

# ON THE MECHANISM OF CARBON DIOXIDE REPLACEMENT IN HETEROTROPHIC METABOLISM<sup>1</sup>

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Ajl and Werkman (1948) have previously reported on the heterotrophic replacement of carbon dioxide and reviewed the literature on the bacterial requirement of the gas for optimal growth. A number of compounds have been found that in the case of both *Escherichia coli* and *Aerobacter aerogenes* substitute for carbon dioxide to give not only normal but, in many instances, enhanced growth. These compounds include the members of the Krebs cycle or their metabolic precursors.

This contribution considers the mechanism of the substitution. The evidence points to an orderly manner in which this phenomenon takes place. Oxalacetic and  $\alpha$ -ketoglutaric acids appear to function as the cardinal compounds in the replacement of CO<sub>2</sub> when added as such or in the form of their precursors. Inhibitors prevent the oxidation or the deamination of the various precursors of oxalacetate and  $\alpha$ -ketoglutarate. Under such conditions no growth is obtained unless oxalacetate or  $\alpha$ -ketoglutarate is present. Cyclohexanol inhibits the deamination of aspartic acid to fumarate; addition of this compound to a CO<sub>2</sub>-free medium containing aspartic acid results in no growth. The addition of oxalacetate now provides abundant growth. Similar results have been obtained with the other members of the Krebs cycle.

## METHODS

In view of the problem two general types of investigations were conducted involving both multiplication experiments and studies with nonproliferating cells. Simultaneous multiplication and resting cell experiments were conducted for confirmatory purposes. 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.

*Growth experiments.* The methods were essentially those employed in previous work (Ajl and Werkman, 1948). The organism was *Aerobacter aerogenes*. A 24-hour culture grown in the basal medium constituted the inoculum. The medium consisted of 0.8 per cent KH<sub>2</sub>PO<sub>4</sub>, 0.4 per cent (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 10 per cent tap water, and 0.8 per cent glucose, final pH adjusted to 6.8. The compounds tested were added before autoclaving, except in the case of oxalacetic acid. A solution of the sodium salt of this acid was sterilized by filtration and added aseptically to the medium.

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*CO<sub>2</sub>-adsorption train.* The air was first passed through a sterile, cotton-filled tube and then freed from CO<sub>2</sub> by being passed through an alkali tower filled with glass beads containing 30 per cent NaOH, with phenolphthalein as an indicator. The reaction flask contained 100 ml of basal medium to which 1 per cent of inoculum by volume was added aseptically, unless otherwise stated. All experiments were conducted at 30 C. Growth was measured by turbidimetric readings on a Klett-Summerson photoelectric colorimeter with 660 m $\mu$  light filter. All readings were made after 18 hours, incubation time. Sterile, uninoculated culture media were used as controls.

*Resting cell experiments.* Large amounts of *A. aerogenes* were grown in the same medium as in growth experiments, plus yeast extract. 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 KH<sub>2</sub>PO<sub>4</sub>, 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 and either used immediately or lyophilized and stored. The Barcroft-Warburg respirometer was used in most of our experiments. The total volume of reactants varied from 2.3 to 4.0 ml. Endogenous values were deducted.

Oxalacetate was determined according to the method of Edson (1935).  $\alpha$ -Ketoglutarate was determined by the ceric sulfate method. The products of this reaction are succinate and carbon dioxide. Cohen's (1939) method was used for glutamic acid, and a modified method of Johnson (1941) was used for ammonia. The sample containing the formed ammonia was transferred from the Warburg vessel into a test tube and to it was added an equal amount of trichloroacetic acid. The cells were centrifuged and filtered. To the filtrate 3 ml of 2 N NaOH, 1 ml of freshly filtered gum arabic, and 0.5 ml of Nessler's reagent were added. The colored solution was mixed and read on the photoelectric colorimeter using a 420 m $\mu$  filter. The range was 10 to 100 micrograms nitrogen; precision,  $\pm$  1.5 micrograms.

#### EXPERIMENTAL RESULTS

*Citric acid.* Since Brewer and Werkman (1939, 1940) found that arsenite, monoiodoacetate, and bisulfite completely, and fluoride partially, inhibited the breakdown of citric acid by *Aerobacter indologenes*, they concluded that the initial breakdown products were oxalacetate and acetate under both aerobic and anaerobic conditions. To determine the mechanism by which citrate replaces carbon dioxide, various concentrations of the foregoing inhibitors were added to cultures of *A. aerogenes* containing citric acid as the substituent for CO<sub>2</sub>. In growth experiments only bisulfite and monoiodoacetate completely inhibited the development of the organism, whereas the growth was normal in the presence of fluoride and nearly normal upon the addition of As<sub>2</sub>O<sub>3</sub> (table 1).

