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Catabolic Enzymes of the Acetogen *Butyribacterium methylotrophicum* Grown on Single-Carbon Substrates

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When grown on formate, formate-CO, and methanol-CO, *Butyribacterium methylotrophicum* contained high levels of tetrahydrofolate (H_4folate) and required enzymes, carbon monoxide dehydrogenase, formate dehydrogenase, and hydrogenase. The activities of methylene-H_4folate reductase were comparable to other H_4 folate activities (which ranged from 0.55 to 9.28 μmol/min per mg of protein) when measured by an improved procedure. The H_4folate activities in formate-grown cells were twice those found in formate-CO-grown cells. This result correlated with a growth yield on formate that was one-half that on formate-CO. The stoichiometry of the formyl-H_4folate synthetase reaction was 1 mol of ATP per 1 mol of formate. The methylene-H_4folate dehydrogenase was NAD^+ dependent. We conclude that *B. methylotrophicum* utilizes these enzymes in homoacetogenic catabolism.

The catabolic reduction of CO_2 to acetic acid is mediated by a diverse group of anaerobic acetogenic bacteria. The oxidation of hydrogen, single-carbon (C_1) substrates, or various saccharides supplies the necessary reducing power. Current models of C_1 catabolism by saccharolytic acetogens (10, 12, 14, 15, 22, 35, 39, 41) portray the simultaneous operation of two CO_2 reduction mechanisms that lead to the initial multicarbon catabolic product acetyl coenzyme A. The carbonyl is formed from CO_2 by [CO], a chemically undefined intermediate whose synthesis and conversion to the C-1 of acetyl coenzyme A (13, 30, 31, 35) is catalyzed in part by carbon monoxide dehydrogenase. The methyl is formed by the reduction of CO_2 to formate, with further reductions occurring via tetrahydrofolate (H_4folate) carrier-bound C_1 units, and yield methyl-H_4folate, the penultimate methyl donor (14, 22). Properties of these enzymes and characteristics of acetogenic organisms have been reviewed extensively (12, 21, 22, 39, 40, 41).

The catabolism of numerous C_1 substrates and substrate mixtures by *Butyribacterium methylotrophicum* has been characterized. This organism performs homoacetogenic fermentations when supplied with more oxidized substrates (e.g., H_2-CO_2, CO alone, HCOOH, HCOOH-CO). In addition, it synthesizes butyrate when it is supplied with more reduced substrates (e.g., CH_3OH-CO_2, CH_3OH-HCOOH) in the absence of CO (9, 17, 18, 24–26, 42). If sufficient levels of acetate are initially present, butyric acid is the sole net reduced product during growth on CH_3OH-CO_2 or CH_3OH-HCOOH (17, 24). Here we describe the levels of oxidoreductases in *B. methylotrophicum* extracts that catabolize formate, formate-CO, or methanol-CO to acetate. An improved radioisotopic assay of methylene-H_4folate reductase is described. The cellular location of some important enzymes was determined, and the levels of H_4folate enzyme activities were correlated with growth yields.

MATERIALS AND METHODS

Gases, chemicals, and isotopes. Nitrogen, CO, H_2, and Ar gases were purchased from the Matheson Gas Co. (Joliet, Ill.) and were greater than 99.998, 99.99, 99.9, and 99.99% pure, respectively. Nitrogen gas was scrubbed of residual oxygen by passage through reduced heated copper columns. *dl*-Tetrahydrofolic acid (77% pure), *dl*-5-formyl-H_4folate (90% pure), *dl*-5-methyl-H_4folate (90% pure), brilliant cresyl blue (64% dye content), and dimedone (5,5-dimethyl-1,3-cyclohexanedione) were purchased from the Sigma Chemical Co. (St. Louis, Mo.). *dl*-5,10-Methenyl-H_4folate was prepared by acidifying a solution of formyl-H_4folate (32), and *dl*-5,10-methylene-H_4folate was prepared by condensing H_4folate and formaldehyde (23) by using a final formaldehyde concentration of 125 mM (38). *dl*-5-[*methyl*-^{14}C] H_4folate (Ba salt; 58.3 mCi/mmol; 50 μCi) was purchased from Amersham Corp. (Arlington Heights, Ill.) and stored anaerobically at −70°C in 4 ml of 6 mg of ascorbate buffer (pH 6.0) per ml (28). Sodium formate was obtained from Mallinckrodt, Inc. (Paris, Ky.), and all other chemicals were of reagent grade.

