EFFECTS OF CARBON DIOXIDE ON THE GROWTH AND AMINO ACID METABOLISM OF *STREPTOCOCCUS BOVIS*

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The position of *Streptococcus bovis* among the *Lactobacteriaceae* is of interest because of the extremely simple nitrogen requirements of this organism in contrast with the other streptococci which have been investigated. Niven, Washburn, and White (1948) studied the nutritive requirements of fifteen strains of *S. bovis* and found that all but two grew in a medium containing arginine as the sole source of amino nitrogen. Further investigation of the metabolic patterns of one strain of *S. bovis* led Washburn and Niven (1948) to report several interesting amino acid antagonisms in the metabolism of this organism when it was grown in a medium in which arginine supplied the nitrogen requirement.

During the course of some experiments on amino acid metabolism in two strains of *S. bovis*, the present authors found that growth in a medium similar to that of Washburn and Niven was often poor and irregular, and a considerable difference between duplicate tubes was frequently observed. The use of younger, heavier inocula resulted in somewhat improved growth. The better results with the heavy inocula suggested the possibility that carbon dioxide was involved in the growth of *S. bovis* in such media since heavy inocula are likely to contain significant amounts of metabolic CO_2. In order to determine whether this were true, an investigation of the role of CO_2 in the growth of *S. bovis* was undertaken.

MATERIALS AND METHODS

Cultures and inocula. The cultures used in this study were *S. bovis* strain ATCC 9809 and a culture from the Cornell University collection, designated *S. bovis* strain P-10. The cultures were maintained in litmus milk containing 0.5 per cent glucose and 0.5 per cent yeast extract. Transfers were made at 6 to 10 day intervals, and the cultures were incubated at 37 C until they became acidic to litmus, which was usually about 18 hours.

The inoculum medium was composed of filtered tomato juice, 20 per cent; Difco peptonized milk, 1 per cent; and Difco tryptone, 1 per cent. Approximately 24 hours before an inoculum was required, a loop transfer was made from the stock culture to a 5 ml portion of inoculum medium and incubated at 37 C. After vigorous growth had occurred (8 to 12 hours), a loop transfer was made from this culture to fresh inoculum medium, which was incubated at 37 C for 8 to 16 hours. The cells from this culture were centrifuged, washed once in sterile distilled water, resuspended in sterile distilled water, and diluted to give a galvanometer reading of 65 to 75 on the Bausch and Lomb photoelectric colorimeter. One drop of this suspension was used to inoculate each assay tube.

Procedure for growth tests. Unless otherwise indicated, growth tests were carried out in culture tubes using the same procedure as previously described (Peters, Prescott, and Snell, 1953). In some experiments, however, 50 ml Erlenmeyer flasks were used as the culture vessels. For these tests, the procedure of Lyman *et al.* (1947) was employed. All experiments were carried out with a total culture volume of 6 ml. Growth was determined turbidimetrically with the Bausch and Lomb photoelectric colorimeter using the 660 mμ filter.

Basal medium. The composition of the basal medium, which is similar to that of Washburn and Niven (1948), is shown in table 1. L-Arginine·HCl or other nitrogen sources were added in amounts dictated by the nature of each experiment.

RESULTS

Under the conditions employed, both strains of *S. bovis* used in the present study were capable of growth in the basal medium supplemented with arginine. Unlike the strain studied by Washburn and Niven (1948), a fairly large amount of arginine was required for maximum growth.