The seemingly contradictory results obtained with arsenious oxide and bisulfite (both prevent the breakdown of  $\alpha$ -keto acids but by different mechanisms, e.g., bisulfite by binding the keto acid and the arsenious oxide by inhibiting its

oxidative decarboxylation) prompted further work to determine more specifically the keto compound that 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 initial compound formed in the dissimilation of citric acid, at least under aerobic conditions. When sodium bisulfite is added to Warburg cups containing sodium citrate and cells, no gas exchange above that of the endogenous is observed for the first few hours (figure 1), but soon afterward an oxygen uptake is noted and at the end of the experiment the R.Q. obtained varies from 1.2 to 1.9. At times a significant gas exchange was observed within the first half-hour of the experiment. The theoretical

TABLE 1  
*Effect of inhibitors on the replacement of carbon dioxide by citric acid*

| INHIBITOR       | CONCENTRATION IN |                     | POINT OF INHIBITION  | GROWTH                    |                        | RESTING CELLS         |                         |
|-----------------|------------------|---------------------|----------------------|---------------------------|------------------------|-----------------------|-------------------------|
|                 | Growth expts.    | Resting cell expts. |                      | Without CO <sub>2</sub> * | With CO <sub>2</sub> † | O <sub>2</sub> uptake | CO <sub>2</sub> evolved |
|                 | mm               |                     |                      |                           |                        | μl                    | μl                      |
| Fluoride        | 0.50             | 0.0027 M            | Phosphoglyceric acid | 310                       | 320                    | 73                    | 235                     |
| Arsenite        | 0.062            |                     | α-Keto acids         | 270                       | 210                    |                       |                         |
| Monoiodoacetate | 0.031            | 0.0027 M            | Dehydrogenases       | 15                        | 120                    | 5                     | 29                      |
| Bisulfite       | 0.05             | 0.0015 M            | α-Keto acid          | 20                        | 375                    | 307                   | 586                     |
| No inhibitor    |                  |                     |                      | 380                       | 425                    |                       |                         |

Growth experiments: Results in terms of turbidity readings. Each flask contained 0.8 per cent KH<sub>2</sub>PO<sub>4</sub>, 0.4 per cent (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.8 per cent glucose, and 10 per cent tap water (for inorganic ions). Total volume, 102 ml. Temperature 30 C. To this were added 2 ml of a 24-hour culture of *A. aerogenes*. Aeration time, 18 hours.

Manometric experiments: Total volume 2.3 ml. Each cup contained 30 mg dry weight of lyophilized *A. aerogenes* grown on citrate medium, 0.5 ml of 0.025 M sodium citrate, 0.5 ml of 0.4 M PO<sub>4</sub> buffer, pH 7.0, NaOH or H<sub>2</sub>SO<sub>4</sub> plus various concentrations of inhibitors as shown in table. Temperature, 30.4 C.

\* Carbon dioxide was removed by passing air through 30 per cent NaOH. These tubes contained 0.5 mm sodium citrate.

† Aeration was with air containing the normal complement of CO<sub>2</sub>. To these tubes no citrate was added.

R.Q. for α-ketoglutaric acid is 2.0 (table 2). 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 fixative. The slight increase of CO<sub>2</sub> 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 CO<sub>2</sub> (Silverman and Werkman, 1940), or acetate and CO<sub>2</sub>. In the presence of bisulfite, pyruvate is fixed. The low value for oxygen uptake in the presence of NaHSO<sub>3</sub> and oxalacetate is not significant since the initial step in the oxidation of the C<sub>4</sub> labile acid involves no oxygen. Objections to these

explanations cannot be raised since the spontaneous decarboxylation of oxalacetate is the same in the presence and absence of bisulfite (indicating no fixation of the keto compound) and pyruvic acid is fixed in the presence of the keto fixative.

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 employed. The results in table 2 clearly show that semicarbazide·HCl is the best of the keto fixatives tested, for in its presence  $\alpha$ -ketoglutarate is scarcely attacked. When this compound is added to citrate

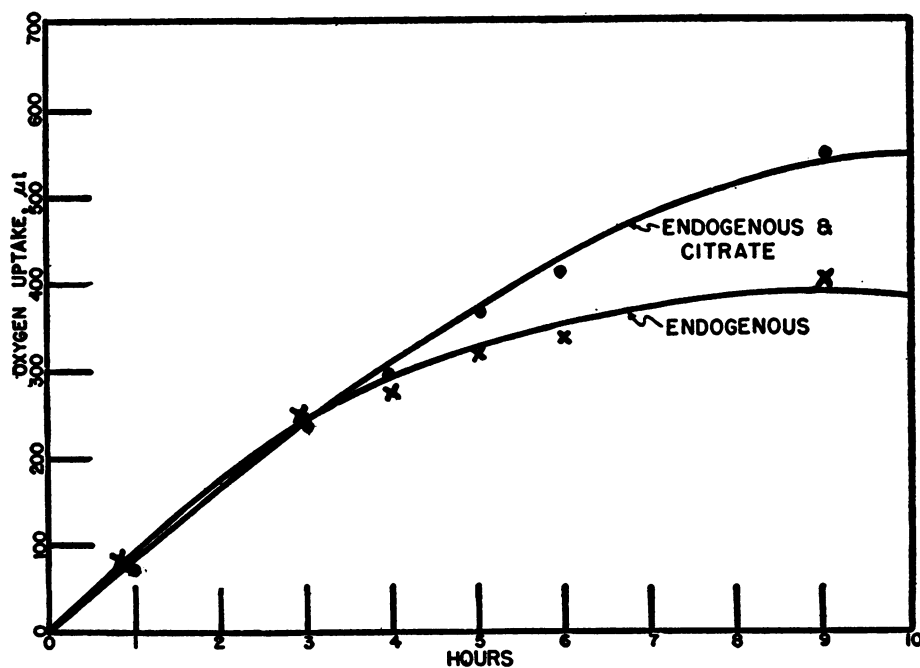


Figure 1. Effect of bisulfite on the oxidation of citric acid. Total volume of reactants 2.3 ml; 0.0054 M sodium citrate, 0.0054 M  $\text{NaHSO}_3$ , 0.047 M phosphate buffer, pH 7.0. Bacterial suspensions were added at the rate of 30 mg per cup dry weight. Temperature, 30.4 C.

