Regulation of the Synthesis of Enzymes Responsible for Glutamate Formation in Klebsiella aerogenes*

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SUMMARY

A mutant of Klebsiella aerogenes lacking glutamine (amide) γ-ketoglutarate (NADP⁺) amidotransferase oxidoreductase (glutamate synthetase) cannot grow in minimal media containing ammonia as the only nitrogen source at a concentration lower than 1 mM. In addition, in a glucose containing medium, it fails to utilize amino acids, such as histidine, that are converted to glutamate, as sources of nitrogen. It can use glutamate as source of nitrogen. Revertants were isolated capable of growth on glucose-histidine, but unable to use ammonia. These revertants still lack glutamate synthetase, but produce glutamine synthetase constitutively (GlnC⁻). They fail to produce glutamate dehydrogenase, and thus cannot use ammonia as a nitrogen source. An analysis of the levels of glutamine synthetase, glutamate synthetase, and glutamate dehydrogenase in the parent organism, in mutants lacking glutamine synthetase, and in mutants producing it constitutively, reveals that glutamine synthetase represses the formation of glutamate dehydrogenase. A lack of glutamate synthetase appears to interfere with the derepression of glutamine synthetase. On the other hand, the loss of glutamate dehydrogenase by mutation does not affect glutamine synthetase. These results, together with those reported in an earlier paper (PRIVAL, M. J., BRENCLEY, J. E., AND MAGASANIK, B. (1973) J. Biol. Chem. 248, 4334-4344), indicate that glutamine synthetase is the key element in the regulation of the synthesis of enzymes capable of supplying the cell with glutamate.

We have previously observed a correlation between the level of glutamine synthetase and the ability of Klebsiella aerogenes to produce histidase and proline oxidase in the presence of glucose (2). These enzymes are required for the conversion of histidine and proline, respectively, to glutamate. We now find that the loss of glutamine (amide): γ-ketoglutarate (NADP⁺) amidotransferase oxidoreductase (glutamate synthetase) prevents the relief of histidase from catabolite repression that normally occurs during starvation for a source of nitrogen (3). This effect caused by the lack of glutamate synthetase also appears to be mediated through glutamine synthetase.

Glutamine synthetase and glutamate synthetase acting in concert catalyze the ATP-dependent conversion of NH₃ and γ-ketoglutarate to glutamate (4).

\[
\text{Glutamate + ATP} + \text{NH}_3 \rightarrow \text{glutamine + ADP + P}_1 + \text{H}_2\text{O} \quad \text{(1)}
\]

\[
\alpha\text{-Ketoglutarate + glutamine + NADPH} + \text{H}^+ \rightarrow 2 \text{glutamate + NADP}^+ \quad \text{(2)}
\]

\[
\text{Sum:} \alpha\text{-Ketoglutarate + ATP + NADPH + NH}_3 + \text{H}^+ \rightarrow \text{glutamate + ADP + P}_1 + \text{NADP}^+ + \text{H}_2\text{O} \quad \text{(3)}
\]

We find that the level of glutamate dehydrogenase is inversely related to the level of glutamine synthetase. Together these observations suggest that glutamine synthetase is a key element in the control of the formation of enzymes able to supply the cell with glutamate.

EXPERIMENTAL PROCEDURE

Cultivation of Bacteria and Phage—The methods used for the cultivation of Klebsiella aerogenes and phage PW-52 have been described (2, 3).

Isolation of Mutants—Many of the mutants used are listed in Table II of an earlier paper (2). Additional strains are listed in Table I. The procedures for mutagenesis have been described (3). Strain MK-19 (pur-1) was isolated from wild type strain MK-1 after mutagenesis with ethylmethanesulfonate. Strain MK-189 (asm-600) was isolated as follows. A culture of MK-19 was treated with ethylmethanesulfonate, washed, and grown in histidine medium. (All media used in the selection were supplemented with adenine.) A penicillin selection was performed for mutants unable to grow in medium containing glucose with 0.2% urocanate as the sole nitrogen source. Surviving cells

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were selected for their ability to grow on histidine medium. Due to the close linkage of the hutC515 mutation to the histidine utilization mutation in the recipient strain, transductants containing the hutC515 mutation were obtained. One such strain was named MK-266. Growth experiments and enzyme assays showed MK-256 to have retained the inability to produce glutamate synthetase and to be constitutive for histidase synthesis.

