

Role of Formaldehyde in the Utilization of C₁ Compounds via the Ribulose Monophosphate Cycle

ELEFTHERIOS PAPOUTSAKIS, HENRY C. LIM, and
GEORGE T. TSAO, *School of Chemical Engineering, Purdue
University, West Lafayette, Indiana 47907*

Summary

Formaldehyde, an intermediate in methanol oxidation, plays an important role in controlling methanol utilization by microorganisms which fix carbon via the ribulose monophosphate (RMP) cycle. The extracellular formaldehyde concentration profiles in batch cultures at low and high starting methanol concentrations with and without the presence of semicarbazide in the media, suggest the role played by formaldehyde. The impact of formaldehyde on growth is demonstrated by the time dependent and the initial methanol concentration-dependent cell-mass-yield coefficient. Kinetic studies of the enzymes involved in the oxidation and incorporation of C₁ units suggest that enzyme inhibition cannot account for the observed growth inhibition. A mechanism is proposed to explain methanol and formaldehyde utilization that stresses the transcriptional regulation of mass flow around formaldehyde in assimilation and oxidation pathways through repression and induction.

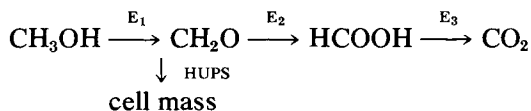
INTRODUCTION

The advantages of methanol as a carbon-energy source for single-cell protein (SCP) production have been well established. Methanol, as most nonsugar substrates, inhibits growth at high concentrations and poses stability problems in continuous reactors (chemostats) for SCP production.¹⁻³ Furthermore, it is thought to be responsible for the cell mass yield which drops with increasing substrate concentrations.³⁻⁵ Finally, the kinetics of substrate inhibited cellular growth is very little understood. There exists no model that can accurately describe the inhibitory growth, although many empirical fits have been proposed.^{1,6,7} These fits deal with modeling of the μ vs. S curve obtained experimentally from batch shake-flask experiments, although this curve is supposed to represent growth under steady-state conditions in a chemostat. No model has

been proposed that describes realistically transient situations. The models that have been proposed for the steady-state μ vs. S curve seem to have been inspired by the usual enzyme-substrate inhibition,¹ i.e., the rate controlling step was presumed to be a substrate-inhibited enzymatic reaction that occupies a key position in the intracellular flow of the limiting substrate. Quite clearly, another presumption has been made that the intracellular concentrations (or specific activity) of the enzyme, which catalyzes this step, is constant; a hypothesis necessary for the derivation of the kinetic expressions. Although this may turn out to be the case in some situations, it is not general, in particular for enzymes in key positions; repression and induction mechanisms regulate the amounts of enzymes—meeting the cellular needs and nutrient availability.

The inhibitory effects are suspected to be due not to the substrate itself, but to an intermediate or final metabolite that is excreted into the cell environment. Harwood and Pirt⁸ removed this inhibitory by-product using the synthetic resin Amberlite CG120 for methane-utilizing bacteria. Likewise, Weaver and Dugan⁹ reported the use of various clays. As summarized by Naguib¹⁰ methanol exerts inhibition on methane oxidizers even at very low concentrations. The use of mixed populations in methane-utilizing cultures has been found effective if the inhibitory or repressive compounds, e.g., methanol, could be utilized by accompanying microorganisms.¹¹

Formaldehyde and formate are intermediates of methanol oxidation by microorganisms. Formaldehyde is the oxidation level at which the C_1 compounds are fixed into cell mass, when the ribulose monophosphate cycle (RMP) is involved.¹² These facts are depicted in the reaction scheme:



where E_1 is methanol oxidase or dehydrogenase, E_2 is formaldehyde oxidase or dehydrogenase, E_3 is formate dehydrogenase, and HUPS is hexulose phosphate synthase. The oxidation level at which carbon is fixed into cell mass is both formaldehyde and formate when the serine pathway^{13,14} is involved.

When formaldehyde is used as a cosubstrate with methanol, it inhibits growth even at very low concentrations. Pilat and Prokop⁵

found that inhibition caused by CH₂O in the presence of 1% CH₃OH, occurs at 0.022mM, while at 0.223mM, the inhibition is complete. Similar effects have been found by the same investigators for formate.⁵ In particular, formaldehyde is 10 times more inhibitory than formate, which is 10 times more inhibitory than methanol. Given the key placement of formaldehyde in the oxidation–assimilation pathways of methanol, its role emerges as potentially critical to the elucidation of the inhibition mechanism.

Various investigators have shown that the enzyme activities (and, thus, the amounts of enzyme per cell) change during the batch cultivation of substrate-inhibited microorganisms and in particular for methanol utilizers.^{15,16} Other investigators^{17,18} have shown that the key enzymes associated with a particular biochemical pathway or with a simple oxidation step, which is further necessary for the assimilation of a given carbon source, are inducible and that clearly the inducers are the substrates (or some intracellular intermediates associated with them) themselves.

It was also shown^{3,5} that the cell mass yield decreases with increasing initial methanol concentrations in batch cultures. In addition, the cell-mass yield is reported⁴ as a function of time during a batch growth experiment. Namely, the yield reaches a maximum at the very early exponential phase (or late lag) and decreases from thereon almost linearly until the end of this phase.

This paper is aimed at elucidating the role of formaldehyde, and a mechanism is proposed for the inhibitory nature of methanol through the impact of formaldehyde on growth. It is believed that the control mechanisms of growth that result in substrate inhibition are localized in the first steps of oxidation and fixation of the C₁ unit. Inferences to this are found in the enzymatic work by Dellweg et al.,¹⁵ and in the following analysis. On the growth curve of μ vs. S_0 , the initial substrate concentration, there exists a maximum point for μ . At this point (although we deal with unsteady transient growth) the maximum capability of cells of incorporating substrate into cell mass is reached. That is, HUPS exhibits its maximum intracellular specific activity. With any further increase in S_0 the carbon incorporation rate (or HUPS specific activity) decreases. This decrease cannot be due to any feedback inhibition by any subsequent intermediate because this would tend to keep the enzyme activity at its maximum and not reduce it; since everything produced after the hexose phosphate (product of the enzymatic step catalyzed by HUPS) will depend on the activity of HUPS. An end-product repression would have the same effect on the HUPS

activity, i.e., keeping it at its maximum value. It should be concluded, therefore, that something else is produced at higher initial methanol concentration that through enzyme inhibition or repression reduces the intracellular activity of HUPS, something whose production does not depend upon the HUPS activity. It is quite clear that the whole subject reduces eventually to understanding the mechanism that controls the intracellular formaldehyde pool.

