The function of carbon dioxide in the metabolism of heterotrophic cells

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THE FUNCTION OF CARBON DIOXIDE IN THE
METABOLISM OF HETEROTROPHIC CELLS

by
Samuel J. Ajl

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
The Requirements for the Degree of
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Signature was redacted for privacy.

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INTRODUCTION

It has been known for a number of years that in the complete absence of carbon dioxide, growth and metabolism of diverse living systems are seriously impaired. Under experimental conditions which would insure (1) the initial presence of only minute quantities of CO₂ and (2) the immediate removal of additional amounts formed during metabolism, the germination of spores and growth of various microorganisms appeared to be greatly retarded, if not altogether prevented. But until now no explanation has been given for these curious phenomena.

In 1935 Wood and Werkman presented the first experimental evidence that heterotrophic bacteria assimilate carbon dioxide. It may be postulated that the function of this gas in cellular metabolism, therefore, is the assimilation of CO₂ or its conversion into several essential metabolites. The question may be raised whether the inability to multiply in the absence of carbon dioxide results from the inability of these essential metabolites to be synthesized. If this hypothesis is correct, presence of the compound should relieve the requirement of CO₂. The purpose of this work was, in part, to determine the validity of the above hypothesis.

Results have been obtained which show that the compounds replacing CO₂ must arise from one or two fixation reactions involving a C₅ and C₁ addition or a C₄ and C₁ addition in which the C₁ compound is carbon dioxide. The C₄ or the C₅ compounds thus formed are essential for the growth of heterotrophic bacteria. In the absence of CO₂, the C₄ or C₅
compound must be supplied to the cells before growth occurs. Finally, investigations will be described showing the mechanism of the C₄ and C₁ addition, as well as the mechanism by which the various compounds replace carbon dioxide.

It is hoped that the experiments described will help to elucidate the function of CO₂ not only in the metabolism of heterotrophic cells but autotrophic cells as well.
HISTORICAL

Beginning with the work of Pasteur and Joubert in 1877, experimental evidence of the influence of carbon dioxide on bacterial cells has been steadily accumulating. Since that time considerable progress has been made in elucidating the effect of this gas on bacteria, but until now no evidence has been presented which explains the necessity of CO₂ for the growth and metabolism of heterotrophic cells.

Carbon Dioxide as a Growth Depressant or Inhibitor

The germicidal effect of high concentrations of carbon dioxide has been studied practically since pure culture methods were developed in bacteriology.

Pasteur and Joubert (1877) observed that *Bacillus anthracis* was killed in the presence of carbon dioxide. No mention is made of the media that were used, nor is it stated how long the exposure to the gas continued or how much CO₂ was required to kill the organism. For these reasons Szpilman (1880) believed that the above claim should be modified since he showed that from 5 to 8 hours' exposure to an almost pure atmosphere of carbon dioxide did not kill the same organism or alter its pathogenicity. Grossmann and Mayerhausen (1877) noted that CO₂ inhibited motility and that small amounts of the gas resulted in increased motility.

Among the earlier workers, Buchner (1885) demonstrated that, when a stream of carbon dioxide is passed through a culture of the cholera...
vibrio, growth of the organism is prevented.

In the following years Schaeffer and Freudenreich (1891) reported that typhoid and anthrax bacilli were unaffected in broth cultures under seven atmospheres of carbon dioxide pressure. Kolbe's (1882) work was an attempt to preserve meat and fruit in an atmosphere of carbon dioxide. He found that beef could be preserved for 18 days, whereas mutton showed spoilage in a very short time.

Liborius (1886) failed to initiate growth of Clostridium foetidum, Clostridium oedematis-venenosum, Clostridium polypiformis (Bacillus polypiformis) and Bacillus pseudo-oedematis when carbon dioxide was passed through the culture medium. However, when the gas was passed over the surface of the medium, some growth did take place. Some erratic experiments were reported by Bolton (1886) with Micrococcus aquatilis and Bacillus erythropus. In some cases inhibition took place, whereas in others it did not. The duration of CO₂ treatment is not given.

The work of Hochstetter (1887) on the behavior of microorganisms in artificial carbonated waters, city water of Berlin and distilled water remains as a source of valuable information and has not been surpassed in extent and comprehensiveness. The experiments included pure culture studies of a number of bacterial species and bacterial and mold spores. The organisms studied fell into three general groups, according to their tolerance for carbon dioxide; namely, (1) those viable for only a short time, (2) those viable for a few weeks, and (3) those which survived for several weeks and even months.
Fränkel (1889) showed that the typhoid bacillus and pneumo-
 bacillus will grow in a medium in contact with an atmosphere of CO₂.
Other organisms were partially inhibited and some saprophytic and
pathogenic forms, including the cholera vibrio, the anthrax bacillus
and Staphylococcus aureus (Micrococcus pyogenes var. aureus) failed to
grow. Some restriction of growth was shown by certain anaerobes.
Fränkel's work is similar to Hochstetter's in comprehensiveness
and, although conducted in a somewhat different manner, the conclusions
drawn are essentially the same.

It should be pointed out that Frankland (1863) also found the
cholera vibrio to be killed within 8 days by an exposure to CO₂.

Chapin (1902) studied the effect of various concentrations of CO₂
upon molds. In an atmosphere of 60 per cent CO₂ spore germination of
Mucor failed; Aspergillus and Penicillium required 100 per cent of CO₂.
Growth of the molds was inhibited at lower CO₂ concentrations, but they
were not killed.

Hoffmann (1906) found that under 50 atmospheres of CO₂, growth of
water bacteria was inhibited. Filtered aqueous suspensions of typhoid,
cholera and dysentery bacilli exposed under these conditions for 3 hours
were destroyed. Berghaus (1907) concluded that some bacteria (as Vibrio)
are killed in 24 hours by 1 atmosphere of pressure of CO₂.

An interesting observation was made by Rockwell and McKann (1921).
These investigators were able gradually to adapt the gonococcus to
grow in contact with relatively high concentrations of CO₂, even to
100 per cent.
Bacteria Requiring Carbon Dioxide as a Nutrient-Autotrophs

During the years 1890-1899 a notable observation was made and proved; namely that, although carbon dioxide exerts injurious effects on certain bacteria, it is actually required by others. Carbon dioxide is utilized as a source of carbon in the synthesis of organic compounds by certain groups of (oligocarbophilous) microorganisms; e.g., those which are either photosynthetic or chemosynthetic.

The first evidence of the existence of oligocarbophilous organisms was furnished by Hueppe (1888) and Hersewus (1886) who concluded that the organisms responsible for nitrification were able to secure all nutrients needed from ammonium carbonate.

Vinogradsky (1890) and Vinogradsky and Omeliansky (1899) in a number of publications showed that the nitrifying organism requires carbon dioxide, utilizing it as a source of carbon.

Meyerhof (1916) expanded our knowledge of the utilization of CO₂ by the nitrifying bacteria. He concluded that carbon dioxide and not bicarbonate is utilized as a carbon source; the latter serves the purpose of a buffer and maintains an alkaline reaction of the medium.

Until 1924 it was assumed that only the autotrophic bacteria consumed carbon dioxide as a metabolite. Up to this date it was found that the hydrogen oxidizing bacteria of the genus Hydrogenomonas (Lebedew, 1909) and certain species of the sulfur-oxidizing groups require the gas. Nathansohn (1902) found those which oxidize hydrogen sulphide or thiosulphate to be chemosynthetic. Knowledge of these forms was markedly extended by Beijerinck (1903). Thiothecillus thiooxidans
capable of oxidizing free sulfur to \( \text{H}_2\text{SO}_4 \) was isolated by Wakeman and Joffe (1922). It grows well at a low pH at which carbonates cannot exist. Inasmuch as this organism utilizes inorganic carbon, it must be in the form of \( \text{CO}_2 \) in solution. Wakeman and Starkey (1922) concluded that this organism derives its \( \text{CO}_2 \) from the atmosphere, but is incapable of taking its carbon from carbonates or organic matter.

Photosynthetic autotrophs may in some cases utilize other carbon compounds. Roach (1926), for example, found that the unicellular alga, *Scedesmus costulatus*, would grow well in the dark in a medium with 1 per cent glucose and retain its green color.

**Utilization of Carbon Dioxide in Heterotrophic Metabolism**

It has been pointed out that \( \text{CO}_2 \) plays an important role in the nutrition of many bacteria. Although it has been known for a long time that autotrophic bacteria require carbon dioxide, the necessity for this gas in the culture medium of heterotrophic bacteria (*Brucella abortus*) became apparent only in 1924, and since that time it has been shown repeatedly that, in the absence of \( \text{CO}_2 \), growth and metabolism of living systems are seriously impaired.

The experiments that led to the conclusion that \( \text{CO}_2 \) is necessary for optimal growth and development of heterotrophic cells started with the work of Nowak (1908). At that time it was claimed by this investigator that *Br. abortus* could be isolated from the host more readily when the concentration of oxygen in the atmosphere was reduced.

Wherry and Oliver (1916) grew the gonococcus and several other
organisms under reduced oxygen tension. It was emphasized that some parasitic species could be isolated or made to grow considerably better under partial tension than under full anaerobic or aerobic conditions. No mention was made of the influence of carbon dioxide. Five bacterial species were used including Bacterium typhosum (Salmonella typhosa). Similar claims for the gonococcus were made by Reudiger (1919) and Swartz and Davis (1920).

Cohen and Markle (1916) reported a successful primary isolation of the meningococcus under partial oxygen tension, but no mention was made of any possible influence of carbon dioxide as such.

Both the gonococcus and the meningococcus were regarded as being microaerophilic on first isolation, but this sensitivity to oxygen was thought to be rapidly lost so that, after a few subcultures, they grew normally in air. We shall see later that this was not a correct assumption.

Wherry and Ervin (1918) furnished some experimental data which indicated that carbon dioxide is necessary for the growth of Mycobacterium tuberculosis. Saprophytic and non-saprophytic strains were employed in the experiments and were found to be alike in their inability to grow in the absence of carbon dioxide. They suggest that the lag period of growth may be the latent period during which CO₂ is being accumulated to the point of stimulation. Since they used solid media, their results were open to criticism (Novy and Soule, 1925) in that absence of growth might be due to excessive drying of the medium by the strong soda.
During the period from 1918 to 1924 a series of articles appeared dealing with the importance of CO₂ for recently isolated organisms, particularly the gonococcus and meningococcus.

Cohen and Fleming (1918) made use of the tandem cultivation method in their meningococcus studies; they also supplied CO₂ gas in amounts varying from 10 to 75 per cent. Their results showed that good growth could be obtained by supplying the gaseous CO₂ or with a culture of *Bacillus subtilis*. The optimum concentration varied from 10 to 30 per cent carbon dioxide. With high concentrations of CO₂ the growth was scanty. The oxygen thereby helping the growth, e.g., the partial pressure phase was strongly emphasized.

Reudiger (1919) confirmed the partial tension idea by obtaining good growth of the meningococcus in stoppered tubes. There was no growth when the tubes were left open. It may be assumed that the CO₂ liberated during respiration of the organism diluted the oxygen.

Chapin (1918), following Wherry's technique in primary isolation of the gonococcus, applied CO₂ directly. He obtained good growth over a wide variety of concentrations. This author suggested that the growth of *B. subtilis* not only diminished the oxygen concentration but supplied CO₂ as well.

Gates (1919) severely criticized the work of Cohen. Gates claimed that ascribing microaerophilic properties to the meningococcus was an error, since the organism would grow successfully in atmospheres of 24, 32, and 40 per cent oxygen. Reducing the amount of oxygen to 15 per cent showed no inhibitive or beneficial influence on control
plates kept under ordinary atmospheric conditions. Growth was equally
good at any point within 10 and 30 per cent carbon dioxide, as long as
the reaction of the medium remained favorable. The author concluded
that if there was any effect from carbon dioxide it was due to the
changing of the reaction of the medium and making it favorable for the
meningococcus.

St. John (1919) denied that there was any influence exerted by
CO₂ on the meningococcus and attributed the beneficial results of the
gas entirely to the increased moisture content in the closed system.
Kohman (1919) supported the view that carbon dioxide is beneficial to
the meningococcus by carefully adjusting the reaction to the optimum
pH range of the meningococcus. By this adjustment a buffer system was
created and further pH deviations due to CO₂ prevented.

The interest in primary cultivation of the gonococcus and other
organisms of this type under increased carbon dioxide tension, or
reduced oxygen tension, has not in the least abated. Herrold (1920)
described a modification of the partial tension method whereby good
results were obtained. This consisted of inverting the plates
inoculated with gonorrheal material over a plate inoculated with
B. subtilis, and joining the two plates with a rubber band. Bicarbonate
was also used instead of B. subtilis in the lower plate. By the addi-
tion of 1 per cent sulfuric acid carbon dioxide was liberated and the
conditions necessary for growth provided.

Hiddleson (1921) concluded that CO₂ stimulated the growth of
Bacterium abortus (Br. abortus) and furnished a satisfactory explanation
of the use of *B. subtilis* in the isolation technique. His observations were in accord with those of Cohen and Fleming (1918) and of Reudiger (1919), with the meningococcus and of Chapin (1918) with the gonococcus. He apparently recognized the importance of carbon dioxide as such in the cultivation of *Bact. abortus*, rather than the necessity of reduced oxygen.

Rockwell and his associates in a series of papers (Rockwell, 1921, 1923, 1924; Rockwell and Highberger, 1926, 1927) not only confirmed Wherry and Irvin's results, but went on to demonstrate the need for carbon dioxide in the growth of many laboratory strains, including anaerobes. By showing that growth occurred normally in cultures incubated over-concentrated sulfuric acid, they argued that growth could not have been prevented by desiccation of the medium.

Theobald Smith (1924, 1926) verified the findings that *Bact. abortus* requires the presence of CO₂ and could grow in contact with air having a concentration of CO₂ as low as 1 per cent. He found that the fewer the bacteria transferred to fresh media in hermetically sealed tubes, the less likely the bacteria were to multiply. He concluded that transferring a large number of living bacteria favors the accumulation of CO₂ to the threshold value. When only a few bacteria were transferred, this limit is not reached, and the bacteria do not multiply.

The work of Rockwell and Theobald Smith was confirmed and extended by Valley and Rettger (1927), who used agar shake-plate cultures incubated over calcium hydroxide in a current of CO₂-free air. They described complete inhibition of growth of all organisms tested,
numbering over 100. The organisms varied in the ease with which inhibition could be produced. Subsequent incubation in the air containing CO₂ gave in most cases little or no growth. Valley and Rettger came to the conclusion that CO₂ is necessary for the growth and development of both aerobic and anaerobic bacteria. In a later paper (Valley, 1928) appeared a comprehensive review of the earlier literature.

The work to this point had been carried out on solid agar media. Walker (1932) used a liquid medium of known composition containing lactose, ammonium tartrate and ammonium phosphate for the growth of *Escherichia coli*. When incubated in a current of CO₂-free air, no growth occurred after 24 hours. If aeration was now stopped, growth took place in a few hours. He regarded the lag phase of bacterial growth as representing the time it takes the organism to produce enough carbon dioxide in its vicinity to allow growth to occur. The production of an optimum concentration was determined by the rate of respiration of the organisms, and the rate of removal of carbon dioxide, which normally occurs by diffusion and chemical combination. Any factor affecting either of these processes will in turn affect growth. It should be pointed out that Rahn (1941a, 1941b) has demonstrated that bacteria as well as protozoa need carbon dioxide not only during the lag phase for purposes of rejuvenation, but also during the period of active growth.

In another paper (Winslow, Walker and Sutermeister, 1932) it was shown that growth could not be prevented, when the synthetic medium was replaced by peptone, or broth containing lactose.
In a study of the importance of a particular factor for growth it is necessary that all other factors should, as far as possible, be known. The early work deals with growth on a highly complex medium, in which the environment of the organism cannot be determined with certainty. It seemed, therefore, desirable to extend the work with the same rigid technique as used by Walker (1932).

Gladstone, et al (1935) selected a number of organisms capable of growing on a synthetic or simple medium and studied the effect on growth of passing CO₂-free air through cultures in these media as compared with the effect of passing through control cultures (air containing 10 per cent CO₂ and atmospheric air). They concluded that their organisms failed to develop in the absence of CO₂, however, the inhibited cultures were not dead. The inhibition was due to rapid removal of CO₂ from the liquid cultures, and the failure to inhibit the growth was due to the production of CO₂ by the organisms at a greater rate than that at which it could be removed. These investigators conclude in their discussion, (p. 347)

... although the present work supports the suggestion that the presence of CO₂ in a culture medium is a prerequisite to bacterial growth, we have no theory to put forward to explain this phenomenon.

Various theories for the function of carbon dioxide have, however, been put forth by various investigators through the years. Prominent among these were that, whereas the autotrophs require the gas as a metabolite, in heterotrophic metabolism carbon dioxide may (1) operate as a factor in altering the reaction of the medium, (2) cause better
retention of moisture, (3) reduce the oxygen tension of the medium or (4) alter the permeability of the cell wall. Rockwell and Highberger (1926) did express the opinion that bacteria, yeasts and molds require CO₂ and that the gas is used as a source of carbon.

No real progress was made in this field until the classical studies of Wood and Werkman from 1935 to 1945.

In 1935 Wood and Werkman advanced the principle of heterotrophic assimilation of carbon dioxide as a definite and experimentally supported concept. These investigators found with several species of Propionibacterium that the total carbon dioxide liberated during the fermentation of glycerol, plus that remaining as carbonate, was less than the CO₂ originally added as carbonate, which served in the medium as a buffering agent. Oxidation-reduction indices and carbon balances supported this view.

In 1938 these same authors showed an equimolar relationship between carbon dioxide fixed and succinic acid formed. In 1940 they found that the inhibition of fixation by sodium fluoride resulted in a corresponding reduction in the formation of succinic acid. For this reason they proposed the C₃ and C₁ addition hypothesis to account for the fixation of carbon dioxide. Pyruvic acid was suggested as the possible C₃ constituent since it could be isolated from the fermentation. The question thus became:

1. CH₃COCOOH + CO₂ → COOHCH₂COCOOH

The formation of succinate results from the stepwise reduction of oxaloacetate through malate and fumarate. Phelps, et al. (1939) confirmed
the work of Wood and Werkman. The observation of Bladen (1938) that
the rate of succinate formation by Escherichia coli was a function of
the concentration of carbon dioxide in the medium constituted indirect
evidence of CO₂ assimilation.

The availability of carbon isotopes at this time greatly facil­
itated further study on the mechanism of the reaction. Fixation of
CO₂ by the propionic acid bacteria fermenting glycerol was confirmed
by Carson and Raben (1940) using radioactive carbon and by Wood, et al
(1940) employing the stable isotope C¹³. When NaH¹³CO₃ was added to
the medium, the excess of heavy carbon was found in the succinic acid
formed during CO₂-fixation.

Wood, et al (1941a) also demonstrated the fixation of carbon
dioxide in the coliform bacteria during the fermentation of galactose,
pyruvate and citrate. In this case the isotope occurred only in the
succinic and formic acids. The latter was formed by the reduction of
carbon dioxide and the former probably by the C₃ and C₁ addition.

Extension of the concept of heterotrophic CO₂ utilization to
animal tissues came about quite logically and in the last decade much
work has been done in this field by several different groups of in­
vestigators. In 1938, when it was proposed that pyruvate was the
possible three-carbon intermediate compound undergoing fixation, Wood
and Werkman also suggested that this fixation might take part in Krebs
citric acid cycle.

The Krebs cycle was originally proposed by Krebs and Johnson
(1937) and modified by Wood, et al (1941b, 1942) and by Evans and
Slotin (1941). It was proposed as a mechanism for the complete oxidation of a molecule of pyruvate to CO₂ and water in mammalian muscle tissue. In the original Krebs scheme one molecule of pyruvic acid combines with oxalacetate to form a seven-carbon compound which then passes through a number of oxidative decarboxylations until the end product, oxalacetate, is reached. Thus the carbon and hydrogen of pyruvic acid is oxidized to CO₂ and H₂O and oxalacetate is again regenerated. The scheme, as presented by Krebs, also included the mechanism for hydrogen transport proposed by Szent-Györgyi and co-workers (1937).

The Krebs cycle has been demonstrated principally in pigeon liver and muscle. However, further accumulation of data also seems to indicate the occurrence of this cycle in sheep heart, testis, brain and guinea pig kidney. Part of this cycle, e.g., the conversion of citrate to α-ketoglutarate has also been observed in plant tissue (cucumber seeds, Krebs, 1943).

No direct evidence has thus far been presented for the occurrence of such a cycle in bacteria.