TABLE 1

Composition of basal medium

Component	Amount per 100 ml Double Strength Medium
	g
Glucose	2
K_2HPO_4	1.2
	mg
Sodium thioglycolate	100
Adenine sulfate	2
Guanine hydrochloride	2
Uracil	2
NaCl	400
$MgSO_4 \cdot 7H_2O$	16
	μg
$FeSO_4 \cdot 7H_2O$	800
$MnSO_4 \cdot H_2O$	320
Calcium pantothenate	80
Nicotinic acid	80
Riboflavin	80
Thiamin	40
p-Aminobenzoic acid	40
Pyridoxal hydrochloride	40
Folic acid	2
Biotin	0.4

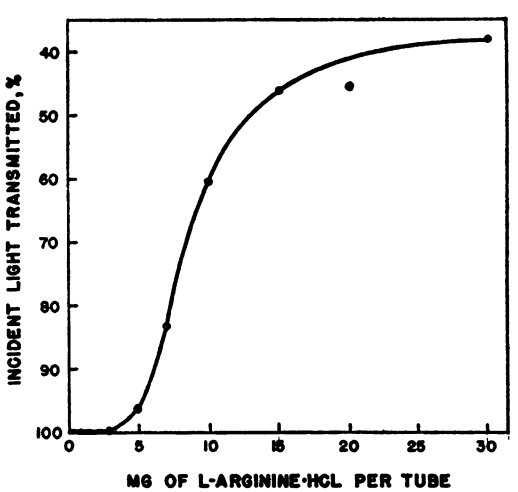

Figure 1. Response of *Streptococcus bovis* strain P-10 to the addition of L-arginine·HCl as the sole source of amino nitrogen. Incubation time, 24 hours.

Figure 1 shows a typical response of strain P-10 to the addition of L-arginine·HCl. Similar results were obtained with strain 9809.

In order to determine the possible effects of CO_2 on the growth of *S. bovis*, the organism was

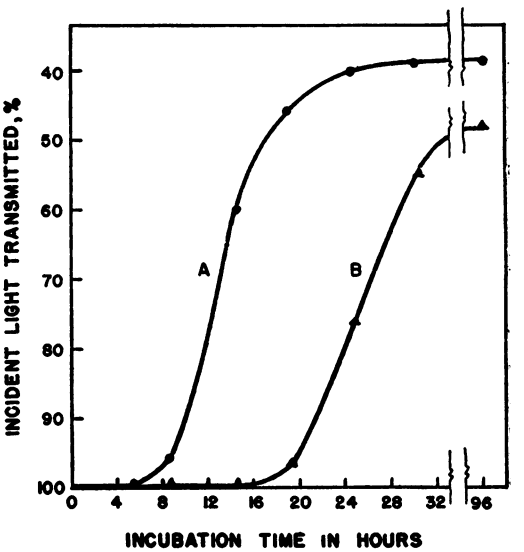

Figure 2. Growth curves for *Streptococcus bovis* strain 9809 in a medium containing 0.05 mM of L-arginine·HCl as the nitrogen source. Curve A, with 0.05 mM of $NaHCO_3$; Curve B, with no addition.

grown in the basal medium with graded levels of arginine, both in the presence and in the absence of 0.05 mM of added carbon dioxide. The CO_2 was supplied by aseptic addition of a sterile solution of $NaHCO_3$ to the autoclaved medium. Growth in the tubes containing added bicarbonate was considerably heavier and much more rapid than that in the tubes containing the same amount of arginine with no added source of CO_2. Identical behavior toward added CO_2 was exhibited by both strains of *S. bovis*. The rapid initiation of growth in the presence of added CO_2 may be seen in the time-growth curves of figure 2.

In repeated experiments with both organisms, growth in the basal medium with arginine as the nitrogen source was consistently heavier, more regular, and more rapid when supplemented with either $NaHCO_3$ or an atmosphere enriched with gaseous CO_2. The effects of adding different amounts of CO_2 on the growth and arginine requirements of *S. bovis* may be seen from the data in table 2. This experiment is illustrative of the occasions upon which the organism failed to grow with small amounts of arginine in the absence of added CO_2, even after prolonged incubation period.

