Control of Carbon and Electron Flow in *Clostridium acetobutylicum* Fermentations: Utilization of Carbon Monoxide to Inhibit Hydrogen Production and to Enhance Butanol Yields

BYUNG HONG KIM,¹ PARA BELLOWS,¹ RATHIN DATTA,² AND J. G. ZEIKUS^{1*}

Department of Bacteriology, University of Wisconsin—Madison, Madison, Wisconsin 53706,¹ and Corn Products, Unit of CPC International, Summit-Argo, Illinois 60501²

Received 21 February 1984/Accepted 14 July 1984

Extracts prepared from non-solvent-producing cells of *Clostridium acetobutylicum* contained methyl viologen-linked hydrogenase activity (20 U/mg of protein at 37°C) but did not display carbon monoxide dehydrogenase activity. CO addition readily inhibited the hydrogenase activity of cell extracts or of viable metabolizing cells. Increasing the partial pressure of CO (2 to 10%) in unshaken anaerobic culture tube headspaces significantly inhibited (90% inhibition at 10% CO) both growth and hydrogen production by *C. acetobutylicum*. Growth was not sensitive to low partial pressures of CO (i.e., up to 15%) in pH-controlled fermentors (pH 4.5) that were continuously gassed and mixed. CO addition dramatically altered the glucose fermentation balance of *C. acetobutylicum* by diverting carbon and electrons away from H₂, CO₂, acetate, and butyrate production and towards production of ethanol and butanol. The butanol concentration was increased from 65 to 106 mM and the butanol productivity (i.e., the ratio of butanol produced/total acids and solvents produced) was increased by 31% when glucose fermentations maintained at pH 4.5 were continuously gassed with 85% N₂–15% CO versus N₂ alone. The results are discussed in terms of metabolic regulation of *C. acetobutylicum* saccharide fermentations to achieve maximal butanol or solvent yield.

The limited supply and escalated price of petroleum has initiated interest in chemical and fuel production from biomass fermentations (23). *Clostridium acetobutylicum* was used previously for the industrial production of ethanol, acetone, and butanol from starch, molasses, and various carbohydrate-based waste fermentations (17, 18, 21). At present, butanol is considered a promising product of biomass fermentations because of its high price and potential industrial use as a solvent, chemical feedstock, and potential liquid fuel.

Current research on acetone, butanol, and ethanol fermentation by *C. acetobutylicum* has focused primarily on three general topics: improvement in strain tolerance towards butanol (10); development of continuous fermentation technology (3, 4); and understanding of the physiological relationships between organic acid metabolism and the initiation of solvent production (2, 5, 13). Another important aspect of this fermentation that warrants more attention is improvement in the amount of solvents produced from a given amount of substrate consumed. In this regard, other fermentation end products (i.e., H₂, CO₂, acetate, and butyrate) that are formed are wastes that require further treatment for depollution. Because the end products of *C. acetobutylicum* are all directly formed via pyruvate metabolism, process-directed control of the enzyme activities associated with carbon and electron flow to fermentation end products could enhance total solvent yield and decrease waste byproduct formation.

Clostridium species produce butyrate and hydrogen by transformation of the intermediary metabolites generated from the pyruvate dehydrogenase phosphoroclastic enzyme(s) complex (8). The hydrogenase present (EC 1.12.1.1) in cell extracts (1) or whole cells (19) of these *Clostridium* species is readily inhibited by carbon monoxide. However, little is known about the enzyme activities mediating carbon

and electron flow to end products in *C. acetobutylicum*. Petitdemange et al. (16) demonstrated that the direction of electron flow mediated by ferredoxin-NAD oxidoreductase in *Clostridium* sp. was reversed when cultures were grown on pyruvate instead of glucose. Simon (20) previously reported that CO addition could inhibit growth and alter starch fermentation of *C. acetobutylicum* Weizmann by causing homolactic acid formation in lieu of solvent production.

