INTERMEDIARY METABOLISM OF CERTAIN HETEROTROPHIC BACTERIA, PARTICULARLY STREPTOCOCCUS PARACITROVORUS AND AEROBACTER INDOLOGENES

by

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TABLE OF CONTENTS

INTRODUCTION

Intermediary metabolism is concerned with the chemical and physical changes associated with the assimilative and dissimilative processes taking place in the living cell. In the last several years, the use of isotopes of carbon and nitrogen has greatly supplemented the usual biochemical technics applied in studies on intermediary metabolism.

The discovery in 1935 by Wood and Werkman of the assimilation of carbon dioxide by the propionic acid bacteria has since been further investigated and extended to many other heterotrophic bacteria and animal tissues by the use of the heavy carbon isotope (C^{13}).

In this thesis studies will be described of the dissimilation of citric acid and glucose, and the assimilation of carbon dioxide by certain heterotrophic bacteria particularly Streptococcus paracitrovorus and Aerobacter indologenes. Experiments will also be described relative to the utilization of acetic acid containing the heavy carbon isotope by Aerobacter indologenes.

HISTORICAL

Fermentation of citric acid

Brewer (1939) has presented a comprehensive review of the dissimilation of citric acid by bacteria, consequently only those references which pertain more specifically to the present investigation will be discussed here.

Deffner (1938) studied the anaerobic breakdown of citrate by an unclassified bacterium isolated from beer yeast, and reported that the citrate molecule was split into oxalacetic and acetic acids. Oxalacetate was reduced in part to succinate or further decomposed to formate, acetate and carbon dioxide. The overall reaction was proposed as follows:

4 citric acid $\longrightarrow$ 7 acetic acid + 5 CO_2 + formic acid + succinic acid

Brewer and Werkman (1939) reported on the anaerobic dissimilation of citric acid by *Aerobacter indologenes*. The fermentation obtained was similar to that announced by Deffner, except that a small amount of lactic acid and of 2,3-butylene glycol, and a trace of acetylmethylcarbinol were also found. A scheme of dissimilation was presented which proposed the occurrence of oxalacetate and pyruvate

as intermediate compounds.

Deffner and Franke (1939) identified the organism used previously as B. lactis-aerogenes. Their results agreed closely with those of Brewer and Werkman (1939).

Brewer and Werkman (1940) investigated the fermentation of a combined substrate of glucose plus citric acid by growing cells of Streptococcus paracitrovorus in a yeast-extract medium. The products obtained were CO_2, formate, acetate, ethyl alcohol, 2,3-butylene glycol and lactate. Pyruvic acid was isolated and suggested as an intermediate but owing to the use of a mixed substrate, proof as to its origin was not conclusive.

S. paracitrovorus is unable to utilize citrate as sole source of carbon for growth; however, in the presence of readily fermentable carbohydrates, the acid is rapidly attacked. This fact was first reported by Knudsen and Sorensen (1929) and later by Rucker and Pedersen (1932). Thus previous attempts made to study the mechanism of the dissimilation of citric acid by this organism have been complicated by the presence of additional carbon sources.

Hammer and his coworkers (1935) have shown that the addition of citric acid to milk cultures of S. paracitrovorus resulted in large increases in the C_4 compounds, diacetyl, acetylmethylcarbinol and 2,3-butylene glycol. The concept was advanced that the latter compounds originated from citric

acid owing to the action of S. paracitrovorus. In order to obtain more evidence for this proposal, Slade and Werkman (1941) prepared cell suspensions of the latter organism which had been grown on citrate plus lactose, and were able to study the mechanism of the fermentation of citric acid in the absence of other carbon sources. It was later found (Slade et al., 1942) that assimilation of CO_2 occurred during this fermentation.

Assimilation of CO_2 by heterotrophic bacteria

The concept of the assimilation of CO_2 by heterotrophic bacteria was first proposed by Wood and Werkman in 1935. Previous to this time the chemosynthetic and photosynthetic autotrophs were the only bacteria known to utilize inorganic carbon. These organisms are able to satisfy the carbon requirements of cell protoplasm from CO_2 and simultaneously obtain their energy by the oxidation of simple inorganic compounds, such as ammonia, nitrite and hydrogen sulfide. In contradistinction to this group of organisms are the heterotrophic bacteria which require organic carbon compounds for growth and cell synthesis. It can no longer be stated, however, that heterotrophic bacteria satisfy their carbon requirements exclusively from compounds containing organic carbon. The significant point of demarcation between autotrophic and heterotrophic bacteria, is the fact that the former can form a carbon to carbon linkage in which both

members are inorganic, while the latter organisms are able to form a carbon to carbon linkage in which one member is organic and one inorganic.

Wood and Werkman (1935) made their discovery while studying the dissimilation of glycerol by various species of Propionibacterium in the presence of $CaCO_3$ as a buffer. It was found that the CO_2 at the conclusion of the fermentation was not equivalent to the $CaCO_3$ added, and hence the fermentation products contained more carbon than was present in the glycerol. It was further proposed (Wood and Werkman, 1936a) that CO_2 acts as a hydrogen acceptor for the glycerol permitting the formation of oxidized products. The formation of the C_4 compound, succinic acid, from the C_3 compound, glycerol, presented proof of a synthesis.

Elsden (1938) presented indirect evidence that utilization of CO_2 was concerned in the formation of succinic acid. The rate of succinate formation by E. coli from a number of substrates was a function of the concentration of CO_2 in the medium.

Wood and Werkman (1938) independently showed by both serial analysis and final analysis of fermentations of glycerol in the presence of $NaHCO_3$, that the CO_2 utilized and succinic acid formed were approximately equimolar. If $NaHCO_3$ was omitted from the medium, little succinic acid was formed. Further studies (1940a) showed that CO_2 was utilized in the dissimilation of a variety of substrates

by Propionbacterium. Sodium fluoride was shown to inhibit the uptake of carbon dioxide, and this inhibition caused a simultaneous decrease in the formation of succinic acid (Wood and Werkman, 1940b).

It was realized at this time that assimilation of CO_2 by heterotrophic bacteria was, in all probability, a general phenomenon. The suggestion was made (Wood and Werkman, 1938) that the synthesis of citric acid by animal tissue involved utilization of CO_2. This suggestion has since been proved correct.

The propionic acid bacteria were particularly adapted to studies on the utilization of CO_2 because more CO_2 was assimilated than was produced, and thus a definite uptake could be demonstrated by manometric methods. The majority of heterotrophic bacteria, however, produce more CO_2 than they assimilate hence it is impossible to demonstrate a CO_2 uptake by non-isotopic methods. The timely availability of the radioactive and heavy carbon isotopes presented a solution to this problem. It has thus been possible to locate the position of the assimilated carbon in the synthesized compounds, and also to extend studies to other heterotrophic bacteria.

The first studies were presented by Wood, Werkman, Hemingway and Nier (1940) in which C^{13} was used, and by Carson and Ruben (1940) in which C^{11} was used. The

assimilated CO_2 was found to reside in succinic acid and, in contrast to previous predictions, also in propionic acid. Wood et al. (1940) proposed the following general reaction to account for CO_2 utilization:

$$CO_2 + CH_3CO.COOH \rightleftharpoons COOH.CH_2.CO.COOH$$

Further studies were made by Wood et al. (1941a) with the coliform bacteria. The dissimilation of galactose and pyruvic acid by Escherichia coli and citric acid by Citrobacter intermedium was found to occur with fixation of CO_2 in succinic acid. Data obtained in the dissimilation of glycerol by Propionibacterium were in agreement with the suggestion that propionic acid was formed by decarboxylation of succinic acid containing fixed C^{13} in one carboxyl group.

To provide additional evidence of the utilization of CO_2 by condensation with a C_3 compound, Wood et al. (1941b) determined the position of the C^{13} fixed in succinic acid synthesized by the above organisms. In agreement with their proposal, fixed C^{13} was found to reside exclusively in the carboxyl groups of succinate. The succinic acid was degraded by two methods: (1) conversion to a mixture of fumarate and malate by a beef heart dehydrogenase preparation and oxidation of the latter acids to CO_2 and acetaldehyde, and (2) oxidation to oxalacetic acid which was subsequently decarboxylated with citric acid and aniline to CO_2 and pyruvoaniline. Also,

calculations made on the basis that the $C^{13}O_2$ available for fixation was equal to the $C^{13}O_2$ at the conclusion of the fermentation, indicated that the fixed carbon was located in only one carboxyl group of succinic acid.

The question naturally arises as to the mechanism involved in assimilation of CO_2. Since the reaction evidently involved carboxylation, i.e., the reverse of decarboxylation, the results of Krampitz and Werkman (1941) are of interest in this connection. An acetone preparation was obtained from Micrococcus lysodeikticus which catalyzed the reaction: $COOH.CH_2.CO.COOH \longrightarrow CO_2 + CH_3.CO.COOH$ Magnesium ions were required for the reaction, whereas cocarboxylase and thiamin had no effect.

The proposal of assimilation of CO_2 by means of the reverse of the above reaction had been made by Wood, Werkman, Hemingway and Nier (1940). By the use of C^{13} and the above preparation a method was available to test the reversibility of the reaction. The recent results of Krampitz, Wood, and Werkman (1942) have proved that a reversible exchange takes place between the CO_2 of the medium and the carboxyl carbon adjacent to the methylene carbon of oxalacetate. No exchange occurred in the other carboxyl group of oxalacetic acid and also little or no exchange took place in the absence of the enzyme. Thus these results constituted the first direct evidence that oxalacetic acid is a

component of the fixation reaction. Pyruvic acid as such is, in all probability, not involved in the reaction; the suggestion of a phosphorylated pyruvic acid seems most likely.

At this time our knowledge of the assimilation of CO_2 involving a carbon to carbon linkage had been limited to several genera of heterotrophic bacteria and the assimilation had been concerned entirely with C_3 and C_1 addition. Therefore, it was the purpose of the investigation reported in a part of this thesis, to determine, first the extent of CO_2 assimilation among heterotrophic bacteria, secondly the extent of C_3 and C_1 addition, and thirdly the possibly of assimilation by other mechanisms. As a means of gaining an insight into possible mechanisms of photosynthesis and autotrophism, it seemed desirable to obtain further knowledge of all types of CO_2 fixation, particularly those involving a carbon to carbon linkage.

The results of this investigation show the existence of assimilation of CO_2 by a wide variety of heterotrophic bacteria with formation of a carbon to carbon linkage. Fixation of CO_2 by C_3 and C_1 addition is a very general reaction. The possibility of CO_2 fixation by other mechanisms must await further investigation.

Wood and Werkman in 1938 made the suggestion that assimilation of CO_2 may constitute a part of the Krebs' cycle of animal tissue respiration. This cycle (Krebs and

Johnson, 1937), which is a series of oxidative reactions concerned in the oxidation of pyruvic acid to CO_2 and water, involves the synthesis of citric acid from pyruvic acid. According to the best evidence at that time, pyruvic and oxalacetic acids condensed to form a 7-carbon intermediate compound which was decarboxylated and oxidized stepwise to citric, isocitric, α-ketoglutaric, succinic and oxalacetic acids; thus the cycle was completed and one molecule of pyruvic acid was oxidized to CO_2 and water.

Evans and Slotin (1940) announced the utilization of CO_2 in the synthesis of α-ketoglutaric acid by pigeon liver from pyruvic acid. Wood, Werkman, Hemingway and Nier (1941c) shortly afterward showed that the synthesized α-ketoglutarate contained fixed carbon exclusively in the carboxyl group adjacent to the carbonyl group. This result eliminated the occurrence of citric acid in the Krebs' cycle, otherwise the α-ketoglutarate would have contained fixed carbon in both carboxyl groups. Later Evans and Slotin (1941) substantiated this finding. It was also found that a part of the non-ketoglutarate radioactivity was released as CO_2 by treatment with ninhydrin and chloramine T. A mechanism of the fixation of CO_2 in amino acids may take place according to the transamination reactions studied by Cohen (1940):

glutamic acid + oxalacetic acid $\rightleftharpoons$ α-ketoglutaric acid + aspartic acid

glutamic acid + pyruvic acid $\rightleftharpoons$ α-ketoglutaric acid + alanine

Wood et al. (1942) extended their previous observations and found that the C_4-dicarboxylic acids were formed by two mechanisms, one reductive through the carbon fixation reaction, the other oxidative by a tentative and modified Krebs' cycle which does not involve citric acid. Thus these results offer direct evidence of the assimilation of CO_2 by animal tissue.

Solomon et al. (1941) have reported some interesting observations on the assimilation of $C^{11}O_2$ after administration of lactic acid to fasted rats. The liver glycogen formed was found to contain 0.3 to 1.1 per cent of the administered C^{11}. Solomon et al. believe that CO_2 is involved in the formation of phosphopyruvate from the C_4-dicarboxylic acids, and that the phosphopyruvate is subsequently synthesized to glycogen by a reverse glycolysis. The reaction may be pictured as follows:

$$C^{11}OOH \cdot \overset{OH}{\overset{\bullet}{C}} : \overset{H}{\overset{\bullet}{C}} \cdot COOH + H_3PO_4 \xrightarrow{-H_2O} C^{11}OOH \cdot \overset{H_2O_3PO}{\overset{\bullet}{C}} : \overset{H}{\overset{\bullet}{C}} \cdot COOH$$

$$\xrightarrow{-CO_2} C^{11}OOH \cdot \overset{OPO_3H_2}{\overset{\bullet}{C}} : CH_2$$

Vennesland et al. (1942) have also found that carbon dioxide-carbon is incorporated in liver glycogen on feeding glucose to fasted rats. However, with rabbit liver slices,

assimilation of CO_2 was not a necessary reaction in the formation of glycogen. Although the above results and the interpretation given are of interest, they present only a working hypothesis for future investigations. The formation of phosphopyruvate from the C_4-dicarboxylic acids remains to be proved. Also it appears that the animal must be synthesizing and depositing glycogen in order for fixation to occur.

Ruben and Kamen (1940) presented qualitative evidence showing the fixation of CO_2 by yeast suspensions and ground barley roots. Attempts made to identify the radioactive compounds were unsuccessful. At the present time little significance can be attached to such experiments.

Foster, Carson, Ruben and Kamen (1941) showed that Rhizopus nigricans fixed $C^{11}O_2$ in fumaric acid during the dissimilation of glucose. The fixed carbon was located in the carboxyl groups of the C_4-dicarboxylic acid by oxidation with $KMnO_4$, which yields three moles of CO_2 and one mole of formic acid per mole of fumarate. Fixed CO_2 was also found in citric acid formed from sucrose by Aspergillus niger. According to the most prevalent theory, citrate is formed by oxalacetic acid and acetic acid condensation, thus the fixed carbon would be located in one carboxyl group, as follows:

$$C^{11}OOH . CH_2 . CO . COOH + CH_3 . COOH \longrightarrow OH . \begin{matrix} CH_2 . COOH \\ \cdot \\ C \\ \cdot \\ CH_2 . COOH \end{matrix} . COOH$$

On oxidation of the radioactive citrate to three moles CO_2 and one mole of pentabromacetone, Carson et al. found that the carbon dioxide contained fifty times more radioactivity than the pentabromacetone. Fixation of CO_2 in lactic acid by Rhizopus could not be demonstrated.

Thus at the present time it is definitely proved that assimilation of CO_2 takes place in plant and animal systems. Also, our present knowledge limits us to the existence of a single reaction responsible for assimilation of CO_2 with the formation of a carbon to carbon linkage, i.e., condensation of CO_2 and a C_3 compound (pyruvic acid?). Part of the material presented in this brief review has been taken from the excellent reviews of Werkman and Wood (1942a, 1942b).

Utilization of acetic acid by Aerobacter

Utilization of organic acids by members of the genus Aerobacter has been known for many years. The methyl red test, used in the bacteriological analysis of water, is based on the fact that species of Aerobacter on continued incubation in glucose-peptone broth will convert the organic acids, formic and acetic, which are formed from glucose, to

alkaline carbonates and bicarbonates. The latter compounds thus cause a reversion of the initial acid pH to an alkaline pH.

Until the report of Ayers and Rupp in 1918, most workers explained the presence of alkaline reactions in fermentations as due to the formation of ammonia or other basic substances from proteins. The above investigators found, however, that the alkaline reaction in milk was caused by the fermentation of salts of citric acid with the formation of alkaline carbonates. The addition of formic and acetic acids to fermentations of glucose by Aerobacter, were found to hasten the reversion to an alkaline pH. Ayers and Rupp were able to show that acetic acid first accumulated in the fermenting medium and subsequently underwent a significant decrease during the fermentation of glucose. It was suggested that acetic acid was changed to bicarbonate.

