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Utilization of CO_2 and Acetate in Amino Acid Synthesis by *Streptococcus bovis*.* (30386)

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Carbon dioxide exerts a marked influence on the metabolic activities and nutritional requirements of *Streptococcus bovis*. A requirement for CO_2 exists when some strains are grown in media containing simple nitrogen sources(1-4); in other strains of *S. bovis*, CO_2 is involved in the production of polysaccharides(5-6), and of destransucrase(7). Acetate also affects the metabolism of *S. bovis*. Bailey and Oxford found that it was required for dextran synthesis(6), and we demonstrated its ability to stimulate growth and to be incorporated into cells growing in an ammonium medium(3).

This paper reports the utilization of CO_2 and acetate in amino acid synthesis by *S. bovis* growing on different nitrogen sources, and elaborates further some of the nutritional requirements for growth of this organism in the absence of CO_2.

Materials and methods. Microbiological. The organism used was *Streptococcus bovis* strain P 10. Procedures for maintenance of cultures, performance of growth tests, and preparation of inocula were identical to those previously described(8). The basal medium was that of Prescott and Stutts(1), supplemented as indicated below with nitrogen sources consisting of (i) *L*-arginine, (ii) ammonium acetate, or (iii), the complex amino acid mixture of Henderson and Snell(9). Some growth experiments requiring removal of CO_2 were performed with special flasks, (10), but usually, cultures were grown in 50 ml Erlenmeyer flasks, stoppered with porous plastic plugs, and incubated over 20% KOH in large vacuum desiccators. Shredded filter paper was immersed in the KOH solution to increase surface area, and the desiccator was evacuated to approximately 40 mm. In some experiments, cultures were incubated over the KOH *in vacuo*; in others, air was readmitted through a trap containing 20% KOH. The CO_2 was supplied to cultures either as a filter-sterilized $NaHCO_3$ solution, or as a gaseous atmosphere. The latter was accomplished by evacuating a desiccator to approximately 40 mm, then slowly admitting filtered CO_2 from a cylinder until a pressure about 10 mm below atmospheric was reached as indicated by an open limb manometer.

C^{14} tracer experiments. Preliminary experi-

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TABLE I. Influence of Nitrogen Source, Surface Area, and Depth of Medium on the Requirement of *Streptococcus bovis* P 10 for CO_2.

Nitrogen source	Container	Galvanometer reading* In air	In CO_2	Over KOH
L-arginine • HCl (1.66 mg/ml)	flasks	100	70	100
	tubes	70	64	55
Ammonium acetate (.83 mg/ml)	flasks	100	45	99
	tubes	95	36	96
Casein hydrolysate (enzymatic) (1.66 mg/ml)	flasks	29	22	30
	tubes	31	24	33
Complex amino acid mixture (1.66 mg/ml)	flasks	40	36	40
	tubes	36	42	34

All cultures had a volume of 6 ml. Surface area of the liquid in the flasks was approximately 9 cm^2, and depth of the liquid was 0.8 cm. In tubes, surface area was 2 cm^2 and depth of liquid was 3.5 cm. Incubation was at 37°C for 100 hr.

* Uninoculated blank = 100.

ments were performed using a variety of methods, but the data reported herein were obtained by the following procedure. Cultures were grown for 12 to 18 hours in basal medium supplemented with 2.8 mg of $NaHCO_3$ per ml and the desired nitrogen source. The actively growing cells were harvested, washed twice in 0.85% NaCl solution, then resuspended in fresh basal medium containing the same nitrogen source and the isotopically labeled nutrient, which was either $NaHC^{14}O_3$ or $CH_3C^{14}OONa$. After 20 to 60 minutes, the cells were collected, washed twice in 0.85% saline solution, packed by centrifugation, and stored at —10°C. Hydrolysis was accomplished by sealing the cells in tubes with 6 N HCl and autoclaving at either 112°C or 121°C for 16 to 18 hours. Excess HCl was removed by repeated vacuum distillation and the insoluble residue was removed by filtration through Whatman No. 50 filter paper. Two-dimensional paper chromatograms were prepared by the procedure of Redfield(11), using Whatman 3 MM filter paper. These were exposed to Eastman Kodak "No-Screen" X-ray film for time periods calculated from counting experiments carried out in a thin window gas flow counter (Nuclear Chicago Model 3037B), which was also used to determine total radioactivity in the cells and hydrolysates.