As a consequence, a study of the enzymes E_1 , E_2 , E_3 , and HUPS was undertaken to determine the levels of substrate inhibition of these enzymes so that mechanisms can be asserted or rejected. For the same reason, free formaldehyde was assayed in the growth medium with methanol as substrate.

EXPERIMENTAL MATERIALS, EQUIPMENT, AND METHODS

Chemicals and Biochemicals

All the chemicals used were of analytical grade except for *para*-formaldehyde which was practical grade (Eastman Kodak Co., N.Y.). Formaldehyde was prepared by heating 0.5 g *para*-formaldehyde in 25 ml water at 115–125°C in a sealed tube. The concentration of the resulting biologically active (i.e., monomer) formaldehyde was determined by using alcohol dehydrogenase (EC 1.1.1.1) from yeast.¹⁹ Thirty-six to 40 hr of heating were found necessary for complete depolymerization of *para*-formaldehyde giving a 95–98% yield. Lithium hydroxypyruvate, 2-mercaptoethanol, chromotropic acid, all the enzymes, coenzymes, sugar phosphate, and bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis).

Preparation of Buffers

The phosphate buffers were prepared by mixing appropriate quantities of Na_2HPO_4 and KH_2PO_4 and bringing to the desired final concentrations. The tris-HCl buffers were prepared by adjusting the pH with HCl.

Chemical Determinations

Formaldehyde was determined by the chromotropic acid method according to West and Sen.²⁰ The method utilizes a one ml solution containing 0.2–20 μg CH_2O , to which 1 ml 1% solution of chromo-

tropic acid in concentrated H₂SO₄ is added and which is mixed thoroughly and cooled, and its extinction is read at 570 nm. A Bausch and Lomb spectrophotometer (Spectronic 70) was used.

Protein was assayed by the Folin-Ciocalteu method with bovine serum albumin as standard.²¹ Methanol was assayed with a flame ionization detector on a Bendix 2300 series dual column gas chromatograph using 3 ft columns, packed with Porapak N, 80–100 mesh, at 130°C. Detector and inlet temperatures were 300–400 and 300°C, respectively. The carrier gas was helium and the output signal was recorded on a Varian Aerograph model A-25 recorder.

Growth of the Microorganism

Methylomonas EP-1 used in this work was obtained as a contaminant of *Methylomonas albus* BG8²² culture which was a gift of Professor J. F. Wilkinson (Edinburgh, Great Britain). It is a short-rod forming, gram-negative bacterium, which is catalase and oxidase positive. It grows well on methanol, poorly on formaldehyde, glucose and ethanol²³ as sole carbon sources. Methanol concentrations up to about 7% (v/v) support growth.³

The working medium contained: MgSO₄·7H₂O, 1 g/liter; CaCl₂, 0.1 g/liter; NH₄Cl, 0.3 g/liter; FeSO₄·7H₂O, 0.01 g/liter; ZnSO₄·7H₂O, 0.025 g/liter; 66 ml/liter of pH 6.9, 0.6M phosphate buffer (final concentration, 0.0396M), and EDTA-disodium salt, 0.04 g/liter. This medium was used for all batch (shaking flasks) experiments in 1000, 500, and 250 ml narrow mouth Erlenmeyer flasks with 500, 100, and 50 ml working volumes, respectively. A New Brunswick Scientific Co. gyratory shaker, model V, was used at 200–250 rpm. The 1000 ml flasks were used for preparing large quantities of organisms for cell-free extract preparations. In continuous culture experiments the above medium was modified using NH₄Cl, 0.9 g/liter and a pH 7.2 buffer instead of 6.9.

Bacterial growth was determined by measurement of extinction at 350, 450, or 550 nm in the aforementioned spectrophotometer. Owing to precipitation of phosphates, corrections had to be made with an appropriate blank for batch experiments. The growth temperature was 30 ± 0.5°C unless stated otherwise.

Preparation of Cell-Free Extracts

Freshly harvested cells which had been grown continuously or in batch (0.1% (v/v) methanol (MeOH)) were washed twice and centrifuged at 8,000 rpm (Beckman model J-21B centrifuge, JA-14

rotor) and were suspended in 3–5 ml/g wet weight of 0.5M, pH 7.0, phosphate buffer and 2mM 2-mercaptoethanol. The addition of α -mercaptoethanol was necessary for retaining the enzymatic activity of formaldehyde dehydrogenase. Cells were disrupted by sonication for 3–4 min at 0–4°C. (Sonifier W-140, Heat Systems Ultrasonics Inc. An aluminum cooling cell, i.d. = 0.94 in., total height 2.70 in., was used with a regular sonication tip.) Sonication times greater than 5 min destroyed the enzyme activities partially; 12 min sonication time destroyed them completely. Disrupted cells were centrifuged for 25 min at 25,000 g and the resulting supernatant was used as cell-free extracts. Pellets were resuspended as much as possible in a small volume of the aforementioned buffer and centrifuged at 25,000 g for 15 min; the thus obtained supernatant will be referred to as a second cell-free extract. The pellet will be referred to as the particulate fraction. All the above operations were carried out at 0–4°C.

