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MECHANISM AND REGULATION OF NITROGEN ASSIMILATION IN *KLEBSIELLA PNEUMONIAE* M5al

BY

MING-LIANG LI

A thesis submitted
in partial fulfillment of the requirements for the
degree Master of Science, major in
Bacteriology, South Dakota
State University

1972

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MECHANISM AND REGULATION OF NITROGEN ASSIMILATION

IN KLEBSIELLA PNEUMONIAE M5a1

This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is acceptable as meeting the thesis requirements for this degree. Acceptance of this thesis does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

Thesis Adviser Date

Head, Bacteriology Department Date

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I wish to express my deepest thanks to Dr. Robert M. Pengra for his continuous encouragement during the course of this investigation and for his suggestion and correction of almost every sentence in this paper.

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As Chinese always say: to be a student will never forget and many thanks to what his teachers have done to him. I like to thank all the staff in Bacteriology Department.

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INTRODUCTION

The pathway of the nitrogen atom after the initial nitrogen fixation reaction has taken place is not known. This study is an attempt to learn something about the enzymes that are involved in amino acid synthesis.

The cell has varying demands for the intermediary metabolites of nitrogen, as the processes of protein and other polymer synthesis vary. In addition, the cell is often confronted with changing supplies of nitrogen and energy, as a function of environmental change. Efficient growth depends upon the optimun utilization of metabolites in response to variation in cellular needs. The bacterium Klebsiella pneumoniae not only has the ability to adapt itself by utilizing various kinds of nitrogen compounds, but also fixes gaseous nitrogen under anaerobic conditions, when other nitrogen compounds are not available (25).

Unless some of the organic substances such as amino acids can pass directly into the cell and go to the amino acid pool immediately, most nitrogen materials must be reduced or deaminated to ammonia before they can be incorporated into amino acids. Two kinds of enzymes responsible for ammonia assimilation found in most organisms are glu-

tamate dehydrogenase and glutamine synthetase. These enzymes are also found in Klebsiella pneumoniae (22). It is interesting that the activity of glutamate dehydrogenase of N_2 grown Klebsiella pneumoniae seems too low to tell if it is responsible for ammonia assimilation. Therefore, attempts to find other enzymes have been made. A new enzyme glutamine: 2-oxoglutarate amidotransferase (NADP oxidoreductase) was found recently (22, 35), which catalyzes the reaction by transferring the terminal amino group from glutamine to α-ketoglutarate resulting in the formation of two moles of glutamic acid. This glutamic acid is ready for either amination or transamination. Thus, the transamination from glutamine or glutamic acid to other keto-acids may also be important in directing ammonia assimilation. Because glutamate dehydrogenase, glutamine synthetase, amino- and amido-transferase are interrelated, their relationships under different growing conditions will be different. The object of this study is try to find these relationships which are still unknown in Klebsiella pneumoniae.

LITERATURE REVIEW

Ammonia as the primary product of nitrogen fixation

Attempts to understand the mechanism of biological nitrogen fixation by identifing the first "stable intermediate" from N_2 were greatly aided by using the ^{15}N tracer technique. Exposure of growing cultures of Azotobacter vinelandii (5), Clostridium pasteurianum (39), photosynthetic bacteria (39), algae (40), or soybean nodules (3) to $^{15}N_2$ or $^{15}NH_4^+$, resulted in high concentrations of isotopic nitrogen in glutamic acid. Since the label from both $^{15}N_2$ and $^{15}NH_4^+$ appeared in the same compounds and in the same proportion after assimilation, it was concluded that N_2 is first reduced to ammonia, then incorporated into glutamic acid. When a strain of Clostridium pasteurianum excreted excess $^{15}NH_4^+$ to medium (43), it gave further evidence that ammonia is the first stable intermediate. By using a short-time exposure of the culture of Azotobacter vinelandii (5), Clostridium pasteurianum (7) or nodules (3, 15) to $^{15}N_2$, it was revealed that 94-96% of the ^{15}N appeared in ammonia. Thus, NH_4^+ appears to be a primary product of biological nitrogen fixation. In long-time exposures to $^{15}N_2$ the percentage

of label in ammonia falls rapidly with time as ammonia is incorporated into α-amino acids.

Whether other unstable intermediates such as hydrazine or hydroxylamine exist between N_2 and ammonia is still in question. Kinetic studies have shown that hydroxylamine reacts more rapidly with oxalacetic acid than with α-ketoglutarate (37). If hydroxylamine is a free intermediate, the accumulation should be into aspartic acid rather than glutamic acid (37). Hydroxylamine is toxic to nitrogen fixers. Even at non-toxic concentrations neither Azotobacter vinelandii nor Clostridium pasteurianum can use it (38). The occurrence of hydroxylamine reductase in Azotobacter vinelandii and Clostridium pasteurianum (12) indicates that if hydroxylamine enters or forms in the cell it would be readily converted to ammonia. This is why no conclusion can be drawn to eliminate it as an intermediate. Attempts to grow Azotobacter vinelandii using hydrazine as the sole nitrogen source have also failed (28). So far numerous studies both in vitro and in vivo made to detect oxidized or reduced intermediates between N_2 and NH_4^+ have been unsuccessful (6).

The absence of isotopic exchange ($^{14}N_2 + {}^{15}N_2 \rightleftharpoons {}^{14}N^{15}N$) indicates no initial cleavage of N_2 to nitrogen

atoms (7). A two steps reduction of N_2 by nitrogenase has been proposed with diimide and hydrazine complexing with nitrogenase forming intermediate enzyme-substrate complexes ultimately releasing ammonia as a stable, free product (13).

Glutamate dehydrogenase and its regulation

The first demonstration of ammonia assimilation was made by Knoop, Embden and Schmitz in animal metabolism in 1910. They showed a reductive formation of amino acids from NH_4^+ and corresponding α-keto acids (21). In 1938, von Euler et al. were able to demonstrate the reaction catalyzed by the enzyme glutamate dehydrogenase. They showed its pyridine nucleotide coenzyme requirement in cell free preparations of animal tissue, Escherichia coli and yeast (1, 2, 11). The following reaction was first shown by Euler in his yeast preparation:

$$NH_4^+ + \alpha\text{-ketoglutarate} + NADPH \rightleftharpoons \text{glutamate} + NADP + H_2O$$

Since then glutamate dehydrogenase has been found widely distributed in a variety of bacteria, fungi, plant and animal tissues. The enzyme was first crystallized from bovine liver by Strecker (34) and by Olson (24) in 1951. The coenzyme specificity appears to depend on the

enzyme source. Only a NAD-dependent glutamate dehydrogenase was found in plants (8); both NAD and NADP-dependent enzymes have been found in animal liver (11), Neurospora crassa (30) and yeast (9). Only NADP-dependent glutamate dehydrogenase was found in Escherichia coli, Klebsiella pneumonia and Rhodospirillum rubrum (1, 22); only NAD-dependent enzyme was found in Clostridium sp. (23) and Azotobacter sp. (4); both NAD and NADP-dependent enzymes were found in Thiobacillus novellus (18).

Glutamate dehydrogenase catalyzes a reversible reaction, so that it is subject to the mechanism of mass action. When Escherichia coli is grown on nutrient broth, or a medium containing glutamate, aspartate or arginine nitrogen, there is a low activity of glutamate dehydrogenase compared to that in cells using ammonia as their nitrogen source (36), whereas cell grown on methionine or alanine had high activity of this enzyme (36). In Neurospora crassa the NADP-dependent glutamate dehydrogenase is repressed by growth on glutamate. In contrast, the NAD-dependent glutamate dehydrogenase is derepressed by growth on glutamate (31). The NAD-dependent glutamate dehydrogenase is chemically different from the NADP-dependent glutamate

dehydrogenase and controlled by different genes. The two enzymes seem to be coordinately regulated (31). The NADP-dependent glutamate dehydrogenase appears to function more for glutamic acid synthesis and NAD-dependent glutamate dehydrogenase more directed toward glutamate degradation. Similar coordinated regulation between NADP and NAD-dependent glutamate dehydrogenase was also observed in baker's yeast (9) and Thiobacillus novellus (18). The kinetics of the NAD-dependent reaction are altered by AMP, whereas those of NADP-dependent reaction are not changed by it (9).

