

**THE EFFECT OF CO ON GROWTH AND PRODUCT FORMATION
IN BATCH CULTURES OF *CLOSTRIDIUM ACETOBUTYLICUM***

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Summary Carbon monoxide sparged in batch fermentations of *C. acetobutylicum* inhibits the production of H₂ by the hydrogenase and enhances the production of solvents by making available larger amounts of NAD(P)H₂ to the cells. CO also inhibits biomass growth and acid formation. Its effect is most pronounced under fermentation conditions of excess carbon- and nitrogen-source supply.

INTRODUCTION

The elucidation of the mechanisms which trigger and control solvent production in fermentations of butyric-acid bacteria is a problem of both fundamental and practical importance, and has recently become the subject of vigorous research activity (Kim et al, 1984; Papoutsakis, 1983). We have recently postulated that solvent production is predominantly regulated by the availability and demand of biosynthetic (ATP) and reduction (NAD(P)H₂, FdH₂) energies (Papoutsakis, 1983; Roos et al, 1985). We have shown that conditions of reduced nitrogen-source availability in batch and continuous cultures of *C. acetobutylicum* (whereby glucose and thus ATP were in excess supply) induce solvent formation (Roos et al, 1985). On the contrary, under glucose-limited conditions, acids are exclusively produced (Roos et al, 1985). The hypothesis that solvent production is controlled by the demand and availability of ATP appears indeed valid. We have also calculated the values of NfF, defined as the amount of NAD reduced by FdH₂ (via the NADH: ferredoxin oxidoreductase) as a function of time for a number of fermentations, using the fermentation equation which has been derived and validated earlier (Papoutsakis, 1984). We found that in all acid fermentations, NfF is negative (i.e. Fd is reduced by NADH₂) throughout the fermentation, while large rates of increase in NfF values and positive NfF values precede and accompany solvent production only (Roos et al, 1985). Thus, the rate of change and values of NfF can predict solvent formation, indicating that, *perhaps*, an excess availability of reduction energy (which is otherwise released as H₂) would induce solvent formation. In order to examine this possibility, we sought a means of making available larger amounts of reduction energy to the cells, to investigate how this would affect the distribution of products. This we accomplished by blocking H₂ production using CO, which is a known inhibitor of the hydrogenase (Gray and Gest, 1965; Mortenson and Chen, 1975). Here we report some of our results with batch cultures under conditions of both excess and limited nitrogen-source supply. A detailed report on the effect of CO on the microbial metabolism in continuous cultures will be forthcoming. The idea of employing CO to probe the metabolism of *C. acetobutylicum* has been investigated, apparently independently of and simultaneously with our own efforts, by Kim et al (1984) [Our first conclusive experiments were performed in the summer of 1983 and were reported in a proposal submitted to the National Science