Lyman *et al.* (1947) found that some lactic

TABLE 2

Effect of $NaHCO_3$ on the growth of Streptococcus bovis strain P-10

L-Arginine·HCl Added	Millimoles of $NaHCO_3$ Added			
	None	0.05	0.1	0.2
	Per cent of incident light transmitted*			
mg				
None	99	99	98	97
0.1	100	99	96	96
0.5	99	97	95	94
0.75	99	93	94	92
1.0	99	39	32	37
2.0	98	35	28	19
3.0	99	35	28	19
5.0	99	34	27	19
7.0	58	33	26	19
10.0	55	32	26	19

* Uninoculated medium = 100; incubation time = 58 hours.

acid bacteria were capable of growing in a medium lacking certain amino acids previously considered essential, provided the cultures were grown in tubes in which the liquid depth of the medium was appreciable. Growth did not occur in containers where a large, shallow surface area of the medium permitted metabolic CO_2 to escape. Substitution of a CO_2-enriched atmosphere above the medium again permitted growth. These results, and those showing a stimulation of the growth of *S. bovis* by added CO_2, suggested that this organism might have an absolute requirement for CO_2 when grown in a medium containing arginine as the sole source of amino nitrogen. This possibility was tested by growing *S. bovis* in tightly stoppered 50 ml Erlenmeyer flasks both in the presence of CO_2 and with CO_2 removed by the method of Lyman *et al.* (1947). That *S. bovis* requires CO_2 for growth in this simple medium was shown by its failure to grow in the absence of CO_2 when the basal medium was supplemented with as much as 30 mg of arginine per 6 ml culture. Heavy growth occurred, however, when flasks containing an identical amount of arginine were incubated under an atmosphere of 6 per cent CO_2. When casein hydrolyzate or a complete mixture of amino acids (Henderson and Snell, 1948) replaced arginine as the nitrogen source, good growth occurred even when CO_2 was removed.

Since *S. bovis* is capable of growth in the arginine medium in which there are very small amounts of CO_2, the possibility exists that the growing cells rapidly liberate CO_2 from the arginine present and utilize this CO_2 in the synthesis of other amino acids. This possibility was tested by suspending washed *S. bovis* cells (strain P-10) in phosphate buffer at pH 6.8 and incubating in the Warburg respirometer in the presence of 0.05 mM of L-arginine·HCl. The production of CO_2 was no greater than that obtained in the control flask which contained no arginine. These results, obtained with resting cells, however, cannot be interpreted to rule out the possibility that growing cells are capable of the enzymatic degradation of arginine to produce CO_2.

DISCUSSION

Previous investigators have established the nutritional requirement of various bacteria for CO_2 under conditions where the organisms were forced to synthesize certain amino acids (Lyman *et al.*, 1947). Others have demonstrated the actual incorporation of $C^{14}O_2$ into amino acids (Abelson *et al.*, 1952*a*,*b*; Huhtanen, Carleton, and Roberts, 1954). The latter group of workers found that one rumen organism incorporated CO_2 into amino acids even in the presence of adequate organic nutrients although they did not establish a definite requirement for CO_2. In the present experiments, the essential nature of CO_2 in the arginine medium and its nonessentiality in the casein hydrolyzate medium indicate that CO_2 is required for the synthesis of amino acids by *S. bovis* in the simple medium.

S. bovis is capable of initiating growth in the arginine medium when very small amounts of CO_2 are present although larger amounts of CO_2 expedite growth considerably. The fact that growth can occur in culture tubes in the absence of added CO_2 may be attributed to the presence of small amounts of CO_2 normally contained in the inoculum and dissolved in the medium.

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SUMMARY

Added CO_2 greatly stimulates the growth of two strains of *Streptococcus bovis* when this organism is grown in tubes with a medium containing arginine as the only source of amino nitrogen. When *S. bovis* was cultured in flasks from which CO_2 was removed, growth in the arginine medium no longer occurred although medium containing a complete mixture of amino acids or hydrolyzed casein supported growth under such conditions. The addition of an atmosphere enriched in CO_2 permitted growth in the flasks containing arginine as the nitrogen source.

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