated per hour
6:30- 8:00 A.M.		36.0
8:00- 9:00	64.4	64.4
9:00-10:00		67.5
10:00-11:00	68.0	68.0
11:00-12:00		68.4
12:00- 1:00 P.M.		64.0
1:00- 2:00		55.0
2:00- 3:00		47.5
3:00- 4:00		40.0
4:00- 5:00	33.5	33.5
5:00- 6:00		30.0
6:00- 7:00		26.0
7:00- 8:00		24.0
8:00- 9:00		21.5
9:00-10:00	18.8	18.8
10:00-11:00		16.5
11:00-12:00		15.0
12:00- 1:00 A.M.		14.0
1:00- 2:00	13.7	13.7
2:00- 3:00		12.0
3:00- 4:00		11.8
4:00- 5:00		11.6
5:00- 6:30	11.5	11.5

Total milligrams CO_2, 770.8

It will be of interest to examine the accompanying table of P_H values determined with the LaMotte standard cell at various times within the 24 hour period during which carbon dioxide data were being collected.

Time at which P_H was determined	P_H values
6:30 A.M.	6.8
6:45	5.8
7:30	6.0
10:00	6.0
1:00 P.M.	6.2
6:00	5.8
11:00	5.8
3:00 A.M.	5.8
6:30	5.8

At the time of adding H_3PO_4, the P_H of the medium was 6.8. Fifteen minutes after the H_3PO_4 was added, the P_H value was 5.8. Just why the values should rise to 6.0 then to 6.2 and then fall and remain at 5.8 is not quite clear. More than casual interest lay in these P_H values for, as has already been mentioned, the organism, when not aerated, was inhibited in its growth for two or three days and it was thought that the P_H might be the cause but the initial value of 6.8 would indicate that the reaction of the medium was well within the range for most organisms. Later work showed that growth was not inhibited under aeration which was employed in order to

permit the microörganisms to grow under a constant oxygen tension at the bottom as well as at the upper surface of the culture.

Experiment 1.

A carbon dioxide determination was made under the following conditions: after sterilization, cooling and attachment to the aerating system, 1 cc H_3PO_4 was added and at same time inoculation was made with 2 cc broth culture of Bacterium coscoroba, 24 hours old; aeration was immediately started and the collection of carbon dioxide begun at once. A chart of the carbon dioxide data may be found by reference to Plate 2.

During the first seven hours most of the carbon dioxide evolved is not from growing bacteria but from the action of H_3PO_4 on $CaCO_3$. After that time the carbon dioxide from chemical action begins to appear in measurable quantity. The amount continues to increase gradually until the 18th hour when the quantity evolved mounts rapidly until a maximum is reached at the 24th hour after which it decreases rather rapidly. In measuring the rapidity of growth by the carbon dioxide evolved it is found that the microörganism reaches its maximum at about the 24th hour. A few later determinations of carbon dioxide are made in order to be sure that the maximum has been reached. This 24 hour maximum agrees well with later determinations.

By comparing the carbon dioxide curves whose points are marked by O and ● (Plate 2), it is evident that they do not

coincide as completely as they should during the first three or four hours of the determination. While it is probable that the experimental errors account for part of the discrepancy between them, yet the points on each of the curves agree within themselves, that is no one point is out of place in reference to others near it. It is scarcely probable that in curve G, the 2 cc of broth culture of organisms added to the medium would be so stimulated by the H_3PO_4 that their early growth would account for the higher values of carbon dioxide represented by this curve when compared to curve O.

The following table contains the carbon dioxide data determined from this particular culture:

Table II.

CO_2 rate curve.
Determinations made after adding 1 cc H_3PO_4 at 8:00 A.M.
2 cc broth culture of Bacterium coscoroba added at 8:00 A.M.

Time interval	Milligrams CO_2 collected per hour	Milligrams CO_2 collected and estimated per hour
8:00- 9:00 A.M.	88.50	88.5
9:00-10:00	96.00	96.0
10:00-11:00	105.50	105.5
11:00-12:00		102.0
12:00- 1:00 P.M.	94.90	94.9
1:00- 2:00		85.0
2:00- 3:00	78.70	78.7
3:00- 4:00		78.7
4:00- 5:00	78.70	78.7
5:00- 6:00		82.5
6:00- 7:00	86.40	86.4
7:00- 8:00		90.0
8:00- 9:00	96.90	96.9
9:00-10:00		97.2
10:00-11:00	97.70	97.7
11:00-12:00		102.0
12:00- 1:00 A.M.		103.5
1:00- 2:00		107.5
2:00- 3:00		112.5
3:00- 4:00	116.90	116.9
4:00- 5:00		126.0
5:00- 6:00		132.0
6:00- 7:00		150.0
7:00- 8:00	176.90	176.9
8:00- 9:00		168.6
9:00-10:00	162.70	162.7
10:00-11:00	132.10	132.1
11:00-12:00	106.30	106.3
	Total milligrams CO_2, 3055.7	

Total milligrams CO_2 collected 3055.7
" " " correction. . . . . 770.8 (Table I)
" " " produced by organism, 2284.9

Now it will be convenient to make an arbitrary standard of reference: it can be stated that one gram molecule of carbon dioxide would be produced from one gram molecule of calcium gluconate by the action of Bacterium coscoroba. On this basis the 20 grams of $Ca(C_6H_{11}O_7)_2.2H_2O$ used would yield 1.888 grams of carbon dioxide; but the actual production was 2.2849 grams, so $\frac{1}{1.888} \times 2.2849 = 1.210$ mole fractions carbon dioxide produced by the organism from calcium gluconate. This relationship is stated merely as furnishing a basis of comparison for all carbon dioxide values discussed hereafter.