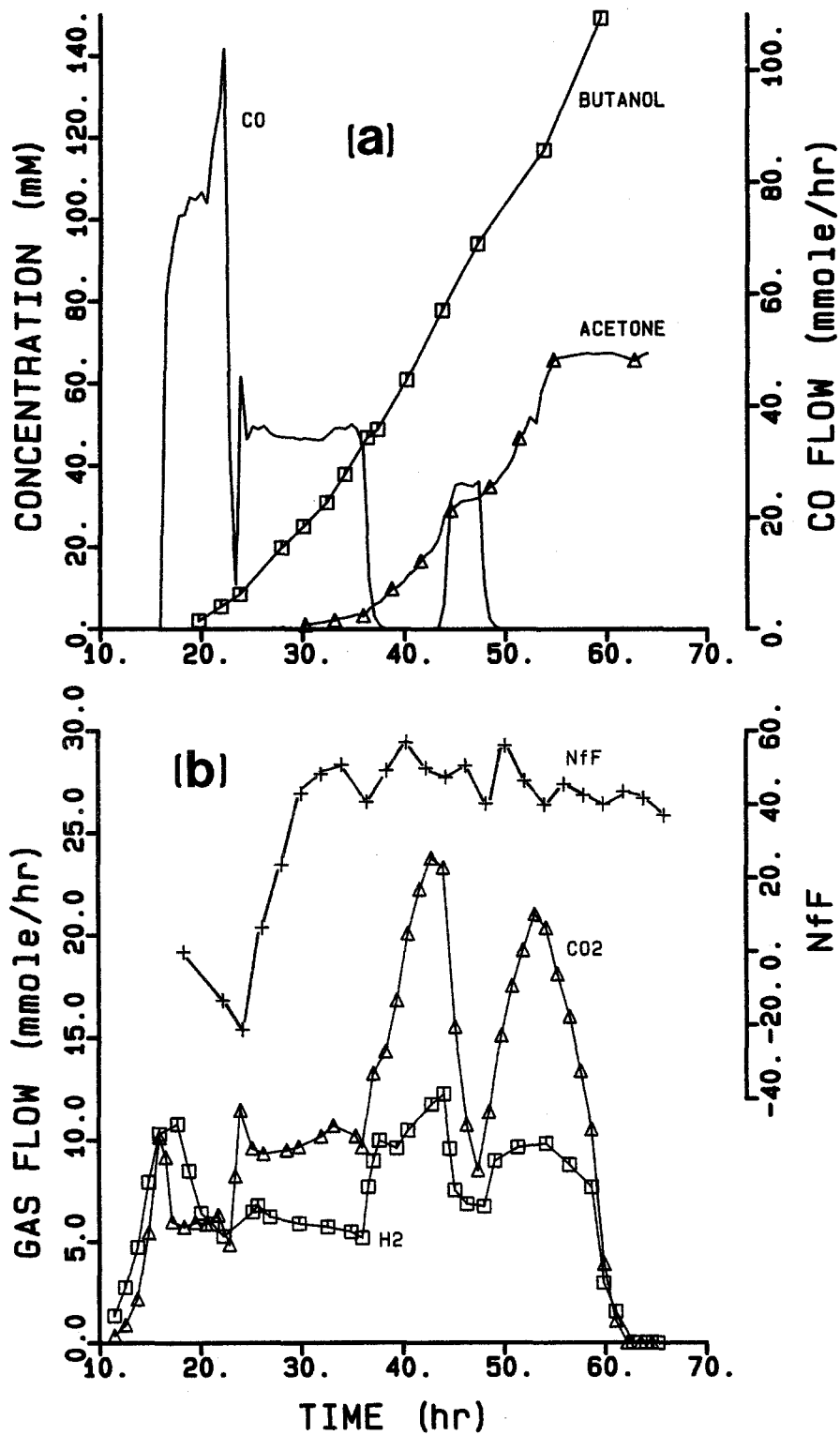


FIGURE 1

Foundation (USA) in December of 1983, and two M.S. theses, Roos (1984) and McLaughlin (1984), in the spring of 1984].

MATERIALS AND METHODS

Clostridium acetobutylicum (ATCC 824) was maintained and grown as described earlier (Roos et al, 1985), except that variable amounts of $(\text{NH}_4)_2\text{SO}_4$, yeast extract and glucose were used as detailed below. The culture was established and maintained at 36.7°C under continuous flow of purified, sterile N_2 in a 2 l Biostat M fermentor (B. Braun Instr.), with a working volume of 0.5-1.4 l, 200-300 rpm stirring, and pH control. The gas phase was analyzed on-line by gas chromatography. The liquid phase was analyzed for products by gas chromatography on- and off-line (Roos et al, 1985). CO was premixed at the desired concentration with N_2 before the mixture was sparged in the reactor. The fermentation equation (Papoutsakis, 1983; 1984) was used to check the consistency of experimental measurements and calculate NFF (mol NAD(P) reduced by FdH_2 per 100 mol glucose fermented) and the Y_{ATP} (g biomass produced per mol ATP utilized).

