EFFECT OF AMMONIUM ION ON GROWTH AND METABOLISM
OF MICROCOCCUS SODONENSIS

J. N. CAMPBELL, JAMES B. EVANS, JEROME J. PERRY, AND C. F. NIVEN, JR.

Division of Bacteriology, American Meat Institute Foundation, and Department of Microbiology,
University of Chicago, Chicago, Illinois

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ABSTRACT

CAMPBELL, J. N. (American Meat Institute Foundation, Chicago, Ill.), JAMES B. EVANS, JEROME J. PERRY, AND C. F. NIVEN, JR. Effect of ammonium ion on growth and metabolism of Micrococcus sodonensis. J. Bacteriol. 82:823–827. 1961.—When Micrococcus sodonensis was grown in a synthetic medium deficient in ammonia, large quantities of α-keto acids, chiefly α-ketoglutaric, accumulated in the medium. The addition of ammonium chloride to this medium prevented such accumulation and also supported increased growth, even in the presence of excess glutamate. Neither potassium nor sodium would substitute for ammonia in producing this effect.

The results indicate that this microorganism has a specific requirement for the ammonium ion in its growth and metabolism. If not supplied exogenously, ammonia is made available by deamination of glutamic acid. The exact route of incorporation of ammonia is unknown.

Ammonia plays a role in the nitrogen metabolism of a large number of microorganisms, and may serve as a sole source of nitrogen for many forms. Several mechanisms whereby ammonia is incorporated into organic material by bacteria are known, the most important of which appear to involve glutamic acid dehydrogenase (Adler et al., 1938), glutamine synthetase (Elliott and Gale, 1948), or carbamyl phosphate synthesis (Jones, Spector, and Lipmann, 1955). In addition, Halpern and Umbarger (1960) demonstrated the importance of aspartase in amino group formation in Escherichia coli, with α-ketoglutaric or succinic acid as the carbon source. If glucose was the carbon source, glutamic acid dehydrogenase activity could account for the formation of amino groups.

Perry and Evans (1960) reported that Micrococcus sodonensis accumulated significant quantities of α-ketoglutaric acid and glutamic acid under their growth conditions. It was known that ammonia was incorporated by, and had a demonstrable metabolic effect upon, this organism. Therefore, investigations were initiated to assess the role of glutamic acid dehydrogenase (i.e., synthesis of glutamic acid) as a route of ammonia disposal.

MATERIALS AND METHODS

M. sodonensis (ATCC 11880) was employed throughout this investigation. For growth response and end-product accumulation experiments, the following basal medium, a modification of that proposed by Aaronson (1955), was used: CaCl2, 1.0 mg; (NH4)2MoO4, 0.08 mg; ZnSO4, 1.0 mg; MnSO4, 0.8 mg; H3BO3, 0.4 mg; CuSO4, 0.1 mg; CoCl2, 0.1 mg; FeSO4, 0.4 mg; MgSO4·7H2O, 20.0 mg; KCl, 50.0 mg; Versene, 0.05 mg; sodium glycerol phosphate, 10.0 mg; biotin, 0.001 mg; lactic acid, 500 mg; amino acid (where indicated), 500 mg; NH4Cl (if present), 50.0 mg; tris(hydroxymethyl)aminomethane (Fisher Scientific Co., Chicago, Ill., 365 mg (final concentration, 0.03 M); and distilled water, to 100 ml. The pH was adjusted to
7.2. In some experiments, 0.01% yeast extract was added. Test media were dispensed in 10-ml aliquots to 50-ml Erlenmeyer flasks.

Unless otherwise designated, washed cells of a 24-hr culture grown in Trypticase soy broth (Baltimore Biological Laboratory, Baltimore, Md.) were used as inocula. The cultures were incubated at 30°C with vigorous aeration on a mechanical shaker. Growth response was measured turbidimetrically at 600 mλ with a Spectronic 20 colorimeter and, in some cases, by dry weight determinations.

The α-keto acids produced were isolated from culture supernatants as the 2,4-dinitrophenylhydrazones by the method of Kun and Hernandez (1957). They were tentatively identified by comparing their behavior with 2,4-dinitrophenylhydrazones of known α-keto acids on paper chromatograms, with absolute ethanol-water (8.3:1.7), and butanol-ethanol-0.5 N ammonium hydroxide (7:1:2) as solvent systems. The material identified chromatographically as the 2,4-dinitrophenylhydrazone of α-ketogluutaric acid was further checked by determining its absorption spectrum from 340 to 500 mλ, using a Beckman DK-2 recording spectrophotometer, and its melting point from material recrystallized from water. The values were compared with those obtained from known α-keto acids.

