THE INFLUENCE OF THE METABOLIC STATE AND OF THE MEDIUM ON CARBON DIOXIDE FIXATION BY SERRATIA MARCESCENS

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The assimilation of carbon dioxide into organic cell materials has been demonstrated many times since the pioneer experiments with propionic acid bacteria (Wood and Werkman, 1938). Fixation has been shown to occur in the cells of pigeon liver (Evans and Slotin, 1941), rabbit liver (Anfinsen *et al.*, 1949), protozoa (van Niel *et al.*, 1942), several species of bacteria including *Clostridium butylicum* (Brown, 1944), *Lactobacillus arabinosus* (Lardy *et al.*, 1949), *Escherichia coli* (Utter *et al.*, 1945), and *Staphylococcus aureus* (Wiken *et al.*, 1947), and, of course, of plants during photosynthesis. The assimilated carbon has been found in many compounds and especially in formic, lactic, pyruvic, oxalacetic, malic, α-ketoglutaric, and aspartic acids as well as in undetermined amino acids.

The inference that the assimilation of carbon dioxide is essential to some metabolic process may be made from the fact that a certain amount of carbon dioxide must be present in the environment if bacteria are to grow (Gladstone *et al.*, 1935; Rahn, 1941; and others) although they may survive in its absence. The quantitative determination of the carbon dioxide need is difficult as most bacteria continuously produce this gas. However, by removing the metabolic carbon dioxide by vigorous aeration, the minimum concentration of carbon dioxide for optimum growth of *E. coli* was shown to be 0.03 per cent (Lwoff and Monod, 1947). By using species producing relatively little carbon dioxide, such as many of the lactic acid bacteria, the need for this compound can be effectively demonstrated (e.g., Lyman *et al.*, 1947).

Some compounds are said to "replace" carbon dioxide since they permit bacterial growth when the carbon dioxide supply is the limiting factor. Both simple substances, such as di- and tricarboxylic acids and certain amino acids, and complex substances, such as peptone and yeast extract, show this effect (Lwoff and Monod, 1947; Lyman *et al.*, 1947; Ajl and Werkman, 1948, 1949). It is generally assumed that such substances decrease fixation because they are related in some way to the compounds normally formed in this manner, and it has been actually demonstrated that fixation can be decreased by the addition of aspartic acid to cultures of *L. arabinosus* (Lardy *et al.*, 1949). However, under the conditions of most of the "replacement" experiments a stimulation of carbon dioxide production would lead to identical observations but was not considered.

The present investigation was undertaken to make a quantitative study of carbon dioxide fixation and to determine the influence on this phenomenon of

factors such as the composition of the medium and the metabolic state of the organism. In addition, a study was made of the relation of fixation to other concomitant cellular activities such as oxygen consumption, carbon dioxide production, and ammonia assimilation.

The organism *Serratia marcescens* is especially suitable for such a study. Not only will it grow on any one of a number of carbon sources combined with a simple nitrogen source, such as ammonia, but also its metabolic state changes relatively rapidly from "growing" to "resting" if the nitrogen source is removed, and to "endogenous" if the carbon source also is removed. Finally, it has the advantage of providing a suspension of small, single, living cells that can be kept relatively well equilibrated with the environment and that can be sampled quantitatively.

MATERIALS AND METHODS

The strain of *S. marcescens* and the synthetic medium were those described previously (McLean and Fisher, 1947). Suspensions free from contamination by the medium were prepared for each experiment by the following procedure: approximately 10^8 cells from 8-hour stock slants were suspended in buffer, spread on the surface of sterile cellophane disks placed over the agar medium (Craigie, unpublished), grown for 16 hours at 30 C, and harvested by removing the cellophane disks and shaking in phosphate buffer (0.07 M, pH 7). The resulting suspensions were diluted to a concentration of 2 to 3 $\times$ 10^9 bacteria per ml (about 40 μg bacterial N per ml).

