MODE OF CO_2 FIXATION BY THE MINUTE STREPTOCOCCI[1]

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The "minute" streptococci (*Streptococcus anginosus*) will grow well in complex media; they require, however, an elevated CO_2 tension when grown in a more chemically defined casein hydrolyzate medium (Niven *et al.*, 1946). In studies with several strains of the minute streptococci, Deibel and Niven (1955) demonstrated that the requirement for increased CO_2 in the casein hydrolyzate medium could be replaced by Tween 80 and that Tween 80, a polyoxyethylene derivative of sorbitan monooleate, owed its biological activity to the oleate moiety of the molecule. The minimum level of oleate required to support maximum growth of the minute streptococci (3 μg per ml) was of such a magnitude as to suggest that oleate functioned catalytically rather than as an essential building block in cell synthesis, and further, tracer studies indicated that CO_2 was not being used for oleic acid or for fat synthesis (Deibel, 1952).

In the present work, short term fixation of CO_2 by minute streptococcal whole cells was studied, and under the conditions employed, it was found that CO_2 was primarily fixed into aspartic acid. From the position of label, and from nutritional studies with oxaloacetic and aspartic acids it appears likely that these microorganisms fix CO_2 primarily by a C_3–C_1 condensation followed by amination.

MATERIALS AND METHODS

The organisms used for this study were "minute" streptococci strains F162 and C628, belonging to Lancefield's serological groups F and G, respectively. The microorganisms were maintained in APT broth (Evans and Niven, 1951), transferred at least twice in this medium, washed and diluted 1:10 in distilled water before use as an inoculum. The casein hydrolyzate medium of Deibel and Niven (1955) was used for both nutritional studies and as a suspending medium for CO_2 fixation experiments. All experiments were conducted at 37C. Tween 80 was added as a source of oleic acid in experiments where indicated. Growth was estimated turbidimetrically with a Beckman model B spectrophotometer at 560 mμ.

For CO_2 fixation studies, the culture was grown for 18 to 24 hr in an air atmosphere containing approximately 75 mm CO_2. The cells were harvested, washed, and resuspended in fresh casein hydrolyzate medium. Cellular suspensions were adjusted to approximately 0.3 mg dry weight of cells per ml. Cell suspensions were exposed to a 10 per cent $C^{14}O_2$ atmosphere in a closed system. $C^{14}O_2$ was generated from $BaC^{14}O_3$. At the end of the exposure period the medium was acidified to approximately pH 1 and $C^{14}O_2$ removed by alkali absorption. The cells were recovered by centrifugation, washed with distilled water, and hydrolyzed with 5 N HCl for 6 hr at 121 C. The hydrolyzates were filtered and reduced to dryness several times to remove HCl.

Aspartic acid was separated from the cellular hydrolyzates chromatographically on columns of Dowex 50 (Moore and Stein, 1951). Aliquots of the fractions were assayed for radioactivity and checked for purity by paper chromatography. Radioactive carbon was assayed as $BaC^{14}O_3$ with a gas flow Geiger counter at a constant geometry. Samples were counted and corrections for absorption made in each case.

RESULTS

$C^{14}O_2$ fixation by minute streptococcal cells. Repeated attempts to induce resting cells to fix $C^{14}O_2$ were unsuccessful; however, when resuspended in a complete casein hydrolyzate growth medium the minute streptococci fixed a significant level of CO_2 within a relatively short period of time (1 hr). The fixed carbon was apparently

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incorporated into a metabolite firmly bound to the cellular protein for it was not extractable by hot or cold trichloroacetic acid, boiling water, or by fat solvents.

Experiments were conducted in which cells suspended in a complete growth medium were exposed to a 10 per cent $C^{14}O_2$ atmosphere. Samples of the cell suspension were withdrawn at intervals without disturbing the gaseous environment. Optical densities taken on each sample indicated that no significant increase in cell numbers had taken place during the experimental time. The cells of each sample were collected by centrifugation, washed twice with distilled water, and hydrolyzed with 6 N HCl.