The reactions in pigeon liver and muscle were observed to be similar but not identical. Malonic acid inhibits the oxidation of succinic acid because of structural similarities between the two dicarboxylic acids. Succinic dehydrogenase activates succinate for the oxidation to fumarate. When malonate is placed in the presence of pigeon breast muscle and pyruvate, the dissimilation of the keto acid is stopped (Krebs and Eggleston, 1940). Upon the addition of fumaric or malic acids, dissimi-
lation of pyruvate will proceed proportionally to the amount of C4 acid added. Inhibition of the succinate oxidation prevents the generation of oxalacetate necessary for the cycle to operate. Addition of fumarate or malate relieves this inhibition owing to the interconversions of these acids to oxalacetic acid. Evans (1940) found that pyruvate dissimilation in pigeon liver is not sensitive to malonic acid inhibition. However, many of the intermediates of the Krebs cycle could be demonstrated; namely, the C4 acids and α-ketoglutaric acid. When the oxidation in liver tissue was compared to that in muscle tissue, it became evident that the liver tissue was capable of synthesizing its oxalacetate by some other mechanism. The only malonate insensitive reaction that has been described in the literature for synthesis of C4 dicarboxylic acids is the fixation reaction of Wood and Werkman.

Independently, Evans and Slotin (1940) and Krebs and Eccleston (1940) and shortly afterwards, Wood, et al. (1941b) presented evidence that carbon dioxide is fixed by a C3 and C1 addition in pigeon liver. Evans and Slotin (1940) showed that carbon dioxide is fixed during the oxidation of pyruvate by the isolation of α-ketoglutaric acid from experiments in which C14O2 was used as a tracer. Wood, et al. (1941b) employed heavy carbon and Evans and Slotin (1941) radioactive carbon; both groups showed the position of the carbon fixed in isolated α-ketoglutaric acid to be in the carboxyl group adjacent to the carboxyl. The original Krebs cycle postulated citric acid as an intermediate. The reactions citrate → αis-aconitate ←→ iso-citrate were considered from evidence obtained by the isolation of citric acid as an intermediate.
Krebs (1941) had predicted that the fixed carbon would be found in both carboxyl groups owing to the symmetry of the carboxyl groups in citric acid. In order to account for the fixed carbon only in the carboxyl group α to the carboxyl group, Wood, et al. (1941b) modified the original Krebs cycle by eliminating citric acid as an intermediate.

Following these indications of the wide biological occurrence of CO₂ fixation, the natural consequence of the investigations which followed was an attempt to elucidate the mechanism by which CO₂ functions in the metabolism of heterotrophic cells.

For a complete resume of the heterotrophic assimilation of carbon dioxide, the reader is referred to the excellent reviews by Workman (1939), Werkman and Wood (1942a), (1942b) and Wood (1946). However, since a new type of CO₂ utilization has been demonstrated in the present work, it is worthwhile to outline the earlier primary fixation reactions discovered by various investigators.

**Initial Reactions in the Fixation of Carbon Dioxide**

By primary fixation is meant (Wood, 1946) the reaction in which the carbon to carbon linkage is first formed. This is in distinction to the overall changes following this initial fixation of carbon dioxide. Presumably the primary fixation reaction initiates fixation which ultimately leads to the inclusion of carbon from carbon dioxide in a large number of derived compounds.
The three primary fixation reactions will be considered in order of their discovery.

**Carbon dioxide fixation by α-oxalacetate carboxylase (C₃-C₁ addition)**

The primary reaction of this system as originally shown by Krampitz and Werkman (1943) involves the reversal of the decarboxylation of oxalacetic acid to pyruvate acid and CO₂. Reaction 2

\[ 2 \text{CH}_2\text{COOH} + \text{CO}_2 \rightleftharpoons \text{CH}_2\text{COO}^\cdot\text{CH}_2\text{COO}^\cdot \]

is catalyzed by oxalacetic α-carboxylase, an enzyme which is found in bacteria and liver and requires magnesium or manganese ions for activation. The equilibrium of the reaction is far to the left. Reversibility has been demonstrated by allowing the enzyme to act on oxalacetic acid in the presence of isotopic carbon dioxide. By stopping the enzyme action when about half of the oxalacetic acid was decarboxylated, the presence of isotopic carbon in the α-carboxyl group was demonstrated. Adenosine triphosphate is an essential component of the reaction. It is entirely probable that a high energy derivative of oxalacetate or pyruvate is formed with which the carboxylation occurs.

**C₂-C₁ addition by the phosphoroclastic reaction**

The proof of C₂ and C₁ addition came from the study of a specific enzyme reaction, the phosphoroclastic reaction of pyruvate which was first demonstrated by Utter, et al (1945) with an extract from E. coli. The C₂ component is almost certainly acetyl phosphate.
3. \( \text{CH}_3\text{COOCOOH} + \text{H}_3\text{PO}_4 \xrightleftharpoons{} \text{CH}_3\text{CO(OPO}_3\text{H}_2) + \text{HCOOH} \)

which condenses with formic acid or carbon dioxide and hydrogen. A corresponding reaction in tissue has not as yet been demonstrated.

C5-C1 addition by oxalosuccinate carboxylase

Ochon and Weisz-Tabori (1945) have recently made an interesting discovery that crude solutions of isocitric dehydrogenase prepared from heart muscle contain an enzyme which catalyzes Reaction 4.

4. \( \text{COOCH}_2\text{CH-COOCOOH} \xrightleftharpoons{} \text{COOCH}_2\text{CH}_2\text{COOCOOH} + \text{CO}_2 \)

Since in these experiments no labeled carbon dioxide was used, it was necessary to remove the oxalosuccinate by reduction to isocitrate (Reaction 5) in order to shift the reaction toward carbon dioxide fixation. Reaction 5 was followed spectrophotometrically.

5. \( \text{COOCH}_2\text{CHOOCOOH} + \text{TPN}^\text{red} \xrightarrow{} \text{COOCH}_2\text{CHOOHCOOH} + \text{TPN}^\text{ox}. \)

In the presence of the heart enzyme the oxalosuccinate for Reaction 5 can be replaced by \( \alpha \)-ketoglutarate and \( \text{CO}_2 \). The \( \alpha \)-ketoglutarate and carbon dioxide combine according to Reaction 4 and the resulting oxalosuccinate is reduced to isocitrate. The reaction can be shifted still further toward carbon dioxide fixation by combining it with a glucose-6-phosphate dehydrogenase system. \( \text{TPN}^\text{red} \) is supplied by the glucose-6-phosphate dehydrogenase system (Reaction 6) for reduction of the oxalosuccinate.

6. \( \text{glucose-6-phosphate} + \text{TPN}^\text{ox} \xleftarrow{} 6\text{-phosphogluconate} + \text{TPN}^\text{red} \)
The net result of the combined systems is the following dismutation:

6a. α-ketoglutarate $\rightarrow$ CO$_2$ + glucose-6-phosphate $\rightarrow$ isocitrate + 6-phosphogluconate

The isocitrate was determined with isocitric dehydrogenase.

The evidence is thus clear that carbon dioxide combines with α-ketoglutarate to form oxalacetic acid.

Other than these primary fixation reactions, no information is at hand regarding exchange reactions involving carbon dioxide, except for the recently discovered reaction of Ochoa, et al (1947) in which malate can be formed from pyruvate and carbon dioxide without the intermediary formation of free oxalacetate, by direct reduction with reduced triphosphopyridine nucleotide (TPNH$_2$). They obtained a protein fraction from pigeon liver which could catalyze the reversible reaction.

7. pyruvate + CO$_2$ + TPNH$_2$ $\rightarrow$ malate + TPN

This reaction was followed spectrophotometrically. Oxalacetate could not oxidize TPNH$_2$, but was, however, decarboxylated by the enzyme. ATP was reported to be without effect on the reaction. To explain previous findings of Utter and of Wood that ATP stimulated the exchange of isotopic carbonate with the δ-carboxyl carbon of oxalacetic acid, it seemed reasonable to suppose that ATP caused the formation of TPN, a reaction which could actually be demonstrated in pigeon liver. Direct tests of the effect of TPN on the exchange reaction revealed, however, that this could not be the explanation since TPN could not substitute for ATP. It was found, however, that TPN could stimulate the decarboxylation of oxalacetic acid—a curious effect for a cofactor thought
to be concerned only with oxidoreductions.

Future work will undoubtedly reveal the actual relationship of these apparently contradictory facts. For the present, the point which deserves emphasis is the intimate association, possibly on the same enzyme molecule, of the decarboxylation and the TPN-mediated oxidoreduction, as demonstrated by Ochoa, et al (1947).

With this information at hand concerning the mechanisms by which CO₂ is utilized in heterotrophic metabolism, very little is known concerning the function of this gas in the metabolism of heterotrophic cells.

Lwoff and Monod (1946) have recently shown that combinations of both succinate and DL-glutamate will replace CO₂ and Lyman, et al (1946) that CO₂ and vitamin B₆ are important factors in modifying the amino acid requirement for some lactic acid bacteria. These investigations are the only attempts thus far made to elucidate the function of CO₂ in bacterial metabolism.
METHODS

General Considerations

The problem under consideration was of such a nature that two general types of investigations had to be conducted involving both growth experiments and studies with non-proliferating and cell-free enzyme systems. In many instances growth and resting cell experiments were carried out simultaneously for confirmatory purposes. This was done with the objective of increasing the validity of the work where non-proliferating cells alone were employed. It is of significance that the results were essentially the same whether a growing bacterial culture represented the enzyme under consideration or a cell deprived of an essential metabolite. For example, if the deamination of aspartic acid in inhibited on the addition of cyclohexanol to a suspension of freshly harvested cells in a buffer solution, the same inhibitor will prevent the amino acid from replacing carbon dioxide in a growing culture of the same organism. Further studies reveal that, before aspartic acid can function in replacing CO₂, it must be deaminated.

Growth Experiments

The organism employed in these experiments was Escherichia coli. Similar but not identical results were obtained with Aerobacter aerogenes. Both organisms were obtained from stock cultures kept in these laboratories.
The basal medium consisted of 0.8 per cent \( \text{K}_2\text{HPO}_4 \), 0.4 per cent \((\text{NH}_4)\text{SO}_4\), 10 per cent tap water (for inorganic ions) and 0.8 per cent glucose, made up with distilled water to the desired volume. The glucose was autoclaved separately to prevent caramelization and added to the medium aseptically. The pH was adjusted to 6.8 unless otherwise indicated.

The compounds to be tested were normally added to the medium before autoclaving, except in the case of oxalacetic acid. A solution of the sodium salt of this acid was sterilized by filtration through a Seitz filter and added aseptically to the medium.

The inoculum was a 24 hour culture grown in 10 ml. of the basal medium. Transfers were made with the same loop (3 mm. in diameter) daily so as to insure uniform growth throughout the experiments. The tube from which the inoculum was made was always checked and adjusted in turbidity to give the same reading on the photoelectric colorimeter. The culture was checked periodically for purity.

The \( \text{CO}_2 \)-absorbing train

The apparatus for the constant removal of \( \text{CO}_2 \) from the air is shown in Fig. 1. The air was first allowed to pass through a sterile, cotton-filled tube and then freed from carbon dioxide by passing it through an alkali tower filled with glass beads and containing 30 per cent \( \text{NaOH} \) with phenolphthalein as an indicator. The air was humidified by introducing a test tube with sterile distilled water prior to the \( \text{CO}_2 \) absorption tower. Any alkali that might spill over would be caught
FIG 1. APPARATUS FOR THE REMOVAL OF CARBON DIOXIDE FROM THE AIR.
in the alkali trap following the tower.

Essentially the same apparatus was employed in anaerobic experiments as well, except that, in order to remove any oxygen, nitrogen from a cylinder was passed over reduced copper gauze heated in an electric furnace. It was then passed through a CO₂-absorbing train and finally through the cultures.

Since the rate of growth not only depends upon the addition of certain compounds but upon the rate of aeration and the porosity of the aeration discs as well, proper precautions were taken to eliminate these variables. The same sintered glass disc was used in all of these experiments and the rate of aeration kept constant by introducing a manometer into the aeration system immediately after the culture flask. Five volumes of air were passed per minute.

The rubber stoppers to which the aeration discs were attached were autoclaved separately before each experiment.

The reaction flask contained 100 ml. of basal medium. To this was added aseptically 1 per cent of the inoculum in all cases, unless otherwise stated. The anaerobic experiments, on the other hand, were carried out with 25 ml. of basal medium and an inoculum of 2 per cent.

All experiments were conducted at either 30 or 37°C. Growth in all cases was measured by turbidimetric readings on the Klett-Summerson photoelectric colorimeter with a 600 nm light filter. All readings were made after 18 hours' incubation unless otherwise stated. Uninoculated controls were used as blanks.
Resting-Cell Experiments

Growth media and cell preparations

*A. aerogenes* and *E. coli* were grown in a medium containing the following constituents, 0.8 per cent glucose, 0.8 per cent KH$_2$PO$_4$, 0.4 per cent (NH$_4$)$_2$SO$_4$, 10 per cent tap water (for inorganic ions), and made to volume with distilled water. The phosphate was dissolved in a small amount of water and adjusted to a pH of 6.8 with 30 per cent sodium hydroxide. The solutions were sterilized by autoclaving for 45 minutes at 15 pounds pressure. The phosphate solution was added aseptically to the other portion of the medium immediately before inoculation.

*A. aerogenes* or *E. coli* were first inoculated into 10 ml. of the above medium; after 18 hours' incubation at 30°C, the 10 ml. were added to 30 ml. of medium. This was also incubated at 30°C for 18 hours and was then added to 800 ml. of medium. The medium was incubated in the same manner as the other aliquots and then used to inoculate 8 liters of medium contained in a 12 liter Florence flask. The medium was aerated for 18 hours by means of compressed air passing through a carborundum aerating ball. The cells were harvested in a Sharples centrifuge and treated according to the experiment. The yield was usually between 35 and 50 grams of cells, although yields as high as 60 and 70 grams per 8 liters of medium often have been obtained.

In some instances it was desirable to increase the activity of the organisms on certain compounds and for this reason such compounds were
added as carbon sources during the growth of the organism.

Since _A. aerogenes_ grown in glucose media in the absence of citrate attacked citrate very weakly in manometric experiments, the cells were grown in a medium of 2 per cent sodium citrate, 0.35 per cent peptone, 0.1 per cent KH₂PO₄ and 10 per cent tap water which was adjusted to pH 6.5. After 48 hours of aeration at 30°C, the cells were harvested in a Sharples centrifuge, lyophilized and stored in the dry state.

Similarly, in order to increase the activity of _E. coli_ juice on succinate, the cells were grown on a medium containing 2 per cent sodium succinate, 0.35 per cent peptone, 0.8 per cent KH₂PO₄ and 10 per cent tap water which was adjusted to pH 7.0. After 18 to 24 hours' aeration, the cells were centrifuged and cell-free juices prepared.

**Obtaining the enzyme preparations.**

Several types of enzyme preparations were employed in this work. When a juice was to be prepared from the bacteria, ground glass was added to the cell paste in a ratio of 2 grams of glass to 1 gram of cells wet weight. The cell-glass mixture was ground in the glass cones described by Kalnitsky, et al., (1945). The bacterial juice was eluted with either 1/15 phosphate buffer or distilled water. One and a half ml. of eluting liquid was used per gram of cells. The cell debris and glass were centrifuged in a refrigerated centrifuge at approximately
0°C. at 12,000 rpm. for 15 minutes. Supernant opalescent bacterial juice was decanted into a container, frozen by means of dry ice and acetone, and kept frozen until used.

**Untreated cells**

The cell paste obtained from centrifugation was made up to a convenient volume so that, when transferred to the reaction vessel, a final concentration of 2.5 per cent (wet weight basis) was obtained.

**Acetone treated cells**

Ten grams of cell paste were mixed with 70 ml. ice-cold acetone and shaken thoroughly for 5 minutes. The cells were filtered off, suspended in 140 ml. ice-cold acetone and shaken vigorously for 10 minutes. The process was repeated. The preparation was then placed in vacuo to remove traces of acetone.

Essentially the same procedure was followed when cells were treated with toluene or n-propyl alcohol.

**Lyophilized preparations**

Cells 18 to 24 hours of age were suspended in a minimum amount of distilled water and transferred into a 500 ml. round-bottom flask. This suspension was frozen while rotating the flask in an acetone-dry
ice bath. The flask containing the frozen culture was evacuated by
use of a vacuum pump. The evacuation of air resulted in sublimation
of the ice in the frozen suspension. The water vapors were extracted
by continuous suction and condensed and frozen in another flask which
was held in an aceton-dry ice bath.

The activity of such preparations remains constant for months
when stored in the refrigerator.

Analytical Procedures


Lactometric methods

The Barcroft-Warburg apparatus was used in most of these experi­
ments. All reactions were carried out at 30.4°C. The conventional
18 ml. flask possessing 2 side-arms was used in all experiments, with
the exception of those in which exchange experiments were conducted
or when large scale fermentations were carried out. In the latter
125 ml., 2 side-arm flasks were employed. The volume of the reactant
mixtures will be given in the appropriate tables and figures.

The following are examples of the use of the Warburg apparatus in
our experiments: (1) Small-scale fermentations, in which only
α-ketoglutaric acid oxidation, CO₂ evolution and oxygen uptake were to
be determined; (2) determination of the activity of enzyme preparations
and the optimum conditions for such activity; (3) determination of the
presence of different enzyme systems in the preparation and components
of the systems (after dialysis); (4) the effect of certain inhibitors on the utilization of various substrates.

Exchange reactions

The exchange reactions were carried out in 125 ml. Erlenmeyer flasks with 2 side-arms which were attached to the Barcroft-Warburg manometers. The final concentration of α-ketoglutarate was 0.01M and that of malonate 0.05M. The sodium salts of both acids were used. Depending on the activity of the juice, a quantity varying between 5 and 10 ml. was added to each flask. Attempts to use more constant concentrations of the enzyme failed since the lyophilized juice showed little or no activity. The mixture was buffered with 0.2M phosphate, pH 6.6 and 0.07M NaHCO₃. Appropriate concentrations of the 2 buffers were mixed with the substrate and enzyme after temperature equilibrium (30.4°C) had been reached. The pH of the resulting mixture was approximately 7.2 and the volume 25 or 30 ml.

The reaction was allowed to continue until approximately half the substrate was utilized. The reaction mixture was then transferred to an aeration apparatus for the determination of residual C¹³O₂. Arrangements of the apparatus were such that the addition of the necessary reagents could be made during the course of aeration and heating. The C¹³O₂ was liberated by the addition of 6 N H₂SO₄ and collected in 8 ml. of 4 N carbonate-free NaOH in specially constructed carbon dioxide absorption tubes.
Determination of α-ketoglutaric acid

Since both malonate and α-ketoglutarate are decarboxylated by the same reagents, it was necessary to remove the former before decarboxylating the keto acid. This was done very effectively by the addition of 10 ml. of 0.025 M sodium bisulfite to the reaction mixture from which the residual CO₂ has been removed, and extracting the deproteinated sample for 72 hours with ether. The ethereal extract contained all of the original malonate, as subsequently determined, plus the other dicarboxylic acid (succinic acid) formed in the course of the reaction. The extracted solution was then transferred to a 500 ml. round-bottom flask and the α-ketoglutaric acid was degraded with ceric sulfate or K₂H₂O₄. The flask was connected to an apparatus in such a fashion that the liberated CO₂, on the addition of the degrading reagent was quantitatively collected into a CO₂ absorption flask. The products of this oxidation are succinate and carbon dioxide. The CO₂ which originates from the carboxyl adjacent to the carboxyl group was collected and the C¹³ content determined on the mass spectrometer.

When residual α-ketoglutaric acid was to be determined, ceric sulfate was kept throughout the experiment in the side arm of the manometric flask and added to the main well after acidification of the reaction mixture.
NaaC$^{12}$O$_3$ rinse

After the solution has been extracted, 0.75 mM of NaaC$^{12}$O$_3$ was added, further acidified with H$_2$SO$_4$, heated and aerated for 15 minutes. The use of C$^{12}$O$_2$ rinse insured that no C$^{13}$O$_2$ remained to interfere subsequently with the isotope determination of the carboxyl carbon. The C$^{13}$ content of the rinse is indicated in the experimental section.

Determination of succinic acid

Succinic acid was determined by the use of a succinic dehydrogenase preparation obtained in the following manner: 2 grams of beef heart tissue freed from fat and connective tissue are blended with 100 ml. of ice-cold 0.9 per cent potassium chloride solution for a period of 4 minutes. The suspension is washed several times with potassium chloride and immediately frozen and lyophilized. The dried preparation is thoroughly ground and, when kept in the refrigerator, quantitatively oxidizes succinic acid even after a number of weeks. This method is simple and eliminates the earlier, laborious procedure used, which involves first grinding and then homogenizing the tissue by the use of the homogenizer described by Potter and Elvehjem (1936).

Determination of oxalacetic acid

This acid was determined according to the aniline-citrate method of Edson (1935). The procedure is as follows. The enzyme preparation plus buffer and water are placed in the main chamber of the Warburg cup,
the substrate in one side arm and 0.3 ml. of a 50 per cent solution
of citric acid in the other side arm. After the substance has been
tipped into the main chamber and the reaction allowed to proceed,
the citric acid is tipped into the main chamber to stop the reaction
and liberate the bound CO₂. The manometers are shaken until all the
bound CO₂ has been evolved (generally 5 to 15 minutes). The manometers
are then taken off the bath and 0.4 ml. of 1:1 mixture of citrate-
aniline quickly added to the side-arm originally containing citric
acid. The manometers are then quickly replaced on the bath, the con­
tents of the cups allowed to come to temperature equilibrium (3 to 5
minutes), readings taken, and the citrate-aniline tipped into the main
compartment of the cup. The CO₂ evolved originates from the carboxyl
group adjacent to the methylene group of oxalacetic acid. The remainder
of the oxalacetate molecule unites with the aniline to form pyruvanilide.

