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AMINO GROUP FORMATION AND GLUTAMATE SYNTHESIS IN *STREPTOCOCCUS BOVIS*

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ABSTRACT

BURCHALL, J. J. (University of Illinois, Urbana), R. A. NIEDERMAN, AND M. J. WOLIN. Amino group formation and glutamate synthesis in *Streptococcus bovis*. J. Bacteriol. **88**:1038-1044. 1964.—Extracts of *Streptococcus bovis* grown on NH_4^+ as a nitrogen source contain a nicotinamide adenine dinucleotide phosphate (NADP)-linked glutamic dehydrogenase and are devoid of alanine dehydrogenase, other amino acid dehydrohygenases, and aspartase. A potential source of reduced nicotinamide adenine dinucleotide phosphate for glutamate synthesis is a NADP and nicotinamide adenine dinucleotide (NAD)-linked glyceraldehyde-3-phosphate dehydrogenase present in the extracts. Experiments with C^{14}-labeled glucose and $NaHCO_3$ indicate that the glutamate carbon skeleton is synthesized by a tricarboxylic acid pathway. The synthesis of the carbon skeleton of glutamate is repressed when glutamate or casein hydrolysate supplement the NH_4^+-containing growth medium. Repression of glutamic dehydrogenase and a NAD-linked isocitric dehydrogenase occurs only when complex nitrogen sources, but not when free amino acids, are added to the growth medium.

Streptococcus bovis uses NH_4^+ as a sole nitrogen source for growth except for trace amounts of nitrogen in the vitamins required by the organism (Wolin, Manning, and Nelson, 1959). *S. bovis* grows best in an anaerobic environment (Wolin and Weinberg, 1960) and, therefore, has a high degree of synthetic ability under anaerobic conditions. We were interested in determining whether the site of incorporation of NH_4^+ into amino acids is similar in *S. bovis* to the sites which have been shown to be operative in more aerobic organisms. In addition, it was of interest to confirm and extend Wright's (1960) observation that the carbon skeleton of glutamate is synthesized by a pathway similar to that of organisms which use the tricarboxylic acid cycle for the synthesis of α-ketoglutarate. Lastly, it was observed by Bryant and Robinson (1961) that the utilization of NH_4^+ by *S. bovis* is prevented by growth on complex media in contrast to results obtained with other rumen bacteria which preferentially used NH_4^+ in the presence of complex nitrogen sources. These results suggested that utilization of NH_4^+ by *S. bovis* is under the control of the nitrogen source supplied. Preliminary investigations of possible control mechanisms were instituted during this present study.

MATERIALS AND METHODS

Organism and culture procedures. S. bovis 7H4 (ATCC 15351) was grown at 40 C in an atmosphere of 80% N_2 + 20% CO_2. The medium used for growth, unless indicated otherwise, was the synthetic medium previously described (Wolin and Weinberg, 1960) with NH_4Cl as the sole source of nitrogen except for nitrogen-containing vitamins. Other nitrogen sources were added to the medium as indicated in the text. For preparation of cell extracts, an inoculum (2%, v/v) of a 16-hr culture was used in 1 or 2 liters of medium. For isotope studies, a 16-hr culture was washed with sterile 0.03 M K_2HPO_4 (pH 7.0) in 0.05% sodium thioglycolate followed by resuspension in the same solution; 1 drop of the washed cells was used to inoculate 10 ml of medium.

Preparation of extracts. Cultures of 1 or 2 liters were harvested by centrifugation, washed twice with 100 ml of 0.1 M K_2HPO_4 (pH 7.4), and resuspended in $\frac{1}{100}$ of their original culture volume with the same buffer. Cells were broken in a French pressure cell (American Instrument Co., Silver Spring, Md.) which had previously been cooled to 4 C. Unbroken cells and debris were removed by centrifugation at 14,500 × *g* for 10 min, and the supernatant solution was the source of the extract used for study.