The purpose of the present paper was to determine whether exogenous CO addition could be used to inhibit hydrogenase activity and thereby enhance solvent production from saccharide fermentations as a consequence of decreasing hydrogen, acetate, and butyrate production by *C. acetobutylicum* ATCC 4259. We report here that hydrogenase activity of *C. acetobutylicum* can be modulated by CO addition to accomplish these goals.

MATERIALS AND METHODS

Chemicals. All chemicals used were reagent grade, and they were purchased from Sigma Chemical Co., St. Louis, Mo., and Mallinckrodt, Paris, Ky. Gases were purchased from Matheson Scientific, Inc., Joliet, Ill. Tritium gas was provided by Paul DeLuca, University of Wisconsin-Madison, Madison.

Bacterial strain and its maintenance. *C. acetobutylicum* ATCC 4259 was used throughout the study. This strain differs from *C. acetobutylicum* Weizmann (ATCC 824) in that it does not produce lactic acid during normal fermentation (20). Preserved soil stock cultures were made by the method described by Walton and Martin (21); these cultures were kept in a desiccator at room temperature until used.

Medium and culture conditions. Anaerobic procedures used from cell cultivation and media preparation were as described previously (9, 12, 14). A complex medium (CAB) was used with either 30 or 60 g of glucose per liter as fermentation substrate. CAB medium contained, in grams per liter of distilled water: yeast extract (Difco Laboratories,

* Corresponding author.

Detroit, Mich.), 4; tryptone (Difco), 1; KH_2PO_4 , 0.7; K_2HPO_4 , 0.7; DL-asparagine, 0.5; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 1.0; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.015; and NaCl, 0.1; the medium also contained 1 ml of 0.2% reazurin. The medium was prepared under an N_2 headspace, and the pH was adjusted to 5.4 before autoclaving. A few grains of spore soil stock were inoculated into 10 ml of CAB medium in an anaerobic pressure tube (Bellco Glass, Inc., Vineland, N.J.). The tube was sealed with a butyl rubber bung and an aluminum crimp before heat shock was applied at 80°C for 2 min. The heat-treated tube was used as inoculum after the culture was grown at 34°C for 48 h. For larger inocula, 3.5 ml of this culture was used to inoculate 70 ml of CAB medium in a 150-ml serum vial sealed with a butyl rubber bung and an aluminum crimp. The serum vial culture was incubated for 24 h, and afterwards, it was used directly as inoculum. Fresh inoculum was made for each experiment, and cultures transferred more than four times from soil culture were not used as inocula.

Growth experiments were performed in pressure tubes or serum vials with a 5% inoculum and were incubated at 34°C for 72 h with or without shaking. For shaken cultures, a reciprocating water bath shaker was used at the speed of 200 strokes per min.

Fermentations were kept at a constant pH in a Multigen fermentor (New Brunswick Scientific Co., Inc., Edison, N.J.) with a working volume of 500 ml. Cells were mass cultivated for enzyme extract preparation in a 14-liter Microferm fermentor (New Brunswick Scientific) with a working volume of 7 liters. Each fermentor was equipped with an automatic pH controller which activated a peristaltic pump to titrate acids produced during the fermentation with 5 N NaOH. The culture vessels were gassed by N_2 or a N_2 -CO gas mixture (when indicated) at the rate of 0.1 volume of gas per volume of medium per min and stirred at 200 rpm. To control excess foaming, we added 1.0 ml of sterile corn oil (baked at 200°C for 10 h) to 1 liter of culture medium.

Quantification of substrate, fermentation products, and growth. Glucose was determined by the glucose oxidase method with Statzyme (Worthington Biochemicals Corp., Freehold, N.J.).

Growth was measured as optical density at 660 nm and was directly read by inserting anaerobic pressure tubes into a Spectronic 20 spectrophotometer (Bausch & Lomb, Inc., Rochester, N.Y.). When serum vials and fermentor cultures were used, samples were taken and diluted when the optical density was above 0.8 to measure culture turbidity. The cell concentration was calculated from turbidity with a standard curve. One optical density unit at 660 nm gave 335 mg/liter (cell dry weight).