Owing to the general instability of oxalacetate, especially at acid
reactions, the determination must be carried out as quickly as possible.
However, care must be taken that all of the CO₂ is driven from the
medium and the cup has reached temperature equilibrium before the aniline-
citrate is tipped into the center well.

When residual oxalacetate was to be determined during growth experimen­
tests when this acid was replacing carbon dioxide, aliquots of culture
medium were removed periodically during the experiments into Warburg
flasks and the CO₂ given off on the addition of aniline-citrate after
the solution had been acidified with citric acid, determined. Proper
blanks to account for the spontaneous decarboxylation of the acid were
subtracted from the values cited in the experimental section.

Determination of glutamic acid

A modification of the method described by Cohen (1939) was used to determine this amino acid. With whole cells, it was not necessary to deproteinate, since small amounts of protein do not interfere. The cells were centrifuged, filtered and analyzed for glutamic acid.

The solution was brought to pH 4.7 by the addition of 1 to 1.5 ml. of citrate buffer. Two ml. of freshly prepared chloramine-T were added and the solutions well mixed by shaking and then placed in a 40°C water bath for 10 minutes and shaken periodically. After 10 minutes shaking, the containers were placed in an ice-bath for 15 to 20 minutes to precipitate most of the p-toluene-sulphonamide formed as a reaction product and most of the unused chloramine-T. The solutions were filtered while cold, the precipitate washed with several small volumes of cold water, and the combined filtrate and washings collected in a large test tube.

Concentrated HCl was added to the filtrate to make a final concentration of not less than 12.5 per cent. The tubes were covered and placed in a boiling water bath for 15 minutes, removed and allowed to cool. Concentrated NaOH was added dropwise until the solution became hot. At this point 0.5 ml. of 5 per cent \( \text{NH}_4\text{Cl} \) solution was added and the contents well mixed. The \( \text{NH}_4\text{Cl} \) decomposes traces of chloramine-T which if present will decolorize the indicator. The solution was cooled and a few drops of phenol red were added. The solution was then made alkaline to a
purple color. A large excess of alkali should be avoided since the p-toluene-sulfonamide forms a salt in strongly alkaline solution. The solution was then extracted with freshly distilled ether for a time sufficient to remove the remaining traces of p-toluene-sulfonamide (usually 1 to 2 hours). The extraction flasks were removed and replaced by clean ones. The contents of the extractors were then acidified with 2 to 3 ml. of 10 per cent H₂SO₄. The phenol red changes to a light yellow-pink color. Additional ether was then added and the extraction resumed for 24 hours.

When the reaction was completed, 1 to 2 ml. of 0.1 N phosphate buffer, pH 7.4, was added to the ether solution and the ether distilled off. The last traces of ether were removed by concentrating the aqueous residue on the steam bath to approximately 0.5 to 1 ml. The residue was then transferred to a 15 ml. graduated centrifuge tube and made up to volume. The solution was now adjusted to a pH of 7.4 by the addition of dilute NaOH. The final volume was adjusted according to the succinic acid concentration.

The succinic acid thus obtained was determined with the lyophilized succinic dehydrogenase described above.

The method for the determination of glutamic acid depends upon its conversion by means of excess chloramine-T to D-cyanopropionic acid, and the hydrolysis of the latter to succinic acid according to the following equations:
Calculation: The $\mu l$ O$_2$ uptakes are converted to mg. of glutamic acid as follows:

$$\frac{\mu l \text{O}_2}{112} \times 1.47 = \text{mg. glutamic acid}$$

Determination of ammonia

Ammonia was determined by a modification of the method of Johnson (1941). The sample was transferred from the Warburg vessel into a test tube and to it was added an equal amount of 10 per cent trichloroacetic acid. The cells were centrifuged and filtered. To the filtrate 3 ml. of 2N NaOH, 1 ml. of gum arabic and 0.5 ml. of Nessler's Reagent were added. The colored solution was mixed and read on the colorimeter.
using a 490 mm filter. Range: 10-100 micrograms nitrogen; precision ± 1.5 micrograms.

The gum arabic solution was prepared by mixing 2 grams of gum arabic, 1 gram of Na$_2$HPO$_4$.12 H$_2$O in 100 ml of distilled water. This solution was allowed to stand for one-half hour, filtered and used.

**Dialysis**

Dialysis of the enzyme preparations was carried out in a collodion bag against distilled water or 0.013 M phosphate buffer, pH 6.8, followed by 0.4 per cent potassium chloride. Biotin was more efficiently removed by the latter treatment.

In the case of biotin the cell-free extracts were dialyzed for 3 hours against 8 liters of 0.013 M phosphate buffer of pH 6.8, followed by a 2 hour dialysis against 8 liters of 0.4 per cent potassium chloride at 15-17°C unless otherwise stated. Continuous mechanical stirring insured rapid diffusion. Control experiments indicated that some enzyme inactivation took place during the period of dialysis.

When the components of α-ketoglutaric acid dehydrogenase were to be determined, dialysis was carried out against distilled water at approximately 1°C for 90 to 120 minutes. The dialyzing apparatus consisted of 2 parts: (1) a motor rotating a glass rod to which the collodion bag was attached with heavy thread; the glass rod extended into (2) a dialyzing chamber at an angle of about 45°C; the capacity of the dialyzing chamber was approximately 10 liters, containing distilled water and ice cubes.
Assay of Biotin

Biotin was determined according to the procedure of Snell et al. (1940) using a stock culture of *Saccharomyces cerevisiae* shown to require biotin for growth.

The basal medium contained: sucrose 20 gm., (NH₄)₂SO₄ 3 gm., K₂HPO₄ 2 gm., MgSO₄·7H₂O 0.25 gm., CaCl₂·2H₂O 0.25 gm., H₃BO₃ 1 mg., ZnSO₄ 1 mg., MnCl₂ 1 mg., TiCl₃ 1 mg., FeCl₃ 0.5 mg., CuSO₄·5H₂O 0.1 mg., KI 0.1 mg., L-aspartic acid 0.1 gm., inositol 5 mg., β-aminolevulinic acid 0.5 mg., thiamin 20μg and pyridoxin 20μg, all added to 1 liter of distilled water.

This medium failed to support the growth of yeast unless biotin or a given quantity of dialyzing medium (after 2 or 3 hours' dialysis) was added.

The response was measured turbidimetrically on the photoelectric colorimeter after 24 hours at 30°C using 600 nm filter.

Preparation of Compounds

1,2-Ethanesulfonate

The procedure of Stone (1936) was used in the preparation of 1,2-ethanesulfonate. One mole ethylene dibromide was added slowly to a hot saturated aqueous solution of 2.5 moles of sodium sulfite. The mixture was heated on a steam plate with stirring until the reaction was complete. This was determined by titration of an aliquot with H₂/₂
silver nitrate solution after slight acidification with acetic acid, and boiling to remove sulfur dioxide. Sodium chromate was added as an indicator. Complete conversion took place in 24 hours. The disulfonate crystallized from the reaction mixture. Purification was accomplished by repeated recrystallizations. The compound was dissolved in a minimum amount of hot water and reprecipitated with ethyl alcohol. The yield was between 30 and 50 per cent. The compound was dried in vacuo over P₂O₅. 1,2-ethanedisulfonate is somewhat hygroscopic. The following are the reactions involved:

\[
\begin{align*}
\text{Br(CH₂)₂Br} + 2\text{Na}_2\text{SO}_3 & \rightarrow \text{NaO}_3\text{S(CH₂)₂SO}_3\text{Na} + 2\text{NaBr} \\
\text{AgNO}_3 + \text{NaBr} & \rightarrow \text{AgBr} + \text{NaNO}_3 \\
\text{Na}_2\text{CrO}_4 + 2\text{AgNO}_3 & \rightarrow \text{Ag}_2\text{CrO}_4 + 2\text{AgNO}_3
\end{align*}
\]

Yellow     Red

**Oxalacetic acid**

To the sodium salt of diethyloxalacetate (100 gm. in 250 ml. water) concentrated H₂SO₄, equivalent to the sodium, was added. The free ester was removed as an oily layer by means of a separatory funnel and dissolved in 80 ml. ether. The ether solution was washed twice with 50 ml. distilled water, and the ether evaporated off. The ester was hydrolyzed by treatment with 3 volumes of concentrated HCl at room temperature.

The acid solution was vigorously shaken mechanically for 2 hours and then placed at -26°C for 24 to 46 hours. The precipitated oxalacetic acid was removed by filtration, dissolved in a requisite amount of warm acetone and recrystallized by cooling and the addition of chloroform.
The HCl solution still contained quantities of the diethyloxalacetate. One additional volume of concentrated HCl was added and the shaking and cold treatment repeated. The purity of the oxalacetate as determined by the aniline-citrate method (Edson, 1935) was approximately 100 per cent.

**α-Ketoglutaric acid**

Commercial preparations of α-ketoglutaric acid were recrystallized by dissolving in acetone and adding excess benzene in the cold. The product was filtered and dried over P₂O₅ and stored in the refrigerator over P₂O₅. Melting point: 112-113°C; 112°C reported in the literature.

**Cyclohexanol**

Commercial cyclohexanol was redistilled and the fraction boiling at 160-161°C collected, appropriate dilutions made, and added to the various experimental setups.

**γ-Ethyl glutamate**

This compound was prepared by the method of Bergmann and Zervas (1933) and modified as suggested by Roper and Mollwain (1948).

Five gms. of finely powdered L-glutamic acid were shaken with 50 ml. of absolute alcohol which contained about 3 gm. HCl (gaseous),
whereupon solution resulted. The volume was made up to 150 ml. with ethanol. Aqueous ammonia was added dropwise until a pH of 2.0, when the precipitated NH₄Cl was removed by filtration. The solution was finally brought to a pH of 6.0, when the ester separated as plates. The whole was kept several hours at 0°C and filtered, the filtrate being tested with Mg for a further precipitation of the ester.

γ-Ethyl glutamate (5 gms.) with 50 per cent (w/w) hydrazine hydrate (10 ml.) and water (5 ml.) were warmed at 70°C for 15 minutes. Absolute ethanol (200 ml.) at 70°C was added slowly and the mixture kept at 0°C for several hours. The fine crystalline precipitate was filtered off and recrystallized from aqueous ethanol. A white crystalline solid having a melting point of 165-166°C was obtained. This is γ-glutamylhydrazine.

δ-Aspartylhydrazine

Asparagine monohydrate (10 gms.) was refluxed with 50 per cent (w/w) hydrazine hydrate (30 ml.) for 20 minutes. The solution was evaporated in vacuo to a gum which was taken up in 15 ml. of water. Ethanol (70 ml.) was added gradually, the precipitated amorphous white solid filtered off, washed well with ethanol and crystallized from aqueous ethanol. It gave fine white crystals. Melting point 194-195°C with decomposition.
Diotin

Contents of an ampule containing the methyl ester were transferred to a 25 ml. Erlenmeyer flask with the aid of 1 N NaOH. The flask was stoppered and the solution was allowed to stand for 1 hour at room temperature. The solution was then neutralized with 1 N HCl and made up to the requisite volume with distilled water. (Alcohol may also be used.) The biotin solution was stored in the refrigerator until used.

Heavy carbon sodium bicarbonate

BaC\(^{13}\)O\(_3\) was prepared from C\(^{13}\)O\(_2\) obtained from methane whose C\(^{13}\) had been concentrated in a thermal diffusion column according to Hier and Bardeen (1941).

A sample containing approximately 2.5 gm. of C\(^{13}\)O\(_2\) (in the form of BaC\(^{13}\)O\(_3\)) was suspended in about 200 ml. of CO\(_2\)-free water. The C\(^{13}\)O\(_2\) was liberated on the addition of H\(_2\)SO\(_4\) and collected in about 50 ml. 1:1 KOH or NaOH. The C\(^{13}\)O\(_2\) content was determined by weight. The C\(^{13}\)O\(_2\) was then liberated in a closed system under a slight vacuum and collected in NaOH equivalent to the C\(^{13}\)O\(_2\) liberated from the concentrated alkali. A manometer was connected to the system during the process of acid addition and collection of C\(^{13}\)O\(_2\). Five normal H\(_2\)SO\(_4\) was used to liberate the carbon dioxide.
EXPERIMENTAL

The Effect of Carbon Dioxide on Bacteria

Since the work of Theobald Smith (1924, 1926) verifying the findings that Bacterium abortus requires the presence of CO₂, attempts have repeatedly been made to show that CO₂ is essential for the growth of many bacteria (Valley and Rettger, 1927, Gladstone, et al., 1935, and others).

Recent work done in these laboratories suggest that this is not always the case. Although the growth of Escherichia coli in a liquid synthetic medium is completely inhibited upon continuous aeration with CO₂-free air during 12 hours, detectable growth occurs after 18 hours and continues to increase under the same experimental conditions to a certain maximum above which no further increase is observed. It is, however, possible to replace the CO₂ by a number of compounds as a result of which not only normal, but, in many instances, enhanced growth results.

Table 1 shows the effect of different atmospheric conditions on the growth of E. coli. Although growth is appreciably reduced in the absence of CO₂, it is not completely inhibited, even when the inoculum is considerably diluted. That the gas is necessary for the optimal growth of bacteria is clearly seen when Table 2 is compared with Table 1. The results show (Table 2) that irrespective of the size of the inoculum, but depending on the time, maximum growth reached in the
Table 1

Effect of Different Atmospheric Conditions on the Growth of Escherichia coli

<table>
<thead>
<tr>
<th>Time in hours</th>
<th>Aeration with air</th>
<th>Aeration with CO₂-free air</th>
<th>Aeration with CO₂-free O₂</th>
<th>No aeration</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>225</td>
<td>0</td>
<td>0</td>
<td>80</td>
</tr>
<tr>
<td>18</td>
<td>230</td>
<td>30</td>
<td>0</td>
<td>80</td>
</tr>
<tr>
<td>24</td>
<td>245</td>
<td>43</td>
<td>2</td>
<td>80</td>
</tr>
<tr>
<td>42</td>
<td>275</td>
<td>57</td>
<td>13</td>
<td>85</td>
</tr>
<tr>
<td>48</td>
<td>300</td>
<td>57</td>
<td>13</td>
<td>85</td>
</tr>
</tbody>
</table>

Results in terms of turbidity readings. 0 indicates no growth.
Total volume, 101 ml. Temperature 30°C.
Table 2

Effect of Size of Inoculum on the Growth of Escherichia coli in the Absence of CO₂

<table>
<thead>
<tr>
<th>Aeration time in hours (CO₂-free air)</th>
<th>12</th>
<th>18</th>
<th>24</th>
<th>30</th>
<th>42</th>
<th>48</th>
<th>53</th>
<th>66</th>
<th>72</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original inoculum</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Dilutions</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:10</td>
<td>0</td>
<td>0</td>
<td>8</td>
<td>9</td>
<td>50</td>
<td>57</td>
<td>57</td>
<td>57</td>
<td>57</td>
</tr>
<tr>
<td>1:100</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>10</td>
<td>25</td>
<td>65</td>
<td>65</td>
<td>65</td>
</tr>
</tbody>
</table>

Results in terms of turbidity readings. 0 indicates no growth.
Total volume, 10 ml. Temperature 30°C.
absence of CO₂ is far less than under the same conditions with CO₂ present. When ordinary air is allowed into the culture flask after maximum growth has been attained, little or no further increase in growth is observed under the same experimental conditions.

The concentration of CO₂ in the medium appears to be an important factor in determining the conditions for optimal growth (Table 3). The concentration of CO₂ in the air, although small, seems to be favorable. In the presence of 5 per cent CO₂, a decrease in growth is noted and a further decrease takes place in 10 per cent CO₂. The pH of the medium in the presence of 10 per cent CO₂ did not change appreciably in 18 hours, indicating that the reduction in growth was due to the CO₂ and not to a change in the hydrogen ion concentration of the medium. Aeration with 100 per cent CO₂ resulted in total inhibition of growth, even though the medium was highly buffered. These results further substantiate the toxicity of carbon dioxide at high concentrations.

**Heterotrophic Replacement of Carbon Dioxide**

Why CO₂ is necessary for optimal growth is not certainly known. However, since Wood and Werkman have shown that CO₂ is fixed by heterotrophic organisms to form oxalacetic acid and subsequently members of the tricarboxylic acid cycle, the replacement of CO₂ by these intermediates or their potential precursors was determined.
Table 3

Effect of CO₂ on the Growth of *Escherichia coli*

<table>
<thead>
<tr>
<th>Concentration of CO₂ in the air</th>
<th>Normal</th>
<th>5%</th>
<th>10%</th>
<th>100%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth</td>
<td>230</td>
<td>122</td>
<td>55</td>
<td>0</td>
</tr>
<tr>
<td>Final pH of the medium</td>
<td>6.0</td>
<td>6.6</td>
<td>6.2</td>
<td>5.6</td>
</tr>
</tbody>
</table>

Results in terms of turbidity readings. 0 indicates no growth. Total volume, 101 ml. Temperature 30°C. Incubation time, 18 hours.
Although Lwoff and Monod (1946) have recently shown that combinations of both succinic and DL-glutamic acids will replace CO₂ and Lyman, et al., (1946) that CO₂ and vitamin B₆ are important factors in modifying the amino acid requirement for some lactic acid bacteria, no reports have thus far appeared in the literature on the extent to which CO₂ can be replaced, in vivo, by substances occurring in the proposed Krebs cycle or their metabolic precursors.

\textbf{C₄ dicarboxylic acids}

Fig. 2 shows the results obtained. The C₄ dicarboxylic acids apparently substitute for CO₂ to the same extent, indicating, in conformity with the proposals of Szent-Györgyi, a similar mode of action of these compounds.

Oxalacetic acid is not immediately spontaneously decarboxylated, even at 30°C (Table 4). The acid may be found unchanged in the reaction flask after 12 hours. The results show that, if the intact molecule of oxalacetic acid is necessary for growth as such, it is present. The data in Table 4 further indicate that oxalacetate is apparently necessary to initiate growth rather than support it. After 12 hours little or no oxalacetate is left in the medium and yet the growth of \textit{E. coli} increases from a colorimetric reading of 55 to approximately 400 at 18 hours. This would seem to substantiate the suggestion made by Kelter (1932) that the log phase of bacterial growth represents the time it takes the organism to manufacture enough carbon dioxide in its vicinity.
Fig. 2. Replacement of CO₂ by C₄ dicarboxylic acids.

Total volume 101 ml. containing 100 ml. of basal medium, 1% inoculum of Escherichia coli and various concentrations of the C₄ dicarboxylic acids. Incubation time, 18 hours. Temp. 30° C. Gas phase CO₂-free air.
Table 4

Relationship of Disappearance of Oxalacetic Acid to Growth

<table>
<thead>
<tr>
<th>Time in hours</th>
<th>0</th>
<th>3</th>
<th>6</th>
<th>9</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxalacetic acid</td>
<td>350</td>
<td>239</td>
<td>124</td>
<td>96</td>
<td>29</td>
</tr>
<tr>
<td>Growth</td>
<td>0</td>
<td>0</td>
<td>20</td>
<td>40</td>
<td>55</td>
</tr>
</tbody>
</table>

Oxalacetic acid was determined on the Warburg respirometer as CO₂. Temp., 30°C. Total volume per cup, 2.3 ml. containing 1.6 ml. test solution, 0.3 ml. citric acid and 0.4 ml. sodium citrate.

Growth measured as turbidity. A reading of 0 indicates no growth. Incubation temp., 30°C. Continuous aeration with CO₂-free air.
to allow growth to occur. In our case, the added oxalacetate would
act as the product normally manufactured from carbon dioxide and
pyruvate.

C₅ dicarboxylic acids, amino acids and asparagine

Since the C₄ acids are members of the Krebs cycle, other members
of this cycle replace CO₂ if the cycle is reversible or if side
reactions are also reversible (Figs. 3 and 4).

α-Ketoglutaric acid and glutamic acid substitute to approximately
the same extent, but considerably less of these compounds is necessary
to give the same amount of growth as obtained with the C₄ acids. These
data, therefore, apparently indicate the possibility of a fixation over
and above the Wood and Warliman reaction.

The effect of asparagine is relatively small compared with the
other compounds. This result may be expected if we assume the substitu-
tive effect of this compound to take place through aspartic acid.

An explanation of the difference in growth resulting from aspartic
acid and from glutamic acid is that the conversion of glutamic acid to
α-ketoglutaric acid is much more efficient than the conversion of
aspartic acid to a C₄ dicarboxylic acid, or that glutamic acid is of
primary importance to the metabolism of the cell and cannot be
synthesized adequately in the absence of CO₂.

