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Effect of pulse-wise addition of "heterotrophic" substrates
to C_1 substrate-limited continuous cultures

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Abstract. The regulation of methylamine and formaldehyde metabolism in *Arthrobacter* P1 was investigated in carbon-limited continuous cultures. To avoid toxic effects of higher formaldehyde concentrations, formaldehyde-limited cultures were established in smooth substrate transitions from choline-limitation. Evidence was obtained that the synthesis of enzymes involved in the conversion of methylamine into formaldehyde and in formaldehyde fixation is induced sequentially in this organism. Compared to growth with methylamine the molar growth yield on formaldehyde was approximately 30% higher. This difference is mainly due to the expenditure of energy for the uptake of methylamine from the medium.

The addition of a pulse of a "heterotrophic" substrate, glucose or acetate, to C_1 substrate-limited continuous cultures resulted in relief of carbon limitation and transient synthesis of increasing amounts of cell material. Concomitantly, a significant decrease in the specific activities of hexulose phosphate synthase was observed. However, the total activity of hexulose phosphate synthase in these cultures remained clearly in excess of that required to fix the formaldehyde that became available in time. The observed strong decrease in the specific activities of this RuMP cycle enzyme strongly suggests that its synthesis is controlled via catabolite repression exerted by the metabolism of "heterotrophic" substrates.

Key words: *Arthrobacter* P1 — Methylamine — Formaldehyde — RuMP cycle of formaldehyde fixation — Regulation — Carbon catabolite repression — Continuous culture — Transient states

For the growth of the facultatively methylotrophic bacterium *Arthrobacter* P1 on methylamine at least four enzymes are required, namely a methylamine transport system, an amine oxidase, and the ribulose monophosphate (RuMP) cycle enzymes hexulose phosphate synthase (HPS) and hexulose phosphate isomerase (HPI) (Dijkhuizen et al. 1982; Levering et al. 1981 a, b, 1982). Following the observation that these enzymes are usually not present in heterotrophically grown cells of *Arthrobacter* P1, we decided

to adopt this organism as a model system to investigate the regulation of the synthesis of RuMP cycle enzymes in facultative methylotrophs. The nature of these regulatory mechanisms was previously investigated in experiments in which *Arthrobacter* P1 was grown on mixtures of methylamine and acetate, both in batch and continuous cultures (Levering and Dijkhuizen 1985). Growth of *Arthrobacter* P1 in batch culture on this mixture resulted in sequential utilization of the two substrates, with acetate as the preferred substrate. It was subsequently shown that this response could be ascribed to the fact that acetate is a strong inhibitor of the methylamine transport system and amine oxidase in this organism. An analysis of the activities of the C_1-specific enzymes indicated that the synthesis of enzymes involved in methylamine oxidation and those of formaldehyde fixation in *Arthrobacter* P1 is most likely induced sequentially by methylamine and formaldehyde, respectively. Such a mechanism of non-coordinately regulated synthesis of C_1-specific enzymes would also explain the enzyme profiles previously observed following growth of the organism on methylamine, ethylamine or choline (Levering et al. 1981 a, b, 1984).

In the course of these batch culture studies it became clear that the presence of acetate also prevented synthesis of HPS (Levering and Dijkhuizen 1985). This has been attributed to the inhibitory effects of acetate on the conversion of methylamine into formaldehyde, thereby preventing the intracellular production of the inducer from methylamine. It remained possible, however, that acetate or its metabolism additionally controlled the synthesis of this RuMP cycle enzyme via catabolite repression. Such a repression resulting from the metabolism of "heterotrophic" substrates is generally observed in facultatively autotrophic bacteria that fix CO_2 via the RuBP- or Calvin cycle (Dijkhuizen and Harder 1984). Because of the analogy between the RuBP- and RuMP cycles (Anthony 1980; Quayle and Ferenci 1978), the involvement of such a control mechanism in *Arthrobacter* P1 was also possible and could not be excluded on the basis of the available evidence. It was therefore decided to investigate the regulation of methylamine and formaldehyde metabolism in this organism in more detail. For this purpose the development of a method for growing *Arthrobacter* P1 on formaldehyde as the sole carbon- and energy source was considered necessary.