Glutamine synthetase and its regulation

The synthesis of glutamine by tissues was first studied by Krebs in 1935, who demonstrated that brain cortex, retina and kidney slices of vertebrates formed glutamine from ammonia and glutamate. In 1947, the glutamine synthesis in cell free system, ATP and Mg^{++} requirement were able to show (38). Glutamine synthetase activity in broken cells of bacteria was first shown by Elliott and Gale (10). Speck was able to establish a stoichometric relationship between NH_4^+ utilized, glutamine formed and inorganic phosphate released. The

reaction has been illustrated as (33):

$$\text{glutamate} + NH_4^+ + \text{ATP} \longrightarrow \text{glutamine} + \text{ADP} + PO_4^{---}$$

Glutamine synthetase from Escherichia coli can be repressed and derepressed by changing the nitrogen source presented to the cell (20, 41). Using a medium with adequate amino or high quantities of ammonia nitrogen to grow the cell, a low activity of enzyme was found. When cells are grown on glutamate or on limiting quantities of ammonia, the specific activity of the enzyme can be increased as much as 20-fold (20, 41). The synthesis of glutamine synthetase is also regulated by adenylylation and deadenylylation in Escherichia coli (16). Thus, the quantitative significance of enzyme activity altered by different nitrogen sources is difficult to assess. The adenylylated and deadenylylated enzyme species have remarkably different activities. This difference was not taken into account in the studies on repression and derepression in the past. Since both adenylylated and deadenylylated species of glutamine synthetase are also found in Klebsiella pneumoniae (22), its regulatory mechanism appears complicated too.

Bacillus, Lactobacillus and Saccharomyces do not

contain the enzyme that catalyzes the adenylylation of glutamine synthetase. Their glutamine synthetase activity has been found to be dependent upon the nitrogen source. Depression of glutamine synthetase in Bacillus subtilis when grown in the present of glutamine and derepression of it when grown with limited nitrogen have been found (27). In Saccharomyces cerevisiae, the level of glutamine synthetase is repressed by growth on either glutamine, asparagine or NH_4^+. Growth on amino acids, such as glutamate, aspartate and methionine leads to an increased synthesis of this enzyme (17).

In all cases cited, it is difficult to tell which is the metabolite directly responsible for the repression. In Lactobacillus, glutamine was found to further repress enzyme synthesis when added to the nitrogen rich medium, therefore it appears probable that glutamine itself is the metabolite responsible for the repression (26).

Transaminases and their regulation

Enzymatic transamination was first described in 1937 by Braunstein and Kritzmann (38). Snell was first to suggest that pyridoxal and pyridoxamine might be interconvertible by transamination and vitamin B_6 might serve

as the coenzyme in enzymatic transamination (32). His suggestion was proved true later. It was found that a number of α-amino acids could transaminate with a number of keto acids in animal and plant tissues and microorganisms.

An amidotransferase which transfers the amido group from glutamine to α-ketoglutarate has been found recently (22, 35). A characteristic of this transaminase is that its activity depends on the pyridine nucleotides as coenzymes and transfers terminal amido group rather than α-amino group. The reaction catalyzed by this enzyme has been established as follow:

2 glutamate + NAD(P) $\rightleftarrows$ α-ketoglutarate + glutamine + NAD(P)H

The importance of this amidotransferase in ammonia assimilation of nitrogen fixers has been described by Nagatani et al. (22). This enzyme has been called glutamine: 2-oxoglutarate amidotransferase (NADP oxidoreductase) or glutamine amide-2-oxoglutarate amino transferase, or glutamate synthetase.

No studies on regulation of transamination by these enzymes have been made so far. Probably their activities are not subject to any kind of regulation.

MATERIALS AND METHODS

Organism

The organism used in this study was isolated from a 2,3-butanediol fermentation by Dr. Elizabeth McCoy, Bacteriology Department, University of Wisconsin. It was first named as Aerobacter aerogenes M5al. and later in 1965 renamed Klebsiella pneumoniae M5al (19). This organism was grown at 30 C and maintained on nutrient agar slants during the study. Culture was stored in the refrigerator.

Medium

Different nitrogen sources were used to grow the organism (42). For N_2 grown cells, nitrogen gas was bubbled through the medium. For NH_4^+ grown cells, various concentrations of NH_4Cl were added. For glutamate grown cells, 0.2g glutamate N/l was added. For cells grown on glutamate without sugar, 15g glutamate/l was added to serve as both the nitrogen and carbon sources.

Inoculum

A loop of the organism from the slant was transferred to 250ml Erlenmeyer flasks containing 50 ml of growth medium. For growth of cells on N_2, the air of the flasks

was removed by evacuation and replaced with high purity nitrogen gas. The inoculum for cells grown on glutamate as the carbon and nitrogen sources was prepared by a successive transfer of cells from a medium of low concentration of glutamate to one of a higher concentration. All the flasks were placed on a rotary shaker and incubated at 30 C for 18-24 hours.

Mass culture

A 2-5% inoculum was transferred to a 4-L Erlenmeyer flask containing 2 liters of growth medium. Air or nitrogen was bubbled through a sintered glass sparger in the culture. The cells were harvested by centrifugation at 20,000g for 10 minutes when the optical density of the culture reached 0.4-0.5. The pellet of the cells was washed twice with normal saline and stored at -20 C.

Cell free extracts

The pellet of cells was resuspended on normal saline at a concentration of 1:2 (W/V), and then poured into a cold French pressure cell which had been precooled at 4 C. The pressure was brought to 10,000 psi, and allowed to equilibrate for 15 minutes before being released from the

pressure cell. The unbroken cells and other debris were removed by centrifugation at 20,000g for 30 minutes. The extract was dialyzed for 8 hours at 4 C against 100 mM Tris buffer (pH 7.8). The dialyzed supernatant fluid containing enzymes was designated as crude extract and stored at -20 C. The protein level was determined by the Biuret method (Figure 4) (14).

Enzymatic assay for glutamate dehydrogenase

A spectroscopic method based on reduced pyridine nucleotide oxidation monitored at 340 nm was used to assay glutamate dehydrogenase (22). The total reaction mixture in a 3ml quartz cuvette contained enzyme (usually added sufficient to allow a linear reaction at least lasting for 3 minutes), 0.1 ml of 0.5 mM NADPH, 0.1 ml of 20 mM α-ketoglutarate, 0.1 ml of 10 mM NH_4Cl and sufficient 100 mM Tris buffer to bring the total volumn to 3.0 ml. The reaction mixture was incubated at room temperature 23 C and the oxidation of reduced pyridine nucleotide was measured using a Beckman DU spectrophotometer. The changes in optical density at 340 nm were referred to a standard NADP curve (Figure 1).

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Enzymatic assay for glutamine: 2-oxoglutarate amidotransferase (NADP oxidoreductase)

The activity was measured using assay conditions similar to the glutamate dehydrogenase reaction except that 0.1 ml glutamine replaced ammonia as the amino donor.