The following is a tabulated set of P_H values determined from the culture at various intervals during the growth of the organism.

Time at which P_H was determined	P_H values
8:00 A.M.	6.8 (Before adding 1cc H_3PO_4)
8:01	4.2 (After adding 1cc H_3PO_4)
9:00	5.8
11:00	5.8
1:00 P.M.	6.2
5:00	6.2
9:00	6.2
8:00 A.M.	6.7
10:00	6.7
12:00	6.8

It will be noted that the P_H goes gradually from a low

value of 4.2 to the initial value of the medium which is 6.8. This gradual adjustment of the P_H values to the initial reading of 6.8 is in contrast to the corresponding determinations made on the sterile mediums where the P_H did not rise higher than 5.8 during the 24 hour period. However, it would be expected that the growing organisms would adjust the P_H value of the medium to near their own optimum (Cluzet, Rochaix and Kofman, 1924).

This culture at the end of the 28 hour period was further examined in a quantitative way for metabolic products. It may be emphasized here that previous qualitative analysis had established the fact that carbon dioxide, formic acid, acetic acid, lactic acid and succinic acid were the metabolic products present and that they were the only ones formed when Bacterium coscoroba ferments calcium gluconate.

The culture was filtered and the residue treated after the manner already given (see page 10); the filtrate from the residue was then added to the original filtrate, the whole of which was made slightly alkaline with KOH and evaporated at 55°C under a reduced pressure of 20 mm.

The distillate was recovered and preserved for further treatment. Low pressure evaporation was thought best in order to avoid as much as possible, the decomposition of any unstable compounds which might be present. The distillate was colorless and odorless and slightly alkaline due to the presence of NH_3 which passed over from the filtered culture. The distil-

late, which represented a volume of 3000 cc, was made neutral and fractionally distilled, under atmospheric pressure, by use of a seven bulb condensing head but the first fractinn gave no reaction with Fehling's solution nor with acid or alkaline permanganate showing absence of carbon compounds. The distillate under fractionation was made acid and distilled with same results just mentioned. When made alkaline and then fractionated, considerable NH_3 came over with first 250 cc of distillate.

The residue from the vacuum distillation flask was transferred to a steam distillation apparatus described by Dyer (1917) and acidified with H_2SO_4 until a drop would produce a blue coloration with a congo red paper. In order to drive out any carbon dioxide that might be present in the residue to be distilled, an upright reflux condenser was fitted to the 500 cc Kjeldahl serving as a distillation flask and the contents of 250 cc were boiled for 20 minutes, cooled, then fitted onto the steam distillation apparatus. Distillation was continued until 10 cc of distillate would give a permanent pink coloration when one-half drop of N/10 NaOH was added, phenolphthalein being used as an indicator. The distillate was collected in a two liter distilling flask protected from the air by a soda-lime tube. A total of about 10 liters was distilled and this was neutralized with NaOH and evaporated to a small volume. This was made up to 500 cc and aliquot of 10 cc taken for titration with $KMnO_4$ (see page 11). This method of titration for formic acid gives very good concordant results;

the errors are within the limits of error allowable for good quantitative values. A 50 cc aliquot was now taken, the formic destroyed by $KMnO_4$, a 500 cc Kjeldahl used as a container. This was fitted to an upright condenser, heated, acidified with H_2SO_4, an excess of oxalic acid added to reduce the manganese compounds present, refluxed for 20 minutes, cooled, steam distilled and titrated for other volatile acids (page 19) not oxidized by $KMnO_4$. Duplicates were run as checks and the calculated total acid checked in the first place beyond the decimal. This acid was now shown to be acetic and none other by making the para toluidine derivative which melts sharply at 147°. That the acid destroyed by $KMnO_4$ was formic and not some other such as pyruvic was shown by the following method: a considerable quantity of the sodium salts of formic and acetic acids from the culture were amassed and evaporated to small volume, acidified with H_2SO_4, extracted for 72 hours in a continuous ether extracter, the ether cautiously evaporated, 25 cc H_2O added, an excess of calcined magnesia placed in contact with the acids (Allen, 1911), the excess water evaporated to small volume, alcohol added and the magnesium acetate is soluble, the formate insoluble and they may be separated by filtration; the formate again extracted with ether, the ether carefully evaporated and the resulting acid gives the characteristic tests for formic such as the reduction of mercuric oxide and the evolution of carbon dioxide by adding concentrated H_2SO_4 to a few drops of the strong acid.

The non volatile residue was made up to a volume of 500 cc and an aliquot of 150 cc taken for extraction. The extracted portion was titrated with standard $Ba(OH)_2$ as has been mentioned (page 12). Another aliquot was examined quantitatively and gave the test for lactic acid (Fletcher and Hopkins, 1907). Succinic acid was also present as was shown by mixed melting point. Oxalic acid was also tested for by the calcium precipitate test but none was found; its absence was also confirmed by the mixed melting point test for succinic acid. The mixed barium salts of the lactic and succinic were evaporated to dryness on a steam bath, 5 cc warm water added, then 25 cc absolute alcohol and let stand for two hours with occasional shaking. The barium succinate is insoluble and the barium lactate is soluble; they are separated by filtration, an excess of H_2SO_4 added to the lactate and the $BaSO_4$ filtered, ignited and weighed and from the weight of the barium the amount of lactic present may be calculated, (Muller, 1896). The succinic acid was calculated by difference.