In view of the toxic nature of formaldehyde, a chemostat culture of *Arthrobacter* P1 was set up with formaldehyde as the growth-limiting substrate. Since repressive effects, exerted by the metabolism of one substrate on the utilization of a second substrate, are generally most clearly discernible

under conditions of carbon excess (Harder and Dijkhuizen 1982; Harder et al. 1984), a pulse of either glucose or acetate was added directly to such a culture and the response of the organism was investigated. For comparison results obtained in similar experiments with methylamine-limited continuous cultures are also presented.

Materials and methods

Organism. Arthrobacter P1 NCIB 11625 and its maintenance have been described previously (Levering et al. 1981a).

Medium and continuous culture experiments. Arthrobacter P1 was grown in carbon-limited chemostat cultures in a fermenter (working volume of 1 l) of the type described by Harder et al. (1974). Incubations were in the dark at a temperature of 30°C and the pH was controlled at 7.0 by automatic adjustment with 1 M NaOH. The medium was as described previously (Levering and Dijkhuizen 1985). Heat-sterilized methylammonium chloride or filter-sterilized formaldehyde were added as carbon sources to the medium reservoir at a final concentration of 25 mM. Formaldehyde was prepared from paraformaldehyde by incubating aqueous solutions in closed bottles at 110°C for 10 h.

In order to establish a formaldehyde-limited continuous culture, the organism was first grown on the mineral medium containing 15 mM filter-sterilized choline. In the mid-exponential growth phase, at an absorbance (433 nm) of approximately 2.0, the culture was switched to a continuous-flow system. This involved addition of fresh medium with a peristaltic pump from a medium reservoir containing 3 mM choline plus 25 mM formaldehyde. Once the culture had reached a steady state, the medium reservoir was replaced by one containing 25 mM formaldehyde only.

When a formaldehyde- (or methylamine-)limited continuous culture of Arthrobacter P1 had reached a steady state at a dilution rate of 0.10 h^{-1}, which was assumed to be the case after at least five volume displacements, the metabolic effects of a pulse-wise addition of the "heterotrophic" substrates glucose (3.3 ml; 5 mM final concentration) or sodium acetate (10 ml; 25 mM final concentration), to these cultures was analyzed. In the ensuing period the addition of the C$_1$ substrate continued at the same rate and various samples were regularly withdrawn from the cultures for analysis.

Growth of the organism was monitored by measuring the absorbance at 433 nm using a Vitatron 280 colorimeter (Vitatron, Dieren, The Netherlands). For the assay of enzyme activities, cells were harvested by centrifugation at 6,000 × g for 10 min at 4°C, washed once with 50 mM potassium phosphate buffer pH 7.0 containing 5 mM MgSO$_4$ and resuspended in this buffer to a concentration of 3 – 10 mg dry weight/ml. These suspensions were either immediately used for enzyme assays or stored at −20°C until required.

Assays of enzyme activity. Preparation of cell-free extracts and assays of activities of C$_1$-specific enzymes were according to Levering et al. (1981a; 1982). The rate of [^{14}C-]methylamine and [^{14}C-]formaldehyde transport by whole cells was measured as described by Dijkhuizen et al. (1982), except that the final concentration of formaldehyde was 2.5 mM (0.63 TBq/mol). Cytoplasmic membrane vesicles

of Arthrobacter P1 were isolated from cells grown in methylamine- or formaldehyde-limited continuous cultures according to Dijkhuizen et al. (1982). Isocitrate lyase (EC 4.1.3.1) was assayed according to Dixon and Kornberg (1959), except that 50 mM imidazol-HCl pH 7.5 was used as a buffer.

Assay of activity of hexulose phosphate isomerase. For the determination of the activity of hexulose phosphate isomerase (HPI), cell-free extracts containing 20 – 30 mg protein/ml were used. The reaction mixture (1 ml) contained triethanolamine − HCl buffer pH 7.7, 100 μmol; magnesium chloride, 2.5 μmol; NADP, 0.4 μmol; phosphoriboisomerase, 1.75 U; ribose-5-phosphate, 5 μmol; formaldehyde, 5 μmol; glucose-6-phosphate dehydrogenase, 1 U; glucose-6-phosphate isomerase, 1 U; hexulose phosphate synthase, 0.1 – 0.2 U. This mixture was incubated at 30°C for 5 min to allow the production of hexulose-6-phosphate, the substrate for HPI. The reaction was started by the addition of cell-free extract. As a source of HPS activity, cell-free extracts from methylamine- or formaldehyde-grown cells of Arthrobacter P1 containing up to 5 mg protein/ml were used. At these protein concentrations HPI activity is usually not detectable in this organism (see Levering et al. 1981a).

