# Nature of Butyrate Oxidation by *Mycoplasma hominis*

P. J. VanDEMARK[1] AND P. F. SMITH

*Division of Bacteriology, New York State College of Agriculture, Cornell University, Ithaca, New York, and Department of Microbiology, School of Medicine, University of South Dakota, Vermillion, South Dakota*

## ABSTRACT

VanDemark, P. J. (Cornell University, Ithaca, N.Y.), AND P. F. SMITH. Nature of butyrate oxidation by *Mycoplasma hominis*. J. Bacteriol. **89**:373-377. 1965.—Cell-free extracts of butyrate-grown *Mycoplasma hominis* strain O7, though lacking thiokinase activity on butyric acid, were found to activate butyrate via an acetyl-butyric thiophorase. These extracts also contained an aceto-coenzyme A (CoA) kinase, a butyryl-CoA dehydrogenase, a crotonase, a reduced nicotinamide adenine dinucleotide-specific β-hydroxybutyryl-CoA dehydrogenase, and a thiolase. Thiolase activity was stimulated by the addition of magnesium ions. The presence of these enzyme activities in this *Mycoplasma* species supports the hypothesis that a fatty acid oxidation represents an energy source for the nonfermentative pleuropneumonia-like organisms.

Recently, workers investigating the problem of *Mycoplasma* in tissue culture have observed the disappearance of arginine from tissue cultures contaminated with these microorganisms (Schimke and Barile, 1963). On the basis of such observations, these investigators have postulated that the dissimilation of arginine, via the arginine dihydrolase pathway, may represent the main energy source for the nonfermentative *Mycoplasma*. The existence of the arginine dihydrolase pathway in the nonfermentative *Mycoplasma* was demonstrated previously by Smith (1960). However, in this latter study it was observed that ornithine accumulated, and its accumulation resulted in the inhibition or repression of further arginine breakdown. In view of these findings it would seem plausible that there exist one or more alternate energy-yielding mechanisms in the nonfermentative *Mycoplasma*.

Lynn (1960) demonstrated that the nonfermentative *Mycoplasma* oxidize short-chain fatty acids and alcohols. The oxidative dissimilation of such substrates when coupled with a heme-terminated respiratory chain, such as previously reported in the nonfermentative *Mycoplasma* (VanDemark and Smith, 1964a) and involving oxidative phosphorylation, would seem to represent a more efficient energy source for these

[1] This work was done while at the University of South Dakota, Vermillion.

strains than the substrate phosphorylation available from arginine breakdown.

If the oxidation of a fatty acid represents an important metabolic pathway and energy source for the nonfermentative *Mycoplasma*, one should be able to demonstrate the enzymes involved in fatty acid oxidation including (i) a system of fatty acid activation, (ii) an acyl dehydrogenase, (iii) an enoly hydrase or crotonase, (iv) a β-hydroxyacyl dehydrogenase, and (v) a thiolase, as well as a tricarboxylic acid cycle. Evidence for the latter cycle in the *M. hominis* strain O7 was previously reported (VanDemark and Smith, 1964b). The present report gives evidence for the presence of those enzymes involved in butyrate oxidation in butyrate-grown cells of this nonfermentative strain.

## MATERIALS AND METHODS

*Culture.* *M. hominis* strain O7 was grown and harvested from butyrate-containing (0.1%, w/v) PPLO broth (Difco), and resting-cell suspensions were prepared as previously described (VanDemark and Smith, 1964b).

*Enzymatic methods.* Cell-free extracts were prepared by sonic oscillation of resting-cell suspensions in a 10-kc Raytheon oscillator for 10 min. The cell debris was removed by centrifugation at 30,000 × *g* for 30 min, and the resulting supernatant fraction was used for enzymatic analysis. The protein level of cell extracts was determined by the trichloroacetic acid method of Stadtman,

Novelli, and Lipmann (1951) with bovine serum albumin as a standard.