A tabulated summary including these and other results will be given later and discussion will be reserved and given at that time.

Experiment 2.

The carbon dioxide curve designated by ⊕ and shown on Plate 2 is deserving of comment. The carbon dioxide values were obtained under the following conditions: the medium, inocula-

tion and all conditions were the same in this run as in the one just discussed with the exception that no H_3PO_4 was added. The evolution of CO_2 indicates that the organisms began to attain considerable growth after a four or five hour period and continued at a rapid rate until the 18th or 19th hour when the maximum rate of carbon dioxide production was reached. It will be noted that after the maximum is attained, the decline in the evolution of carbon dioxide is much less marked than in the curve which it crosses in its decline; its maximum rise is reached about six hours earlier than when H_3PO_4 is added to the medium. Even though the presence of the H_3PO_4 may possibly stimulate the early growth of Bacterium coscoroba it seems that the low P_H resulting from the presence of the acid, delays the maximum carbon dioxide but the maximum although slower in being reached is considerably greater. The accompanying table gives the determined and estimated carbon dioxide values for the full 26 hours that the culture was incubated.

TABLE III.

CULTURE INOCULATED AT 1:00 P.M.
2 cc broth culture 24 hours old.
No H_3PO_4 was added

Time interval	Milligrams CO_2 collected per hour	Milligrams CO_2 collected and estimated per hour
1:00- 2:00 P.M.		2.5
2:00- 3:00		5.0
3:00- 4:00	25.02	9.0
4:00- 5:00		12.5
5:00- 6:00		16.0
6:00- 7:00		19.0
7:00- 8:00	23.71	23.7
8:00- 9:00		32.0
9:00-10:00		40.0
10:00-11:00	57.6	57.6
11:00-12:00		70.0
12:00- 1:00 A.M.		82.5
1:00- 2:00		93.0
2:00- 3:00		102.0
3:00- 4:00	122.3	122.3
4:00- 5:00		126.0
5:00- 6:00		133.0
6:00- 7:00		140.0
7:00- 8:00	143.6	143.6
8:00- 9:00		140.0
9:00-10:00	136.0	136.0
10:00-11:00	132.5	132.5
11:00-12:00	127.4	127.4
12:00- 1:00 P.M.		123.5
1:00- 2:00		120.0
2:00- 3:00	118.1	118.1

Total milligrams CO_2, 2127.2
Mole fraction CO_2, 1.1270

The P_H values obtained at various times during the growth period are constant as might be expected.

Time of determination	P_H value
1:00 P.M.	6.8
11:00	6.8
10:00 A.M.	6.8
3:00 P.M.	6.8

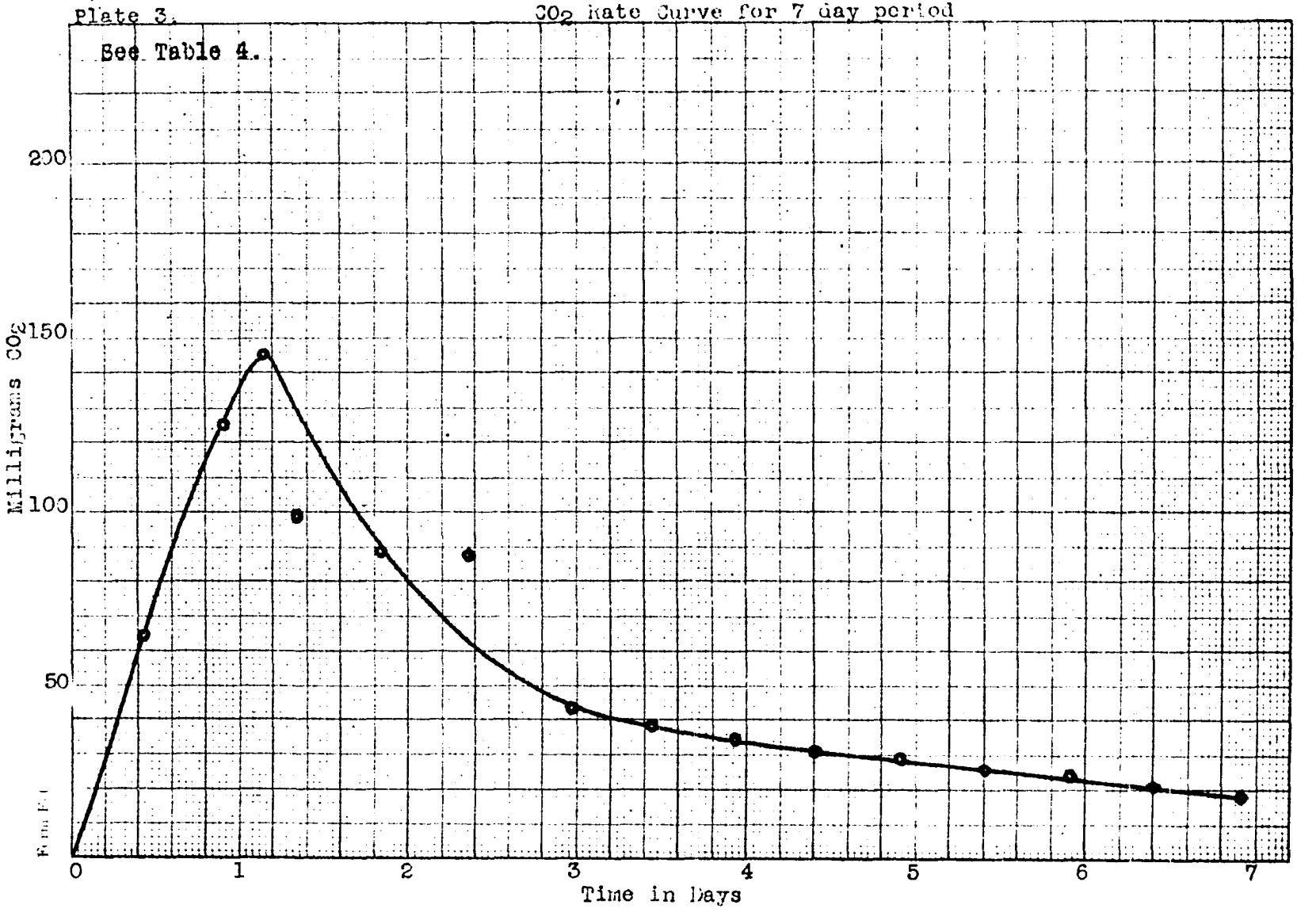
It has long been known that all of the coli-aerogenes group have optimum hydrogen ion concentration which is not far removed from P_H 6.8; they grow well at this P_H value. It can be said that P_H is not the cause of the two or three day inhibition period that this organism undergoes when the medium is not aerated. This experience in having aeration stimulate the growth of microörganisms confirms the work of earlier investigators in this connection (Hausen, 1880; Hoppe-Seylor, 1883; Buchner, 1884; Brown, 1892; Kayser and Demolon, 1909).