The various anaerobic reactions listed in the literature which are concerned with the utilization of acetic acid by heterotrophic systems may be summarized as follows:

(1) reduction and condensation to acetylmethylcarbinol followed by reduction to 2,3-butylene glycol.

(2) oxidative condensation to succinic acid.

(3) reduction to ethyl alcohol.

(4) conversion to acetoacetic acid, β-hydroxybutyric acid and acetone.

(5) condensation with oxalacetic acid to form citric acid. The first three reactions will be discussed in this thesis.

The first evidence that acetic acid may be converted to 2,3-butylene glycol was presented by Reynolds and Werkman in 1937. By serial analyses of fermentations of glucose by Aerobacter indologenes, it was shown that a reciprocal relationship existed between acetic acid and 2,3-butylene glycol. The former compound was continuously destroyed during the fermentation while the latter was being formed. The reduction of acetate to ethyl alcohol was not suggested to occur because of the linear relationship between glucose fermented and alcohol formed.

A short time later Reynolds, Jacobsson and Werkman (1937) presented additional evidence of the conversion of acetate to glycol. Acetic acid was added to fermentations of glucose by Aerobacter, and it was found in comparison to the control, that the yield of glycol had increased, ethyl alcohol had decreased and hydrogen was reduced to zero. It was suggested that in the presence of a competing hydrogen acceptor, i.e., acetic acid, acetaldehyde is converted to glycol instead of the alcohol. Ninety-eight per cent of the increase in glycol above the control was accounted for by the acetate and alcohol utilized. Also molecular hydrogen was not activated to serve in the reduction of acetic acid.

Mickelson and Werkman (1938) investigated the influence

of an acid and alkaline pH on the fermentation of glucose plus acetic acid by Aerobacter. At an alkaline pH (above 6.3), acetic acid accumulated and the yield of glycol was reduced in comparison to the yield at an acid pH (below 6.3) at which acetate was utilized and glycol increased. This result was attributed to the necessity of free, dissociated acid and available hydrogen for the reduction of acetate to glycol. Under alkaline conditions, CO_2 was the better hydrogen acceptor resulting in the formation of formic acid; under acid conditions CO_2 could not compete with acetate and the latter was reduced to the glycol.

Cell suspensions of Aerobacter were not able to reduce acetic acid in the presence of formic acid as a hydrogen donator, but an experiment with E. coli resulted in the formation of 2,3-butylene glycol from acetylmethylcarbinol in the presence of formic acid.

It should be mentioned at this time (see Mickelson (1939) for review of the literature) that acetaldehyde was considered by most workers to be the principal intermediate in the formation of acetylmethylcarbinol. Direct evidence, however, was lacking. Therefore, Mickelson and Werkman (1939) presented results obtained on the addition of aldehydes and fatty acids to fermentations of glucose by Aerobacter. Increases in the yields of 2,3-butylene glycol and ethyl alcohol were noted when acetaldehyde was added under acid con-

ditions. Acetic and propionic acids when added were reduced, resulting in increases in glycol and a decrease in gaseous hydrogen. Propionic acid was reduced to propyl alcohol. Propionaldehyde and butyraldehyde were likewise reduced to the corresponding alcohols. It was suggested that acetic acid itself does not enter into the formation of 2,3-butylene glycol, but shifts the O/R balance in such a manner so that other C_2 intermediates are converted to the glycol.

Silverman and Werkman (1941) by the use of a cell-free preparation from Aerobacter, were unable to show the necessity of acetaldehyde in the formation of acetylmethylcarbinol from pyruvic acid. If acetaldehyde is a necessary intermediate, it must be concluded that the bacterial preparation did not possess the ability to activate the added aldehyde under the experimental conditions.

Green et al. (1942) have recently shown that the addition of acetaldehyde to pyruvic acid in the presence of washed cells of Aerobacter and Escherichia, resulted in large increases in the yield of acetylmethylcarbinol and CO_2. A preparation was also obtained from pig heart that produced a similar effect. It was suggested that pyruvic acid and acetaldehyde condense and that the resulting compound is subsequently decarboxylated to the carbinol.

Thunberg (1920) postulated the formation of succinic acid by B. coli communis by an oxidative condensation of acetic

acid. The reaction may be pictured as follows:

$$\begin{array}{lcl} CH_3.COOH & & CH_2.COOH \\ + & \xrightarrow{-2H} & \\ CH_3.COOH & & CH_2.COOH \end{array}$$

Wood (1934) has extensively reviewed the work of Virtanen (1925). Wieland and Sonderhoff (1932) claimed that 'starved' yeast formed succinic acid and citric acid from acetate under aerobic conditions. Succinate accounted for five per cent and citrate ten per cent of the acetate oxidized. Large concentrations of acetate were used. The suggestion was made that acetate was oxidized through succinate even though the oxidation of succinate was only 30 per cent as rapid as the oxidation of acetate. Citrate was proposed to be formed by a condensation of acetic and oxalacetic acids.

Wood and Werkman (1936b) suggested the formation of succinate from acetate in fermentations of glucose by Propionibacterium because yields of CO_2 were in excess of the yields of acetic acid. In some cases the yield of CO_2 was greater or less than that required on the basis of C_3 condensation. It was admitted that such evidence was not conclusive.

Stone, Wood and Werkman (1936) found that acetic and propionic acids were activated by the propionic acid bacteria to reduce methylene blue at pH 5-7. Wood, Stone

and Werkman (1937) have also shown that slight decreases in acetic acid and slight increases in the yield of succinic acid occurred during the dissimilation of glucose by Propionibacterium.

Weil-Malherbe (1937) proposed the formation of succinate by way of acetate condensation from data obtained with several animal tissues. An increase in the yield of succinate as compared to the control, was obtained aerobically when acetate was added. An increase in succinate under anaerobic conditions was demonstrated only after the addition of pyruvate and α-ketoglutarate.

Sonderhoff and Thomas (1937) investigated the metabolism of trideuteroacetic acid by yeast. Sodium trideuteroacetate containing 86 atoms per cent of deuterium was incubated with yeast and the succinate formed contained 40.6 atoms per cent deuterium. It was concluded that the acetic acid had undergone dehydrogenation to succinate. It is safe to conclude that the succinate was derived from the acetate but the mechanism is probably more complex than simple dehydrogenation, otherwise the succinate would have contained the same percentage deuterium as the acetate. The unfermented acetate contained the same percentage deuterium as the original.

It is difficult to evaluate the results of Sonderhoff and Thomas. The labilization of organic hydrogen atoms is

difficult to predict even though the hydrogen of methyl groups is usually considered stable and no enzymes have been reported which merely labilize carbon-bound hydrogen atoms without further involving the substance in a chemical reaction. Confirmation by other investigators has never appeared. The other quantitative data discussed above are hardly conclusive. It is evident that additional investigations employing the isotopes of carbon are necessary before conclusive evidence of the reaction can be obtained.

Therefore, it can be safely stated that there is no positive evidence for the formation of succinate by acetic acid condensation, or the condensation of C_2 compounds to a C_4 dicarboxylic acid. Evidence will be presented in the experimental section of this thesis which strongly indicates the condensation of two C_2 compounds originating from acetic acid to C_4 dicarboxylic acid.

Reilly et al. (1920) were probably the first to notice the reduction of aliphatic acids to the corresponding alcohols by heterotrophic bacteria. Their work on the acetone-butyl alcohol fermentation of starches showed a rapid production of butyric acid in the early stages of the fermentation which later decreased with a corresponding increase in butyl alcohol. This result has been confirmed by Speakman (1920) and Osburn, Brown and Werkman (1937, 1938).

With other bacterial fermentations, ethyl alcohol has

usually been considered to arise by a reduction of acetaldehyde with a simultaneous oxidation of another molecule of aldehyde to acetic acid (Cannizzaro reaction), or a similar reaction with the oxidation of a molecule other than acetaldehyde. Mickelson and Werkman (1939) found that acetaldehyde, when added to glucose fermentations by Aerobacter under alkaline conditions, was quantitatively converted to ethyl alcohol and acetic acid. Propionic acid was reduced to propyl alcohol and a similar reaction was postulated for acetic acid, although the quantitative results obtained when acetic acid was added, do not definitely prove this conclusion.

Evidence will be presented in the experimental section of this thesis which proves the reduction of acetic acid to ethyl alcohol. This evidence has been obtained by the use of $CH_3.C^{13}OOH$ and $C^{13}H_3.C^{13}OOH$.

METHODS

General Considerations

In the experiments to be described, the non-proliferating cell suspension technic was generally used. In this procedure, the organism is grown on a favorable medium, removed from this medium, and suspended in a nitrogen-free substrate-buffer solution. In the absence of nitrogen, the organism

will not reproduce yet it will attack the added substrate, under suitable conditions of cell permeability to the substrate. By the use of cell suspensions, enzymatic processes associated with reproduction are eliminated, hence it is convenient to study the dissimilation of specific substrates and compounds considered to be intermediates in the breakdown of the compound being investigated. Also the technic is well adapted to the study of the effect of cell inhibitors and poisons on enzymatic reactions concerned in bacterial metabolism. Wilson (1938) has adequately discussed the advantages of the cell suspension technic.

Cell suspensions were obtained by centrifuging the bacteria from liquid media by means of either a Sharples or Swedish 'angle head' centrifuge. The cell paste was removed from the centrifuge and transferred to a sterile tared test tube or an Erlenmeyer flask containing glass beads. After addition of sterile water to the desired concentration, the organisms were resuspended and an aliquot removed to the experimental flask. The concentration used was generally 2 per cent, calculated on a weight-weight basis. In the case of solid media, the cells were removed by scraping the agar surface with a rubber policeman in the presence of a little water; the suspension was then filtered through glass wool and centrifuged.

In the above procedure strict asepsis was not practised,

however, gross contamination can be prevented by the observance of the general principles of bacteriological technic. An incubation period of several days does not require a cell preparation harvested under aseptic conditions, provided the remaining constituents of the reaction mixture are sterilized, however if longer incubation periods are required greater care must be exercised.

The growth media employed with the various bacteria will be described in the respective sections to follow. In all cases 10 per cent by volume of tap water was used. Acidic substrates were brought to neutrality with NaOH before sterilization. All materials were sterilized at 20 lbs. pressure for a period of time depending on the amount of medium. Because solutions of K_2HPO_4 and KH_2PO_4 attack carbohydrates during autoclaving, the phosphate buffer solution was sterilized separately and added to the medium at the time of inoculation. Brom-thymol blue was used as an indicator.

Actively growing cultures, either in liquid media or on agar slants, were used as source of inoculum. Purity of the cultures was checked at periodic intervals by the Gram stain and other characteristic tests.

All experimental reactions were carried out under an atmosphere of nitrogen. All constituents of the reaction mixture, insofar as possible, were autoclaved at 20 lbs.

pressure for 30 minutes. Pyruvic acid was sterilized by Seitz filtration. Oxalacetic acid was added to the reaction mixture without sterilization since it is quite unstable. Phosphate buffer mixtures in a final concentration of approximately 0.1 M were generally employed. A concentration of substrate was chosen so that complete fermentation was reasonably assured, thus the determination of unfermented substrate was avoided.

In general, the experiments were of two types: (1) manometric and (2) non-manometric. In both cases micro- and macro-technics were used. The micro-manometric experiments were conducted in a manner similar to the usual Warburg technic. An incubation temperature of 30°C was used in all cases. The substrate and buffer were placed in the main chamber of the manometric flask, and the cell suspension and any other additional constituent of the reaction mixture, were placed in each of two side arms of the vessel. When it was necessary to absorb evolved CO_2, 40 per cent NaOH was placed in the alkali chamber of the Warburg flask plus folded filter paper to increase the absorbing surface. The total volume was either 2 or 2.3 ml. The air in the manometer was then replaced by nitrogen, stopcocks were closed and the apparatus placed in the water bath. After temperature equilibration, the cell suspension was tipped into the substrate-buffer mixture and the initial reading

recorded. Controls without substrate were used to check endogenous fermentation. In those experiments in which it was necessary to record H_2 as well as CO_2 evolution, two manometers were used, one containing alkali for absorption of CO_2; the difference between the two values was a measure of the H_2 produced in the dissimilation of the substrate.

Macro-manometric experiments were carried out in 125 ml. Erlenmeyer flasks equipped with two side arms, each of which was capable of containing 4 ml. The technic employed was similar to that described above for the micro-manometric experiments. The total volume in all cases was 30 ml. At the conclusion of the fermentation, the reaction mixture was poured into a centrifuge tube, acidified to Congo red with 1:1 H_2SO_4 and centrifuged. The supernatant liquid was analyzed as described in the following section.

Non-manometric experiments were carried out in Erlenmeyer flasks which contained a reaction mixture of 30, 60, 200 or 300 ml. Two bottles were attached to the reaction flask in series for the collection of CO_2 and H_2. The first bottle contained normal carbonate-free NaOH for the absorption of CO_2. The amount of alkali displaced into the second bottle of the gas train is determined by the quantity of H_2 evolved. Before addition of the cell suspension to the reaction mixture, the outlet to the gas train was clamped off and the air above the mixture was replaced

with nitrogen by means of a glass tube entering through the rubber stopper and extending below the surface of the reaction mixture. Immediately after addition of the cell suspension, the flow of nitrogen was stopped, and the tube to the gas train opened. The train was connected to the reaction flask by means of rubber-glass tube which entered through the rubber stopper. At the conclusion of the fermentation, a known amount of 5N carbonate-free NaOH containing dry phenolphthalein was added to the reaction mixture by means of a third opening in the rubber stopper. Sufficient alkali was added to ensure an alkaline reaction as evidenced by the indicator. The reaction flask with its attached gas bottles was then placed in the ice-box overnight to ensure the complete absorption of CO_2.

Quantitative determinations of fermentation products are expressed as millimoles per 100 millimoles of fermented substrate. The amount of carbon recovered in the fermentation analysis was calculated by multiplying the millimoles of products by the number of carbon atoms in the respective compounds, and dividing this total value into the value obtained from the millimoles of substrate fermented times its number of carbon atoms. The product obtained times 100 expresses the carbon recovery on a percentage basis.

Oxidation-reduction balances were calculated according to Erb, Wood and Werkman (1936). They represent

$\frac{\text{total oxidation value}}{\text{total reduction value}}$ and a perfect balance is indicated by a value of one.

By use of both carbon recovery and O/R index, the fermentation analyst has two values by which to judge the accuracy of his analysis. Interpretation of the carbon recovery is obvious. The O/R index is based upon the fact that, in an anaerobic experiment, the only source of hydrogen and oxygen, in addition to substrate, is the water present. These two elements exist in water in a ratio of 2:1, and if carbohydrates are used as substrate they also exist in the same ratio. Since there is an equivalent reduction for each oxidation, the ratio of hydrogen to oxygen in the final products should be the same as in the unfermented substrate. An O/R value in excess of one indicates a deficiency of reduced products or an excess of oxidized products, and vice versa.

In order to determine the C^{13} content of the respective compounds isolated from fermentations in which the isotope was added, it was first necessary to convert them to CO_2. The oxidation was performed according to Osburn and Werkman (1932) and the CO_2 evolved collected in 1.5 N carbonate-free NaOH. Compounds such as iodoform, which are not readily oxidized by the above procedure, were oxidized by the method of Friedemann and Kendall (1929). The CO_2 was then liberated

from the alkali, and the C^{13} content determined by mass spectrometer analysis as described by Nier (1940). In many cases this procedure was varied and the CO_2 was precipitated as $BaCO_3$ by the addition of $BaCl_2$. The $BaCO_3$ was washed several times, centrifuged, and dried at 110°C. The carbonate was then treated with 4 N lactic acid and the liberated CO_2 analyzed by the mass spectrometer. Carbon dioxide, enriched with C^{13}, was prepared by oxidation of methane, in which the C^{13} had been concentrated in a thermal diffusion column as described by Nier and Bardeen (1941). Nier and Gulbransen (1939) have found that all naturally occurring materials contain approximately 1.09 per cent C^{13}. In the course of the following study, $NaHCO_3$, acetic acid, and succinic acid were enriched with C^{13} and added to various fermentations. Inasmuch as any one of the above compounds was the only material in the reaction mixture which contained an excess of the carbon isotope, the presence of C^{13} in any other compound at the conclusion of the fermentation in excess of 1.09 $\pm$ 0.02 per cent indicated the C^{13} had its origin in the compound added.

