INFLUENCE OF CASEIN HYDROLYZATES AND AMINO ACIDS ON GLUCOSE FERMENTATION BY PROPIONIBACTERIUM FREUDENREICHII

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Although the pathways by which propionibacteria ferment glucose have not been completely established, it has been noted by several investigators that relatively large numbers of bacterial cells are required for reasonable reaction rates. During studies on glucose fermentation by intact cells of Propionibacterium freudenreichii we have found that the addition of the medium components described by Delwiche (1950) resulted in marked stimulation of the rate of glucose utilization. The present paper reports an investigation of this phenomenon with the finding that the stimulatory materials reside in the nitrogenous components of the medium.

MATERIALS AND METHODS

The organism employed was P. freudenreichii strain ATCC 6207. Cells for metabolic experiments were grown in a complex medium consisting of 1 per cent each of glucose and yeast extract (Difco) and 0.5 per cent each of casitone (Difco) and K$_2$HPO$_4$ adjusted to pH 6.7 to 6.9. After incubation at 30 C for 42 to 48 hr, the cells were harvested by centrifugation, washed twice with deionized water and resuspended to a turbidity equivalent to 11 mg of dried cells per ml. Carbon dioxide was measured manometrically by conventional Warburg techniques in which the following experimental conditions were employed: temperature, 37 C; atmosphere, nitrogen; pH 5.9; final volume, 3 ml. Unless otherwise indicated, additions to the main compartment included potassium phosphate buffer, 0.033 M; glucose, 50 µmoles and other desired test materials. The bacterial cells (5.5 mg dry weight) were tipped from the side arm after gassing and equilibration. For glucose determinations, the fermentation was stopped at the desired time interval by the addition of 0.3 ml of 10 n H$_2$SO$_4$ from the second side arm. The fermentation mixtures were transferred to graduated centrifuge tubes, the volume brought to 10 ml and after thorough mixing, the cells were removed by centrifugation. Residual glucose was measured quantitatively with anthrone reagent by the method of Morris (1948). Adequate fermentation and zero time controls were included in all experiments.

RESULTS AND DISCUSSION

Following the preliminary observation that the addition of the chemically defined growth medium to the Warburg vessel markedly enhanced glucose utilization by P. freudenreichii, it seemed desirable to test the individual components of the medium. Of these, all were inactive except the casamino acids (Difco). Representative data in figure 1 demonstrate the marked increase in glucose utilized or carbon dioxide produced after the addition of 10 µg of acid hydrolyzed casein to the vessel. Subsequent titrations revealed maximum effect at the 10 µg per cup level. It should be noted that the endogenous carbon dioxide evolution from flasks containing the hydrolyzed casein was slightly higher than that produced in its absence. In each case, this value was subtracted from the fermentation vessels. Nevertheless, stimulation of carbon dioxide production was always accompanied by a corresponding increase of glucose utilization.

The stimulatory effect of acid hydrolyzed casein always occurred within 20 min after the cells were tipped and did not seem to result from further growth or enzyme synthesis in the Warburg vessel. Thus, the addition of penicillin (1 µg) or tetracycline (100 µg) to the Warburg vessel or pretreatment of the cells with ultraviolet irradiation for 1 to 5 min had no effect on the degree of stimulation. Attempts to eliminate the stimulation by growth in high levels of casein

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hydrolyzed casein during the actual fermentation process.

Of interest was the finding that the addition of enzymatically hydrolyzed casein or a mixture of 19 purified amino acids resulted in only part of the activity of acid hydrolyzed casein (figure 2). Furthermore, treatment of the enzymatically hydrolyzed casein or amino acid mixture with constant boiling hydrochloric acid (21 per cent) resulted in activity equal to that of the commercial casamino acids. These results suggest, therefore, that two factors may be involved in enhanced glucose utilization: (1) one or more of the amino acids, and (2) a product of acid hydrolysis. It should also be mentioned that purified isoelectric casein was inactive when tested in the unhydrolyzed form and that stimulation equal to the commercial acid hydrolyzed casein resulted after acid hydrolysis for 24 hr. Hydrolysis for 6 and 12 hr periods produced lesser amounts of activity.