Results and discussion. Relationship of nitrogen source to essentiality of CO_2. The mere omission of CO_2 frequently fails to eliminate the growth of *S. bovis* in media containing simple nitrogen sources, although growth is less vigorous than when CO_2 is supplied(1,2). Niven *et al*(12) observed growth of several strains of *S. bovis* (including strain P 10) under these conditions. These workers neither added nor removed CO_2, but grew their cultures in tubes with considerable depth of liquid. We have suggested that the depth of liquid and small surface area in culture tubes permits retention of CO_2 present in the inoculum and medium (1)(cf. Lyman *et al*(10)). To test this possibility, the effects of nitrogen source, surface area, and depth of liquid in the culture vessel were determined, with the results shown in Table I.

These data show the effects of active removal of CO_2, as contrasted to its mere omission from the medium or atmosphere, and emphasize that CO_2 is essential, not merely stimulatory, for the growth of *S. bovis* strain P 10 on simple nitrogen sources. This organism apparently utilizes small amounts of CO_2 more effectively in the arginine medium than in the ammonium medium, and can grow in low concentrations of CO_2 when conditions for its retention are favorable. We earlier observed that removal of CO_2 from cultures growing rapidly in a simple medium did not halt growth(13), and Barnes *et al* demonstrated that some strains of *S. bovis* produce appreciable amounts of exogenous CO_2 when growing in a complex medium(14). From the present results and from these earlier observations, it is evident that cultural conditions markedly affect the ability of this organism to grow without added CO_2. In any experiment designed to test the essentiality of CO_2 or the ability of a compound to replace it, it is mandatory actively to remove CO_2 rather than simply to omit it from the medium. The data in Table I and the results of Barnes *et al*(14) indicate that if there is a metabolic need for CO_2 with a complex array of amino acid in the medium, it can be met by endogenous production.

Nutritional requirements for amino acids

TABLE II. Effects of Omission of Individual Amino Acids of Complex Mixture on Growth of *Streptococcus bovis* P 10.

Amino acid omitted	Galvanometer reading*	
	CO_2 removed†	CO_2 added‡
L-arginine	100	41
L-glutamic acid	100	26
L-leucine	100	48
DL-isoleucine	100	38
DL-valine	100	37
None	44	43

* Uninoculated blank = 100.

† Growth was not affected by omission of *L*-alanine, *DL*-aspartic acid, *L*-cystine, glycine, *L*-histidine, *DL*-methionine, *DL*-phenylalanine, *L*-proline, *DL*-serine, *DL*-threonine, *DL*-tryptophan or *L*-tyrosine.

‡ Moderate to heavy growth occurred in presence of CO_2 irrespective of any amino acid omissions; CO_2 was supplied as a gaseous atmosphere.

in absence of CO_2. Because a complex mixture of amino acids eliminates the requirements of *S. bovis* for exogenous CO_2 (Table I), a number of nutritional experiments were performed to determine the minimum amino acid requirements in the absence of CO_2. These requirements were tested first by single omissions from the complex amino acid mixture, with the results shown in Table II. With added CO_2, no individual amino acid was essential for growth. These results agree with those of Niven *et al*(12), since these workers did not remove CO_2 and it must be presumed that enough of this compound was present to support growth (*vide supra*). In our experiments, the removal of CO_2 caused complete growth failures when arginine, glutamic acid, leucine, isoleucine or valine were omitted individually from the complex mixture. A mixture of these 5 indispensable amino acids, however, was incapable of supporting growth of *S. bovis*. Snell(15) has pointed out that the failure of mixtures containing only "essential" amino acids to support growth is not unusual. The essentiality of arginine and glutamic acid in the absence of CO_2 is not surprising, as arginine is a good nitrogen source for this strain, and glutamic acid stimulates the growth of this and other strains of *S. bovis*, even the presence of CO_2(13). These results implicated CO_2 in the synthesis of arginine, a view reinforced by results of the tracer experiments reported below. The route of arginine synthesis was therefore investigated by nutritional experiments in which citrulline and ornithine were tested for their ability to replace arginine in the absence of CO_2. Citrulline was consistently capable of replacing arginine, but ornithine was not, thus implying that failure to synthesize arginine was due to an inability to convert ornithine to citrulline, possibly because of the absence of carbamyl phosphate. Addition of filter-sterilized dilithium carbamyl phosphate alone did not permit growth, and when it was added with ornithine, inconsistent results were observed, with growth in some experiments but no growth in others. This variable response to carbamyl phosphate plus ornithine might result from any of several factors, *e.g.*, impermeability of *S. bovis* cells to carbamyl phosphate, or the liability of carbamyl phosphate in solution. The data do not indicate the reason for the failure of carbamyl phosphate plus ornithine to provide consistently for arginine synthesis by *S. bovis;* however, growth of this organism lags considerably when CO_2 is removed, even with complex amino acid mixtures in the medium, and it seems likely that standing in the medium during the extended lag phase could cause destruction of the carbamyl phosphate (16).

