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Short Communications

Synthesis of Glutamate in *Aerobacter aerogenes* by a Hitherto Unknown Route

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In order to grow in a simple salts medium in which ammonia provides the sole source of nitrogen, micro-organisms must possess some mechanism for the synthesis of amino acids from ammonia and intermediary metabolites. In many bacteria this requirement is fulfilled by the enzyme glutamate dehydrogenase (EC 1.4.1.4), which reductively aminates 2-oxoglutarate to glutamate. In other bacteria (particularly in some species of *Bacillus*) analogous amino acid dehydrogenases (e.g. alanine dehydrogenase and leucine dehydrogenase) functionally replace glutamate dehydrogenase and glutamate is then formed by an aminotransferase reaction.

Available kinetic data indicate that amino acid dehydrogenases generally have high K_m values for ammonia (above 5mM; see Sanwal & Zink, 1961; Wiame, Pierard & Ramos, 1962), and one might therefore expect that when bacteria are growing in a simple salts medium the intracellular free ammonia concentration always would be relatively large. When growth takes place in the presence of a large excess of ammonia, the intracellular free ammonia content is difficult to assess without washing the organisms (which is undesirable, since it may cause changes in 'pool' composition; see Britten, 1965). But, when organisms are cultured in a chemostat under conditions where growth is limited by the availability of ammonia, the intracellular free ammonia can be easily determined by extracting the organisms with 0.25% $HClO_4$ and applying the extracts to the columns of a Technicon automatic amino acid analyser; washing these organisms is unnecessary since the extracellular free ammonia concentration is negligible (Herbert, 1958). With ammonia-limited chemostat cultures of *Aerobacter aerogenes*, growing at a dilution rate of $0.3\,h^{-1}$ in the medium of Tempest (1965) at pH 6.5 and 35°C, the intracellular free ammonia concentration was found to be less than 0.005% of the bacterial dry weight (below 0.5mM), well below the K_m value for ammonia (3–4mM) of *A. aerogenes* glutamate dehydrogenase. This observation prompted a detailed quantitative study of the relationship between growth condition and bacterial glutamate dehydrogenase activity of

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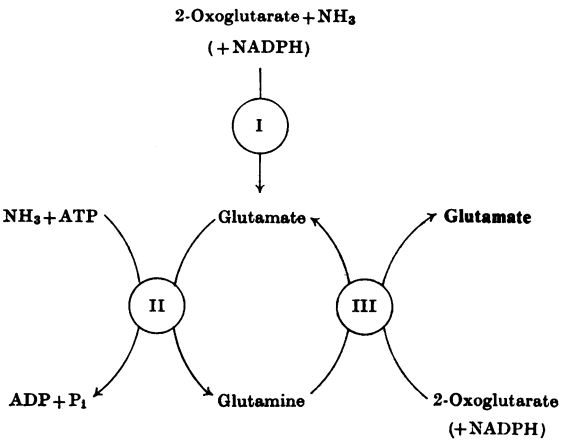

Scheme 1. Synthesis of glutamate from ammonia in *A. aerogenes*. Overall reactions:

(I) Glutamate dehydrogenase:

$$NH_3 + \text{2-oxoglutarate} + NADPH \rightarrow \text{glutamate} + H_2O + NADP^+$$

(II) Glutamine synthetase:

$$NH_3 + \text{glutamate} + ATP \rightarrow \text{glutamine} + ADP + P_i + H_2O$$

(III) Glutamine amide–2-oxoglutarate aminotransferase (oxidoreductase, NADP):

$$\text{Glutamine} + \text{2-oxoglutarate} + NADPH \rightarrow 2\ \text{glutamate} + NADP^+$$

Sum of reactions (II) and (III):

$$NH_3 + \text{2-oxoglutarate} + ATP + NADPH \rightarrow \text{glutamate} + ADP + P_i + NADP^+ + H_2O$$

A. aerogenes, and this in turn led to the discovery of a novel pathway of synthesis of glutamate possessing an overall low K_m value for ammonia. Details of this pathway, which involves a hitherto undescribed enzyme or enzyme system [glutamine amide–2-oxoglutarate aminotransferase (oxidoreductase, NADP)], are given in Scheme 1; the experimental evidence supporting this finding is described below.

Substantial amounts of glutamate dehydrogenase (at least 80nmol of NADPH oxidized/min per mg of protein) could be detected in extracts (prepared by the method of Hughes, 1951) of *A. aerogenes* organisms that had been grown in chemostat cultures (see Herbert, Phipps & Tempest, 1965) with growth limited, successively, by the availability of glucose, phosphate and Mg^{2+} (dilution rate of $0.3\,h^{-1}$ at 35°C at pH 6.5). But when these organisms were limited in their growth by the availability of ammonia the glutamate dehydrogenase activity present in the extracts was much decreased (less than 10nmol of NADPH oxidized/min per mg of protein). This indicated that glutamate synthesis in these ammonia-limited organisms probably proceeded by some other route. Since examination of these organisms showed that aspartate ammonia-lyase, alanine dehydrogenase and leucine dehydrogenase activities (known routes of ammonia assimilation in bacteria) were absent, it was concluded that ammonia incorporation into amino acids occurred via some hitherto undescribed pathway in these ammonia-limited *A. aerogenes* organisms.