and nonproliferating cells, an R.Q. of 1.6 or higher is obtained. If oxalacetate is the initial intermediate and it is fixed with the semicarbazide, thus leaving acetate as the only product to be attacked according to the scheme presented by Brewer and Werkman (1939) for citrate dissimilation, an R.Q. of 1.0 should be obtained. Further, acetate is not attacked by our strains of *A. aerogenes*.

The manometric results with bisulfite vary and the reason is not clear. Paret-sky (1948) 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, i.e., there is no replacement of  $\text{CO}_2$  by citrate in the presence of  $\text{NaHSO}_3$ .

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 CO<sub>2</sub> was given off. Aniline citrate decomposes oxalacetate to pyruvate and carbon dioxide even in the presence of the foregoing fixatives. The explanation, therefore, that citrate is not attacked in the presence of carbonyl reagents due to the binding of oxalacetate cannot be accepted as valid.

Ochoa (1948) describes a soluble pyridine protein enzyme of heart muscle that catalyzes the oxidation of isocitrate to oxalsuccinate, which in turn is decar-

TABLE 2  
*Oxidation of citrate, α-ketoglutarate, and oxalacetate in the presence of keto fixatives*

|                             | SUBSTRATE |                    |      |                                     |                        |      |                    |                                     |                        |      |                    |                                     |                        |             |
|-----------------------------|-----------|--------------------|------|-------------------------------------|------------------------|------|--------------------|-------------------------------------|------------------------|------|--------------------|-------------------------------------|------------------------|-------------|
|                             | Citrate*  |                    |      | α-Ketoglutarate*                    |                        |      | Oxalacetate†       |                                     |                        |      |                    |                                     |                        |             |
|                             | Inhibitor |                    |      |                                     |                        |      |                    |                                     |                        |      |                    |                                     |                        |             |
|                             | None      | NaHSO <sub>3</sub> |      | NH <sub>2</sub> NHCONH <sub>2</sub> | NH <sub>2</sub> OH·HCl | None | NaHSO <sub>3</sub> | NH <sub>2</sub> NHCONH <sub>2</sub> | NH <sub>2</sub> OH·HCl | None | NaHSO <sub>3</sub> | NH <sub>2</sub> NHCONH <sub>2</sub> | NH <sub>2</sub> OH·HCl |             |
| Oxygen uptake,<br>μl        | 199       | 1                  | 150  | 155                                 | 111                    | 191  | 100                | 15                                  | 49                     | 268  | 111                | 171                                 | 41                     |             |
|                             |           | 2                  | 151  |                                     |                        |      |                    |                                     |                        |      |                    |                                     |                        |             |
|                             |           | 3                  | 307  |                                     |                        |      |                    |                                     |                        |      |                    |                                     |                        |             |
|                             |           | 4                  | 214  |                                     |                        |      |                    |                                     |                        |      |                    |                                     |                        |             |
| CO <sub>2</sub> evolved, μl | 236       | 1                  | 194  | 246                                 | 140                    | 249  | 88                 | 77                                  | 19                     | 802  | 630                | 358                                 | 183                    |             |
|                             |           | 2                  | 215  |                                     |                        |      |                    |                                     |                        |      |                    |                                     |                        |             |
|                             |           | 3                  | 586  |                                     |                        |      |                    |                                     |                        |      |                    |                                     |                        |             |
|                             |           | 4                  | 308  |                                     |                        |      |                    |                                     |                        |      |                    |                                     |                        |             |
| R.Q.                        | 1.20      | 1                  | 1.30 | 1.60                                | 1.26                   | 1.30 |                    |                                     |                        | 2.99 |                    |                                     |                        |             |
|                             |           | 2                  | 1.42 |                                     |                        |      |                    |                                     |                        |      |                    |                                     |                        | Avg<br>1.51 |
|                             |           | 3                  | 1.90 |                                     |                        |      |                    |                                     |                        |      |                    |                                     |                        |             |
|                             |           | 4                  | 1.43 |                                     |                        |      |                    |                                     |                        |      |                    |                                     |                        |             |

Total volume of reactants 2.3 ml, 0.047 M phosphate buffer, pH 7.0. Bacterial suspensions were added at the rate of 30 mg dry weight per cup. Temperature 30.4 C.

\* 0.0054 M sodium citrate and α-ketoglutarate, respectively; 0.0054 M concentration of inhibitors.