The failure of *S. bovis* to grow in the absence of CO_2 on complex amino acid mixtures from which valine, leucine, and isoleucine were individually omitted was unexpected, as each of these amino acids retards growth of strain P 10 on simple nitrogen sources(13). Washburn and Niven observed complex antagonisms involving these 3 amino acids and glutamic acid, methionine, cystine, threonine, and norleucine, when another strain of *S. bovis* was grown on an arginine nitrogen source(17). The essentiality of valine, leucine, and isoleucine for growth in the complex amino acid mixture in our experiments may therefore indicate an imbalance or an antagonism produced by the absence of any one of these three. Indirect evidence for this explanation is found in the ability of *S. bovis* to grow in the absence of CO_2 on simpler mixtures of amino acids containing no valine, leucine, or isoleucine (*vide infra*).

Growth in simple mixtures of amino acids. Several amino acids stimulate the growth of *S. bovis* in the presence of CO_2, in either the arginine or the ammonium medium(3,13). Various combinations of these amino acids with arginine were tested for their ability to support growth with CO_2 removed. A mixture of *L*-arginine, *DL*-aspartic acid, *L*-glutamic acid, *L*-histidine, *L*-proline, and *L*-lysine gave moderately heavy growth, when added in the same ratios as those in the complex mixture, or when equal concentrations of each amino acid were supplied. When each of these componds was omitted individually, only arginine and glutamic acid were indispensable; removal of the others singly reduced growth but did not entirely eliminate it. Efforts to culture *S. bovis* in media containing only arginine and glutamic acid in the absence of CO_2 were unsuccessful, however, even when widely varying ratios of these two compounds were used.

Ability of miscellaneous compounds to replace CO_2. Various compounds other than amino acids were tested for the ability to support the growth of strain P 10 with simple nitrogen sources in flasks with CO_2 removed. Tween 80, oleic acid, oxaloacetic acid, uracil and acetate all were ineffective under these conditions. Barnes *et al*(14) found that light growth of 2 other strains was supported by several compounds, including Tween 80, uracil, aspartic acid, malic acid, or sodium acetate, when CO_2 was omitted. This apparent disagreement may be explained by a difference in (i) strains, or (ii) cultural conditions. We infer that these workers grew their cultures in tubes with 10 ml volumes, which would be expected to permit retention of some adventitious CO_2 and support light growth (Table I).

The ability of *S. bovis* to utilize ammonium salts as its nitrogen source in the presence of CO_2, and the ability of rapidly growing cultures to continue growth when CO_2 was suddenly removed(13), raised the possibility that CO_2 was required only for initiation of growth, and that the requirement might be overcome by adding small quantities of a complete mixture of amino acids to the medium, in addition to the simple primary nitrogen source. Levels of acid hydrolyzed casein up to 50 μg per ml were ineffective in initiating growth in the ammonium acetate medium with CO_2 removed, however, and the light growth which occurred was due entirely to the added casein hydrolysate.

TABLE III. Radioactivity of Hydrolysates of *Streptococcus bovis* P 10 Cells Grown on Different Nitrogen Sources in the Presence of $NaHC^{14}O_3$.

Nitrogen source	Specific activity* Exp 1†	Exp 2‡§
Ammonium acetate	40,955	14,136
L-arginine	27,645	19,548
Complex amino acid mixture	11,185	7,633

* Disintegrations per minute per leucine equivalent, corrected for counting efficiency.
† 60 min growth in radioactive media.
‡ 20 min growth in radioactive media.
§ The 2 experiments were performed on completely different batches of medium, with a lapse of several months between experiments.

Incorporation of $NaHC^{14}O_3$ by growing cells. Incubation of rapidly dividing cells of *S. bovis* with media containing $NaHC^{14}O_3$ resulted in an appreciable uptake of radioactivity. The nitrogen source in the medium exerted a pronounced effect on CO_2 incorporation (Table III). Utilization of CO_2 was considerably greater in the ammonium and arginine media, which required the organism to synthesize all, or all save one, of its cellular amino acids. There was a definite incorporation of CO_2 into cells grown in the medium containing complex mixture of amino acids, however, indicating that CO_2 is utilized by strain P 10 even under the most favorable nutritional conditions. Hydrolysates of cells grown in the ammonium and arginine media showed labeling principally in the spots corresponding to glutamic acid and aspartic acid, although several lighter areas of radioactivity were evident, some of which corresponded closely to the R_f values of various purines and pyrimidines, as one would expect. In radioautograms of cell hydrolysates from the ammonium medium, one spot coincided with the R_f values for arginine, providing further evidence for participation of CO_2 in the synthesis of this compound.

Cells growing on arginine or ammonia must necessarily synthesize both aspartate and glutamate, therefore the radioactivity found in

TABLE IV. Relative labeling of Aspartic Acid and Glutamic Acid in Hydrolysates of *Streptococcus bovis* P 10 Cells Grown in Presence of $NaHC^{14}O_3$ with Different Nitrogen Sources.