Assays. Amino acid dehydrogenases were assayed in a reaction mixture which contained

33.3 mM tris(hydroxymethyl)aminomethane (tris) buffer (pH 7.2); 0.33 mM nicotinamide adenine dinucleotide (NAD) or nicotinamide adenine dinucleotide phosphate (NADP); 1.65 mM β-mercaptoethanol; *S. bovis* extract; and 3.3 mM of the amino acid substrate, which was used to start the reaction, in a total volume of 3.0 ml. Pyridine nucleotide reduction was measured at 340 mμ. A similar spectrophotometric assay was used for measuring glyceraldehyde-3-phosphate dehydrogenase in a reaction mixture which contained 0.53 mM DL-glyceraldehyde-3-phosphate, 0.33 mM NAD or NADP, 3.3 mM cysteine, 6.7 mM tris (pH 7.2), 20 mM Na_2HASO_4 (pH 7.2), and extract in a total volume of 3.0 ml. Isocitric dehydrogenase was measured in an assay system identical with that used for the amino acid dehydrogenases except for the substitution of 3.3 mM DL-isocitrate for the amino acid, the addition of 3.3 mM $MgCl_2$, and the omission of β-mercaptoethanol. Aspartase activity was measured in an assay system identical with that used for the amino acid dehydrogenases, except for the omission of pyridine nucleotide and the measurement of ammonia as the product of enzyme activity.

Ammonia was determined by nesslerization as described by Johnson (1941) after separation from reaction mixtures by microdiffusion in a Conway (1940) cell. Protein was estimated by the method of Lowry et al. (1951) with crystalline bovine serum albumin as a standard.

Tracer studies. To separate, serum-cap stoppered, 20-ml bottles which contained 10 ml of synthetic medium were added 200 μc of acetate-*1-*C^{14}, 50 μc of glucose-*U-*C^{14}, and 200 μc of $NaHC^{14}O_3$. After incubation for 20 hr, cultures were acidified with 0.5 ml of 10 N H_2SO_4, and any $C^{14}O_2$ present was trapped in Primene according to the procedure of Oppermann et al. (1959), except that a nitrogen sweep of 60 ml/min was employed which passed through a -80 C trap placed between the culture bottle and a train of three traps, each containing 15 ml of 0.5 M Primene.

Cells were washed twice with 0.5 N H_2SO_4, and cell fractionation, protein hydrolysis, separation of amino acids by paper chromatography, and their location by radioautography were performed as described by Roberts et al. (1957). Extracellular amino acids were also isolated by the methods of Roberts et al. (1957). Aspartic and glutamic acid were eluted from chromatograms with 0.01 N KOH. The α- and β-carboxyl groups of aspartic acid and the α-carboxyl of glutamic acid were removed with ninhydrin as described by Greenberg and Rothstein (1957), except that no carrier was added, and the CO_2 was trapped in Primene.

Measurements of radioactivity were made in liquid scintillation spectrometers (models 314X and 3214, Packard Instrument Co., La Grange, Ill.) at tap or gain settings which gave maximal efficiencies. Areas of paper chromatograms containing amino acids located by radioautography were cut out and placed in 10 ml of scintillator (3 g of 2,5-diphenyloxazole in 1 liter of redistilled toluene), and radioactivity was determined (Davidson, 1962). Aqueous samples were counted in a similar manner after cutting out an area of Whatman no. 1 filter paper which had been spotted with 0.05 to 0.2 ml of sample. Radioactivity of the Primene-$C^{14}O_2$ complex was measured as described by Oppermann et al. (1959). In one experiment (Table 3), cells were plated in thin layers and radioactivity was determined with a gas-flow counter (Packard Instrument Co., La Grange, Ill.) equipped with a scaler (model 1090, Nuclear-Chicago Corp., Des Plaines, Ill.).

Chemicals. Acetate-*1-*C^{14}, glucose-*U-*C^{14}, and $NaHC^{14}O_3$ were obtained from New England Nuclear Corp., Boston, Mass. Primene was obtained from Rohm & Hass Co., Philadelphia, Pa. DL-Isocitrate was prepared from the allo-free DL-isocitrate lactone (Calbiochem). DL-Glyceraldehyde-3-phosphate was prepared from the barium salt of DL-glyceraldehyde-3-phosphate diethylacetal (Schwarz Bio Research, Orangeburg, N.Y.) by heating the diethylacetal for 3 min at 100 C in the presence of Dowex 50 (H^+). Casein hydrolysates were vitamin-free preparations from Nutritional Biochemicals Corp., Cleveland, Ohio.