Enzymatic assay for glutamine synthetase

Activity of glutamine synthetase was determined by measuring the amount of phosphate released from ATP (41). This assay mixture contained 0.05 ml crude extract and 0.15 ml of 1.5 μM ATP, 20 mM L-glutamate, 0.01 mM NH_4Cl and 0.01 mM $MgCl_2$ in 0.05 M imidazole buffer (pH 7). The reaction was stopped by the addition of 1.8 ml of a freshly prepared solution contained 0.8% $FeSO_4.7H_2O$ in 0.05 N H_2SO_4 followed by the addition of 0.15 ml of a solution contained 6.6% $(NH_4)_6Mo_7O_{24}.7H_2O$ in 7.5 N H_2SO_4. After several minutes delay for color development, the coagulated protein was removed by centrifugation and optical density of the supernatant solution was read at 660 nm. A control mixture containing ATP and crude extract was measured to correct for the ATPase interference. A standard quantitative curve (Figure 2) was prepared for converting optical density to μM of phosphate.

Enzymatic assay for glutamic acid-keto acid and glutamine-keto acid transferases

Activity of transaminase was assay by the method described by Roswell (29). The assay mixture contained 0.5 ml enzyme, 0.5 ml of 0.08 M glutamate or glutamine, 0.25 ml of 0.08 M pyruvate, 0.25 ml of 0.08 M oxalacetate and 0.1 ml pyridoxal phosphate (200 µg/ml). The mixture was incubated at temp. (23 C) for 60 minutes. The reaction was stopped by boiling for 5 minutes and the coagulated protein was removed by centrifugation. Twenty ul of supernatant was applied to the Whatman No.1 chromatographic filter paper. The solvent system consisted of 1-butanol-formic acid- water (67:20:17 V/V/V). After development was complete, the chromatograms were dried in air and sprayed with 0.5% ninhydrin in 70% ethanol, followed by heating to 65 C for 22 minutes. The alanine and aspartate spots were cut on rectangles of equal area and from each chromatogram a blank retangle was taken. Each rectangle was cut into small pieces which were placed in a 10ml conical centrifuge tube and 5.0 ml 70% ethanol was added. Tubes were agitated for about 5 minutes until all the purple color was extracted from the paper and evevly dispersed in solution. The paper sedimented to the bottom

and supernatant was decanted for optical density measurement at 575 nm. Alanine and aspartate formed by transamination were calculated by comparing the optical density with the standard run on the same chromatogram and taking into account the paper blank (Figure 3). The enzyme without substrate was run also to account for the original amino acids in the crude extracts.

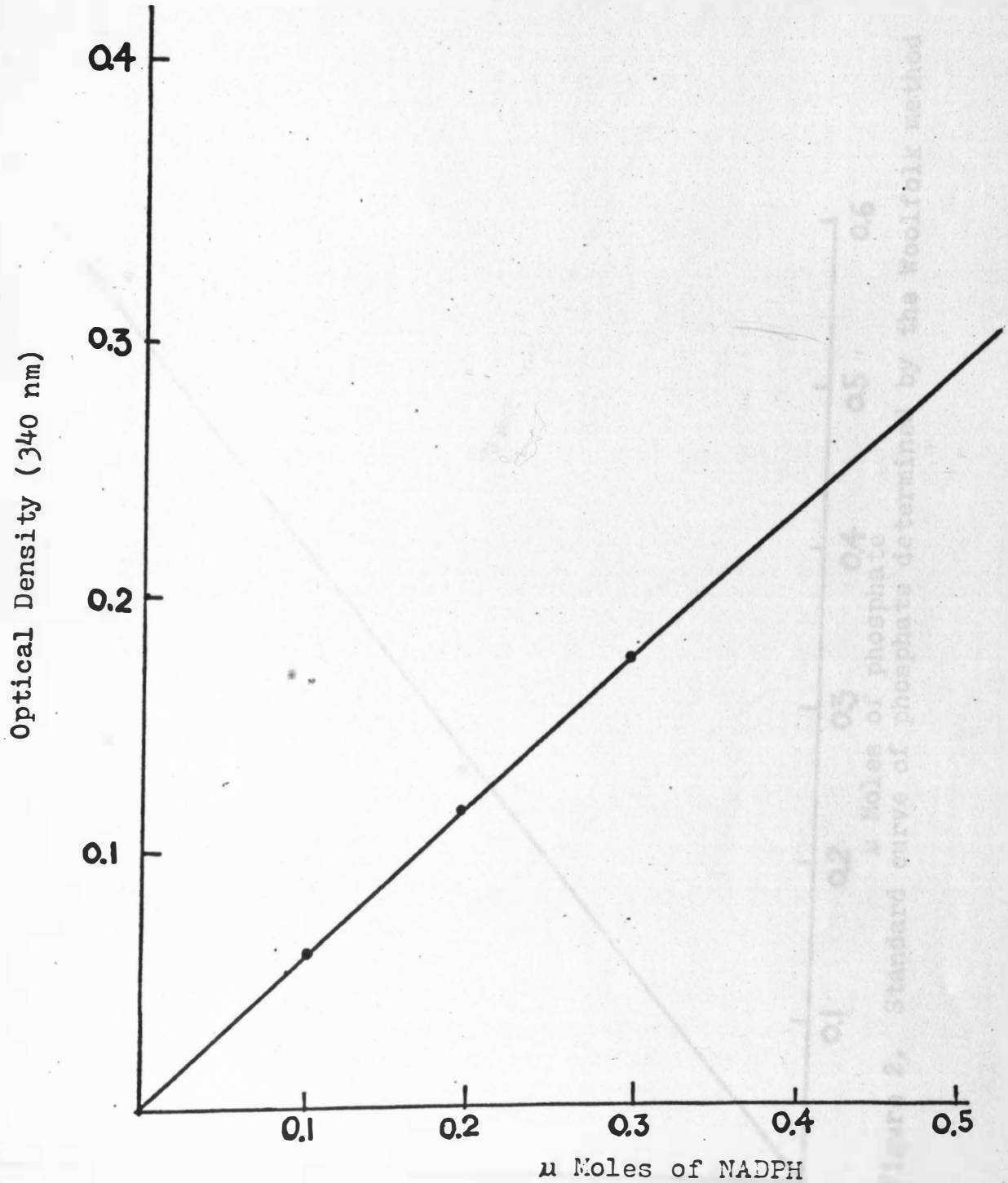


Figure 1. Standard NADPH curve measured at 340 nm in 3ml Tris buffer.

