Relationship of Carbon Dioxide to Aspartic Acid and Glutamic Acid in *Actinomyces naeslundii*

B. B. BUCHANAN† AND LEO PINE‡

Department of Microbiology, Duke University, Durham, North Carolina

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**ABSTRACT**

Buchanan, B. B. (Duke University, Durham, N.C.), and Leo Pine. Relationship of carbon dioxide to aspartic acid and glutamic acid in *Actinomyces naeslundii*. J. Bacteriol. 89:729-733. 1965.—CO₂, which was essential for the fermentation of glucose to succinic acid by *Actinomyces naeslundii*, was also required for the synthesis of internal aspartic acid, for which the cells lack a permease.

In a study of the factors influencing the fermentation and growth of *Actinomyces naeslundii*, Buchanan and Pine (1963) observed that malate, fumarate, or succinate partially satisfy the requirements for CO₂, although the total cell yield is greater when CO₂ is added. Previous results had shown that added CO₂ is fixed into the carboxyl carbons of succinate (Pine, 1956, 1960). On the basis of the fermentation products formed anaerobically in the presence and absence of CO₂, as well as the growth yields under these conditions, it was suggested that CO₂ and pyruvate function as a hydrogen-accepting system to form succinate. CO₂ thereby permits the oxidation of additional glucose to formate and acetate. In the presence of added CO₂, more energy is made available for growth, as evidenced by increased cell yields (Buchanan and Pine, 1963).

Acetate and formate did not substitute for CO₂; because CO₂ was consistently required to initiate growth and to give maximal cell yields under both aerobic and anaerobic growth conditions, it was also suggested that CO₂ had a function in addition to that of a hydrogen acceptor. The experiments reported here were conducted to determine the second function of CO₂.

**MATERIALS AND METHODS**

*A. naeslundii* (*A. bovis* ATCC 10049), described previously (Buchanan and Pine, 1963), was grown on the medium of Pine and Watson (1959). In general, the methods of analyses and isolation of products were those described before (Buchanan and Pine, 1965). Organic acids and amino acids were separated on a Dowex-1 column (Busch, Hurlbert, and Potter, 1952). Individual amino acids were separated by paper chromatography. Purity was established by a combination of paper chromatography (phenol-water, 80:20) and radioautography run alone and with known amino acids. Specific protocols are given in the tables to which they apply. All radioactivity was measured with a continuous-flow counter equipped with a "micro-mil" window. "Q-gas" (98.4% He, 1.6% butane) was used as the ionizable gas. All samples were measured at infinite thinness or were corrected for background and self-absorption by reference to a standard curve obtained with known amounts of BaCO₃.

**RESULTS**

The observation that succinate, malate, or fumarate partially replace CO₂ under anaerobic growth conditions suggests that CO₂ is required for the synthesis of an essential metabolite, perhaps an intermediate in the synthesis of succinate. Christie and Porteous (1959; 1962a, b) and Keir and Porteous (1962) determined the growth-factor requirements for the Wills strain of *A. israelii*; serine, cysteine, lysine, glutamic acid, leucine, isoleucine, and tryptophan most probably constitute the minimal amino acid requirements for this organism. The similarities of growth requirements for strains of *A. israelii* and *A. naeslundii*, reported by Howell and Pine (1956), and the observation that the strain of *A. naeslundii* presently under study shows a cysteine requirement (Buchanan and Pine, 1963) similar to that reported by Christie and Porteous (1962a), suggested that this strain might also require the other listed amino acids. The observation that aspartic acid is not required suggested that it is synthesized by the cell when the amino acid is omitted from the growth medium. But
whether aspartic acid supplied externally is used for growth remained to be determined.

To test its capacity to use aspartic acid supplied in the growth medium, A. naeslundii was grown in the Castitone medium with added DL-aspartic acid-4-C\(^{14}\) or DL-glutamic acid-2-C\(^{14}\). Since Christie and Porteous (1962a) and Keir and Porteous (1962) reported that the Wills strain required glutamic acid, a control culture was grown with DL-glutamic acid-2-C\(^{14}\). Virtually no radioactivity was incorporated into the cells grown with C\(^{14}\)-aspartic acid, whereas 0.5% of the added C\(^{14}\)-glutamic acid was assimilated by cells grown under the same conditions (Table 1). Failure of the cells to incorporate C\(^{14}\)-aspartic acid demonstrated that the aspartic acid of the medium was not used for growth. The low incorporation of C\(^{14}\)-glutamic acid showed that this amino acid, although used, was not incorporated to the extent reported by Keir and Porteous (1962) for the Wills strain. The Wills strain used 42.4% of the glutamic acid (added at a concentration of 0.0068 M); on this basis, we had anticipated five times the incorporation of glutamic acid actually found (based on the incorporation of L isomer of C\(^{14}\)-glutamic acid).