Thiokinase activity was measured as the rate of hydroxamic acid formation by use of the method of Jones et al. (1953). Thiophorase activity was measured spectrophotometrically by the method of Stadtman (1953) as the decrease in optical density at 232 mμ resulting from the arsenolysis of acetyl coenzyme A (CoA) (formed when butyryl-CoA is incubated with acetate), the O7 enzyme preparation, and arsenate. Butyryl-CoA dehydrogenase was measured as the reduction of dichlorophenol-indophenol (DCPIP) at 600 mμ upon incubation of the enzyme with butyryl-CoA, DCPIP, and phenazine methosulfate, as described by Hauge (1956). Crotonase activity was based on the method of Stern (1955) as the decrease in absorption at 263 mμ upon incubation of the enzyme with crotonyl-CoA. β-Hydroxy-butyryl-CoA dehydrogenase was determined by following the oxidation of reduced nicotinamide adenine dinucleotide (NADH₂) with acetoacetyl-CoA and the sonic preparation. Thiolase activity was measured as the decrease in absorption at 305 mμ when acetoacetyl-CoA was incubated with the Mycoplasma enzyme.

Materials. Butyryl-CoA and crotonyl-CoA were prepared from their respective acid anhydrides according to the method of Simon and Shemin (1953). Acetoacetyl-CoA was prepared from diketene according to the method of Wieland and Rueff (1953). The diketene was purchased from the K & K Laboratories, Plainview, N.J., and was purified by distillation immediately prior to its use. NADH₂, reduced nicotinamide adenine dinucleotide phosphate (NADPH₂), CoA, and phenazine methosulfate were purchased from the Sigma Chemical Co., St. Louis, Mo. All other chemicals were commercial
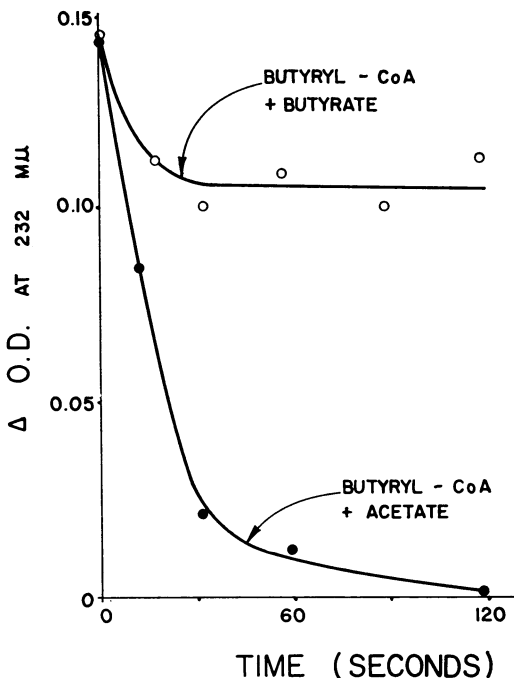


Fig. 1. Evidence for an acetyl-butyric thiophorase in Mycoplasma hominis O7. The cuvettes contained 100 μmoles of histidine buffer (pH 6.8), 0.5 mg of enzyme protein, 0.5 μmole of butyryl-CoA, and, where indicated, 20 μmoles of potassium acetate or butyrate.

TABLE 1. Thiokinase activity of butyrate-grown Mycoplasma hominis O7

| Fatty acid | Hydroxamic acid* (μmoles per hr per mg of enzyme protein) |
|---|---|
| Formate | <0.01 |
| Acetate | 1.97 |
| Propionate | 0.65 |
| Butyrate | 0.06 |
| β-Hydroxybutyrate | 0.03 |
| Isobutyrate | 0.01 |
| Acetoacetate | 0.12 |
| Valerate | 0.05 |
| Isovalerate | <0.01 |