RESULTS

Enzyme studies. Since amino acid dehydrogenases have been shown to be an important site of ammonia fixation into amino acids in other organisms, a survey was made to see if certain amino acid dehydrogenases could be detected in crude extracts of *S. bovis*. A 0.5-mg portion of extract protein was incubated with either NAD or NADP, the amino acid was tested, and pyridine nucleotide reduction was measured. L Glu-

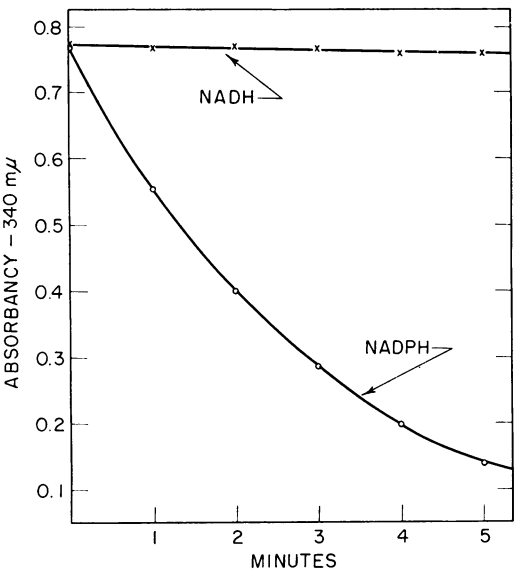

FIG. 1. *Pyridine nucleotide specificity of glutamic dehydrogenase. Oxidation of reduced pyridine nucleotides was measured by use of 3.0-ml reaction mixtures containing 33.3 mM tris (pH 7.2), 0.13 mM NADPH or NADH, 3.3 mM NH_4Cl, 0.5 mg of Streptococcus bovis extract protein, and 3.3 mM α-ketoglutarate which was used to start the reaction.*

tamate, L-alanine, L-glutamine, L-aspartate, L-leucine, L-serine, L-arginine, L-methionine, L-cysteine, and L-phenylalanine were tested in this manner, and pyridine nucleotide reduction was observed only when L-glutamate and NADP were used as substrates. Pyridine nucleotide reduction was only observed with glutamate and NADP as substrates when (pH 10) tris was substituted for the pH 7.2 tris used in the assay system. The pyridine nucleotide specificity of the glutamic dehydrogenase of *S. bovis* is shown in Fig. 1.

Attempts to demonstrate aspartase, another possible site of fixation of ammonia into amino acids, gave completely negative results. Crude extracts were incubated with L-aspartate, and measurements of ammonia showed that ammonia was not produced from L-aspartate.

Thus, this enzyme survey suggested that the primary site of fixation of ammonia into amino acids was an NADP-linked glutamic dehydrogenase. The enzyme survey was continued to see whether enzymes were present which could be presumed to be involved in the formation of reduced nicotinamide adenine dinucleotide phosphate (NADPH) for glutamate formation from α-ketoglutarate.

It was found that glyceraldehyde-3-phosphate could serve as a source of NADPH for glutamate synthesis because NAD or NADP were reduced when glyceraldehyde-3-phosphate was added to crude extracts (Fig. 2). It is not known whether the lack of pyridine nucleotide specificity of the glyceraldehyde-3-phosphate dehydrogenase is due to the presence of more than one enzyme. No reduction of NAD or NADP was obtained with glucose-6-phosphate as substrate. An isocitric dehydrogenase which was specific for NAD reduction was found in crude extracts (Fig. 3).

If *S. bovis* synthesizes glutamate by reactions involving the use of some of the tricarboxylic acid cycle enzymes, isocitric dehydrogenase would be expected to be present in the organism. In addition, it would be expected that condensing enzyme and aconitase would be present. Assays for aconitase and condensing enzyme, however, gave consistently negative results.