Soluble fermentation products were analyzed by chromatographic methods with a Packard 419 gas chromatograph (Packard Instrument Co., Inc., Downers Grove, Ill.), equipped with a flame ionization detector and a glass column (2 mm [inside diameter] by 182.9 cm). Acetone, acetate, butanol, and butyrate were measured with a column packed with Super Q (80/100 mesh; Alltech Assoc. Inc., Deerfield, Ill.). Ethanol was measured by using a glass column packed with Chromosorb 101 (80/100 mesh; Supleco, Inc., Bellefonte, Pa.). N_2 carrier gas was passed through the column at the flow rate of 40 ml/min. The temperatures for injector, column, and detector were 200, 170, and 220°C, respectively. Lactic acid was determined by gas chromatography after methyl ester derivatization by methods described elsewhere (6). Fermentation broth was directly analyzed for acids and solvents after acidification to pH 1.0 with phosphoric acid.

H_2 was measured on a Packard 800 gas chromatograph (Packard Instruments) with a thermal conductivity detector with a stainless steel column (3.2 mm [inner diameter] by 182.9 cm) packed with Porapak N (80/100 mesh; Waters Associates, Inc., Milford, Mass.). The flow rate of N_2 carrier gas was 30 ml/min, and the instrument was operated at room temperature. CO_2 and CO were measured by gas chromatographic methods with a thermal conductivity detector as described previously (12, 15).

In vivo hydrogenase assay. Anaerobic pressure tube cultures were used to measure in vivo hydrogenase activities by the tritium exchange technique (19). Cultures were grown to mid-exponential phase when 0.25 ml of tritium gas with a specific activity of $25.75 \pm 0.25 \mu\text{Ci/ml}$ was added. The pressure tubes were shaken slowly (50 rpm) at 34°C, and 0.25-ml samples were removed from the liquid phase every 10 min. The samples were placed into scintillation vials that contained 5 ml of Instagel (Packard Instruments) as scintillant, and the amount of tritium incorporated into water was measured with a Packard Tri-Carb liquid scintillation counter (Packard Instruments).

Enzymatic procedures. Anaerobic procedures were used for the preparation of cell extracts and the assay of enzyme activities, as described elsewhere (9, 12). Non-solvent-producing cells grown to the mid-exponential phase at pH 7.0 were collected by centrifugation and were suspended in anaerobic distilled water containing 3 mM dithiothreitol and 2 μg of DNase per ml. A French press cell (American Instrument Co., Inc., Silver Spring, Md.) connected to a centrifuge tube sealed with a flanged rubber bung was gassed with N_2 for 30 min before the cell suspension was passed through the cell at a pressure of 1,400 kg/cm². The cell lysate was centrifuged anaerobically at $10,000 \times g$ for 30 min. The supernatant was transferred to N_2 -containing glass vials that were sealed with rubber bungs. Cell extract was stored at -20°C until used. The protein content of cell extracts was measured by the method of Lowry (11) with bovine serum albumin as standard.

Hydrogenase and carbon monoxide dehydrogenase activities were assayed spectrophotometrically with an Eppendorf recording spectrophotometer (Brinkmann Instruments, Inc., Westbury, N.Y.) by procedures described elsewhere (12). Activity was measured by the reduction of an artificial electron acceptor, methyl viologen, at 578 nm ($E_{578 \text{ nm}} = 9.18 \text{ mM}^{-1} \text{ cm}^{-1}$). Assays were performed at 37°C in 1.6-ml cuvettes that were sealed with grey rubber stoppers. The cuvettes were made anaerobic by repeated flushing and evacuation under N_2 gas and by the addition of enough buffered dithionite to turn the reaction mixture light blue. The anaerobic reaction mixtures (1 ml) contained the following: 2 mM methyl viologen, 2 mM dithiothreitol, and 0.1 M Tris hydrochloride buffer (pH 7.8) in 1 atm of H_2 for hydrogenase; and 0.1 mM phosphate buffer (pH 6.8) containing 2 mM methyl viologen, 2 mM dithiothreitol, and 1 atm of CO for carbon monoxide dehydrogenase. The reaction was started by adding cell extract to anoxic prewarmed cuvettes. One enzyme unit represents 1 μmol of substrate oxidized per mg of protein per min.