Mutants lacking glutamate dehydrogenase were isolated by the following procedure. Strain MK-189 was treated with ethylmethanesulfonate and mutants unable to grow without added glutamate were isolated as described previously (3). One of these mutants, strain MK-201, failed to grow not only on glucose, but also on acetate, succinate, or citrate, unless the medium was supplemented with glutamate. Extracts of this mutant, in contrast to those of the parent strain, did not contain glutamate dehydrogenase activity (see Table VI). The strain, like its parent strain MK-189, lacks glutamate synthetase (see Table VI). Strain MK-261 was treated with a phage lysate of strain MK-1, and transductants capable of growth on glucose-ammonia medium were isolated. Among the transductants, some had also acquired the ability to grow on glucose-histidine. Extracts of cells of these transductants, such as strain MK-270, were found to contain glutamate synthetase, but not glutamate dehydrogenase activity.

**Enzyme Assays**—Whole cell assays as described by Prival and Magasanik (3) were used for histidase and β-galactosidase determinations. Enzyme activity was related to the optical density at 420 nm of dilutions of the untreated cell suspension. Cells were sonically disrupted and extracts were prepared for the glutamate dehydrogenase, glutamate synthetase, and glutamine synthetase assays (2). The assays for glutamate dehydrogenase and glutamate synthetase are those described by Meers et al. (4). The glutamine synthetase assay was the “transferase” reaction described by Stadtman et al. (5) and in detail in an earlier paper (2).

Protein determinations on extracts were carried out by the method of Lowry et al. (6).

Because assays of mutant MK-189 showed some residual glutamate synthetase activity even though there was no growth on media containing less than 1 mM ammonia (indicating that the mutation was not leaky), we wanted to determine the cause of this activity. We considered the possibility that the activity measured was in fact glutamate synthetase, but not glutamate dehydrogenase. This possibility is a likely one, since the assay mixtures for the two enzymes are identical, except that 40 mM NH₄Cl replaces 5 mM glutamine when the assay is for glutamate dehydrogenase. The crude extracts used and the glutamine solution are not entirely free of NH₄⁺. We used DON, the kind gift of Dr. S. Hartman of Boston University. This compound is an analogue to glutamine, known to inhibit the transfer of the amide group of glutamine (7), and should therefore inhibit glutamate synthetase but not glutamate dehydrogenase. We carried out the assays for these two enzymes, after preincubation of the mixture containing the cell extract with increasing concentration of DON for 10 min before addition of the substrate. The results, summarized in Table II, show that with extracts from strain MK-94, which lacks glutamate dehydrogenase (see Table VI), 0.1 mM DON completely inhibits glutamate synthetase. With extracts from strain MK-53 where the specific activity of glutamate synthetase is approximately 100 units per mg of protein, the specific activity of glutamate synthetase was

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*The abbreviation used is: DON, 6-diazo-5-oxo-1-norleucine.*
apparently reduced by 0.02 to 0.1 mM DON from 96 to 10 units per mg of protein. We assume that this residual activity is actually due to glutamate dehydrogenase. Consequently, we have corrected all reported assays for glutamate synthetase by subtracting 10% of the measured specific activity of glutamate dehydrogenase.

We have also found substantial instability of the glutamate synthetase activity during preparation and with assay conditions described making the observed fluctuations difficult to interpret (8). However, the assay is reliable enough to distinguish the reproducible low activity found in MK-189 extracts from the higher levels found in other strains.

RESULTS

Mutant with CnS Phenotype—We have presented evidence that mutants of _K. aerogenes_ isolated as glutamine requireurs are unable to synthesize glutamine synthetase (GlnC) and are also unable to relieve the catabolite repression of histidase under conditions of nitrogen limitation (CnS) (2). A revertant of one of these glutamine requireurs overproduces glutamine synthetase under all growth conditions tested (GlnC+), and its histidase is not subject to catabolite repression (CnS) (2). We now set out to determine whether a mutant could be isolated which is unable to relieve its catabolite repression of histidase (CnS) during nitrogen limitation but which does not require glutamine (GlnC+). Such a CnS mutant should be unable to use histidine or urocanic acid effectively as a source of nitrogen in the presence of glucose, but should grow in a medium containing histidine as sole carbon source.

The CnS mutant was isolated as described under “Experimental Procedure”; strain MK-189 grows normally on histidine alone, but grows in the glucose-histidine medium with a generation time of 312 min while its parent grows in this medium with a generation time of 78 min (Fig. 1). The regulation of histidase in MK-189 is shown in Table III. Histidase synthesis is induced by histidine and repressed by glucose as it is in wild type strain MK-1. However, when the supply of nitrogen is limited by growth on histidine as sole nitrogen source in the presence of glucose, histidase synthesis is derepressed in the wild type strain MK-1 but not in the mutant strain MK-189. Thus, MK-189 is CnS for histidase synthesis, while still being GlnC+.