Organism cultivation. The C_1 catabolism by both the Marburg (9, 17, 25, 26, 42) and the CO-adapted (24) strains of *B. methylotrophicum* have been described previously. The preparation of phosphate-buffered saline-reduced anaerobic medium which contained 0.05% yeast extract was outlined previously (17). Cultures were routinely maintained on medium (pH 7.0) supplied with sodium formate (100 mM under a N_2 headspace), formate-CO (100 mM formate, 101 kPa of 100% CO at 25°C), CH_3OH-CO (50 mM methanol, 101 kPa of 100% CO headspace), CO alone (172 kPa of 100% CO at 25°C), or CH_3OH-acetate-CO_2 (100 mM CH_3OH, 25 mM sodium acetate, 12.5 mM $NaHCO_3$, N_2 headspace) and were incubated on their sides with shaking (160 rpm) at 37°C (17).

B. methylotrophicum was mass cultured on medium con-

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taining CH_3OH-acetate-CO_2 in 18-liter glass carboys which contained 15 liters of sterilized medium. Prior to inoculation, the sterile medium was sparged for 1 h with N_2 (passed through sterile glass wool filters), and additions of sterile solutions of phosphate buffer (pH 7) to 46 mM, CH_3OH to 100 mM, sodium acetate to 25 mM, and $NaHCO_3$ to 12.5 mM were made. The medium was reduced by the addition of 10 to 15 ml of 15% Na_2S, warmed to 37°C, and inoculated with 50 to 100 ml of a log-phase culture maintained on CH_3OH-acetate-CO_2. When the CO-adapted strain was grown on CH_3OH-acetate-CO_2, the inoculum consisted of two serum vials containing 50 ml of medium supplemented with CH_3OH-acetate-CO_2 that was inoculated with 1 ml of the CO-adapted strain maintained on CO. Mass culture on sodium formate, CO alone, formate-CO, and CH_3OH-CO was accomplished in 14 liter fermentors (New Brunswick Scientific Co., Inc., Edison, N.J.) containing 12 liters of sterile reduced medium at 37°C. Phosphate buffer (to 60 mM) and, depending on the substrate used, sodium formate (to 150 mM when it was the sole substrate to 100 mM when CO was supplied) or CH_3OH (to 100 mM) were added. CO was supplied to the vessel headspace with a transfer hose connected to an 18-liter carboy containing 100% CO. Anaerobic water from a second carboy (slightly pressurized with argon) was fed into the CO-containing carboy as the CO was consumed, thereby maintaining a constant supply of CO to the fermentor. (We are grateful to Harland Wood for suggesting this procedure.) The medium was stirred at 400 rpm to ensure good mixing of the poorly soluble CO. The pH was maintained automatically at 7.0 by the addition of 4 M NaOH during growth with CH_3OH-CO or formate-CO as substrates or the addition of 3 M formic acid during growth with sodium formate as the substrate. Fermentors were inoculated with 50 to 100 ml of log-phase cultures maintained on the appropriate substrate(s). The same lot of trace mineral stock solution was used for all media (16) to prevent changes in enzyme levels resulting from medium variations (1). Large-scale cultures were harvested in the mid- to late-exponential phase.

Harvesting. A continuous flow apparatus (Sorvall model KSB; DuPont Instruments, Bridgeport, Conn.) operated at 17,400 × *g* and 5°C was used to harvest cells anaerobically. During the procedure, the rotor chamber was continuously flushed with N_2 with a hose attached to the centrifuge drain. Media in the feed and effluent lines to the centrifuge remained colorless, which indicated that the redox indicator resazurin remained reduced. During harvesting (4 to 5 h) the growth vessel was stirred, cooled on ice, and slightly pressurized with N_2 or CO. CO was used only when CO-formate was the growth substrate. Under these conditions, oxidation of the remaining formate increased the medium pH in the absence of CO. Cells were transferred under N_2 into tared glass tubes which were sealed with butyl rubber bungs and frozen at −70°C until use.

