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Inhibition of Sporulation of *Clostridium thermosaccharolyticum*

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Studies were undertaken to determine the effect of chemical inhibitors on the sporulation of thermophilic anaerobes of which *Clostridium thermosaccharolyticum* is a representative organism. Since fluoroacetic acid (R. S. Hanson, Ph.D. Thesis, Univ. of Illinois, Urbana, 1962), diethyl malonate (6), and α-picolinic acid (pyridine-6-carboxylic acid; reference 3) have been found to inhibit the sporulation of various species of bacilli, it was decided to use these inhibitors plus malonic acid in the preliminary stages of this investigation.

C. thermosaccharolyticum, National Canners Association strain 3814, was grown in either 0.5% L-arabinose medium to obtain sporulating cultures or in 0.5% glucose medium to obtain vegetative cultures by the procedure of Pheil and Ordal (8). Various levels of each inhibitor were added to the media at time of inoculation (except fluoroacetic acid, which was added 2 hr after inoculation) of the cultures in order to determine the concentration of inhibitor required to obtain complete inhibition of sporulation without inhibiting vegetative growth. It was found that 5 mM levels of fluoroacetic acid (FAA) and α-picolinic acid and 40 mM levels of malonate and diethyl malonate were required for the complete inhibition of the sporulation of this organism in L-arabinose medium. However, at the levels of inhibitors required to obtain complete inhibition of sporulation, FAA was found to be the only inhibitor which did not adversely affect the growth rates or *p*H changes of vegetative cultures grown in glucose medium. For this reason, FAA (5 mM) was used in further experiments on the overall effects of the inhibitor on the metabolism of vegetative and sporulating cells.

FAA (7), diethyl malonate (11), and α-picolinic acid (P. Fortnagel and E. Freese, Bacteriol. Proc., p. 117, 1968) have been found to inhibit acetate utilization and the tricarboxylic acid (TCA) cycle in sporulating bacilli and other biological systems. FAA and α-picolinic acid have been found to inhibit the TCA cycle in the aconitase enzyme, and the site of action of the malonate inhibiton was the succinate dehydrogenase enzyme complex. Therefore, part of this study was directed to finding evidence for or against the operation of the TCA cycle during sporulation of *C. thermosaccharolyticum*.

The end products found in supernatant fluids of vegetative and sporulating cultures grown in the presence of ^{14}C-labeled substrates were determined by silica gel column chromatography (9). Various specifically labeled ^{14}C-glucose compounds (^{14}C in carbons 1, 2, 3-4, or 6) were used as the tracers in these experiments. The labeled compounds were added to glucose-grown cultures 1 hr after inoculation of the cultures. In the L-arabinose-grown cultures, the labeled compounds were added 12 hr after culture inoculation, to determine the end products formed during the period of altered metabolic activity of sporulation. The labeled end products recovered were butyric acid, ethyl alcohol, acetic acid, and lactic acid. The specifically labeled glucose compounds were used in these experiments to determine the effects of FAA on the utilization of specific carbons of glucose during glucose catabolism. However, no specific localized effects of FAA were observed, so that the values obtained from the experiments with specifically labeled ^{14}C-glucose compounds were used to calculate the theoretical distribution of uniformly labeled ^{14}C-glucose in the various end products to provide a basis of comparison of the effect of FAA on the metabolism of vegetative and sporulating cells (M. F. Campbell, Ph.D. Thesis, Univ. of Illinois, Urbana, 1968). The results of these calculations are shown in Table 1. It was found that the addition of FAA reduced the amount of added ^{14}C incorporated into end products by about 50% in both culture systems. The more specific effects of FAA were an increase in the amount of lactic acid and a decrease in the amount of butyric acid formed during the growth of FAA-inhibited sporulating cultures. The addition of FAA to vegetative cultures resulted in an approximately 50% decrease in the amount of butyric acid, lactic acid, and acetic acid formed.

Fractionation (10) of the cells obtained from the above cultures grown in the presence of the vari-

TABLE 1. *Results of calculation[a] of theoretical utilization of uniformly labeled ^{14}C-glucose to metabolic end products by glucose (vegetative cells)- and L-arabinose (sporulating cells)- grown cultures of C. thermosaccharolyticum in the presence and absence of 5 mM FFA[b]*

Substrate	Butyric acid	Ethyl alcohol	Acetic acid	Lactic acid	Recovered in end products
					%
Glucose-grown cells[c]					
Uniformly labeled ^{14}C-glucose	22.39	2.23	32.23	4.37	61.22
Uniformly labeled ^{14}C-glucose + 5 mM FAA	10.71	1.94	13.36	2.28	28.29
L-Arabinose-grown cells[d]					
Uniformly labeled ^{14}C-glucose	16.21	1.57	35.30	0.69	53.77
Uniformly labeled ^{14}C-glucose + 5 mM FAA	1.23	1.19	25.97	3.95	32.34

[a] Values were calculated from actual data from individual experiments utilizing glucose labeled with ^{14}C in carbon 1, 2, 3-4, or 6. The experiments were terminated when the evolution of $^{14}CO_2$ from glucose-*3,4-^{14}C* ceased, at which time it was assumed all of the glucose had been utilized by the culture. The 3-4 values were multiplied by 2 to give values for both carbons. Data from the results for carbon 2 were assumed to be equal to carbon 5. Thus, the equation used was: [(% from C-1) + 2(% from C-2) + 2(% from C-3,4) + (% from C-6)]/6 = theoretical distribution of ^{14}C from uniformly labeled ^{14}C-glucose in specific end product.

[b] FAA added to appropriate cultures at 2 hr after inoculation of culture. Results are given as percentage of total ^{14}C initially added to the culture.

[c] After 8 hr of growth in presence of uniformly labeled ^{14}C-glucose. Labeled compounds added 1 hr after inoculation of culture.

[d] After 3 hr of growth in presence of uniformly labeled ^{14}C-glucose. Labeled compounds added 12 hr after inoculation of culture.

ous ^{14}C-glucose compounds indicated that the addition of FAA resulted in a reduction of about 50% the amount of ^{14}C incorporated into cellular fractions. There was no overall pattern of localized effect on the assimilation of the various carbons of glucose into cellular material as evidenced by the incorporation of ^{14}C.

It has been reported (1, 2) that citrate accumulated in cultures of *Rhodospirillum rubrum* grown in the presence of FAA. This was owing to the inhibition of the TCA cycle at the aconitase enzyme, with the resultant accumulation of citrate. The inhibition was thought (5) to be due to the fluorocitrate which was produced from fluoroacetyl coenzyme A and oxalacetate. We observed no accumulation of citric acid, as determined by direct chemical analysis (4) of the supernatant fluids of L-arabinose-grown FAA-inhibited cultures of *C. thermosaccharolyticum* at 4, 12, 24, 30, 36, or 48 hr after culture inoculation. Column chromatography of sporulating cultures grown in the presence of acetate-*2-^{14}C* and FAA did not show any accumulation of ^{14}C-citric acid at 6, 12, 24, 30, 36, or 48 hr after culture inoculation. Therefore, if the accumulation of citric acid as a result of the inhibition of the TCA cycle by FAA was used as a criterion for the judgment of the presence or absence of the TCA cycle, the result would be that no TCA cycle was involved during the sporulation of *C. thermosaccharolyticum*. Thus, the evidence accumulated from this preliminary investigation indicated that FAA partially inhibited the glucose catabolism of vegetative cells and completely inhibited the formation of refractile spores of *C. thermosaccharolyticum* at a site other than the aconitase enzyme; however, the site of inhibitory action was not determined.

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