The per cent C^{13} is an expression of the quantitative relationship of C^{12} to C^{13} and is not an expression of the actual amount of C^{13}. The amount of C^{13} present was calculated as: per cent C^{13} x millimoles of compound x number of carbon atoms in compound.

Sodium bicarbonate containing excess C^{13} was prepared by liberating $C^{13}O_2$ from $K_2C^{13}O_3$ by the addition of 5 N H_2SO_4 in a closed system under a vacuum; the $C^{13}O_2$ was collected in an equivalent amount of N carbonate-free NaOH. Other details of technic relating to the use of C^{13} will be described in the sections to follow.

Analytical Methods

Qualitative procedures.

The presence of reducing sugars was detected by heating 1 ml. of fermented medium with 1 ml. each of Fehling's A and B solution. A red precipitate of Cu_2O is a positive test under the experimental conditions.

Citric acid was detected by heating 2 ml. of substrate plus 2 ml. of Denigès' reagent (50 gm. HgO, 75 ml. concentrated H_2SO_4 and H_2O to one liter) plus 1 ml. 1:1 H_2SO_4 in a steam bath for several minutes. The solution was filtered when necessary, strong $KMnO_4$ was added in excess and 30 per cent H_2O_2 until decolorization. A white precipitate indicated the presence of citric acid. This test will detect 10 mg.

To test for pyruvic acid, $(NH_4)_2SO_4$ was added to 2 ml. of substrate until saturated, plus four drops of 2 per cent nitroprusside and 1 ml. of concentrated NH_4OH. A blue color

constitutes a positive test. Other keto-acids, such as α-ketoglutaric and oxalacetic, will give color reactions.

Acetylmethylcarbinol or diacetyl can be detected by adding 1 ml. 40 per cent NaOH and a pinch of creatine to 1 ml. substrate. A pink color develops on standing.

Compounds which contain the group CH_3-C = O attached to C or H, or compounds which are oxidized to this group, can be detected by the addition of dilute NaOH followed by weak iodine. The odor of iodoform, CHI_3, is characteristic of the above group.

Formic acid can be detected by heating an alkaline $AgNO_3$ solution in a clean test tube. The formation of a silver mirror is a positive test.

Quantitative procedures.

The use of macro-fermentations (total volume 300 ml.) and semi-micro-fermentations (total volume 30 or 60 ml.) in this study determined the course of the analysis. In the former case, sufficient fermented medium was available to use several aliquots for the analysis, while in the latter case, the complete analysis was performed on the entire fermentation. This necessitated a change in the order of separation of the components of the fermented medium although the quantitative methods for determination of the respective compounds remained the same. In regard to distillation

procedures, the liquor from macro-fermentations was usually distilled to 20 ml., and that from semi-micro fermentations was distilled to 5 ml. before beginning the steam distillation.

Gaseous products. Carbon dioxide was determined either gravimetrically or manometrically. In the first case the absorbing agent was either KOH (1 volume saturated KOH + 1 volume water + dry phenolphthalein), or ascarite. When large fermentations were analyzed, an aliquot (50 - 100 ml.) was acidified to congo red with 1:1 H_2SO_4 and the liberated CO_2 aerated into a Bowen bulb containing 1:1 KOH. In semi-micro-experiments the CO_2 was aerated into the alkali originally contained in the gas train and then made to convenient volume. An aliquot containing about 50 mg. CO_2 was removed and aerated into a small ascarite tube.

Hydrogen was determined by volume displacement of alkali as described in the preceding section.

Carbohydrates. Glucose was determined by the micro-method of Stiles, Peterson and Fred (1926). The clarification procedure as given by them was used when it was impossible to run a control simultaneously which contained all the constituents of the reaction mixture except substrate.

Citric acid. This organic acid was determined by the pentabromacetone method of Reichard (1934). The cells should be removed by centrifugation before performing the oxidation.

Ethyl alcohol. The residue of the CO_2 determination was

cooled and placed in a Kjeldahl flask. This material was directly distilled to half volume to remove ethyl alcohol and part of the volatile acids. The distillate was then neutralized to dry phenolphthalein with NaOH and directly distilled to half volume; the distillate was collected in a volumetric flask immersed in cold water. The distillate was brought to a convenient volume and an aliquot removed for determination of ethyl alcohol. Acetylmethylcarbinol, but not 2,3-butylene glycol, also will be present in the distillate.

An aliquot of the distillate was placed in a Kjeldahl flask, 15 ml. of oxidizing solution (135 gm. $K_2Cr_2O_7$ plus 675 ml. 10 N H_2SO_4 plus water to 1 liter) added and the volume made to 65 ml. A rubber stopper was wired in the flask which was then placed in a steam bath for ten minutes. The acetic acid was then removed from the oxidizing solution by direct distillation to 5 or 15 ml., followed by ten volumes of steam distillate. The distillate was refluxed and an aliquot titrated with 0.02 N NaOH. The presence of acids other than acetic was checked by the partition method of Osburn, Wood and Werkman (1936).

The alcoholic distillate before oxidation was tested for acetylmethylcarbinol. This compound is completely oxidized to two moles of acetic acid and a separate determination must be made on the distillate and the necessary

correction applied to the alcohol value. Small quantities can be removed by the addition of 2,4-dinitrophenylhydrazine in 2 N H_2SO_4.

Acetylmethylcarbinol. A separate aliquot of the fermentation liquor was generally used for this determination. The method of Stahly and Werkman (1936) was employed. A steam distillation of seven volumes is sufficient to remove the compound quantitatively. A 50 ml. aliquot was distilled to 20 ml. and an additional 140 ml. collected by steam distillation.

2,3-Butylene glycol. A separate aliquot was taken for this determination when sufficient medium was available. When semi-micro-fermentations were being analyzed, the glycol was removed after the distillation of the neutral volatile compounds. To the glycol-containing solution was added 10 gm. anhydrous Na_2SO_4 per 50 ml. of liquid; the solution was directly distilled to the saturation point and 14 volumes were then removed by steam distillation.

The oxidation of the glycol was carried out according to a modification of the Brockman and Werkman (1933) method. An aliquot of the distillate containing not more than 0.6 millimole of glycol was oxidized with 5 ml. of KIO_4 reagent (5.75 gm. KIO_4 in 100 ml. of 3.6 N H_2SO_4) and the aldehyde distilled into $NaHSO_3$. The aldehyde-bisulfite complex was then titrated according to Clausen (1922).

In the presence of acetylmethylcarbinol, the glycol value must be corrected. One mole of carbinol yields one mole of acetaldehyde and one mole of acetic acid, thus it was necessary to subtract one-half the carbinol value from the glycol value. Citric acid and sugars interfere in this determination and were removed by use of $CuSO_4$-$Ca(OH)_2$ as proposed by Hewitt (1931). Fifteen ml. of 20 per cent $CuSO_4$ and 15 ml. of 20 per cent $Ca(OH)_2$ will remove 0.5 gm. of glucose.

Volatile acids. The residue of the original neutral volatile distillation was combined with the residue of the alcohol distillation and the whole acidified to Congo red, directly distilled to 20 ml. or five ml. and 14 volumes of steam distillate recovered. The distillate was refluxed, an aliquot removed and titrated for total volatile acids with 0.05 N NaOH.

Formic acid was determined on a separate aliquot of the distillate after neutralization with NaOH, in the absence of phenolphthalein, according to the total volatile acid titration. The method of Auerbach and Zeglin (1922) was used.

Formic acid in the distillate from semi-micro experiments was oxidized to CO_2 according to Osburn et al. (1933) and the CO_2 collected in 1.5 N carbonate-free alkali. The CO_2 contained in the alkali was determined manometrically;

a control is necessary.

In studies in which C^{13} was used, it was necessary to recover the acetic acid from the oxidation residue in order to determine the C^{13} content of the acid. This was done by filtration (to remove MgO), neutralization and evaporation to a small volume, acidification and distillation.

Non-volatile acids. The acids were removed from solution by continuous ether extraction for 24 hours and then taken up in water. Volatile acids when present were removed by distillation. Total non-volatile acids were then determined by titration with 0.05 N NaOH, and saturated $Ba(NO_3)_2$ added to precipitate phosphates and sulfates. If the latter were present, the above titration could not be used as a measure of total non-volatile acids. The precipitate was filtered off, a slight excess of 10 per cent $AgNO_3$ added, and the precipitate of silver succinate was filtered and washed in a tared Gooch crucible. The salt was dried at 110° C. for four hours and weighed.

Lactic acid was determined according to Friedemann and Graeser (1933). Compounds which yield acetaldehyde on oxidation with $KMnO_4$ will interfere in this determination.

In the case of semi-micro-fermentations, succinic and lactic acids were determined on the residue of the volatile acid distillation, provided 2,3-butylene glycol had been

previously removed. When separate aliquots were taken for succinate determination, lactic acid could not be determined on the residue because of the presence of the glycol; in such cases, lactate was determined on the residue of the glycol distillation.

EXPERIMENTAL

Anaerobic Dissimilation of Citric Acid by Cell Suspensions of *Streptococcus paracitrovorus*

Mechanism and intermediary metabolism.

Three theories have been advanced at the present time in regard to the mechanism of fermentation of citric acid by heterotrophic bacteria. First, cleavage of the citrate molecule into oxalacetic and acetic acids (Deffner, 1938; Brewer and Werkman, 1939), secondly, by cleavage into pyruvic acid, acetic acid and CO_2 (van Beynum and Pette, 1939) and thirdly, decarboxylation and dehydrogenation to acetone dicarboxylic acid (Butterworth and Walker, 1929). Evidence obtained in the present investigation supports the first theory.

Streptococcus paracitrovorus is a hetero-fermentative lactic acid organism associated with *S. lactis* in butter

cultures. In milk culture it is thought to ferment citric acid with the subsequent production of diacetyl, the principal flavor and aroma constituent of cultured butter. S. paracitrovorus is unable to utilize citrate as sole source of carbon for growth; however, in the presence of readily fermentable carbohydrates, the acid is rapidly attacked.

All previous attempts made to study the mechanism of the dissimilation of citric acid have been complicated by the presence of additional carbon sources. Thus in order to obtain definite information of the dissimilation, it appeared necessary to obtain a bacterial preparation which would be able to ferment citrate without the addition of carbohydrates. The results of this work are described below.

The cell suspensions were prepared by growing the organism on a medium of 1 per cent sodium citrate, 0.5 per cent lactose, 0.5 per cent peptone, 0.2 per cent peptonized milk, 0.1 per cent yeast extract, 4 per cent filtrate of tomato juice, 0.05 per cent $MgCl_2$, 0.05 per cent K_2HPO_4, 10 per cent tap water and distilled water to volume. The cells were harvested by centrifugation after 48 hours' growth at 30°C. and resuspended in distilled water. Strain Mu29 was used.

The fermentation mixture consisted of a concentration of substrate as indicated (Table 1), 2 per cent cell suspension, 0.125 M phosphate buffer pH 6.6, and 0.05 per cent $MgCl_2$. The

experiments were conducted in Erlenmeyer flasks containing 300 ml. of medium under an atmosphere of nitrogen. The flasks were held at 30°C. for four to five days.

The quantitative distribution of the products obtained from citric, oxalacetic, and pyruvic acids is presented in Table 1. The following points are to be noted. A comparison of the products from citrate with those from oxalacetate reveals that the former is the source of practically 100 mM of additional acetic acid, indicating the formation of one mole of acetate per mole of citrate fermented. Thus it is possible that the citrate molecule is cleaved into C_3 and C_4 fractions. If such is the case, oxalacetate may be the necessary intermediate compound. Reference to Table 1 will demonstrate that the products from oxalacetate are qualitatively and quantitatively similar (with the exception of the acetate difference mentioned above) to those formed from citrate.

Experiments will be described which prove that cell suspensions of S. paracitrovorus fix carbon dioxide in the carboxyl group of succinic acid during the fermentation of citric acid. This assimilation of CO_2 is believed to take place according to the Wood and Werkman reaction. Thus, under these conditions, it appears that the citrate molecule is fermented according to the following reactions:

TABLE 1.

ANAEROBIC DISSIMILATION OF CITRIC, OXALACETIC AND PYRUVIC ACIDS BY CELL SUSPENSIONS OF S. PARACITROVORUS

Substrate	Substrate fermented per liter mM	CO_2	H_2	Formic acid	Acetic acid	Lactic acid	Succinic acid	O/R index	Carbon recovered %
Citric acid	49.20	124.9	13.9	20.9	157.3	14.3	24.1	0.94	99.8
" "	46.90	128.8	20.6	18.1	158.7	14.0	24.8	0.94	100.1
Oxalacetic acid	43.71	129.6	24.2	18.2	59.8	15.6	20.5	0.92	97.8
Pyruvic acid	47.12	51.9	32.7	15.8	68.3	17.0	4.1	0.93	90.1

Products expressed as mM per 100 mM substrate fermented.

citric acid $\longrightarrow$ acetic acid + oxalacetic acid

oxalacetic acid $\rightleftharpoons$ pyruvic acid + CO_2

oxalacetic acid + 4H $\rightleftharpoons$ succinic acid + H_2O

pyruvic acid $\underset{-2H}{\overset{+2H}{\rightleftharpoons}}$ lactic acid

pyruvic acid + H_2O $\longrightarrow$ formic acid + acetic acid

formic acid $\rightleftharpoons$ H_2 + CO_2

Further analysis is justified in light of the above reactions. The entire CO_2 produced in the fermentation is pictured as being derived initially from oxalacetic acid. Thus, if succinate is formed by reduction, the total CO_2 cannot be greater than twice the difference between the millimoles of citrate fermented and millimoles of succinate formed. In addition the formation of succinate in this manner, rather than by acetic acid condensation, requires the yield of CO_2 plus formate to be less than the acetate. The data are essentially in agreement with these contentions.

With regard to the yield of acetic acid, the theoretical value formed from citrate according to the above reactions is 161.2 mM. The experimental value is 158.7 mM. Likewise, from oxalacetate, the theoretical value is calculated to be 63.9 mM, while the experimental value is 59.8 mM. On the other hand, the hydrogen formed from the three substrates was in excess of that calculated, indicating there may be another source of this element.

The carbon recovery of the dissimilation of pyruvic acid was not completely satisfactory. It appeared that part of the pyruvic acid was converted to a substance which did not give a positive nitroprusside test. This material may be a polymer of pyruvic acid. A carbon recovery of 90 per cent was obtained when a ceric sulfate oxidation (Fromageot and Desnuelle, 1935) was performed at 50° to 55°C. on the residue of the 2,3-butylene glycol distillation. A correction was necessary for the lactic acid present.

The succinate produced from pyruvate was probably formed by means of C_3 and C_1 addition, however, a balance still existed between the C_2 and C_1 compounds. An explanation of this result is not at hand but a C_2 compound must have broken down to CO_2 in sufficient quantity to balance the amount of CO_2 fixed in succinic acid.

The argument may be presented that the data do not support the proposal of the formation of oxalacetic acid in the initial cleavage of the citrate molecule. It is doubtful whether this can ever be definitely proved, since oxalacetate is quickly reduced or decarboxylated in fermentation mixtures and thus has never been shown to accumulate. Also, the possible formation of oxalacetic acid by C_3 and C_1 addition probably would confuse the origin of the latter compound, if it were ever isolated and identified.

It is well known that oxalacetic acid decarboxylates

spontaneously to form CO_2 and pyruvic acid. Thus, the point may be presented that in the experiment in which oxalacetate was added, pyruvic acid was the actual substrate and oxalacetate served in part as a hydrogen acceptor. There seems little doubt but that the pyruvic acid formed by spontaneous decarboxylation was actually fermented in a manner similar to added pyruvate. However, the proof obtained of the fixation of CO_2 in succinic acid by S. paracitrovorus (Slade et al., 1942) when taken with the work of Krampitz et al. (1942), strongly indicates that the enzyme necessary for the decarboxylation of oxalacetate is present in this organism. It is improbable also, that a living cell would rely on the spontaneous, non-enzymatic decarboxylation of a substrate, e.g. oxalacetic acid, in order for that substrate to be metabolized.

Brewer (1939) did not obtain good agreement between the end-products recovered from fermentations of oxalacetic and citric acids by Aerobacter. In contrast to citrate, no ethyl alcohol, hydrogen and little 2,3-butylene glycol were formed from oxalacetate. The data in Table 1 agree well with the proposed cleavage of citrate to oxalacetate and acetate.

Other experiments were performed by use of the Warburg technic to test the effect of certain inhibitors on the dissimilation of citrate and its proposed intermediary com-

pounds. The reaction mixture was composed as follows: 0.025 M substrates, 0.125 M phosphate buffer pH 6.6, 2 per cent cell suspension, and 0.005 to 0.01 M inhibitor in a total volume of 2 ml.