Extract preparation. Frozen cells were suspended (1:3) in cold anaerobic water containing 4 mM dithiothreitol and disrupted under H_2 by a single pass through a French pressure cell at 20,000 lb/in^2 (13,800 N/cm^2). An estimated 20 to 40% of the cells were broken by this procedure. The effluent was fed directly into an anaerobic, polycarbonate, thick-walled centrifuge tube (1 by 3.5 in [2.5 by 8.9 cm]; Spinco Div. of Beckman Instruments, Inc., Arlington Heights, Ill.) sealed with a Noryl cap. Cell debris was removed by centrifuging the extract for 35 min at 10,000 × *g* and 4°C, and the clear brown supernatant was transferred to anaerobic glass vials that were kept on ice.

For the preparation of extracts used to assay the enzyme distribution, the initial centrifugation at 10,000 × *g* was shortened to 25 min, and the resulting crude supernatant was carefully transferred into an anaerobic glove bag (Coy Laboratory Products, Inc., Ann Arbor, Mich.) into a polycarbonate ultracentrifuge tube (5/8 by 3 in [1.6 by 7.6 cm]; Spinco), which was then tightly sealed and ultracentrifuged at 150,000 × *g* for 90 min (4°C). In the glove bag, the supernatant was transferred into one anaerobic glass vial, and the viscid membrane pellet was suspended in anaerobic buffer (0.1 potassium maleate [pH 7.3], 5 mM 2-mercaptoethanol) and transferred to a second anaerobic glass vial. The extract fractions were left overnight on ice to allow for the gradual solubilization of the membrane pellet.

Enzyme analyses. Enzyme levels were assayed in fresh extracts within 24 h of preparation, with the exception of the assay to determine the level of radioisotopic methylene-H_4folate reductase, which was performed within 48 h of extract preparation. Unless otherwise specified, for all transfers of extracts and anaerobic reagents N_2-flushed glass syringes were used. The syringes were either disposable or, for smaller volumes, gas-tight microliter syringes (Unimetrics Corp., Anaheim, Calif.). Assays were performed at 37°C, and a spectrophotometer (Varian Cary 219) was used for all spectrophotometric measurements. Specific activities were calculated from linear reactions and were constant over a range of extract concentrations. Protein was determined by the Lowry method (8), with bovine serum albumin used as a standard. Hydrogenase, formate dehydrogenase, and carbon monoxide dehydrogenase were determined in anoxic cuvettes containing 1 ml of phosphate buffer (55 mM, pH 7.2, 35°C) plus methyl viologen (5 mM). The addition of a few microliters of a solution of about 20 mM dithionite caused a slight reduction of methyl viologen to a light blue color and ensured anaerobic conditions. Substrates (5 mM sodium formate, 1 atm of 100% CO or H_2) were added, and the cuvettes were shaken vigorously and warmed to 37°C. Reactions were initiated by the addition of extract (freshly diluted in the reaction mixture if necessary), and the increase in the A_{578} was recorded. Specific activities (micromoles of substrate oxidized per minute per milligram of protein, equivalent to 2 μmol of methyl viologen reduced per min per mg of protein) were calculated by using an extinction coefficient for methyl viologen of 9.78 ml/μmol per cm.

Formyl-H_4folate synthetase activity was assayed in a two-step procedure (5) in which the enzyme-dependent synthesis of formyl-H_4folate from formate, ATP, and H_4folate was followed by the conversion at low pH of formyl-H_4folate to methenyl-H_4folate$^+$, which absorbs at 350 nm (ε = 24.9 ml/μmol per cm). The enzymatic reaction was stable in the presence of 100 mM 2-mercaptoethanol and was performed with 0.5-ml reaction mixtures contained in 1.5-ml plastic microfuge tubes. The synthesis of formyl-H_4folate exhibited a 1:1 stoichiometric dependence on the level of ATP supplied (Fig. 1).

Methenyl-H_4folate cyclohydrolase activity was determined in anaerobic cuvettes by following the decrease in the A_{356} (pH 7.0) resulting from methenyl-H_4folate$^+$ hydrolysis (23). Corrections amounting to 40 to 70% of the total reaction rate were made for the spontaneous hydrolysis of the substrate in the pH 7.0 potassium maleate buffer.