RESULTS

Influence of CO on hydrogenase activity in cell extracts. Experiments were initiated to confirm the presence of hydrogenase activity in *C. acetobutylicum* and to assess whether CO inhibited hydrogenase or whether it was oxidized by cell extracts. In a comparison of the levels of H_2 - and CO -

oxidizing activity present in cell extracts prepared from non-solvent-producing cells grown at pH 7.0, hydrogenase activity was high (20.0 μmol of H_2 oxidized per mg of protein per min), whereas carbon monoxide dehydrogenase activity was not detectable ($<1 \times 10^{-3}$ μmol of CO oxidized per mg of protein per min).

Figure 1 shows hydrogenase activity as a function of CO partial pressure in the cuvette headspace. The CO concentration-dependent inhibition of hydrogenase displayed first-order kinetics for concentrations of CO of up to 10%, suggesting high specificity for inhibition of this enzyme activity.

Influence of CO on growth and hydrogen metabolism. Experiments were begun to examine the influence of CO addition on the growth physiology of *C. acetobutylicum*. Figure 2 shows that the specific growth rate of the organism in sealed, unshaken anaerobic pressure tubes was significantly inhibited by low partial pressures of CO (2 to 10% in the culture headspace). In these experiments, the specific growth rates were calculated from the initial exponential phase, and the total initial gas pressure was 1 atm of N_2 or the N_2 -CO mixture indicated. The specific growth rate at 5% CO was only one-third of that observed in the absence of CO.

In analogous experiments, the effect of culture headspace CO concentration on hydrogen production by cells grown without shaking in pressure tubes was determined. Figure 3 shows the ratio of hydrogen produced after 72 h of incubation by cultures with CO to that produced by the controls without the growth inhibitor. A sharp decrease in hydrogen production was observed at a concentration of 1% CO, followed by a first-order decrease at higher CO partial pressures. The CO concentration remained constant in all tubes, indicating the absence of significant CO-consuming

FIG. 1. Effect of CO partial pressures on in vitro hydrogenase activity of *C. acetobutylicum*. Extracts prepared from non-solvent-producing cells were used to assay the H_2 -dependent reduction of methyl viologen in the presence or absence of the indicated cuvette headspace concentration of CO. One unit of activity represents 1 μmol of H_2 consumed per mg of protein per min at 37°C.

FIG. 2. Effect of initial CO concentration on the growth rate of *C. acetobutylicum*. Cells were grown at 34°C without shaking in anaerobic pressure tubes that contained 10 ml of CAB medium (pH 5.4), 3.0% glucose, and 1 atm of 100% N_2 or the N_2 -CO gas mixture indicated.

enzyme activity which is present in some anaerobic bacterial species (12).

A tritium exchange assay was employed to demonstrate that low concentrations of CO specifically inhibited hydrogenase in viable, solventogenic cells. Cultures were grown to the mid-solvent-producing phase in anaerobic pressure tubes that contained 1 atm of either 100% N_2 , 96% N_2 -4% CO, or 92% N_2 -8% CO. Tritiated hydrogen was added to the tubes, and in vivo hydrogenase activity was monitored with time. Figure 4 illustrates that 4 and 8% CO inhibited the rate of in vivo hydrogenase activity by 54 and 65%, respectively. These results are consistent with the interpretation that the addition of CO at low concentrations specifically inhibits in vivo hydrogenase during the acidogenic or solventogenic

FIG. 3. Effect of initial CO concentration on hydrogen production by *C. acetobutylicum*. Cells were grown without shaking in anaerobic pressure tubes that contained 10 ml of CAB medium (pH 5.4), 3.9% glucose, and 1 atm of 100% N_2 or the N_2 -CO gas mixture indicated.