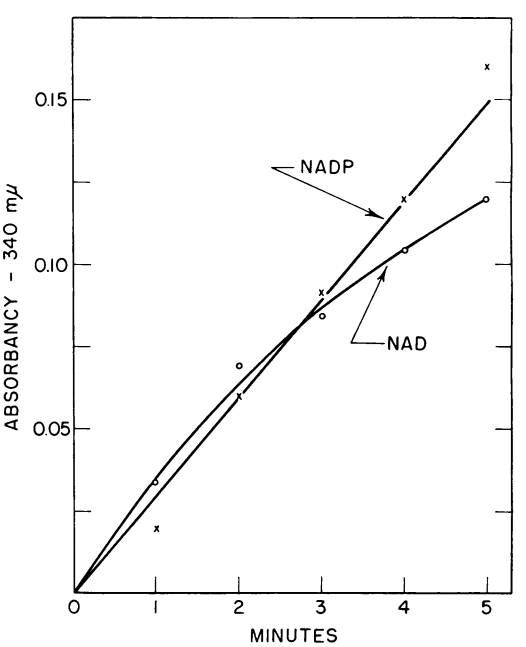

FIG. 2. *Glyceraldehyde - 3 - phosphate dehydrogenase activity with NAD and NADP. Reduction of pyridine nucleotides was measured by use of 3.0-ml reaction mixtures containing 0.33 mM NAD or NADP, 3.3 mM cysteine, 6.7 mM tris (pH 7.2), 20 mM Na_2HSO_4 (pH 7.2), 10 μg of extract protein, and 0.53 mM DL-glyceraldehyde-3-phosphate which was used to start the reaction.*

Tracer studies. Although enzyme surveys provided some preliminary evidence that glutamic dehydrogenase is the primary site of fixation of ammonia into amino acids, the lack of condensing enzyme and aconitase in extracts raised questions concerning the synthesis of α-ketoglutarate. The tracer studies of Wright (1960) indicated that glutamate synthesis by *S. bovis* proceeds via a tricarboxylic acid-cycle type of synthesis. Since Wright's studies were performed with cells grown on complex nitrogen sources, we decided to determine whether similar results would be obtained when NH_4^+ was used as the major nitrogen source for growth.

Glutamate was isolated from the protein of *S. bovis* grown in media containing C^{14} as uniformly labeled glucose and $NaHC^{14}O_3$ with NH_4Cl as the primary nitrogen source. The glutamate was decarboxylated with ninhydrin, and the radioactivity of the CO_2 and the decarboxylation product were compared with the activity of the nondecarboxylated glutamate. The results in Table 1 show that the sole source of the α-carboxyl of glutamate is exogenous CO_2 and that CO_2 is not incorporated to any significant extent in carbon atoms other than the α-carboxyl carbon. Carbons 2 to 5 are primarily formed from glucose. Ninhydrin degradation of isolated L-aspartate from cells grown on $NaHC^{14}O_3$ (Table 1) showed that the label was present only in the α- or β-carboxyl groups, or both, whereas only 38% of the total label in aspartate from cells grown on glucose-*U*-C^{14} was in the α- or β-carboxyl groups, or both. No acetate-*1*-C^{14} was incorporated into aspartic acid, but acetate-*1*-C^{14} was incorporated into glutamate. It should be pointed out that, in general, acetate-*1*-C^{14} was poorly incorporated into cell material by *S. bovis*. Presumably, the acetyl units derived from glucose are used in preference to free acetate for synthetic reactions.

TABLE 1. *Ninhydrin degradation of C^{14}-labeled glutamate and aspartate*

C^{14} source in culture medium	Amino acid	Radioactivity*		
		Before ninhydrin	After ninhydrin	
			CO_2	Residue
Glucose-*U*-C^{14}	Glutamic	2.5 $\times 10^3$	0.1 $\times 10^3$	2.2 $\times 10^3$
$NaHC^{14}O_3$	Glutamic	1.3 $\times 10^4$	1.2 $\times 10^4$	0.2 $\times 10^4$
Glucose-*U*-C^{14}	Aspartic	1.3 $\times 10^3$	0.5 $\times 10^3$	0.8 $\times 10^3$
$NaHC^{14}O_3$	Aspartic	4.8 $\times 10^3$	4.7 $\times 10^3$	0.2 $\times 10^3$

* Expressed as disintegrations per minute.