α-Ketoglutaric acid was measured quantitatively by the chromatographic-spectrophotometric method of Kun and Hernandez (1957). Optical density values were converted to micrograms of α-ketoglutaric acid by application to a standard curve, prepared in like manner from known concentrations of α-ketoglutaric acid. By this technique, a linear relationship between optical density and concentration was obtained for values between 1.5 and 35 μg α-ketoglutarate.

In labeled experiments, D.L-lactic acid-2C¹⁴ and D.L-glutamic acid-1C¹⁴ were employed as substrates. Counting was conducted in a gas-flow counter, using infinitely thin samples to avoid error due to self-absorption.

RESULTS

Amino acid requirements. Experiments designed to test the ability of M. sodonensis to utilize single amino acids as the sole source of nitrogen showed that satisfactory growth was obtained using the basal synthetic medium plus any one of the following: glutamic acid, aspartic acid, glycine, histidine, or, to a lesser extent, arginine or ornithine. Also tested but found unable to support growth when added singly were: alanine, valine, serine, lysine, leucine, isoleucine, threonine, methionine, cystine, cysteine, proline, phenylalanine, tyrosine, tryptophan, and citrulline.

Effect of added NH₄⁺ upon growth. Although M. sodonensis will not utilize ammonia as a sole source of nitrogen, its addition (as NH₄Cl) to the otherwise complete synthetic medium yielded an increased growth response regardless of the amino acid utilized as the organic nitrogen source (Table 1). If arginine or ornithine was the sole amino acid present, the addition of ammonia was obligatory for significant growth to occur.

Table 2 demonstrates growth response of this microorganism to varying molar concentrations and combinations of potassium and ammonium ions. These experiments were performed to determine whether another source of monovalent cation would substitute for ammonia in producing growth stimulation. If potassium ion alone were added, an optimum concentration of approximately 0.05 M was evidenced (Perry and Evans, unpublished data).

In the second group in Table 2, the molar levels of K⁺ were maintained near the optimal level, and varying levels of NH₄⁺ were added. Growth response was stimulated even at ionic concentrations which were inhibitory if they were added as NH₄Cl.

<table>
<thead>
<tr>
<th>Additions to basal medium</th>
<th>Optical density (600 mλ)</th>
<th>Dry weight of cells per ml medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamate</td>
<td>1.00</td>
<td>1.20</td>
</tr>
<tr>
<td>Glutamate + NH₄Cl</td>
<td>2.00</td>
<td>3.78</td>
</tr>
<tr>
<td>Aspartate</td>
<td>0.62</td>
<td>—</td>
</tr>
<tr>
<td>Aspartate + NH₄Cl</td>
<td>1.70</td>
<td>—</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.51</td>
<td>1.05</td>
</tr>
<tr>
<td>Glycine + NH₄Cl</td>
<td>1.52</td>
<td>2.04</td>
</tr>
<tr>
<td>Arginine</td>
<td>0.13</td>
<td>—</td>
</tr>
<tr>
<td>Arginine + NH₄Cl</td>
<td>1.70</td>
<td>—</td>
</tr>
<tr>
<td>Ornithine</td>
<td>0.05</td>
<td>—</td>
</tr>
<tr>
<td>Ornithine + NH₄Cl</td>
<td>1.10</td>
<td>—</td>
</tr>
</tbody>
</table>

* Aaronson's modified medium as previously described was used, plus NH₄Cl (0.05% or amino acid (0.5%) or both, as indicated. Cultures were incubated in duplicate and the optical density values averaged.
TABLE 2. Growth response of Micrococcus sodonensis to varying combinations of potassium and ammonium ions

<table>
<thead>
<tr>
<th>K⁺</th>
<th>NH₄⁺</th>
<th>Total</th>
<th>Growth at 28 hr</th>
<th>OD units at 600 μg</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.053</td>
<td>-</td>
<td>0.053</td>
<td>1.16</td>
<td></td>
</tr>
<tr>
<td>0.066</td>
<td>-</td>
<td>0.066</td>
<td>0.53</td>
<td></td>
</tr>
<tr>
<td>0.100</td>
<td>-</td>
<td>0.100</td>
<td>0.46</td>
<td></td>
</tr>
<tr>
<td>0.050</td>
<td>0.003</td>
<td>0.053</td>
<td>5.30</td>
<td></td>
</tr>
<tr>
<td>0.050</td>
<td>0.016</td>
<td>0.066</td>
<td>6.00</td>
<td></td>
</tr>
<tr>
<td>0.050</td>
<td>0.050</td>
<td>0.100</td>
<td>4.80</td>
<td></td>
</tr>
<tr>
<td>0.100</td>
<td>0.003</td>
<td>0.103</td>
<td>3.50</td>
<td></td>
</tr>
<tr>
<td>0.100</td>
<td>0.017</td>
<td>0.117</td>
<td>4.00</td>
<td></td>
</tr>
<tr>
<td>0.100</td>
<td>0.033</td>
<td>0.133</td>
<td>3.40</td>
<td></td>
</tr>
<tr>
<td>0.100</td>
<td>0.100</td>
<td>0.200</td>
<td>3.50</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.200</td>
<td>0.200</td>
<td>0.00</td>
<td></td>
</tr>
</tbody>
</table>

*The basal medium was prepared with 0.5% glutamic acid and 0.01% yeast extract, but lacking NH₄⁺ and K⁺ salts. These (as the chlorides) were added to yield the designated concentrations.