Oxygen consumption was measured at 30 C in the Warburg respirometer (Umbreit *et al.*, 1945) with air as the gas phase. Since growth of the bacteria under certain conditions depends on an appreciable concentration of carbon dioxide in the atmosphere, the alkali was replaced by 0.6 ml of an ethanolamine-acid-carbonate solution (Pardee, 1949), which maintains a constant partial pressure of carbon dioxide of approximately 0.5 per cent. This solution consumes oxygen at a slow steady rate and a correction for this was applied.

Carbon dioxide output was determined by the direct method of Warburg (Umbreit *et al.*, 1945). "Bound" carbon dioxide was liberated quantitatively by adding 0.3 ml of 0.5 N potassium acid phthalate, which served also to stop metabolism. Phthalate proved more satisfactory than stronger acids as it did not cause agglutination of the bacteria but left them in a suspension suitable for sampling.

In order to follow carbon dioxide fixation, 1.5×10^{-3} M bicarbonate containing about 1/30 microcurie per ml of C^{14} was added to the bacteria. Preliminary work indicated that this dose of radiation had no effect on the metabolic processes measured in these experiments.

No exchange of C^{14} occurred between the bicarbonate and the other components of the medium since, in the absence of bacteria, the C^{14} was completely liberated by the addition of acid to the medium even after 5 hours' incubation with the usual amount of labeled bicarbonate.

The ammonia remaining in the bacteria-free medium was determined by the addition of alkali to the medium, aeration into 0.02 N H_2SO_4, and nesslerization. Clouding of Nessler's reagent was prevented by the addition of a solution described by Gentzkow (1942). Total initial nitrogen measurements were made by digesting samples of the original bacterial suspension essentially as described by Snell and Snell (1936), and the ammonia thus formed was determined as in the bacteria-free medium.

In a typical experiment the contents of the vessels were as follows (all amounts are expressed as the final concentration or quantity in the vessel): in the main part, the bacterial suspension (3×10^9 cells), phosphate buffer (0.035 M, pH 7), and $MgSO_4 \cdot 7H_2O$ (0.05 per cent); in the first side arm, an excess of citrate or other carbon source (0.3 to 0.5 per cent), NH_4Cl (50 μg N) in the vessels used to study growth, and $NaHC^{14}O_3$ (1.5×10^{-3} M) in the vessels used to study CO_2 production and fixation. Each vessel was made up to a volume of 2 ml with these solutions. In addition, those vessels used to measure O_2 consumption contained the CO_2 absorbent in the inset, but the others used to measure CO_2 production and fixation contained acid in the second onset.

At zero time, when the bacteria were metabolizing at an endogenous rate, the contents of the first side arm were added. At intervals, acid was tipped from the second side arm, the CO_2 liberated was measured, and the vessel was then removed from the bath. The contents were quantitatively transferred to small tubes and centrifuged for 10 minutes at 10,000 rpm (13,000 *g*) in the multispeed attachment for the International centrifuge. (Washing and recentrifugation were found to be unnecessary since unused bicarbonate was completely removed from the bacteria and medium by the addition of acid.) The supernatant, which was kept for later determination of the ammonia uptake, was withdrawn by suction. This involved a fairly constant loss of about 10 per cent of the bacteria. The bacterial pellet was resuspended in 0.5 ml of distilled water and duplicate 0.1-ml aliquots were plated on stainless steel disks by distributing the sample in about 30 small drops and drying under an infrared lamp. The samples were counted in a methane flow counter developed from the α-counter described by Simpson (1947). The samples were so thin that self-absorption was negligible, and samples of twice the usual weight deposited on the same area gave double the counting rate.

EXPERIMENTAL OBSERVATIONS

The observations of the uptake of C^{14}, as an indication of carbon dioxide assimilation, and of the decrease of ammonia in the medium, as an indication of growth, are shown in figure 1 for a typical experiment. During the conversion of ammonia from the external medium to bacterial nitrogen, carbon dioxide is rapidly assimilated. After about 3 hours, when the external ammonia is exhausted, the rate of carbon dioxide fixation decreases. This is the time at which the rate of oxygen consumption also decreases to a resting value (McLean and Fisher, 1947). Observations of carbon dioxide uptake by cells lacking a supply of am-

monia, i.e., resting cells, are included in figure 1 and these also indicate that the fixation of carbon dioxide is slow in the absence of growth.