One possible explanation for the slow growth of MK-189 on the glucose-histidine medium might be a mutation within the _hut_ region altering the response of the _hut_ enzymes to catabolite repression. However, transduction experiments failed to reveal any linkage of the CnS phenotype to the _hut_ region making this explanation unlikely.

MK-189 and MK-1 were tested for their ability to utilize each of the 20 common amino acids as a source of nitrogen in the presence of glucose. The wild type, MK-1, grows on media containing glucose plus any of the amino acids tested except lysine, isoleucine, or leucine; MK-189 grows well only on glucose medium containing glutamic acid, glutamine, aspartic acid, or asparagine as sole source of nitrogen. Growth of the mutant on glucose plus other amino acids is significantly slower than that of the wild type (Table IV). When MK-189 was transduced with a phage lysate grown on MK-1, transductants selected for their ability to grow on the glucose-histidine medium were found to

![Graph](http://www.jbc.org/)

**TABLE II**

| Strain | DON addition | Glutamate dehydrogenase | Glutamate synthetase |
|--------|--------------|-------------------------|----------------------|
| MK-53  | None         | 90.5                    | 95.8                 |
|        | 0.01 mM      | <1                      | 15.1                 |
|        | 0.02 mM      | <1                      | 9.2                  |
|        | 0.10 mM      | 10.0                    | 11.0                 |
| MK-94  | None         | <1                      | 107.0                |
|        | 0.10 mM      | <1                      | <1                  |
|        | 0.50 mM      | <1                      | <1                  |

* Not done.
Regulation of histidase synthesis in strain MK-189

The cells from overnight cultures of the wild strain MK-1 and the mutant strain MK-189 were collected by centrifugation, washed in 0.85% NaCl, resuspended in the original volume, and diluted 1:100 into 10 ml of the indicated media. These cultures were incubated with shaking at 37°C. When the growth had reached 100 Klett units, the cultures were chilled, and the cells were collected by centrifugation, washed once with 0.85% NaCl and suspended in 0.01 M phosphate buffer, pH 7.5. The cell suspensions were assayed for histidase and their density was measured in a Zeiss spectrophotometer at 420 nm. The results are given as nanomoles of urocanate formed per min per ml of cell suspension with an optical density of 1.0 at 420 nm.

| Experiment | Composition of medium | Histidase level |
|------------|-----------------------|----------------|
|            | Citrate | Glucose | (NH₄)₂SO₄ | Histidine | MK-1 | MK-189 |
| 1          | +       | +       | -        | -        | 52   | 78    |
| 2          | -       | +       | +        | -        | 0.6  | 0.6   |
| 3          | +       | -       | +        | +        | 28   | 29    |
| 4          | -       | -       | +        | +        | 0.2  | 0.3   |
| 5          | +       | -       | +        | -        | 6.5  | 5.2   |
| 6          | -       | -       | -        | +        | 44   | 2.9   |

Utilization of different nitrogen sources for growth by strain MK-189 and by its revertants and transductants

The amount of growth on minimal agar plates containing the indicated compounds as sources of carbon and nitrogen was compared after 2 to 3 days of incubation. These compounds were present in a concentration of 0.2%, except when histidine was used as sole source of carbon and energy at a concentration of 0.4%.

| Composition of medium | Strain |
|-----------------------|--------|
|                       | MK-189 | MK-247 | MK-204b | MK-203b | MK-204b | MK-208b |
| Glucose              | +       | +       | +       | +       | +       | +       |
| NH₄                  | +       | +       | +       | +       | +       | +       |
| Histidine            | +       | +       | +       | +       | +       | +       |
| Histidine            | -       | -       | -       | -       | -       | -       |
| Tryptophan           | -       | -       | +       | +       | +       | +       |
| Cysteine             | -       | -       | +       | +       | +       | +       |
| Glycine              | +       | +       | +       | +       | +       | +       |
| Arginine             | -       | -       | +       | +       | +       | +       |
| Glutamate            | +       | +       | +       | +       | +       | +       |

a Transductant obtained by treatment of MK-189 with phage P-52 grown on the wild strain MK-1, selected for ability to grow on glucose-histidine.

b Revertant of strain MK-189 selected for ability to grow on glucose-histidine.

grow well on glucose plus any of the other amino acids tested (Table IV, compare strains MK-189 and MK-202); also some spontaneous revertants of MK-189, selected for the ability to grow on the glucose-histidine medium, grew on all the media tested (Table IV, strain 203). Thus, the inability of MK-189 to grow on glucose plus any of these amino acids is probably due to a single mutation. However, other revertants had only recovered the ability to use a more limited group of amino acids (MK-204, MK-208). These will be discussed in the next section.