† 0.022 M oxalacetate and inhibitors, respectively.

boxylated to α-ketoglutarate and CO<sub>2</sub>. Oxalsuccinate may be an intermediary in the oxidation of citrate by *A. aerogenes*, but as yet there is no direct evidence. If oxalsuccinate is formed and fixed by either bisulfite or semicarbazide, no CO<sub>2</sub> will be evolved since the change from citrate to oxalsuccinate involves only a dehydrogenation, which should be observed 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 were similar to those obtained with bisulfite added to the culture medium containing citric acid or the various keto acids as substituents for CO<sub>2</sub> (table 3). This keto fixative completely inhibits the ability of

citrate to replace carbon dioxide. In the presence of  $\text{NaHSO}_3$ ,  $\alpha$ -ketoglutarate is not effective, whereas with oxalacetate, growth takes place in the presence of 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 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 may play a role in its breakdown. In growth experiments, however, fluoride is not effective (table 1), and this indicates that no such esters

TABLE 3  
Replacement of carbon dioxide in the presence of bisulfite

| COMPOUND REPLACING CARBON DIOXIDE | CONCENTRATION | BISULFITE CONC. | GROWTH                  |                            |
|-----------------------------------|---------------|-----------------|-------------------------|----------------------------|
|                                   |               |                 | Without $\text{CO}_2^*$ | With $\text{CO}_2^\dagger$ |
|                                   | MM            | MM              |                         |                            |
| Citrate                           | 0.50          | —               | 380                     |                            |
| Citrate                           | 0.50          | 0.50            | 20                      | 425                        |
| $\alpha$ -Ketoglutarate           | 0.25          | —               | 390                     |                            |
| $\alpha$ -Ketoglutarate           | 0.25          | 0.25            | 0                       | 350                        |
| Oxalacetate                       | 0.25          | —               | 315                     |                            |
| Oxalacetate                       | 0.25          | 0.25            | 250                     | 290                        |

Results in terms of turbidity readings. Each flask contained 0.8 per cent  $\text{KH}_2\text{PO}_4$ , 0.4 per cent  $(\text{NH}_4)_2\text{SO}_4$ , 0.8 per cent glucose, and 10 per cent tap water (for inorganic ions). Total volume 102 ml. Temperature, 30 C. To this were added 2 ml of a 24-hour culture of *A. aerogenes* and the compounds as indicated in the table. Aeration time, 18 hours.

\* Carbon dioxide was removed by passing air through 30 per cent  $\text{NaOH}$ .

† Aeration was with normal air; tubes did not contain any added compounds.

are involved in the partial breakdown of citrate for  $\text{CO}_2$  replacement. Monoiodoacetate, on the other hand, inhibits the oxidation of citric acid both in growth and resting cell experiments. This is expected if  $\alpha$ -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 oxalsuccinate to  $\alpha$ -ketoglutarate would result in a loss of the ability of citric acid to replace carbon dioxide. Again, if oxalacetate is the initial product of the breakdown of citric acid, it would replace  $\text{CO}_2$ , 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

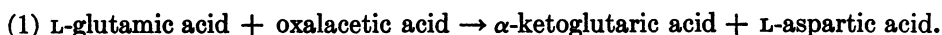
such inhibition with *Streptococcus (Leuconostoc) paracitrovorus* under anaerobic conditions, with citrate as the substrate. Brewer and Werkman (1939) report some inhibition with *Aerobacter indologenes* using 0.02 M  $\text{Na}_4\text{P}_2\text{O}_7$  aerobically. Under aerobiosis pyrophosphate completely inhibits the oxidation of citrate in the first three hours of the experiment.

A series of organic acids structurally related to citric acid was tested manometrically with *Aerobacter aerogenes* to determine fermentability and ability to replace  $\text{CO}_2$ . The acids tested were aconitic, tricarballic, citraconic, itaconic, and  $\alpha$ -hydroxy-*iso*-butyric. Of these, tricarballic and itaconic were not attacked. Citraconic and  $\alpha$ -hydroxy-*iso*-butyric acid were oxidized slowly, whereas only  $\alpha$ -hydroxy-*iso*-butyric acid failed to substitute for carbon dioxide.

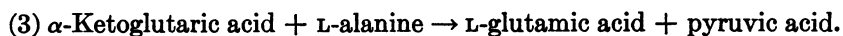
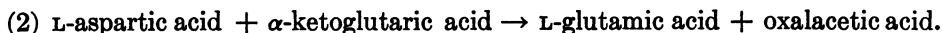
The behavior of aconitic acid is of interest because of its structural relationship to citric acid. Whereas the *cis* form is both active in replacing  $\text{CO}_2$  and functioning as a substrate in manometric experiments, the *trans* form is completely inactive. Even as high a concentration as 4 mM will not replace carbon dioxide to any appreciable extent.