Residual substrate concentrations. For the determination of substrate concentrations samples were rapidly withdrawn from the cultures, filtered through a Millipore filter (0.2 μm pore size) and immediately placed on ice. These cell-free samples were used directly or stored at −20°C until required. Formaldehyde was assayed with the method of Nash (1953). Acetate and methylamine concentrations were analyzed as described by Levering et al. (1984). Glucose concentrations were determined with the GOD-Perid Method (Boehringer GmbH, Mannheim, FRG).

Protein and dry weight determinations. Protein was determined by the method of Lowry et al. (1951), using bovine serum albumin as a standard. Dry weight of bacterial suspensions was determined with a carbon analyzer (Beckman, model 915A), connected to an infrared analyzer (Beckman, model 865). Carbon contents were multiplied by a factor of 2 to obtain dry weight.

Calculations. Following addition of a pulse of "heterotrophic" substrate to a C$_1$ substrate-limited continuous culture of Arthrobacter P1, the supply of fresh medium containing the C$_1$ substrate continued at the set dilution rate. A partial wash-out of the "heterotrophic" substrate will therefore occur in time. The kinetics of this wash-out (Tempest 1978) assuming that the organism would be unable to use the added "heterotrophic" compound, was calculated using the formula $C_t = C_0 \cdot e^{-Dt}$, where C_t is the concentration (mM) at time t(h), C_0 is the concentration (mM) at zero time and D the dilution rate (h^{-1}).

Biochemicals and enzymes. [^{14}C-]methylamine and [^{14}C-]formaldehyde were obtained from the Radiochemical Centre (Amersham, UK). Catalase was purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other biochemicals and enzymes were obtained from Boehringer, Mannheim, FRG.

Table 1. Enzyme activities in cell-free extracts and molar growth yields (Y_{mol}) of *Arthrobacter* P1 grown in methylamine- or formaldehyde-limited continuous cultures at a dilution rate of $0.10\ h^{-1}$. Activities are expressed as nmol $\cdot$ min$^{-1}\cdot$ mg^{-1} protein, except for catalase; $\Delta E_{240}\cdot$ min$^{-1}\cdot$ mg^{-1} protein. Y_{mol} is expressed as g dry weight of cells produced/mol substrate consumed

| Enzyme	Growth-limiting substrate ($S_R = 25$ mM)	
	Methylamine	Formaldehyde
[^{14}C-]Methylamine transport	290	0
Amine oxidase	1,365	0
Catalase	150	20
Hexulose phosphate synthase	1,170	1,830
Hexulose phosphate isomerase	–	+
Transketolase	800	1,075
Y_{mol}	9.0–9.2	11.5–12.0

0, not detectable
–, not determined
+, present but fluctuating activities (see text)