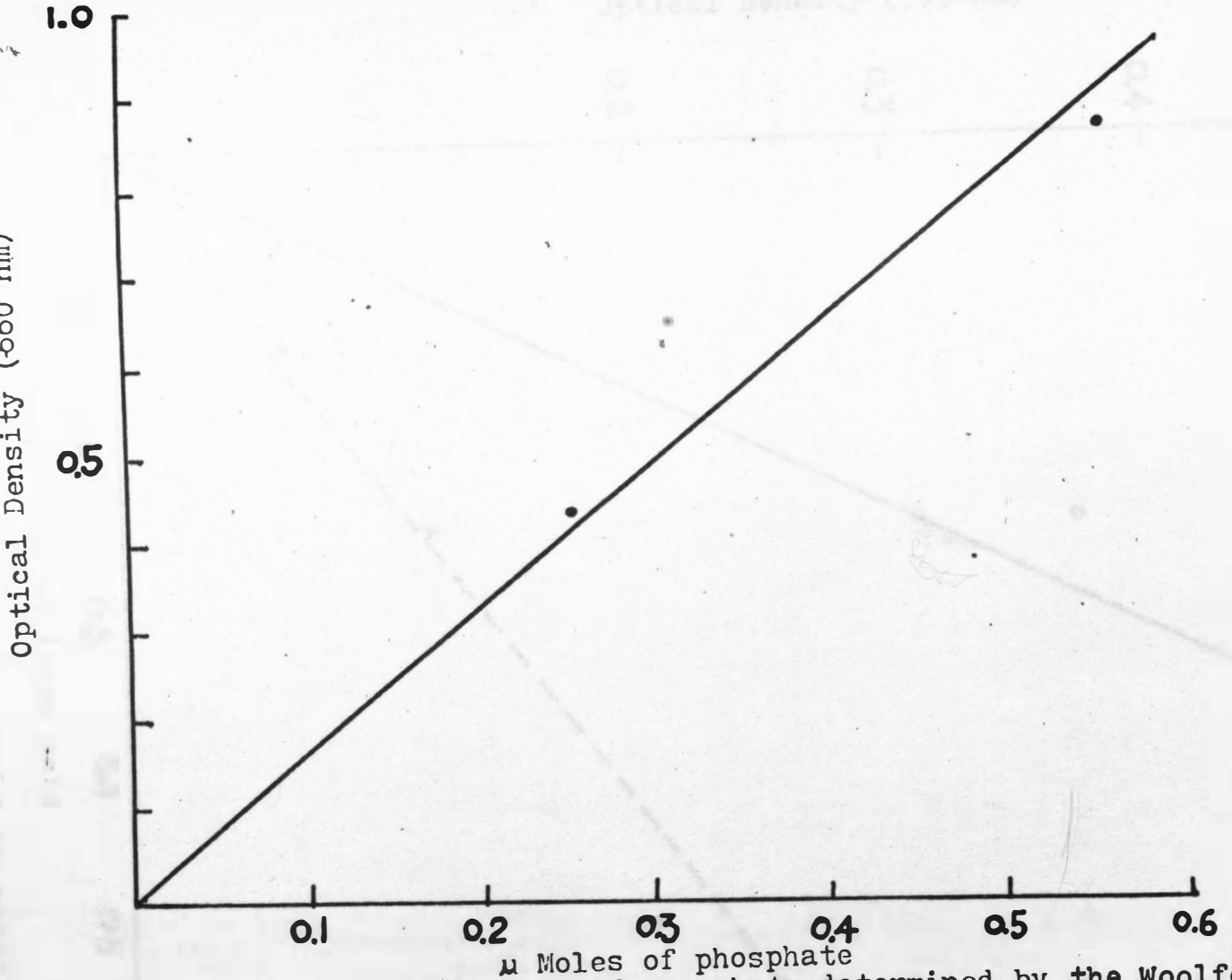


Figure 2. Standard curve of phosphate determined by the Woolfolk method

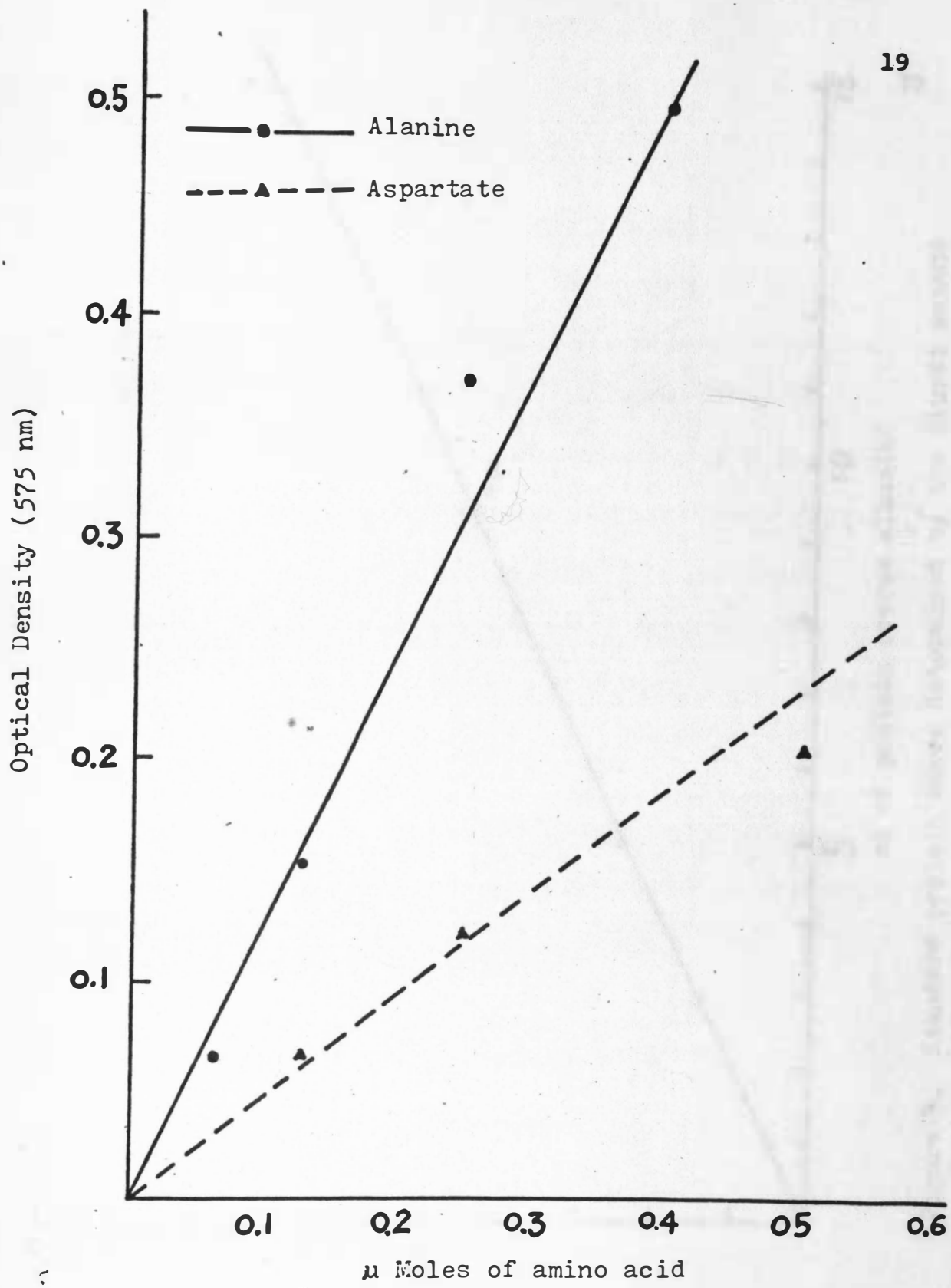


Figure 3. Standard amino acid curve determined by reacting known amounts of the amino acid with ninhydrin on paper chromatogram.