Table 2 shows that sodium azide, sodium iodoacetate, and sodium arsenite are very effective inhibitors of the breakdown of citrate, oxalacetate, and pyruvate by S. paracitrovorus. Sodium fluoride had a weak inhibitory action. It is clear that each inhibitor possessed a similar effect on each of the three substrates. These results support the occurrence of oxalacetic and pyruvic acids as intermediates in the dissimilation of citrate. Inasmuch as the proposed intermediates are both keto-acids, the effect of a single inhibitor on both compounds would be expected to be the same. Also, since no gas is evolved in the initial cleavage of the citrate molecule, the per cent inhibition of both citrate and oxalacetate would likewise be expected to be the same, provided that the initial cleavage of citrate was not inhibited. Sodium pyrophosphate had no effect on any of the substrates.

Effect of pH on formation of C_4 compounds.

Hammer et al. (1935) have shown that in butter cultures, S. paracitrovorus produced varying amounts of the C_4 compounds, diacetyl, acetylmethylcarbinol, and 2,3-butylene

TABLE 2.

EFFECT OF INHIBITORS ON THE ANAEROBIC DISSIMILATION OF CITRATE, OXALACETATE, AND PYRUVATE BY CELL SUSPENSIONS OF S. PARACITROVORUS

Inhibitor	% Inhibition		
	Citrate	Oxalacetate	Pyruvate
.01 M sodium azide	88	94	96
.01 M sodium iodoacetate	96	95	91
.005 M sodium arsenite	100	100	97
.025 M sodium fluoride	14	16	12
.01 M sodium pyrophosphate	0	0	0

Duration of experiment, twenty four hours. Inhibition calculated on basis of total gas evolution as compared to the control.

glycol. At a neutral pH (6.5), the carbinol and the glycol predominate while at an acid pH (4.5) the latter compounds were converted to diacetyl.

van Beynum and Pette (1939) state that the addition of citric acid to a lactose, peptone, yeast autolysate medium did not result in the formation of acetylmethylcarbinol at a neutral pH, while at an acid pH, acetylmethylcarbinol was formed.

Brewer and Workman (1940) found that Streptococcus paracitrovorus produced principally ethyl alcohol and lactic acid from glucose, but on the addition of citric acid, acetic acid and 2,3-butylene glycol were also formed. Thus, it appears that citric acid, in the presence of lactose or glucose, is fermented by butter culture streptococci with the formation of acetylmethylcarbinol. The latter compound is reduced to 2,3-butylene glycol as the alkalinity of the medium increases.

The results expressed in Table 1 show that neither acetylmethylcarbinol nor 2,3-butylene glycol was formed by cell suspensions of S. paracitrovorus at a pH of 6.6. In order to determine whether an acid reaction will affect the production of these compounds, analyses were made of fermentations of citric acid at increasingly acid reactions.

The reaction mixture consisted of approximately 0.05 M citric acid, 0.1 M phosphate buffer mixtures, and 2 per cent

cell suspension in a total volume of 200 ml. A pH of 3.6 was obtained by the addition of 10 per cent H_2SO_4 to a 0.1 M KH_2PO_4 solution. It is not necessary to buffer the salts of organic acids as strongly as carbohydrates because they serve as good buffers. In undisturbed fermentations in which CO_2 is not removed, little change in pH has been found to occur.

The results expressed in Table 3 show that an acid pH (3.6 to 5.7) influenced the dissimilation of citric acid in such a manner that the C_4 compounds, acetylmethylcarbinol and 2,3-butylene glycol were formed. As the reaction became increasingly acid, the carbinol was reduced to the glycol in increasing amounts. This result is in essential agreement with the results of Hammer et al. (1935) on skimmilk cultures. However, a substantial amount of C_4 compound existed as the glycol at the most acid reaction.

The absence of the compounds, acetylmethylcarbinol and diacetyl at a neutral pH, is in agreement with the work of van Beynum and Pette (1939). The latter authors have attempted to construct a scheme of dissimilation of citric acid to account for the effect of pH on the products formed. The present results are not in agreement with this scheme on the following points: (1) absence of ethyl alcohol, (2) presence of lactic and succinic acids, and (3) variation in the yield of CO_2 plus formic acid at an acid pH and at a neutral pH.

TABLE 3.

EFFECT OF pH ON THE DISSIMILATION OF CITRIC ACID BY CELL SUSPENSIONS OF STREPTOCOCCUS PARACITROVORUS

Initial pH	Substrate fermented per liter mM	CO_2	H_2	Formic acid	Acetic acid	Lactic acid	Succinic acid	Acetylmethyl carbinol	2,3-Butylene glycol	O/R index	Carbon recovered %
6.3	46.90	128.8	20.6	18.1	158.7	14.0	24.8	0	0	0.94	100.1
5.7	52.1	167.9	18.1	0.7	150.5	19.8	9.6	0	11.7	0.98	102.4
5.0	50.5	171.7	21.2	1.4	146.4	20.2	9.1	2.4	9.9	0.99	102.0
4.6	49.5	176.9	5.2	2.2	141.8	21.9	2.7	3.5	13.2	1.02	104.7
3.6	49.5	175.6	1.6	3.5	134.9	19.8	3.6	6.5	14.8	1.00	104.6

Products expressed as mM per 100 mM of citric acid fermented.

It is interesting to note in Table 3 the decrease in yield of succinic acid and increase in yield of acetylmethylcarbinol and 2,3-butylene glycol as the pH became increasingly acid. The total amount of C_4 compound formed in each fermentation, however, was approximately the same. Apparently the pH has a preferential effect on the various systems concerned. The effect on the yield of succinic acid is particularly marked. It will be shown that a certain relationship exists in regard to the formation of succinic acid and 2,3-butylene glycol from added acetic acid.

It is interesting to compare the effect of pH noted here with the results obtained by Silverman and Werkman (1941) with a cell-free enzyme preparation obtained from Aerobacter. The latter enzyme converts pyruvate to acetylmethylcarbinol and CO_2 and has a pH optimum of 5.6 to 6.0, although some of the carbinol was obtained as high as pH 7.2. Aerobacter cells grown at a pH of 7.5 did not produce an enzyme system capable of evolving CO_2 from pyruvate at pH 5.6. Thus an acid pH appeared to affect primarily the quantity or activity of the enzyme produced.

It is possible that a similar enzymatic system is involved in the formation of the carbinol and the glycol by S. paracitrovorus. The sharp reduction in yield of formic acid between pH 6.3 and pH 5.7 indicates that the hydroclastic enzyme does not possess optimum activity at an acid reaction,

in agreement with Silverman and Werkman (1941).

From a preliminary report of the work of Green et al. (1942), it is not clear at what pH A. aerogenes was able to couple acetaldehyde and pyruvate. It is probably more alkaline than the system of Silverman and Werkman. The mechanism of formation of these compounds will be discussed in more detail in a subsequent section.

Summary and conclusions.

Streptococcus paracitrovorus is a heterofermentative lactic acid organism which is unable to utilize citric acid as a sole source of carbon for growth. In the presence of fermentable carbohydrates, the acid is rapidly attacked. All previous attempts made to study the dissimilation of citric acid by this organism have been confused by the presence of carbohydrate.

It has been found that cells of S. paracitrovorus, which have been grown on citrate plus lactose, when suspended in a nitrogen-free medium, are able to ferment citrate in the absence of carbohydrate. Analyses of fermentations of citrate, oxalacetate, and pyruvate are presented. The data support the occurrence of oxalacetic and pyruvic acids as intermediary compounds in the dissimilation of citrate. A series of reactions are presented to illustrate the probable intermediary mechanism of the fermentation of citric acid.

The compounds, sodium azide, sodium arsenite, and sodium iodoacetate effectively inhibit the fermentation of citric, oxalacetic, and pyruvic acids.

An acid reaction (pH 4 to 6) has been shown to be necessary for the formation of acetylmethylcarbinol and 2,3-butylene glycol from citric acid by cell suspensions of S. paracitrovorus. As the yield of the latter compounds increased, the yield of succinic acid and acetic acid was found to decrease. The total yield of C_4 compounds, however, remains approximately the same. A relationship probably exists in the methods of formation of these compounds.

Assimilation of Heavy Carbon Dioxide by Certain Heterotrophic Bacteria

The concept of the assimilation of carbon dioxide by heterotrophic bacteria is due, in large measure, to the investigations of Wood and Werkman (1935, 1936a, 1938,) and Wood et al. (1941a). Species of the genera Propionibacterium, Escherichia, and Citrobacter were used. The proposal was advanced that CO_2 was fixed in heterotrophic systems according to the following reaction:

$$CO_2 + CH_3.CO.COOH \longrightarrow COOH.CH_2.CO.COOH$$

This reaction was not intended to give a detailed picture of the actual fixation mechanism, but it was intended to serve

as a working hypothesis for future investigations. It is commonly referred to as C_3 and C_1 addition. Studies with C^{13} by the above workers have confirmed the principle of this reaction.

Aside from the above investigations on the propionic acid and coliform bacteria, studies on the location of the CO_2 assimilated in compounds synthesized by heterotrophic bacteria have not been made. Since such information is essential for an understanding of the mechanism of assimilation of CO_2, it is of primary importance that these studies be extended to other bacteria. Therefore, it has been the purpose of the following investigation to determine, first the extent of assimilation of CO_2 among heterotrophic bacteria, secondly the extent of C_3 and C_1 addition, and thirdly the possibility of assimilation by other mechanisms. As a means of gaining an insight into possible mechanisms of photosynthesis and autotrophism, it is desirable to have further knowledge of all types of assimilation of CO_2, particularly those involving a carbon to carbon linkage.

Briefly it can be stated that the results of this investigation show the existence of assimilation of CO_2 with formation of a carbon to carbon linkage by a wide variety of genera of heterotrophic bacteria (Aerobacter, Proteus, Staphylococcus, Streptococcus, Clostridium). Evidence obtained does not allow definite conclusions to be

drawn in regard to the existence of other mechanisms of fixation of CO_2 besides that of C_3 and C_1 addition.

General distribution of fixed carbon.

With the exception of Clostridium acetobutylicum, non-proliferating cell suspensions of the respective organisms were used. The majority of the experiments were carried out in large manometric flasks with a capacity of 125 ml., and possessing two side arms. The substrate and $NaHC^{13}O_3$ were placed in the main chamber of the vessel, and in each of the two side arms, the cell suspension and phosphate buffer (pH 6.3). The vessels were attached to standard Warburg manometers and the enclosed air was replaced by oxygen-free nitrogen. The phosphate was tipped into the substrate-bicarbonate mixture just before the manometers were placed in the water bath. The addition of the phosphate lowered the pH of the reaction mixture to approximately 7, and simultaneously CO_2 was liberated from the bicarbonate. Thus the acid phosphate ensured the presence of a partial carbon dioxide atmosphere above the reaction mixture and also a favorable pH. After equilibration, the cell suspension was tipped into the main chamber of the vessel, and the initial reading recorded. When the fermentation, as measured by gas evolution, had ceased, the vessel was removed, and the contents acidified to Congo red with 1:1 H_2SO_4 and centrifuged.

It must be remembered that all naturally occurring materials contain approximately 1.09 per cent C^{13}. Inasmuch as the $NaHCO_3$ was the only material in the reaction mixture which contained an excess of the carbon isotope, the presence of C^{13} in a compound in excess of 1.09 ± 0.02 per cent indicates assimilation of CO_2 from the $NaHCO_3$.

The exact composition of the various reaction mixtures, growth media, etc., used in the experiments presented in Table 4, is as follows:

Fermentation 1. Reaction mixture, 0.125 M glucose, 0.153 M $NaHCO_3$ (9 per cent C^{13}), 2 per cent cell suspension, volume 60 ml.; incubation period 7 hours at 30°C.; pH maintained above 7 by addition of NaOH; cells grown for 24 hours at 30°C. on 1 per cent glucose, 0.3 per cent peptone, 10 per cent tap water.

Fermentation 2. Reaction mixture, 0.1 M glucose, 0.125 M $NaHCO_3$ (5.29 per cent C^{13}), 0.066 M phosphate buffer pH 6.3, 2 per cent cell suspension, volume 30 ml.; incubation period 10 hours at 30°C.; growth medium same as Fermentation 1.

Fermentation 3. Reaction mixture, 0.1 M citric acid, 0.0625 M $NaHCO_3$ (5.29 per cent C^{13}), 0.02 M phosphate buffer pH 6.3, 3 per cent cell suspension, volume 30 ml.; incubation period 24 hours at 30°C.; cells grown for 48 hours at 30°C. on 1 per cent lactose, 0.5 per cent Na_3 citrate, 0.5 per cent

TABLE 4.

DISTRIBUTION OF ASSIMILATED $C^{13}O_2$ AMONG FERMENTATION

PRODUCTS OF HETEROTROPHIC BACTERIA

Fermentation No.		Aerobacter indologenes (1)*	Aerobacter indologenes (2)	Streptococcus para-citrovorus (3)	Streptococcus para-citrovorus (4)	Clostridium welchii (5)	Clostridium aceto-butylicum (6)	Proteus vulgaris (7)	Staphylococcus candidus (8)
Acetic acid	mM	62.7	16.0	137.0	30.8	22.5	34.5	22.7	54.7
	%C^{13}	1.33	1.19	1.10	1.11	1.24	1.11[x]	1.10	1.09
Butyric acid	mM					8.9	33.9		
	%C^{13}					1.11	1.11[x]		
Ethyl alcohol	mM	40.3	64.0		68.0	7.7	28.7	18.0	27.6
	%C^{13}	1.11	1.10		1.08	1.09[+]	1.10[+]	1.08	1.09
Butyl alcohol	mM					0.3	41.4		
	%C^{13}					1.09[+]	1.10[+]		
Acetone	mM						25.7		
	%C^{13}						1.10		
Lactic acid	mM	1.60		14.3	73.3	10.0	17.1	92.1	20.0
	%C^{13}	1.36		1.09	1.17	1.31	1.16	1.18	1.19
Succinic acid	mM	32.0	15.4	21.9	2.4			21.3	11.1
	%C^{13}	1.68	1.42	1.31	1.27			1.50	1.25
2,3-Butylene glycol	mM		43.0		27.2				
	%C^{13}		1.09		1.09				

Yield of products expressed as mM per 100 mM substrate fermented, except Cl. acetobutylicum which is expressed as mM per liter.

*68 mM formic acid were also produced which contained 3.13 per cent C^{13}.

[+]Per cent C^{13} in combined alcohol distillate.

[x]Per cent C^{13} in combined volatile acid distillate.

Underscored figures denote assimilation of $C^{13}O_2$.

peptone, 0.2 per cent peptonized milk, 0.1 per cent yeast extract, 4 per cent filtrate of tomato juice, 0.05 per cent $MgCl_2$, 0.05 per cent K_2HPO_4, 10 per cent tap water.

Fermentation 4. Reaction mixture, 0.1 M glucose, 0.0625 M $NaHCO_3$ (4.79 per cent C^{13}), 0.02 M phosphate buffer pH 6.3, 3 per cent cell suspension, volume 30 ml.; incubation period 30 hours at 30°C.; cells grown for 48 hours at 30°C. on medium of Fermentation 3.

Fermentation 5. Reaction mixture, 0.05 M glucose, 0.0625 M $NaHCO_3$ (4.36 per cent C^{13}), 0.02 M phosphate buffer pH 6.3, 3 per cent cell suspension, volume 30 ml.; incubation period 6 1/2 hours at 40°C.; cells grown for 20 hours at 38°C. on 1 per cent glucose, 0.5 per cent peptone, 0.1 per cent yeast extract, 0.5 per cent K_2HPO_4 adjusted to pH 7 with H_2SO_4.

Fermentation 6. Reaction mixture, 25 ml. of 5 per cent corn mash, 5 ml. of 0.375 M $NaHCO_3$ (10.64 per cent C^{13}), 2 ml. of 0.2 M phosphate buffer pH 6.3; inoculum consisted of 2 ml. of 24 hour corn mash culture; bicarbonate and phosphate added 5 hours after inoculation; incubation period 2 1/2 days at 38°C.

Fermentation 7. Reaction mixture, 0.05 M glucose, 0.0625 M $NaHCO_3$ (4.36 per cent C^{13}), 0.02 M phosphate buffer pH 6.3, 3 per cent cell suspension, volume 30 ml.; incubation period 30 hours at 30°C.; cells grown for 24 hours at 30°C.

on 1 per cent glucose, 0.3 per cent beef extract, 0.5 per cent peptone, 0.4 per cent NaCl, 2 per cent agar.