Methylene-H_4folate dehydrogenase activity was assayed (23) in anaerobic stoppered glass vials by quantifying the NAD^+-dependent oxidation of methylene-H_4folate to methenyl-H_4folate$^+$, which was then measured at 350 nm (after acidification to oxidize reduced pyridine nucleotides

and to convert any formyl-H_4folate formed subsequent to methenyl-H_4folate$^+$ back to methenyl-H_4folate$^+$).

Methylene-H_4folate reductase was assayed both radioisotopically and spectrophotometrically. The radioisotopic assay was derived from the assay that is routinely cited (assay A in reference 19), in which the oxidation of [*methyl*-^{14}C]H_4folate to [*methylene*-^{14}C]H_4folate is coupled with the reduction of menadione. Our most significant modifications of this procedure were the substitution of menadione with brilliant cresyl blue (BCB) and the replacement of the phosphate buffer (pH 6.7) with maleate buffer (pH 7.0). Assays were performed in glass tubes sealed with rubber bungs under an atmosphere of N_2 gas. The reaction mixture was prepared from the following anaerobic solutions, with volumes indicated in microliters double distilled water, 251.5; 600 μM FAD, 5; 3% bovine serum albumin, 60; 1.0 M potassium maleate (pH 7.0), 62.5; 40 mM EDTA (pH 7.0), 15; 6.4 mM BCB, 80; 0.1 M 2-mercaptoethanol, 6; 2.6 mM 5-[*methyl*-^{14}C]H_4folate (2,300 dpm/nmol), 100. The 5-[*methyl*-^{14}C]H_4folate was prepared by combining 0.76 ml of the stock solution (1.28×10^5 dpm/nmol; see above) with 0.0056 g of *dl*-methyl-H_4folate dissolved in 2.84 ml of anaerobic 0.1 M Tris hydrochloride buffer (pH 7.0) containing 1 mM 2-mercaptoethanol. The reaction was initiated by the addition of 20 μl of freshly diluted extract (1/100 to 1/400 in anerobic reaction mixtures lacking substrate) to a warmed (37°C) tube containing the assay components. The assay was terminated after 1 to 3 min by injecting 0.3 ml of dimedone reagent (3 mg/ml in 1 M sodium acetate [pH 4.5]). Vented tubes were heated to 95°C for 5 min and allowed to cool at room temperature. After the addition of 3 ml of toluene, each tube was vigorously vortexed for 15 s and then centrifuged to separate the phases. The radioactivity extracted into 500 μl of the organic phase was counted in 5-ml vials (Pico-vials; Packard Instrument Co., Inc., Downers Grove, Ill.) containing scintillation cocktail (Instagel; Packard) using a ^{14}C quench, correction-programmed liquid scintillation counter (PLD Prias; Packard). Data were corrected for blank values (enzyme and dimedone were added simultaneously at the end of the incubation period), and the 82% extraction efficiency was determined by adding known amounts of [^{14}C]formaldehyde to reaction mixtures lacking [*methyl*-^{14}C]H_4folate.

The spectrophotometric assay depended on following the enzyme-dependent reduction of BCB at 624 nm. This dye was less metachromatic than methylene blue (36), and its absorptivity more closely adhered to the Beer-Lambert relationship (data not shown), with an extinction coefficient of 64 ml/μmol per cm through 2.5 absorbance units. The assay was prepared from the solutions used in the radioisotopic assay and was performed in anaerobic cuvettes that contained the following, in microliters: potassium maleate, 62.5; EDTA, 25; bovine serum albumin, 100; FAD, 8; BCB, 6; double distilled water, 678.5; 2-mercaptoethanol, 10; 2.59 mM *dl*-5-methyl-H_4folate, 100. *dl*-5-Methyl-H_4folate was prepared by dissolving 0.0056 g of substrate in 3.5 ml of anaerobic 0.1 M Tris hydrochloride containing 1 mM 2-mercaptoethanol (final pH, 7.0). The addition of diluted extract initiated the reaction. Although maximum reaction velocities were observed in the pH range of 7.7 to 8.3, less variability occurred under the conditions specified here. Lower BCB concentrations were used in the spectrophotometric assay compared with the radioisotopic assay to maintain the Beer-Lambert relationship.