Stetten and Schoenheimer (1944) fed isotopic L (−)-proline to
rats and isolated various amino acids from the carcass and organ
Fig. 3. Effect of various concentrations of amino acids on the growth of E. coli.

Same conditions as in Fig. 2.
Fig. 4. Effect of various concentrations of the $C_3$, $C_4$, $C_5$, and $C_6$ compounds tested on the growth of *Escherichia coli*.

Same conditions as in Fig. 2.
proteins. They found deuterium as well as $^1^5$H in the isolated glutamic acid. Krebs (1939) suggests that arginine may be converted through ω-keto-S-aminovaleric acid to ω-ketoglutaric acid. These compounds were, therefore, tested as substituents for CO$_2$. It is of interest that arginine and proline do, whereas histidine and lysine do not, replace CO$_2$ in the metabolism of E. coli (Table 5).

Experiments have been conducted to indicate whether the effects of substituting compounds are additive (Table 6). When 2 such compounds are added to the culture flask, each acts as if it were present alone.

Acids with carbon atoms less than 4

None of the C$_3$ compounds tested substituted for CO$_2$, since there was no CO$_2$ in the medium to permit an initial fixation reaction, which apparently is necessary for optimal growth (Fig. 4).

Since Slade, et al., (1943) have shown that 2 molecules of acetic acid can be condensed by *Aerobacter indologenes* to form succinic acid, attempts have been made to use acetic acid to substitute for CO$_2$. No satisfactory results have been obtained. Equimolar amounts of pyruvic acid and acetic acid also gave negative results.

The action of C$_6$ acids

The action of the C$_6$ acids depended upon the organism (Table 7). Whereas cis-aconitic acid can substitute for carbon dioxide to some extent with both E. coli and *Aerobacter aerogenes*, citric acid is
Table 5

Effect of Arginine, Proline, Histidine, and Lysine on the Growth of *Escherichia coli* in the Absence of CO₂

<table>
<thead>
<tr>
<th>Compound</th>
<th>Amount added in mL</th>
<th>Growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arginine</td>
<td>1</td>
<td>150</td>
</tr>
<tr>
<td>Proline</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Histidine</td>
<td>1</td>
<td>30</td>
</tr>
<tr>
<td>Lysine</td>
<td>1</td>
<td>30</td>
</tr>
</tbody>
</table>

A reading of 30 indicates no growth due to the addition compound. Total volume, 101 mL. Temp., 30°C. Incubation time, 18 hours. Aerated with CO₂-free air.
### Table 6

Additive Effects of Compounds Substituting for CO₂ in *Escherichia coli* Metabolism

<table>
<thead>
<tr>
<th>Compound</th>
<th>Succinic acid</th>
<th>Fumaric acid</th>
<th>Succinic+α-ketoglutaric acid</th>
<th>Succinic+α-ketoglutaric acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amount added; ml.</td>
<td>1</td>
<td>1</td>
<td>0.035</td>
<td>1 of succinic+α-ketoglutaric</td>
</tr>
<tr>
<td>Growth</td>
<td>120</td>
<td>140</td>
<td>305</td>
<td>220</td>
</tr>
</tbody>
</table>

Total volume, 101 ml., temp. 30°C, 18 hours' incubation period. Aerated with CO₂-free air.
Table 7

Effect of Cis-aconitic Acid and Citric Acid on the Growth of *Escherichia coli* and *Aerobacter aerogenes* in the Absence of CO₂

<table>
<thead>
<tr>
<th>Organism</th>
<th>Compound</th>
<th>% of compound</th>
<th>Growth</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em></td>
<td>Cis-aconitic</td>
<td>1</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>A. aerogenes</em></td>
<td>Cis-aconitic</td>
<td>1</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Citric acid</td>
<td>1</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Citric acid</td>
<td>1</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

A reading of 50 indicates no growth due to the addition compound.
Total volume, 101 ml. Temp., 30°C. 18 hours' incubation time.
effective only for the latter. The effect of both of these compounds is low as compared to the C\textsubscript{4} and C\textsubscript{5} acids.

In all cases where growth was retarded in the absence of CO\textsubscript{2}, abundant growth did take place upon the addition of any of these compounds which were shown to substitute for CO\textsubscript{2}. This phenomenon was observed even when no further increase of growth resulted upon the introduction of ordinary air (Table 8).

Anaerobic Replacement of Carbon Dioxide

Studies thus far have dealt exclusively with the aerobic metabolism, largely because the compounds substituting for CO\textsubscript{2} were known to occur in the normal aerobic metabolism of bacteria. Further research in this field, however, reveals first that organisms normally requiring CO\textsubscript{2} when growing aerobically require it anaerobically, and secondly, that the same compounds will replace carbon dioxide under anaerobic conditions.

To remove any oxygen that it might contain, nitrogen from a cylinder was passed over reduced copper gauze heated in an electric furnace. It was then passed through a CO\textsubscript{2} absorbing train and finally through the cultures. Greater inocula were used in this work - 2 per cent of a 24 hour culture of \textit{E. coli} and the time of aeration reduced from 18 to 12 hours.

Neither \textit{E. coli} nor \textit{A. aerogenes} will grow anaerobically in the absence of carbon dioxide. Atmospheric CO\textsubscript{2} can be replaced by NaHCO\textsubscript{3}.
Table 8

Effect of Air and α-Ketoglutaric Acid on the Growth of
Escherichia coli after Maximum Growth has been reached in the Absence of CO₂

<table>
<thead>
<tr>
<th>Maximum growth in absence of CO₂</th>
<th>Addition of CO₂</th>
<th>Addition of α-ketoglutarate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>65</td>
<td>80</td>
</tr>
</tbody>
</table>

Total volume of solution 96 ml. (5 ml. removed to check turbidity).
Amount of α-ketoglutarate added is equivalent to 2 mg. per ml.
Readings were made 8 hours after maximum growth in the absence of CO₂ took place.

Temp., 30°C.
(Table 9). These results are in accord with Gladstone, et al (1935). Since no growth takes place in the absence of CO₂ the conclusion can be drawn that the gas has a definite function under anaerobic conditions.

The compounds and the extent to which they replace CO₂ are listed in Table 10. The results are similar to those obtained aerobically with the exception of oxalacetic acid which most effectively replaces CO₂ in the complete absence of oxygen. It is therefore possible that oxalacetic is a key compound in the anaerobic metabolism of the cell.

α-Ketoglutaric acid (as well as glutamic acid) substitutes anaerobically to a greater extent than any of the other compounds, except oxalacetate. Similar results were obtained aerobically. These facts again suggest a possible further fixation over and above the Wood and Werkman reaction. Such a fixation would in normal metabolism (in the presence of carbon dioxide) yield C₅ acids which are more essential to the cell. Indirect evidence has been obtained (Table 11). More abundant growth is obtained with bicarbonate and succinate, malate or fumarate than with any of the acids alone.

A number of compounds metabolically related to those of Table 10 will also replace carbon dioxide anaerobically (Table 12).

In the case of A. aerogenes the function of citric acid under anaerobic conditions should be stressed. Whereas, the effect is comparatively small aerobically, in the absence of oxygen the organism uses this acid very effectively in place of CO₂ (Table 13). E. coli will not utilize citric acid under any of the above conditions.
Table 9

Effect of $\text{NaHCO}_3$ on the Growth of *Escherichia coli* under Anaerobic Conditions

<table>
<thead>
<tr>
<th>Basal medium with</th>
<th>$2 \text{ mM NaHCO}_3$</th>
<th>$0 \text{ mM NaHCO}_3$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30</td>
<td>5</td>
</tr>
</tbody>
</table>

The extent of growth is indicated by turbidimetric readings on the photoelectric colorimeter. A reading of 3 to 9 indicates the amount of growth in the absence of $\text{CO}_2$.

*Total volume, 25.5 cc. Incubation time, 12 hours. Temp. 37°C.*
Table 10

Effect of the Addition of Various Compounds on the Anaerobic Growth of *Escherichia coli* in the Absence of CO\(_2\)

<table>
<thead>
<tr>
<th>C$_3$-Compounds</th>
<th>Growth</th>
<th>C$_4$-Compounds</th>
<th>Growth</th>
<th>C$_5$-Compounds</th>
<th>Growth</th>
<th>C$_6$-Compounds</th>
<th>Growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>DL-Alanine</td>
<td>6</td>
<td>Succinate</td>
<td>30</td>
<td>α-Ketoglutarate</td>
<td>85</td>
<td>Citrate</td>
<td>9</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>4</td>
<td>Fumarate</td>
<td>40</td>
<td>Glutamate</td>
<td>200</td>
<td>Cis-aconitate</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Malate</td>
<td>35</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Oxalacetate</td>
<td>220</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Aspartate</td>
<td>62</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

All of the compounds were used in 2 ml quantities. The extent of growth is indicated by turbidimetric readings on the photoelectric colorimeter.

Control experiments give readings from 3 to 9.

Total volume, 25.5 cc. Incubation time, 12 hours. Temp., 37°C.
### Table 11

**Effect of Added Carbonate on the Growth of *Escherichia coli* in the Presence of C4 Compounds**

<table>
<thead>
<tr>
<th>Compound</th>
<th>With NaHCO₃</th>
<th>Without NaHCO₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>Succinic</td>
<td>55</td>
<td>30</td>
</tr>
<tr>
<td>Fumaric</td>
<td>65</td>
<td>40</td>
</tr>
<tr>
<td>Malic</td>
<td>60</td>
<td>45</td>
</tr>
</tbody>
</table>

All of the compounds including NaHCO₃ were added in 2 ml quantities. The extent of growth is indicated by turbidimetric readings on the photoelectric colorimeter.

Control experiments give readings from 3 to 9.

Total volume, 25.5 ml. Incubation time, 12 hours. Temp. 37°C.
Table 12

The Effect of Glutamine, Asparagine, Arginine and Proline on the Anaerobic Growth of Escherichia coli in the Absence of CO₂

<table>
<thead>
<tr>
<th>Compound</th>
<th>Glutamine</th>
<th>Asparagine</th>
<th>Arginine</th>
<th>Proline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth</td>
<td>125</td>
<td>40</td>
<td>55</td>
<td>30</td>
</tr>
</tbody>
</table>

The extent of growth is indicated by turbidimetric readings on the photoelectric colorimeter.

Compounds were added in 2 ml quantities.

Total volume, 25.5 cc. Incubation time, 12 hours. Temp. 37°C.
Table 13
Replacement of CO₂ by Citric Acid under Aerobic and Anaerobic Conditions

<table>
<thead>
<tr>
<th>Organism</th>
<th>Citric Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nitrogen with O₂ : Air with CO₂ and CO₂ removed</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>9</td>
</tr>
<tr>
<td>Aerobacter aerogenes</td>
<td>180</td>
</tr>
</tbody>
</table>

The extent of growth is indicated by turbidimetric readings on the photoelectric colorimeter.

2 ml quantities of citric acid were used.

Total volume, 25.5 cc. Incubation time, 12 hours. Temp., 37°C.
Enzymatic Fixation of Carbon Dioxide in α-Ketoglutaric Acid

Introductory remarks

During investigations on the heterotrophic assimilation of CO₂ by *E. coli* and *A. aerogenes*, it was shown that the C₅ compounds, α-ketoglutaric acid and its precursor, glutamic acid, were more effective than the other members of the Krebs cycle or their precursors in replacing CO₂ in the metabolic requirements of the organisms. It was proposed that a fixation over and above the Wood and Werkman reaction takes place and that this reaction may be of great importance to the cell. Results have been obtained which show an enzymatic exchange of C¹³O₂ with the carboxyl group of α-ketoglutaric acid, thus revealing a new type of heterotrophic fixation of CO₂ involving a C₄ and C₁ addition.

Enzyme preparations

A cell-free enzyme preparation from *E. coli* decarboxylates α-ketoglutarate to succinate and CO₂ in the presence of malonic acid as an inhibitor of succinate oxidation (Table 14). The reaction is similar to that reported by Ochoa (1944) using cat heart as the source of α-ketoglutaric dehydrogenase. When the bacterial succinic dehydrogenase is blocked by sodium malonate 1 molecule of succinate and 1 molecule of CO₂ are formed for each atom of oxygen taken up in the oxidative decarboxylation of the keto acid. Succinate determinations
Table 14

Oxidative Decarboxylation of α-Ketoglutarate by a Cell-free Enzyme Preparation of Escherichia coli

<table>
<thead>
<tr>
<th>Material Determined</th>
<th>With Malonate</th>
<th>R.Q.</th>
<th>Without Malonate</th>
<th>R.Q.</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Ketoglutarate utilized, ml</td>
<td>0.02</td>
<td></td>
<td>0.002</td>
<td></td>
</tr>
<tr>
<td>Succinic acid formed, ml</td>
<td>0.017</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O₂ uptake, μl</td>
<td>109</td>
<td>2.0</td>
<td>103</td>
<td>1.25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.1</td>
<td></td>
<td>1.30</td>
</tr>
<tr>
<td>CO₂ liberated, μl</td>
<td>239</td>
<td></td>
<td>135</td>
<td></td>
</tr>
</tbody>
</table>

Total volume of reactants 2.8 ml. 0.0035 M α-Ketoglutarate, 0.05 M PO₄ buffer pH 6.8, 1 cc. bacterial juice per cup. Temp. 30.4°C.

All values are corrected for enzyme blanks.

1 0.031 M sodium malonate

2 Time, 2 hours.
by the silver salt method or by the oxidation with succinic dehydro-
genase obtained from beef heart agreed well with the values for oxygen uptake and CO₂ evolved. No attempts were made to determine the products when malonate was omitted since the respiratory quotient of 1.2 to 1.3 obtained (theoretical 1.25) indicated a complete oxidation of α-ketoglutarate to CO₂ and water.

The cell-free enzyme preparations are generally not highly active on α-ketoglutarate or succinate but were used because malonic acid does not block the oxidation of succinate when the intact bacterial cell is employed (Table 15). It was assumed that malonate did not penetrate the cell, therefore the bacteria were treated with various solvents to increase the permeability of the cell wall to the inhibitor. However, no satisfactory results were obtained. Quastel and Wooldridge (1922) have shown that exposure of E. coli to toluene brings about a number of inactivations. The enzymes for lactic, succinic and formic acids were found intact. Our results are in agree-
ment. Toluene and acetone treated cells remain active on succinate and become permeable to malonate; however, the same cells show no activity on α-ketoglutarate. n-Propyl alcohol destroys both enzymes.

1,2-Ethanedisulfonate

Recently, Klotz and Tietze (1947) reported on the inhibition of succinic acid oxidation by structurally related sulfonic acids using rat liver homogenates. No such inhibition was noted with bacterial cells (Table 16). However, in the case of bacterial juices, high
Table 15

Effect of Malonic Acid on Succinic Dehydrogenase

<table>
<thead>
<tr>
<th>Enzyme Preparation</th>
<th>Methods</th>
<th>Substrates</th>
<th>Manometric; Oxygen uptake μl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Succinate</td>
<td>Succinate + Malonate</td>
</tr>
<tr>
<td>Suspension of washed Escherichia coli</td>
<td>446</td>
<td>516</td>
<td>186</td>
</tr>
<tr>
<td>Toluene treated suspension of E. coli</td>
<td>103</td>
<td>3</td>
<td>18</td>
</tr>
<tr>
<td>Acetone treated suspension of E. coli</td>
<td>201</td>
<td>63</td>
<td>62</td>
</tr>
<tr>
<td>n-Propyl alcohol treated suspension of E. coli</td>
<td>-6</td>
<td>-2</td>
<td>23</td>
</tr>
<tr>
<td>Cell-free enzyme preparation of E. coli</td>
<td>93</td>
<td>65</td>
<td>257</td>
</tr>
</tbody>
</table>

Manometric data: Total volume of reactants 2.3 ml. 0.0043 M α-keto-glutarate buffer, pH 7.0. Bacterial suspension were added at the rate of 30 mg. per cup. Temp. 30.4°C.

10.0066 M sodium malonate.

Thunberg data: Total volume 6.0 ml. 0.0033 M succinate, 0.0066 M malonate, suspensions - 0.5 cc. of a 10% suspension wet weight. Dilution of methylene
### Table 15

**Ionic Acid on Succinic Dehydrogenase**

<table>
<thead>
<tr>
<th>Methods of Investigation</th>
<th>Thunberg; Reduction of M.B.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Time</td>
</tr>
<tr>
<td></td>
<td>Hours</td>
</tr>
<tr>
<td><strong>Substrates</strong></td>
<td></td>
</tr>
<tr>
<td>Succinate + Malonate</td>
<td></td>
</tr>
<tr>
<td><strong>Substrates</strong></td>
<td></td>
</tr>
<tr>
<td>α-Ketoglutarate</td>
<td></td>
</tr>
<tr>
<td>α-Ketoglutarate + Malonate</td>
<td>30</td>
</tr>
<tr>
<td>Succinate</td>
<td>516</td>
</tr>
<tr>
<td>Malonate</td>
<td>-10</td>
</tr>
<tr>
<td>α-Ketoglutarate</td>
<td>62</td>
</tr>
<tr>
<td>Malonate</td>
<td>23</td>
</tr>
<tr>
<td>α-Ketoglutarate + Malonate</td>
<td>-6</td>
</tr>
<tr>
<td>Succinate</td>
<td>65</td>
</tr>
<tr>
<td>Malonate</td>
<td></td>
</tr>
</tbody>
</table>

0.0043 M α-ketoglutarate and succinate respectively, 0.05 M PO₄, led at the rate of 30 mg. per cup dry weight, bacterial juice, 1 cc. per succinate, 0.0066 M malonate, 0.066 M PO₄ buffer, pH 7.2. Bacterial weight. Dilution of methylene blue 1/5000.
Table 16

Oxidation of Succinic Acid in the Presence of 1,2-Ethane Disulfonic Acid

<table>
<thead>
<tr>
<th>Enzyme Preparation</th>
<th>Substrate</th>
<th>Oxygen Uptake</th>
<th>Time (hours)</th>
<th>Per cent Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suspension of washed Escherichia coli</td>
<td>Succinate</td>
<td>312</td>
<td>0.6</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>Succinate+ Inhibitor</td>
<td>352</td>
<td>0.3</td>
<td>95</td>
</tr>
<tr>
<td>Toluene Treated E. coli</td>
<td>Succinate</td>
<td>107</td>
<td>2</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>Succinate+ Inhibitor</td>
<td>103</td>
<td>2</td>
<td>80</td>
</tr>
<tr>
<td>Cell-free Enzyme Preparation of E. coli</td>
<td>Succinate</td>
<td>224</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Succinate+ Inhibitor</td>
<td>60</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Manometric data: Total volume 2.3 ml. 0.0043 M succinate, 0.05 M PO₄ buffer, pH 7.2. 0.08 M 1,2-ethane disulfonic acid. Bacterial suspensions were added at the rate of 30 mg. per cup dry weight. Bacterial juice, 1 cc. per cup. Temp. 30.4°C.

Thunberg data: Total volume, 6.0 ml. 0.0033 M succinate, 0.16 M inhibitor, 0.066 M PO₄ buffer, pH 7.0. Bacterial suspensions, 0.5 cc. of a 10 per cent suspension wet weight. Dilution of methylene blue 1/5000.
concentrations are effective. The endogenous activity of the juice remains essentially unchanged even when a concentration as high as 0.08 M of inhibitor is used. Malonate not only inhibits succinic dehydrogenase but reduces the endogenous activity as well. Since toluene treated cells remain active on succinic acid, this treatment does not alter permeability to 1,2-ethanedisulfonic acid. Type of the inhibition is yet to be determined. Apparently the sulfonate ion forms an enzyme complex of almost the same strength as that with malonate.

Exchange reaction defined as fixation reaction

Any exchange reaction involving CO₂ and resulting in the formation of a carbon-to-carbon linkage is considered to be a fixation reaction. Relative to the problem at hand, if the enzymatic decarboxylation of α-ketoglutarate is reversible, exchange with carbon dioxide should occur during decarboxylation. Regardless of how far the equilibrium point of the reaction is to the side of decarboxylation, part of the succinate thus formed will be carboxylated to α-ketoglutarate, owing to the dynamic nature of equilibria.

In general, the method employed was to allow the reactions to occur in the presence of NaHCO₂ until approximately one-half of the original substrate remained. The residual substrates were degraded and the C₁³ content of the fragments determined on the mass spectrometer.
Fixation of carbon dioxide in \( \alpha \)-ketoglutaric acid

Because of the difficulties with intact cells, it was necessary to employ a cell-free preparation which not only decarboxylated \( \alpha \)-ketoglutarate to succinate and \( \text{CO}_2 \) but under the proper experimental conditions carboxylated succinate to form the \( \text{C}_5 \) keto acid (Table 17).

It is apparent that the decarboxylation of \( \alpha \)-ketoglutaric acid is reversible. The per cent excess of \( \text{C}^{13} \) in the \( \alpha \)-carboxyl of the keto acid was much higher when the reaction was conducted in the presence of malonate and adenosine triphosphate. In the absence of malonate the products of the reaction were water and \( \text{CO}_2 \). As soon as succinate was formed, it was oxidized. No detectible carboxylation of succinate occurred, consequently the reaction is not recognized as reversible. Since the error of the mass spectrometer is \( \pm 0.02 \), a per cent excess of 0.06 per cent is questionable.