The metabolic organic acids determined in this culture are given in Table VI.

The microörganisms growing under the influence of aeration and H_3PO_4 added to the medium produce carbon dioxide. The carbon dioxide chart has its own characteristics up to the 28th hour. In order to follow the curve beyond this period a plan was evolved which would allow for the continuous collection

Plate 3. CO_2 Rate Curve for 7 day period

See Table 4.



of carbon dioxide for seven days (Plate 3).

Experiment 3.

The amount of calcium gluconate used was 5 grams instead of 20 grams and the other components of the medium reduced accordingly. Instead of 1 cc H_3PO_4 added, only 0.25 cc was used in order to have comparative conditions. The amount of calcium gluconate was reduced in order to collect carbon dioxide over longer periods of time without changing the absorption apparatus. The maximum carbon dioxide production occurs about the 27th hour and from this time there is a rapid decline in carbon dioxide evolved until the third day is reached when the rate decreases very slowly and continues so to the seventh day.

Table IV contains the carbon dioxide data for this curve.

TABLE IV.

Seven day culture
5 gram sample of calcium gluconate
0.25 cc H_3PO_4 added

Hours of continuous collection	Milligrams CO_2 collected	Milligrams CO_2 per hour
10 hours	161.3	16.1
11	345.9	31.4
6	219.7	36.6
8	198.1	24.8
12	131.7	19.8
12	131.6	19.7
12	130.4	10.9
12	115.7	9.6
12	103.2	8.6
12	92.9	7.7
12	87.4	7.3
12	78.3	6.5
12	73.8	6.2
12	65.0	5.4
12	55.2	4.7

Total CO_2 for 7 days = 1990.2
Total CO_2 for 20 gram culture = 7960.8 milligrams for 7 days
Total CO_2 for first 27 hours = 726.9 milligrams (values from table)
Total CO_2 for first 27 hours for 20 gram culture = 2907.6 milligrams.
770.8 milligrams correction factor for CO_2 when 1 cc H_3PO_4 used.

$\frac{770.8}{4}$ " " " " " 0.25 cc " "

2907.6 milligrams total CO_2 evolved
-192.7 " correction
2714.9 " CO_2 produced by organisms.