Except as noted in the footnotes to Tables 1 and 2, the procedure of mass culturing an organism on a substrate, harvesting, extract preparation, and enzyme quantification was repeated three or four times; and the reported values are averages ± standard deviations.

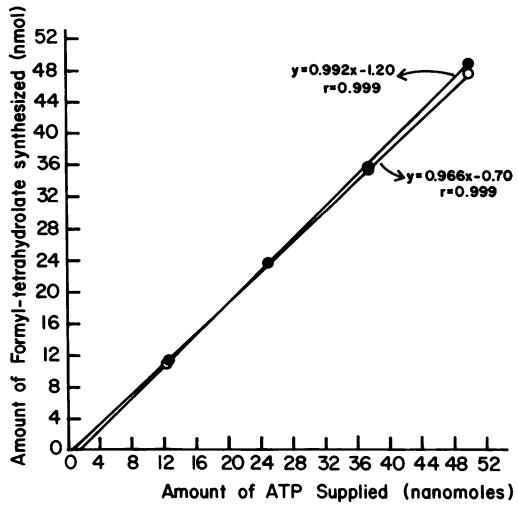

FIG. 1. Stoichiometric dependence of formyl-H_4folate synthesis on the level of ATP supplied. The H_4folate-dependent formation of formyl-H_4folate was measured in an assay system in which ATP was limiting. In one set of reaction mixtures (○), 1.8 mM H_4folate and 12.5 to 50 nmol of ATP were present initially, and the reaction was started by adding 10 μg of crude protein extract. The reactions were stopped after 20 min at 37°C by the addition of acid. In a second set of reaction mixtures (●) intended to test for the extent of ATP hydrolysis by non-H_4folate-dependent enzymes, 10 μg of crude protein extract was added to reaction mixtures that were supplied with ATP but that lacked H_4folate and incubated at 37°C for 20 min. Then, H_4folate (to 1.8 mM) was added, and the mixtures were incubated for an additional 20 min at 37°C before the reaction was halted.

RESULTS

Enzyme properties. ATP (1 mol) was used by formyl-H_4folate synthetase for each mol of product formed (Fig. 1). The reaction required $MgCl_2$ and was enhanced in the presence of KCl (data not shown). The methylene-H_4folate dehydrogenase was NAD^+ dependent. In the presence of NAP^+, 1.3% of the NAD^+ activity was obtained. The specific activity for methylene-H_4folate reductase was enhanced nearly 20-fold by replacing menadione (e.g., specific activity, 0.20 μmol/min per mg of protein) with more soluble flavin-like electron acceptors with standard midpoint reduction potentials closer to 0 mV (e.g., BCB [2.80 μmol/min per mg of protein] methylene blue, [3.65 μmol/min per mg of protein]). Dyes with similar structures but with lower E_0 values were inferior electron acceptors (e.g., neutral red). Although BCB was less active than methylene blue, its absorbance characteristics at higher concentrations more closely approximated the Beer-Lambert relationship. Therefore, BCB was routinely used in the spectrophotometric and radioisotopic assays.

Enzyme levels. Extracts of formate-grown cells contained high levels of formate dehydrogenase, hydrogenase, and the enzymes that act on C_1 units bound to H_4folate carriers (Table 1). Slightly lower activities were found in cells grown on formate-CO and methanol-CO. The spectrophotometric methylene-H_4folate reductase assay consistently yielded 20 to 40% of the activity found in the radioisotopic assay, which

TABLE 1. Specific activities of catabolic enzymes in *B. methylotrophicum*

Enzyme activity	Sp act (μmol/min per mg of protein) on the following growth substrates:		
	HCOOH	HCOOH-CO	CH_3OH-CO
Formate dehydrogenase	1.87 ± 0.52	0.38 ± 0.04	1.28 ± 0.81
Hydrogenase	3.38 ± 0.30	1.46 ± 0.25	2.10 ± 0.23
Formyl-H_4folate synthetase	8.76 ± 0.96	4.59 ± 0.64	2.61 ± 0.26
Methenyl-H_4folate cyclohydrolase	1.16 ± 0.41	0.42 ± 0.06	0.55 ± 0.03
Methylene-H_4folate dehydrogenase	7.08 ± 1.07	3.92 ± 0.27	2.21 ± 0.23
Methylene-H_4folate reductase[a]	9.28 ± 1.12	3.60 ± 0.65	2.66 ± 0.26

[a] A radioisotopic assay was used. With the exception of the methylene-H_4folate reductase results, the procedure of culturing *B. methylotrophicum*, harvesting extract preparation, and enzyme quantification was repeated three times with cells grown on each substrate, and the data presented are averages (±standard deviations) of these independent assays. The data for methylene-H_4folate reductase are averages of two independently harvested cell batches.