RESULTS AND DISCUSSION

Fig. 1 shows the substrate, biomass, product, CO and the cumulative NFF profiles for a batch fermentation with 10 g/l yeast extract, and 6 g/l $(\text{NH}_4)_2\text{SO}_4$ (the molar ratio of ammonia to glucose was 0.292) as nitrogen sources in addition to the standard 2 g/l asparagine. The pH was controlled at 4.5. In view of the plentiful supply of nitrogen sources (Roos et al, 1985), this fermentation was controlled by the presence of CO which inhibits both H_2 and biomass production as is clearly demonstrated in Figs. 1b and 1d. The inhibition of biomass growth (as better demonstrated by the inhibition of CO_2 production) and H_2 production via the hydrogenase are clearly reversible and almost instantaneous (Fig. 1b). The ratio of H_2/CO_2 is approximately 1 in the early part of the fermentation, and drops to ca. 0.5 as more and more solvents are produced. The effect of CO on the metabolism is demonstrated most dramatically by the direction and magnitude of flow of electrons as measured by the sign and magnitude of NFF. In the early part of the fermentation and before CO sparging, NFF is negative (NAD(P)H_2 reduces Fd to be used for H_2 production) and decreasing. CO sparging reverses this trend and causes NFF to increase to high positive values (FdH_2 reduces NAD(P) to produce solvents), where it remains for the rest of this solvent fermentation. The effect of CO on the flow of electrons results in an early induction of butanol formation. Butanol concentration passes the 1 mM level at an OD_{600} value of 2.2, a butyrate concentration of 8.5 mM and an acetate concentration of 6 mM. In typical butanol/acetone fermentations, butanol formation is induced at much higher biomass concentrations (OD_{600} between 4 and 6), butyrate concentrations (between 20 and 40 mM) and acetate concentrations (between 20 and 40 mM). It is also interesting to note that acetone formation is induced 12.5 hours after butanol formation, unlike the typical butanol/acetone fermentation where the formation of the two solvents is simultaneously induced. We shall report shortly that under continuous fermentation conditions with CO sparging, no acetone is formed during butanol production. The final concentrations of butanol, acetone and ethanol were 150, 66 and 28 mM respectively. The butyrate profile (Fig. 1c) shows a decrease in butyrate production or butyrate reuptake with increasing CO sparging and an immediate increase of the butyrate-formation rate when CO sparging is temporarily stopped. Butyrate concentration remained below 22 mM during the fermentation. Similarly,