*$\alpha$ -Ketoglutaric acid.*—Arsenious oxide prevents the oxidative decarboxylation of  $\alpha$ -ketoglutaric acid and yet is only partially effective in preventing this compound from replacing carbon dioxide. The chief mechanism by which the keto acid replaces  $\text{CO}_2$  may, however, involve a reaction other than an oxidative decarboxylation. An oxidation may also be involved, particularly during the lag phase when the development of the organisms in the absence of  $\text{CO}_2$  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  $\alpha$ -ketoglutaric acid replaces carbon dioxide by functioning as a substrate for amination or transamination. Adler *et al.* (1938) state that suspensions of *E. coli* form oxalacetic acid from  $\alpha$ -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) reported negative results in a more elaborate study. On the other hand, Lichstein and Cohen (1945) showed a small but definite transaminase activity in *E. coli*, which catalyzed the following reaction:



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



Since reactions 2 and 3 proceed slowly, the products will not accumulate in determinable quantities. Evidence will be presented that these reactions do occur and furthermore that reaction 3 is involved in protein synthesis.

No growth of *A. aerogenes* occurs in a medium of 0.8 per cent  $\text{KH}_2\text{PO}_4$ , 0.8

per cent glucose, and 10 per cent tap water, with 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. The addition of  $\alpha$ -ketoglutarate results in normal development. Other amino acids, e.g., glycine,  $\beta$ -alanine, and serine, also known to be deaminated by *A. aerogenes* do not replace alanine.  $\alpha$ -Ketoglutaric acid alone—without alanine or ammonium

TABLE 4  
*Replacement of carbon dioxide by  $\alpha$ -ketoglutaric acid in the presence of various nitrogen sources*

| ADDITIONS TO BASAL MEDIUM                       | CONCENTRATION  | GROWTH IN THE ABSENCE OF CO <sub>2</sub> |
|-------------------------------------------------|----------------|------------------------------------------|
| No additions                                    | —              | 5                                        |
| Ammonium sulfate                                | 0.4%           | 10-15                                    |
| Ammonium sulfate<br>$\alpha$ -Ketoglutaric acid | 0.4%<br>0.5 mM | 325                                      |
| DL-Alanine                                      | 4 mM           | 50                                       |
| DL-Alanine<br>$\alpha$ -Ketoglutaric acid       | 4 mM<br>0.5 mM | 425                                      |
| Glycine                                         | 2 mM           | 5-10                                     |
| Glycine<br>$\alpha$ -Ketoglutaric acid          | 2 mM<br>0.5 mM | 10-20                                    |
| $\beta$ -Alanine                                | 2 mM           | 10                                       |
| $\beta$ -Alanine<br>$\alpha$ -Ketoglutaric acid | 2 mM<br>0.5 mM | 30                                       |
| DL-Serine                                       | 4 mM           | 25                                       |
| DL-Serine<br>$\alpha$ -Ketoglutaric acid        | 4 mM<br>0.5 mM | 35                                       |

The basal medium contained 0.8 per cent KH<sub>2</sub>PO<sub>4</sub>, 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 *A. aerogenes*. It was aerated with CO<sub>2</sub>-free water for 18 hours at 30 C; growth was expressed in terms of turbidimetric readings on the photoelectric colorimeter using 660 m $\mu$  filter.

sulfate—will not support growth (table 4). It may be concluded that the basic mechanism responsible for protein synthesis or growth in the absence of CO<sub>2</sub> 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 very significant function in replacing CO<sub>2</sub>. However, the ammonia given off by glycine,  $\beta$ -alanine, or serine apparently cannot be utilized 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 or 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 with a twofold purpose: (1) to prevent the oxidative

TABLE 5

*Evidence for amination and transamination by A. aerogenes in the presence of As<sub>2</sub>O<sub>3</sub> as measured by residual  $\alpha$ -ketoglutaric acid*

| ADDITIONS TO $\alpha$ -KETOGLUTARIC ACID | FINAL CONCENTRATION  | $\alpha$ -KETOGLUTARIC ACID RESIDUAL,<br>MEASURED AS $\mu$ l CO <sub>2</sub> EVOLVED ON<br>ADDITION OF CERIC SULFATE AT<br>END OF EXPERIMENT |              |
|------------------------------------------|----------------------|----------------------------------------------------------------------------------------------------------------------------------------------|--------------|
|                                          |                      | Experiment 1                                                                                                                                 | Experiment 2 |
| Arsenious oxide                          | 0.0011 M             | 201                                                                                                                                          | 205          |
| Arsenious oxide<br>DL-Alanine            | 0.0011 M<br>0.0044 M | 184                                                                                                                                          | 199          |
| Arsenious oxide<br>DL-Aspartic acid      | 0.0011 M<br>0.0044 M | 143                                                                                                                                          | 143          |
| Arsenious oxide<br>Ammonium chloride     | 0.0011 M<br>0.0044 M | 90                                                                                                                                           | 155          |
| Arsenious oxide<br>Ammonium sulfate      | 0.0011 M<br>0.0044 M | 140                                                                                                                                          | 153          |

Total volume of reactants was 2.8 ml. Each cup contained 30 mg (dry weight) *A. aerogenes*, 0.5 ml of 0.2 M phosphate buffer, pH 7.0, 0.5 ml of 0.02 M  $\alpha$ -ketoglutaric acid, and 0.5 ml of neutralized arsenious oxide. Temperature, 30.4 C.