Of the 19 amino acids in the mixture, only aspartic acid (figure 3) was active. In the case of this amino acid, however, a concentration (0.5 mg per vessel) approximately equivalent to that in the mixture (0.6 mg aspartate per 10 mg mixture) had very little effect. Thus, an addition of at least 5 mg per cup was required for activity equal to that produced by the amino acid mixture. The reasons for this are not clear, but may be due to interaction of other amino acids with aspartate. Figure 3 also presents data for asparagine which was found to be more active than aspartic acid on a weight basis. It was also noticed that a considerable amount of carbon dioxide was evolved from the former compound, while a very low rate of gas was produced from higher levels of aspartate. This difference in utilization possibly resulted from permeability differences to the cell membrane and may also explain the greater activity of asparagine on glucose utilization. Whether active utilization of aspartate or asparagine is necessary for enhanced glucose utilization by P. freudenreichii is not known. However, Nesslerization of fermented mixtures indicated that ammonia was produced from both compounds during fermentation. Of interest in this connection is the finding of McIlwain (1946a, b) that resting cells of several β-hemolytic streptococci convert glutamine to glutamic acid and ammonia only during active glycolysis and that this conversion is accom-

**Figure 1.** Effect of acid hydrolyzed casein on glucose fermentation by Propionibacterium freudenreichii. 1 = CO2 produced; 2 = glucose utilized; O = no additions; ● = 10 mg acid hydrolyzed casein per vessel.

**Figure 2.** Effect of acid and enzymatically treated casein and amino acid mixtures on glucose fermentation by Propionibacterium freudenreichii. A = no additions; B = amino acid mixture (10 mg); C = enzymatically hydrolyzed casein (10 mg); D = B after acid treatment (10 mg); E = C after acid treatment (10 mg); F = acid hydrolyzed casein (10 mg).
panied by a simultaneous increase in the rate of glycolysis. In a more recent paper, Pierce and White (1955) noted that the conversion of arginine to ornithine stimulated glycolysis by cell free extracts of Streptococcus pyogenes. Since arginine could substitute for adenosine triphosphate (ATP) in glucose fermentation, they suggested that the dissipation of arginine supplied ATP for the phosphorylation of glucose.

The effect of metabolic inhibitors on glucose fermentation by P. freudenreichii in the presence and absence of aspartate was studied in the hope that the results might contribute to an understanding of the stimulatory effect of this compound. It can be seen (table 1) that each of the inhibitors at the concentrations tested resulted in significantly less inhibition in flasks containing aspartate than in those with the carbohydrate alone. For example, the addition of $2 \times 10^{-4} \text{M}$ iodoacetate resulted in almost complete inhibition of glucose dissimilation, while in the presence of 25 $\mu$moles aspartate only 81.8 per cent inhibition was observed. A similar or greater effect was observed with each level of fluoride and semicarbazide tested. Similar results were obtained using acid hydrolyzed casein in place of aspartate.

Although the use of inhibitors may provide only indirect evidence, especially in an organism whose glycolytic pathways are not completely elucidated (Wood et al., 1955), the results indicate that the presence of aspartic acid or casein hydrolyzate induces at least a portion of the carbohydrate to be dissimilated by pathways not sensitive to the action of iodoacetate, fluoride, and semicarbazide. It should also be emphasized that aspartate may possibly interfere in some manner with the action of these inhibitors. McIlwain et al. (1948), while studying the relationship between the metabolism of glucose and glutamine by streptococci, observed that the increased utilization of glucose in the presence of glutamine could be accounted for as lactic acid. The addition of fluoride and iodoacetate inhibited both the dissimulation of glucose and glutamine, suggesting that reactions of glutamine may be coupled with one or more reactions in the glycolytic scheme and that enhanced glucose utilization did not involve alternate pathways of metabolism. With P. freudenreichii, on the other hand, the results indicate that the effect of aspartate is independent of the action of these inhibitors.
SUMMARY

The enhanced utilization of glucose by washed cells of Propionibacterium freudenreichii in the presence of the chemically defined growth medium could be accounted for in the acid hydrolyzed casein of this medium. Enzymatically hydrolyzed casein and amino acid mixtures were only partially active. Of the amino acids, aspartate was partially effective at higher levels, whereas asparagine was much more active on a weight basis. The greater activity of asparagine might be explained on the basis of increased permeability to the cell. Experiments with glycolytic inhibitors revealed that the fermentation of glucose by P. freudenreichii was inhibited by fluoride, iodoacetate, and semicarbazide to a lesser extent in the presence of aspartic acid than in its absence. A possible mechanism for this phenomenon was discussed.

REFERENCES