Enzyme Assays

Spectrophotometric assays were carried out in a recording Perkin–Elmer model 124 double-beam spectrophotometer at $26 \pm 2^\circ\text{C}$.

i) **Hexose phosphate synthase.** The enzyme assay system used was that of Lawrence et al.,²⁴ scaled to a total volume of 3 ml. The reaction was stopped with 0.2 ml 4N HCl. Three ml of 20% TCA were added, mixed thoroughly, and centrifuged immediately at 14,000 rpm (JA-20 rotor) for 40 min. The supernatant was assayed for formaldehyde. The amounts of formaldehyde consumed were determined at different reaction times. The reaction was linear for about 2 min with 1.9 mg protein in the reaction system. The initial rate was determined as the average for $\frac{1}{2}$ and 1 min reaction times. For these short reaction times no CH_2O was found to react in appropriately incomplete reaction systems. A discussion on this subject follows later.

ii) **D-glycerate–NAD⁺ oxidoreductase (EC 1.1.1.29) (hydroxypyruvate reductase).** This enzyme was assayed at pH 6.5, 7.0, and 7.5 as described by Large and Quayle.²⁵

iii) **Methanol oxidase (EC 1.1.3.13).** This enzyme was assayed in accordance with the method of Janssen and Ruelius²⁶ with peroxidase and *o*-dianisidine with various amounts of MeOH and cell extracts; alternatively, it was assayed in a simple reaction mixture containing 250 μmol phosphate buffer, pH 6.5, 7.0, and 7.5, different amounts of MeOH, and extract (or particulate fraction) up

to 3 ml. The reaction was stopped with 0.2 ml 4N HCl, and, after adding 3 ml 20% trichloroacetic acid (TCA), the mixture was centrifuged at 14,000 rpm (JA-20 rotor) for 25 min and the supernatant was assayed for formaldehyde.

In both reaction systems the reaction was allowed to go for a wide range of times, ranging from 1 min up to 1.5 hr.

iv) **Methanol dehydrogenase.** NAD-linked methanol dehydrogenase (EC 1.1.1.1) was assayed according to Wenger and Bernofsky²⁷ or Racker²⁸ with various amounts of substrate and at various pH (appropriate phosphate buffers were used).

(PMS) Phenazine methosulfate- and 2,6-dichlorophenolindophenol (DCPIP)-linked or only PMS-linked methanol dehydrogenase (EC 1.1.99.8), with or without NH₄⁺, with or without KCN at pH 9.0, 8.5, 7.5, and 7.0 (according to Kato et al.²⁹) were assayed according to Anthony and Zatman.³⁰

Cytochrome-c-linked methanol dehydrogenase was assayed according to Kato et al.²⁹ but, in addition, with varying amounts of cytochrome c and at various pH values (7.0, 7.5, 8.0). Methylene-blue-dependent methanol dehydrogenase was assayed according to Sakaguchi et al.¹⁷

v) **Formaldehyde dehydrogenase (EC 1.2.1.1) and formate dehydrogenase (EC 1.2.1.2).** NAD-linked dehydrogenases were assayed in a reaction system as was done by Johnson and Quayle³¹ with and without glutathione (GSH) and with and without KCN for formaldehyde dehydrogenase. The reaction system contained 1.5 μmol NAD, 210 μmol phosphate buffer of different pH values, 42 μmol formate or 15 μmol formaldehyde, 4.0 μmol GSH, and 4.5 μmol KCN, the last two added only for the formaldehyde dehydrogenase system. The final volume was 3 ml.

vi) **NADH₂ oxidizing enzyme activity.** Reaction was followed spectrophotometrically, by the decrease in extinction at 340 nm, in a simple reaction system consisting of 100–200 μmol, pH 7.5 or 6.5, phosphate buffer, 0.4–1 μmol NADH₂, and 0.1 ml extract in a total volume of 3 ml.

RESULTS

Enzyme Studies

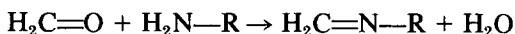
i) **Hexose phosphate synthase (HPS).** Under standard assay conditions this enzyme gave a specific activity of 3027 (*n* mol CH₂O) (min)⁻¹ (mg protein)⁻¹.

The natural substrate for hexulose phosphate synthase (HUPS)

is D-ribulose-5-phosphate,¹² but it also accepts D-ribose-5-phosphate as substrate exhibiting 10% less activity.¹²

Figure 1 shows a Lineweaver-Burk plot for HPS. For higher formaldehyde concentrations, higher sugar-phosphate concentrations were used. The apparent K_m for formaldehyde is approximately 13mM. Considering that the sugar phosphate concentration was not kept absolutely constant, the affinity of the enzyme for CH_2O does not depend much upon the sugar concentration.

Formaldehyde reacts with all compounds that bear an $-\text{NH}_2$ group; namely,



and as such it reacts with proteins without any specificity. For this reason, blank runs had to be made for the enzyme assay. For less than 60–80 μmol CH_2O in the reaction system the unspecific reaction of CH_2O is almost zero during the first 2 min and approaches 8–10% at 10 min, depending upon the exact CH_2O concentration. For amounts higher than 100 μmol CH_2O in the system, however, this reaction becomes so severe that it was almost impossible to determine accurately the enzymatically reacting amounts of CH_2O . Therefore, we could not reach the CH_2O concentrations where enzyme inhibition could probably occur. For up to 100 μmol /cuvet (33.3mM CH_2O), no inhibition was observed for this enzyme.