At the end of the experiment, 0.3 ml of 12 N H<sub>2</sub>SO<sub>4</sub> were added to liberate bound CO<sub>2</sub>. Then 0.5 ml of saturated ceric sulfate were added to oxidatively decarboxylate  $\alpha$ -ketoglutarate. When the proper corrections are made for the oxidative decarboxylation of other keto acids that might have formed (e.g., pyruvate from alanine) and when the proper controls are employed, the CO<sub>2</sub> liberated on the addition of ceric sulfate becomes a measure for residual  $\alpha$ -ketoglutaric acid.

decarboxylation of  $\alpha$ -ketoglutarate, thus forcing the reaction in the desired direction, and (2) to show that As<sub>2</sub>O<sub>3</sub> does not inhibit transamination (the explanation why both citrate and  $\alpha$ -ketoglutarate replace CO<sub>2</sub> in its presence). The results obtained with ammonium sulfate, DL-alanine, and DL-aspartic acid are shown in table 5.

To show that residual  $\alpha$ -ketoglutarate is a valid measure of amination (or transamination) large-scale experiments were set up, and the product of amination was measured directly. The results (table 6) obtained clearly show a considerable inhibition of the oxidation of  $\alpha$ -ketoglutarate by As<sub>2</sub>O<sub>3</sub> and, furthermore, that increased amounts of glutamic acid form in its presence.

*C<sub>4</sub> dicarboxylic acids.* The chief manner in which these acids act in replacing carbon dioxide is by serving as precursors of oxalacetic acid (table 7). 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

TABLE 6  
*Synthesis of glutamic acid by Aerobacter aerogenes*

|                              | α-KETOGLUTARIC ACID |               | GLUTAMIC ACID |
|------------------------------|---------------------|---------------|---------------|
|                              | Original conc.      | Oxygen uptake |               |
| With arsenious oxide.....    | 0.0176 M            | μl<br>1,420   | mg<br>24      |
| Without arsenious oxide..... | 0.0176 M            | μl<br>5,186   | mg<br>4       |

Each flask contained 300 mg (dry weight) of cells, 5 ml of 0.1 M ammonium sulfate, 5 ml of 0.005 M As<sub>2</sub>O<sub>3</sub>, and 5 ml of phosphate buffer, pH 7.4. Final volume, 30 ml. Aerobic. Time, 2.5 hours; temperature, 30.4 C.

TABLE 7  
*Mechanism of carbon dioxide replacement by C<sub>4</sub> dicarboxylic acids*

| C <sub>4</sub> ACID ADDED | CONC. | INHIBITOR              | CONC. OF INHIBITOR | GROWTH IN                  |                                                      | GROWTH (AFTER NO GROWTH IN 12-14 HR) ON ADDITION OF |             |       |
|---------------------------|-------|------------------------|--------------------|----------------------------|------------------------------------------------------|-----------------------------------------------------|-------------|-------|
|                           |       |                        |                    | Absence of CO <sub>2</sub> | Presence of CO <sub>2</sub> , no C <sub>4</sub> acid | CO <sub>2</sub>                                     | Oxalacetate | Conc. |
|                           | mM    |                        |                    |                            |                                                      |                                                     |             | mM    |
| A                         |       |                        |                    |                            |                                                      |                                                     |             |       |
| Succinate.....            | 1     | Iodo-<br>ace-<br>tate  | 0.031 mM           | 35                         | 170                                                  | 140                                                 | 270         | 1     |
| Fumarate.....             | 1     |                        |                    | 13                         |                                                      |                                                     |             |       |
| Malate.....               | 1     |                        |                    | 10                         |                                                      |                                                     |             |       |
| Oxalacetate.....          | 1     |                        |                    | 145                        |                                                      |                                                     |             |       |
| B                         |       |                        |                    |                            |                                                      |                                                     |             |       |
| Fumarate.....             | 1     | Cyclo-<br>hexa-<br>nol | 0.25%              | 45                         | 100                                                  | 150                                                 | 150         | 0     |
| Succinate.....            | 1     |                        |                    | 65                         |                                                      |                                                     |             |       |
| Malate.....               | 1     |                        |                    | 185                        |                                                      |                                                     |             |       |
| Oxalacetate.....          | 1     |                        |                    | 190                        |                                                      |                                                     |             |       |

Results are in terms of turbidity readings. 0-30 indicates no growth due to the added compound. Total volume, 101 ml. Temperature, 30 C. Basal medium consisted of 0.8 per cent KH<sub>2</sub>PO<sub>4</sub>, 0.4 per cent (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 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 *A. aerogenes*.

B: Basal medium plus 0.2 per cent glucose, 0.5 ml of a 24-hour culture of the organism.

the addition of carbon dioxide or oxalacetate, an observation that may be used to support a role of the Szent-Györgyi cycle in bacterial respiration, at least in the direction of oxidation. If the cycle operates, the inhibition of any one 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 the addition of oxalacetate. Similarly, the C<sub>4</sub> 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 that 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  $\alpha$ -ketoglutaric acid as well as to 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