The amount of carbon dioxide taken up by the cells cannot be measured directly from the increase in activity since the specific activity of the carbon dioxide absorbed is constantly decreasing, primarily as a result of the production of inactive carbon dioxide by the bacteria. The dilution of the active carbon dioxide at any given time may be calculated from the total volume of carbon dioxide present divided by the activity remaining in the medium. The dilution factor thus determined increases to 8 times its initial value in 4 hours. The total amount of fixed carbon dioxide corresponding to the counts observed can be

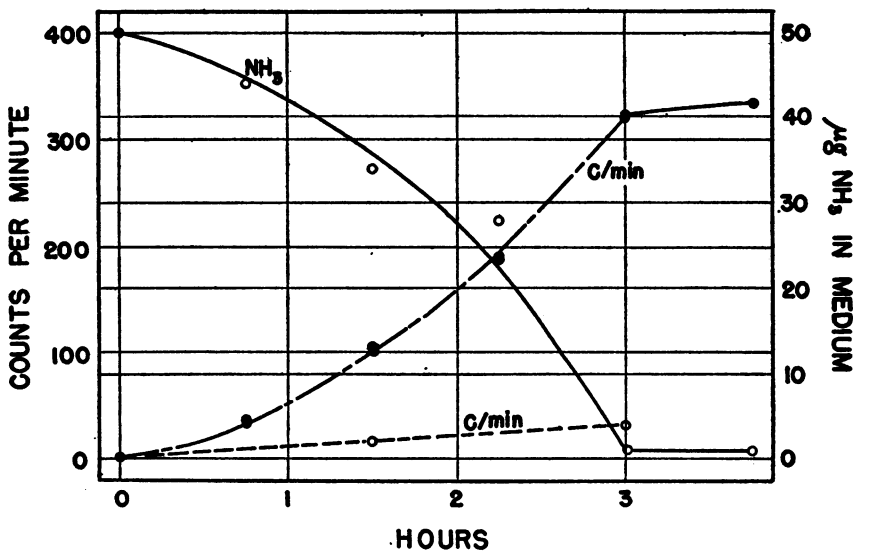

Figure 1. The time course of the uptake of C^{14} from bicarbonate by *S. marcescens* and of the removal of ammonia from the medium. The activity of growing cells is shown by solid circles and that of resting cells by open circles. The solid curve shows the ammonia remaining in the external medium.

calculated by taking the area under the curve relating the dilution factor (μl CO_2 per c per min) and the observed activity (c per min) in the bacteria.

The total carbon dioxide uptake in four comparable experiments is shown in figure 2. An examination of these data reveals that the total amount of carbon dioxide fixed during the assimilation of 50 μg of nitrogen supplied as ammonia is relatively constant, although the rate of fixation varies considerably from one culture to another.

The four lower curves show the fixation expected if the bacterial protoplasm increased at the same rate as in the upper curves but the cells remained in a physiologically resting state. They were determined by first calculating the average fixation (μl CO_2 per hr per mg bacterial N) by resting cells from the total bacterial nitrogen and the rate of uptake of counts. This value was then used to predict the "resting" fixation for the growing culture on the basis of its

nitrogen content at any time. The nitrogen content of the growing culture was estimated by adding the amount of nitrogen assimilated from ammonia to the initial nitrogen, since preliminary experiments had shown that no nitrogen was excreted.

The fixation that appears to be dependent on growth processes can be estimated by subtracting the calculated resting fixation from the observed total fixation at the time when all the ammonia has been removed. The amounts of net fixation associated with the assimilation of 50 μg of nitrogen are shown in figure 3 as