Because of the known relationships of aspartic acid to four-carbon dicarboxylic acids synthesized through CO\(_2\) fixation, an attempt was made to determine the mechanism of aspartic acid synthesis by A. naeslundii. An experiment was done to determine whether glutamic acid was converted to aspartic acid. Cells (15 g, wet packed weight) were incubated aerobically for 3 hr at 37 C in 50 ml of the glucose-Casitone medium with 5 \(\times\) 10\(^4\) count/min of glutamic acid-2-C\(^{14}\). After incubation, the cells were harvested by centrifugation, washed, and hydrolyzed in 5 N HCl by autoclaving for 1 hr at 120 C. The radioactivity recovered in the acid-ether extract of both the fermentation liquor and the cell hydrolysate was negligible. Since the presence of succinate in readily determinable amounts within the cells had been demonstrated in previous experiments, it was concluded that glutamic acid was not significantly converted to succinate or to

<table>
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<tr>
<th>TABLE 1. Incorporation of C(^{14})-glutamic acid and C(^{14})-aspartic acid into growing cells of Actinomyces naeslundii*</th>
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<tr>
<td><strong>C(^{14})-amino acid added</strong></td>
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<tr>
<td>--------------------------------</td>
</tr>
<tr>
<td>DL-Aspartic acid-4-C(^{14})</td>
</tr>
<tr>
<td>DL-Glutamic acid-2-C(^{14})</td>
</tr>
</tbody>
</table>

* Tubes containing 25 ml of glucose-Casitone medium with 2.25 \(\times\) 10\(^4\) count/min of DL-aspartic acid-4-C\(^{14}\) or DL-glutamic acid-2-C\(^{14}\) were inoculated with two drops of a 72-hr culture and incubated 72 hr under Na\_2CO\(_3\)-pyrogallol seals. Cells were harvested by centrifugation, washed five times with distilled water, and counted at infinite thinness. On the basis of a concentration of 22.4% glutamic acid and 7.1% aspartic acid in casein hydrolysate (Hawk, Oser, and Summerson, 1954), the molar concentration of these amino acids in the medium was calculated as: glutamic acid, 0.0061 M; aspartic acid, 0.0020 M.

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<th>TABLE 2. Specific activities of C(^{14}) compounds isolated from Actinomyces naeslundii incubated with Na_2C(_{14}O_4)*</th>
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<tr>
<td><strong>Expt</strong></td>
</tr>
<tr>
<td>----------</td>
</tr>
<tr>
<td>I (aerobic)</td>
</tr>
<tr>
<td>II (anaerobic)</td>
</tr>
</tbody>
</table>

* Values for specific activity are expressed as counts per minute per \(\mu\)mole of the compound isolated from hydrolyzed cells. In Experiment I, 15 g (wet weight) of unwashed cells were incubated in air on a rotating shaker in 95-ml test tubes with 50 ml of glucose-Casitone medium containing 5 \(\times\) 10\(^4\) count/min of Na\_2C\(_{14}O_4\). After 3 hr of incubation at 37 C, cells were collected by centrifugation, and were hydrolyzed in 2 N HCl by autoclaving for 60 min at 15 psi. In Experiment II, cells were grown in 1 liter of glucose-Casitone medium containing 2.5 \(\times\) 10\(^4\) count/min of Na\_2C\(_{14}O_4\) sealed with Na\_2CO\(_3\)-pyrogallol. The initial specific activity of the C\(_{14}O_2\) in the medium was approximately 5,000 counts per min per \(\mu\)mole. After 4 days of incubation, cells were collected by centrifugation, washed three times with distilled water, and hydrolyzed in 5 N HCl by autoclaving for 60 min at 15 psi. After acid hydrolysis, C\(_{14}\)-labeled compounds were isolated from the cells, identified, and their specific activities determined. The nonvolatile acids and amino acids were separated on a Dowex-1 column (Buchanan and Pine, 1963; Busch et al., 1952), and were purified by combinations of paper chromatography (phenol-water, 80:20), radioautography, and water elution. Cochromatography of isolated C\(_{14}\)-glutamate and aspartate with authentic glutamic and aspartic acids, respectively, gave single homogeneous radioactive spots which coincided with the spots obtained with ninhydrin spraying. Specific activities were calculated by determining the concentration of amino acids with the procedure of Trol and Cannan (1958) and by counting known samples at infinite thinness.