In the presence of malonate succinic acid accumulates to enhance the fixation of \( \text{CO}_2 \). At approximately half time of the reaction carboxylation is readily detectable.

Adenosine triphosphate

In common with other carboxylations, this one is endergonic as is borne out by enhancement of the carboxylation by adenosine triphosphate (Table 17).
Table 17

Exchange of Heavy Carbon Dioxide in the Carboxyl Gr
of α-Ketoglutaric Acid during Enzymatic Decarboxyla

<table>
<thead>
<tr>
<th>Expt. No.</th>
<th>ATP Added</th>
<th>α-Ketoglutarate Added</th>
<th>α-COOH of α-Ketoglutarate Recovered</th>
<th>Excess C(^{13}) Fixed</th>
<th>Residual NaHC(^{13}O)</th>
<th>Rinse</th>
<th>Excess C(^{13}) Recovered</th>
<th>Excess C(^{13})</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.25 mg.</td>
<td>0.25 mM</td>
<td>0.16 mM</td>
<td>0.171 mM</td>
<td>2.7</td>
<td>2.79</td>
<td>0.75</td>
<td>0.00</td>
</tr>
<tr>
<td>2</td>
<td>0.25</td>
<td>0.15</td>
<td>0.00</td>
<td>0.00</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.25</td>
<td>0.09</td>
<td>0.06</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>4a**</td>
<td>0.25</td>
<td>0.18</td>
<td>0.102</td>
<td>1.3</td>
<td>1.3</td>
<td>1.3</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>4b</td>
<td>0.25</td>
<td>0.19</td>
<td>0.372</td>
<td>7.0</td>
<td>7.0</td>
<td>7.0</td>
<td>7.0</td>
<td></td>
</tr>
</tbody>
</table>

Reactions were carried on in 125 ml. Erlenmeyer flasks with 2 side arms which were attached manometers. The final concentration of the α-ketoglutarate was 0.01 M and that of malate activity of the juice, a quantity varying between 5 and 10 ml. were added to each flask with 0.2 M phosphate, pH 6.6 and 0.07 M NaHC\(^{13}O\) (3.113% excess). Appropriate concentration of the substrate and enzyme after temperature equilibration (30°C) has been re-
mixture was approximately 7.2 and the volume 25 or 30 ml.

*mg* represents amounts recovered after treatment with NaHC\(^{12}O\).

**Experiments 4a and 4b were carried out simultaneously.
### Table 17

Heavy Carbon Dioxide in the Carboxyl Group
of L-Aspartate during Enzymatic Decarboxylation

<table>
<thead>
<tr>
<th>Sample</th>
<th>Residual NaHCO$_3$</th>
<th>Rinse</th>
<th>Malonate</th>
<th>COOH Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fixed Excess C$_{13}$</td>
<td>Recovered</td>
<td>Excess Malonate</td>
<td>Recovered</td>
</tr>
<tr>
<td>1</td>
<td>$10^{-3}$ m$^3$</td>
<td>%</td>
<td>mM</td>
<td>%</td>
</tr>
<tr>
<td>1.25</td>
<td>2.7</td>
<td>2.79</td>
<td>0.75</td>
<td>0.00</td>
</tr>
<tr>
<td>0.5</td>
<td>0.0</td>
<td>1.25</td>
<td>Not added</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>1.8</td>
<td>1.25</td>
<td>1.23</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>7.0</td>
<td>1.25</td>
<td>1.25</td>
<td></td>
</tr>
</tbody>
</table>

Flasks with 2 side arms which were attached to the Warburg-Barcroft apparatus. The concentration of L-ketoglutarate was 0.01 M and that of malonate 0.05 M. Depending on the concentration, 5 and 10 ml. were added to each flask. The mixture was buffered to pH 7.5 (3.113% excess). Appropriate concentrations of the 2 buffers were added to each sample when equilibrium (30.4°C.) has been reached. The pH of the resulting mixture was 7.5. The mixture was then titrated with NaHCO$_3$. The reaction was carried out simultaneously.

---

**Calculations:**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Residual NaHCO$_3$</th>
<th>Rinse</th>
<th>Malonate</th>
<th>COOH Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fixed Excess C$_{13}$</td>
<td>Recovered</td>
<td>Excess Malonate</td>
<td>Recovered</td>
</tr>
<tr>
<td>1</td>
<td>$10^{-3}$ m$^3$</td>
<td>%</td>
<td>mM</td>
<td>%</td>
</tr>
<tr>
<td>1.25</td>
<td>2.7</td>
<td>2.79</td>
<td>0.75</td>
<td>0.00</td>
</tr>
<tr>
<td>0.5</td>
<td>0.0</td>
<td>1.25</td>
<td>Not added</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>1.8</td>
<td>1.25</td>
<td>1.23</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>7.0</td>
<td>1.25</td>
<td>1.25</td>
<td></td>
</tr>
</tbody>
</table>

---

**Notes:**

- Flasks with 2 side arms were attached to the Warburg-Barcroft apparatus.
- The concentration of L-ketoglutarate was 0.01 M and that of malonate 0.05 M.
- Depending on the concentration, 5 and 10 ml. were added to each flask. The mixture was buffered to pH 7.5 (3.113% excess).
- Appropriate concentrations of the 2 buffers were added when equilibrium (30.4°C.) was reached.
- The pH of the resulting mixture was 7.5.
- The mixture was then titrated with NaHCO$_3$.

---

**Additional Information:**

- Calculations were performed to determine the amount of carbon dioxide in the carboxyl group.
- The reaction was carried out simultaneously.

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**Reference:**

- Table 17: Heavy Carbon Dioxide in the Carboxyl Group of L-Aspartate during Enzymatic Decarboxylation.
It should be pointed out that at times reversibility could not be demonstrated. This may have been due to faulty procedure in the preparation of the juice. For example, when the juice was not centrifuged long enough to remove all of the unground cells, enough of them will be present to oxidize much of the succinate formed since their succinic dehydrogenase is not blocked by malonate, and consequently no CO₂ can be shown to be fixed. Partial denaturation of the enzyme or some component of the enzyme system may also account for experiment 2 in Table 17. However, the discrepancies in this experiment are not too serious when compared with the overwhelming evidence for the reversibility (Expts. 1, 3, 4a and 4b).

Components of the Enzyme System

**Inorganic phosphate, adenosine triphosphate and magnesium ions**

It is at present difficult to ascertain whether one or more enzymes are involved in the oxidative decarboxylation of the keto acid. Some of the components involved have been identified by activating the enzyme after it has lost its entire activity as a result of dialysis. Inorganic phosphate, adenosine triphosphate and magnesium ions make up for all that has been dialized out in 3 hours against ice-cold distilled water (Table 18). The addition of biotin to the enzyme system containing phosphate, Mg and ATP increased the oxygen uptake from 204 μl as given in Table 18 to 252 μl, a value almost equal to
Table 18

Some Components of α-Ketoglutaric Acid Dehydrogenase

<table>
<thead>
<tr>
<th>Expt.</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>57</td>
<td>74</td>
<td></td>
</tr>
<tr>
<td>73</td>
<td>74</td>
<td></td>
</tr>
<tr>
<td>143</td>
<td>204</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>3.0 ml</td>
<td>3.0 ml</td>
<td></td>
</tr>
</tbody>
</table>

Additions to dialized juice:

- HO₄⁻ : PO₄³⁻ : Mg²⁺ : ATP : PO₄³⁻ + Mg²⁺ : PO₄³⁻ + Mg²⁺ + ATP + boiled juice

Total volume of reactants varied from 2.3 to 3.0 ml.

Expt. 1 - 0.0043 M α-ketoglutaric acid, 0.043 M PO₄ buffer pH 7.0, 0.0043 M MgCl₂, 0.001 M ATP, 1 cc. of dialized juice.

Expt. 2 - 0.0033 M α-ketoglutaric acid, 0.033 M PO₄ buffer pH 7.0, 0.0033 M MgCl₂, 0.001 M ATP, 1 cc. of dialized juice and 0.5 cc. of boiled (10 min.) juice.

Temp. 30°C. Time, 3 hours.

In both experiments 0.003 M malonate was used.

Appropriate enzyme blanks were deducted from substrate values.
the oxygen uptake in the presence of boiled juice. Since malonate is not a perfect block for the oxidation of \( \alpha \)-ketoglutarate to succinate and \( \text{CO}_2 \), it is difficult to ascertain accurately whether this vitamin is actually involved in the oxidative decarboxylation of the keto acid or in some subsequent step during this oxidation. Since experiments already conducted indicate the involvement of biotin in succinic acid oxidation, little attention ought to be given to the increased oxygen uptake in the presence of either biotin or boiled juice as far as \( \alpha \)-ketoglutaric acid dehydrogenase is concerned. The enzyme components may therefore considered to be phosphate, magnesium, and ATP.

\[
\text{Zn}^{++}, \text{Co}^{++}, \text{Ni}^{++}, \text{In}^{++}
\]

In an attempt to replace some of the above components of \( \alpha \)-ketoglutaric dehydrogenase with other substances it was found that manganese and to a less extent nickel can be used instead of magnesium, whereas other divalent ions such as zinc and cobalt are inhibitory even in minute concentrations (Table 19). It is doubtful whether nickel has any physiological function but its partial replacement for magnesium is significant from a theoretical standpoint in that it may indicate a rather general function of divalent ions in metabolism.
Table 19

Replacement of Magnesium by Divalent Ions

<table>
<thead>
<tr>
<th>Ions</th>
<th>Mg$^{++}$</th>
<th>Ca$^{++}$</th>
<th>Co$^{++}$</th>
<th>Zn$^{++}$</th>
<th>Mn$^{++}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conc.</td>
<td>0.0043 M</td>
<td>0.0004 M</td>
<td>0.0043 M</td>
<td>0.0004 M</td>
<td>0.0043 M</td>
</tr>
<tr>
<td>O$_2$ uptake $\mu$l</td>
<td>120</td>
<td>41</td>
<td>-3</td>
<td>-28</td>
<td>-49</td>
</tr>
</tbody>
</table>

Total volume of reactants 2.3 ml. The various ions were added to 0.0043 M $\alpha$-ketoglutarate, 0.0033 M malonate, and 1 cc. of dialized juice.
Bacterial \(\alpha\)-ketoglutaric acid dehydrogenase—an \(-\text{SH} \) enzyme

The question of zinc was further investigated. Barron and Kalnitsky (1947) succeeded in inhibiting succinoxidase by heavy metals such as zinc and reactivate the enzyme by the addition of dithiols. A similar experiment was conducted using glutathione and cysteine as the reactivating agents after poisoning the \(\alpha\)-ketoglutaric dehydrogenase with \(\text{ZnCl}_2\). The results are given in Table 20. The fact that glutathione almost completely reverses the inhibition due to zinc strongly suggests that the inhibition produced by this heavy metal is a result of tying up some of the \(-\text{SH} \) groups of the protein moiety of the enzyme system involved.

Diphosphothiamin

Stumpf, et al. (1947) reported the involvement of diphosphothiamin both in the oxidation of \(\alpha\)-ketoglutarate as well as pyruvate. Under our conditions thiamin pyrophosphate has no function whatsoever (Table 21). It can also be seen from the results in the same table that adenylic acid will not replace ATP in \(\alpha\)-ketoglutaric acid dehydrogenase.

Hydrogen carriers

The possible route of hydrogen transfer was also investigated. The function of the \(\text{C}_4 \) dicarboxylic acids as hydrogen carriers in metabolic systems as postulated by Szent-Györgyi is still questionable.
**Table 20**

Inhibition (by ZnCl$_2$) and Reactivation (by glutathion) of α-Ketoglutaric Acid Dehydrogenase

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Activator</th>
<th>Oxygen uptake µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>Zn</td>
<td>-</td>
<td>39</td>
</tr>
<tr>
<td>Zn</td>
<td>glutathione</td>
<td>85</td>
</tr>
<tr>
<td>Zn</td>
<td>cysteine</td>
<td>-32</td>
</tr>
</tbody>
</table>

Total volume 2.8 ml. 0.0036 M α-ketoglutarate, 0.0021 M malonate, 0.0056 M PO$_4$ buffer, pH 7.0, 0.0003 M ZnCl$_2$, 0.001 M glutathione, 0.001 M cysteine. NaOH in center well. 1 cc. of Escherichia coli juice. Temp. 30.4°C.
Table 21

Function of Coenzyme, Adenosine Triphosphate and Adenylc Acid in α-Ketoglutaric Acid Dehydrogenase

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Coenzyme</th>
<th>Mg++</th>
<th>Coenzyme</th>
<th>Mg++</th>
<th>PO_4^-</th>
<th>PO_4^-</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A.T.P. + Hg++</td>
<td>A.A. + Hg++</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>-3</td>
<td>73</td>
<td>-10</td>
<td>75</td>
<td>57</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Total volume of reagents 5.0 mL. 0.0033 M α-ketoglutaric acid, 0.033 M PO_4 buffer, pH 7.0, 0.0035 M MgCl_2, 0.001 M ATP, 25 gamma per cup coenzyme, 0.013 M adenylc acid and 0.035 M malonate. 1 cc. dialized juice. Temp. 30.4°C.
particular in bacterial respiration. However, in our experiments a noticeable increase in oxygen uptake was observed when fumarate and malate were added to \( \alpha \)-ketoglutaric acid (Table 22). Oohoe (1944) reports no such increase. Our results are of particular interest since malate or fumarate were not appreciably attacked by the enzyme preparation and yet when added to the keto acid increased oxygen utilization. It may be assumed that a function of the \( C_4 \) dicarboxylic acids, e.g., succinate, malate and fumarate, is the catalytic hydrogen transfer from substrate to oxygen.

Other possible hydrogen carriers have also been determined. The poisoning of the entire system by cyanide indicates that cytochrome is involved (Table 23). Moniodoacetate also completely inhibits the enzyme system presumably by blocking some dehydrogenase and the inhibition due to fluoride suggests that a phosphorylated compound (or compounds) plays a part.

**Properties of enzyme**

The affinity between \( \alpha \)-ketoglutaric acid dehydrogenase obtained from *E. coli* and substrate is moderate. The amount of oxygen uptake may vary from 7 to 150 \( \mu \)l in the first hour.

The enzyme is relatively stable over a period of weeks when kept frozen. Lyophilizing the juice results in loss of activity. When the oxidation is carried out in an atmosphere of oxygen the rate of the reaction is increased. Optimum activity is at pH 7.0, approximately.
Table 22

$C_4$ Dicarboxylic Acids

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Concentration; M</th>
<th>Oxygen uptake</th>
<th>ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Succinate</td>
<td>0.010</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>Fumarate</td>
<td>0.010</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>Malate</td>
<td>0.010</td>
<td>-5</td>
<td></td>
</tr>
<tr>
<td>α-Ketoglutarate</td>
<td>0.010</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>α-Ketoglutarate</td>
<td>0.010</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td>Fumarate</td>
<td>0.0017</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-Ketoglutarate</td>
<td>0.010</td>
<td>39</td>
<td></td>
</tr>
<tr>
<td>Malate</td>
<td>0.0017</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Total volume 2.3 ml. 0.054 M $PO_4$ buffer, pH 7.0
0.0017 M malonate. 1 cc. *Escherichia coli* juice.
Temp. 30.4°C.
### Table 23

Effect of Inhibitors on the Oxidation of α-Ketoglutaric Acid by *Escherichia coli* Juice

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration</th>
<th>Point of Inhibition</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyanide</td>
<td>0.0003 M</td>
<td>Cytochrome</td>
<td>Completely inhibits</td>
</tr>
<tr>
<td>Iodoacetate</td>
<td>0.008 M</td>
<td>Dehydrogenases</td>
<td>Completely inhibits</td>
</tr>
<tr>
<td>Fluoride</td>
<td>0.008 M</td>
<td>Phosphoric esters</td>
<td>Inhibits</td>
</tr>
</tbody>
</table>

Total volume 2.8 ml. 0.0088 M α-ketoglutarate, 0.0036 M malonate, 0.017 M PO₄ buffer, pH 7.0. 1 cc. *E. coli* juice. Temp. 30.4°C.
Other Reactions of α-Ketoglutarate

In the presence of malonate 1 mole of succinate and 1 mole of carbon dioxide are obtained from the oxidation of 1 mole of α-ketoglutarate according to the following equation:

\[
\text{COOHCH}_2\text{CH}_2\text{COOCOOH} + \text{O} \rightleftharpoons \text{COOHCH}_2\text{CH}_2\text{COOH} + \text{CO}_2
\]

Stumpf, et al (1947) obtained from animal tissues enzyme preparations which in the presence of coenzyme A and magnesium catalyzed the anaerobic decarboxylation of α-ketoglutarate to succinate semialdehyde; its oxidative decarboxylation may be a result of the following 2 reactions:

\[
\text{COOHCH}_2\text{CH}_2\text{COOCOOH} \rightarrow \text{COOHCH}_2\text{CH}_2\text{CHO} + \text{CO}_2
\]

\[
\text{COOHCH}_2\text{CH}_2\text{CHO} + \frac{1}{2} \text{O}_2 \rightarrow \text{COOHCH}_2\text{CH}_2\text{COOH}
\]

This possibility was investigated with our enzyme preparation. If the aldehyde of succinic acid is an intermediate in this reaction, then 1 mole of CO₂ should be liberated for each mole of α-ketoglutarate under anaerobic conditions. Our results are similar to those of Ochoa (1944). In the presence of oxygen-free nitrogen, the cell-free preparation of E. coli, or the intact cells themselves, whether freshly harvested or lyophilized and stored, fail to catalyze the anaerobic decarboxylation of α-ketoglutarate with or without malonate (Table 24). This fact also excludes the possibility of α-ketoglutaric acid dismutating as reported by Weil-Kalherbe (1937) for some animal tissues.
Table 24

Anaerobic Decarboxylation of α-Ketoglutaric Acid

<table>
<thead>
<tr>
<th>Enzyme preparation</th>
<th>Washed suspension</th>
<th>Lyophilized cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO₂ evolved (μl)</td>
<td>2</td>
<td>17</td>
</tr>
</tbody>
</table>

1. Total volume of reactants 2.3 ml. anaerobic; 0.0043 M α-ketoglutaric acid, 0.043 M PO₄ buffer, pH 7.0, temp. 30.4°C, 1.0 cc of cell-free juice.

2. Total volume 2.0 ml. anaerobic; 0.0125 M α-ketoglutaric acid, 0.043 M PO₄ buffer, pH 7.0, temp. 30.4°C, 10 per cent suspension of cells (0.5 cc).

3. Same as 2. Lyophilized cells were used at the rate of 30 mg./cup.

Note: All of the above preparations oxidized α-ketoglutaric acid in the presence of oxygen.
Involvement of Biotin in Succinic Acid Oxidation

Introductory remarks

As was already pointed out, an increased oxygen uptake was noted when biotin was added to a dialyzed cell-free extract of E. coli in the presence of α-ketoglutaric acid. This increase was appreciably lower when malonate was added. It was, therefore, likely that the vitamin functions in some step involving the oxidation of succinic acid. This was found to be the case. Dialysis against phosphate and potassium chloride results in an appreciable reduction of activity of succinic dehydrogenase. On addition of biotin activity of this enzyme increases and in some instances up to three fold. Evidence will be presented that succinic dehydrogenase is affected by biotin and not the formation of a precursor or immediate product of oxidation of the dicarboxylic acid.

Oxidation of succinic acid with and without biotin

The activity of bacterial succinic dehydrogenase is appreciably reduced when a cell-free extract of E. coli is dialyzed against phosphate buffer and dilute potassium chloride solutions. This activity can be largely restored on the addition of small amounts of biotin (Table 25). In some instances the addition of as little as 0.025Y biotin resulted in a considerable activation of the enzyme. The endogenous O₂ uptake has been included in Table 25 to point out that
### Table 25

**Function of Biotin in Succinic Acid Oxidation**

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Additions to Succinate</th>
<th>Oxygen uptake, ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Endogenous</td>
</tr>
<tr>
<td>1</td>
<td>Dialyzed juice</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Dialyzed juice + 0.05γ biotin</td>
<td>8</td>
</tr>
<tr>
<td>2</td>
<td>Dialyzed juice</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Dialyzed juice + 0.05γ biotin</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>Non-dialyzed juice</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Non-dialyzed juice (kept in dialysis bath)</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>Dialyzed juice</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Dialyzed juice + 0.025γ biotin</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Dialyzed juice + 0.05γ biotin</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>Dialyzed juice + 0.075γ biotin</td>
<td>7</td>
</tr>
<tr>
<td>3</td>
<td>Dialyzed juice</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Dialyzed juice + 0.05γ biotin</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Dialyzed juice + 0.1γ biotin</td>
<td>16</td>
</tr>
</tbody>
</table>

Total volume of reactants = 2.0 ml, conc. of succinate 0.013 M, 0.5 ml. of juice obtained from cells grown on a medium which contained succinate as the sole source of carbon.