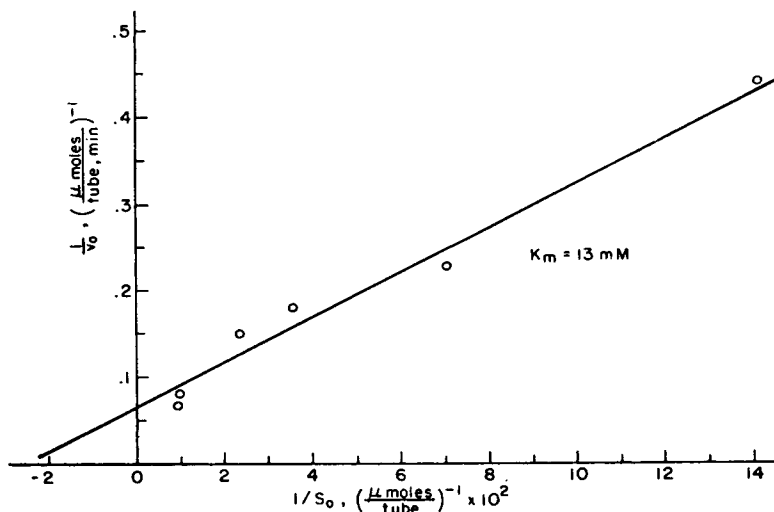


Fig. 1. Lineweaver-Burk plot for HPS, $T = 30^\circ\text{C}$.

ii) **Hydroxypyruvate reductase (HPR).** No HPR activity has been detected either at pH 6.5, 7.0, or 7.5. Instead, a strong NADH-oxidizing activity independent of any other substrate was detected. Details are found elsewhere.²³

iii) **Methanol oxidase; methanol dehydrogenase.** No enzyme has been found, either in cell-free extracts or in the particulate fraction, which is responsible for the oxidation of CH₃OH to CH₂O. It is speculated that it is a dehydrogenase for which the electron acceptor is yet to be found.³

iv) **Formaldehyde dehydrogenase.** Only NAD-linked formaldehyde dehydrogenase has been detected in cell-free extracts. Its activity has been found independent of the presence of GSH in contrast to what is usually reported in the literature.¹⁶ Its pH optimum was at 7.5. Figure 2 shows a Lineweaver-Burk plot for the enzyme from one batch of extracts prepared from batch grown cells. A concentration of 100mM CH₂O is required to cause 20% inhibition of the enzyme even at the low NAD concentration of 0.5mM. NAD stabilizes the enzyme activity at higher concentrations.

v) **Formate dehydrogenase (NAD-linked).** The enzyme had a pH optimum from 6.8–8.0. Figure 3 shows that 100mM formate is needed to cause 25% inhibition of this enzyme, even at very low NAD concentration (0.5mM).

The ratio FF = (formate dehydrogenase)/(formaldehyde dehydrogenase) for three different batches of extract²³ was as follows:

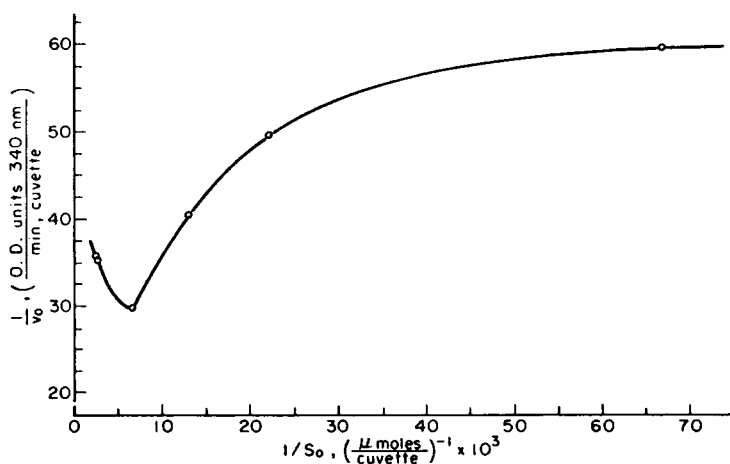


Fig. 2. Lineweaver-Burk plot for formaldehyde dehydrogenase. pH = 7.5; $T = 26 \pm 2^\circ\text{C}$; NAD = 1.5 $\mu\text{mol/cuvet}$.

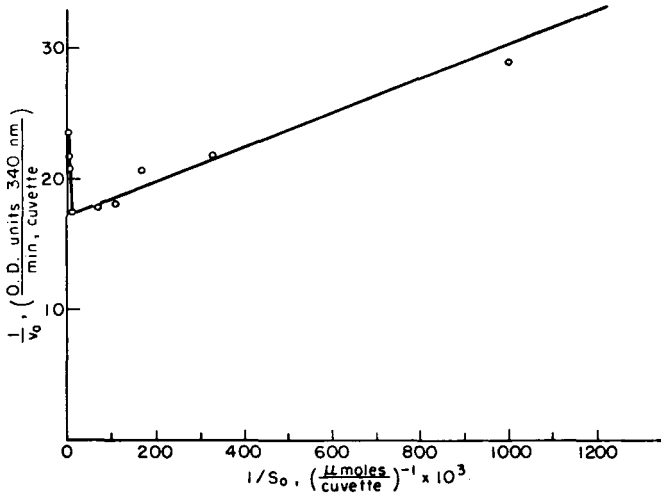


Fig. 3. Lineweaver-Burk plot for formate dehydrogenase. pH = 7.0; $T = 26 \pm 2^\circ\text{C}$; NAD = $1 \mu\text{mol/cuvet}$.

a) Batchwise grown cells (mid-exponential phase), FF = 1.98; b) batchwise grown cells (end of exponential phase), FF = 3.35; c) continuously grown cells, FF = 1.00.

Recalling that the batch grown cells have been harvested at the mid to late exponential phase, we conclude that the first two values express an unbalanced growth, while the third value, according to the mass flow balance, indicates balanced growth at a steady state. There is no need for more formate dehydrogenase than formaldehyde dehydrogenase because all formaldehyde oxidized to formate will be eventually oxidized to carbon dioxide. It is apparent from the above that the microorganism utilized the RMP cycle and not the serine pathway.

Detection of Free Formaldehyde

Preliminary experimental findings had shown that free formaldehyde was excreted from the methanol-grown cells into the nutrient medium and that the concentration of free formaldehyde changed with time during the course of a batch culture. Moreover it had been shown that the rate of change and the absolute amounts of free formaldehyde depend somehow on the initial methanol concentration. It was thus decided to follow the free formaldehyde concentration during the course of batch cultures. Two initial methanol concentrations corresponding to substantially different

initial specific growth rates were chosen so that by following the CH₂O concentration in the reaction mixture during batch growth, we should be able to observe differences in absolute amounts, amounts per optical density (OD) unit, or the rates of CH₂O production; these were 0.2% and 3% (v/v).