TABLE 8  
*Action of cyclohexanol upon glutamic and aspartic acids*

| COMPOUND           | MANOMETRIC DATA*        |                         |                         |                         | AMMONIA PRODUCED |                | GROWTH EXPERIMENTS† |                |
|--------------------|-------------------------|-------------------------|-------------------------|-------------------------|------------------|----------------|---------------------|----------------|
|                    | With cyclohexanol       |                         | Without cyclohexanol    |                         | With cyclo.      | Without cyclo. | With cyclo.         | Without cyclo. |
|                    | CO <sub>2</sub> evolved | O <sub>2</sub> utilized | CO <sub>2</sub> evolved | O <sub>2</sub> utilized |                  |                |                     |                |
|                    | $\mu$                   | $\mu$                   | $\mu$                   | $\mu$                   | $\mu$ EN         | $\mu$ EN       |                     |                |
| Aspartic acid..... | -9                      | -14                     | 402                     | 317                     | 18               | 200            | 3                   | 200            |
| Glutamic acid..... | 0                       | -18                     | 522                     | 432                     | 7                | 263            | 100                 | 350            |
| Glucose.....       | 431                     | 302                     | 521                     | 540                     |                  |                |                     |                |

\* Total volume of reactants 2.8 ml. The following additions were made in the manometer cup: 30 mg lyophilized *A. aerogenes*, 0.5 ml of 0.05 M DL-aspartic acid (or 0.5 ml of 0.025 M D-glutamic acid or 0.5 ml of 0.025 M glucose), 1 ml of a 1:50 dilution of cyclohexanol, 0.5 ml of 0.4 M phosphate buffer of pH 7.0. Alkali or acid in the respective cups. Temperature, 30.4 C.

† Results in terms of turbidity readings; 0 indicates no growth. Total volume 101 ml containing basal medium plus 0.8 per cent glucose, 1 mM of either D-glutamate or 2 mM of DL-aspartate, 0.2 per cent cyclohexanol, 1 ml of a 24-hour inoculum of *A. aerogenes*. Temperature, 30 C. Incubation time, 18 hours. Aerated with CO<sub>2</sub>-free air. In the presence of CO<sub>2</sub>, 0.2 per cent cyclohexanol showed no inhibition of growth.

and glutamic acids (table 8) by nonproliferating lyophilized cells of *A. aerogenes*. When suitable amounts of this inhibitor are added to cultures in which aspartate or glutamate functions as a 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 its addition to cells of *A. aerogenes* incubated with amino acids results in a decrease in the oxygen uptake below that of the endogenous respiration in the presence of cyclohexanol incubated with glucose. There was no poisoning effect for the first 30 to 60 minutes; then a considerable reduction in gas exchange took place. Similar results were obtained with arsenious oxide. When amino acids served as substrate, the inhibition with cyclohexanol was immediate. The inhibition of deam-

ination (and oxidation) found when the bacteria are incubated with aspartic or glutamic acid is, therefore, not a general poisoning of the cells.

*Asparagine and glutamine.* Both of these compounds replace CO<sub>2</sub> through their respective amino acids. McIlwain *et al.* (1948) synthesized a compound

TABLE 9  
*The effect of inhibitors on the replacement of CO<sub>2</sub> by glutamine and asparagine*

| SUBSTRATE  | INHIBITION          | CONC. OF INHIBITOR |                     | GROWTH                    |                        | RESTING CELLS         |                         |
|------------|---------------------|--------------------|---------------------|---------------------------|------------------------|-----------------------|-------------------------|
|            |                     | Growth expts.      | Resting cell expts. | Without CO <sub>2</sub> * | With CO <sub>2</sub> † | O <sub>2</sub> uptake | CO <sub>2</sub> evolved |
|            |                     | mm                 | M                   |                           |                        | μl                    | μl                      |
| Glutamine  | γ-Glutamylhydrazine | 0.125              | 0.0054 M            | 10                        | 100                    | 17                    | 33                      |
| Glutamine  | —                   | —                  | —                   | 370                       | 400                    | 368                   | —                       |
| Asparagine | β-Aspartylhydrazine | 0.125              | 0.0054 M            | 17                        | 125                    | 10                    | 26                      |
| Asparagine | —                   | —                  | —                   | 175                       | 300                    | 245                   | —                       |

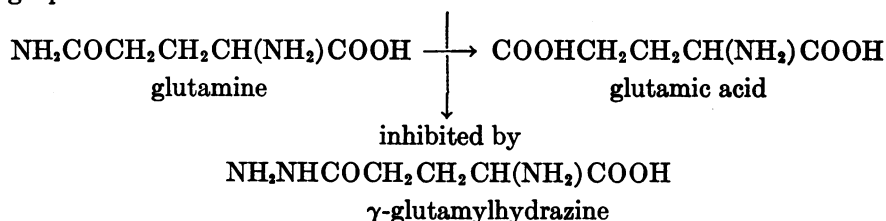
Growth experiments: Results are in terms of turbidity readings. Each flask contained 0.8 per cent KH<sub>2</sub>PO<sub>4</sub>, 0.4 per cent (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.8 per cent glucose, and 10 per cent tap water (for inorganic ions). Total volume, 101 ml. Temperature, 30 C. To this was added 1 ml of a 24-hour culture of *A. aerogenes*. Aeration time, 18 hours.

Manometric experiments: Total volume 2.3 ml. Each cup contained 30 mg dry weight of lyophilized *A. aerogenes*, 0.5 ml of 0.025 M glutamate or asparagine, 0.5 ml of 0.4 M PO<sub>4</sub> buffer, pH 7.0, NaOH or H<sub>2</sub>SO<sub>4</sub>, plus various concentrations of inhibitors as shown in table. Temperature, 30.4 C.