The sample withdrawn at 30 min after the start of the experiment was the first to show any significant increase in radioactivity; the 1-, 1½-, and 2-hr samples each showed proportionately greater increases in activity. Paper chromatograms of samples representing ½, 1½, and 2 hr of exposure were developed on Whatman no. 1 paper with a phenol-water solvent system. Authentic samples of several amino acids were also developed on the same chromatogram. Autoradiograms prepared from this chromatogram indicated that the radioactivity was primarily confined to one substance having an R_f of 0.2, identical to that of the authentic aspartic acid control. Traces of other radioactive substances were present in the 1½- and 2-hr samples but these were minor compared to the area located at R_f 0.2. Two-dimensional paper chromatograms of cellular hydrolyzates were developed by phenol-water in one direction and butanol-acetic acid-water in the other direction. Autoradiograms were prepared and the main radioactive area corresponded in each chromatogram to a ninhydrin positive spot identical in R_f to that of the aspartic acid controls. The substance containing virtually all of the carbon from short term $C^{14}O_2$ fixation by both strains used was therefore identified as aspartic acid.

Washed cells of strain F163 previously grown under CO_2 were resuspended in fresh casein hydrolyzate medium and exposed to an atmosphere of 10 per cent $C^{14}O_2$ for 1 hr. The cells were harvested, washed, and hydrolyzed in the previously described manner. Radioactive aspartate was separated chromatographically on a Dowex 50 column, mixed with inactive L-aspartic acid, and crystallized as the copper salt.

Copper aspartate was degraded by (a) combustion (Van Slyke and Folch, 1940) (b) treatment with ninhydrin, whereby both carboxyl groups were liberated as CO_2 (Van Slyke *et al.*, 1943), and (c) treatment with chloramine-T whereby the α-carboxyl group is preferentially liberated as CO_2 at a ratio of 4:1 (Ehrensvard *et al.*, 1951). The data derived from the degradation of copper aspartate clearly show that the aspartic acid was labeled exclusively in the β-carboxyl carbon (table 1). Also included in table 1 are data from a separate experiment in which oleate was added to the suspending medium during 1 hr exposure to $C^{14}O_2$. The position of label is exactly the same as in the asparate derived from cells exposed without oleate.

Effect of oxaloacetic acid upon growth. The pattern of CO_2 incorporation found by the degradation of aspartate is most simply accounted for by the well-established carboxylation of pyruvic acid leading to the formation of oxaloacetic acid, followed by amination. Therefore, the effect of oxaloacetate upon growth in the absence of an increased CO_2 tension and oleate was tested. For this nutritional experiment, the medium was acidified to pH 5.8 to prevent an accumulation of bicarbonate due to oxaloacetate decarboxylation. Oxaloacetate was sterilized by filtration and added to the growth medium just prior to inoculation. Estimation of growth was made turbidimetrically after 20 hr incubation with the one exception noted.

The results as presented in table 2 show that oxaloacetate at a concentration of 0.2 mg per ml

TABLE 1

*C^{14} distribution in aspartic acid isolated from the minute streptococci**

	Cpm per μmole of carbon	
	Oleate present†	No oleate†
Cu-aspartate	19	31
Carboxyl carbons	36	60
α-Carboxyl carbon	0	1

* Resting cells (0.3 mg per ml) were suspended in a complete growth medium, containing oleate where indicated, and exposed to an atmosphere of 10 per cent $C^{14}O_2$ for 1 hr.

† These data represent two independent experiments and thus the specific activities of the two treatments are not comparable.

TABLE 2

Growth response of the minute streptococci to graded levels of oxaloacetic acid in the absence of oleate or increased CO_2 tension

Oxaloacetic Acid Conc	Optical Density × 100 after 24 hr		
	No oleate or CO_2	Oleate	CO_2
mg/ml			
0	0	86	85
0.05	0	—	—
0.1	65*		
0.2	80		
0.3	79		
0.4	72		
0.5	78		

* Reading at 48 hr.

TABLE 3

Growth response of the minute streptococci to graded levels of L-aspartate in the absence of oleate or increased CO_2 tension

L-Aspartate	Optical Density × 100		
	No oleate or CO_2*	Oleate†	10% CO_2†
mg/ml			
0	0	95	90
0.5	0	110	110
1.0	0	110	110
2.5	62	110	110
5.0	110	110	110
8.0	110	110	110

* Readings at 48 hr.
† Readings at 20 hr.

will support complete growth of the minute streptococci without added oleate or CO_2. The possibility that this growth response is due to the participation of CO_2 derived from a decarboxylation of oxaloacetate cannot be ruled out completely. Separate growth experiments were conducted, however, in which the initial molar concentration of bicarbonate required to support maximum growth was approximately two times greater than that shown for oxaloacetate (1.5 μmole oxaloacetate per ml to 3.5 μmole sodium bicarbonate per ml). The initial pH of the bicarbonate growth medium was approximately 7.0.