A trapping agent for CH₂O was required so that we could trap the CH₂O produced and see the effect of its trapping on growth. This trapping agent should release CH₂O under strong reaction conditions so that CH₂O can be assayed by chromotropic acid. We discussed earlier that CH₂O reacts with compounds that possess an amine group, the reaction being reversible. From this group of compounds semicarbazide (SC), H₂N—NH—CONH₂, was chosen because it meets the specified requirements.³² Since its reaction with CH₂O is not quantitative, we expected to sufficiently trap 10 mg/liter CH₂O (i.e., 0.33 μmol/liter) by 1 μmol/liter SC in our reaction system, an amount chosen purposely low to avoid any possible inhibitory effects. One μmol/liter SC was used with both 0.2% and 3% (v/v) initial methanol concentrations ((MeOH)₀).

Figure 4 shows the results of the experiment with 0.2% (v/v) (MeOH)₀ in duplicates. Figure 5 shows the corresponding curves on 3% (MeOH)₀. Figure 6 shows the curves corresponding to 0.2% and 3% (MeOH)₀ with SC in the reaction systems. For comparison the four formaldehyde concentration profiles (one from each of Figs. 4 and 5, and those of Fig. 6) were superimposed in Figure 7. The formaldehyde concentration scale is logarithmic and the time scale has an arbitrary origin, so that neither the absolute nor the relative log-phase duration is shown.

As shown in Figure 4 the free formaldehyde concentration increases initially, it reaches a maximum at early to mid-exponential phase (OD = 0.3–0.4), and then decreases steadily to depletion (one more point close to zero is not shown owing to the logarithmic scale). Methanol is also completely exhausted by the end of growth. We don't have any other choice but to assume that formaldehyde is excreted from the cells by a diffusion mechanism owing to an intracellularly formed CH₂O pool and that it is eventually retransported into the cells when the intracellular concentration becomes lower than the extracellular concentration. Thus, treating the system as a two-phase system with a biotic and an abiotic phase (the nutrient medium), we can write the following mass balance for CH₂O:³

$$\frac{dS_2}{dt} = K_1X(C_2 - S_2) \quad (1)$$

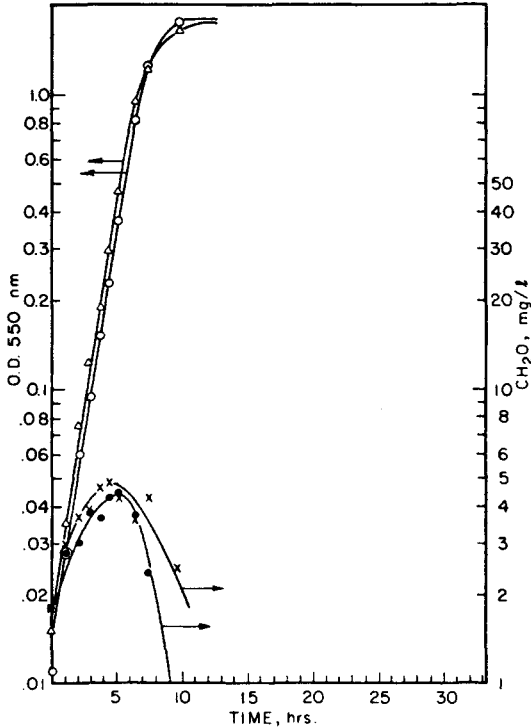


Fig. 4. Formaldehyde concentration during growth on 0.2% (v/v)(MeOH)₀.

where S_2 is the free formaldehyde concentration, C_2 is the intracellular formaldehyde concentration, X is the dry biomass concentration, and K_1 is a physical constant of the system. Clearly initially $C_2 > S_2$, when (CH_2O) reaches its peak $dS_2/dt = 0$ and thus $C_2 = S_2$ and from thereon $C_2 < S_2$. It is easily seen that the maximal dS_2/dt occurs at the very early exponential phase indicating a maximal difference, $C_2 - S_2$. As it is shown in the appendix, C_2 follows a profile similar to that of S_2 with a phase difference, that is, its peak occurs earlier than that of S_2 . These profiles of C_2 and thus of S_2 are apparently caused by a desynchronization of intracellular CH_2O production and uptake.

Similar phenomena appear to occur in Figure 5 with 3% (MeOH)₀. However, CH_3OH is not exhausted in the growth medium by the end of exponential phase, apparently causing the intracellular C_2 to start increasing again. Indeed at the observed minimum of S_2 , $dS_2/dt = 0$ and $S_2 = C_2$, while from thereon $dS_2/dt > 0$ and $C_2 > S_2$. It was found later that above an OD of 2.5, the

growth was limited by the nitrogen source (ammonium salt). It is observed that with 3% (MeOH)₀, the maximal S_2 is higher than that corresponding to 0.2% (MeOH)₀ (5.65–6.56 and 4.45–4.85 mg CH₂O/liter, respectively). It is not very difficult to show also that for the same S_2 the corresponding C_2 is higher for 3% (MeOH)₀ than for 0.2% (MeOH)₀ by calculating the quantity $(1/X)(dS_2/dt)$ (approximated by the quantity $(1/OD)(\Delta S_2/\Delta t)$, appropriately discretizing the time) as suggested by eq. (1) and for $OD \geq 0.050$ (where the calculations are accurate). Finally it is noted that the maximum S_2 occurs at a lower OD (0.2–0.3) for 3% (MeOH)₀ as compared to an OD of 0.3–0.4 for 0.2% (MeOH)₀.