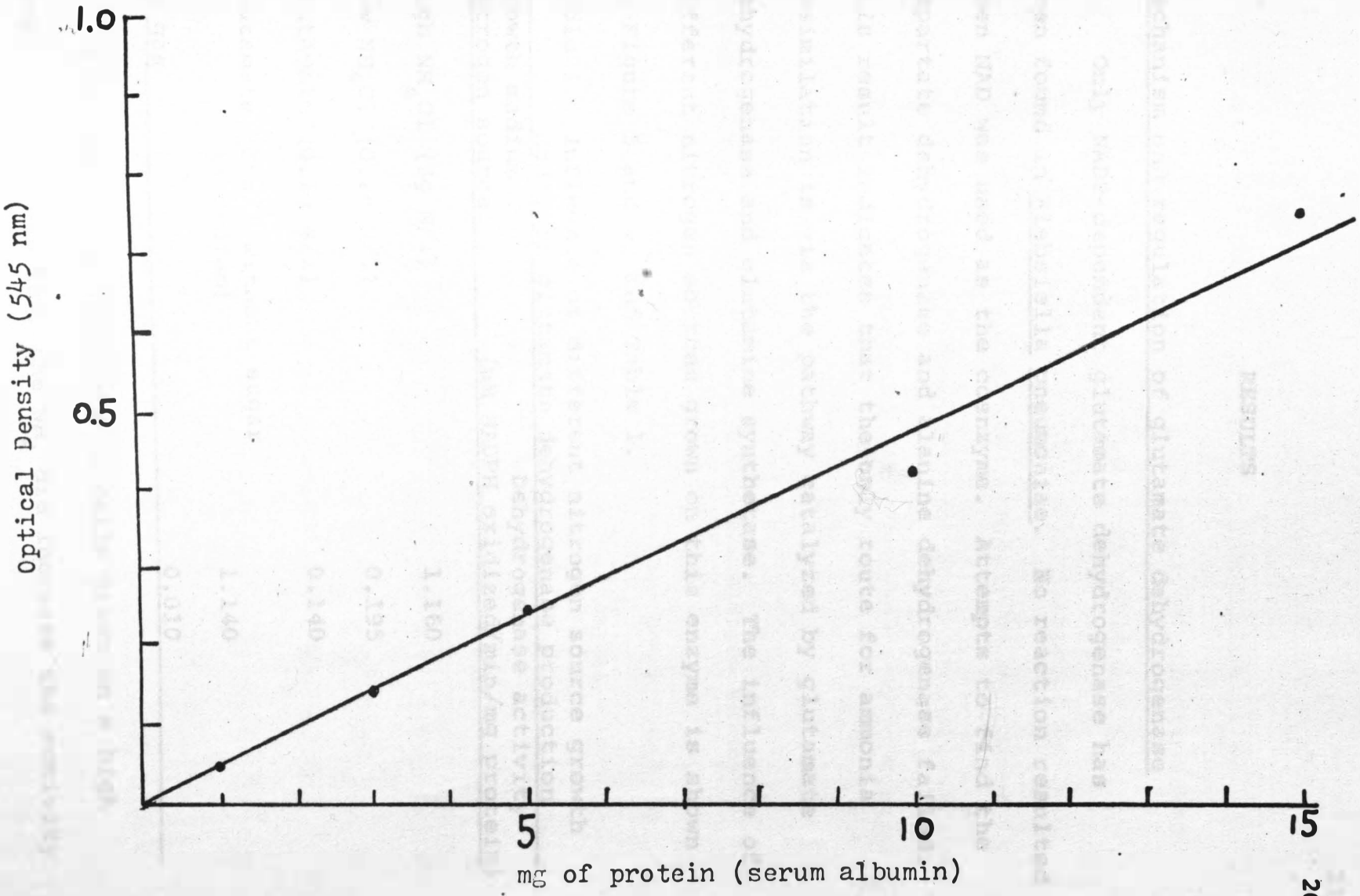


Figure 4. Standard protein curve determined by the Biuret method

RESULTS

Mechanism and regulation of glutamate dehydrogenase

Only NADP-dependent glutamate dehydrogenase has been found in Klebsiella pneumoniae. No reaction resulted when NAD was used as the coenzyme. Attempts to find the aspartate dehydrogenase and alanine dehydrogenase failed. This result indicates that the only route for ammonia assimilation is via the pathway catalyzed by glutamate dehydrogenase and glutamine synthetase. The influence of different nitrogen sources grown on this enzyme is shown in Figure 5 and 6, and Table 1.

Table 1. Influence of different nitrogen source growth media on glutamate dehydrogenase production

Growth medium nitrogen source	Dehydrogenase activity (μM NADPH oxidized/min/mg protein)
High NH_4Cl (3g N/l)	1.160
Low NH_4Cl (0.2g N/l)	0.195
Glutamate (0.2g N/l)	0.140
Glutamate (15g/l without sugar in medium)	1.140
N_2 gas	0.010

Although the growth rate of cells grown on a high level of NH_4Cl was low, the NH_4^+ did increase the activity

of glutamate dehydrogenase. Even a minute amount of glutamate showed a repression of glutamate dehydrogenase formation if the energy requirement of cells was not dependent on glutamate only. Using glutamate as both nitrogen and carbon sources, the activity of glutamate dehydrogenase increased sharply. The N_2 grown cells showed a lower activity of glutamate dehydrogenase when compared to other nitrogen sources grown cells.

Mechanism and regulation of glutamine: 2-oxoglutarate amidotransferase (NADP oxidoreductase)

Only NADP dependent transferase have been found in Klebsiella pneumoniae. No NAD dependent enzyme was shown with cells grown under different growing conditions. The enzyme is specific for glutamine, glutamate and α-keto-glutarate. When oxalacetate and pyruvate were used as the amino acceptors, the assay reaction did not proceed. However, by using asparagine or arginine as the amino donor, the reaction was catalyzed only by the extract from cells growing on glutamate as both their nitrogen and carbon sources. This indicates that the glutamine: 2-oxo-glutarate amidotransferase is specific for dibasic amino acid glutamine only. The NADP dependent reaction using arginine and asparagine is probably catalyzed by the new

unknown enzymes. The influence of different nitrogen sources for growth of the cells used to produce the enzyme is shown in Figures 5 and 6, and Table 2.

Table 2. Influence of different nitrogen source growth media on glutamine: 2-oxoglutarate amidotransferase production

Growth medium nitrogen source	Amidotransferase activity (μM NADPH oxidized/min/mg protein)
High NH_4Cl (3g N/l)	1.000
Low NH_4Cl (0.2g N/l)	0.200
Glutamate (0.2g N/l)	0
Glutamate (15g/l without sugar in medium)	0
N_2 gas	0.170

As the concentration of NH_4^+ in the growth medium was increased, the formation of the enzyme increased. When glutamate was used in the growth medium, formation of the enzyme decreased. A considerable amount of this enzyme is present in N_2 grown cell.

Mechanism and regulation of glutamine synthetase

Glutamine synthetase is specific for glutamate and ammonia. The reaction requires ATP and Mg^{++}. The influence of different nitrogen sources for growth of the cells used to produce the enzyme is shown in Figure 7 and

Table 3.

Table 3. Influence of different nitrogen source growth media on glutamine synthetase production

Growth medium nitrogen source	Synthetase activity (μM phosphate released/15 min/mg protein)
High NH_4Cl (3g N/l)	3.05
Low NH_4Cl (0.2g N/l)	17.50
Glutamate (0.2g N/l)	18.50
Glutamate (15g/l without sugar in medium)	2.50
N_2 gas	5.00

Repression of glutamine synthetase by high concentration of ammonia and glutamate is shown. Relatively high enzyme production is exhibited in N_2 grown cells.

Mechanism and regulation of glutamic acid-keto acid transaminases and glutamine-keto acid transaminases

The transamination requires pyridoxal phosphate. The influence of different nitrogen sources in growth medium on the production of transaminases is shown in Table 4 and Table 5.

Table 4. Influence of different nitrogen source growth media on glutamic acid-keto acid transaminases production

Growth medium nitrogen source	Transaminases activity (μM amino acid produced/hour/mg protein)	
	Alanine	Aspartate
High NH_4Cl (3g N/l)	0	Trace
Low NH_4Cl (0.2g N/l)	0	Trace
Glutamate (0.2g N/l)	0	0.05
Glutamate (15g/l without sugar in medium)	0	0.03
N_2 gas	0.01	0.10

Table 5. Influence of different nitrogen source growth media on glutamine-keto acid transaminases production

Growth medium nitrogen source	Transaminases activity (μM amino acid produced/hour/mg protein)	
	Alanine	Aspartate
High NH_4Cl (3g N/l)	0.20	0.83
Low NH_4Cl (0.2g N/l)	0.74	2.76
Glutamate (0.2g N/l)	0.43	2.16
Glutamate (15g/l without sugar in medium)	0.10	0.26
N_2 gas	0.63	2.63

Comparing the data in Table 4 to Table 5, transamination activity from glutamine is higher than from glutamate. Extract from cells grown on N_2 had a higher transaminases activity than extracts from cells grown on other nitrogen

sources.