Plate 4. CO_2 rate Curve for 31 day period

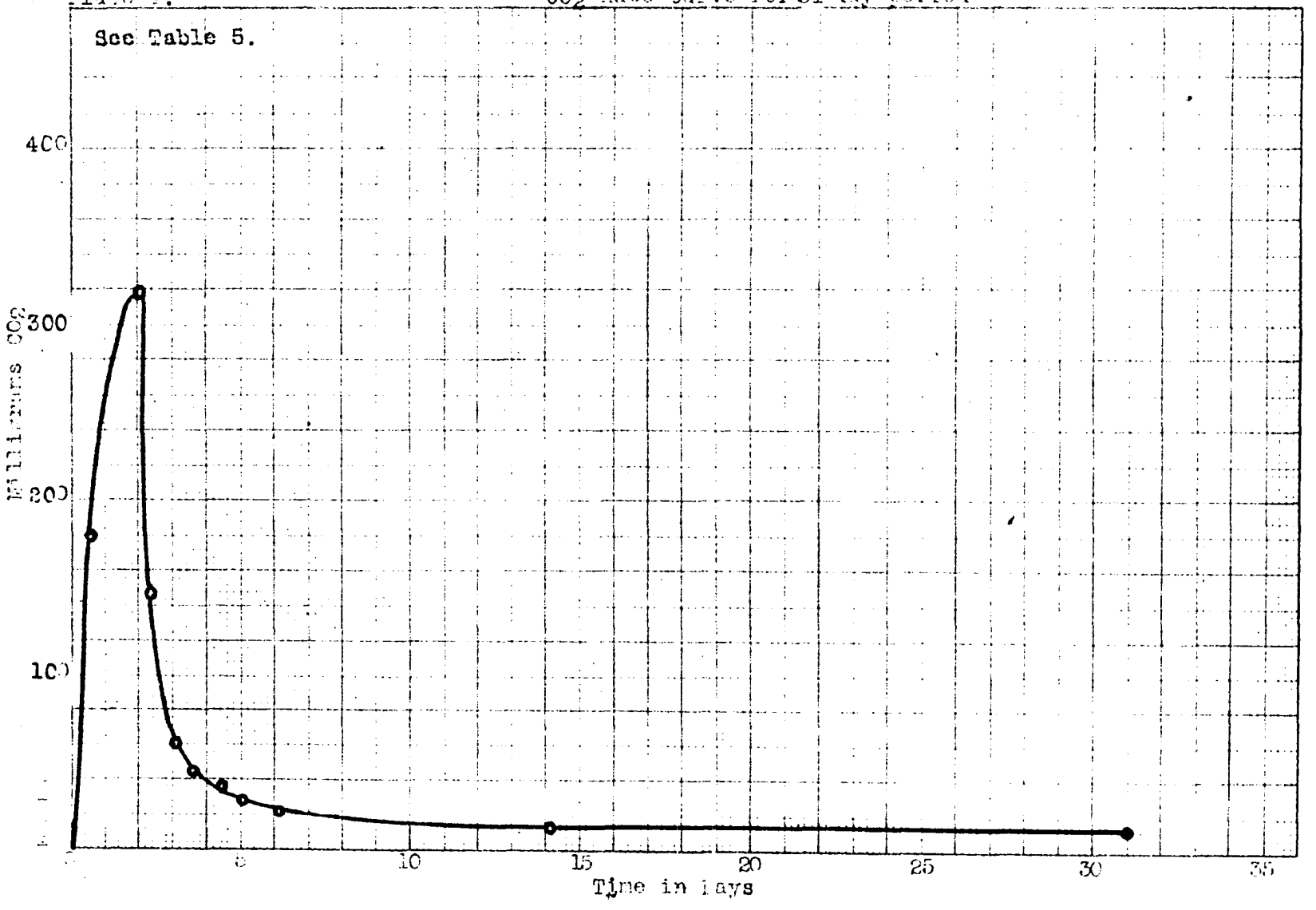


TABLE V.

Thirty-one day culture
40 grams calcium gluconate, 1 cc H_3PO_4 added,
1 hour later flask inoculated with
4 cc broth culture of Bacterium coscoroba

Time interval between collections of CO_2		Hours of collection of CO_2	Milligrams CO_2 collected	Milligrams CO_2 per hour	Total CO_2 evolved (calculated)
Hours	Min.				
13	0	1	179.5	179.5	2333.5
12	0	1/2	159.4	318.8	3825.6
5	0	1/2	735.7	147.1	735.7
8	0	1	604.3	60.4	483.4
13	30	4	179.5	44.9	605.8
9	35	3	95.7	31.9	305.6
14	45	4	108.7	23.2	400.8
9	43	2	56.3	23.2	273.6
16	0	5	147.4	29.5	472.7
23	15	5	117.2	23.4	545.0
24	5	5-1/2	122.7	22.3	536.9
20	30	3-1/2	94.1	26.8	551.0
26	5	3	51.7	17.3	449.9
27	7	6	121.9	20.3	550.9
23	5	6	121.9	20.3	469.0
25	48	5	110.2	22.0	568.6
21	55	6	118.0	19.7	432.0
23	55	6	97.7	16.3	389.7
24	0	6	80.9	13.5	323.1
24	2	6	77.5	12.9	310.7
23	48	6	93.4	15.6	375.9
24	15	6	72.3	12.1	292.4
26	33	6	55.7	9.3	246.5
21	8	6	55.8	9.3	197.8
24	3	6	95.8	15.9	384.1
24	0	6	67.7	11.3	270.7
24	15	6	88.4	24.2	357.5
23	50	6	53.4	23.8	212.1
23	27	6	65.8	10.9	258.9
26	30	6	88.4	14.7	381.5
23	30	7-1/2	97.1	12.9	380.2
22	30	6	63.1	15.2	236.6
24	0	6	83.9	14.0	335.7
22	0	4	42.9	10.7	236.2
25	0	5	62.2	12.4	308.9

Milligrams CO_2, 19038.5

Mole fraction CO_2 for 30 day period is 4.8378

Experiment 4.

Carbon dioxide values over a still longer period have been taken at irregular times each day for 31 days. (Table V). In this work 40 grams of calcium gluconate were used and the other components of the medium were increased proportionately. Inoculation was made with 4 cc broth culture of Bacterium coscoroba 24 hours old; only 1 cc H_3PO_4 was added. By reference to Plate 4 it will be evident that the maximum evolution of carbon dioxide is twice that when only 20 grams of calcium gluconate is needed; the slope on each side of the maximum appear much steeper than in the curves of Plate 2 and even Plate 3 for the time unit must be condensed in order to find sufficient horizontal space on one sheet. The chart for carbon dioxide in the 31 day culture shows that after the 10th day there is practically no change in the rate of carbon dioxide evolution. This may indicate the slow fermentation of some of the metabolic products formed earlier in the growth period.