Fermentation 8. Reaction mixture, same as for Fermentation 7; incubation period 7 hours at 30°C.; cells grown for 48 hours at 30°C. on 1 per cent glucose, 0.3 per cent beef extract, 0.3 per cent peptone, 0.2 per cent NaCl, 0.2 per cent K_2HPO_4.

The cells were harvested from the respective growth media by centrifugation, washed once, and resuspended in distilled water. Cell suspensions of Cl. acetobutylicum were not found to be satisfactory in regard to the production of neutral compounds (acetone, alcohols); so that a growing culture in 5 per cent corn mash containing $NaHC^{13}O_3$ was used.

The general distribution of the assimilated CO_2 among the fermentation products of the various bacteria is indicated in Table 4. Two species of homofermentative lactic acid bacteria, Streptococcus lactis and Lactobacillus plantarum, were found to fix no CO_2. It is interesting to note that such compounds as 2,3-butylene glycol, butyric acid, and butyl alcohol, which are probably formed by synthesis, contained no assimilated carbon dioxide. On the other hand, succinic acid contained fixed C^{13} in every case in which it was formed. The other compounds which contained fixed C^{13} are acetic acid and lactic acid.

The location of the assimilated CO_2 in succinic, lactic and acetic acids obviously presented the problem of determining the position of the C^{13} in the respective molecules.

Fixation of carbon dioxide in succinic acid.

The degradation of succinic acid was performed by a succinic dehydrogenase-fumarase preparation from beef heart according to Krebs (1937). Succinate, obtained as the silver salt from the fermented medium, was acidified, extracted with ether, and then converted to the sodium salt. The reaction mixture consisted of approximately 0.02 M succinate, 0.04 M phosphate buffer (pH 7.4), and 10 ml. of enzyme preparation. The oxidation was performed aerobically at 30°C. in large manometric flasks. Succinate was oxidized by this preparation to a mixture of malate and fumarate. The former compound when oxidized with $KMnO_4$ yields $CH_3.CHO$ and CO_2 and fumarate yields CO_2 and a trace of $CH_3.CHO$, thus the presence of fumarate does not introduce an error. Carbon dioxide originates from the carboxyl groups and acetaldehyde from the methylene carbons of succinic acid. The CO_2 and $CH_3.CHO$ were subsequently treated as in the lactic method. The reactions are:

$$COOH.CH_2.CH_2.COOH \xrightarrow{-2H} COOH.CH{:}CH.COOH \underset{-H_2O}{\overset{+H_2O}{\rightleftharpoons}}$$

$$COOH.CHOH.CH_2.COOH \xrightarrow{1/2\ O_2} CO_2 + H_2O + \left[COOH.CH_2.CHO\right]$$

$$\left[COOH.CH_2.CHO\right] \longrightarrow CO_2 + CH_3.CHO$$

Table 5 shows that the succinic acid produced by Aerobacter, Streptococcus and Proteus contained fixed carbon dioxide in the carboxyl groups. A similar fixation of CO_2 in the carboxyl groups of the C_4-dicarboxylic acids produced by Escherichia and Propionibacterium has also been found by Wood et al. (1941b). The following reactions were proposed by the latter authors (1940) to explain the synthesis of succinate.

$C^{13}O_2 + CH_3.CO.COOH \rightleftharpoons C^{13}OOH.CH_2.CO.COOH$

$C^{13}OOH.CH_2CO.COOH + 4H \rightleftharpoons C^{13}OOH.CH_2.CH_2.COOH + H_2O$

It is probable that the same or similar reactions are involved in the present study.

The above reactions postulate the formation of succinate containing fixed carbon in one carboxyl group. Since succinic acid is a symmetrical molecule, the carboxyl groups no longer possess their original orientation in respect to the original fixation reaction, and hence it is not possible to prove that the fixed C^{13} resides in only one carboxyl group. However, by quantitative methods it is possible to show with some certainty that a symmetrical C_4-dicarboxylic acid contains fixed carbon in one or both of its carboxyl groups. To accomplish this the per cent C^{13} in the CO_2 available to the cell for fixation should be known. The best method available at present is to employ the concentration of C^{13} in the final CO_2 at the conclusion of the fermen-

TABLE 5.

POSITION OF ASSIMILATED $C^{13}O_2$ IN SUCCINIC ACID SYNTHESIZED BY HETEROTROPHIC BACTERIA

Bacteria	Succinic acid % C^{13}	α and β carbon % C^{13}	COOH carbon % C^{13}	Calculated* COOH carbon % C^{13}
Streptococcus paracitrovorus	1.27	1.10	1.36	1.45
Proteus vulgaris	1.50	1.12	1.82	1.91
Aerobacter indologenes	1.42	1.13	1.61	1.75
Aerobacter indologenes	1.37	1.14	1.84	1.61

*Calculated by means of equation: 2 x 1.09 + 2X = 4 x per cent C^{13} in succinic acid; X = average per cent C^{13} in carboxyl groups of succinic acid.

tation. It is admitted however, that this value is not ideal; at best it is a minimum value. There is no assurance that the carbon dioxide produced within the cell reaches complete equilibrium with the carbon dioxide in the medium before it is utilized. However, assuming complete equilibrium was reached the per cent C^{13} available for fixation would be greater in the early stages than in the later stages of the fermentation. In the extreme it is hardly to be expected that the C^{13} available to the cell would be as great as the C^{13} added in the bicarbonate.

Thus with these limitations in mind the calculation can be made in the following manner. If the concentration of C^{13} in the carboxyl groups as determined by experimentation, is greater than the average value calculated on the basis that one carboxyl group contained 1.09 per cent C^{13} and the other a concentration equivalent to the final $C^{13}O_2$, fixation of CO_2 in both carboxyl groups has occurred in some of the C_4 acid molecules. If the average calculated value is greater than or equal to the C^{13} concentration of the carboxyl groups as determined by experimentation, it cannot be stated that any one molecule of dicarboxylic acid contained fixed C^{13} in more than one carboxyl group.

In most of the experiments the concentration of C^{13} was not determined in the bicarbonate and CO_2 at the conclusion of the fermentation. However, calculations have

been made on the basis of an equivalent formation of C_1 and C_2 compounds. In the fermentations by Staphylococcus candidus, Streptococcus paracitrovorus, and Proteus vulgaris, the C^{13} content of the final CO_2 was too high to indicate a stoichiometric fixation of CO_2 by C_3 and C_1 addition. However, in one fermentation in which Aerobacter indologenes was used (Table 4, fermentation 1) the C^{13} content of the final CO_2 was determined. This value amounted to 3.13 per cent and the succinate formed in the experiment contained 1.68 per cent C^{13}. On the basis that all fixed carbon in succinate resides in one carboxyl group, the calculated value for this carboxyl carbon is 3.45 per cent C^{13}. It is possible that some of these succinic acid molecules contained fixed C^{13} in both carboxyl groups. In view of recent results to be described later with this organism, which have shown the condensation of C_2 compound to C_4 dicarboxylic acid and the reverse reaction, the above result is entirely possible.

Although succinate contained fixed carbon in every case in which it was formed, it does not necessarily follow, however that this is the only mechanism of succinate formation. It does indicate that the formation of succinic acid by means of fixation of carbon dioxide is a general reaction among heterotrophic bacteria.

Fixation of carbon dioxide in lactic acid.

The $KMnO_4$ method of Friedemann and Graeser (1933) was

used to degrade lactic acid to CH_3CHO and CO_2. The aldehyde was trapped in $NaHSO_3$ and the CO_2 collected in 1.5 N carbonate-free NaOH. Calcium carbonate was added to the aldehyde-bisulfite complex and the liberated aldehyde distilled into ice cold water. $Ba(OH)_2$ was added to the distillate and the precipitated carbonate filtered off; the aldehyde was then oxidized to CO_2 with persulfate (Osburn and Werkman, 1932). The aldehyde originates from the α, β-carbon atoms and CO_2 from the carboxyl group of lactic acid. The reaction is as follows:

$$CH_3.CHOH.COOH + 1/2\ O_2 \longrightarrow CH_3.CHO + CO_2 + H_2O$$

The data presented in Table 6 show that in every case in which fixation of carbon dioxide occurred in lactic acid, the fixed carbon was found in the carboxyl group. Of the number of possible mechanisms for this fixation, either C_2 and C_1 addition or a secondary conversion of a C_4-dicarboxylic acid containing fixed carbon in the carboxyl group seems most probable. Lactic acid is generally accepted to be formed by the reduction of pyruvic acid, so that any scheme which accounts for assimilation of CO_2 in the carboxyl group of pyruvic acid provides a probable source of lactic acid containing fixed carbon in the carboxyl group. The exchange of CO_2 with the carboxyl group of pyruvic acid through the action of carboxylase seems a likely mechanism for fixation of the heavy carbon. Thus far, however, all experimental evidence indicates that decarboxylation of pyruvic acid by

TABLE 6.

POSITION OF ASSIMILATED $C^{13}O_2$ IN LACTIC ACID SYNTHESIZED BY HETEROTROPHIC BACTERIA

Bacteria	Lactic acid* % C^{13}	COOH carbon % C^{13}	α and β carbon % C^{13}
Staphylococcus candidus	1.19	1.34	1.11
Streptococcus paracitrovorus	1.17	1.33	1.09
Clostridium welchii	1.31	1.68	1.13
Clostridium acetobutylicum	1.16	1.22	1.13
Proteus vulgaris	1.18	1.34	1.11
Aerobacter indologenes	1.36	1.81	1.13

*Calculated by means of equation: 2 x per cent C^{13} in α, β carbons + per cent C^{13} in carboxyl carbon = 3 x X; X = per cent C^{13} in lactic acid molecule.

carboxylase is irreversible. This point has been investigated both by Evans (1942) and Carson et al. (1941a) with C^{11} and the yeast carboxylase system and by Krampitz et al. (1942) with C^{13} and an acetone preparation of Micrococcus lysodeikticus which oxidatively converts pyruvate to acetate and carbon dioxide. Carson et al. state that they have isolated a carbonyl compound which possessed a very small amount of radioactivity. However, the amount of CO_2 assimilated by this reaction was far too small to account for the CO_2 utilized in many systems. Thus it can be concluded that, according to our present knowledge, the decarboxylation of pyruvate is an irreversible reaction.

In regard to the possibility of C_3 and C_1 addition, the work of Lipmann (1941) is of importance. The latter investigator has found that cells of Lactobacillus delbrueckii, when dried with acetone or over P_2O_5 in a vacuum, were able to oxidize pyruvate to acetone and CO_2. The system contained no iron oxidation catalysts, and possessed the following components: (1) thiaminpyrophosphate, (2) flavin-adenine-dincleotide, (3) Mn^{++}, Mg^{++} or CO^{++}, (4) protein and (5) inorganic phosphate. Lipmann has proposed the occurrence of acetyl phosphate in the reaction:

$$CH_3.CO.COOH + H_3PO_4 \rightleftharpoons CH_3.\underset{\displaystyle OPO_3H_2}{\overset{\displaystyle OH}{\overset{|}{C}}}.COOH \xrightarrow{-2H} CH_3.\underset{\displaystyle OPO_3H_2}{\overset{\displaystyle O}{\overset{\|}{C}}} + CO_2$$

Lipmann believes that acetylphosphate, due to the fact that

phosphorylation has raised the O/R potential of the system, is possibly the intermediate in C_2 and C_1 addition to form pyruvic acid. Such a reaction involving acetylphosphate, would provide a means of generating phosphopyruvate, the only compound in glucolysis not regenerated by a reversible reaction.

It should be realized that no reliable evidence exists at the present time in support of the hypothesis of C_2 and C_1 addition involving carbon dioxide.

An alternative mechanism for the fixation of CO_2 in lactic acid, i.e., by formation of lactate from a C_4-dicarboxylic acid, offers more attractive possibilities but there are at present certain unexplained facts.

The following series of reactions serves to illustrate a possible mechanism.

$C^{13}O_2 + CH_3.CO.COOH \rightleftharpoons C^{13}OOH.CH_2.CO.COOH \rightleftharpoons C^{13}OOH.CH:COH.COOH \rightleftharpoons C^{13}OOH.COH:CH.COOH \rightarrow C^{13}OOH.CO.CH_3 + CO_2 \overset{+2H}{\underset{-2H}{\rightleftharpoons}} C^{13}OOH.CHOH.CH_3 + CO_2$

This scheme incorporates the suggestion of Meyerhof (1942) that there exists a dynamic, non-enzymatic interchange of H and OH between the alpha-carbon and the beta-carbon of enol oxalacetic acid. The results of Krampitz et al. (1942), however, have shown that the above interchange does not occur due to chemical influences, neither does it occur in the

presence of an enzyme which brings about a reversible exchange of CO_2 and the carboxyl carbon adjacent to the methylene carbon of oxalacetate.

Carson et al. (1941b) have proposed the reversible conversion of pyruvic acid and CO_2 to the symmetrical molecule, fumarate, as a possible mechanism to explain the formation of pyruvic acid containing fixed carbon in the carboxyl group. Lactic acid would then arise by reduction of the latter compound. Such a mechanism would lead to the formation of succinate containing fixed carbon in both carboxyl groups, since part of the pyruvate containing heavy carbon would again condense with $C^{13}O_2$ and be converted to succinate.

The following are some observations which at first sight may seem inconsistent with the above theories. Lactic acid formed from glucose by Streptococcus paracitrovorus contained fixed carbon, whereas that formed from citrate did not. On the other hand, the succinate formed by both fermentations contained fixed carbon (cf. Table 4). It follows that the lactate was formed from citrate by a mechanism which differs from that responsible for the formation of lactate containing C^{13} from glucose. Assuming oxalacetate was formed containing fixed carbon in both fermentations, and the lactate containing C^{13} was formed by the fermentation of glucose from oxalacetate, there must have been a mechanism present in the fermentation of citrate which prevented the breakdown

of oxalacetate to yield pyruvate containing heavy carbon. Moreover, in other fermentations there does not seem to be any relationship between the concentration of C^{13} in the carboxyl groups of succinate and lactate, as might be expected if both had a common origin. Recent results (Slade and Werkman, 1942) have shown an additional means of formation of succinate, hence it is doubtful whether the above relationship would occur. Also, the discrepancy may be caused by oxalacetate being removed prior to its breakdown to pyruvate more completely in some fermentations than in others.

Clostridium welchii produced lactic acid containing fixed carbon but there was no C_4-dicarboxylic acid formed. If the latter compounds function in the formation of lactate in this organism, it would be necessary to assume that all the C_4 acid formed was broken down to compounds containing fewer carbon atoms.

The homofermentative lactic acid bacteria produce substantially only lactic acid, and little C_2 compounds from glucose. Hence, it is logical to suppose that these bacteria have a weak carboxylase activity. It is apparent therefore, that these bacteria might be used to test the formation of lactic acid by C_2 and C_1 addition. However, Streptococcus lactis and Lactobacillus plantarum were found to fix no carbon dioxide in lactic acid. These results are in agreement with those of Carson et al. (1941a). As was

pointed out above, pyruvate carboxylase probably has no connection with fixation of CO_2, on the basis of the evidence available. These negative results, however, do not exclude the possibility that assimilation of CO_2 is involved in some unknown manner, in the metabolism of these organisms.

It is apparent that the above reactions discussed do not assign an essential role to the fixation of CO_2 in the carboxyl group of lactic acid, i.e., lactate would be formed regardless of the fixation of carbon dioxide. It is admitted also, that the mechanism of this fixation is largely a matter of speculation. No definite evidence exists for either C_2 and C_1 addition or a secondary conversion of a C_4-dicarboxylic acid. The statement can be made in favor of the latter mechanism however, that the reversibility of the reactions concerned has been proved in the case of animal tissues (Szent-Györgyi, 1939) and bacteria (Krebs and Eggleston, 1941).

Fixation of carbon dioxide in acetic acid.

The procedure used for the degradation of acetic acid was similar to that of Ardagh et al. (1924). Wood et al. (1941d) have proved the reliability of this method to determine the position of fixed CO_2 in propionic acid. After recovery of the acetic acid from the fermented medium, its

purity was checked by the partition method of Osburn, Wood and Workman (1936). In the presence of brom-thymol blue, $Ba(OH)_2$ was added to neutrality and the solution evaporated to dryness. The dry $Ba(CH_3.COO)_2$ was placed in a 25 ml. distilling flask and held at 460°C. in a Wood's alloy metal bath for 50 minutes. The flask was continuously flushed with oxygen-free and CO_2-free nitrogen. The reaction is as follows:

$$(CH_3COO)_2Ba \longrightarrow CH_3CO.CH_3 + BaCO_3$$

The carbonate originates from the carboxyl group of acetate. Acetone was collected in ice-cold water. The CO_2 was liberated from the residual carbonate by addition of 2 N HCl.