In order to substantiate the CnS character of strain MK-189 and to eliminate the need of adding histidine to the growth media, we changed the control of its hut system from inducible to constitutive. This was accomplished as described under "Experimental Procedure," by isolating a HutC- mutant of MK-189 and transducing it to HutC+ with phage grown on a strain MK-53 (hutC515). One such transductant, strain MK-256, produced histidase constitutively but retained the CnR character of its parent. This is illustrated in Fig. 2 where histidase production is measured in glucose containing media with glutamine as sole nitrogen source. The rate of histidase production is not significantly different in media containing excess glutamine, limiting glutamine, or in a medium containing both glutamine and ammonia. It has been shown in the preceding paper that in a HutC-constitutive strain with normal susceptibility to catabolite repression, Cn+, growth on excess or limiting glutamine in the absence of added ammonia leads to derepression of histidase (2). The results with MK-256 also demonstrate that ammonia accumulates in the medium even when glutamine is limited; in the Cn+ strain no accumulation of ammonia occurs under these growth conditions.

CnR Revertants of CnS Mutant—Some of the spontaneous revertants of the CnS strain MK-189 selected for their ability to grow on glucose-histidine had not recovered the full range of ability of the wild type to use amino acids as sources of nitrogen. In addition, these revertants (strains MK-204 and MK-208, Table IV) appeared to have the CnS character when subjected to the "tryptophan" spot test (2) in which Cn+ strains produce a yellow color on glucose-ammonia-tryptophan medium while Cn+ strains do not. We compared the sensitivity of these revertants to catabolite repression with that of their parent MK-189 (Table IV). It can be seen that in all three strains β-galactosidase responds normally to repression by glucose; histidase on the other hand in strains MK-204 and MK-208 is insensitive to repression by glucose even in media containing ammonia. Thus, the control of histidase in strains MK-204 and MK-208 differs from that in their CnS parent (repressed in glucose-ammonia-histidine media) and to a limited extent with its derivatives as sole nitrogen source at a concentration of 0.4%.

| Experiment | Strain | Regulation | Carbon source | Nitrogen source | Enzyme level |
|------------|--------|------------|---------------|----------------|-------------|
| 1          | MK-189 | CnS        | Glucose       | Histidine      | 48          |
| 2          | MK-189 | CnS        | Citrate       | Histidine      | 42          |
| 3          | MK-189 | CnS        | Glucose-NH₄   | Histidine      | 52          |
| 4          | MK-204 | CnR        | Glucose       | Histidine      | 25          |
| 5          | MK-204 | CnR        | Citrate       | Histidine      | 52          |
| 6          | MK-204 | CnR        | Glucose-NH₄   | Histidine      | 48          |
| 7          | MK-208 | CnR        | Glucose       | Histidine      | 41          |
| 8          | MK-208 | CnR        | Glucose       | Histidine      | 44          |
| 9          | MK-206 | CnR        | Glucose-NH₄   | Histidine      | 45          |
| 10         | MK-53s | Cn+        | Glucose-NH₄   | Histidine      | 3.4         |
| 11         | MK-53s | Cn+        | Glucose-NH₄-lim | Histidine     | 61          |
| 12         | MK-257s| CnR        | Glucose-NH₄-lim |              | 22          |

a Strain is HutC-

b Ammonia was added at a growth-limiting rate.
medium and in glucose-histidine medium) and from that in a Cn\textsuperscript{R} wild strain such as MK-53 (repressed in glucose-ammonia-histidine, but not in glucose-histidine). Strains MK-204 and MK-208 have the Cn\textsuperscript{R} phenotype associated with some revertants of a glutamine-requiring mutant (2).

We had previously shown that the genetic site responsible for the Cn\textsuperscript{R} character (\textit{glnC}) of the revertant of the glutamine re-quirer is closely linked to a site where mutations lead to the loss of glutamine synthetase (\textit{glnA}). We examined whether the mutation responsible for the Cn\textsuperscript{R} phenotype of strain MK-204 is due to a mutation at this site by using phage grown on MK-204 cells to transduce strain MK-604, a lysogenic derivative of the glutamine requiring strain MK-104 (\textit{glnA6 hutC515}), to glutamine independence. The transductants, 15/15, were found to have the Cn\textsuperscript{R} character by the tryptophan test. One trans-ductant, strain MK-257, was examined further and found to have a high constitutive level of histidine when grown on glucose-ammonia (Table V, Experiment 12). Thus, it has retained the Hut constitutive phenotype of the recipient and acquired the Cn\textsuperscript{R} phenotype of the donor. Apparently, a mutation in the \textit{glnC} site is responsible for the Cn\textsuperscript{R} character of the revertants of the glutamine independent Cn\textsuperscript{R} strain described here and of the glutamine requiring Cn\textsuperscript{R} strain described in the previous paper. This finding is particularly interesting considering that entirely different procedures were used to obtain these mutants. The former revertants were selected for their ability to use histidine as a nitrogen source in the presence of glucose, while the latter were selected for their ability to grow in the absence of glutamine.