The quantitative data determined in Experiments 1, 2, 3 and 4 just discussed have been supplemented by values secured from other runs. A table summarizing all such quantitative data follows. (Table VI).

TABLE VI.

Designation of experiment	Duration of experiment	Grams CO_2	Grams formic	Grams acetic	Grams lactic	Grams succinic
7	24 hrs.	--	2.83	5.485	--	--
10	24*	--	3.095	6.257	2.08	0.941
2	26	2.1272	2.275	3.30	1.422	0.523
1	28	2.2845	2.13	5.212	1.78	0.834
5	36	--	1.86	5.67	--	--
3	7 days	1.9902	--	--	--	--
8	7	--	0.7912	5.32	0.570	0.960
4	31	4.8378	--	--	--	--
9	7	--	0.2772	5.463	--	--

*This culture was aerated with 1-1/2 bubbles of air per second; all other cultures aerated with 1 bubble per second. This may account for the increased production of acids both volatile and non volatile.

Discussion of Results

Examination of the above table will show that the values listed in the vertical columns, for instance the one containing formic acid data, exhibit considerable variation even when the duration of growth is nearly the same and all other conditions are identical. No series of cultures grown under identical conditions will always produce the same quantity of individual metabolic products. The causative factor here

concerned may be due to inability to control conditions. Also certain small errors enter during the preliminary preparation of the culture for analysis and during analysis as well.

Another point will be noticed: the longer the culture is allowed to grow, the less formic acid remains. Under the conditions obtaining, formic acid is available for Bacterium coscoroba. This is not surprising since the formate ion as the only source of carbon has been found to be available (page 8). It will be noted that the quantity of formic acid found in Experiment 2 is low, and the same applies for acetic, lactic and succinate acids. It may be recalled that in this particular culture no H_3PO_4 was added and consequently there was no stimulation of growth due to H_3PO_4 or the high carbon dioxide tension resulting.

A study of the data contained in the acetic acid column will convince one that, due to the conditions within the growing culture, the acetate ion is not available to Bacterium coscoroba, at least beyond a certain stage. The amount of acetic acid, present at the end of 7 days is approximately the same as at the end of only 24 hours. It would be pertinent to determine the acetic acid present at the end of 6, 8, 10, 15 or 18 hours after inoculation of the medium. It is quite possible that the amounts of this acid remaining at the end of 24 hours or at any other period thereafter does not represent the total amount produced. The results of earlier experiments (page 15) would indicate that some of the acid originally formed would be later used by the growing microörganisms.

The amounts of lactic and succinic acids present in any case are small when compared with the quantities of formic and acetic acids. The results of previous experiments (pages 17 and 21) point out the strong probability that the amounts of these two acids found at the 24 hour period and thereafter represent only a portion of each formed before the end of 24 hours. Lactic acid has been found to be a good source of carbon for Bacterium coscoroba. Grey (1924) has reported that succinic acid, in presence of calcium formate, is readily available for Bacterium coli commune. Now calcium formate is present in all the cultures under discussion and it is not at all improbable that the amount of succinic found represents the difference between that produced and that used before the end of the 24 hour period. Kunz (1906) has found that the succinic acid present in alcoholic fermentation is probably due to a digestive process set up in the cells of microörganisms but if this were the case here the amount of the succinate should increase as the culture grows older.

The question arose as to how much unchanged calcium gluconate might remain unchanged in the culture at the period of maximum carbon dioxide production or at some time thereafter. Gluconic acid is non volatile, insoluble in ether so it would remain in the acid residue after lactic and succinic acids were removed. This residue was evaporated over a water bath and dried for several days thereafter, and the residue ground in a mortar with hot alcohol. Prolonged heating changes the

acid into a lactone which is easily soluble in hot alcohol (Fischer, 1890). It was found that under the conditions this method of determining gluconic acid was by no means quantitative for too much of the inorganic residue present was soluble in the hot alcohol. However, it was determined that nearly all the gluconic acid molecule had been split into simpler acid compounds. In no case more than 2 grams were left undecomposed out of the 20 grams used and this was in the comparatively slow growing culture marked as experiment 2. (See Table VI.)

The carbon dioxide values are all corrected to represent that gas produced only by bacterial actions and excludes that formed by H_3PO_4 on $CaCO_3$.

The carbon dioxide evolved by the growing microörganisms has two sources: (1) that produced as a result of the action of the acids formed on calcium carbonate; (2) that produced by the complete oxidation of these acids. The amount of carbon dioxide from the first source could not be accurately determined for some of it would react with the calcium carbonate and remain as bicarbonate which would be in unstable equilibrium with the carbon dioxide constantly evolved by the growing Bacterium coscoroba. If aeration were continued for a sufficiently long period after growth stopped this carbon dioxide so held in unstable equilibrium could all be removed but this was not attempted. The carbon dioxide from the second source is likewise impossible to determine because it is not known how much of each acid has been converted to carbon dioxide by Bac-

terium coscoroba within a specified length of time. Consequently, the carbon dioxide actually determined cannot be related to the metabolism of the organism in any definite quantitative way. However, the relative vigor of growth of two or more cultures over a definite period can be determined by comparing the total carbon dioxide evolved.