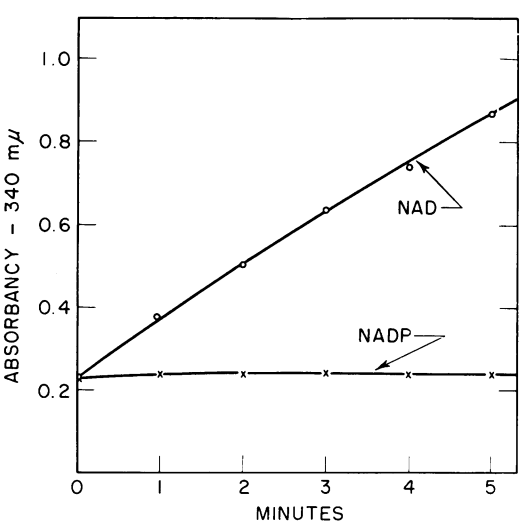

FIG. 3. *Pyridine nucleotide specificity of isocitric dehydrogenase. Reduction of pyridine nucleotides was measured by use of 3.0-ml reaction mixtures containing 33.3 mM tris (pH 7.2), 0.33 mM NAD or NADP, 3.3 mM $MgCl_2$, 0.3 mg of extract protein, and 3.3 mM* DL*-isocitrate which was used to start the reaction.*

We found that the amount of $C^{14}O_2$ incorporated into glutamate is decreased when *S. bovis* is grown with 10 mM nonradioactive L-glutamate, L-glutamine, or 1.0% (w/v) acid-hydrolyzed casein added to the NH_4^+-containing synthetic medium. Table 2 also shows results obtained with 10 mM L-aspartate or L-asparagine

Control of glutamate synthesis. Bryant and Robinson (1961) showed that no net uptake of NH_4^+ was detected if *S. bovis* was grown in a complex medium, suggesting that amino acid synthesis was inhibited by growth on complex media. Wright (1960) reported, however, that significant amounts of $C^{14}O_2$ were incorporated into aspartic and glutamic acids when *S. bovis* was grown in a complex medium. Although Wright's results showed that *S. bovis* is impermeable to L-aspartic acid, there were no results presented concerning the permeability of the cells to glutamic acid.

TABLE 2. *Radioactivity of aspartate and glutamate of cells grown on various nitrogen sources and $NaHC^{14}O_3$*

Nitrogen source in growth medium	Specific activity*		Specific activity of glutamate / specific activity of aspartate
	Glutamate	Aspartate	
None	13.8	11.4	1.21
L-Glutamate	2.5	5.5	0.45
L-Glutamine	3.2	11.0	0.29
Acid-hydrolyzed casein	3.1	5.2	0.60
L-Aspartate	14.3	10.0	1.43
L-Asparagine	14.0	4.7	2.98

* Expressed as disintegrations per minute per microgram of protein.

TABLE 3. *Enzyme activity and acetate-1-C^{14} incorporation of cells on different media**

Medium	Specific activity		
	Acetate incorporation†	Glutamic dehydrogenase‡	Isocitric dehydrogenase‡
NH_4^+-grown (11 hr)	120	32	20
Casein-grown (11 hr)	60	11	9
NH_4^+-grown (16 hr)	103	30	22
Casein-grown (16 hr)	61	12	7
Brewer Thioglycollate-grown		6.7	0

* Cells were grown on 500 ml of NH_4^+-containing synthetic medium without and with 0.6% acid-hydrolyzed casein; 35 μc of acetate-1-C^{14} were added where indicated. Washed cells were dried in thin layers and counted in a gas-flow counter.

† Expressed as counts per minute per milligram of protein.