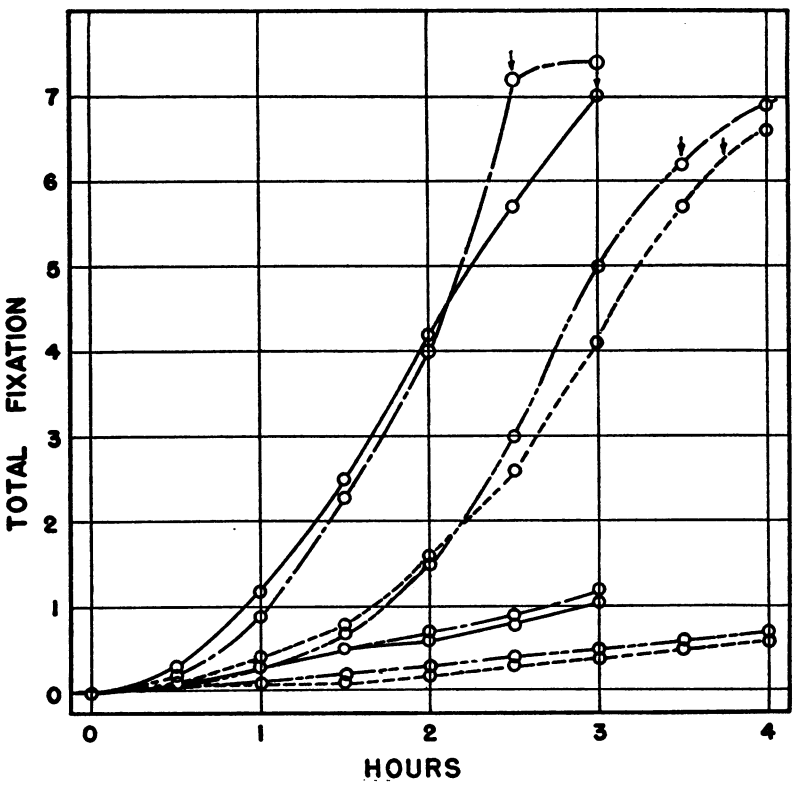

Figure 2. The time course of the fixation of CO_2 by *S. marcescens* in four experiments with citrate as the organic carbon source. The upper curves show the μl CO_2 fixed by growing bacteria and the lower ones the calculated fixation for an equivalent amount of bacterial nitrogen in the resting state. The arrows indicate the time at which the ammonia (50 μg N) added to the medium is exhausted.

solid bars and the corresponding resting values as crosshatched bars. The results plotted in figure 2 are those shown in figure 3 with citrate as the substrate.

In comparable experiments endogenous cells, i.e., cells lacking a carbon source (with or without ammonia), were found to fix carbon dioxide at a rate that was less than one-tenth that of resting cells.

Since the nature of the carbon substrate has previously been found to have a qualitative influence on the carbon dioxide need (e.g., Ajl and Werkman, 1948),

experiments were conducted to determine the quantitative effect of replacing the citrate with glycerol, glucose, pyruvate, or malate. In each case, as with citrate, the rate of fixation increased when the ammonia was being assimilated and decreased when it was exhausted. The values of the net and resting fixation with these substrates are given in figure 3.

The data on the net fixation during growth can be divided into two groups. The first includes glycerol, glucose, and pyruvate, which show high fixation; the second includes malate and citrate, which show only about two-thirds as much