* Hydroxamic acid formation was determined by the method of Jones et al. (1953) upon incubation of 20 μmoles of the sodium salt of fatty acid, 60 μmoles of tris(hydroxymethyl)aminomethane buffer (pH 7.4), 20 μmoles of ATP, 10 μmoles of MgCl₂, 20 μmoles of glutathione, 0.13 μmole of CoA, 600 μmoles of hydroxylamine with 2 mg of enzyme protein.
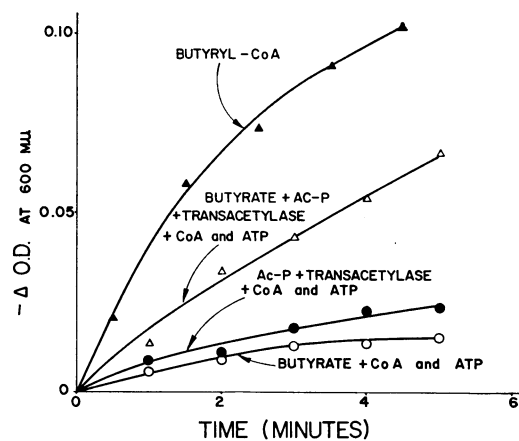


Fig. 2. Evidence for an acetyl-butyric thiophorase and butyryl-CoA dehydrogenase in the oxidation of butyrate by extracts of Mycoplasma hominis O7. The cuvettes contained 67 μmoles of Tris buffer (pH 7.3), 1 mg of enzyme protein, 2 mg of phenazine methosulfate, 0.05 mg of DCPIP, 0.1 mg of CoA, 10 μmoles of ATP, and, where indicated, 20 μmoles of potassium butyrate, 20 μmoles of dilithium acetyl phosphate, 2 μmoles of butyryl-CoA, and 5 units of transacetylase.

preparations of reagent grade. Transacetylase was prepared from a laboratory culture of *Escherichia coli* according to the method of Ochoa, Stern, and Schneider (1951).

## RESULTS AND DISCUSSION

*Nature of butyrate activation.* Previous studies of fatty acid activation by acetate-grown *M. hominis* O7 indicated that the kinase activity of these cells was relatively specific for acetate, with negligible activation of the other fatty acids tested (VanDemark and Smith, 1964b). Butyrate-grown cells showed a similar pattern of fatty acid activation, with thiokinase activity limited to acetate and propionate (Table 1). In the absence of a specific kinase for butyrate, it would seem plausible that butyrate activation is accomplished by means of an acetyl-butyric thiophorase analogous to that reported in *Clostridium kluyveri* by Stadtman (1953). Butyrate activation via thiophorase activity in the *Mycoplasma* enzyme preparation is illustrated in Fig.
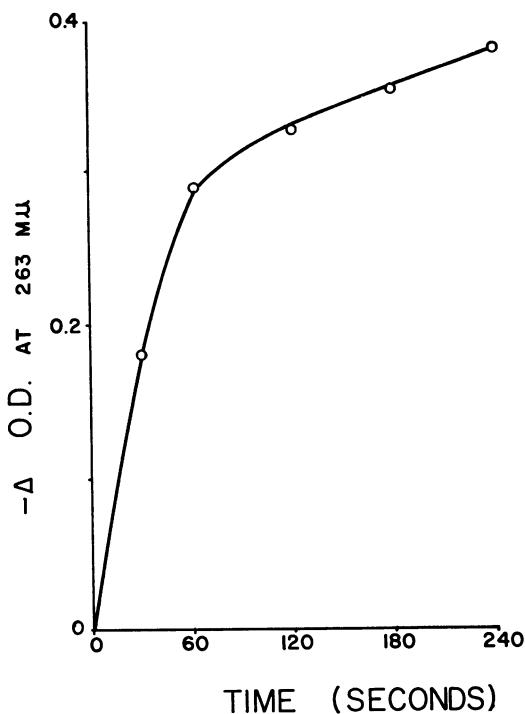
FIG. 4. *Evidence for a NADH₂-linked β-hydroxybutyric dehydrogenase and its stimulation by Mg⁺⁺ in Mycoplasma hominis O7. Cuvettes contained 67 μmoles of Tris buffer (pH 7.4), 0.5 mg of enzyme protein, 0.2 μmole of acetoacetyl-CoA, and where indicated 1 mg of NADH₂ or NADPH₂, and 5 μmoles of MgCl₂. The blank cuvette contained all components except acetoacetyl-CoA.*