\* Carbon dioxide removed by passing air through 30 per cent NaOH. These tubes contained 0.125 mm glutamine or asparagine.

† Aeration with air containing normal complement of CO<sub>2</sub>. These tubes contained the inhibitors but no substrate other than basal medium.

that inhibited the liberation of ammonia from glutamine according to the following equation:



Similarly, β-aspartylhydrazine prevents the deamidation of aspartic acid. When these inhibitors are added to cultures in which 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 CO<sub>2</sub> by yielding their respective amino acids (table 9). Manometrically, the oxidation of both glutamine and asparagine is inhibited by their hydrazines.

#### DISCUSSION

The evidence points to an orderly manner in which the substitution of the various compounds for carbon dioxide takes place. The results show that the com-

pounds replacing  $\text{CO}_2$  must arise from one or two fixation reactions involving a  $\text{C}_3$  and  $\text{C}_1$  addition or a  $\text{C}_4$  and  $\text{C}_1$  addition in which the  $\text{C}_1$  compound is carbon dioxide. The  $\text{C}_4$  or the  $\text{C}_5$  compounds thus formed are essential for the growth of heterotrophic bacteria. In the absence of the gas, the  $\text{C}_4$  or  $\text{C}_5$  compound must be supplied to the cells before growth occurs. The essential  $\text{C}_4$  and  $\text{C}_5$  compounds appear to be oxalacetic and  $\alpha$ -ketoglutaric acids, respectively.

It is tempting to assume that citric acid replaces  $\text{CO}_2$  by yielding  $\alpha$ -ketoglutarate. No direct evidence has yet been obtained. However, from our data it cannot be concluded that the initial breakdown products of citrate are oxalacetate and acetate, although the oxalacetic acid thus formed could replace  $\text{CO}_2$ . If the  $\text{C}_4$  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  $\text{CO}_2$  by citric acid since oxalacetate continues to function as a carbon dioxide substituent even in the presence of this keto fixative. The replacement of  $\text{CO}_2$  by  $\alpha$ -ketoglutarate in the presence of bisulfite is completely inhibited. Second, monoiodoacetate completely inhibits citric acid from replacing carbon dioxide. If oxalacetate is the intermediate through which citrate functions in replacing  $\text{CO}_2$ , the iodoacetate should not interfere. On the other hand, if a 5-carbon keto acid is one of the initial breakdown products, a dehydrogenation is involved and monoiodoacetate should, theoretically at least, inhibit the function of citric acid in replacing  $\text{CO}_2$ . Further, if oxalacetate is an initial breakdown product of the  $\text{C}_5$  acid and it is bound by bisulfite, then on the addition of aniline citrate,  $\text{CO}_2$  should evolve. No such gas evolution was obtained when citrate was being slowly oxidized in the presence of  $\text{NaHSO}_3$ .

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

$\alpha$ -Ketoglutaric acid replaces  $\text{CO}_2$  by serving as a substrate for amination or transamination. Evidence that a transamination is involved in initiating growth in systems completely devoid of  $\text{CO}_2$  is presented in the experimental section.

The  $\text{C}_4$  dicarboxylic acids replace carbon dioxide by functioning as precursors of oxalacetate. Support of the premise that any of the four dicarboxylic acids may function in the absence of  $\text{CO}_2$  as a hydrogen carrier is indicated by the resumption of growth on the addition of oxalacetate after multiplication has been stopped by the inhibition of any one of the  $\text{C}_4$  acids.

The amino acids function in the absence of carbon dioxide by either yielding  $\text{C}_4$  dicarboxylic acids or serving as compounds that would normally be formed during the metabolism of the cell. For example, aspartic acid serves solely as a source of fumarate, whereas the function of glutamate is apparently twofold. First, it yields  $\alpha$ -ketoglutaric acid, and, secondly, it serves as an essential amino acid whose formation would depend upon the presence of atmospheric carbon dioxide.

The criticism may be offered that if growth is prevented because of the inhibition of any one compound that replaces CO<sub>2</sub>, the same inhibition should result when a given inhibitor is added to a culture that is aerated with normal air. For example, if bisulfite binds  $\alpha$ -ketoglutarate and consequently prevents the latter from replacing CO<sub>2</sub>, should not bisulfite also inhibit the growth of the organisms in the presence of carbon dioxide? Several explanations may be offered. First, in normal metabolism, e.g., in the presence of CO<sub>2</sub>, more of the keto acid may form than the amount of NaHSO<sub>3</sub> 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. Secondly, in the absence of CO<sub>2</sub> 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 CO<sub>2</sub>, alternate mechanisms may operate, and, when  $\alpha$ -ketoglutarate, for example, is blocked, the organism may use a different compound whose function is independent of keto fixatives in the medium.

Evidence that those compounds do not function simply as sources of CO<sub>2</sub> has been given in a previous publication (Ajl and Werkman, 1948).

#### SUMMARY

All compounds replacing CO<sub>2</sub> function by yielding keto acids, which in turn by amination, transamination, or similar reactions serve as substituents for carbon dioxide.

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