Because, when growing in an ammonia-limited chemostat culture, the rate of amino acid synthesis is limited by the rate of supply of ammonia, it was possible that a clue to the pathway of incorporation of ammonia into amino acids could be obtained by adding a pulse of ammonia to the culture (thereby disturbing the steady state) and studying the transient changes in the intracellular concentrations of free amino acids. When ammonia (final concn. 10mM) was added rapidly to a steady-state ammonia-limited culture of *A. aerogenes* and samples of organisms were taken within 1min of adding the pulse, a 25-fold increase in the free glutamine concentration was observed in the 'pools' extracted with 0.25M-$HClO_4$ and analysed with a Technicon automatic amino acid analyser. This indicated that ammonia incorporation into amino acids here might proceed via the amidation of glutamate, and examination of ammonia-limited organisms showed that their glutamine synthetase (EC 6.3.1.2) activity was greatly increased (80-fold greater than that found in correspondingly grown glucose-limited organisms; see also Wu & Yuan, 1968; Rebello & Strauss, 1969). The measured K_m for ammonia incorporation into glutamine was found to be less than 1mM, and it therefore seemed feasible that glutamine may lie on the pathway of synthesis of amino acids from ammonia, particularly when the intracellular concentration of ammonia is low. But, if this is so, organisms would need to possess an enzyme or enzyme system capable of transferring the amide nitrogen of glutamine to the α-amino position of some amino acid; and if this reaction involved a 2-oxo acid as the amino acceptor molecule (as does glutamate dehydrogenase) then the pathway would include a coupled oxidoreduction step.

The simplest route whereby the glutamine amide nitrogen could be incorporated into an amino acid would be by a reaction analogous to that effected by glutamate dehydrogenase, namely:

$$\text{Glutamine} + \text{2-oxo acid} + \begin{matrix}\text{NADH}\\ \text{or}\\ \text{NADPH}\end{matrix} \rightarrow \text{glutamate} + \text{amino acid} + \begin{matrix}\text{NAD}^+\\ \text{or}\\ \text{NADP}^+\end{matrix}$$

Incubation of cell-free extracts of ammonia-limited *A. aerogenes* (again prepared by the procedure of Hughes, 1951) with glutamine, NADH, NADPH and either pyruvate or oxaloacetate produced no net synthesis of amino acid, although some glutamate (plus an equivalent amount of ammonia) was formed owing to the presence of glutaminase (EC 3.5.1.2) in the extracts. However, incubation of bacterial extracts with glutamine, NADH, NADPH and 2-oxoglutarate led to a considerable net synthesis of glutamate without concomitant formation of ammonia. Subsequent spectrophotometric assays (following the change in E_{340}) showed that the cofactor required was NADPH.

Thus net synthesis of glutamate in ammonia-limited *A. aerogenes* organisms is effected by a two-stage process that involves first the synthesis of glutamine and then the reductive transfer of the amide group to the 2-position of 2-oxoglutarate, thereby forming two molecules of glutamate (Scheme 1). The relationship between this pathway of synthesis of glutamate and that involving glutamate dehydrogenase is also shown in Scheme 1; overall, the two reactions are similar except that the one functioning in low-ammonia environments involves the participation of ATP. Presumably this expenditure of energy, associated with the synthesis of glutamine, is the 'price that must be paid' by the bacteria in order to assimilate low intracellular concentrations of ammonia. In this context, it is significant that the glutamine pathway of glutamate synthesis is almost totally absent from glucose-limited *A. aerogenes* organisms where ammonia is present in considerable excess of requirement but the supply of energy is severely restricted.

Britten, R. J. (1965). *Symp. Soc. gen. Microbiol.* **15**, 57.
Herbert, D. (1958). In *Recent Progress in Microbiology: Proc. 7th int. Congr. Microbiol., Stockholm*, p. 381. Ed. by Tunevall, G. Stockholm: Almqvist and Wiksell.
Herbert, D., Phipps, P. J. & Tempest, D. W. (1965). *Lab. Pract.* **14**, 1150.
Hughes, D. E. (1951). *Br. J. exp. Path.* **32**, 97.
Rebello, J. L. & Strauss, N. (1969). *J. Bact.* **98**, 683.
Sanwal, B. D. & Zink, M. W. (1961). *Archs Biochem. Biophys.* **94**, 430.
Tempest D. W. (1965). *Biotechnol. & Bioengng*, **7**, 367.
Wiame, J. M., Pierard, A. & Ramos, F. (1962). In *Methods in Enzymology*. vol. 5, p. 673. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Wu, C. & Yuan, L. H. (1968). *J. gen. Microbiol.* **51**, 57.