The acetone was distilled from the original solution and then converted to iodoform (Goodwin, 1920) by treatment with successive aliquots of NaOH and I_2. The solution was allowed to stand half an hour in an ice bath; a slight excess of H_2SO_4 was added and the liberated I_2 titrated with $Na_2S_2O_3$. The degradation reaction is as follows:

$$CH_3.CO.CH_3 + 3I_2 + 4NaOH \longrightarrow CHI_3 + CH_3COONa + 3NaI + 3H_2O$$

Iodoform originates from the methyl carbon atom of acetate. The latter compound was filtered onto a sintered glass disk, dried over $CaCl_2$ at room temperature, and oxidized to CO_2 with chromic-phosphoric acid according to Friedemann and Kendall (1929).

The yields of CO_2 ranged between 90 to 95 per cent of

theoretical, and acetone 45 to 50 per cent. The recovery of iodoform is practically quantitative.

In order to secure sufficient acetate to perform the degradation, stationary fermentations by Aerobacter (Table 4, number 1) and Clostridium were carried out in a volume of 60 ml. In the latter case the reaction mixture consisted of 0.1 M glucose, 0.065 M $NaHCO_3$ (7 per cent C^{13}), and 2 per cent cell suspension; the incubation temperature was 30°C.

In the case of fermentations produced by Clostridium welchii, it was necessary to separate a mixture of acetic and butyric acids before the former could be degraded. The benzene distillation method of Schicktanz et al. (1940) was used. A three foot still packed with porcelain saddles was employed. After the distillation, the acetic acid was recovered from the benzene solution by alkaline evaporation, acidification with H_2SO_4, and subsequent steam distillation. The purity of the acetic acid was checked according to Osburn et al. (1936).

The residue containing butyric acid, benzene, and p-toluenesulfonic acid was filtered, water added, and the mixture neutralized and evaporated to a small volume. After a second filtration, the solution was acidified and steam distilled, and the distillate neutralized, evaporated to a small volume, acidified, and again steam distilled. The

partition constant of the distillate indicated the presence, in addition to butyric acid, of traces of an unknown acid.

The results in Table 7 show that Aerobacter indologenes and Clostridium welchii fixed $C^{13}O_2$ exclusively in the carboxyl group of acetic acid. The per cent C^{13} fixed in acetate by the latter organism has been consistently lower when compared to Aerobacter. In a single experiment these values would be questionable but from repeated experiments it is concluded that none of the fixed carbon is located in the methyl group. In an additional experiment with Clostridium welchii, the acetic acid formed contained 1.24 per cent C^{13} and the acetone derived therefrom contained 1.19 per cent C^{13}. Calculating all the fixed C^{13} to reside in the carboxyl group of the acetic acid, a value of 1.19 per cent C^{13} was obtained for the acetone which agrees exactly with the experimental value. In this experiment, the CO_2 originating from the carboxyl group of the acetic acid was lost.

According to most schemes of fermentation, acetic acid is believed to arise by oxidation of pyruvic acid, as follows:

$$CH_3.CO.COOH + H_2O \longrightarrow CH_3.COOH + CO_2 + 2H$$

In this reaction the carboxyl carbon is transformed to CO_2; therefore even though there was fixed carbon in the carboxyl group of pyruvate, the acetic acid would be devoid of fixed carbon dioxide. It thus is apparent that two mechanisms exist for the formation of acetic acid, one in which the acetate

TABLE 7.

POSITION OF ASSIMILATED $C^{13}O_2$ IN ACETIC ACID SYNTHESIZED BY HETEROTROPHIC BACTERIA

Bacteria	Acetic acid $\%C^{13}$	Methyl carbon $\%C^{13}$	Carboxyl carbon $\%C^{13}$	Calculated* carboxyl carbon $\%C^{13}$
Aerobacter indologenes	1.33	1.11	1.34	1.57
Aerobacter indologenes	1.17	1.10	1.19	1.23
Clostridium welchii	1.13	1.11	1.15	1.15

*Calculated by means of equation: $1.09 + X = 2 \times$ per cent C^{13} in acetic acid; X = per cent C^{13} in carboxyl group of acetic acid.

contains fixed carbon in the carboxyl group and the other in which it does not.

Slade et al. (1942) suggested that the reaction of fixation may not be directly concerned in the formation of acetic acid, in that the acid may arise by secondary conversions following the initial fixation of CO_2, e.g., by C_3 and C_1 addition. The recent results of Slade and Werkman (1942) have shown that this suggestion is substantially correct. It appears that the fixation takes place by means of the Wood and Werkman reaction, and the acetate is subsequently derived from the C_4-dicarboxylic acid by a splitting of the molecule into two C_2 fragments. This material will be discussed in a following section of this thesis.

There are several other possibilities which may be considered in regard to the fixation of CO_2 in the carboxyl group of acetic acid. The possibility of an exchange reaction seems remote. If such a reaction did occur it would involve a new type reaction of fundamental importance, i.e., C_1 and C_1 addition in which one component is not CO_2 or formed from CO_2. The possibility also exists of a separation of a C_2 fragment from an intermediate compound which contains more than four carbon atoms. One of the six carbon or seven carbon atom compounds which occur in the modified Krebs cycle (Wood et al., 1942) may be cited as an example. Finally, it

is possible to theorize that acetate may arise by alpha decarboxylation of oxalacetate to malonic aldehyde, followed by oxidation and subsequent decarboxylation.

$$C^{13}OOH.CH_2.CO.COOH \xrightarrow{-CO_2} C^{13}OOH.CH_2.CHO \xrightarrow[-2H]{+H_2O}$$

$$C^{13}OOH.CH_2.COOH \longrightarrow C^{13}OOH.CH_3 + CO_2$$

Little evidence is available to support these possibilities.

It should be emphasized that the type of fixation of CO_2 in acetic acid discussed here, differs fundamentally from that reported by Barker et al. (1940a) and Wieringa (1940). The former have found that Clostridium acidi-urici is able to reduce CO_2 to acetic acid in the presence of uric acid as a hydrogen donator. Radioactive carbon was used as a tracer and the CO_2 was found in both methyl and carboxyl groups. The methyl carbon possessed twice as much radioactivity as the carboxyl carbon. A direct reduction of CO_2 to acetic acid would be expected to result in an equal distribution of the radioactivity between the two carbon atoms. Thus it is possible that the individual acetate molecules synthesized by this organism did not originate entirely from carbon dioxide.

Wieringa (1940) found that Clostridium aceticum was able to reduce carbon dioxide to acetic acid with gaseous hydrogen as a reducing agent. This organism and also that used by Barker et al., are considered as representative of

types intermediate between the autotrophic and heterotrophic groups of bacteria. It is obvious that the type of fixation found with Aerobacter indologenes and Clostridium welchii is fundamentally distinct from that of the two species mentioned above.

In passing it may be of interest to point out that, in fermentation 1, Table 4, 68 mM of formic acid were produced which contained 3.13 per cent C^{13}. The concentration of C^{13} in the final carbon dioxide was also 3.13 per cent. Evidently, under the expressed conditions, complete equilibrium was reached between formic acid and CO_2, as in the following reaction: $HCOOH \rightleftharpoons CO_2 + H_2$. Woods (1936) has shown the reversibility of the above reaction.

Mickelson (1939) found a cell-free, non-volatile, non-ether-soluble residue from alkaline fermentations of glucose by Aerobacter. This material has not been identified but is known not to contain adjacent hydroxyl groups. In one experiment (Table 4, fermentation 1), the cell-free residue remaining after steam distillation and ether extraction was found to contain fixed carbon (1.24 per cent C^{13}). Barker et al. (1940b) in experiments with cell suspensions of Methanobacterium in the presence of $C^{11}O_2$, have found fixed carbon in the fermentation residue which contained cell material and probably other non-volatile substances. Barker et al. believe those results show the incorporation of CO_2

into cell material. The results reported here indicate the possibility that the fixation may have been in other than cell material.

Summary and conclusions.

Assimilation of carbon dioxide with the formation of a carbon to carbon linkage is established as a general phenomenon among heterotrophic bacteria. It is shown by the use of heavy carbon, C^{13}, as a tracer, that the fixed carbon is located in the carboxyl groups of succinic, lactic, and acetic acids. The assimilated carbon is distributed as follows: Aerobacter indologenes, acetate, lactate, and succinate; Proteus vulgaris, Streptococcus paracitrovorus, and Staphylococcus candidus, lactate and succinate; Clostridium welchii, acetate and lactate; Clostridium acetobutylicum, lactate.

Succinic acid contained assimilated carbon in every case in which it was formed. This fixation of carbon dioxide in the carboxyl groups of succinate is believed to arise by C_3 and C_1 addition according to the Wood and Werkman reaction.

Conversely, lactic acid did not contain fixed carbon in every case. Streptococcus paracitrovorus produced lactate from glucose which contained fixed carbon, while lactate produced from citric acid did not contain fixed carbon.

Two possible mechanisms are suggested to explain the fixation of carbon dioxide in the carboxyl group of lactic acid, (1) C_2 and C_1 addition, and (2) a secondary conversion of a C_4 dicarboxylic acid containing fixed carbon in the carboxyl group. No concrete evidence is at present available to support either possibility, however, the latter appears most likely.

Acetic acid containing fixed carbon dioxide in the carboxyl group was produced by Aerobacter indologenes and Clostridium welchii. The suggestion is made that the reaction of fixation may not be directly concerned in the formation of acetic acid, in that the acetic acid may arise from a cleavage of a C_4 dicarboxylic acid containing fixed carbon in the carboxyl groups into two C_2 molecules. Several other possibilities are presented.

The homofermentative lactic acid bacteria, Streptococcus lactis and Lactobacillus plantarum did not fix carbon dioxide in their products of the fermentation of glucose.

A cell-free, non-volatile, non-ether-soluble residue from alkaline fermentations of glucose by Aerobacter was found to contain fixed carbon dioxide.

Assimilation of Acetic Acid and Succinic Acid Containing Heavy Carbon by Aerobacter indologenes

Slade et al. (1942) have shown the fixation of carbon

dioxide in the carboxyl group of acetic acid during the fermentation of glucose by Aerobacter indologenes and Clostridium welchii. The suggestion was made that the acetic acid may arise by a cleavage of a C_4 dicarboxylic acid containing fixed carbon in the carboxyl groups into two C_2 molecules. It has been the purpose of this investigation, to prove the occurrence of this reaction by the use of synthetic heavy acetic acid and biological succinic acid containing heavy carbon as a tracer, in fermentations of glucose by cell suspensions of A. indologenes.

Briefly, definite evidence has been obtained for (1) the formation of succinic acid by the condensation of two C_2 molecules, probably those of acetic acid, and (2) the reverse reaction, i.e., the cleavage of succinic acid into two C_2 molecules which are isolated as acetic acid. In addition, the first positive proof has been obtained for the conversion of acetic acid to 2,3-butylene glycol and to ethyl alcohol.

Synthesis of acetic acid.

Two types of acetic acid were synthesized; the heavy isotope of carbon was contained in (1) the carboxyl group (type 1) and (2) in both the methyl and carboxyl groups (type 2). Type 1 acetate was synthesized according to the following reactions:

$$CH_3I + Mg \xrightarrow[\text{ether}]{\text{dry}} CH_3MgI$$

$$CH_3MgI + C^{13}O_2 \longrightarrow CH_3.C^{13}OOMgI$$

$$CH_3.C^{13}OOMgI + H_2O \longrightarrow CH_3.C^{13}OOH + MgIOH$$

The Grignard reagent was prepared by adding cold methyl iodide in dry ether to magnesium turnings under an atmosphere of nitrogen. At the conclusion of the reaction, the reagent was filtered into a Woulff flask which was then connected to the flask containing the $Na_2C^{13}O_3$ solution. The delivery tube entered below the surface of the Grignard reagent and a drying tube was placed in the line between the two flasks to prevent the entrance of water into the reaction mixture. The system was placed under a vacuum and the CO_2 released from the carbonate by the addition of H_2SO_4. The Grignard reagent was vigorously stirred during the reaction period. By means of a small mercury manometer attached to the reaction flask, the uptake of CO_2 was followed. In this manner the CO_2 was released from the carbonate as needed. Approximately 0.08 M CO_2 was taken up in four hours. Water was then added to destroy the excess CH_3MgI and to convert the acetic acid complex to the magnesium salt. The solution was acidified and distilled to one-half volume to remove acetone and ether. The distillate was made alkaline and evaporated to a small volume, acidified and combined with the residue of the original distillation. The acetic acid was then removed

by distillation. The distillate was again made alkaline, evaporated, acidified, and redistilled. The distillate contained pure acetic acid when checked according to Osburn et al. (1936), and gave a negative test for iodides. A yield of 60.6 per cent was obtained.

Acetic acid containing C^{13} in the methyl and carboxyl groups (type 2) was synthesized by a modification of the method of Cramer and Kistiakowsky (1941). The reactions are as follows:

$$BaC^{13}O_3 + Mg \xrightarrow{700°C.} BaC_2^{13}$$

$$BaC_2^{13} + H_2O \longrightarrow C_2^{13}H_2$$

$$C_2^{13}H_2 + H_2O \xrightarrow[Fe_2(SO_4)_3]{HgSO_4} C^{13}H_3.C^{13}HO$$

$$C^{13}H_3.C^{13}HO + 1/2\ O_2 \xrightarrow{K_2Cr_2O_7} C^{13}H_3.C^{13}OOH$$

All materials used in the preparation of the acetylide must be perfectly dry. Fifty mM $BaCO_3$ and one mole Mg(40-80 mesh) were ground carefully in a mortar, and the mixture placed in the center portion of an iron pipe 1 3/8 inches in diameter. Dry hydrogen gas was passed through the tube for several minutes, and the tube was then placed in a previously heated oven at about 700°C. until the tube reached a red heat. The heating was then continued for 5 to 10 minutes longer. Passage of the hydrogen was continued during this procedure, and the tube then cooled in running water.

The BaC_2-Mg mixture was placed in a dry Claissen flask which had been previously swept out with nitrogen. A salt-ice bath was placed about the flask, and then 60 ml. of water were added slowly to the mixture during vigorous stirring. After addition of the water, the mixture was warmed gently until three liters of gas were obtained. The acetylene and hydrogen gases were collected in a three liter flask over water, and then passed through a sintered glass disk which was immersed in 100 ml. of the catalytic solution (2 gm. $HgSO_4$ plus 4.2 gm. $Fe_2(SO_4)_3.9H_2O$ plus 5 gm. H_2SO_4 plus water to 100 ml.). The solution was placed in a boiling water bath during the passage of the acetylene. A reflux condenser was placed above the catalytic solution and the aldehyde displaced into 30 ml. of five per cent $NaHSO_3$. Because 10 per cent of the acetylene escapes conversion to the aldehyde, the gas from the bisulfite tower was collected and again passed through the hot catalytic solution. The yield of acetaldehyde was 58.4 per cent of theoretical. The aldehyde was then released from the bisulfite solution and oxidized to acetic acid as in the determination of ethyl alcohol.

Condensation of acetic acid to succinic acid and the reverse reaction.

Aerobacter indologenes was grown for 24 hours at 30°C.

on a medium consisting of 1 per cent corn sugar, 0.3 per cent peptone, 0.3 per cent $(NH_4)_2SO_4$, 1 per cent K_2HPO_4, 10 per cent tap water, and distilled water to volume. Strain 23B was used.

The experiments listed in Table 8 were conducted in 0.1 M phosphate buffer (pH 6.2) in the presence of a 2 per cent cell suspension. The experiment to which succinic acid was added contained 0.05 M glucose in 30 ml.; that to which type one acetate was added contained 0.05 M glucose in 60 ml., and that to which type two acetate was added contained 0.1 M glucose in 60 ml.

Succinic acid, containing C^{13} exclusively in the carboxyl group, was obtained as the silver salt from various bacterial fermentations, acidified and extracted with ether. The acids were neutralized to brom-thymol blue with a carbonate-free solution of NaOH before being added to the reaction mixtures.

It should be indicated that all naturally occurring carbon materials contain 1.09 per cent C^{13}, and for the purpose of this discussion, such values will be considered normal with respect to C^{13} content.