A further comparison of the data contained in Table VI may be had by converting the grams of carbon dioxide, formic, acetic, lactic and succinic acids into mole fractions. This conversion will be found in Table VII which follows.

TABLE VII.

Designation of experiment	Duration of run	Mole fraction CO_2	Mole fraction formic	Mole fraction acetic	Mole fraction lactic	Mole fraction succinic
7	24 hrs.	--	1.431	2.118	--	--
10	24	--	1.565	2.429	0.5627	0.1840
1	28	1.10	1.077	2.022	0.4598	0.1618
2	26	1.13	1.150	1.281	0.3821	0.1006
5	36	--	0.9416	2.204	--	--
6	7days	1.13	--	--	--	--
3	7	4.646	--	--	--	--
8	7	--	0.3999	2.064	0.1476	0.1871
9	7	--	0.14	2.119	--	--
4	31	5.042	--	--	--	--

*The mole fraction of CO_2 given is on basis of 20 grams calcium gluconate for duration of 24 hours.

As a matter for comparison, the mole fractions are computed on the arbitrary standard of reference that one gram molecule of calcium gluconate would produce one gram molecule of carbon dioxide, one of formic, acetic, lactic and succinic acids. The 466 grams which is the molecular weight of the calcium gluconate is reduced to the 20 grams of the calcium salt used and calculations are made accordingly. On this basis of calculation 20 grams of calcium gluconate will produce theoret-

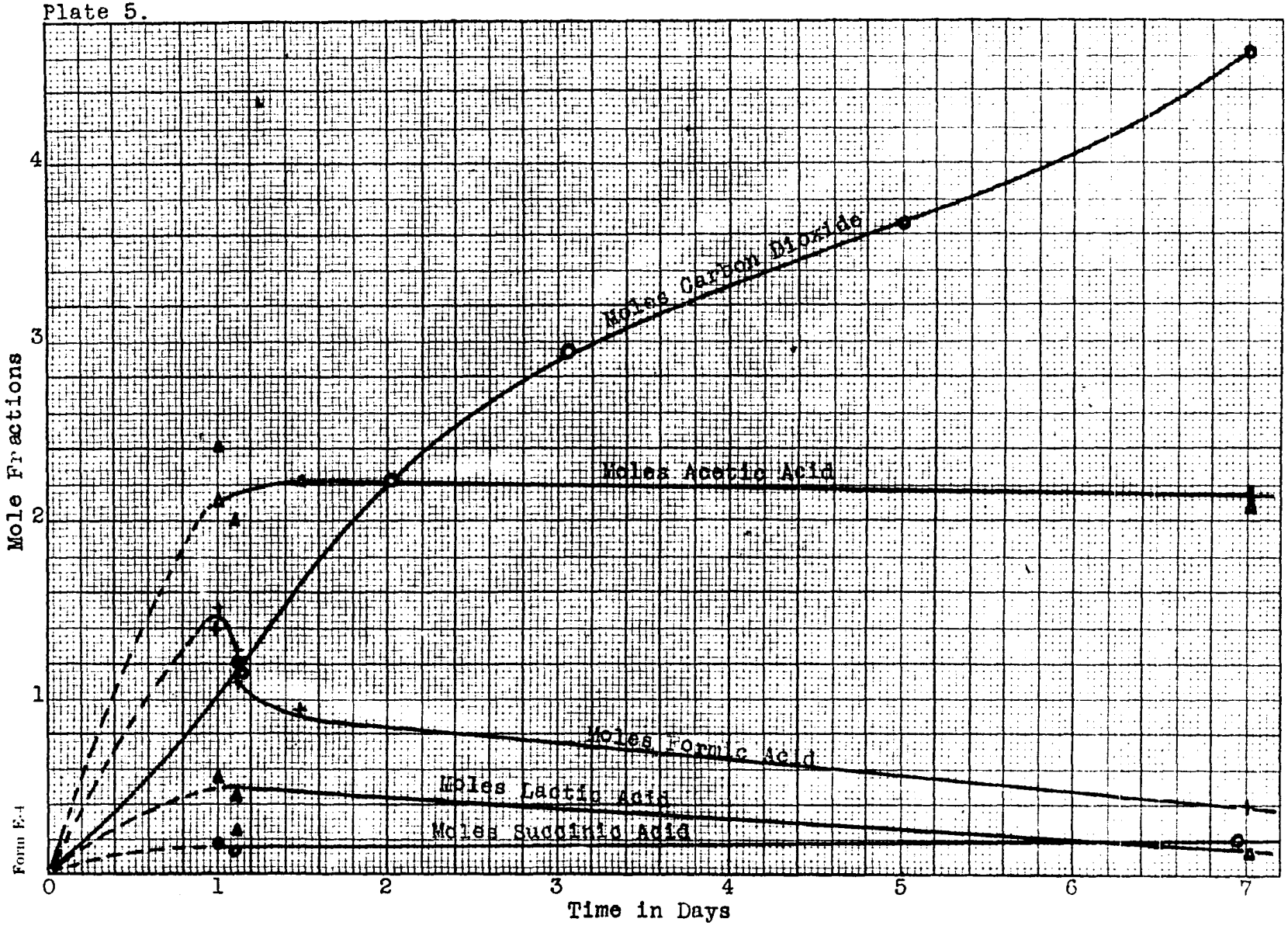
ically 1.974 grams of formic acid and corresponding amounts of acetic, lactic and succinic acids. The actual amount of such acids produced is divided by the theoretical quantity and will give in each case the mole fractinn of that particular product.