† Percentage of added counts recovered in the nonvolatile fraction of the cells and the fermentation liquor; nd = quantity not determined.
other dicarboxylic acids. When the ether-extracted residue of the cell hydrolysate was subjected to chromatography on a Dowex-1 column, all of the radioactivity was recovered in a single set of ninhydrin-positive fractions. Paper chromatography of these combined fractions revealed several unidentified ninhydrin-positive compounds with traces of activity, but glutamic acid was the only compound significantly active as determined by radioautography. The isolated aspartic acid was not labeled. It was thus concluded that aspartic acid was not formed from glutamic acid under the conditions of the experiment, and that essentially all of the C\textsubscript{4}-glutamic acid assimilated by the cells resided in the internal glutamic acid pool or was incorporated into cellular proteins.

Experiments were then done to determine whether succinate, formed as a result of glucose utilization and C\textsubscript{4}O\textsubscript{2} fixation, was in equilibrium with cellular aspartic acid, and whether the labeling patterns of aspartic acid and glutamic acid were commensurate with their formation by way of known reactions involving intermediates of the tricarboxylic acid cycle. In the aerobic experiment (Table 2), an attempt was made to balance the conditions to permit maximal fixation into glutamate and minimal dilution of the added C\textsubscript{4}O\textsubscript{2}. Greater growth was anticipated aerobically and, by limiting the total volume of the medium, the amount of CO\textsubscript{2} produced would be small. In this case, the specific activity of the succinate formed was approximately 30 times that found in aspartic acid and glutamic acid, but the specific activities of the aspartic and glutamic acids were essentially the same. This high specific activity of succinate relative to that of the amino acids was not expected, in view of the high initial cell concentration; the amino acids of the cells diluted those formed as a result of CO\textsubscript{2} fixation. Approximate equilibration of cellular succinate and aspartate was achieved under anaerobic conditions; the aspartic acid and succinate formed anaerobically had essentially equal specific activities (Table 2). The results also show that a significant amount of the glutamic acid of the cell was derived from the fixation of CO\textsubscript{2}. Although C\textsubscript{4}-glutamic acid was not converted to C\textsubscript{4}-aspartic acid in the previous experiment, the similar specific activities of the two amino acids indicated that a close relationship existed between them under aerobic conditions when the source of radioactivity was C\textsubscript{4}O\textsubscript{2}. Presumably, oxaloacetate was the common precursor.

The glutamic acid and the aspartic acid isolated in the anaerobic experiments were degraded to determine the extent of labeling of their respective carbon atoms (Table 3). Of the total radioactivity found in aspartic acid, 77\% was recovered in the \(\beta\)-carboxyl carbon and 23\% in the \(\alpha\)-carboxyl carbon. These results were in agreement with the synthesis of aspartic acid from oxaloacetate, formed by a Wood-Werkman condensation of CO\textsubscript{2} and pyruvate (or a derivative thereof). The labeling of the \(\alpha\)-carboxyl carbon was explained by the partial equilibration of the aspar-

### Table 3. Results of the degradation of C\textsubscript{4}-aspartate and C\textsubscript{4}-glutamate isolated from Actinomyces naeslundii grown with Na\textsubscript{2}C\textsubscript{4}O\textsubscript{2}\textsuperscript{a}

<table>
<thead>
<tr>
<th>C\textsubscript{4}-Aspartate\textsuperscript{b}</th>
<th>C\textsubscript{4}-Glutamate</th>
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<tbody>
<tr>
<td>Average total</td>
<td>(\alpha)-COOH</td>
</tr>
<tr>
<td>294</td>
<td>70</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Values are expressed as counts per minute per \(\mu\) mole of the isolated BaCO\textsubscript{2}. The C\textsubscript{4}-aspartate and C\textsubscript{4}-glutamate were isolated from cells grown anaerobically in the presence of Na\textsubscript{2}C\textsubscript{4}O\textsubscript{2} and had the specific activities given in Table 2. Glutamate and aspartate were degraded after 100 mg of the unlabeled L amino acids were added as carrier.