‡ A unit of dehydrogenase is that amount of enzyme catalyzing a change in absorbancy of 0.001 per min at 340 mμ. Results are expressed as units per milligram of protein.

added to the medium. These results show that the cells are at least partially permeable to L-glutamate, L-glutamine, and L-asparagine, and impermeable to L-aspartate. The depression of incorporation of $C^{14}O_2$ into cellular glutamate could have been due to dilution of synthesized glutamic or an inhibition of glutamate synthesis by feedback inhibition or repression of enzyme synthesis. Analysis of crude extracts for enzyme activity showed that there was repression of synthesis of glutamic dehydrogenase and isocitric dehydrogenase only when a complex nitrogen source, such as casein hydrolysate, was used. No repression of these enzymes was observed if the complement of free amino acids found in casein, L-glutamate, or L-glutamine were added to the growth medium, even though these nitrogen sources were capable of depressing $C^{14}O_2$ incorporation into glutamate. Table 3 shows the activities of glutamic and isocitric dehydrogenases in extracts of cells grown on NH_4Cl, acid-hydrolyzed casein, and a complex medium. Included in Table 3 are data showing a decrease in incorporation of acetate-1-C^{14} into cells grown with acid-hydrolyzed casein as compared with the incorporation into cells grown with NH_4Cl as a nitrogen source. Table 4 shows the specific activity of glutamic dehydrogenase in extracts of cells grown with various levels of acid-hydrolyzed casein added to the synthetic medium. Enzyme-hydrolyzed casein also caused repression of glutamic and isocitric dehydrogenases.

An experiment was performed to determine the amount of ammonia-nitrogen which disap-

TABLE 4. *Repression of glutamic dehydrogenase by acid-hydrolyzed casein*

Percentage of acid-hydrolyzed casein	Specific activity of glutamic dehydrogenase*
0	44
0.02	55
0.04	27
0.20	18
0.40	21

* Expressed as units per milligram of protein. See Table 3 for definition of a unit.

TABLE 5. *Uptake of NH_3-nitrogen on various media*

Medium	NH_3-N			No. of cells	μg of NH_3-N per ml / mg of cells per ml
	Initial	Final	Δ		
	$\mu g/ml$	$\mu g/ml$		mg/ml	
Brewer Thioglycollate	207	219	+12	0.23	+52
Acid-hydrolyzed casein	261	261	0	0.69	00
Complete amino acid	222	180	−42	0.89	−47
NH_4^+ synthetic	210	105	−105	0.67	−157

peared from various media during cell growth. No ammonia was taken up from Brewer Thioglycollate Medium (Difco), small amounts of NH_3-nitrogen were used when free amino acids or acid-hydrolyzed casein were used as nitrogen sources, and large amounts were used when NH_4Cl was the sole nitrogen source (Table 5).

The decrease in incorporation of $C^{14}O_2$ into glutamate when glutamate, glutamine, or casein hydrolysate was added to the growth medium was not due to a simple dilution of the glutamate pool by the unlabeled additions. All of the glutamate synthesized from $C^{14}O_2$ with NH_4Cl as a sole nitrogen source was recovered from the cell protein and glutamate found in the medium after acidification and removal of CO_2. The total radioactivity incorporated into the protein glutamate and the glutamate in the medium was considerably lower when L-glutamate or casein hydrolysate was added to the medium. Table 6 shows the results of these experiments. Thus, the addition of L-glutamate or casein hydrolysate inhibited synthesis of L-glutamate from $C^{14}O_2$. L-Asparagine, however, did not cause an inhibition of aspartate synthesis, although L-aspartate radioactivity was diluted when L-asparagine was added to the synthetic medium (Table 2).

DISCUSSION

The results strongly suggest that a NADP-linked glutamic dehydrogenase is the sole site of incorporation of NH_3 into amino acid nitrogen in *S. bovis*. Halpern and Umbarger (1960) showed that the same situation is true for glucose-grown *Escherichia coli*, and that aspartase only becomes active in amination and deamination reactions in *E. coli* when other carbon sources (e.g., α-ketoglutarate, L-glutamate, or casein hydrolysate) are added to the growth medium. A NADP-linked glutamic dehydrogenase has been implicated in amino-nitrogen formation in *Neurospora crassa* (Fincham, 1951). NAD-linked alanine dehydrogenases appear to be important sites of amino-nitrogen formation from NH_3 in the genus *Bacillus* (Hong, Shen, and Braunstein, 1959).