FIG. 3. *Crotonase activity of cell-free extracts of Mycoplasma hominis O7. The assay is based on the method of Stern (1955), with the cuvettes containing 400 μmoles of Tris buffer (pH 7.4), 0.4 mg of egg albumin, 6 μmoles of potassium ethylenediaminetetraacetate (pH 7.4), 0.002 mg of enzyme protein, with and without 0.3 μmole of crotonyl-CoA. The blank cuvette contained 0.3 mg of adenylic acid in place of crotonyl-CoA.*
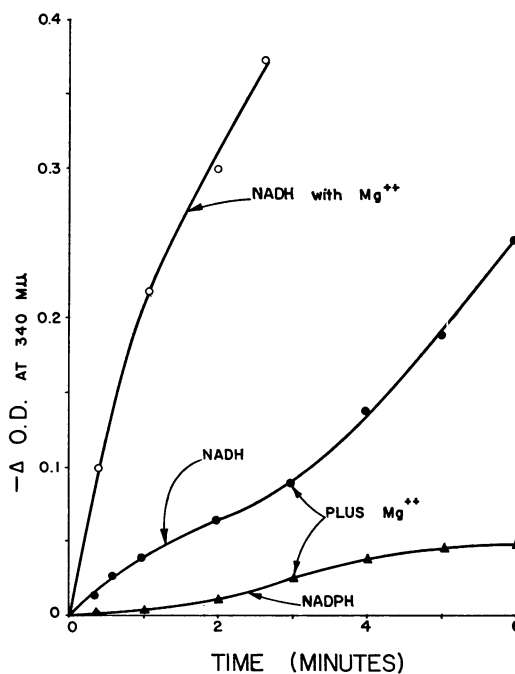
1. The decrease in optical density at 232 mμ results from the loss of thiol ester absorption due to the arsenolysis of the acetyl-CoA formed via the transfer of CoA from butyryl-CoA to acetate by the thiophorase of the *M. hominis* O7 enzyme preparation.

Because of the high acetyl-CoA deacylase activity in these enzyme extracts (VanDemark and Smith, 1964b), the reverse reaction, starting with acetyl-CoA and butyrate, could not be demonstrated. For this reason, no attempt has been made at this time to demonstrate the substrate specificity of this transphorase.

*Butyryl-CoA dehydrogenase and crotonase activity.* Butyryl-CoA dehydrogenase activity, measured as the rate of DCPIP reduction, is illustrated in the upper curve of Fig. 2. These data indicate that dye reduction occurs when butyryl-CoA is incubated with the enzyme preparation and DCPIP. Negligible dye reduction occurs when butyrate, CoA, and adenosine triphosphate (ATP) are incubated with the *M. hominis* O7 enzyme (Fig. 2). Butyrate serves as a hydrogen donor for dye reduction by this

organism only upon the addition of acetyl phosphate, transacetylase, CoA, and ATP, confirming the previous observations that prior to the oxidation of butyryl-CoA, butyrate is not activated via a kinase-type enzyme but by means of a CoA transphorase.

The crotonyl-CoA formed as a result of this oxidation is hydrated to $\beta$-hydroxybutyryl-CoA by the action of a crotonase present in this *Mycoplasma* enzyme preparation (Fig. 3). The incubation of the sonic enzyme preparation with crotonyl-CoA results in a rapid decrease in absorption at 263 m$\mu$ due to the hydration of the substrate to form $\beta$-hydroxybutyryl-CoA (Fig. 3).