When SC is added in the growth medium, the lag phases are prolonged considerably for both 0.2% and 3% (MeOH)₀. The specific growth rate μ is not affected in the case of 0.2% (MeOH)₀, while it is lowered in the case of 3% (MeOH)₀ (0.42 hr⁻¹ without

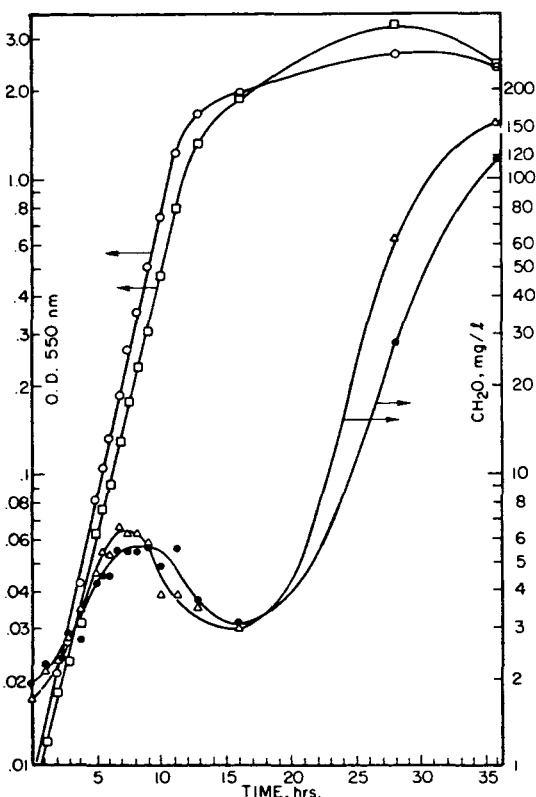


Fig. 5. Formaldehyde concentration during growth on 3% (v/v)(MeOH)₀.

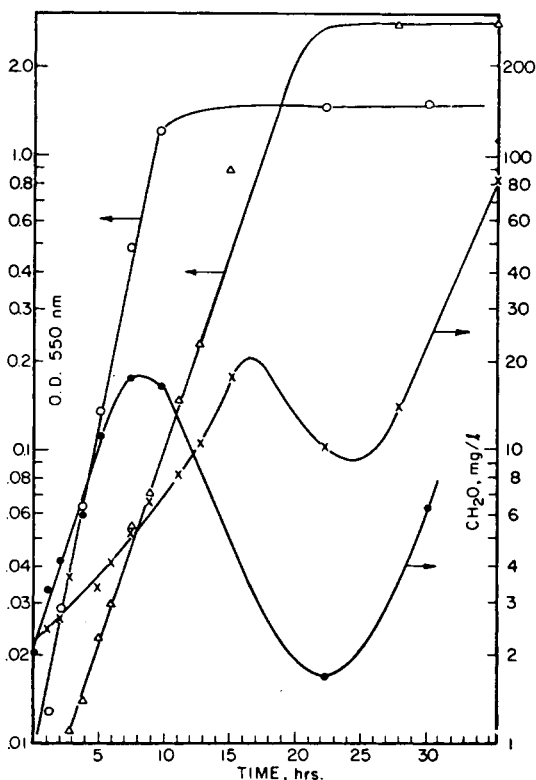


Fig. 6. Formaldehyde concentration during growth on (●) 0.2% and (×) 3% (v/v) (MeOH)₀ with SC.

SC versus 0.30 hr^{-1} with SC). Semicarbazide cannot be responsible for the inhibition of growth by itself since if it should be the case, inhibition should occur in the 0.2% (MeOH)₀ case as well. Clearly these experiments with SC demonstrate that the growth is directly associated with the rate of production and the concentration of CH₂O since these two quantities are only affected by the presence of SC. The CH₂O profiles obtained with SC are remarkably different than the ones obtained without SC. Formaldehyde is accumulated in the medium at significantly higher concentrations; the maximal S_2 for 0.2% and 3% are 17.63 and 17.75 mg CH₂O/liter, respectively as compared to the corresponding 4.65 and 6.1 mg/liter without SC. Apparently, these high CH₂O concentrations occur because CH₂O reacts with SC and thus the driving (diffusional) force $C_2 - S_2$ remains high, and therefore more CH₂O diffuses out

of the cells. When the trapping capacity of SC is exhausted, the same qualitative phenomena occur as without SC.

During continuous cultivation with CH₃OH very small amounts of CH₂O were detected in the reaction system, 0.2–0.7 mg/liter. This indicates that for balanced growth very small CH₂O concentrations are needed intracellularly to support growth.

DISCUSSION

Mechanism of Methanol Utilization

Dellweg et al.¹⁵ have shown experimentally that (E₁), (E₂), and (E₃) change with time during a batch culture of *Kloeckera* yeast species. Similarly, Ogata et al.¹⁶ have shown that (E₂) changes during a batch culture of the same yeast species. Clearly, there must be a mechanism to account for these changes in enzyme concentrations.

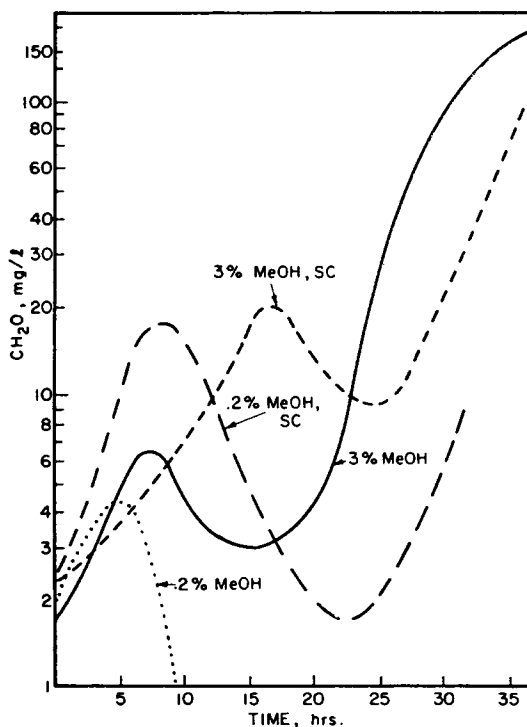


Fig. 7. Formaldehyde concentration profiles during growth on 0.2% and 3% (v/v)(MeOH)₀ with and without SC.