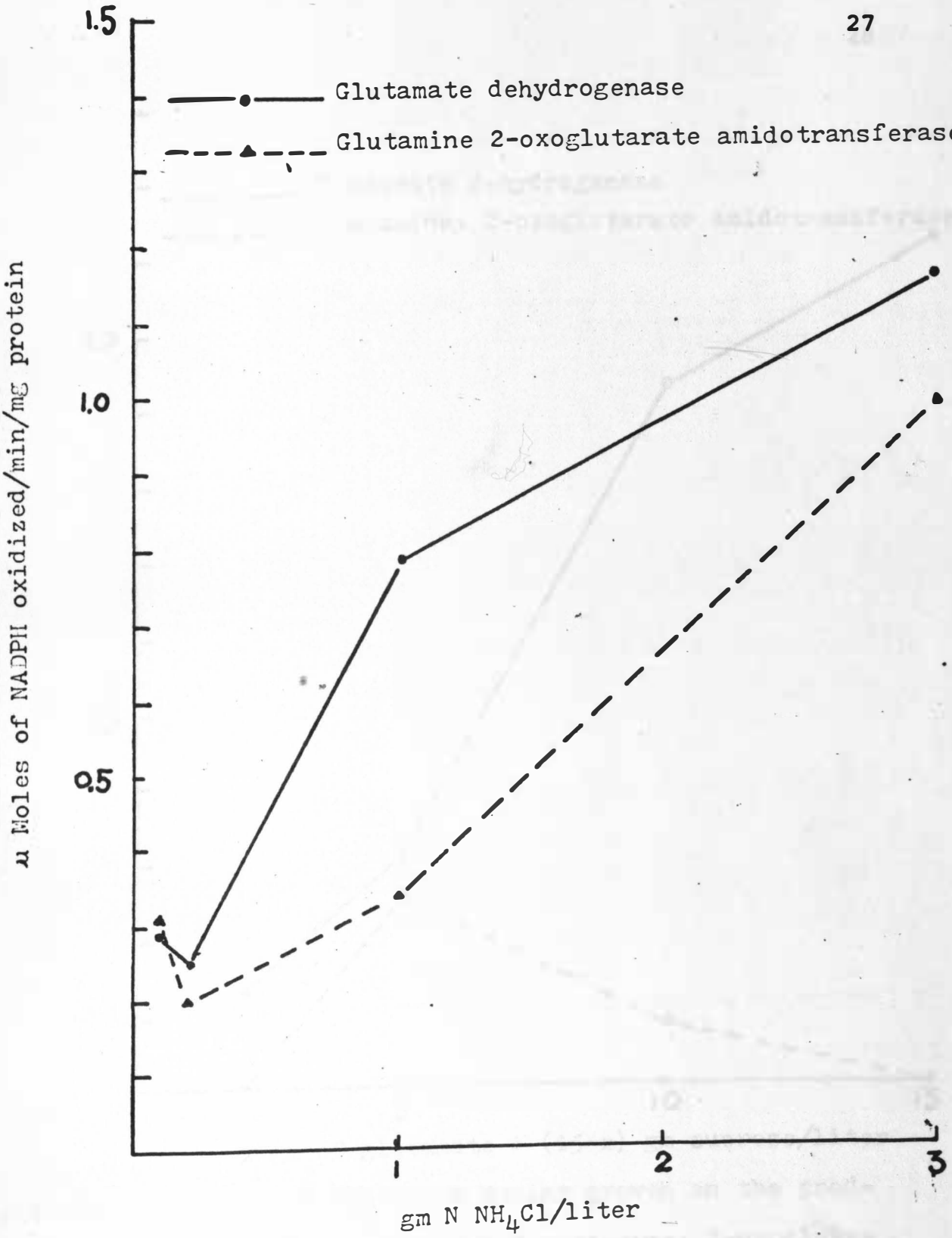


Figure 5. Influence of NH_4^+ during growth on the production of glutamate dehydrogenase and glutamine: 2-oxoglutarate amidotransferase.

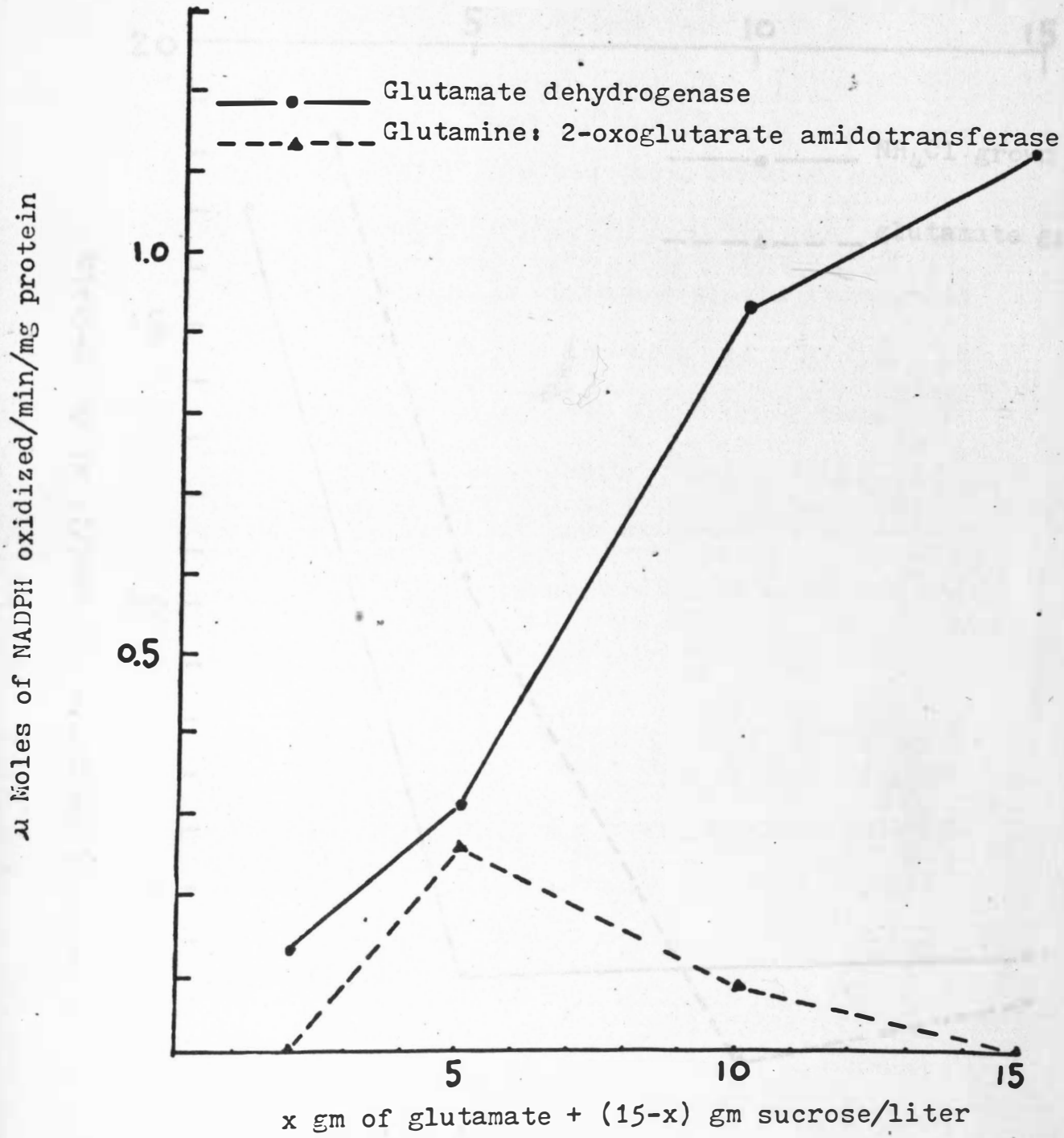


Figure 6. Influence of glutamate during growth on the production of glutamate dehydrogenase and glutamine: 2-oxoglutarate amidotransferase.