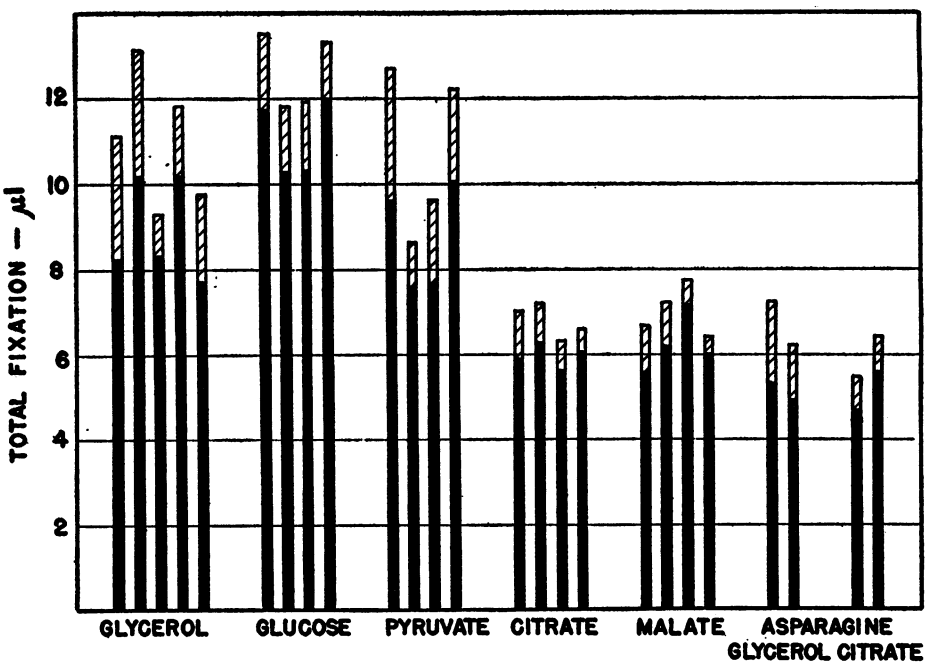

Figure 3. The total amount of CO_2 fixed during the assimilation of 50 µg of nitrogen in the presence of different organic carbon sources. Ammonia was used as the nitrogen source in all but the last four experiments; in these it was replaced by asparagine. The cross-hatched portion represents the fixation expected for an equivalent amount of bacterial nitrogen in a resting state. The solid portion represents the net fixation associated with growth.

fixation. The results for the five substrates were compared in pairs by the "*t*" test. It was found that no two within one group differ significantly ($P > 0.1$), whereas the fixation with any substrate in the first group is significantly higher than with any one in the second group ($P < 0.02$).

An equivalent amount of asparagine was substituted for ammonia as a nitrogen source since earlier work proved that asparagine nitrogen is used completely by these bacteria (McLean and Fisher, 1949). Glycerol, representing the first group of substrates, and citrate, representing the second group, were used as sources of carbon. The results, included in figure 3, indicate that, in glycerol, fixation

is decreased to a value characteristic of group two, but in citrate it is unchanged. The asparagine could not act as the main carbon source since more than four times the amount of carbon supplied as asparagine is usually consumed during the experiment.

Although the carbon and nitrogen sources do have a considerable influence on fixation, the change of the metabolic state from endogenous to resting or growing causes increases of much greater magnitude. Since the metabolic state of the cell governs both the rate of oxygen consumption and carbon dioxide production, it is important to see whether the changes in the rate of fixation can be correlated with these factors. The values of the rates of carbon dioxide fixation and production and of oxygen consumption in growing cells have been converted to μl per hr per mg bacterial N and are given in table 1, along with their standard deviations, as an index of the spread of the values. The growth rates, expressed as mg N assimilated per hr per mg bacterial N, are also included. Because the

TABLE 1

Metabolic activities of S. marcescens growing in NH_4Cl plus the carbon substrate specified

(The values given are the average of at least four determinations. The standard deviations are included to indicate the spread of the values. Growth rates are expressed as mg new bacterial nitrogen formed per hr per mg bacterial N and gas exchange as μl per hr per mg bacterial N)

SUBSTRATE	RATE OF GROWTH	RATE OF CO_2 FIXATION	RATE OF CO_2 PRODUCTION	RATE OF O_2 CONSUMPTION
Glycerol	0.80 ± .14	96 ± 33	1,330 ± 340	1,760 ± 130
Glucose	0.55 ± .11	140 ± 65	1,880 ± 190	1,560 ± 160
Pyruvate	0.53 ± .08	90 ± 16	2,790 ± 420	1,680 ± 190
Citrate	0.73 ± .18	52 ± 4	3,380 ± 390	1,590 ± 80
Malate	0.98 ± .22	92 ± 26	5,700 ± 1,200	2,990 ± 1,130

growth lag is variable, the rates are calculated at the time when one-half of the added nitrogen is assimilated and the bacteria appear to be growing logarithmically.

The fixation rates in different carbon substrates do not vary in the same manner as the total fixations. The reason is that the *rate* of fixation is influenced by the growth rate whereas *total* fixation is not. For example, although the total fixation in malate is the same as in citrate, the fixation rate in malate is much higher since growth also is proceeding more rapidly and the ammonia is consumed in a shorter time.