Growth Characteristics and Enzymatic Composition of Cn\textsuperscript{R} and Cn\textsuperscript{S} Strains—As already noted, the Hut constitutive derivative of the Cn\textsuperscript{R} strain MK-189 accumulates ammonia in the medium during growth on glutamine as sole source of nitrogen (see Fig. 2). This behavior suggested that the cells experience difficulty in the utilization of ammonia present at low concentration. Indeed, the Cn\textsuperscript{R} strain MK-189 was found to be incapable of growth in media containing ammonia at concentrations lower than 1 \textmu M as sole nitrogen source, whereas the prototroph can grow in media containing 0.01 \textmu M ammonia as sole nitrogen source.

Recent work by Meers et al. (4) has implicated two enzymes as necessary for synthesis of glutamate in \textit{K. aerogenes} growing at low ammonia concentrations: glutamine synthetase and glutamine (amide) \alpha-\ketoglutarate amidotransferase oxido-reductase (NADP) (glutamate synthetase). Enzymatic analysis of extracts prepared from cells grown under a variety of conditions revealed that the glutamate synthetase activity is lacking in mutant MK-189 (Table VI, Experiments 6 and 7). Mixing experiments with extracts from MK-247, a prototroph, and MK-189 did not cause any decrease in MK-247 activity indicating that the absence of activity in the MK-189 extract is not due to an inhibitor.

Nagata et al. (9) isolated a mutant of \textit{Klebsiella} unable to fix elementary nitrogen due to an inability to synthesize the glutamate synthetase. When we compared this mutant with its parent M5A1, we found that the mutant had also lost the ability to utilize histidine as a sole nitrogen source in the presence of glucose as well as its ability to grow on glucosamine medium, where nitrogen assimilation is limited by the slow hydrolysis of glucosamine. Nagata et al. refer to the genetic lesion in their mutant as \textit{asm-1}. Since MK-189 apparently has the same characteristics as \textit{asm-1}, we refer to the mutation in MK-189, which resulted in its inability to grow on limiting concentrations of ammonia, as \textit{asm-200}.

Examination of the Cn\textsuperscript{R} revertants of MK-189 (\textit{asm-200}) revealed that these strains, MK-204 and MK-208, had lost the

| TABLE VI |

| Glutamine synthetase, glutamate synthetase, and glutamate dehydrogenase in wild type and mutants |

The cells were grown in media containing 0.2% of glucose as source of carbon and 0.2% of the indicated sources of nitrogen. Where indicated the nitrogen compound was added to the culture at a growth-limiting rate. The cells were disrupted and the extracts assayed for the three enzymatic activities. Enzyme units are nanomoles of product formed per min. Gln-S, glutamine synthetase; Glut-S, glutamate synthetase; Glut-D, glutamate dehydrogenase.