In order that the above values may be further visualized they have been charted to scale and designated as plate 5 which follows.

Summary

1. The gluconic acid molecule exhibits a much more stable structure when subjected to sterilization than the glucose molecule. It has no tendancy to caramalize during sterilization as does glucose.
2. The addition of 1 cc H_3PO_4 to the medium stimulates the growth of Bacterium coscoroba either directly or indirectly through the consequent production of CO_2 which is a growth stimulant. However, the resulting P_H may be a stimulant.
3. By selecting different periods of growth for Bacterium coscoroba it has been shown that formic acid is readily available for the organism.
4. Acetic acid under the conditions is not readily available as food for Bacterium coscoroba. Considerable quantities of the acid remain constant in the culture for seven days.
5. It is quite probable that lactic and succinic acids are also available as food for the organism but at present sufficient data have not been collected to prove this conclusively.
6. In presence of $CaCO_3$, the carbon dioxide evolved and determined from a growing culture cannot be linked up in a quantitative way with the other metabolic products. The carbon dioxide so determined serves as a comparative indication of growth during the log phase.

Plate 5.



Form E-4

Theoretical Discussion

An earlier experiment (page 4) of growing Bacterium coscoroba in a medium containing calcium gluconate gave results as follows: the gluconate was fermented with the formation of acid and no gas. In later work under different conditions the metabolic products were determined as carbon dioxide, formic, acetic, lactic and succinic acids. There are several different ways in which these products may be split off from the gluconate chain which contains six carbon atoms. Thus it is that the total carbon atoms of any combination of metabolic products so split off must be neither greater nor less than six.

The possibilities are:

(1) 2 lactic acid molecules.
(2) 1 " " , 1 formic and 1 acetic acid.
(3) 1 " " and 3 formic acids.
(4) 1 " " and 2 " " , and 1 CO_2.
(5) 1 " " , 1 formic and 2 CO_2.
(6) 1 " " , 1 acetic and 1 CO_2.
(7) 1 " " and 3 CO_2.
(8) 1 succinic acid and 1 acetic acid.
(9) 1 " " " 2 formic acids.
(10) 1 " " " 1 " " and 1 CO_2.
(11) 1 " " " 2 CO_2.

Obviously the results of the experiment (page 4) would rule out six of the above possibilities of splitting because

they contain at least one molecule of carbon dioxide.

It is impossible to have one succinic and one lactic acid molecule from the same gluconate molecule. Since these two acids are present as metabolic products it is essential to consider that at least two of the above listed splitting actions occur simultaneously.

Does this work on gluconic acid cast any light on the problem of the decomposition of sugar by microörganisms? The questions may be answered in the affirmative when one considers the following points. The point of attack by microörganisms on the glucose molecule is not the aldehyde group. Gluconic acid has a carboxyl group in place of an aldehyde group and the molecule is readily available as a source of carbon for bacteria. In this laboratory Bacterium coscoroba was grown in a medium containing the calcium -potassium salt of saccharic acid which was available for the organism and this compound has a carboxyl group at both ends. This confirmed the point just made that the aldehyde group of glucose is not the vulnerable point for bacterial attack. Moreover, this experiment showed that the primary alcohol group (CH_2OH) at the other end of the glucose molecule was not essential for bacterial action. Then it must be that one or more of the central carbons of the glucose chain that bacteria first attack and so break the chain. An experiment concerning the action of various bacteria on 3-methyl glucose carried out in this laboratory by the writer some time ago will throw light on this particular phase of the problem. Recently this 3-methyl glucose experiment has

been expanded by Coles (1926). It has been shown that the replacement of the hydrogen, of the hydroxyl group in position 3, by a methyl group will effectually inhibit bacterial action on the molecule. Only about half a dozen organisms out of 125 will attack it at all. This indicates that the carbon in position 3 is a particularly weak place in the glucose chain. By placing the methyl group in 2, 4, 5, or 6 much more specific information regarding other possible vulnerable positions in the glucose chain might be obtained. Now that carbon 3 in the glucose chain has been found to be a point of attack by most bacteria, it may be covered by a methyl group and the molecule split by a selected bacterium. Carbon 3 is now well marked by a stable OCH_3 group which will serve to identify any fractionated molecule which contains it. When 3 methyl glucose is ruptured by bacterial action at the third carbon atom, there are two possible ways in which this may occur: the carbon holding the OCH_3 group may form a compound containing three carbon atoms such as

$$\begin{array}{llllllll} & H & H & H & H & H & H & \\ OC & -C & -C & -C & -C & -C & -CH & \\ OH & & OCH_3 & OH & OH & OH & & \end{array}$$

and the possibilities are a methoxy derivative of lactic acid and one lactic acid molecule containing the four carbon atoms or if it were again split between, say, the fourth and fifth carbons, the location of this rupture could as easily be determined.

Following this same line, the third carbon in glucose or gluconic acid could be marked by the substitution of a chlorine or other halogen, by the reduction of the (OH) to an H,

by introduction of an amino group, sulphonic or phosphonic group. The substitution of some of these groups such as an amino or phosphoric would presumably make the molecule much more available than is the case with the presence of the OCH_3 group.

It would seem that work conducted along such lines as suggested above would yield results which would materially aid in solving the problem of how glucose is split by the action of bacteria.

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