It is obvious that there is no close relationship between the metabolic rate, as indicated by oxygen consumption, and the fixation rate. For example, the fixation rates in pyruvate and malate are almost identical, whereas the rate of oxygen consumption is almost twice as high in malate.

Nor does the fixation rate appear to be related to the rate of carbon dioxide production, since, although the former is almost identical in glycerol and malate,

the latter is four times higher in malate. Since this correlation cannot be found, it is probable that low rates of fixation are not due to high rates of production of carbon dioxide and therefore intracellular utilization of unequilibrated carbon dioxide cannot be an important source of error in this work. The actual amount of carbon dioxide fixed varies from 1.5 to 7.5 per cent of the carbon dioxide produced by growing cells and is below 1 per cent in the case of resting cells.

Since biotin (2 mμg per ml) is needed for carbon dioxide fixation in *L. arabinosus* (Lardy *et al.*, 1949), this vitamin was added to *S. marcescens* in concentrations up to 50 mμg per ml. It had no effect, as might be expected, since this organism needs no external biotin for growth and presumably synthesizes an ample supply.

DISCUSSION

The most striking observation in this work is the large increase in carbon dioxide fixation accompanying the reactions associated with growth. This implies that the carbon must ultimately be found in some compound or compounds laid down preferentially during growth but does not preclude the possibility that the first product of fixation may be the same in resting and growing cells provided that it is removed more rapidly in the latter.

Although the final destination of the fixed carbon cannot be determined unequivocably from the present data, the indications are that it is used for protein formation. Not only is protein the main product of ammonia assimilation, but in addition, preliminary chemical analyses of the bacteria have shown that the bulk of the activity is not in the lipid or carbohydrate fraction and that a considerable amount is in the protein fraction. Also there are several reports in the literature of the fixation of carbon dioxide in protein (Evans and Slotin, 1941; Kritzmann, 1947; MacLeod and Lardy, 1949; Anfinsen *et al.*, 1949).

When the relative fixations of carbon dioxide and of ammonia are calculated, it is found that with substrates of group one (glycerol, glucose, and pyruvate) on the average 9 nitrogen atoms are assimilated from ammonia for every carbon assimilated from carbon dioxide, and with group two (malate and citrate) the ratio is 13 nitrogens per carbon. This suggests that, even if all the fixed carbon is in protein, it is only in certain of the amino acids.

The increased ratio of nitrogen to carbon in the presence of group two substrates indicates that some compound or compounds formed from group one substrates by fixation are formed from group two substrates without fixation. One such compound might be aspartic acid, which is known to be formed metabolically from citrate and malate without fixation, and from glucose, glycerol, and pyruvate by a fixation reaction of the Wood-Werkman type. In addition, the fixation in glycerol is reduced if the amide of aspartic acid, asparagine, is present.

Since the presence of asparagine only decreases fixation to the value observed with citrate and malate, the carbon must be fixed in other compounds in addition, but these cannot be postulated from the data available at present.

SUMMARY

Carbon dioxide fixation by *Serratia marcescens* was studied using bicarbonate labeled with C^{14}.

The rate of fixation of carbon dioxide by growing cells was about ten times higher than that by resting cells. There was no evidence of fixation by endogenous cells.

In the five substrates tested, the amount of carbon dioxide fixed by growing cells was less than 10 per cent of that produced and showed no correlation with the oxygen consumption and carbon dioxide production.

The total amount of carbon dioxide fixed during the assimilation of 50 μg of nitrogen from ammonium chloride was approximately 9.5 μl when the organic carbon source was glucose, glycerol, or pyruvate and 6 μl when it was citrate or malate.

When the same amount of nitrogen was assimilated from asparagine, the amount of carbon dioxide fixed with glycerol as substrate was the same as with citrate and approximately that value characteristic of citrate or malate in the presence of ammonium chloride. These results suggest that some of the carbon dioxide fixed when the substrate is glucose, glycerol, or pyruvate is necessary for the formation of aspartic acid or some related compound.

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