| Experiment | Strain | Relevant genotype | Regulation | Nitrogen source | Enzyme level |
|------------|--------|------------------|------------|----------------|--------------|
|            |        |                  |            |                | Gln-S | Glut-S | Glut-D |
| 1          | MK-53  | Wild             | Cn\textsuperscript{+} | NH\textsubscript{3} | 270   | 109   | 244   |
| 2          | MK-53  | Wild             | Cn\textsuperscript{+} | NH\textsubscript{4}-lim | 880   | 64    | 274   |
| 3          | MK-53  | Wild             | Cn\textsuperscript{+} | Glutamate-lim | 950   | 49    | 24    |
| 4          | MK-71  | glnC\textsuperscript{3} | Cn\textsuperscript{+} | NH\textsubscript{4} + glutamate | 160   | 110   | 231   |
| 5          | MK-71  | glnC\textsuperscript{3} | Cn\textsuperscript{+} | NH\textsubscript{4} + glutamate-lim | 990   | 105   | 205   |
| 6          | MK-189 | asm-200          | Cn\textsuperscript{8} | NH\textsubscript{3} | 380   | <1    | 110   |
| 7          | MK-189 | asm-200          | Cn\textsuperscript{8} | NH\textsubscript{4} + glutamate | 230   | <1    | ND    |
| 8          | MK-206 | asm-200          | Cn\textsuperscript{8} | Glutamate-lim | 450   | ND    | ND    |
| 9          | MK-204 | asm-200; glnC\textsuperscript{4} | Cn\textsuperscript{R} | NH\textsubscript{4} + glutamate | 1140  | <1    | 0.4   |
| 10         | MK-204 | asm-200; glnC\textsuperscript{4} | Cn\textsuperscript{R} | NH\textsubscript{4} + glutamate-lim | 1180  | <1    | 1.8   |
| 11         | MK-208 | asm-200; glnC\textsuperscript{4} | Cn\textsuperscript{R} | NH\textsubscript{4} + glutamate | 1880  | <1    | 0.6   |
| 12         | MK-208 | asm-200; glnC\textsuperscript{4} | Cn\textsuperscript{R} | NH\textsubscript{4} + glutamate-lim | 1000  | <1    | 1.9   |
| 13         | MK-204 | glnA\textsuperscript{6} | Cn\textsuperscript{8} | Glutamate-lim | 0     | 126   | 228   |
| 14         | MK-267 | glnC\textsuperscript{4} | Cn\textsuperscript{R} | NH\textsubscript{3} | 740   | 40    | 2.0   |
| 15         | MK-93  | glnB\textsuperscript{3} | Cn\textsuperscript{8} | Glutamate-lim | 0     | 408   | 518   |
| 16         | MK-94  | glnB\textsuperscript{3}, glnC\textsuperscript{4} | Cn\textsuperscript{R} | NH\textsubscript{3} | 1400  | 35    | 0.1   |
| 17         | MK-94  | glnB\textsuperscript{3}, glnC\textsuperscript{4} | Cn\textsuperscript{8} | NH\textsubscript{4}-lim | 840   | 32    | 1.2   |
| 18         | MK-261 | asm-200; glnD\textsuperscript{1} | Cn\textsuperscript{8} | NH\textsubscript{4} + glutamate | 470   | <1    | 0.6   |
| 19         | MK-270 | gdhD\textsuperscript{1} | Cn\textsuperscript{+} | NH\textsubscript{3} | 280   | 121   | 0.5   |

*Accounted for by enzyme present in original inoculum.
ability to grow in media containing ammonia as sole nitrogen source, even when it was present at high concentration (33 mM) (Table IV). The cells will grow in media supplemented with L-glutamate, or with amino acids such as histidine, arginine, proline, or tryptophan which can provide glutamate either by their degradation or transamination reactions. Thus, it appears that the CnR strains MK-204 and MK-208 have lost the ability to use ammonia for the synthesis of glutamate.

The examination of cell extracts of MK-204 and MK-208 revealed the cause of the glutamate requirement (Table VI). These strains, like their parent MK-189, still lack glutamate synthetase activity (Experiments 6, 7, 9 to 12), and in addition, lack glutamate dehydrogenase activity. Presumably, it is the presence of glutamate dehydrogenase in strain MK-189 (Experiments 6 and 7) that enables this organism to grow in media containing NH₃ at concentrations greater than 1 mM without glutamate supplementation. The low level of glutamate dehydrogenase in strains MK-204 and MK-208 is apparently not due to the addition of glutamate to the medium since cells grown in a medium to which glutamate is added slowly to limit the growth rate (Experiments 9 to 12) also lack this enzyme activity. Furthermore, another glutamate requiring mutant presumably blocked in the citric acid cycle, strain MK-71, has normal levels of glutamate dehydrogenase under both conditions (Experiments 4 and 5).

Regulation of Enzymes Involved in Glutamate Synthesis—The experimental results summarized in Table VI reveal interesting relationships between glutamine synthetase, glutamate synthetase, and glutamate dehydrogenase.

We have previously confirmed the results of other investigations that cells whose growth rate is limited by the availability of a nitrogen source contain more glutamate synthetase than those growing with the nitrogen source in excess (2). This is illustrated by Experiments 1 to 3, in which cells with normal glutamine synthetase were used. We can see that the level of this enzyme is higher in cells growing on limiting ammonia or limiting glutamine as the sole source of nitrogen than in cells growing on excess ammonia. The level of glutamate synthetase is not greatly altered, but the level of glutamate dehydrogenase is greatly reduced by growth in the nitrogen-deficient medium. This confirms an earlier report by Meers et al. (4).

Experiments 4 and 5, in which a glutamate-requiring mutant was used, show that glutamate does not affect the production of these enzymes; their levels are approximately those found in the wild type growing with excess ammonia whether glutamate was provided in excess or as growth-limiting nutrient.

A deficiency in glutamate synthetase appears to prevent the large increase in the level of glutamine synthetase which occurs when glutamine is supplied as the growth rate-limiting nitrogen source (compare Experiments 3 and 8).