†The optical density units take into account a dilution factor, where necessary, to give comparable values.

consisted of K⁺ alone. The third group in the table contained a still higher level of K⁺, and shows the inhibitory effect of excess cation, but yet the growth response was superior with NH₄⁺ present than in the medium containing K⁺ alone. The second and third groups in Table 2 demonstrate that an optimal concentration of NH₄⁺ (0.01 to 0.02 M) exists but is somewhat less sharply defined than that found for K⁺ (Perry and Evans, unpublished data). These results show that K⁺ will not substitute for NH₄⁺ in the role of growth stimulant for M. sodonensis, nor will NH₄⁺ substitute for K⁺, the requirement for which is absolute in this organism.

Further experiments showed this organism to be insensitive to reasonable fluctuations in Na⁺ level, and this ion will not substitute for either K⁺ or NH₄⁺. The stimulatory effect of NH₄⁺, therefore, appears real and unique, and not just a reflection of a favorable alteration in monovalent cation level.

Effect of added NH₄⁺ on metabolic processes. Chromatographic examination of boiling-water extracts of this organism showed glutamic acid to be the chief constituent of the intracellular amino acid pool under the growth conditions employed. Experiments were conducted using cell-free and resting-cell preparations in an attempt to demonstrate an incorporation of NH₄⁺ into glutamic acid or glutamine in the presence of α-ketoglutaric acid or glutamic acid, respectively. The results of the experiments were inconclusive. It was observed, however, that if the cells were grown in the absence of exogenous NH₄⁺, α-keto acids accumulated to a marked degree in the growth medium. On the other hand, if NH₄⁺ were initially present, no such accumulation occurred. The α-keto acids which accumulated varied somewhat, depending upon the amino acid used as the source of nitrogen, but in all cases α-ketoglutaric acid was the major one that accumulated (Table 3). Since these growth experiments employed long incubation periods (48 hr), the establishment of direct correlations between the accumulation of α-keto acids, other than α-ketoglutaric acid, with pathways of metabolism of the respective amino acids was not attempted.

The identity of material isolated from supernatants of cultures grown on glutamic acid or glycine and identified chromatographically as the 2,4-dinitrophenylhydrazone of α-ketoglutaric acid was verified by comparison of absorption spectra and melting points with similar values obtained using known α-keto acids. By all the criteria employed, the behavior of the material isolated from the culture medium was indis-
tanguishable from that of the 2,4-dinitrophenyl-
hydrazone of a-ketoglutaric acid.

Table 4 gives the results of a quantitative
estimation of a-ketoglutaric acid accumulated
in the presence and absence of added NH₄⁺.

**Substrate sources of accumulated a-ketoglutaric acid.** For the sake of simplicity, further studies
were limited to media with glutamic acid as the
sole source of nitrogen. Perry and Evans (1960)
showed that *M. sodonensis* possesses the enzymes
of the Krebs cycle. Nevertheless, the use of C¹⁴-
labeled substrates showed that both lactic acid
and glutamic acid function as sources of the
accumulated a-ketoglutaric acid (Table 5). Whether the a-ketoglutaric acid originating
from glutamate was a result of deaminase or
transaminase activity is not apparent from these
data.

**Studies with depleted and nondepleted cells.** Two
groups of cells were prepared: "Nondepleted" cells
were obtained by growth in the synthetic
medium with glutamic acid as the sole source of
organic nitrogen. After harvesting and washing,
these cells were found to contain high levels of
endogenous glutamic acid. "Depleted" cells were
obtained similarly, but were subsequently incubated with shaking in distilled water at 30
C for 4 hr. This treatment did not reduce the
viability of the cells significantly, but it did
effectively deplete the internal pool of glutamic
acid and suppress endogenous metabolism, as
estimated by paper chromatography of boiling-
water extracts and by respirometry, respectively.

When these cells were inoculated separately
into the synthetic medium in which NH₄Cl
served as the sole source of nitrogen, it was
observed that "nondepleted" cells achieved
only slight but detectable growth, which subsided
after a few hours (estimated visually and by plate counts). On the other hand, the "depleted"
cells exhibited no detectable growth in the same
medium. The addition of a-ketoglutarate to the
medium did not support further growth from
either inoculum.