FIG. 4. Effect of CO concentration on in vivo hydrogenase activity in solventogenic cultures of *C. acetobutylicum*. Cells were grown without shaking to mid-solventogenic phase in anaerobic pressure tubes that contained 10 ml of CAB medium (pH 5.4), 3.0% glucose, and an initial headspace that contained 1 atm of 100% H₂ or the N₂-CO mixture indicated. At this time, ³H₂ was added, and ³H₂O formation was monitored. The results represent the average of three tubes for each set of conditions.

phases and total H₂ production in *C. acetobutylicum* fermentations.

Influence of CO on solvent production. To determine the influence of low CO partial pressures on the glucose fermentation balance, we grew *C. acetobutylicum* in shaken anaerobic pressure tubes that contained 1 atm of either 100% N₂ or 94% N₂-6% CO. Table 1 shows the results of glucose fermentation after 72 h of incubation. Hydrogen production and growth were both inhibited by CO, but not as dramatically as in fermentation tubes that were not shaken (see above). This difference appears to be associated with enhanced glucose consumption (50%) in shaken cultures (data not shown). CO dramatically altered the fermentation balance by inhibiting H₂, CO₂, acetone, acetate, and butyrate formation while stimulating ethanol and butanol production.

The effect of CO on enhancement of solvent production was discovered to be even more significant in pH-controlled fermentors that were continually mixed and gassed. Figure 5 shows the glucose fermentation time course of *C. acetobutylicum* when grown at pH 4.5 in CAB medium containing 6% glucose and gassed by either 100% N₂ or an 85% N₂-15% CO gas mixture. Solvent production occurred as a secondary metabolic process in the presence or absence of CO. The culture conditions used were not maximized for gas transfer

into the liquid, and the presence of 15% CO in the gas phase only increased the lag period from 1.5 to 3 h and decreased the growth rate from 0.35 to 0.32/h. However, the presence of 15% CO significantly enhanced the rate of butyrate reconsumption and butanol production. It was not possible to accurately measure CO₂ and H₂ production because of the high gassing rates used in the cultures.

Table 2 summarizes the effect of continuous gassing with 15% CO on solvent and acid production from glucose by *C. acetobutylicum* after 32 h of incubation in fermentors maintained at pH 4.5. It should be noted that fermentation balances were not calculated because of the inability to measure gases accurately and because of the evaporation of solvents. Solvent yield was much higher in the culture gassed with CO than in the control cultures, whereas the total acid concentration was lower. The total solvent/total acid ratio was 63:37 for the control culture and 87:13 for the culture gassed with CO. CO increased the butanol productivity (i.e., moles of butanol produced per total moles of acids and solvents produced) by 31% without inhibiting glucose consumption.

DISCUSSION

C. acetobutylicum ferments saccharides to multiple end products with different individual oxidation-reduction states. It is well known that the ratio of fermentation end products formed by this organism can be altered by changing the environmental culture conditions (2-5, 7, 13). The switch from acid production to solvent production is the best documented example of alteration of carbon and electron flow caused when the glucose fermentation is shifted from a primary to a secondary metabolic process. The present data provide the first evidence to show that the addition of exogenous CO can alter H₂ metabolism by inhibiting hydrogenase and that this enhances solvent production by alteration of the carbon and electron flow pathways present in *C. acetobutylicum*. The practical utility of this discovery in an improved industrial process for butanol production from starch is described elsewhere (R. Datta and J. G. Zeikus, Dev. Ind. Microbiol., in press). The present data cannot exclude the possibility that CO may also inhibit other metalloenzymes or coenzymes present and this also contributes to higher solvent yield. In a subsequent paper (B. H. Kim and J. G. Zeikus, submitted for publication), we will show that hydrogenase activity decreases and is regulated in normal fermentations when the organism shifts from the acidogenic to the solventogenic phase.