Of particular interest are the effects of mutations in MK-204 and MK-208 which endow these revertants of the glutamate synthetase-deficient mutant with the ability to grow on glucose-histidine. These strains, which are CnR, contain a high level of glutamine synthetase, even when grown in media containing excess ammonia (Experiments 9 to 12). They, like their parent MK-189, are deficient in glutamate synthetase, but in contrast to their parent they also lack glutamate dehydrogenase.

We have shown in the previous section that phage grown on the CnR strain MK-204 transduce the CnR character to the glutamine-requiring CnR strain MK-104 together with glutamine independence. We find now that the transductant strain MK-257 differs from the recipient by the manner in which its ability to produce glutamine synthetase is regulated (compare Experiments 13 and 14). It produces this enzyme at a high level even in the presence of excess ammonia; it resembles in this respect the donor strain MK-204 (compare Experiments 9 and 14). It also resembles the donor, and differs from MK-104, by its deficiency of glutamate dehydrogenase (compare Experiments 1, 9, 13, and 14). It resembles the recipient and differs from the donor by its ability to produce glutamate synthetase (compare Experiments 9, 13, and 14). In summary, the transductant selected for glutamine independence received from the donor the ability to produce high levels of glutamate synthetase even in the presence of excess ammonia, the CnR character, and the inability to produce glutamate dehydrogenase. We assume therefore that these characteristics may all be attributed to a single genetic trait, glsC15, present in strain MK-204 that is linked to the site responsible for the inability to produce glutamine synthetase in MK-104 (glnA8). On the other hand, the transductant has retained the ability of the recipient, absent in the donor, to produce glutamate synthetase, this trait (asw-200) therefore does not appear to be linked to the gla4 site nor is its absence necessary for expression of the CnR character. It is the presence of the glutamate synthetase in strain MK-257 that permits it, in spite of the lack of glutamate dehydrogenase, to grow in media not supplemented with glutamate. Thus, as long as the ammonia concentration is greater than 1 mM, either glutamate synthetase or glutamate dehydrogenase can provide glutamate.

This correlation between high glutamine synthetase and low glutamate dehydrogenase levels is not limited to the strains derived from the awsm-200 mutant since another strain with the CnR phenotype, selected as the revertant of glutamine requiring mutant strain MK-93 (2), also contains a high level of glutamine synthetase, but no glutamate dehydrogenase when grown on excess ammonia (Experiment 16). Therefore, it appears that the lack of glutamate dehydrogenase is generally associated with a high level of glutamine synthetase (Experiments 2, 3, 9 to 12, 14, 16, and 17). Conversely, a low level of glutamine synthetase is associated with a high level of glutamate dehydrogenase. Thus, in strains with normal regulation of glutamine synthetase formation the level of glutamate dehydrogenase is high only when the cells are grown with excess ammonia (Experiments 1, 4 to 7). In strains deficient in glutamine synthetase, the level of glutamate dehydrogenase is high, even when the cells are grown with the nitrogen source supplied at a growth-limiting rate (Experiments 13 and 15).

The correlation of the reduced level of glutamate dehydrogenase with the elevated glutamine synthetase raises the question whether a deficiency in glutamate dehydrogenase is responsible for excessive production of the glutamine synthetase. To answer this question, we isolated from the glutamate synthetase-less mutant strain MK-189, mutants requiring glutamate, as described under "Experimental Procedure." One of these mutants, MK-261, lacks both glutamate synthetase and glutamate dehydrogenase (Table VI, Experiment 18). We find that strain MK-261 has the CnR character of its parent MK-189, and produces glutamine synthetase at the usual low level in a medium containing excess ammonia. Some of the transductants obtained by treatment of cells of this strain with phage grown on wild type and selected for the ability to grow on a medium without glutamate had recovered only the glutamate synthetase (strain MK-270, Experiment 19). Strain MK-270 has the CnR character of the wild type, and also resembles the wild type in its low level of glutamine synthetase on excess ammonia (Experiment 19). It appears therefore that a deficiency in glutamate
dehydrogenase does not necessarily lead to excessive production of glutamine synthetase and the CnR phenotype.

**DISCUSSION**

It has long been recognized that glutamic acid is the key intermediate in the synthesis of nitrogenous compounds. Glutamic acid not only provides a portion of the carbon skeleton for the amino acids of the "glutamic acid family," but also the amino groups of several amino acids via transamination plus some of the nitrogen atoms of purine and pyrimidine nucleotides through reactions with glutamine. It has generally been assumed that an essential step in the utilization of ammonia by bacteria is its incorporation into glutamic acid. This assumption is fully confirmed by the results reported in this paper: the loss of the two enzymes which provide alternative routes for the conversion of α-ketoglutarate to glutamate results in a requirement of glutamate for growth.