Nitrogen source	Relative radioactivity*			
	Exp 1†		Exp 2‡	
	Aspartate	Glutamate	Aspartate	Glutamate
L-arginine	+++	++++	++++	++++
Ammonium acetate	++	+++	++++	+++
Complex amino acid mixture	+++	+	++	+

* Estimated by visual comparison of spots on radioautograms.
† 60 min growth period in radioactive medium.
‡ 20 min growth period in radioactive medium.

these compounds would be explicable on the basis of $C^{14}O_2$ fixation into oxaloacetate, from which these amino acids might arise by amination and reactions of the citric acid cycle as suggested by Wright(7). Appreciable densities were observed in the radioautographic spots for both aspartic acid and glutamic acid in hydrolysates of cells grown on both the arginine and ammonium media. However, the cells grown in the complex mixture of amino acids showed considerably greater radioactivity in aspartate than in glutamate (Table IV). Wright found that $C^{14}O_2$ incorporation by *S. bovis* strain 1, growing on a complex nitrogen source, was principally into aspartic acid (7). Strain 1 differs from strain P 10 in that it does not grow in chemically defined media containing a single, simple nitrogen source; the experiments of Wright necessarily involved the use of a casein hydrolysate which was analogous to our complex amino acid mixture. Wright further demonstrated that exogenous aspartic acid is incorporated poorly by strain 1, and that its incorporation is antagonized by the presence of glutamic acid (7). Our experiments suggest that strain P 10 also has a limited ability to incorporate exogenous aspartate, as poor assimilation of this amino acid would necessitate that synthesis at least partially replace its incorporation from the medium; hence, the greater radioactivity in aspartate than in glutamate, which apparently can be taken up from the medium.

Incorporation of acetate-C^{14} into amino acids. When grown for short periods in media containing carboxyl-labeled acetate, strain P 10 incorporated appreciable radioactivity from the arginine medium, but considerably less from the complex amino acid medium (Table V).§ Cells from the arginine medium were labeled principally in glutamic acid, with smaller amounts of radioactivity in leucine. No detectable radioactivity was associated with aspartic acid, possibly because sufficient aspartate was provided by the synthetic pathway involving CO_2. The data do not furnish an explanation of the incorporation of acetate into glutamic acid, but this may proceed *via* reactions of the citric acid cycle to α-ketoglutarate, thence to glutamate by amination. In the medium containing the complex amino acid mixture, glutamate is presumably incorporated from the exogenous source and its synthesis would not be required. A mechanism such as that proposed by Strassman *et al*(18) for synthesis of leucine, and substantiated by the experiments of Umbarger, Gross, and coworkers(19,20), could account for the labeling in this amino

TABLE V. Effect of Nitrogen Source on Labeling of Aspartic Acid and Glutamic Acid in Hydrolysates of *Streptococcus bovis* P 10 Cells Grown in Presence of Sodium Acetate Carboxyl C^{14}.

Nitrogen source	Specific activity of hydrolysate*	Relative radioactivity†	
		Aspartic acid	Glutamic acid
L-arginine	5180	—	++
Complex amino acid mixture	1370	—	±

* Disintegrations per minute per leucine equivalent.
† Estimated by visual comparison of the spots on radioautograms.

§ Utilization of acetate in the ammonium medium was not studied in these experiments, but an earlier experiment performed under slightly different conditions showed labeling exclusively into glutamic acid.

acid, but direct evidence is not available from our experiments.

The study of the effects of CO_2 on bacterial metabolism entails certain difficulties. In testing the essentiality of CO_2 for growth, it is obviously insufficient merely to omit it from the medium, and considerable care must be exercised to remove it. The possibility always exists that traces of CO_2 have not been removed; therefore, any experiments which show a positive growth response in the supposed absence of this ubiquitous compound must be re-checked several times in order to insure that the CO_2 has really been removed. In fact, it is risky to claim the existence of specific nutritional requirements in the complete absence of CO_2, since such a condition can never be proved unequivocally, and all that the investigator can do is to reduce the level of adventitious CO_2 to a minimum.

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Effect of Gamma Irradiation on Response of Fasted Rats to Erythropoietin.* (30387)

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We have reported(1) that the fractional reduction of iron uptake by erythropoietic tissue, produced by irradiation, was much less in rats exposed when their erythropoiesis was maximally stimulated than when it was normal(1). In this same report it was postulated that the high endogenous levels of erythropoietin (EP) observed(2) in the stimulated rats might explain in part the results obtained. Furthermore, preliminary results(1) showed that the response to erythropoietin was much greater when EP was given 5 hours before irradiation than when it was given five hours after. The object of

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