**DISCUSSION**

The accumulation of large amounts of a-
ketoglutaric acid by *M. sodonensis* growing in a
synthetic medium with a single amino acid as
the sole source of nitrogen, coupled with the
fact that the addition of NH₄⁺ (as NH₄Cl) to
this medium prevented such accumulation,
might lead to the supposition that glutamic acid
synthesis may be involved in the stimulatory
effect of NH₄⁺ upon the growth of this organism.
Glutamic acid dehydrogenase is held to be a
key system in the incorporation of ammonia into
organic material within the cell. One may,
therefore, assume that in the presence of exog-
enous NH₄⁺ this system catalyzes the synthesis
of glutamic acid from the a-ketoglutaric acid
formed via the Krebs cycle, thus stimulating
growth. However, this explanation is incom-
patible with the results obtained. First, the
addition of NH₄⁺ to the medium stimulated
growth and prevented accumulation of a-keto-
glutarrlic acid by the culture even in the presence
of excess glutamic acid, i.e., under conditions
wherein the rate of formation of glutamic acid
could not be the limiting reaction. Second, if
the glutamic acid formed was sufficient to
produce the observed increased growth response,
it should have been sufficient to support some
growth response with the depleted cells in the
medium containing all the requirements for
synthesis of a-ketoglutaric acid via the Krebs
cycle. Such was not the case, however, and even
the undepleted cells grew only until their en-
dogenous glutamic acid was exhausted.

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**TABLE 4. Quantity of a-ketoglutaric acid produced in
the presence and absence of exogenous NH₄⁺**

<table>
<thead>
<tr>
<th>Additions to basal medium</th>
<th>a-Ketoglutaric acid produced in µg/ml dry wt of cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamic acid</td>
<td>508</td>
</tr>
<tr>
<td>Glutamic acid + NH₄Cl</td>
<td>0.37</td>
</tr>
<tr>
<td>Glycine</td>
<td>625</td>
</tr>
<tr>
<td>Glycine + NH₄Cl</td>
<td>0.98</td>
</tr>
</tbody>
</table>

* NH₄Cl (0.05%) or the designated amino acid,
or both, were added and the cultures incubated
48 hr. The a-ketoglutaric acid formed was isolated
as the 2,4-dinitrophenylhydrazone.

**TABLE 5. Incorporation of substrate C¹⁴ into
accumulated a-ketoglutaric acid**

<table>
<thead>
<tr>
<th>Additions to basal medium</th>
<th>Substrate</th>
<th>Specific activity (counts per min per mmole)</th>
<th>Isolated a-keto-glutaric acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lactate-2C¹⁴ + glutamate</td>
<td>42.8 × 10⁵</td>
<td>25.0 × 10⁵</td>
</tr>
<tr>
<td></td>
<td>Lactate + glutamate-1C¹⁴</td>
<td>72.2 × 10⁵</td>
<td>8.3 × 10⁵</td>
</tr>
</tbody>
</table>
These data suggest that, in addition to its absolute requirement for a source of organic nitrogen, for which one of several amino acids suffices, this organism has a specific requirement for NH$_4^+$ If none is available exogenously, the organism deaminates some of its supply of glutamic acid. The resultant α-ketoglutaric acid, in addition to that synthesized from lactic acid, is more than can be metabolized, and an excess accumulates. Thus the glutamic acid dehydrogenase (or a similar system) functions as a method of obtaining, rather than fixing, NH$_4^+$ in the absence of an exogenous supply.

The formation of NH$_4^+$, rather than synthesis of glutamic acid, is rate-limiting. The presence of exogenous NH$_4^+$ thus spares the deamination of glutamic acid and prevents the accumulation of α-ketoglutaric acid and whatever reversing influence this accumulation might have upon Krebs cycle equilibria. The net result is an increased growth response. In contrast with this picture, Strauss (1956) reported that dicarboxylic acid-requiring mutants of Neurospora crassa accumulated acetylmethyl carbinol, pyruvic acid, and α-ketoisovaleric acid, when grown in the presence of ammonia or nitrate salts. This accumulation was prevented by the addition of dicarboxylic acids or by the reduction in the amount of added inorganic nitrogen. It was postulated that the nitorgenous salts diverted dicarboxylic acids, already in short supply, from the Krebs cycle to other synthetic reactions, resulting in accumulation of intermediates.

It has been reported frequently that ammonia exerts a stimulatory effect upon Krebs cycle enzymes in some systems. It is possible that ammonia may play some similar role in the growth and metabolism of M. sodonensis.

The route of incorporation of ammonia by this organism is under investigation. Preliminary work seems to indicate that the synthesis of glutamic acid, aspartic acid, glutamine, or asparagine is not a major route. An attractive possibility, in view of the ability of this organism to synthesize excessive quantities of deoxyribonucleic acid (Campbell et al., 1961), would be via carbamyl phosphate formation into pyrimidine and ultimately into nucleic acid synthesis.

LITERATURE CITED