TABLE 2. Specific activity of carbon monoxide dehydrogenase in *B. methylotrophicum* grown on different substrates

Strain and growth substrate	Doubling time (h)	Sp act (μmol/min per mg of protein)[a]
Marburg strain		
CH_3OH-acetate-CO_2	7	47.2 ± 12.0
HCOOH	17	23.3 ± 0.3
HCOOH-CO	12	9.4 ± 2.5
CH_3-OH-CO	14	4.1 ± 1.3
CO-adapted strain		
CH_3OH-acetate-CO_2	12	41.6 ± 5.5
CO	11	13.3 ± 9.0

[a] Anaerobically prepared crude extracts of *B. methylotrophicum* were assayed for their methyl viologen-linked carbon monoxide dehydrogenase activity (pH 7.2 at 37°C), as described in the text. The data (±standard deviation) represent averages of three or four independent measurements for each substrate.

was probably the result of the lower BCB levels used in the spectrophotometric assay. Nevertheless, the measured activities were much higher than those obtained with menadione as the electron acceptor.

The levels of carbon monoxide dehydrogenase activity did not correlate with the presence of CO as a growth substrate or the adaptation of *B. methylotrophicum* to growth on CO (Table 2). Indeed, the inclusion of CO as a cosubstrate consistently resulted in reduced carbon monoxide dehydrogenase levels compared with those of cells grown in its absence. The oxidoreductase activities were predominantly (≥80%) found in the soluble extract fractions.

DISCUSSION

As is typical of other acetogens (1–4, 7, 11, 15, 20, 29, 33, 37), the levels of formate dehydrogenase, hydrogenase, carbon monoxide dehydrogenase, and the enzymes that act on C_1 units bound to H_4folate carriers are remarkably elevated in extracts of *B. methylotrophicum*.

The substrate-level phosphorylation catalyzed as part of the formyl-H_4folate synthetase activity consumed 1 mol of ATP per mol of formyl-H_4folate formed (Fig. 1), and the possibility (15) that the enzyme of an acetogen might be energetically more parsimonious is unlikely. The methylene-H_4folate dehydrogenase activity couples with NAD^+, like the enzymes from two other mesophilic acetogens (27, 34). Clark and Ljungdahl (6) recently purified the iron-sulfur flavoprotein methylene-H_4folate reductase from *Clostridium formicoaceticum* and reported similar electron acceptor specificities when this enzyme catalyzed methyl-H_4folate oxidation.

Results of previous studies on the growth of *B. methylotrophicum* on C_1 compounds (17, 18, 24, 25, 26, 42) indicate that fruitful comparisons of enzyme levels might be obtained from cells grown on formate, formate-CO, and methanol-CO. Thus, the enzyme level and cell growth substrate seem to correlate when cells grown in formate and formate-CO are compared. The molar growth yield for acetate production by cells grown on formate is half that of cells grown on formate-CO, and on average, the specific activities of the H_4folate enzymes of the cells grown on formate are double those of the cells grown on formate-CO.

In other cases, however, the relationship between growth substrate and enzyme level seems less straightforward. For example, while growth on methanol-CO proceeds with little apparent substrate oxidation (17), substantial levels of all the measured enzymes were found. High levels of H_4folate enzymes in *Clostridium thermoautotrophicum* grown on CH_3OH-CO_2 has been inferred to support a function of these enzymes in CH_3OH oxidation (7), and a similar explanation could hold for the results of this study. Similarly, while high levels of formate dehydrogenase and carbon monoxide dehydrogenase were predictably found in cells grown on formate or CO, respectively, even higher levels of formate dehydrogenase were found in cells grown on CO and even higher levels of carbon monoxide dehydrogenase were found in cells grown on formate. We postulate that higher levels of formate and carbon monoxide dehydrogenases are required when they function in vivo as carbon dioxide reductases.

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