Temp. 30.4°C. Time 30 min.

Juice dialyzed against 0.013 L phosphate buffer, pH 6.8 for 3 hours followed by a 2 hour dialysis against 0.4 per cent potassium chloride at 15-17°C.
the increased activity of succinic dehydrogenase due to the added biotin is specific and not the result of a general activation.

Apparently it was not possible to remove biotin completely, thus the rate of $O_2$ uptake is the same for the first few minutes for juice with and without added biotin (Fig. 5). When the oxygen uptake is plotted against time, the curves for juice alone and juice with added biotin are super-impossible and only after 15 minutes is an appreciable difference noted. There is apparently enough biotin remaining in the cell-free extract after dialysis to initiate the reaction, but soon after additional amounts are required to permit the reaction to continue at an appreciable rate.

**Some components of succinic dehydrogenase**

Axelrod, et al (1941) have shown that succinic dehydrogenase is activated by calcium ions. The oxidation of succinic acid by bacteria is apparently also dependent upon this divalent ion (Table 26). The necessity for phosphate in the dehydrogenation of the dicarboxylic acid is questionable.

Extracts from _E. coli_ have proved to be relatively inactive aerobically. The activity of such extracts on succinic acid has been increased by growing the organisms on media which contained the dicarboxylic acid (Table 27). The increase in succinic acid oxidation due to the added biotin is also significantly greater when the source of the enzyme is a cell-free preparation from organisms grown on succinate medium.
Fig. 5. Comparative utilization of oxygen by a cell-free extract of *Escherichia coli* with and without biotin.

Total volume of reagents 2.0 ml. Conc. of succinate 0.013 M, 0.5 ml. of juice obtained from cells grown on medium with succinate as source of carbon, 0.05 \( \mu \)g biotin per cup. Temp. 30.4°C. Time 30 min.

Dialysis against phosphate and KCl as in Table 25.
Table 26

Some Components of Succinic Dehydrogenase

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Additions to dialyzed juice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>None</td>
</tr>
<tr>
<td>1</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

Total volume of reactants 2.8 ml. Conc. of succinate 0.0069 M, 0.5 ml. of juice obtained from cells grown on a medium which contained in addition to ammonium sulfate and potassium phosphate, glucose and yeast extract, 0.029 M P₀₄ buffer pH 7.0, 0.0004 M calcium hydroxide, 0.5 ml. boiled juice (10 min.). Temp. 30.4°C.

Juice dialyzed against distilled water for 3 hours at 1-3°C.
### Table 27

**Effect of Growth Medium on the Activity of Succinic Dehydrogenase**

| Carbon source in medium | Substrate       | Oxygen uptake, µl |  |  |  |  |
|-------------------------|-----------------|------------------|------------------|------------------|------------------|
|                         | Non-dialyzed    | Dialyzed juice   | With biotin      | Without biotin   | Kept in dialysis |
|                         | juice           |                  | biotin           |                  | bath             |
| 0.6% glucose            | Sodium          | 57               | 54               | 49               | 60               |
| 0.4% yeast extract: succinate | (vary from 57-78) |                  |                  |                  |                  |
| 2% succinate            | Sodium          | 167              | 107              | 68               | 116              |
| 0.35% peptone           | succinate       | (vary from 137-171) |                  |                  |                  |

Total volume of reactants 2.0 ml. Conc. of succinate 0.015 M, 0.5 ml. of juice obtained from cells grown on medium as indicated in Table, 0.05% biotin per cup. Temp. 30.4°C. Time, 30 min.

Juice dialyzed as in Table 25.
Dialysis

It might be well to consider dialysis as employed to obtain a biotin-deficiency. Microbiological assays of the medium before and after dialysis showed a definite increase in the biotin content. Thus in a typical experiment, addition of a given quantity of dialysate to a biotin deficient medium failed to support the growth of Saccharomyces cerevisiae at the start of the experiment. After 2 or 3 hours' dialysis addition of the same quantity of dialysate did support growth.

Distilled water or 0.013 M phosphate buffer, pH 6.8, followed by 0.4 per cent potassium chloride were used as dialyzing media. Biotin was more efficiently removed by the latter. After 5 hours' dialysis against water, an increase of 19 μL O₂-uptake was obtained on the addition of 0.1N biotin, whereas an increase of 56 μL resulted with a juice dialyzed against phosphate and KCl.

Enzyme studies

The rate of oxygen utilization is increased with increased vitamin concentrations (Fig. 6). When the quantity of substrate or enzyme is varied with constant biotin concentration, similar results are observed.

When the concentration of substrate is too low to saturate the enzyme surface, the rate is low. When an excess of substrate is added (e.g., 0.5 cc. of 0.04 N succinate) the rate decreases (Fig. 7).
Fig. 6. Effect of increased concentrations of biotin upon the oxygen uptake.

Total volume of reactants 2.3 ml. Conc. of succinate 0.01 M, 0.5 ml. of juice obtained from cells grown on media with succinate as source of carbon. Temp. 30.4°C. Time 30 min. Juice dialyzed against phosphate and KCl as in Table 25.
Fig. 7. Effect of increased substrate concentration upon the oxygen uptake.

Total volume of reactants 2.3 ml. 0.1 M biotin per cup, 0.5 ml. juice obtained from cells grown on media with succinate as source of carbon. Temp. 30.4°C. Juice dialyzed against phosphate and KCl as in Table 25.
The results shown in Fig. 8 indicate that succinate dehydrogenase activity in *E. coli* juice is directly proportional to the concentration of juice within the limits of 0.1 and 0.3 ml. of bacterial cell-free extract. Beyond 0.3 ml. activity decreases.

**The specificity of biotin action**

The degree of specificity of biotin toward succinate oxidation has been determined by using various substrates (Table 28). Since biotin has been reported to play a role in the Wood and Werkman reaction, its effect on fumarate and malate metabolism — precursors of oxaloacetic acid — was determined. The results clearly confirm the specificity of the biotin for succinate dehydrogenation, since dialyzed juice hardly attacks either fumarate or malate, and the rate of oxidation is hardly altered on the addition of biotin.

It should be mentioned that occasionally cell-free preparations have been obtained on which the effect of biotin can be demonstrated prior to dialysis. This effect may result from insufficient amounts of biotin in the preparation (Table 28).

Various biotin analogues were tested for possible biotin-like activity (Table 29). DL-homobiotin and desthiobiotin are effective.
Fig. 8. Effect of increase enzyme concentration upon the oxygen uptake.

Total volume of reagents 2.3 ml. Conc. of succinate 0.01 M, 0.1 N biotin per cup. Juice obtained from cells grown on media with succinate as source of carbon. Temp. 30.4°C. Time 30 min. Juice dialyzed against phosphate and KCl as in Table 25.
Table 28

Effect of Biotin on the Oxidation of Various Substrates

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Succinate</th>
<th>Fumarate</th>
<th>1-Malate</th>
<th>Citrate</th>
<th>Aspartate</th>
<th>Acetate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dialed^1 cell-free prep.</td>
<td>With biotin</td>
<td>93</td>
<td>0</td>
<td>21</td>
<td>16</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>Without biotin</td>
<td>55</td>
<td>21</td>
<td>7</td>
<td>9</td>
<td>12</td>
</tr>
<tr>
<td>Non-dialed cell-free prep.</td>
<td>With biotin</td>
<td>184</td>
<td>8</td>
<td>5</td>
<td>14</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Without biotin</td>
<td>123</td>
<td>8</td>
<td>11</td>
<td>23</td>
<td>6</td>
</tr>
</tbody>
</table>

Total volume of reactants 2.3 ml. Conc. of substrate 0.012 M. 0.5 ml. of juice obtained from cells grown on media containing succinate as source of carbon. 0.1 N biotin per cup. Temp. 30.4°C. Time, 30 min.

^1Juice dialyzed against 0.013 M phosphate buffer, pH 6.8 for 3 hours followed by a 2 hour dialysis against 0.4 per cent potassium chloride at 15-17°C.
Table 29

Effect of Biotin Analogues on Succinic Acid Oxidation

<table>
<thead>
<tr>
<th>Biotin and Biotin Analogues</th>
<th>No additions: Biotin</th>
<th>$\gamma$-(3,4-ureylene-cyclohexyl): DL butyric acid</th>
<th>ha</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxygen: Dialyzed cell-free prep.</td>
<td>50</td>
<td>95</td>
<td>45</td>
</tr>
<tr>
<td>Oxygen: Non-dialyzed cell-free prep.</td>
<td>123</td>
<td>184</td>
<td>144</td>
</tr>
</tbody>
</table>

Total volume of reactants 2.3 ml. Conc. of succinate 0.012 M, 0.5 ml. juice containing succinate as source of carbon. Conc. of biotin 0.1 M/cup. Conc. of butyric acid 0.1 M/cup.

Time, 30 min.

1 Juice dialyzed as in Table 25.
Table 29
Effect of Biotin Analogues on Succinic Acid Oxidation

<table>
<thead>
<tr>
<th>Compound</th>
<th>Succinate Conc.</th>
<th>Biotin Conc.</th>
<th>Homologues Conc.</th>
<th>Temp. 30.4°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>(3,4-dureylene cyclohexyl)</td>
<td>95</td>
<td>45</td>
<td>100</td>
<td>59</td>
</tr>
<tr>
<td>DL-butyric acid</td>
<td>184</td>
<td>144</td>
<td>202</td>
<td>160</td>
</tr>
<tr>
<td>Homobiotin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-oxo-4-imidazolidine-caproic acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Desthio-biotin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Conc. of succinate 0.012 M, 0.5 ml. juice obtained from cells grown on media containing Succinate. Conc. of biotin 0.1 Y/cup. Conc. of homologues 0.5 Y/cup. Temp. 30.4°C.
Citric Acid

Since Brew and Werkman (1940, 1939) found arsenite, moniodoacetate and bisulfite completely, and fluoride partially, inhibited the breakdown of citric acid by *A. indologenes*; they concluded that the initial breakdown products of this acid were oxalacetate and acetate under both aerobic and anaerobic conditions. To determine the mechanism by which citrate replaces carbon dioxide, various concentrations of the above inhibitors were added to cultures of *A. aerogenes* containing citric acid as the substituent for CO₂. In growth experiments only bisulfite and moniodoacetate completely inhibited the development of the organism, whereas the growth was normal in the presence of fluoride and nearly normal upon the addition of As₂O₃ (Table 30).

The seemingly contradictory results obtained with arsenious oxide and bisulfite (both prevent the breakdown of α-keto acids but by completely different mechanisms, e.g., bisulfite by binding the keto acid and arsenious oxide by inhibiting the oxidative deoxyxylalation) prompted further research to determine more specifically the keto compound which determines the ability of citrate to replace carbon dioxide. From resting-cell and particularly from cell multiplication experiments it appears that oxalacetate may not be the compound formed as the initial breakdown product of citric acid, at
Table 30

Effect of Inhibitors on the Replacement of Carbon Dioxide by Citric Acid

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration in</th>
<th>Point of Inhibition</th>
<th>Growth</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>growth expts.</td>
<td>resting cell expts.</td>
<td>Without CO₂</td>
</tr>
<tr>
<td>Fluoride</td>
<td>0.50 mL</td>
<td>0.0027 M</td>
<td>Phosphoglyceric acid</td>
</tr>
<tr>
<td>Arsenite</td>
<td>0.062</td>
<td>α-Keto acids</td>
<td>270 : 210</td>
</tr>
<tr>
<td>Moniodoacetate</td>
<td>0.031 mL</td>
<td>0.0027 M</td>
<td>Dehydrogenases</td>
</tr>
<tr>
<td>Bisulfite</td>
<td>0.05 mL</td>
<td>0.0015 M</td>
<td>α-Keto acid</td>
</tr>
<tr>
<td>No inhibitor</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Manometric expts. - Total volume 2.3 ml. Each cup contained 30 mg. dry weight of lyophilized cells grown on citrate medium, 0.5 ml. of 0.025 M sodium citrate, 0.5 ml. of 0.1 M PB buffer, pH 7 plus various concentrations of inhibitors as shown in table. Temp. 30.4°C.

Growth expts. - Results in terms of turbidity readings. Each flask contained 0.8% KH₂PO₄, glucose and 10% tap water (for inorganic ions). Total volume, 102 ml. Temp. 30°C. To this 24 hour culture of A. aerogenes. Aeration time 18 hours.

1 Carbon dioxide removed by passing air through 30% NaOH. These tubes contained 0.5 ml sodium bicarbonate.

2 Aeration with air containing normal complement of CO₂. To these tubes no citrate was added.
Table 30
the Replacement of Carbon Dioxide by Citric Acid

<table>
<thead>
<tr>
<th>Point of Inhibition</th>
<th>Growth Without CO₂</th>
<th>Growth With CO₂</th>
<th>0₂ uptake</th>
<th>CO₂ evolved</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphoglyceric acid</td>
<td>310</td>
<td>320</td>
<td>73</td>
<td>235</td>
</tr>
<tr>
<td>α-Keto acids</td>
<td>270</td>
<td>210</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dehydrogenases</td>
<td>15</td>
<td>120</td>
<td>5</td>
<td>29</td>
</tr>
<tr>
<td>α-Keto acid</td>
<td>20</td>
<td>375</td>
<td>307</td>
<td>586</td>
</tr>
</tbody>
</table>

A cup contained 30 mg. dry weight of lyophilized Aerobacter aerogenes sodium citrate, 0.5 ml. of 0.1 M PO₄ buffer, pH 7.0, NaOH or H₂SO₄, shown in table. Temp. 30°C.

Readings. Each flask contained 0.8% KH₂PO₄, 0.4% (NH₄)₂SO₄, 0.8% NaCl. Total volume, 102 ml. Temp. 30°C. To this were added 2 cc. of a solution 18 hours.

1. 30% NaOH. These tubes contained 0.5 mM sodium citrate.
2. 0.4% CO₂. To these tubes no citrate was added.
least under aerobic conditions. When sodium bisulfite is added to Warburg cups containing sodium citrate and cells (grown on citrate medium), no gas exchange above that of the endogenous is observed for the first few hours (Fig. 9), but soon thereafter an oxygen uptake is noted and at the end of the experiment the R.Q. values obtained vary from 1.2 to 1.9. At times a significant gas exchange was observed within the first half hour of the experiment. The theoretical R.Q. for α-ketoglutaric acid to be the intermediate fixed is 2.0 (Table 31). It is possible that the reason the R.Q. is not higher is that α-ketoglutarate is attacked by the resting cells even in the presence of equivalent amounts of bisulfite although to a less extent than in its absence. Oxalacetate, on the other hand, is attacked almost as readily in the presence as in the absence of this keto fixative. The slight increase of CO₂ evolution in the absence of bisulfite from oxalacetic acid is because of the attack on pyruvate, arising from the decarboxylation of the former, probably forming acetyl methylcarbinol and CO₂ or acetate and CO₂. In the presence of bisulfite, pyruvate is fixed. The low value for oxygen uptake in the presence of NaHSO₃ and oxalacetate is not significant since the initial step in the oxidation of the labile C₄ acid involves no oxygen. Objections to these explanations cannot be raised since the spontaneous decarboxylation of oxalacetate is the same in the presence as in the absence of bisulfite (indicating no fixation of the keto compound) and pyruvic acid is fixed in the presence of the keto fixative.
Fig. 9. Effect of bisulfite on the oxidation of citric acid.

Total volume of reactants 2.3 ml. 0.0054 M sodium citrate, 0.0054 M NaHSO₃, 0.047 M phosphate buffer, pH 7.0. Bacterial suspensions were added at the rate of 30 mg/cup dry weight. Temp. 30.4°C.
Table 31
Oxidation of Citrate, α-Ketoglutarate and Oxalacetate in the Presence of Various Inhibitors

<table>
<thead>
<tr>
<th>Substrate:</th>
<th>Citrate</th>
<th>α-Ketoglutarate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inhibitor:</td>
<td>NaHSO₃</td>
<td>NH₂NHCONH₂:NH₂OH·HCl</td>
</tr>
<tr>
<td>Oxygen uptake</td>
<td>199:1:150</td>
<td>155:111:191</td>
</tr>
<tr>
<td>μl</td>
<td>4:214</td>
<td>:</td>
</tr>
<tr>
<td>CO₂ evolved</td>
<td>236:1:194</td>
<td>246:140:249</td>
</tr>
<tr>
<td>μl</td>
<td>4:308</td>
<td>:</td>
</tr>
<tr>
<td>R.Q.</td>
<td>1.20:1:30:1:42:4:43</td>
<td>1.60:1.26:1.30:1.45:1.50</td>
</tr>
</tbody>
</table>

Total volume of reactants 2.3 ml. 0.047 M phosphate buffer, pH 7.0. Bacteria mg. per cup dry weight. Temp. 30.4°C.

10.0054 M sodium citrate and α-ketoglutarate respectively, 0.0054 M concentration of oxalacetate and inhibitors respectively.
Table 31
rate, $\alpha$-Ketoglutarate and Oxalacetate in the Presence of Keto Fixatives

<table>
<thead>
<tr>
<th></th>
<th>$\alpha$-Ketoglutarate$^1$</th>
<th>Oxalacetate$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCO$_2$:NH$_2$:OH.HCl:</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>155</td>
<td>111</td>
</tr>
<tr>
<td></td>
<td>246</td>
<td>140</td>
</tr>
<tr>
<td></td>
<td>1.60</td>
<td>1.26</td>
</tr>
</tbody>
</table>

$^1$ 0.047 M phosphate buffer, pH 7.0. Bacterial suspensions were added at the rate of 30°C.

$^2$ 0.0054 M concentration of inhibitors.

$\alpha$-Ketoglutarate respectively, 0.0054 M concentration of inhibitors.
These results do not conflict with those of Brewer and Werkman (1939) since they reported the bisulfite inhibition of citrate breakdown under anaerobic conditions only and made no mention as to the effect of this fixative aerobically.

Other carbonyl reagents were also employed. The results in Table 31 clearly show that semicarbazide·HCl is the best of the keto fixatives tested for in its presence α-ketoglutarate is hardly attacked. When this compound is added to citrate and non-proliferating cells, an R.Q. of 1.6 or higher is obtained. If oxalaeate is the initial intermediate and it is fixed with the semicarbazide thus leaving acetate as the only product to be attacked according to scheme presented by Brewer and Werkman for citrate dissimilation, R.Q. values of 1.0 should be obtained. Further, acetate is not attacked by our strain of A. aerogenes.

The manometric results with bisulfite vary and the reason is not clear. Paretsky (1940) reported similar observations with bisulfite. However, the fixative gave consistent results in a number of experiments to validate the data obtained by its use. In growth experiments uniform results are always obtained, e.g., no replacement of CO₂ by citrate in the presence of NaHSO₃.

When the R.Q. for citrate was between 1.4 and 1.8 in the presence of bisulfite or semicarbazide, aniline-citrate was added at the end of the experiment and no CO₂ was given off. Aniline-citrate decomposes oxalaeate to pyruvate and carbon dioxide even in the presence of the above fixatives. The explanation, therefore, that citrate is not
attacked in the presence of carboxyl reagents due to the binding of oxalacetate cannot be accepted as valid.

Ochoa (1947) has shown a soluble pyridine protein enzyme of heart muscle which catalyzes the oxidation of isocitrate to oxalacetate, which in turn is decarboxylated to \( \alpha \)-ketoglutarate and \( \text{CO}_2 \). Oxalacetate may be an intermediary in the oxidation of citrate by \( A. \) aerogenes, but as yet there is no direct evidence. If oxalacetate is formed and fixed by either bisulfite or semiacetate, no \( \text{CO}_2 \) should be evolved, since the change from citrate to oxalacetate involves only a dehydrogenation which should be observed, under the conditions, in terms of an oxygen uptake. It is possible that as soon as the compound is fixed it is attacked and consequently not detectable.

The manometric results are similar to those obtained with bisulfite added to the culture medium containing citric acid or the various keto acids as substitutents for \( \text{CO}_2 \) (Table 32). This keto fixative completely inhibits the ability of citrate to replace carbon dioxide. In the presence of \( \text{Na}_{2}\text{SO}_3 \), \( \alpha \)-ketoglutarate does not function, whereas with oxalacetate, growth takes place with or without bisulfite. These results show that citrate replaces \( \text{CO}_2 \) by first being converted to a keto compound, probably \( \alpha \)-ketoglutaric acid, and that oxalacetate is not the initial product, at least in the process of replacing carbon dioxide. If oxalacetate were the intermediate then citrate should replace \( \text{CO}_2 \) in the presence of bisulfite since the latter compound has no effect on the labile \( \text{C}_4 \) dicarboxylic acid. The keto compound formed
Table 52

Replacement of Carbon Dioxide in the Presence of Bisulfite

<table>
<thead>
<tr>
<th>Compound replacing carbon dioxide</th>
<th>Concentration</th>
<th>Bisulfite conc.</th>
<th>Growth Without CO₂</th>
<th>Growth With CO₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrate</td>
<td>0.50 mM</td>
<td>--</td>
<td>380</td>
<td></td>
</tr>
<tr>
<td>Citrate</td>
<td>0.60 mM</td>
<td>0.50</td>
<td>20</td>
<td>425</td>
</tr>
<tr>
<td>α-Ketoglutarate</td>
<td>0.25</td>
<td>--</td>
<td>390</td>
<td></td>
</tr>
<tr>
<td>α-Ketoglutarate</td>
<td>0.25</td>
<td>0.25</td>
<td>0</td>
<td>350</td>
</tr>
<tr>
<td>Oxalacetate</td>
<td>0.25</td>
<td>--</td>
<td>315</td>
<td></td>
</tr>
<tr>
<td>Oxalacetate</td>
<td>0.25</td>
<td>0.25</td>
<td>250</td>
<td>290</td>
</tr>
</tbody>
</table>

Results in terms of turbidity readings. Each flask contained 0.3 per cent KH₂PO₄, 0.4 per cent (NH₄)₂SO₄, 0.8 per cent glucose and 10 per cent tap water (for inorganic ions). Total volume 102 ml. Temp. 30°C. To this were added 2 cc. of a 24 hour culture of Aerobacter aerogenes and the compounds as indicated in the table. Aeration time, 18 hours.