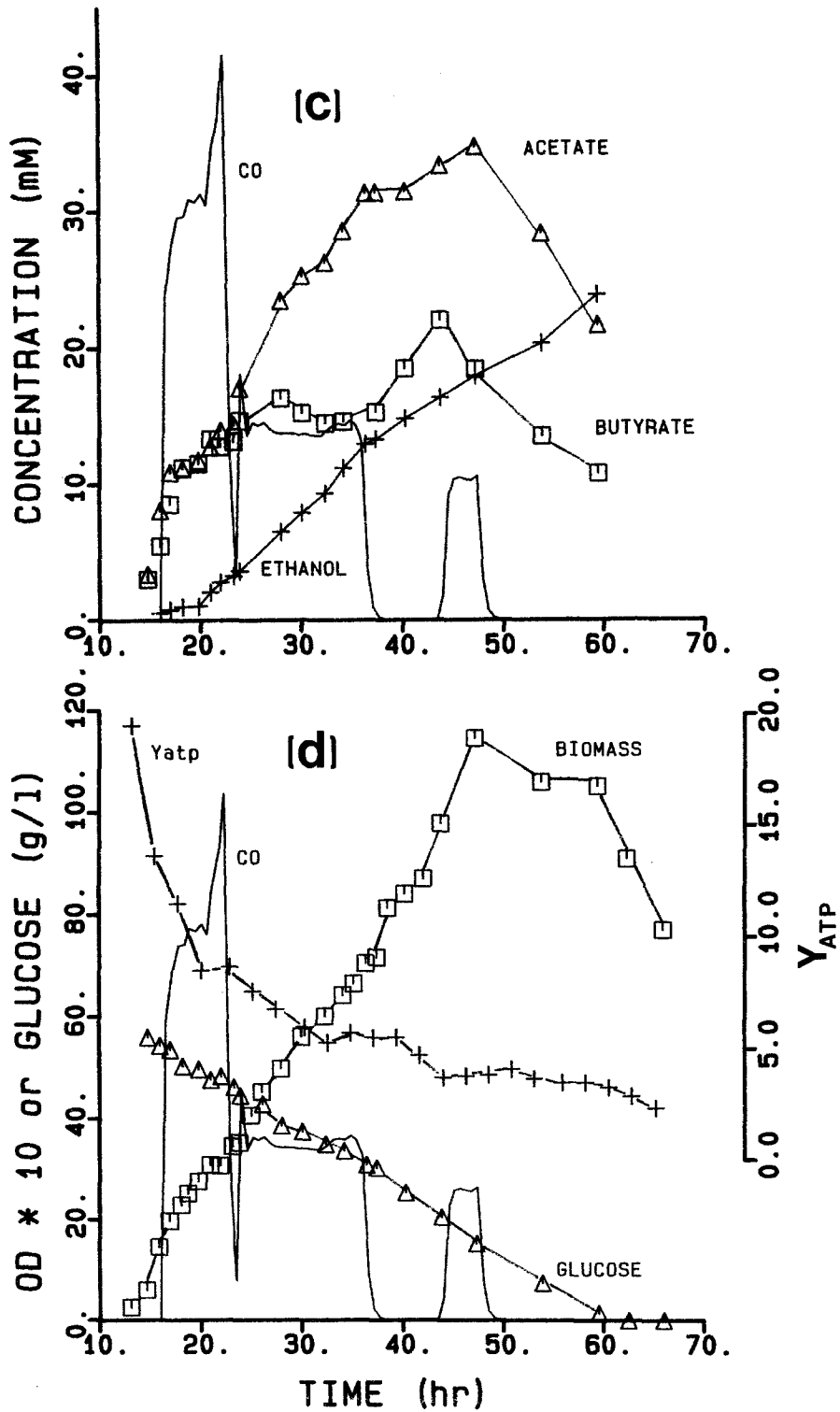


FIGURE 1

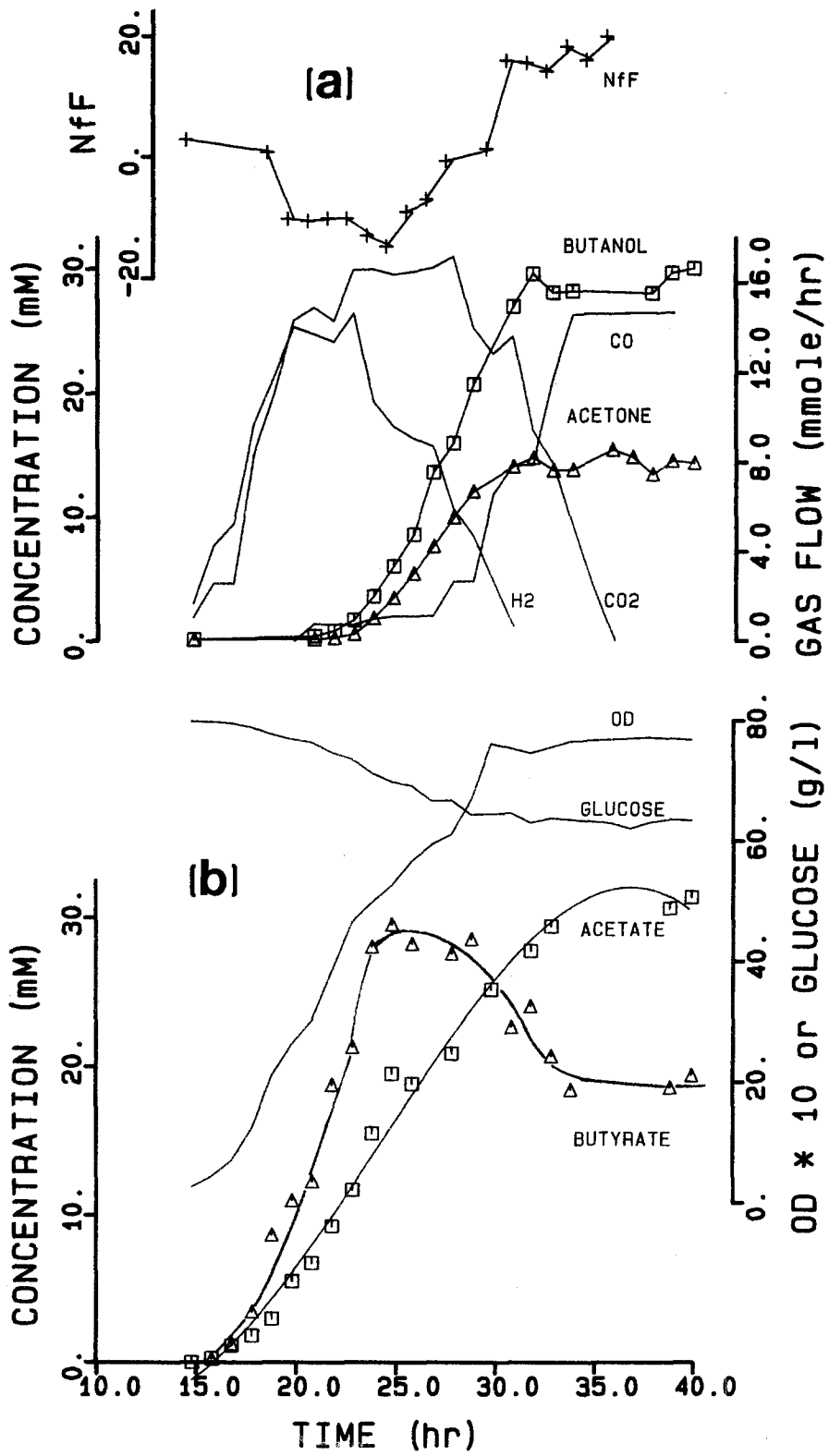


FIGURE 2

acetate formation was reversibly inhibited by the CO sparging as shown in Fig. 1c. The Y_{ATP} decreased continuously during the fermentation from a value of 19 at the early stage of the fermentation down to 2.5 at the end of the fermentation (Fig. 1d).

The effect of CO sparging on a fermentation with reduced nitrogen-source availability is shown in Fig. 2. Yeast extract was added at 5 g/l in the medium, the ratio of NH_4^+ to glucose was 0.0256 with an initial glucose concentration of 80 g/l⁴ (444 mM), and the pH was controlled at 3.7. Because pH values below 3.8 reduce drastically the availability of organic and inorganic nitrogen, this fermentation was eventually limited by the nitrogen supply (Roos et al, 1985). Although CO sparging had an immediate effect in inhibiting H_2 production, reversing the flow of electrons (NfF from negative and decreasing became positive and increasing) and inducing solvent formation, production of solvents could not be sustained, apparently due to nitrogen limitation of growth.

In conclusion, it appears that an increased availability of reduction energy not released as H_2 enhances solvent formation, as has been originally postulated. CO sparging may potentially be used for production of solvents at high concentrations under conditions of excess carbon- and nitrogen-source supplies. Our results are, qualitatively, in agreement with those reported recently by Kim et al (1984).

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Figure 1. The effect of CO on a batch fermentation of *C. acetobutylicum* under conditions of excess carbon- and nitrogen-source supplies at pH = 4.5. Initial glucose conc. was 310 mM and initial molar (NH_4^+)/Glucose was 0.292. For clarity, only a small number of experimental-data points are shown. However, curves were drawn employing all the experimental data. The CO scale in 1c and 1d is the same with that of 1a. OD is optical density at 600 nm.

Figure 2. The effect of CO on a batch fermentation of *C. acetobutylicum* under conditions of limited nitrogen-source supply at pH = 3.7. Initial glucose conc. was 444 mM and the initial molar (NH_4^+)/Glucose was 0.0256. For clarity, the experimental points of the gas, glucose and OD (at 600 nm) data are not shown on the figures.