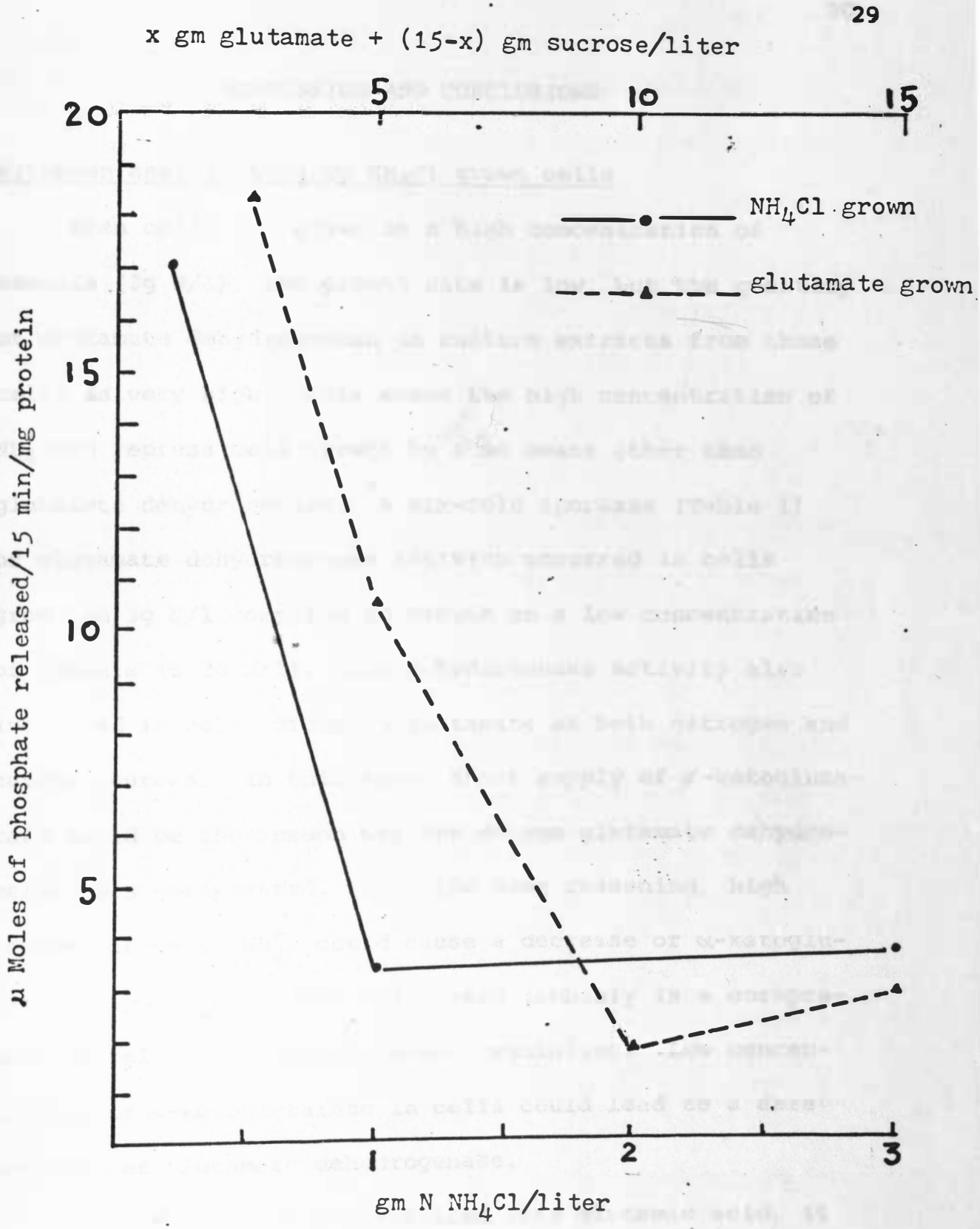


Figure 7. Influence of NH_4^+ and glutamate during growth on the production of glutamine synthetase.

DISCUSSION AND CONCLUSIONS

Nitrogen assimilation by NH_4Cl grown cells

When cells are grown in a high concentration of ammonia (3g N/l), the growth rate is low, but the quantity of glutamate dehydrogenase in culture extracts from these cells is very high. This means the high concentration of NH_4^+ did repress cell growth by some means other than glutamate dehydrogenase. A six-fold increase (Table 1) of glutamate dehydrogenase activity occurred in cells grown on 3g N/l compared to growth on a low concentration of ammonia (0.2g N/l). The dehydrogenase activity also increased in cells grown on glutamate as both nitrogen and carbon sources. In this case, short supply of α-ketoglutarate could be the reason why the enzyme glutamate dehydrogenase was derepressed. With the same reasoning, high concentration of NH_4^+, could cause a decrease of α-ketoglutarate supply. Alpha-ketoglutarate probably is a corepressor for glutamate dehydrogenase regulation. Low concentration of α-ketoglutarate in cells could lead to a derepression of glutamate dehydrogenase.

After ammonia is incorporated into glutamic acid, it will be further metabolized into other amino acids.

Generally, the glutamic acid-keto acid transaminases activities are low in NH_4^+ grown cells. Only small amounts of glutamate amino nitrogen was found transferred into aspartic acid (Table 4). No glutamic acid-pyruvate tranaminase activity was detected. Most glutamate metabolized is used to synthesize glutamine by two different pathways.

1) glutamate + NH_4^+ + ATP $\longrightarrow$ glutamine + ADP + PO_4^{---}

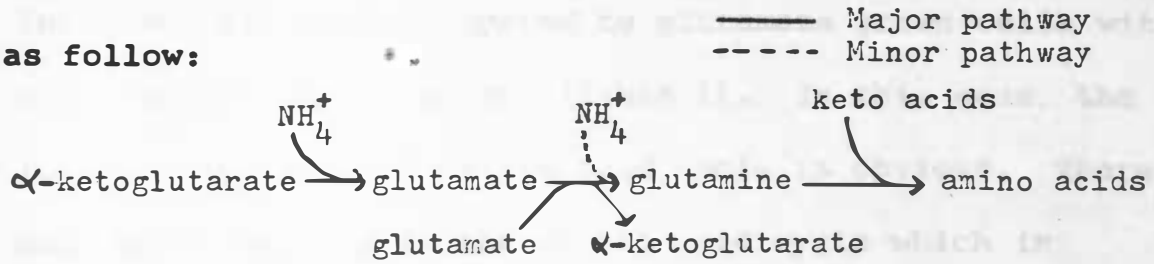
2) glutamate + NADP $\rightleftharpoons$ glutamine + α-ketoglutarate + NADPH

Pathway 1 is catalyzed by glutamine synthetase. This enzyme is repressed by high concentrations of NH_4^+ (Table 3). It has six-fold less activity than glutamine synthetase found in cell free preparation from cells grown on low concentration of NH_4^+. Comparing the activity of glutamine synthetase to glutamate dehydrogenase, the two enzymes seem to coordinately regulate each other. Cells with a high activity of glutamate dehydrogenase have a lower activity of glutamine synthetase; cells with a low activity of glutamate dehydrogenase have a higher activity of glutamine synthetase. Pathway 2 is catalyzed by NADP-dependent glutamine: 2-oxoglutarate amidotransferase. It is a reversible reaction. Figure 5 shows a parallel

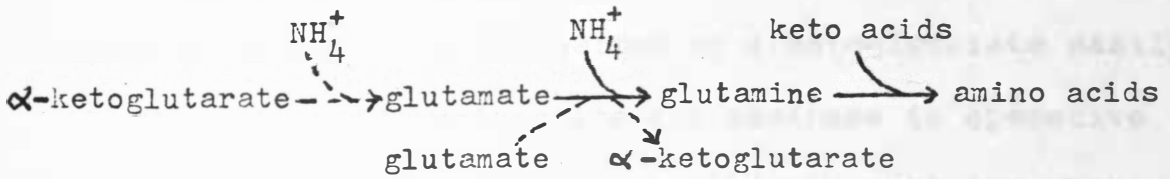
increase of activity of this enzyme and glutamate dehydrogenase. Cells grown on a high amounts of NH_4^+ have a low activity of glutamine synthetase. Pathway 2 provides an alternate way to synthesize glutamine.