We have shown that C_2 follows a profile similar to that of S_2 with time. A mass balance on the biotic phase of the system for CH_2O gives,³

$$\frac{dC_2}{dt} = r_{12} - r_{22} - r_{32} - r_{42} - \mu C_2 \quad (2)$$

where r_{12} is the rate of CH_2O production caused by CH_3OH oxidation, r_{22} is the rate of CH_2O conversion to HCOOH , r_{32} is the rate of CH_2O incorporation into cell mass, r_{42} is the rate of CH_2O diffusion to the abiotic phase, and μC_2 is the term that takes care of C_2 dilution caused by the expansion of biomass. It can be shown from the data of the present experiments that μC_2 is of the order of magnitude of $10^{-6}r_{32}$, and that r_{42} decreases steadily until almost the end of exponential phase and is always less than 1.2% of r_{32} . Moreover, it can be shown³ that

$$r_{32} = K_{II}\mu \quad (3)$$

where K_{II} is a physical constant of the system. Thus, eq. (2) simplifies to

$$\frac{dC_2}{dt} = r_{12} - r_{22} - K_{II}\mu - r_{42} \quad (4)$$

Since $dr_{42}/dt < 0$, it is not difficult to see that during the fast rising portion of the C_2 profile the dominant term is r_{12} , while after C_2 reaches its maximum the term r_{22} dominates, since (dC_2/dt) becomes negative while μ remains apparently constant. In other words, during the early part of growth CH_3OH oxidation is not accompanied by CH_2O oxidation while later a stronger CH_2O oxidation is observed.

Since dC_2/dt can be estimated to be less than 0.01% of r_{32} , from eq. (2) we can write

$$r_{12} = r_{22} + r_{32} \quad (5)$$

while for the incorporation of CH_2O into cell mass we can write,



where $\text{C}_4\text{H}_8\text{O}_2\text{N}$ represents the dry cell mass. Then, we can write from eqs. (5) and (6) and by definition for the cell-mass yield:

$$Y = (\text{mol CH}_2\text{O converted to C}_4\text{H}_8\text{O}_2\text{N}) \left(\frac{102}{4} \right) /$$

$$(\text{mol CH}_3\text{OH consumed})(32) = 0.80 \frac{r_{32}}{r_{32} + r_{22}} = \frac{0.80}{1 + r_{22}/r_{32}} \quad (7)$$

Reuss et al.⁴ have shown experimentally how Y changes with time during a batch culture; namely, that Y has a maximum at late lag or early exponential phase and decreases from thereon until the end of the exponential phase. The maximum Y is apparently achieved by the initial increase of $r_{32} = K_{11}\mu$ until the growth enters the exponential phase where $\mu = \text{constant}$, while the subsequent decrease of Y can only be attributed to an increase of r_{22} as eq. (7) suggests. It has also been shown^{3,4} that the overall yield decreases with increasing initial methanol concentration. It is then clear that for higher $(\text{MeOH})_0$, r_{22}/r_{32} is higher, that is, more C₁ units are oxidized eventually to CO₂ than incorporated into cell mass. For higher $(\text{MeOH})_0$, the corresponding μ are lower and, as we have deduced in the present work, the intracellular CH₂O concentration is higher. Thus, higher CH₂O concentrations are accompanied by lower rates of CH₂O fixation (r_{32}) and higher rates of CH₂O oxidation (r_{22}). Since the presence of CH₂O as substrate inhibits growth on CH₃OH,⁵ we can conclude that CH₂O is the cause for the lower rates of its fixation at higher $(\text{MeOH})_0$.

Since the enzymes E₁, E₂, E₃, and HUPS are not constitutive, CH₃OH must induce E₁ while HUPS could be induced by either CH₃OH or CH₂O. If CH₂O can induce HUPS, then it will be able to serve as sole substrate; if not, CH₃OH must induce HUPS. Among methanol-utilizing yeasts *none* have been found to utilize CH₂O or HCOOH as sole carbon sources. All methanol-utilizing yeasts follow the RMP cycle. All the CH₂O and/or HCOOH-utilizing bacteria have been found to follow the serine pathway.^{13,33} Therefore, CH₂O cannot induce HUPS.

Our enzymatic studies demonstrated that very high CH₂O concentrations, of the order of 35 to 100mM, are needed to cause even a relatively small percentage of inhibition of the key enzymes involved in CH₃OH utilization, while growth inhibition owing to CH₂O in the presence of 1% $(\text{MeOH})_0$ occurs at 0.022mM and the inhibition is complete at 0.233mM CH₂O.⁵ Pure enzyme inhibition cannot thus account for the growth inhibition observed. We thus postulate that E₂ is induced by CH₂O to account for the changing rates r_{12} , r_{22} , and ratio r_{22}/r_{32} in batch and continuous cultures³ as demonstrated by considerable changes in cell-mass yield (eqs. (5) and (7)).

The above-reported ratios of formate to formaldehyde dehydrogenases show that the enzymes responsible for the complete oxidation of CH₃OH are sequentially produced with lags from one to the next. This causes higher E₃ activities in batch culture as can

dehydrogenase shows high specific activities during batch growth, and that it functions optimally over a broader pH range.

The repressive affect of CH₂O on E₁ and HUPS can be visualized either by CH₂O binding to the repressor protein-CH₃OH complex and making it again active to repress the transcription of the appropriate m-RNAs, or by CH₂O competing with CH₃OH for the binding site(s) on the repressor without inactivating it. Alternatively, this repression may be closely associated with energy production and the intracellular concentrations of ATP and cyclic AMP (c-AMP), which frequently has a secondary control function in promoting the transcription of the genes through the *p*-locus of the genome. That is, a decreased concentration of c-AMP represses the production of certain enzymes. An increased CH₂O concentration results in high production rates and high activities of E₂ and consequently in high rates of energy production through NADH from the oxidation of CH₂O to CO₂, and thus in high ATP and low c-AMP pools. Dellweg et al.¹⁵ reported the concentrations of ATP, c-AMP, and other adenylates during a batch culture of a *Kloeckera* yeast species on CH₃OH and their results are in agreement with the ideas exposed above.