Addition of CO to carbohydrate fermentations of *C. acetobutylicum* Weizmann was previously shown to inhibit growth and to cause homolactic acid production (20). The present data suggest that this metabolic response occurred as a consequence of inhibiting more than hydrogenase activity (i.e., pyruvate dehydrogenase was also inhibited). Our observation that CO addition can enhance solvent yield

TABLE 1. Influence of CO on the glucose fermentation balance of *C. acetobutylicum*^a

CO concn (%, in headspace)	Growth (final OD ₆₆₀) ^b	Glucose consumed (mM)	End products formed (mM/100 mM glucose)							Oxidation- reduction balance ^c	Carbon recovery (%)
			H ₂	Ethanol	Acetone	Acetate	Butanol	Butyrate	CO ₂		
0	2.0	146.6	139.1	3.5	19.2	18.3	28.3	17.0	175.8	1.06	96
6	1.85	130.4	117.8	4.0	15.0	17.4	32.0	12.6	162.5	1.05	105

^a Anaerobic pressure tubes that contained 10 ml of CAB medium (pH 5.4) and 3.0% glucose were incubated with shaking at 34°C for 48 h after inoculation.

^b OD₆₆₀, Optical density at 660 nm.

^c The oxidation-reduction balance was calculated by the method of Wood (22).

FIG. 5. Glucose fermentation kinetics in pH (4.5)-controlled fermentor cultures continuously gassed with 100% N_2 (A) or 85% N_2 -15% CO (B). The experiments were performed in 1-liter fermentors that contained 500 ml of CAB medium and 6% glucose. The fermentors were gassed at a flow rate of 100 ml/liter of culture per min, and the incubation temperature was 34°C.

in lieu of lactate production by *C. acetobutylicum* ATCC 4259 differs from that reported previously (20) because we used conditions whereby CO did not completely inhibit hydrogenase activity or stimulate a potential pyruvate reductase activity (i.e., no lactate was produced) by our strain.

Figure 6 provides a tentative carbon and electron flow pathway to illustrate the proposed biochemistry of saccharide transformation by *C. acetobutylicum*. Careful analysis of several coordinated enzymatic activities (Fig. 6, I to V) is needed to understand the effect of CO on alteration of

intracellular carbon and electron flow during glucose fermentation. Glucose is dehydrogenated via glyceraldehyde 3-phosphate dehydrogenase (Fig. 6, I) and NADH is generated. Pyruvate is oxidized via pyruvate dehydrogenase (Fig. 6, II), and reduced ferredoxin is formed. Electron flow between the pyridine nucleotides and ferredoxin is interconnected (16) such that reduced ferredoxin can reduce NADP via ferredoxin-NADP oxidoreductase activity (Fig. 6, IV) and NADH can reduce ferredoxin via NAD-ferredoxin oxidoreductase activity (III). The acetyl coenzyme A generated by pyruvate dehydrogenase can be either a precursor of oxidized products (i.e., acetate, acetone, or CO_2) or a precursor of reduced products (i.e., butanol, butyrate, or ethanol) when coupled to the oxidation of reduced pyridine nucleotides. The reduced electron carrier ferredoxin plays a pivotal function in that it can donate electrons to form H_2 via hydrogenase (Fig. 6, V) or it can donate electrons to the pyridine nucleotides via the pyridine nucleotide-ferredoxin oxidoreductase (Fig. 6, III and IV).

The present data indicate that hydrogenase can function to control the direction of carbon and electron flow in *C. acetobutylicum*, because inhibition of this activity by CO resulted in both increased solvent production and decreased acid production. The specific biochemical mechanisms proposed here to explain this effect (Fig. 6) suggest that enhanced levels of reduced ferredoxin are generated by inhibition of hydrogenase (V). This alters electron flow by enhancing NADPH and NADH pools which leads to increased levels of solvent production. It is thought that the function of ferredoxin-NADPH reductase is only anabolic and that ferredoxin-NAD oxidoreductase only functions in reduced ferredoxin generation during glucose metabolism (16). However, this clearly is not the case during glucose fermentation by *C. acetobutylicum* (ATCC 4259) in the presence of CO , because electron flow to H_2 was decreased while electron flow to butanol and ethanol was increased, and this process must occur by the flow of electrons from reduced ferredoxin to the pyridine nucleotides. At present, the exact *in vivo* electron donors (i.e., NADH, HADPH, or other compounds) for butyrate, butanol, or ethanol production have not been documented.