A higher activity of glutamine-keto acid transaminases was found than glutamic acid-keto acid transaminases. This indicates that most amino groups or amido groups are finally transferred to other amino acids from glutamine.

For cells grown with a high level of ammonia in the medium, the pathway of nitrogen assimilation is illustrated as follow:



For cells grown with a low level of ammonia in the medium, the pathway is illustrated below:



Nitrogen assimilation by glutamate grown cells

Cells using glutamate as the nitrogen source could transport glutamate directly into the amino acids pool. They have no need for glutamate dehydrogenase to catalyze

a synthetic reaction. If the energy source is sufficient, there is no need for glutamate dehydrogenase to catalyzed a deamination reaction. Therefore, glutamate dehydrogenase is not only regulated by nitrogen compounds but may also be regulated by the compounds of the citric acid cycle. The formation of glutamate dehydrogenase is repressed in cells grown using glutamate for their nitrogen source (0.2g glutamate N/l) and sugar for their carbon source. If they use glutamate as both nitrogen and carbon sources, the activity of glutamate dehydrogenase is increased six times compared to glutamate grown cells with sugar as the carbon source (Table 1). In this case, the lack of compounds of citric acid cycle is obvious. There must be a compound in the citric acid cycle which is responsible for derepression of glutamate dehydrogenase formation. That compound could be α-ketoglutarate or other compounds which can be converted to α-ketoglutarate easily.

Glutamic acid-oxalacetate transaminase is operative in cells grown on glutamate with a higher activity than NH_4^+ grown cells (Table 4). Whether synthesis of this enzyme is derepressed by glutamate is uncertain. Because glutamate content of N_2 grown cells would not exceed that of the glutamate grown cells, then their transaminase

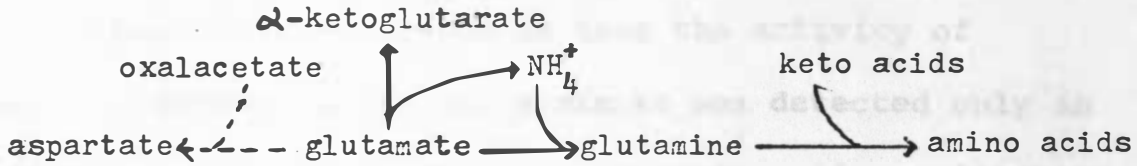
activity would be expected to be greater than glutamate grown cells.

The glutamate in glutamate grown cells is ready for glutamine synthesis. The synthesis of glutamine synthetase is repressed in cells grown in the presence of glutamate without sugar. The synthesis is not repressed in cultures grown on low concentrations of glutamate with sugar. Whether the repression is caused by glutamate or by glutamine itself is not certain. The glutamine synthetase in this study is restricted to adenylylated species. A report from Nagatani et al. showed the deadenylylated species is not important in Klebsiella pneumonia (22).

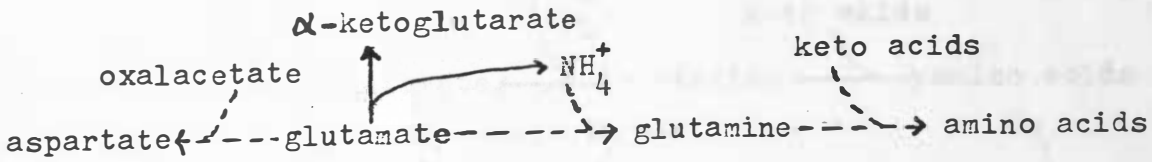
Activity of glutamine: 2-oxoglutarate amidotransferase is lost in cells grown on small amounts of glutamate (0.2g glutamate N/l and 15g sucrose) and in cells grown on glutamate (15g glutamate/l and without sugar). Glutamate serves both for nitrogen and carbon sources in culture grown without sugar. Some of its activity has been regained when cells are grown on 5g of glutamate and 10g of sucrose (Figure 6). This indicates that glutamate itself is not the metabolite responsible for glutamine: 2-oxoglutarate amidotransferase regulation. Which factors regulate this enzyme are unknown.

The transamination activity from glutamine is higher than from glutamate. This implies that amino group transfer from glutamine is more important than from glutamate.

In cells grown on small amounts of glutamate (0.2g glutamate N/l), it is probable that a molecule of glutamate is deaminated and the resulting ammonia is used in the amination catalyzed by glutamine synthetase. Simultaneously, a small amounts of the glutamic acid nitrogen is transaminated to oxalacetate forming aspartic acid. The pathway is expressed by the following illustration:



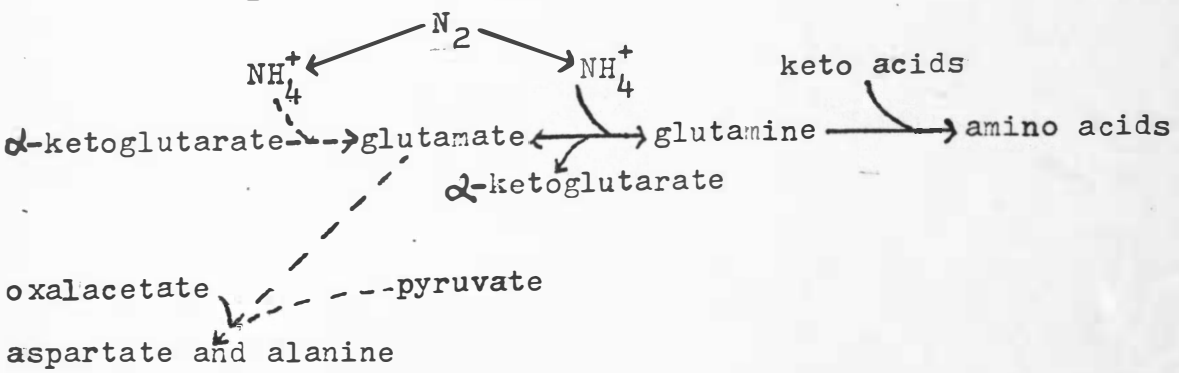
In cells grown on glutamate without sugar in medium, most glutamate is deaminate to yield α-ketoglutarate. Only a minute amounts of glutamate is aminated to synthesize glutamine and transaminated to synthesize aspartic acid. Their pathway is illustrated below:



Nitrogen assimilation by N_2 grown cells

A very low activity of glutamate dehydrogenase is present in N_2 grown cells. Therefore, most of the NH_4^+ is used in the amination reaction to form glutamine catalyzed by glutamine synthetase (Table 1 and 3). The repression of glutamate dehydrogenase is probably due to the accumulation of glutamate in the cells. This accumulation is a result of high activity of glutamine: 2-oxoglutarate amidotransferase which produces the glutamate by transferring its terminal amino group from glutamine to a molecule of α-ketoglutarate.

A peculiar phenomenon is that the activity of glutamic acid-pyruvate transaminase was detected only in N_2 grown cells and the highest glutamic acid- oxalacetate transamination is present in these cells also. What factors contribute to this regulation are unknown. The pathway of nitrogen assimilation after converting to ammonia in N_2 grown cells is showed below:



Many facts have been revealed in this study, but among them some are difficult to explain, such as why the glutamic acid-pyruvate transaminase is only detected in N_2 grown cells; why the activity of glutamic acid-oxalacetate transaminase is higher in N_2 grown cells; and what factors are involved in their regulation.

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