Effect of increased levels of L-aspartate upon growth. Although the amount of casein hydrolyzate in the medium should contain a sufficient level of aspartate for growth, results of CO_2 fixation studies led to further nutritional studies designed to test the effect of increased amounts of aspartic acid on growth of the minute streptococci. For these studies high purity D- and L-aspartic acids were used. The results obtained with added levels of L-aspartate are shown in table 3. The growth response obtained with D-aspartate was negative at all levels tested and these data are not included. It will be noted that complete growth in tubes containing increased aspartate alone was not reached for approximately 20 hr after that of the controls. The same growth lag was noted when cells from the media containing an additional 0.5 per cent aspartic acid were used as inocula to subculture media containing the same level of added aspartate. Oleate and CO_2 controls showed maximum growth in 20 to 24 hr.

DISCUSSION

The results presented are interpreted to mean that without oleate the minute streptococci require increased CO_2 tensions for synthesis of cellular aspartic acid. The incorporation of CO_2 into aspartic acid by minute streptococci is merely an extension to another organism of an already well-established reaction. Fixation of significant quantities of $C^{14}O_2$ into aspartate by a metabolically related organism, *Lactobacillus arabinosus*, was observed by Lardy (1949), and by Lynch and Calvin (1952) with *Lactobacillus casei*. More recently MacDonald (1958) demonstrated that CO_2 fixation into aspartic acid by *L. arabinosus* occurred via a C_3–C_1 condensation to oxaloacetic acid followed by amination. The data presented in this study as to the probable mechanism of aspartate synthesis in the minute streptococci are in good agreement with those reported by MacDonald.

Of interest in the present study is the implication of a possible oleic acid function in aspartic acid metabolism. For growth without oleate in a medium containing aspartic acid (approximately 0.35 mg per ml), these organisms require an elevated CO_2 tension; the CO_2 being utilized primarily for aspartate synthesis. When oleate is added, however, aspartate synthesis is apparently not required. Presumably, under these conditions, the aspartate present in the medium is sufficient to support growth. Relationships between as-

partic and oleic acids have been observed by other investigators. Potter and Elvehjem (1948) demonstrated that aspartic acid exerted a biotin sparing effect in the nutrition of *L. arabinosus* and that, in the absence of biotin, a combination of oleate and aspartate was required for an optimal growth response. More recently, Firestone (1959) observed that aspartate partially replaced the biotin requirement for several strains of *Candida albicans* and the inclusion of both oleate and aspartate under biotin-free conditions resulted in a significant growth stimulation. Oleic acid alone does not spare the biotin requirement for these organisms. It should be kept in mind, however, that all media used in the present study contained biotin.

Just how oleate is related to aspartic acid in the nutrition of the minute streptococci is not clear. An attractive explanation is that oleate affects cellular permeability either to aspartate or to another metabolite(s) which must otherwise be synthesized from aspartate. The slow growth response obtained when the minute streptococci were grown in a high aspartate medium without increased CO_2 or added oleate would seem to indicate a slow aspartate uptake in the absence of oleic acid, and that a relatively high extracellular aspartic acid concentration is required to make aspartate available to the cell. Whether oleate exerts its growth promoting effect on the minute streptococci by increasing cellular permeability to aspartate or by yet another mechanism, cannot be determined on the basis of the present experimental evidence.

SUMMARY

The minute streptococci require either oleic acid or an elevated CO_2 tension for growth in a casein hydrolyzate medium. Tracer studies indicate that the route of CO_2 fixation is via a C_3–C_1 condensation (Wood-Werkman reaction).

Washed CO_2-grown whole cells fixed detectable quantities of $C^{14}O_2$ only when resuspended in a complete growth medium. Essentially all radioactivity fixed by cells treated under these conditions was found in β-labeled aspartic acid. Either oxaloacetic acid or elevated levels of aspartic acid replaced the CO_2 or oleate requirement in the nutrition of the minute streptococci. The results suggest that oleate may exert its CO_2 sparing effect by facilitating the assimilation of aspartate from the growth medium by some unknown mechanism.

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