\textsuperscript{b} The two carboxyl carbons were removed by ninhydrin decarboxylation (Greenberg and Rothstein, 1957), the \(\alpha\)-carboxyl by chloramine-T decarboxylation (Ehrensvar et al., 1951), and the relative activity of each carboxyl carbon was calculated by solving simultaneous equations based on these values; values for C\textsubscript{1}-C\textsubscript{2} were obtained by difference.

\textsuperscript{c} Obtained with glutamic decarboxylase.

\textsuperscript{d} Obtained by chloramine-T decarboxylation (Hendler and Afćnsen, 1954).

\textsuperscript{e} Isolated and counted as the 2,4-dinitrophenylhydrazone of succinic semialdehyde obtained after chloramine-T decarboxylation.

\textsuperscript{f} Obtained by the Schmidt method of decarboxylation of the isolated \(\gamma\)-aminobutyric acid produced after decarboxylation with glutamic decarboxylase (Phares and Long, 1955).

\textsuperscript{g} Isolated and counted as the dipieric derivative, after Schmidt decarboxylation of \(\gamma\)-aminobutyric acid (Hendler and Afćnsen, 1954).
tate precursor with a symmetrical compound such as fumarate or succinate.

Table 3 shows that 88% of the total activity of glutamic acid synthesized from C14O2 was recovered as the α-carboxyl carbon obtained with chloramine-T decarboxylation. This labeling pattern was in agreement with the synthesis of glutamic acid from α-ketoglutarate, formed from β-labeled oxaloacetate (produced from C14O2 fixation) and acetyl coenzyme A by known reactions of the tricarboxylic acid cycle. The formation of C14-glutamate from succinate-1,4-C14 and C14O2 by a reversal of the α-ketoglutarate dehydrogenase reaction seemed unlikely, since carbon atoms 2 and 5 of the isolated C14-glutamic acid had very little activity. Similar considerations eliminated the formation of C14-glutamic acid from α-ketoglutarate produced from succinate-1,4-C14 and glyoxalate by a reversal of the isocitratase reaction and isocitric dehydrogenase. The observation that the specific activity of the α-carboxyl carbon, obtained enzymatically with glutamic acid decarboxylase, was approximately one-half that obtained by chloramine-T decarboxylation indicates that A. naeslundii synthesized a mixture of the N and L isomers of glutamic acid and that the two isomers were not in equilibrium. This is based on the knowledge that glutamic acid decarboxylase is specific for the L isomer.

It was concluded that the aspartic acid of the cell was synthesized from oxaloacetate produced by a Wood-Werkman condensation reaction and that these cells lacked a permease required for the assimilation of aspartic acid from the environment. On the assumption that oxaloacetate also served as a precursor of α-ketoglutarate, it appeared that one-third of the glutamic acid of the cell was synthesized as a result of CO2 fixation under anaerobic conditions. The remainder of the glutamic acid required for growth was presumably assimilated from that supplied in the growth medium.

DISCUSSION

The strain of A. naeslundii used in the present experiments was chosen because it showed all of the physiological characteristics relating to the strains of A. israelii and A. naeslundii studied previously (Howell and Pine, 1956). With the exception of A. propionicus (Buchanan and Pine, 1962), all strains of other Actinomyces species have shown requirements for CO2 to initiate the growth of small inocula and to effect maximal cell yields. All strains of A. naeslundii have shown a requirement for CO2, even if grown aerobically when CO2 is a product of glucose oxidation. The results suggest that the CO2 was needed for aspartic acid synthesis. Extending these results with radioactive experiments for a single species of Actinomyces to members of the genus as a whole is precarious, but in view of their similar fermentation products and growth requirements it is not unreasonable to assume that CO2 is used similarly by other species of the genus. Consequently, their marked requirement for CO2 and their failure to utilize exogenous aspartic acid appear to us to be a reflection of the absence of an aspartic acid permease in these organisms.

ACKNOWLEDGMENT

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LITERATURE CITED


Howell, A., Jr., and L. Pine. 1956. Studies on