One of the two enzymes, glutamate dehydrogenase (see Reaction 3), has long been known to be present in enteric organisms and many other species of bacteria. However, it was recently shown by Mears et al. (4) that this enzyme, whose \( K_m \) for ammonia is high, is not present at a sufficiently high level in cells of *K. aerogenes* cultured in an ammonia-limited chemostat to account for the growth of the cells. They discovered a new enzyme, glutamate synthetase, which, acting in concert with glutamine synthetase whose \( K_m \) for ammonia is very low, could catalyze the ATP-dependent conversion of \( \text{NH}_3 \) and α-ketoglutarate to glutamate (see Reactions 1 and 2).

Our results confirm the essential nature of Reaction 2 for the growth of *K. aerogenes* in a medium containing ammonia at a concentration lower than 1 mM. A mutant lacking the enzyme catalyzing Reaction 2 requires a higher concentration of ammonia in the medium than the wild strain. The loss of both glutamate dehydrogenase, catalyzing Reaction 3, and glutamate synthetase catalyzing Reaction 2, produces a mutant requiring glutamate for growth at all ammonia concentrations. Mutants lacking only glutamate dehydrogenase can grow without glutamate at high or low concentrations of ammonia.

It is quite apparent from these results that the cell does not require glutamine synthetase for the production of glutamate in media containing a sufficiently high concentration of ammonia to allow glutamate dehydrogenase to operate effectively. On the other hand, the cell does not require glutamate dehydrogenase when the exogenous concentration of ammonia is too low for its effective operation. It is therefore not surprising that the cell has evolved appropriate control mechanisms for the synthesis of these enzymes: limitation of the nitrogen source increases the level of glutamine synthetase and greatly decreases the level of glutamate dehydrogenase.

Our observations suggest that the decrease in the level of glutamate dehydrogenase is a consequence of the increase in the level of glutamine synthetase. Mutants lacking glutamine synthetase (Gln\(^-\)) contain a high level of glutamate dehydrogenase even when grown in a nitrogen-limited medium, while mutants whose level of glutamine synthetase is always high (Gln\(^+\)) produce very little glutamate dehydrogenase even when grown with excess ammonia. A Gln\(^+\) mutant which also lacks glutamate synthetase (Asm\(^-\)) consequently requires glutamate for growth because of the absence of both glutamate synthetase and glutamate dehydrogenase activities. The inverse relation between the levels of glutamine synthetase and glutamate dehydrogenase in Gln\(^+\), Gln\(^-\), and Gln\(^+\) cells grown with different nitrogen sources is illustrated in Fig. 3.

We have shown in the preceding paper that the ability of *K. aerogenes* to produce histidase and proline oxidase in the presence of glucose depends on a high level of glutamine synthetase. Our present finding strongly supports this idea. In a mutant deficient in glutamine synthetase (Reaction 2), the level of glutamine synthetase does not increase during growth on glutamine, a limiting nitrogen source, and histidase fails to be relieved from catabolite repression (CnR). The reason for this failure may be that derepression of glutamine synthetase requires a low internal pool of glutamine; the loss of the enzyme catalyzing the reaction of glutamine with ketoglutarate may prevent the drop of the intracellular glutamine concentration.

Whatever the reason for the CnR character of the Asm\(^-\) mutant, it is of great significance that some of the revertants selected for the ability to use histidine as nitrogen source with glucose as the major source of carbon, still are Asm\(^-\), but form glutamine synthetase constitutively (ClnC\(^-\)). These revertants have the CnR phenotype and were found to have a genetic defect in the same region as GlnC\(^-\) mutants previously selected as revertants of a glutamine requiring mutant with a defect in the *ghnB* site.

The observations we have reported in this and an earlier paper (2) suggest that *K. aerogenes* has evolved an elegant control system for the enzymes capable of supplying the cell with glutamate. Glutamine synthetase plays the most important part. Its synthesis is controlled by repression: a decrease in the level of ammonia and glutamine leads to rapid synthesis of the enzyme. The increased level of glutamine synthetase brings about a dramatic decrease in the level of glutamate dehydrogenase, an enzyme not capable of contributing to glutamate synthesis when the exogenous concentration of ammonia is low. At the same time, the increased level of glutamine synthetase relieves the catabolite repression of enzymes capable of forming glutamate from other amino acids, such as histidine or proline. These enzymes are inducible and can now be formed when the appropriate amino acids are available in the growth medium. Formation of these enzymes would be superfluous as long as the cell is supplied with ammonia in high concentration and glucose: in such a medium, glutamate is most efficiently produced by means of glutamate dehydrogenase.

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\(^2\) J. E. Brenchley and B. Magasanik, unpublished observation.
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