TABLE 6. *Total radioactive glutamate produced from $NaHC^{14}O_3$ with different nitrogen sources in medium*

Nitrogen source in growth medium	Total DPM in glutamate (intra- and extracellar)*
None	6.0×10^6
L-Aspartate (10 mM)	6.3×10^6
Acid-hydrolyzed casein (1%, w/v)	7.0×10^5
L-Glutamate (10 mM)	7.1×10^5
L-Glutamine (10 mM)	4.1×10^6

* DPM = disintegrations per minute.

It appears likely that the NADPH for glutamate synthesis is supplied by a glyceraldehyde-3-phosphate dehydrogenase in *S. bovis*. The absence of a NAD- or NADP-linked glucose-6-phosphate dehydrogenase is somewhat surprising, because it was expected that pentose phosphate might be formed by such a reaction. It is possible that pentose phosphates are formed by a nonhexose monophosphate pathway such as the one described by Bernstein (1953).

The C^{14} experiments confirm and extend Wright's (1960) experiments and conclusion that tricarboxylic acid cycle reactions are involved in glutamate synthesis in *S. bovis*. It should be emphasized, however, that there is no cycle as such, but only similar reactions for the synthesis of α-ketoglutarate. Practically no carbon from uniformly labeled glucose is incorporated into the α-carboxyl of glutamate, acetate-*1*-C^{14} is not incorporated into aspartate, and there is no significant production of radioactive CO_2 from glucose-*U*-C^{14}. The labeling data are in accord with a reaction sequence involving citrate, isocitrate, oxalosuccinate, and α-ketoglutarate formation, but the enzymatic basis for citrate and isocitrate formation has not yet been established.

Until the enzymatic basis of α-ketoglutarate formation is established, the repression of isocitric dehydrogenase formation and inhibition of NH_4^+ utilization when complex nitrogen sources are used for growth is only suggestive of a possible mechanism for control of NH_4^+ utilization. A better argument can be made for a relationship between repression of glutamic dehydrogenase and control of NH_4^+ utilization because of the evidence for the importance of the enzyme in amino nitrogen formation. It is unusual that the repression of isocitric and glutamic dehydrogenases is not observed if *S. bovis* is grown with a complete mixture of amino acids, glutamic acid, or glutamine added to the growth medium, whereas acid- and enzyme-hydrolyzed casein cause repression. Extracts of NH_4^+-grown cells mixed with extracts of casein hydrolysate-grown cells show additive isocitric and glutamic dehydrogenase activities, indicating that inhibitors of

the enzymes are not present in casein hydrolysate-grown cells. Addition of *N*-acetyl-L-glutamate, arginyl-L-glutamate, glycyl-L-glutamate, or isoglutamine to NH_4^+-containing growth medium does not cause repression of isocitric and glutamic dehydrogenases.

Although addition of glutamate and addition of a complete mixture of free amino acids does not cause repressions of isocitric and glutamic dehydrogenases, these additions do cause an inhibition of glutamate synthesis (Tables 2 and 6). Perhaps a feedback control mechanism is operative under these growth conditions.

Whatever the growth condition, inhibition of the synthesis of the carbon skeleton of glutamate never is complete, and inhibition of ammonia utilization during growth is only complete when a highly complex medium such as Brewer Thioglycollate (Difco) is used. The incompleteness of inhibition of carbon skeleton synthesis may be a reflection of a permeability barrier to glutamate, which is suggested by the data in Table 6. The specific activity of the cell protein glutamate of cells grown with nonradioactive glutamate added to the medium is much higher than would be expected if the internal pool of the cell were in concentration equilibrium with the external glutamate. Thus, the potential for control may be limited by the amount of glutamate capable of entering the cells.

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