Further Evaluation of the Proposed Mechanism

It is quite clear that the induction-repression level of the above discussed key enzymes in the inoculum cells affects greatly the lag phases of the inoculated cultures. If, in particular, the size of the inoculum is large, carrying a considerable concentration of the repressor metabolite (CH₂O), it may prolong the lag phases instead of shortening them. This may be the case reported recently,¹⁰ especially if the cells of the large size inocula were not washed.

Some researchers^{3,15} reported that under certain conditions the first exponential phase is followed by a second one provided that no other nutrients were limiting. It is not difficult to explain such a phenomenon by reduced HUPS concentrations owing to excess CH₂O concentration. Clearly also, the second exponential phase μ will be closer to the corresponding steady-state μ since the system proceeds to that phase without any appreciable changes in environmental conditions but owing to intracellular changes toward the steady-state conditions. It appears, therefore, that batch experiments with substrate-inhibited microorganisms more closely resemble pulse experiments than steady-state experiments. This may be the reason that batch growth rates cannot be approached in continuous cultures.^{3,34}

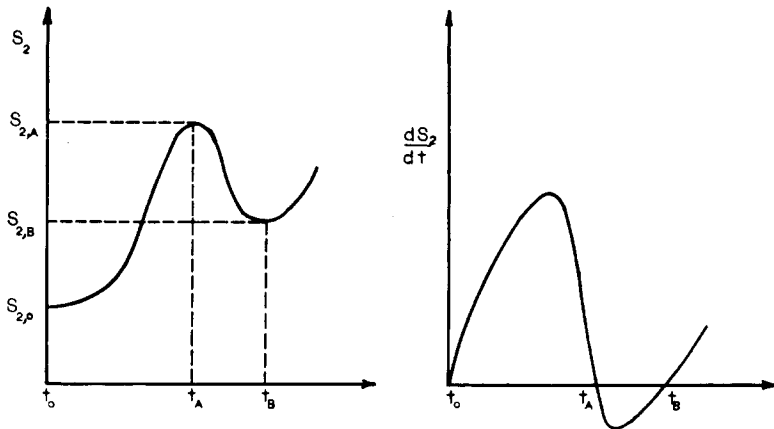


Fig. 9. Time profiles of S_2 and dS_2/dt .

CONCLUSIONS

The enzymatic studies demonstrated that simple inhibition of the key enzymes involved in CH_3OH utilization is not very likely to occur under usual growth conditions. The profiles of free CH_2O in batch cultures on low and high CH_3OH concentrations with and without semicarbazide led us to conclude that the concentrations of the key enzymes are regulated by induction-repression mechanisms. An analysis of the cell-mass yield as a function of time and the initial methanol concentration showed the importance of the above transcriptional phenomena. The mechanism proposed to explain the growth of C_1 -compound utilizers is in agreement with the known facts of C_1 -compound utilization, although some of the mechanism postulates are somewhat arbitrary. It was also concluded that continuous reactor design should not be based on data obtained from batch data, for they may be highly misleading.

APPENDIX

We want to prove that C_2 follows a profile similar to that of S_2 with phase differences for extrema.

Proof: From eq. (1) we obtain

$$C_2 = \frac{1}{K_1 X} \frac{dS_2}{dt} + S_2 \quad (8)$$

and from eq. (8) we obtain by differentiation

$$\frac{dC_2}{dt} = -\frac{\mu}{K_1 X} \frac{dS_2}{dt} + \frac{1}{K_1 X} \frac{d^2 S_2}{dt^2} + \frac{dS_2}{dt} \quad (9)$$

$$\text{for } t \rightarrow t_0^+, \quad \frac{dS_2}{dt} \rightarrow 0, \quad \text{and thus} \quad S_{2,0} = C_{2,0} \quad (10)$$

where t_0 is some time after inoculation. At $t = t_A$ and $t = t_B$, $dS_2/dt = 0$ and therefore

$$S_{2,A} = C_{2,A} \quad \text{and} \quad S_{2,B} = C_{2,B} \quad (11)$$

where $S_{2,0} < S_{2,A}$ and $S_{2,B} < S_{2,A}$.

Since at $t = t_A$, $(d^2S_2/dt^2) < 0$ (Fig. 9) and $dS_2/dt = 0$, we obtain from eq. (9) that $dC_2/dt < 0$. Therefore, C_2 increases from $C_{2,0}$ to $C_{2,A}$ and has at least a maximum at t , $t_0 < t < t_A$. Similarly for $t = t_B$, $d^2S_2/dt^2 > 0$ and $dS_2/dt = 0$ and thus $dC_2/dt > 0$, that is, C_2 decreases from $C_{2,A}$ to $C_{2,B}$ and has at least a minimum at t , $t_A < t < t_B$. Thus, the simplest profile for C_2 is similar to that of S_2 . This completes the proof.

Nomenclature

C_2	intracellular CH ₂ O concentration, g-mol CH ₂ O/liter
c-AMP	cyclic AMP
DCPIP	2,6-dichlorophenolindophenol
E_1	methanol dehydrogenase or oxidase
E_2	formaldehyde dehydrogenase
E_3	formate dehydrogenase
GSH	glutathione
HPR	hydroxypyruvate reductase
HPS, HUPS	hexose, hexulose phosphate synthase
K_1	constant, (hr-g-mol dry biomass) ⁻¹
K_{11}	constant, g-mol CH ₂ O/liter
μ	specific growth rate, hr ⁻¹
MeOH	methanol
OD	optical density
PMS	phenazine methosulfate
RMP	ribulose monophosphate (cycle)
r_{12}	rate of CH ₂ O production based on biomass volume, g-mol CH ₂ O/hr-liter
S_2	extracellular CH ₂ O concentration, g-mol CH ₂ O/liter
S, S_0	substrate, initial substrate concentration
SC	semicarbazide
SCP	single-cell protein
TCA	trichloroacetic acid
t	time, hr
V_0	initial rate of an enzymatic reaction
X	dry-biomass concentration, g-mol dry biomass/liter
Y	cell-mass yield, g dry biomass/g CH ₃ OH
(), () ₀	concentration, initial concentration of a chemical
[]	constant pool of a chemical

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