The effect of CO on growth and glucose metabolism of *C. acetobutylicum* is greatly influenced by the exact experiment culture conditions employed. Under agitated conditions, for example, 6% CO in the headspace inhibited growth and metabolism in sealed tubes, but growth and metabolism were not inhibited in our fermentor system by a 15% CO gas mixture. This can be explained because in sealed tubes, the dissolved CO concentration remains constant as total gas pressure increases during the fermentation. In the fermentor in which constant total gas pressure was maintained, however, the CO partial pressure was lowered by hydrogen and CO_2 production.

TABLE 2. Influence of continuous gassing with CO on acid and solvent production from glucose by *C. acetobutylicum* in fermentors maintained at pH 4.5

CO concn (% in gas phase)	Glucose consumption (mM)	Acid and solvent production (mM)					Butanol/total soluble products ratio (mol/mol)
		Ethanol	Acetone	Acetate	Butanol	Butyrate	
0	177.5	5.1	29.8	31.8	65.4	27.4	0.41
15	180.8	13.4	37.0	16.8	105.6	6.8	0.59

^a Fermentors (1 liter) contained 500 ml of CAB medium and 6.0% glucose and were continuously gassed with either 100% N_2 or 85% N_2 -15% CO at 100 ml of gas per liter of culture per min. pH was maintained at 4.5 by the continuous addition of NaOH. The cultures were incubated at 34°C for 32 h before fermentation product analysis.

FIG. 6. Tentative carbon and electron flow scheme to explain the influence of CO on the coordinated activity of key oxidoreductases in *C. acetobutylicum*. Enzymes: I, glyceraldehyde 3-phosphate dehydrogenase; II, pyruvate ferredoxin oxidoreductase; III, NAD-ferredoxin oxidoreductase; IV, NADP-ferredoxin oxidoreductase; and V, hydrogenase. Low partial pressures of CO inhibit hydrogenase, and this alters electron flow towards reduced pyridine nucleotide formation whose oxidation is coupled to proton consumption and acetyl coenzyme A and butyryl coenzyme A reduction. See the text for additional explanations.

The present findings indicate that solvent yields (i.e., grams of solvent produced per gram of substrate consumed) of *C. acetobutylicum* fermentations can be significantly improved by modulation at the enzymatic level. This points the direction for future research at: documenting the exact enzymes involved in the carbon and electron flow path; understanding how the key enzymes (e.g., hydrogenase) which control carbon and electron flow are regulated in primary versus secondary metabolism; isolation of mutant strains with specific enzyme modifications; and discovering more selective and less toxic modulators than CO to control key electron flow enzymes. In the latter regard, Hongo (7) reported that the addition of 6 mM neutral red to *C. acetobutylicum* saccharide fermentations resulted in a shift in the acetone-butanol-ethanol ratio from 26:63:11 to 7:90:3. The physiological basis for this phenomenon was not ex-

plained at the enzymatic level, but the present studies suggest that it may occur as a consequence of altering intracellular hydrogen (i.e., electron) metabolism. Perhaps saccharide fermentation can be modified to yield the maximal theoretical chemical yield of 1 mol of butanol and 2 mol of CO₂ produced per mol of glucose consumed, once sufficient understanding on metabolic regulation of *C. acetobutylicum* is achieved.

ACKNOWLEDGMENTS

This research was supported by the College of Agriculture and Life Sciences, University of Wisconsin, and by a grant from CPC International to J.G.Z.

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