1 Carbon dioxide removed by passing air through 30 per cent NaOH.

2 Aeration with normal air - tubes did not contain any added compounds.
from citrate probably by amination or transamination replaces carbon dioxide.

The partial inhibition of the oxidation of citric acid by fluoride suggests that phosphate esters play a role in its breakdown. In growth experiments, however, fluoride is not effective (Table 30) and these results indicate that no such esters are involved in the partial breakdown of citrate for CO2 replacement. Monoiodoacetate, on the other hand, inhibits the oxidation of citric acid both in growth and resting-cell experiments. This is expected if α-ketoglutarate is one of the initial compounds formed from citrate before the latter can replace carbon dioxide in which case a dehydrogenation is involved. Preventing the dehydrogenation of isocitrate to oxaloacetate to α-ketoglutarate would result in a loss of the ability of citric acid to replace carbon dioxide. Again, if oxalacetate is the initial breakdown product of citric acid, it would replace CO2, thus permitting growth to occur in the presence or absence of monoiodoacetate.

Further evidence for an initial dehydrogenation of citric acid has been obtained with pyrophosphate. Adler, et al (1939) found that isocitric dehydrogenase is inhibited by pyrophosphate. Slade and Werkman (1941) found no such inhibition with Streptococcus paracitrovorus under anaerobic conditions with citrate as the substrate. Brewer and Werkman (1939) report some inhibition with A. indolores using 0.02 M Na4P2O7 aerobically. Under our experimental conditions (aerobiosis) pyrophosphate completely inhibits the oxidation of citrate during the first 3 hours of the experiment.
A series of organic acids structurally related to citric acid were tested manometrically to determine their fermentability by *Aerobacter* and their CO₂ replacing ability. The acids tested were: citric, aconitic, tricarballylic, citraconic, itaconic and α-OH-iso-butyric. Of these tricarballylic and itaconic were not attacked. Citraconic and α-OH-iso-butyric acid were oxidized slowly, whereas only α-OH-iso-butyric acid failed to substitute for carbon dioxide.

The behavior of aconitic acid is of interest because of its structural relationship to citric acid. Whereas the cis form is active in replacing CO₂ and functions as a substrate, the trans form is completely inactive. Even at high a concentration as 4 mM will not replace carbon dioxide to any appreciable extent.

**α-Ketoglutaric acid**

Arsenious oxide prevents the oxidative decarboxylation of α-ketogluteric acid and yet is only partially effective in preventing this compound from replacing carbon dioxide. The chief mechanism by which the keto acid replaces CO₂ may, however, involve a reaction other than an oxidative decarboxylation. An oxidation may also be involved particularly during the log phase when the development of the organisms in the absence of CO₂ may depend upon hydrogen carriers, intermediately formed, e.g., succinate, fumarate, which would otherwise have to be formed from a synthesis involving atmospheric carbon dioxide.
Experimental evidence has been obtained that α-ketoglutaric acid replaces carbon dioxide by functioning as a substrate for transamination or transamination. Transamination in bacteria has not been extensively studied although it is known to occur in muscle, liver and other tissues. Adler, et al. (1938) state that suspensions of E. coli form oxalacetic acid from α-ketoglutaric acid and aspartic acid; and that lactic acid bacteria are capable of transamination, though less rapidly than E. coli. No experimental data are given. Diczfalusy (1942) working with the same organism reported negative results in a more elaborate study. On the other hand, Lichstein and Cohen (1945) showed a small but definite transaminase activity by E. coli which catalyzed the following reaction:

\[ L(+)\text{-glutamic acid} + \text{oxalacetic acid} \rightarrow \alpha\text{-ketoglutaric acid} + L(-)\text{-aspartic acid.} \]

Diczfalusy's (1942) failure to demonstrate transaminase activity in E. coli with the following reactions is understandable:

\[ L(-)\text{-aspartic acid} + \alpha\text{-ketoglutaric acid} \rightarrow L(+)\text{-glutamic acid} + \text{oxalacetic acid} \]
\[ \alpha\text{-Ketoglutaric acid} + L(+)\text{-alanine} \rightarrow L(+)\text{-glutamic acid} + \text{pyruvic acid} \]

Since the second and third reactions proceed slowly, the end products will not accumulate in determinable quantities. Evidence will be presented that these reactions do occur, and furthermore that the last reaction is actually involved in protein synthesis.

No growth of A. aerogenes occurs in a medium of 0.8 per cent KH₂PO₄, 0.8 per cent glucose, 10 per cent tap water and no nitrogen
source in the presence or absence of carbon dioxide. When alanine is added as a source of nitrogen, still no growth is obtained in the absence of carbon dioxide. Addition of \(\alpha\)-ketoglutarate results in normal development. Other amino acids, e.g., glycine, \(\beta\)-alanine, serine known to be deaminated in the presence of *A. aerogenes* do not replace alanine. \(\alpha\)-Ketoglutaric acid without alanine or ammonium sulfate will not support growth (Table 33). From these and similar results it may be concluded that the basic mechanism responsible for protein synthesis or growth in the absence of \(CO_2\) is a transamination reaction between \(\alpha\)-ketoglutarate and alanine or an amination reaction of this \(\alpha\)-keto acid to yield glutamic acid, which in turn has a significant function in replacing \(CO_2\). However, the ammonia given off by glycine, \(\beta\)-alanine or serine cannot apparently be utilized by the organism for the direct amination of \(\alpha\)-ketoglutaric acid. Ammonium sulfate must constitute the nitrogen source.

Manometric experiments have been conducted to show indirectly that ammonium sulfate can be utilized for the amination of \(\alpha\)-ketoglutarate and that *A. aerogenes* can cause a transamination between this keto acid and some amino acids in the presence and absence of arsenious oxide.

The method employed is based upon the fact that less residual \(\alpha\)-ketoglutarate is left after a given incubation period in the presence of cells, ammonium sulfate, or a potential transaminating amino acid than without these additions. Arsenious oxide is also added for a twofold purpose: (1) to prevent the oxidative decarboxylation of
Table 33

Replacement of Carbon Dioxide by α-Ketoglutaric Acid in the Presence of Various Nitrogen Sources

<table>
<thead>
<tr>
<th>Additions to basal medium</th>
<th>Concentration</th>
<th>Growth in the absence of CO₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>No additions</td>
<td>-</td>
<td>5</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>0.4%</td>
<td>10-15</td>
</tr>
<tr>
<td>Ammonium sulfate plus α-ketoglutaric acid</td>
<td>0.4%</td>
<td>325</td>
</tr>
<tr>
<td>DL-alanine</td>
<td>4 mM</td>
<td>50</td>
</tr>
<tr>
<td>DL-alanine plus α-ketoglutaric acid</td>
<td>4 mM</td>
<td>425</td>
</tr>
<tr>
<td>Glycine</td>
<td>2 mM</td>
<td>5-10</td>
</tr>
<tr>
<td>Glycine plus α-ketoglutaric acid</td>
<td>2 mM</td>
<td>10-20</td>
</tr>
<tr>
<td>L-Alanine</td>
<td>2 mM</td>
<td>10</td>
</tr>
<tr>
<td>L-Alanine plus α-ketoglutaric acid</td>
<td>2 mM</td>
<td>30</td>
</tr>
<tr>
<td>DL-serine</td>
<td>4 mM</td>
<td>25</td>
</tr>
<tr>
<td>DL-serine plus α-ketoglutaric acid</td>
<td>4 mM</td>
<td>35</td>
</tr>
</tbody>
</table>

1Basal medium contained 0.8 per cent KNO₃, 0.8 per cent glucose, 10 per cent tap water (for inorganic ions) made up to 100 ml. with distilled water.

To this were added the various compounds listed plus 1 ml. of a 24 hour culture of Acrobacter aerogenes. Aerated with CO₂-free water for 18 hours at 30°C C; growth expressed in terms of turbidimetric readings on the photoelectric colorimeter using 660 μm filter.
α-ketoglutarate, thus causing the bulk of the reaction to proceed in
the desired direction and (2) to show that As₂O₃ does not inhibit
transamination (explanation why both citrate and α-ketoglutarate
replace CO₂ in its presence). The results obtained with ammonium
sulfate, DL-citram and DL-aspartic acid are shown in Table 34.

To ascertain that residual α-ketoglutarate is a valid measure
of amination (or transamination) large scale experiments were run
and the product of amination was measured directly. The results
(Table 35) obtained clearly show a considerable inhibition of the
oxidation of α-ketoglutarate by As₂O₃, and furthermore, increased
amounts of glutaric acid formed in its presence.

_BUILDING_ C₄ DICARBOXYLIC ACIDS

The chief manner in which these acids act in replacing carbon
dioxide is by serving as precursors of oxalacetic acid (Table 36).
If the oxidation of succinate, fumarate or malate is prevented by
the addition of various inhibitors, the bacteria fail to develop.
This inhibition can be reversed on the addition of carbon dioxide
or oxalacetate, an observation which may support the occurrence of
the Szent-Györgyi sequence in bacterial respiration at least in a
unidirectional manner. If the sequence operates, the inhibition of
any one member of the hydrogen carriers should prevent respiration
of the cells. This was found to be the case. When cyclohexanol
disturbs the fumarate-malate equilibrium, growth takes place on
Table 34
Evidence for Ammoniation and Transamination by Aerobacter aerogenes in the Presence of As$_2$O$_3$ as Measured by Residual α-Ketoglutaric Acid

<table>
<thead>
<tr>
<th>Additions to α-Ketoglutaric Acid</th>
<th>Final Concentration</th>
<th>Residual α-Ketoglutaric acid measured as μl CO$_2$ evolved on addition of ceric sulfate at end of experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Experiment 1</td>
</tr>
<tr>
<td>Arsenious oxide</td>
<td>0.0011 M</td>
<td>201</td>
</tr>
<tr>
<td>Arsenious oxide plus DL-alanine</td>
<td>0.0011 M</td>
<td>184</td>
</tr>
<tr>
<td>Arsenious oxide plus DL-aspartic acid</td>
<td>0.0044 M</td>
<td>145</td>
</tr>
<tr>
<td>Arsenious oxide plus ammonium chloride</td>
<td>0.0044 M</td>
<td>90</td>
</tr>
<tr>
<td>Arsenious oxide plus ammonium sulfate</td>
<td>0.0044 M</td>
<td>140</td>
</tr>
</tbody>
</table>

Total volume of reactants 2.6 ml. Each cup contained 50 mg. (dry weight) A. aerogenes, 0.5 ml. of 0.2 M phosphate buffer, pH 7.0, 0.5 ml. of 0.02 M α-ketoglutaric acid and 0.5 ml. of neutralized arsineous oxide. Temp. 30.4°C.

At the end of experiment, 0.3 ml. of 12 N H$_2$SO$_4$ was added to liberate bound CO$_2$. Then 0.5 ml. of saturated ceric sulfate was added to oxidatively decarboxylate α-ketoglutarate. When the proper corrections are made for the oxidative decarboxylation of other keto acids that might have formed (e.g., pyruvate from alanine) by having the proper controls, the CO$_2$ liberated on the addition of ceric sulfate becomes a measure for residual α-ketoglutaric acid.
### Table 35

**Synthesis of Glutamic Acid by *Aerobacter aerogenes***

<table>
<thead>
<tr>
<th></th>
<th>(\alpha\text{-Ketoglutaric Acid} )</th>
<th>Glutamic Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>\text{Original conc.}</td>
<td>\text{Oxygen uptake}</td>
</tr>
<tr>
<td><strong>With</strong> arsenious oxide</td>
<td>0.0166 M</td>
<td>1420</td>
</tr>
<tr>
<td><strong>Without</strong> arsenious oxide</td>
<td>0.0166 M</td>
<td>5186</td>
</tr>
</tbody>
</table>

Each flask contained 300 mg (dry weight) cells, 5 ml. of 0.1 M ammonium sulfate, 5 ml. of 0.005 M \(\text{As}_2\text{O}_3\) and 5 ml. of phosphate buffer, pH 7.4. Final volume 30 ml. *Aerobic* Time, 2.5 hours. Temp. 30.4°C.
Table 36

Mechanism of Carbon Dioxide Replacement by C₄ Dicarboxylic Acids

<table>
<thead>
<tr>
<th>Acid Added</th>
<th>Conc. of Inhibitor</th>
<th>Conc. of Inhibitor</th>
<th>Absence of CO₂</th>
<th>Presence of CO₂</th>
<th>CO₂ Oxalacetate</th>
<th>Growth (after no growth in 12-14 hrs.) on addition of CO₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Succinate</td>
<td>1 ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fumarate</td>
<td>1 ml</td>
<td>Iodoacetate 0.031 ml</td>
<td>13</td>
<td>170</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malate</td>
<td>1</td>
<td></td>
<td>10</td>
<td></td>
<td>140 270 1</td>
<td></td>
</tr>
<tr>
<td>Oxalacetate</td>
<td>1</td>
<td></td>
<td>145</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Succinate</td>
<td>1 ml</td>
<td>Cyclohexanone 0.25%</td>
<td>65</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malate</td>
<td>1</td>
<td></td>
<td>165</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxalacetate</td>
<td>1</td>
<td></td>
<td>190</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Results in terms of turbidity readings. 0-30 indicates no growth due to the added compound. Total volume, 101 ml. Temp. 30°C. Basal medium consisted of 0.8 per cent KH₂PO₄, 0.4 per cent (NH₄)₂SO₄, 10 per cent tap water. Time of aeration, 18 hours.

A Basal medium plus 0.4 per cent glucose, 1.0 ml. of a 24 hour culture of *Aerobacter aerogenes*.
B Basal medium plus 0.2 per cent glucose, 0.5 ml. of a 24 hour culture of the organism.
addition of oxaloacetate. Similarly, the C₄ labile dicarboxylic acid can reverse the inhibition obtained with iodoacetate on malate.

The results with succinate are indirect since no inhibitor has yet been found which is effective against succinate dehydrogenase of intact cells.

**Aspartic and glutamic acids**

Both of these amino acids replace carbon dioxide by acting as precursors of some of the constituents of the Krebs cycle. A difference, however, exists. Whereas aspartic acid functions merely as a source of fumarate, the function of glutamic acid is to supply α-ketoglutaric acid as well as serve as an amino acid which would otherwise have to be synthesized before growth could take place.

For example, cyclohexanol in addition to affecting the fumarate-malate equilibrium also prevents the deamination of aspartic and glutamic acids in *in vitro* experiments (Table 37) by non-proliferating lyophilized cells of *A. aerogenes*. When suitable amounts of this inhibitor are added to cultures in which aspartate or glutamate functions as carbon dioxide replacement compound, growth is completely inhibited in the case of aspartic acid and only partially prevented in the presence of glutamate.

Cyclohexanol is of interest in that in almost all cases the addition to cells of *A. aerogenes* incubated with amino acid results in a decrease in the oxygen uptake below that of the endogenous
Table 37

Action of Cyclohexanol on Glutamic and Aspartic Acids

<table>
<thead>
<tr>
<th>Compound</th>
<th>Manometric Data</th>
<th>Ammonia Produced</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>With Cyclohexanol</td>
<td>Without Cyclohexanol</td>
</tr>
<tr>
<td></td>
<td>CO₂ evolved</td>
<td>O₂ utilized</td>
</tr>
<tr>
<td>Aspartic Acid</td>
<td>-9</td>
<td>-14</td>
</tr>
<tr>
<td>Glutamic Acid</td>
<td>0</td>
<td>-16</td>
</tr>
<tr>
<td>Glucose</td>
<td>431</td>
<td>302</td>
</tr>
</tbody>
</table>

¹Total volume of reactants 2.6 ml. The following additions were made in the manometer cups: Aerobacter aerogenes, 0.5 ml. of 0.05 M DL-aspartic acid (or 0.5 ml. of 0.025 M D-glucose), 1 ml. of a 1:50 dilution of cyclohexanol, 0.5 ml. of 0.4 M phosphate buffer. Temp. 30.4°C.

²Results in terms of turbidity readings; 0 indicates no growth. Total volume 101 ml. per cent glucose, 1 mM of either D-glutamate or 2 mM of DL-aspartate, 0.2% cyclohexanol showed no inhibition of growth.
Table 37

Glutamyl and Aspartic Acids

<table>
<thead>
<tr>
<th>Data</th>
<th>Ammonia Produced</th>
<th>Growth Experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without Cyclohexanol</td>
<td>With Cyclo.</td>
<td>Without Cyclo.</td>
</tr>
<tr>
<td>CO₂ evolved</td>
<td>O₂ utilized</td>
<td>N</td>
</tr>
<tr>
<td>402</td>
<td>317</td>
<td>18</td>
</tr>
<tr>
<td>522</td>
<td>432</td>
<td>7</td>
</tr>
<tr>
<td>521</td>
<td>540</td>
<td></td>
</tr>
</tbody>
</table>

Owing additions were made in the manometer cup: 30 mg. lyophilized aspartic acid (or 0.5 ml. of 0.025 M D-glutamic acid or 0.5 ml. of 0.025 M cyclohexanol, 0.5 ml. of 0.1 M phosphate buffer of pH 7.0. Alkali or acid in...
respiration in the presence of cyclohexanol incubated with glucose. There is no poisoning effect for the first 30-60 minutes, then a considerable reduction in exchange takes place. Similar results were obtained with arsenious oxide. When amino acids serve as substrate, the inhibition with cyclohexanol is immediate. The inhibition of desamination (and oxidation) found when the bacteria are incubated with aspartic or glutamic acids is, therefore, not a general poisoning of the cells.

Asparagine and glutamine

Both of these compounds replace CO₂ through their respective amino acids. McIlwein, et al (1948) synthesized a compound which inhibited the liberation of ammonia from glutamine according to the following equation:

\[
\text{glutamine} \rightarrow \text{glutamic acid}
\]

inhibited by

\[
\text{glutamylhydrazine}
\]

Similarly, 3-aspartylhydrazine prevents the deamination of asparagine. When these inhibitors are added in cultures where glutamine or asparagine replaces carbon dioxide, no growth is obtained. It can, therefore, be assumed that the amides of the dicarboxylic amino acids function in replacing CO₂ by yielding their respective amino acids.
(Table 38). Nanostructurally, the oxidation of both glutamine and asparagine is inhibited by their hydrazines.
### Table 38

**Glutamine and Asparagine**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Inhibition</th>
<th>Conc. of Inhibitor</th>
<th>Growth</th>
<th>Growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamine</td>
<td>γ-glutamyl-hydrazine</td>
<td>0.125</td>
<td>0.0054 M</td>
<td>10</td>
</tr>
<tr>
<td>Glutamine</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>370</td>
</tr>
<tr>
<td>Asparagine</td>
<td>β-aspartyl-hydrazine</td>
<td>0.125</td>
<td>0.0054 M</td>
<td>17</td>
</tr>
<tr>
<td>Asparagine</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>175</td>
</tr>
</tbody>
</table>

Manometric expts. - Total volume 2.3 ml. Each cup contained 30 mg. dry weight of A. aerogenes, 0.5 ml. of 0.025 M glutamate or asparagine, 0.5 ml. of 0.1 M PO₄, plus various concentrations of inhibitors as shown in table. Temp. 30.4°C.

Growth expts. - Results in terms of turbidity readings. Each flask contained 0.8% glucose and 10% tap water (for inorganic ions). Total volume 101 ml. Each flask contained 1 cc. of a 24 hour culture of A. aerogenes. Aeration time, 16 hours.