The addition of succinic acid containing 1.57 per cent C^{13} fermentations of glucose by Aerobacter resulted in the formation of acetic acid containing 1.22 per cent C^{13} (Table 8). This result

TABLE 8.

FERMENTATION OF GLUCOSE PLUS ORGANIC ACIDS BY CELL SUSPENSIONS OF AEROBACTER INDOLOGENES

		Acid added	CO_2	H_2	Formic acid	Acetic acid	Lactic acid	Succinic acid	Ethyl alcol
Control	mM	0	149.0	48.5	18.7	3.4	1.2	18.1	56.(
$CH_3 \cdot C^{13}OOH$	mM	68.6	186.0	50.1	4.0	35.3	3.5	11.0	51.(
	% C^{13}	2.39	1.08			2.01		1.21	1.(
$C^{13}H_3 \cdot C^{13}OOH$	mM	64.6	179.8	45.3	4.3	26.2	2.0	12.2	54.(
	% C^{13}	4.51	1.12		1.10	3.27	1.07	1.33	2.(
$COOH \cdot CH_2 \cdot CH_2 \cdot C^{13}OOH$	mM	130.0	133.0			5.3		126.0	6.(
	% C^{13}	1.57	1.09			1.22		1.37	1.(

Products expressed as mM per 100 mM of glucose fermented.

TABLE 8.

TATION OF GLUCOSE PLUS ORGANIC ACIDS BY CELL

SUSPENSIONS OF AEROBACTER INDOLOGENES

	CO_2	H_2	Formic acid	Acetic acid	Lactic acid	Succinic acid	Ethyl alcohol	2,3-Butylene glycol	Carbon recovered %	O/R index
	149.0	48.5	18.7	3.4	1.2	18.1	56.0	58.6	99.5	1.00
	186.0	50.1	4.0	35.3	3.5	11.0	51.4	70.1	94.1	1.10
2	1.08			2.01		1.21	1.64	1.21		
	179.8	45.3	4.3	26.2	2.0	12.2	54.3	69.6	92.7	1.04
1	1.12		1.10	3.27	1.07	1.33	2.19	1.24		
	133.0			5.3		126.0	6.6	44.6		
7	1.09			1.22		1.37	1.18	1.15		

as mM per 100 mM of glucose fermented.

proves a cleavage of the succinate molecule into acetic acid or into two molecules of a C_2 compound which are converted into acetic acid.

The formation of acetate from succinate by way of the C_4 dicarboxylic acids, i.e., by oxidation of the succinate to pyruvic acid, would result in the formation of acetate not containing C^{13} because the carboxyl groups of the succinate would be converted to $C^{13}O_2$ in the oxidation process. Also, a necessary reaction in this process, i.e., oxidation of succinate to fumarate, has never been shown to occur under anaerobic conditions. Thus, if acetic acid cannot be formed from succinic acid by a removal of C_1 compounds, the only possibility remaining is a process involving a cleavage of the succinate to two C_2 compounds.

If acetic acid were formed entirely by a cleavage of succinate, the acetic acid should contain approximately the same per cent C^{13} as the final succinic acid. The acetic acid contained 1.22 per cent C^{13} while the final succinate contained 1.37 per cent (Table 8). It is probable that acetic acid was formed in this experiment by an oxidation of pyruvate as well as a cleavage of succinate. The question arises as to what extent was acetate formed by the latter mechanism. By means of calculations it will be shown that the reaction is of quantitative significance. The calculations have been made on the assumption that the C^{13} content of the

final succinic acid represents the concentration of C^{13} in the succinic acid available to the cell for the cleavage reaction. It is admitted that this value involves an assumption. There is no assurance that the succinic acid formed in the cell comes completely to equilibrium with the succinate dissolved in the medium before being utilized. Undoubtedly, cell permeability is an important factor in this respect. However, the calculation may be made in the following manner: let X = per cent acetic acid formed from a substrate containing normal carbon, and 1 - X = per cent acetic acid formed from succinic acid containing C^{13} carbon. The equation is, per cent C^{13} in final acetic acid = 1.09 X + per cent C^{13} in final succinic acid x (1 - X). Using the values expressed in Table 8, 1.22 = 1.09 X + 1.37 (1 - X), X = 53 per cent and 1 - X = 47 per cent. This means that 53 per cent of the acetic acid was formed from a substrate containing normal carbon, and 47 per cent was formed from the succinate containing C^{13} carbon. In other words, if there were 100 molecules of acetic acid, 53 of which were formed from normal carbon, and 47 of which were formed from C^{13} carbon of succinate, a value of 1.22 per cent C^{13} would be obtained for the final acetic acid. Hence, under the experimental conditions, apparently one-half of the acetate was formed by a cleavage of the succinate.

A net loss of 4 mM of added succinate occurred during

the dissimilation (cf. Table 8). Reynolds et al. (1937) have reported a decrease of 15 mM of added succinate per 100 mM of glucose fermented by growing cells of Aerobacter. In the present experiment, however, succinate was formed from the glucose. This is evident because the concentration of C^{13} in the succinate decreased from 1.57 to 1.37 per cent. Slade et al. (1942) have shown the formation of succinate by Aerobacter by means of the Wood and Werkman reaction.

The addition of type 1 acetate, ($CH_3.C^{13}OOH$) resulted in its condensation to succinic acid. The added acetate contained 2.39 per cent C^{13} while the succinate formed contained 1.21 per cent C^{13} (cf. Table 8). If succinic acid was formed in this dissimilation by acetic acid condensation, the succinate should contain C^{13} exclusively in the carboxyl groups. Table 9 shows that the degradation of this succinic acid resulted in the location of the C^{13} exclusively in the carboxyl groups (1.25 per cent), while the methylene carbon atoms were negative (1.10 per cent). Hence this result demonstrates that the succinic acid was formed by means of a carbon to carbon linkage involving the methyl carbon atom of acetic acid. This reaction may be represented as follows:

$$\begin{matrix} CH_3.COOH \\ + \\ CH_3.COOH \end{matrix} \underset{+2H}{\overset{-2H}{\rightleftharpoons}} \begin{matrix} CH_2.COOH \\ \cdot \\ CH_2.COOH \end{matrix}$$

The above reaction represents C_2 condensation and is not intended to express a clear-cut picture of the reaction.

TABLE 9.

LOCATION OF THE HEAVY CARBON OF ASSIMILATED ACETIC ACID IN COMPOUNDS SYNTHESIZED BY AEROBACTER INDOLOGENES

Product isolated	$CH_3.C^{13}OOH$ added (type 1)	$C^{13}H_3.C^{13}OOH$ added (type 2)
Succinic acid	1.20	1.33
methylene carbon	1.10	1.32
carboxyl carbon	1.25 (1.31)	1.29
2,3-Butylene glycol	1.21	1.24
methyl carbon	1.10	1.23
hydroxyl carbon	1.27 (1.33)	1.22
Ethyl alcohol	1.64	2.19
methyl carbon	---	2.16
hydroxyl carbon	---	2.17

The values are given in per cent C^{13}. The values in parentheses were calculated by the following equation: $2X + (2 \times 1.09) = 4 \times$ per cent C^{13} in whole molecule, X = average per cent C^{13} in carboxyl carbon atoms of succinic acid, or hydroxyl carbon atoms of 2,3-butylene glycol.

From the results of these experiments it is not possible to determine positively the actual nature of the C_2 molecules involved in the condensation reaction. It is highly probable however, that acetic acid or a phosphorylated derivative is involved.

The succinic acid was not formed by a synthesis of pyruvic acid by way of C_1 and C_2 ($CH_3.C^{13}OOH$) addition, followed by C_3 and C_1 addition to oxalacetic acid, for this would have resulted in the formation of succinic acid containing C^{13} in the methylene carbon atoms. Actually, the succinate contained C^{13} exclusively in the carboxyl carbon atoms (cf. Table 9). Also, the cleavage of succinate to acetate supports the proposal of the formation of succinate by C_2 and C_2 addition rather than by C_2 and C_3 addition. There is no mechanism known for the oxidation of succinate by which single carbon atoms may be split off leaving a C_2 residue containing a carboxyl carbon atom of the original succinate. The most plausible explanation of the formation of C^{13} acetate from succinate is to assume cleavage of succinate to two C_2 fragments by a mechanism which is the reverse of acetic acid condensation.

Calculations to show the amount of succinate formed by acetic acid condensation may be made in a manner similar to that shown previously with added succinate. Let X = per cent succinic acid formed from a substrate containing normal carbon,

and 1 - X = per cent succinic acid formed from acetic acid containing C^{13} carbon. The equation is, per cent C^{13} in final succinic acid = 1.09 X + per cent C^{13} in final acetic acid x (1 - X). Using the values expressed in Table 8, 1.21 = 1.09 X + 2.01 (1 - X). This means that 13 per cent of the succinic acid was formed by C_2 condensation and 87 per cent by other mechanisms. Thus this approximate calculation shows that C_2 condensation is a reaction which possesses quantitative significance.

On addition of type 2 acetate, succinic acid was formed containing C^{13} (Table 8). The condensation of two molecules of acetic acid containing C^{13} in both methyl and carboxyl groups, would result in the formation of succinate containing C^{13} in both methyl and carboxyl groups. Table 9 shows that the succinate (1.33 per cent C^{13}) formed in the presence of type 2 acetate contained C^{13} equally distributed between the methylene carbon atoms (1.32 per cent C^{13}) and the carboxyl carbon atoms (1.29 per cent C^{13}). The fact that the methylene carbon atoms of succinate contained C^{13} is conclusive proof that the succinate was not formed by the CO_2 fixation reaction, i.e., the acetic acid was not oxidized to CO_2 and the resulting heavy carbon fixed in the C_4 dicarboxylic acids. If this reaction had occurred the C^{13} would be exclusively in the carboxyl carbon atoms. It is thus definitely established that under anaerobiosis there

exists at least two mechanisms of succinic acid formation, one involving fixation of CO_2 (Wood and Werkman reaction), and the other some form of condensation of acetic acid or its derivative.

In this experiment in which type 2 acetate was added 11 per cent of the succinate was formed by C_2 condensation. This value agrees with the amount of succinate formed on the addition of type one acetate (13 per cent). The calculation of the equilibrium constant for the condensation reaction must await the isolation of the cell-free enzyme system and the consequent elimination of the various "side" reactions which existed in the present experiments.

A net decrease in added acetate occurred in both experiments; 35.3 mM in the case of type 1 acetate, and 38.4 mM in the case of type 2 acetate. However, there has been a simultaneous production of acetic acid from the glucose. This is evident from the C^{13} content of the initial and final acetic acids. The per cent C^{13} of type 1 acetate was diluted from 2.39 to 2.01, and type 2 acetate was diluted from 4.51 to 3.27 per cent C^{13}. Mickelson and Werkman (1939) have shown a loss of 36 mM of acetic acid added to a fermentation of glucose by growing cells of Aerobacter.

In a preceding section it was shown that Clostridium welchii formed acetic acid which contained fixed $C^{13}O_2$ in the carboxyl group, although no succinate was formed. In

view of the formation of acetate from succinate by Aerobacter, it would be important to determine whether Cl. welchii is able to perform the same reaction, if not, an additional mechanism of formation of acetate in that fermentation would have to be assumed.

The oxidative condensation of acetic acid to succinic acid requires the participation of a hydrogen acceptor. The identity of such a molecule in these experiments is not known. However, the increased yield of 2,3-butylene glycol on the addition of acetate, indicates that the reduction and condensation of the latter to the glycol, may serve as hydrogen acceptor for the condensation of acetic acid to succinate. Also, it has been found (unpublished data) that the addition of acetate to fermentations of oxalacetate by Citrobacter results in an increase in the yield of succinate. The condensation of acetate may take place under anaerobic conditions with the participation of oxalacetate as a hydrogen acceptor.

Krebs and Eggleston (1941) in experiments with Propionibacterium shermanii reject the possibility that succinate can be formed by a condensation of acetate, but they were able to demonstrate an increase in succinate on the addition of acetate to fermentations of oxalacetate. They believe that acetate may be oxidized to CO_2 with the reduction of oxalacetate to succinate, although no evidence exists for

the anaerobic oxidation of acetate to CO_2, and no possible mechanisms were suggested. Such an oxidation in the present experiments would have resulted in the formation of $C^{13}O_2$, and also an unequal distribution of the C^{13} between the methylene and carboxyl carbon atoms of the succinate formed on the addition of type 2 acetate.

Wood, Stone and Werkman (1937) have shown the utilization of acetic and succinic acids during fermentations of glucose by Propionibacterium. The suggestion was made that succinic acid was formed by acetic acid condensation and the former compound decarboxylated to propionic acid and CO_2. Wood and Werkman (1940b) in experiments with Propionibacterium on glucose and glycerol, suggested that, in addition to the succinate formed by C_3 and C_1 condensation, a mechanism was also present which involved acetic or pyruvic acid condensation.

In a preceding section it was shown that $C^{13}O_2$ was fixed in the carboxyl carbon atoms of lactic acid formed by Aerobacter. The possibility was presented that the lactate arose by a reversible conversion of the C_4 dicarboxylic acids, to pyruvate and CO_2, i.e., fumarate $\underset{-H_2O}{\overset{+H_2O}{\rightleftharpoons}}$ malate $\underset{+2H}{\overset{-2H}{\rightleftharpoons}}$ oxalacetate $\rightleftharpoons$ pyruvate + CO_2. In the present experiments, the lactate containing C^{13} would necessarily have to originate from succinate instead of fumarate. As previously indicated, the anaerobic oxidation of succinate to fumarate

may not occur. Thus the formation of lactic acid containing C^{13} would be a definite indication of the existence of such a reaction, and also the formation of lactate by way of the C_4 dicarboxylic acids. The lactate formed on the addition of type 2 acetate did not contain C^{13} (Table 8). This negative result cannot be considered conclusive because of the low yield of lactate, and also because the pyruvate formed from glucose has probably diluted the pyruvate formed from the C_4 dicarboxylic acids. This problem could possibly be settled by the addition of fumarate and malate containing C^{13} to fermentations of glucose by Aerobacter or Escherichia. The anaerobic oxidation of succinate to fumarate (if it occurs) would thus be avoided and lactic acid containing C^{13} might result.

Nishina, Endo, and Nakayama (1941), with the aid of $C^{11}O_2$, have shown a conversion of ethyl alcohol to radioactive malic acid by E. coli. Such a conversion may involve C_2 condensation to succinate. By oxidation of the succinate to oxalacetate, $C^{11}O_2$ may enter the latter molecule by exchange, and the oxalacetate containing $C^{11}O_2$ then be reduced to malate.

The aerobic oxidation of pyruvic acid has long been a problem to cell physiologists. In animal tissues such as brain and livor, and in bacteria such as the lactic acid bacteria and gonococci, acetic acid has been found as an

oxidation product of pyruvic acid. This is especially true when injured cells or cell-free enzyme systems were employed. Such results indicate that acetic acid or a similar C_2 compound is an intermediate in the oxidation process. The main problem thus resolves itself into an oxidation of acetic acid to carbon dioxide and water. Oppenheimer and Stern (1939) state, "The decisive key reaction consisting in the dehydrogenation of acetic acid to succinic acid has long been a theoretical postulate." This postulate involves the condensation of two molecules of acetic acid to succinic acid, followed by oxidation of the latter acid to pyruvic acid and CO_2. Thus one molecule of pyruvate has been completely oxidized and the other has been recovered.

Although the present results do not demonstrate necessarily a direct condensation of acetic acid to succinate, proof has been obtained of the condensation of C_2 compounds originating from acetic acid to a C_4 compound which is isolated as succinic acid. Inasmuch as the initial compound is acetic acid and the end product is succinic acid, the reaction in question, probably involves acetic acid condensation. Thus these results present the most direct evidence obtained so far for this reaction, with the implication that the reaction may function as a part of an oxidation cycle in bacteria and animal tissues.

Conversion of acetic acid to 2,3-butylene glycol.

Reynolds, Jacobsson and Werkman (1937) have shown that the addition of acetic acid to fermentations of glucose by Aerobacter results in an almost quantitative increase in 2,3-butylene glycol. The suggestion was made that the added acetic acid was reduced and condensed to the glycol. The results to be presented support their suggestion in that the glycol does arise in part from acetic acid. It is doubtful whether the greater portion of the glycol arises from acetic acid. In other words, the conversion of acetate to 2,3-butylene glycol probably is not an essential reaction in the formation of the glycol.