*$\beta$-Hydroxybutyryl-CoA dehydrogenase and thiolase activities.* Evidence for a $\beta$-hydroxybutyryl-CoA dehydrogenase is shown in Fig. 4, in which the reduction of acetoacetyl-CoA is measured as rate of NADH$_2$ oxidation at 340 m$\mu$ when acetoacetyl-CoA and NADH$_2$ are incubated
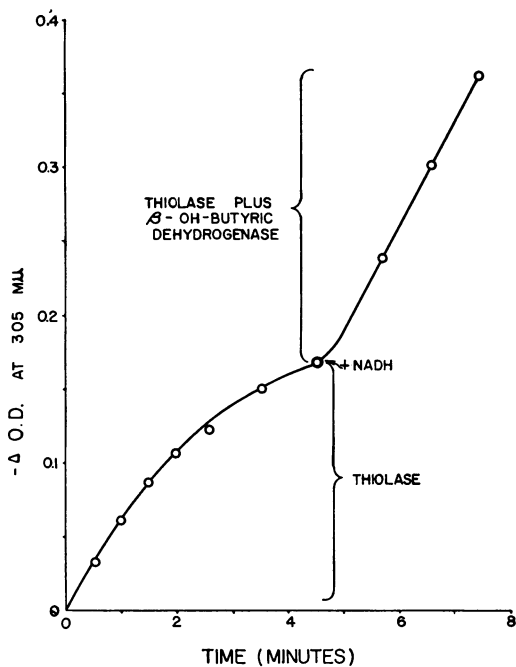


FIG. 6. *Postulated cycle of butyrate oxidation in Mycoplasma hominis O7.*

with the enzyme preparation. The reaction is specific for NADH$_2$ and is markedly stimulated by the addition of Mg ions.

Thiolase activity is illustrated in Fig. 5. The initial portion of the plot represents the decrease in the thiol ester absorption at 305 m$\mu$ due to the enzymatic cleavage of acetoacetyl-CoA to yield two molecules of acetyl-CoA. The addition of NADH$_2$ to the reaction mixture results in an increase in the rate of acetoacetyl-CoA disappearance due to the additive action of the $\beta$-hydroxybutyryl-CoA dehydrogenase and thiolase activities present in the crude sonic preparations.

The postulated pathway of butyrate oxidation based on this demonstration of the specific enzymes as described in this paper is illustrated in Fig. 6. In this cyclic mechanism, the resultant acetyl-CoA serves either as the CoA donor for the activation of butyrate by transphorase, or it enters the tricarboxylic and glyoxylic acid cycles which have previously been described in this strain (VanDemark and Smith, 1964*b*).



FIG. 5. *Thiolase and $\beta$-hydroxybutyric dehydrogenase activity of sonic extracts of Mycoplasma hominis O7. Cuvettes contained 67 $\mu$moles of Tris buffer (pH 7.4), 5 $\mu$moles of MgCl$_2$, 0.1 mg of enzyme protein, approximately 0.2 $\mu$mole of acetoacetyl-CoA, and 1 mg of NADH$_2$ where indicated. The reaction was followed as the decrease in optical density at 305 m$\mu$ due to the disappearance of acetoacetyl-CoA. The blank cuvette contained all the reaction components except acetoacetyl-CoA.*
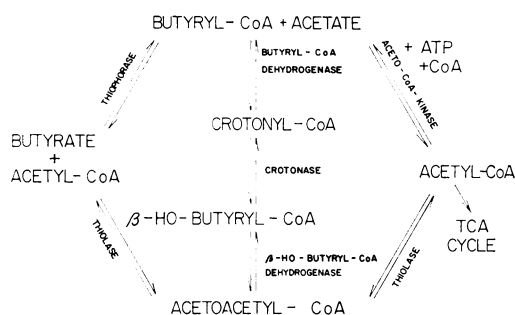
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