1 Carbon dioxide removed by passing air through 30% NaOH. These tubes contained asparagine.

2 Aeration with air containing normal complement of CO₂. These tubes contained substrate other than basal medium.
Table 38
Glutamine and Asparagine

<table>
<thead>
<tr>
<th>Conc. of Inhibitor</th>
<th>Growth</th>
<th>Resting Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without</td>
<td>With</td>
</tr>
<tr>
<td>expts.</td>
<td>cell expts.</td>
<td></td>
</tr>
<tr>
<td>nM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.125</td>
<td>0.0054 U</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td></td>
<td>370</td>
</tr>
<tr>
<td>0.125</td>
<td>0.0054 U</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td></td>
<td>175</td>
</tr>
</tbody>
</table>

.3 ml. Each cup contained 30 mg. dry weight of lyophilized Aerobacter
umate or asparagine, 0.5 ml. of 0.4 M PO₄ buffer, pH 7.0, NaOH or H₂SO₄
itors as shown in table. Temp. 30.4°C.

 turbidity readings. Each flask contained 0.8% KH₂PO₄, 0.4% (NH₄)₂SO₄,
 inorganic ions). Total volume 101 ml. Temp. 30°C. To this were added
rogenes. Aeration time, 16 hours.

air through 30% NaOH. These tubes contained 0.125 mU glutamine or
il complement of CO₂. These tubes contained the inhibitors but no
DISCUSSION

The results obtained in this study permit us to make a few definite statements concerning the mechanism by which certain compounds replace carbon dioxide. Experimental data indicate that the gas functions as a metabolite and not merely as an environmental factor, e.g., by affecting the pH of the medium or the permeability of the cell wall.

The evidence points to an orderly manner in which the substitution of the various compounds takes place. That this is not a general effect is indicated by the fact that compounds such as alanine, pyruvic acid, histidine, lysine or citric acid cannot substitute for carbon dioxide with *Escherichia coli*. *Aerobacter aerogenes*, which normally utilizes citric acid as a carbon source, can use this acid instead of carbon dioxide.

This last observation may prove to be of considerable importance. If it be assumed that the results indicate further evidence for the operation of the proposed Krebs cycle in *E. coli*, it may be that citric acid occurs in that cycle in the case of *A. aerogenes* and not in *E. coli*.

It is of considerable interest that, in addition to the compounds normally occurring in the Krebs cycle, a number of amino acids can also be used by these organisms to replace carbon dioxide. The manner in which they substitute cannot be stated with certainty. However, in the case of aspartic and glutamic acids, 2 main paths of
action are possible.

1. The amino acids substituting for carbon dioxide are converted by transaminations or similar reactions to the various dicarboxylic acids and eventually to oxaloacetic acid, which is required by the cells as a catalyst in normal aerobic metabolism. This implies a catalytic function of these compounds. Dependence of growth on the amount of amino acid present suggests that the oxaloacetate eventually formed serves as an outlet from carbohydrate breakdown into protein synthesis. This is particularly possible under anaerobic conditions where the replacement of oxaloacetate is very striking; e.g., more growth occurs in the presence of this labile C₄ acid than in the case of any of the other C₄ dicarboxylic or amino acids.

2. The substitution of the C₅ acids, e.g., glutamic and α-ketoglutaric acid, to a greater extent than any of the other compounds tested is suggestive of a further fixation of CO₂ over and above the Wood and Werkman reaction in normal metabolism, involving the formation of a C₅ compound which can be used more efficiently by the cell. It is possible, therefore, that many of the compounds supplied in the absence of CO₂, particularly the amino acids, function in more than one way. They may supply either oxaloacetic acid or α-ketoglutaric acid and function as products which would normally arise during the metabolism of the cell.

That a C₅ compound may be formed as a result of a C₄ and C₁ addition has been demonstrated in this study. Cell-free extracts
of *E. coli* reversibly and oxidatively decarboxylate α-ketoglutarate to succinate and CO₂. As the Krebs cycle is now presented, every reaction except the one studied has been shown to be reversible. According to the present findings, a complete reversal of the oxidative degradation of foodstuff would not be possible. By carboxylation and reduction, α-ketoglutaric acid can be converted to citric acid and the latter may split into acetic and oxalacetic acids. Further, oxalacetic acid can be reduced to succinic acid by way of malic and fumaric acids, and the succinate thus formed could be converted to α-ketoglutaric acid by reductive carboxylation. In this way, α-ketoglutaric acid would constantly be regenerated. We would have a system whereby CO₂ and H₂ entering at various points during metabolism would emerge as pyruvate which could then be used for the synthesis of carbohydrates.

In a system devoid of CO₂, α-ketoglutaric acid may function in 1 or 2 ways. First, it may be oxidatively decarboxylated to yield succinic acid and CO₂, the former being utilized by the organism in place of CO₂ and secondly, the keto acid may function as a substrate for transamination, a mechanism responsible for the interconversion of proteins and carbohydrates. (That this mechanism operates in initiating the growth of *A. aerogenes* has been proved in this work.) Since growth depends on the presence of carbon dioxide and since α-ketoglutaric acid replaces the gas to a much greater extent than any of the C₄ acids, it is entirely possible that, during the normal metabolic processes of the bacterial cells, the C₁ to C₄ addition
reported in this investigation may be of greater importance to the
cell than some of the other fixation reactions known to occur.

The mechanism of the reversibility may be represented:

\[
\begin{align*}
&\text{COOH} & \text{COOH} & \text{COO} \\
&\text{CO} & \text{HOC-PO}_3\text{H}_2 & \text{COO} + \text{CO}_3\text{H}_2 \\
&\text{CH}_2 & \text{H}_2\text{PO}_4 & \text{CH}_2 \\
&\text{CH}_2 & \text{CH}_2 & \text{CH}_2 + \text{ADP} \\
&\text{COOH} & \text{COOH} & \text{COOH} \\
& & & \text{CH}_2 + \text{CO}_2 + \text{ATP} \\
&\text{XH}_2 & & \text{COOH} \\
&\text{YH}_2 & & \text{PO}_3\text{H}_2 \\
&\text{H}_2\text{O} & & \text{PO}_3\text{H}_2
\end{align*}
\]

where X and Y are intermediary hydrogen acceptors.

The fact that phosphate increases the activity of the dialyzed
dye on d-ketogluatate strongly supports the validity of the sug-
gested mechanism. The first step of this reaction is apparently a
phosphorylation of d-ketogluatotic acid. This phosphate is then
utilized for the formation of an energy-rich compound and a potential
source of adenosine triphosphate. Such oxidative decarboxylations
are of extreme importance to cells since they offer means by which
the organism can utilize the energy produced by the oxidation of a
given substrate.
Of the 80 energy rich phosphate bonds that are generated when 1 molecule of glucose is completely oxidized, not more than 2 are formed during the initial stages that lead to the formation of lactic acid. The residual 48 bonds, therefore, must arise during the subsequent oxidative stages. Twenty-four (24) must be generated from each molecule of lactate. Lactate is dehydrogenated to pyruvate and the keto acid oxidatively degraded through the Krebs cycle. Of all the reactions involved, only 2 are known to undergo oxidative decarboxylations yielding energy rich phosphate bonds, e.g., the oxidative decarboxylation of pyruvate and of α-ketoglutarate. It follows that about 22 additional energy-rich phosphate bonds are formed as a result of other reactions. It is postulated that the additional bonds are formed during the transfer of electrons from the primary acceptor to oxygen. If such bonds are generated, their energy could conceivably be used to reverse the intermediary steps of the reaction studied. That this may actually be the case is particularly borne out by the fact that ATP enhances the reversibility by increasing the concentration of energy rich phosphate bonds.

The dehydrogenation of α-ketoglutaric acid proceeds through a series of hydrogen carriers. The evidence obtained that fumate and malate are involved in the dehydrogenation of the keto acid should be stressed for it differentiates our enzyme system from that of Ochoa (1944) and places emphasis on the C₄ dicarboxylic acids as potential hydrogen carriers in bacterial metabolism.
Our enzyme system does not decarboxylate \( \alpha \)-keto glutaric acid under anaerobic conditions. This makes our enzyme distinct from the one reported by Stumpf et al (1947) and Wein-Baltese (1937). However, this keto acid replaces \( \text{CO}_2 \) under anaerobic conditions which apparently indicates its function under such conditions to be involved in transamination and/or amiation.

That nickel can partially replace magnesium or manganese in this reaction is of theoretical importance only. It may be used to support the hypothesis that, when divalent ions are involved in certain enzyme systems, their function is merely to bind the protein and prosthetic groups together due to their divalency. Many exceptions, however, are noted. Zinc, for instance, although a divalent metal, does not function in a similar manner since it apparently ties up \(-\text{SH}\) groups of the protein moiety of the enzyme. It is very difficult to decide the kind of linkage formed between the heavy metal and the \( \text{SH} \) groups of the protein molecule. However, the mechanism suggested by Barron and Kalnitsky (1947), where two \(-\text{SH}\) groups combine with the heavy metal, e.g., Protein\( \text{metal} \) forming cyclic compounds, seems to be a very plausible one.

An increase in oxygen uptake was noted when biotin was added to a dialyzed cell-free extract of \( E. \text{coli} \) in the presence of \( \alpha \)-keto glutaric acid. This increase was lower when malonate was added. It was, therefore, likely that the vitamin functions in some step during the oxidation of succinic acid. On addition of biotin activity of succinic dehydrogenase increases and in some instances up to three fold.
This finding adds to the growing weight of evidence assigning to biotin the role of a respiratory catalyst.

Recent studies by Kosar, et al (1942), Stokes, et al (1947) and Lardy, et al (1947) have implicated biotin in the reversible \(\beta\)-carboxylation of pyruvate to oxalacetate. The effect of biotin on succinate may be ascribed to the oxidation of oxalacetate, arising from succinate; however, the results showed this not to be the case. First, fumarate, arising from the oxidation of succinic acid, is not appreciably attacked by E. coli juice either with or without biotin. Secondly, carbon dioxide is not formed from succinate. Thirdly, the oxidation of L-malate or sodium acetate shown to arise from succinic acid, is not increased on the addition of the vitamin.

That biotin has a single function in metabolism is doubtful in view of the studies of Lichstein and Umbreit (1947) on the deaminase content of biotin-deficient E. coli, of Hadden and Gavin (1941) on the synthesis of fatty acids in rats supplemented with biotin, and of Potter and Elvehjem (1948) on oleic acid-biotin interrelationships in Lactobacillus arabinosus. Our results with cell-free juice show some increase in the deamination of the aspartic acid on the addition of biotin.

The mechanism of the action of biotin is not clear. Slight variations in the structure of the vitamin can be introduced and the activity maintained. DL-homobiotin as well as desthiobiotin increase succinic acid oxidation to the same extent as biotin. The non-specificity of biotin action is thus further indicated.
In growth experiments a similar function of biotin could not be demonstrated. If the oxidation of succinic acid depends upon the presence of this vitamin, then the replacement of CO$_2$ by this C$_4$ dicarboxylic should also be dependent upon the presence of biotin. This, however, could not be shown, due to the synthesis of biotin during growth of *E. coli* to satisfy all its requirements for the vitamin.

That succinic acid as well as fumarate and malate must be oxidized to oxalacetate prior to becoming effective as CO$_2$ substituents, further stresses the importance of the Wood and Werkman reaction. That the formation of oxalacetate is not the only way by which CO$_2$ enters into cellular metabolism is clear from a consideration of the following established facts:

1. The reduction of CO$_2$ to formic acid by *E. coli* (Woods, 1936).
2. The production of CH$_4$ from CO$_2$ in the methane fermentation (Barkat, et al. 1940, 1936).
3. The utilization of CO$_2$ in the formation of urea (Krebs and Henseleit 1932, Evans and Slotin 1940).

However, the formation of this C$_4$ labile dicarboxylic acid appears to be of greater metabolic significance than any of the above mentioned mechanisms by which CO$_2$ may enter into cellular metabolism. If the formation of formate were an essential reaction and if its formation depended upon gaseous CO$_2$, why does not formic acid replace CO$_2$? Is it not, therefore, clear that the Wood-Werkman reaction involved in
formation of oxalacetate is more fundamental; upon its presence depends the survival of the cell.

The complex manner in which urea appears to be formed from NH₃ and CO₂ indicates that the CO₂ is initially built into a larger organic molecule (ornithine-citrulline-arginine cycle). In essence, this is somewhat similar to the already discussed mechanism for the formation of oxalacetate, although not giving rise to a new carbon to carbon linkage. It is not unlikely that this particular reaction involving CO₂ may be significant in some forms of life but in E. coli the function of arginine in replacing CO₂ is probably to yield α-ketoglutaric acid.

According to our present knowledge it appears that the biological degradation of a substrate leads to a series of reactions in which electrons or hydrogen atoms are transferred to the ultimate acceptors by way of a rather extensive series of oxido-reduction reactions. In this chain of events the reversible hydrogenation of oxalacetate to malate and of the latter (via fumarate) to succinate, plays the part of a catalytic hydrogen transporting system in various mammalian and avian tissues, and also in microorganisms. Hence, it has been proposed that the C₄ dicarboxylic acids comprise a catalytic sequence functioning in hydrogen transport.

This hypothesis implies that the metabolic capacity of a system depends in part upon the presence of sufficient amounts of the participating C₄ dicarboxylic acids. In growing cells the necessary increase in metabolic capacity must be brought about by a synthesis of one or
more of the components of the catalytic cycle and from the foregoing discussion it appears that such a synthesis can occur from CO$_2$ and pyruvic acid. If this were the only or most important way in which the C$_4$ dicarboxylic acids originate, it is obvious that CO$_2$ should be an indispensable component of the medium in which growth takes place.

One significant observation, however, does not permit us to conclude that the chief function of the C$_4$ dicarboxylic acids is catalytic in nature and involves hydrogen transport only. If this were the case, the inhibition of any one of the 4 acids should result in an inhibition of respiration or, under our conditions, no growth should take place. This, however, was found not to be the case. When the oxidation of succinate, fumarate or malate was stopped by the addition of various specific and non-specific inhibitors, growth in the absence of CO$_2$ was also stopped. On the addition of either atmospheric carbon dioxide or oxalacetate, the development was normal. It may be argued that not the oxalacetate but the CO$_2$ coming off as a result of the spontaneous decarboxylation of the acid is actually the cause of the resumed growth. This may be the case. However, the medium is aerated at the rate of 5 volumes of air per minute, previously shown to be sufficient for the removal of all of the CO$_2$ from the medium as soon as it is formed.

From our results it appears probable that the chief mechanism by which the dicarboxylic acids function in replacing CO$_2$ is to yield oxalacetate which, in turn, may be directly aminated or transaminated
to yield amino acids -- thus initiating protein synthesis.

Whereas these contentions point to the fundamental role of a reaction by which oxalacetate is synthesized from CO₂ and pyruvate, it does not necessarily follow that this reaction constitutes the only important function of CO₂ in metabolism. The formation of other keto compounds involving CO₂ may be just as significant.

The mechanism by which citrate replaces CO₂ has been traced to a keto compound (not oxalacetate). It is tempting to assume that α-ketoglutarate is the compound which arises from citrate in the absence of CO₂ and that the latter, by transamination, serves as an outlet into protein synthesis, thus initiating growth. No direct evidence has yet been obtained. However, that the keto acid undergoesamination or transamination and that these in turn are actually responsible for initiating growth in a system devoid of carbon dioxide, has been shown in the experimental section.

Brewer and Werkman (1939) concluded that, under anaerobic conditions at least, oxalacetate and acetate are the initial breakdown products of citric acid. From our data it cannot be concluded that this occurs aerobically, although the oxalactic acid thus formed could replace CO₂. If the C₄ labile dicarboxylic acid was one of the initial breakdown products during the oxidation of citrate, then several conditions should be met. First, bisulfite should not inhibit the replacement of CO₂ by citric acid since oxalacetate continues to function as a carbon dioxide substituent in the presence of this keto fixative. The replacement of CO₂ by α-ketoglutarate in the presence of bisulfite is
completely inhibited. Secondly, monoiodoacetate completely prevents replacement of carbon dioxide by citric acid. If oxaloacetate were the intermediate through which citrate functions in replacing CO₂, the iodoacetate should not interfere. On the other hand, if a five carbon keto acid is one of the initial breakdown products, a dehydrogenation is involved and the monoiodoacetate should theoretically at least, inhibit the function of citrate in replacing CO₂. Finally, if oxaloacetate is an initial breakdown product of the C₆ acid and it is bound by bisulfite then, on the addition of aniline citrate, CO₂ should evolve. No such gas evolution was obtained when citrate was being slowly oxidized in the presence of NaHSO₃.

Evidence for an initial dehydrogenation of citrate has been obtained manometrically by the use of pyrophosphate which inhibits iso-citric dehydrogenase. In its presence, citrate is not attacked during the first 3 hours of the experiment, indicating that aerobically at least an initial dehydrogenation is involved during citrate oxidation.

The 2 chief criticisms of the work involving the replacement of carbon dioxide in heterotrophic metabolism may be:

If growth is prevented because of the inhibition of any one compound which replaces CO₂, the same inhibition should result when a given inhibitor is added to a culture which is aerated with air. For example, if bisulfite binds L-ketoglu tarate and consequently prevents the latter from replacing CO₂, should not bisulfite also inhibit the growth of the organisms in the presence of carbon dioxide?
Secondly, what are the important experimental data to support the conclusion that the various compounds replacing carbon dioxide do not do so simply by furnishing the gas to the medium?

The first criticism may be answered by assuming that the various inhibitors function differently in the presence and absence of CO₂. First, in normal metabolism (in the presence of CO₂) more of the keto acid may form than the amount of NaHSO₃ present in the medium and consequently growth will not be inhibited. This is substantiated since large concentrations of the keto fixative will inhibit the development of the bacteria both in the presence and absence of carbon dioxide.

Similarly, the addition of excessive amounts of cyclohexanol not only will inhibit the degradation of aspartic acid but the growth of the organism as well. Secondly, in the absence of CO₂ we deal with a "controlled metabolism", e.g., growth will or will not occur, depending upon the presence or absence of one compound and the inhibition of the action of that compound results in an inhibition of growth. In the presence of carbon dioxide alternate mechanisms may operate and when ß-ketoglutarate, for example, is blocked, the organism may choose a different compound whose function is independent of keto fixatives in the medium.

That the compounds do not function simply by supplying carbon dioxide to the medium is indicated by the fact that glucose, otherwise a good source of CO₂, cannot support growth in the absence of the gas. Also, the inclusion of carbon dioxide after maximum growth has been
obtained in its absence, very little further increase in turbidity results. On the addition of a C₄ or C₅ acid, growth is abundant.

The general inferences that can be drawn from the preceding considerations lead, we believe, to a unified and rather simple concept of the role played by carbon dioxide in cellular metabolism.
A number of compounds have been found which, in the case of *Escherichia coli*, substitute for carbon dioxide and give not only normal but, in instances, enhanced growth. These compounds include the amino acids: arginine, proline, aspartic and glutamic acids; the dicarboxylic acids: succinic, fumaric, malic, oxalacetic and \( \alpha \)-ketoglutaric acids, and 1 tricarboxylic acid, namely, cis-aconitic acid. Citric acid replaces carbon dioxide only in *Aerobacter aerogenes*. All of these compounds are constituents of the Krebs oxidation cycle, or their metabolic precursors. That this is not a general effect is indicated by the fact that compounds such as alanine, lysine, pyruvic acid or citric acid and histidine cannot be used by *E. coli* in the place of carbon dioxide.

The results indicate that the degree of substitution of the various compounds is quantitatively significant.

Our experimental data warrant the conclusion that the compounds added do not function merely by supplying carbon dioxide to the organism. If this were the case, the inclusion of carbon dioxide, after maximum growth has been obtained in its absence, should give a corresponding increase. This was found not to be the case. Also glucose which is potentially a good source of carbon dioxide is included in all of our media.

The compounds which have been found to replace carbon dioxide aerobically will do so anaerobically as well. The 6 compounds replace
carbon dioxide to a greater extent than any of the other compounds, both in the presence and absence of oxygen. Oxalosuccinic acid is used very effectively under anaerobic conditions in the place of carbon dioxide, indicating that this acid may be the chief substrate for amination or transamination under such conditions.

As a result of the special effect obtained with the C₅ acids, the suggestion was made that a C₅ compound may be formed in normal metabolism involving a C₄ and C₁ synthesis, and that this reaction may be of great importance to the cell.

Reversibility of the following reaction has been demonstrated with a cell-free enzyme preparation of E. coli.

\[
\text{COOHCH₂CH₂COOH + O} \rightleftharpoons \text{COOHCH₂CH₂COO}^- + \text{CO₂}
\]

Adenosine triphosphate enhances the reversibility.

Occurrence of this reaction explains, in part, the function of α-ketoglutaric acid in replacing carbon dioxide.

The components of the enzyme system include phosphate, adenosine triphosphate and magnesium. The last mentioned component can be fully replaced by manganese and only partially replaced by nickel ions. Evidence that the protein moiety has -SH groups has been obtained.

Some work was done on the oxidation of succinic acid by cell-free enzyme systems. The oxidation of this dicarboxylic acid is appreciably reduced as a result of dialysis. The activity is increased on the addition of biotin. DL-homobiotin and desthiobiotin replace the free vitamin. The increase in succinic acid oxidation due to biotin is not the effect of the vitamin on oxalosuccinate or any of the other known
intermediates in succinic acid oxidation.

The mechanism by which the compounds replace carbon dioxide has been investigated and the evidence thus far obtained points to an orderly manner in which this phenomenon takes place.
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