For many years acetaldehyde has been considered an intermediate in the formation of acetylmethylcarbinol by yeast. Neuberg and Kobel (1925) suggested that one molecule of synthetic acetaldehyde condenses with one molecule of nascent acetaldehyde formed by yeast preparations from glucose or pyruvic acid to form the carbinol. Dirscherl (1931) suggested the possibility of acetaldehyde coupling with pyruvic acid prior to decarboxylation to the carbinol.

On the other hand, little work has been performed on the formation of acetylmethylcarbinol and 2,3-butylene glycol by bacteria. In view of the results of Reynolds et al. (1937), Mickelson and Werkman (1939) attempted to obtain evidence of

the conversion of fatty acids and aldehydes to the corresponding glycols during the fermentation of glucose by Aerobacter. On the addition of acetaldehyde, increases in acetylmethylcarbinol, ethyl alcohol and acetic acid were obtained. However, the organism was not able to synthesize the homologous glycol or carbinol on addition of propionaldehyde and butyraldehyde. The latter compounds were reduced to the corresponding alcohols with a decrease in the yield of ethyl alcohol. Added propionic acid was reduced to the alcohol and caused an increase in the yield of 2,3-butylene glycol. It was suggested that added acetic acid was assimilated in a similar manner, without itself being transformed to the glycol. It was also suggested that perhaps synthetic acetaldehyde was condensed with some intermediate other than acetaldehyde during the formation of the carbinol.

Hammer (1930) found increases in the yield of acetylmethylcarbinol on the addition of homologues of acetaldehyde to cultures of Streptococcus liquefaciens. Homologues of acetylmethylcarbinol were not formed.

Silverman and Werkman (1941) were not able to show the participation of acetaldehyde in the formation of acetylmethylcarbinol by a cell-free enzyme preparation of Aerobacter. Recently, Green et al. (1942) demonstrated large increases in the carbinol upon the addition of acetaldehyde to pyruvic acid in the presence of cell suspensions of

Aerobacter. The proposal was made that a condensation product of pyruvic acid and acetaldehyde was involved in the formation of the carbinol.

Thus it can be stated that little definite evidence exists for the occurrence of acetaldehyde as an intermediate in the formation of acetylmethylcarbinol and 2,3-butylene glcyol by bacteria. The results of the present investigation prove that a C_2 compound, probably acetaldehyde or a closely related derivative formed by a reduction of acetic acid, is involved in the synthesis of 2,3-butylene glycol. It is also shown that the carbon to carbon linkage created in the condensation reaction, involves the carboxyl carbon atom of acetic acid. These results present the most direct evidence for the occurrence of acetaldehyde as an intermediate in the formation of 2,3-butylene glycol by intact cells of Aerobacter.

The addition of type 1 acetate (2.39 per cent C^{13}) to fermentations of glucose by Aerobacter results in the formation of 2,3-butylene glycol containing 1.21 per cent C^{13} (Table 8). According to a condensation reaction involving two molecules of acetaldehyde or a molecule of acetaldehyde and a molecule of pyruvic acid, the glycol formed in the above experiment should contain C^{13} exclusively in the hydroxyl carbon atoms.

To determine the position of the C^{13}, the degradation of the glycol was carried out by a preliminary oxidation to acetaldehyde by periodic acid as described in the procedure for its determination. The aldehyde was collected in ice-cold water, and the iodoform reaction performed as in the procedure for the degradation of acetone. The reaction is as follows:

$$CH_3.CHO + 3I_2 + 4NaOH \longrightarrow CHI_3 + HCOONa + 3NaI + 3H_2O$$

Iodoform originates from the methyl carbon atoms and formic acid from the hydroxyl carbon atoms of the glycol. The residue remaining after filtration of the iodoform, was acidified and the formic acid recovered by distillation. However, it was found that hyposulfurous acid which originates in the titration of iodine by thiosulfate, decomposed during the distillation, probably according to the following reaction:

$$H_2S_4O_6 \longrightarrow H_2SO_4 + SO_2 + 2S$$

Thus, the distillate was filtered to remove sulfur and refluxed four hours to remove sulfur dioxide, made alkaline, evaporated to a small volume, acidified and redistilled. The formic acid was oxidized with mercuric oxide according to Osburn, Wood and Werkman (1933).

The hydroxyl carbon atoms contained 1.27 per cent C^{13} (Table 9), whereas the methyl carbon atoms were normal. This result proves that acetic acid took part in a condensation

reaction, with the creation of a carbon to carbon linkage, at least one of the carbon atoms was originally the carboxyl carbon atom of acetic acid. Calculations indicate that approximately 13 per cent of the glycol was formed from acetic acid containing C^{13}.

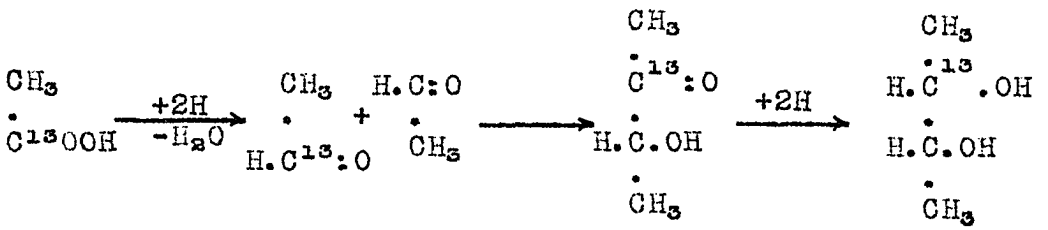
On the addition of type 2 acetate, 2,3-butylene glycol was formed which contained 1.24 per cent C^{13}. Likewise, according to a condensation reaction, this glycol should contain C^{13} equally distributed between the methyl and hydroxyl carbon atoms. Table 9 shows that the methyl carbon atoms contained 1.23 per cent and the hydroxyl carbon atoms 1.22 per cent C^{13}. Calculations indicate that approximately 7 per cent of the glycol was formed from acetic acid containing C^{13}.

The addition of succinic acid also resulted in the formation of 2,3-butylene glycol containing C^{13}. This experiment is comparable to those in which acetic acid was added, because of the cleavage of succinic acid to acetic acid. Calculations indicate that approximately 47 per cent of the glycol was formed from acetic acid containing C^{13}, although this value is not reliable because of the small amount of C^{13} present in the glycol.

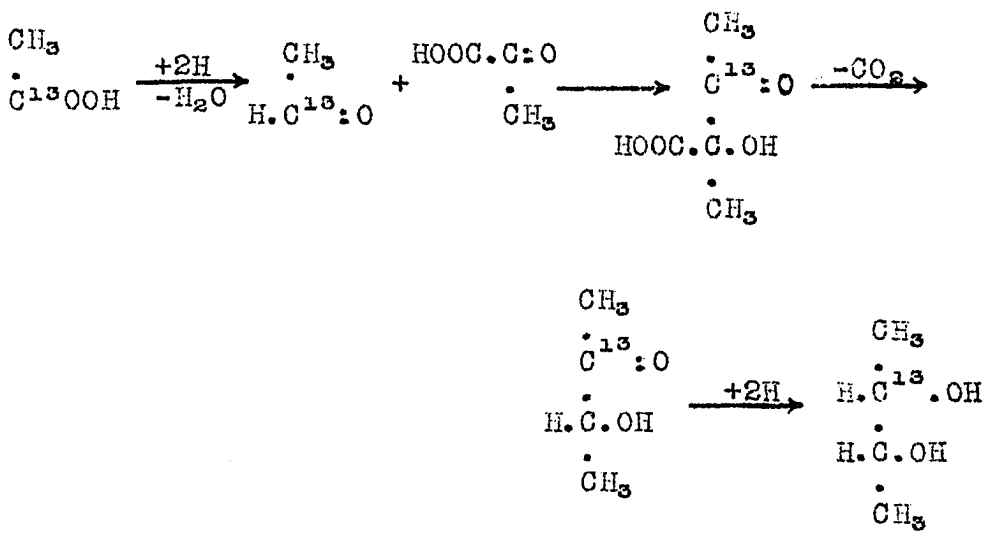
In regard to the mechanism of formation of the glycol in these experiments, it appears that either (1) two molecules of acetaldehyde, or (2) one molecule of acetaldehyde

and molecule of pyruvic acid are involved. However, it is not possible to determine which mechanism occurred on the basis of the position of the C^{13} in the glycol. The following reactions will illustrate:

(1)



(2)



In view of the results of Green et al. (1942), it is likely that in the present experiments, the 2,3-butylene glycol was formed by a mechanism similar to that represented by equation (2) above. The C_5 compound in the equation represents a possible structure of the intermediate compound formed in

the condensation reaction. The acetaldehyde necessary for this condensation was probably formed by the reduction of acetic acid. In the next section it will be shown that added acetic acid is reduced to ethyl alcohol by cell suspensions of Aerobacter, and the conclusion is unavoidable that acetaldehyde is an intermediate in that reduction.

The recent results obtained in this laboratory by Gross, Wood and Werkman are of interest in respect to the intermediate mechanism of glycol formation. On the addition of $C^{13}H_3.C^{13}HO$ to pyruvic acid and the Aerobacter preparation of Silverman and Werkman (1941), it was found that little of the added aldehyde was utilized in the synthesis of acetylmethylcarbinol. This result is in agreement with the results of the latter workers. Thus the conclusion is necessary that two mechanisms exist for the formation of acetylmethylcarbinol by the preparations of Silverman and Werkman and Green et al. In the former case, the enzyme preparation did not possess the ability to activate the added acetaldehyde or to convert it to the necessary intermediate form, while in the latter case the intact cell was able to perform this function. Thus, it is evident that it is necessary to use caution in correlating results obtained with intact cells with those obtained with cell-free preparations. It can be safely stated that a reaction which occurs in a cell-free preparation will

probably occur in the intact cell, but the reverse will not always be true because the enzyme may have been destroyed in the extraction procedure.

It can be briefly stated that the present results prove that a C_2 compound, probably acetaldehyde or a closely related derivative formed by a reduction of acetic acid, is involved in the synthesis of 2,3-butylene glycol. It is also shown that the carbon to carbon linkage created in the condensation reaction, involves the carbon atom of acetic acid originally present in the carboxyl group. These results strongly indicate that acetaldehyde is involved in the synthesis of acetylmethylcarbinol and 2,3-butylene glycol by the intact cell of Aerobacter.

Conversion of acetic acid to ethyl alcohol.

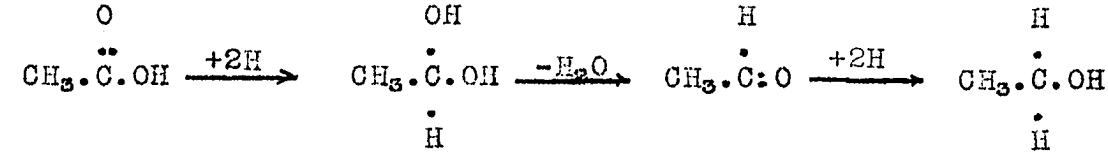
The reduction of aliphatic acids to the corresponding alcohols has been postulated for several genera of heterotrophic bacteria (Osburn, Brown and Werkman, 1938; Mickelson and Werkman, 1939). The latter authors added propionic acid to fermentations of glucose by Aerobacter and found a formation of propyl alcohol. The alcohol was very likely formed by a reduction of the corresponding acid. A similar reaction was suggested in the case of acetic acid. Wood et al. (1941a) have presented evidence which indicates that Propionibacterium pentosaceum is able to reduce pro-

pionic acid to the alcohol. In a fermentation of glycerol to which $C^{13}O_2$ was added, both the propionic acid and the propyl alcohol formed contained fixed C^{13}. Inasmuch as propionic acid has been shown by the latter authors to arise by a decarboxylation of succinic acid, it is reasonable to suppose that the propyl alcohol was formed by a reduction of the propionic acid.

Ethyl alcohol containing C^{13} was formed on the addition of acetic acid and succinic acid to fermentations of glucose by Aerobacter (Table 8). A reduction of added type 2 acetate to ethyl alcohol should result in an equal distribution of the C^{13} between the methyl and hydroxyl carbon atoms. To determine the position of the C^{13} in the alcohol, the latter was recovered from the fermentation, and oxidized with potassium dichromate to acetic acid as in the method for the determination of ethyl alcohol. The resulting acetic acid was degraded by dry distillation of the barium salt as previously described. The alcohol formed on the addition of type 2 acetate contained 2.19 per cent C^{13}, and after degradation, the methyl carbon atoms were found to contain 2.16 per cent C^{13} and the hydroxyl carbon atoms 2.17 per cent C^{13} (Table 9).

These results prove a reduction of acetic acid to ethyl alcohol by Aerobacter. The alcohol could not have arisen by oxidation of succinic acid because it contains a greater

percentage of C^{13} than the percentage of C^{13} in the final succinic acid. The reduction may be pictured as follows:



Calculations made on the basis of the C^{13} content of the final acetic acid, indicate that on the addition of type 1 acetate, 60 per cent of the alcohol was formed from acetic acid containing C^{13}; on the addition of type 2 acetate, 51 per cent of the alcohol was formed from acetic acid containing C^{13}; and on the addition of succinic acid, 69 per cent of the alcohol was formed from acetic acid containing C^{13}. Thus the reduction of acetic to ethyl alcohol is a reaction possessing quantitative significance, i.e., under the existing experimental conditions.

Summary and conclusions.

Acetic and succinic acids containing C^{13} were added to fermentations of glucose by cell suspensions of Aerobacter indologenes (23B). The addition of succinic acid ($COOH.CH_2.CH_2.C^{13}OOH$) results in the formation of acetic acid, ethyl alcohol, and 2,3-butylene glycol, each containing C^{13}. Acetic acid is formed by a cleavage of succinic acid to two molecules of a C_2 compound which are isolated as acetic acid.

The addition of type 1 acetic acid ($CH_3.C^{13}OOH$), results in the formation of succinic acid, 2,3-butylene glycol, and ethyl alcohol, each containing C^{13}. Succinic acid, isolated from the fermentation, contains C^{13} exclusively in the carboxyl carbon atoms. Hence, the succinic acid is formed by means of a carbon to carbon linkage involving the methyl carbon atom of acetic acid. The general reaction may be represented as follows:

$$\begin{matrix} CH_3.COOH \\ + \\ CH_3.COOH \end{matrix} \underset{+2H}{\overset{-2H}{\rightleftharpoons}} \begin{matrix} CH_2.COOH \\ . \\ CH_2.COOH \end{matrix}$$

The reaction is presented with the reservation that acetic acid as such may or may not be the actual compound involved in the condensation reaction.

The addition of type 2 acetic acid ($C^{13}H_3.C^{13}OOH$), also results in the formation of succinic acid, 2,3-butylene glycol, and ethyl alcohol, each containing C^{13}. Succinic acid, isolated from the fermentation, contains C^{13} equally distributed in the methyl and carboxyl carbon atoms. Thus in this fermentation, the succinic acid was not formed by means of the CO_2 fixation reaction, i.e., the acetic acid was not oxidized to $C^{13}O_2$ and the resulting heavy carbon fixed in the C_4 dicarboxylic acids, otherwise the C^{13} would have been found exclusively in the carboxyl carbon atoms.

Although these results do not demonstrate a direct condensation of acetic acid to succinate, proof has been

obtained of the condensation of C_2 compounds originating from acetic acid to a C_4 compound which is isolated as succinic acid. Inasmuch as the initial product is acetic acid and the end-product is succinic acid, the reaction probably involves acetic acid condensation. Thus these results present the most direct evidence obtained so far for this reaction, and the implication is made that the reaction may function as apart of an aerobic oxidation cycle in bacteria and animal tissues.

The addition of type 1 acetate results in the formation of 2,3-butylene glycol containing C^{13} exclusively in the hydroxyl carbon atoms. This result proves that a carbon to carbon linkage was created in the synthesis of the glycol which involves the carbon atom of acetic acid originally present in the carboxyl group. This indicates the participation of acetaldehyde in the condensation reaction.

The addition of type 2 acetate results in the formation of 2,3-butylene glycol containing C^{13} equally distributed between the methyl and hydroxyl carbon atoms. Also the addition of succinic acid results in the formation of the glycol. The latter result is comparable to those in which acetic acid was added, because of the cleavage of succinic acid to acetic acid.

In regard to the mechanism of the formation of 2,3-butylene glycol, it appears that either (1) two molecules

of acetaldehyde, or (2) one molecule of acetaldehyde and one molecule of pyruvic acid are involved. The latter possibility appears more probable.

The results prove that a C_2 compound probably formed by a reduction of acetic acid, is involved in the synthesis of the glycol by the intact cell of Aerobacter.

Proof is presented of the reduction of acetic acid to ethyl alcohol. It is highly probably that acetaldehyde is an intermediate in this reduction.

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