Results

Growth of *Arthrobacter* P1 on formaldehyde in continuous cultures

To establish a formaldehyde-limited culture of *Arthrobacter* P1, cells were pregrown on choline and, subsequently, on a mixture of choline plus formaldehyde in a chemostat (see Methods). This procedure was adopted since we previously (Levering et al. 1981 b) observed that growth of *Arthrobacter* P1 on choline leads to the synthesis of the RuMP cycle enzyme HPS, probably because formaldehyde is an important intermediate in choline metabolism. As was expected, at $D = 0.05$ or $0.10\ h^{-1}$, a change-over from growth on choline to formaldehyde occurred smoothly and did not result in (transient) accumulation of formaldehyde (limit of detection 0.05 mM). The cultures thus obtained were stable for at least 50 volume displacements at dilution rates up to $0.20\ h^{-1}$, which is in the same order of magnitude as the μ_{max} on methylamine (0.28 h^{-1}; Levering et al. 1981 a). Under no conditions was residual formaldehyde detectable in culture supernatants. After the culture had reached a steady state at $D = 0.10\ h^{-1}$, cells were removed and activities of some key enzymes of C$_1$ metabolism in *Arthrobacter* P1 were measured (Table 1). For a comparison methylamine-limited chemostat cultures were also investigated. As can be seen from Table 1 in formaldehyde-grown cells activities of the methylamine transport system and amine oxidase were absent, whereas methylamine-grown cells contained high levels of these enzymes. Catalase, which plays an important role in the oxidation of methylamine to formaldehyde by degrading the hydrogen peroxide produced (Levering et al. 1981 a; van Vliet-Smits et al. 1981), showed a considerably enhanced activity in methylamine-grown cells as compared to cells grown under formaldehyde-limitation. The first key enzyme of the RuMP cycle, hexulose phosphate synthase (HPS), was present in high activities in cells grown on either of these two substrates (Table 1). As previously observed in cells grown under methylamine-limitation (Levering and Dijkhuizen 1985), the activities of amine oxidase and HPS in cells grown in methylamine- or formaldehyde-limited chemostats at a fixed dilution rate varied considerably in
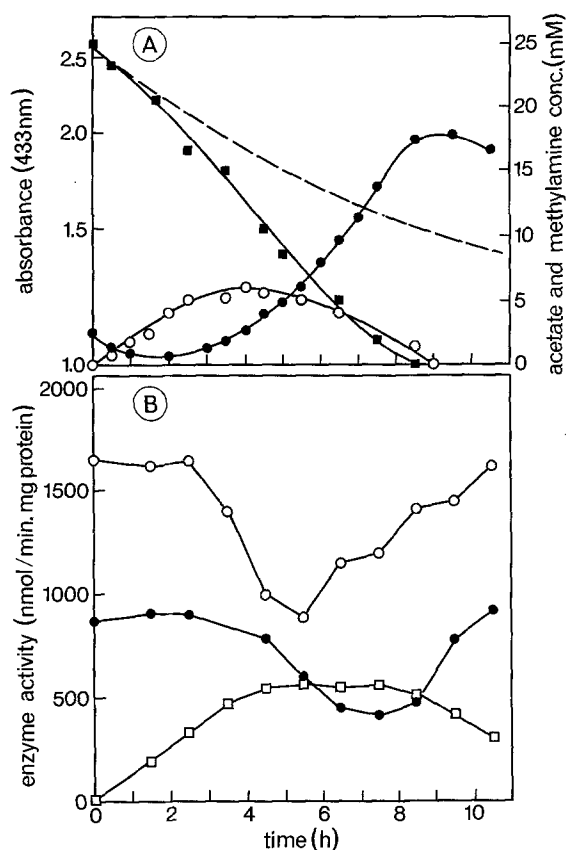
Fig. 1A, B. Addition of acetate (25 mM) to a methylamine-limited ($S_R = 25$ mM) continuous culture of *Arthrobacter* P1 growing at $D = 0.10\ h^{-1}$. **A** ●, absorbance; ○, methylamine concentration; ■, acetate concentration; ––––, wash-out kinetics for acetate; **B** ●, amine oxidase; ○, hexulose phosphate synthase; □, isocitrate lyase

independent experiments. The activities of these enzymes as presented in Table 1 are mean values of at least eight measurements performed on cells harvested from various independent cultures in steady state. These activities ranged from $1-2\ \mu$mol $\cdot$ min$^{-1}\cdot$ mg^{-1} protein in the case of amine oxidase and from $1-2.5\ \mu$mol $\cdot$ min$^{-1}\cdot$ mg^{-1} protein for HPS.

Cells of *Arthrobacter* P1 grown on formaldehyde, methylamine or choline rapidly incorporated [^{14}C-]formaldehyde. In further experiments it became evident that a strong correlation existed between the rate of [^{14}C-]formaldehyde uptake by whole cells and the specific activities of HPS measured in extracts prepared from these cells (data not shown). The observed incorporation of [^{14}C-]formaldehyde might therefore simply be due to passive diffusion into the cell followed by its fixation catalyzed by HPS. To study this in more detail, cytoplasmic membrane vesicles devoid of the cytoplasmic enzyme HPS were isolated from cells of *Arthrobacter* P1 grown on methylamine or formaldehyde. Although these membrane vesicles were able to accumulate [^{14}C-]amino acids, energy-dependent uptake of [^{14}C-]formaldehyde was not observed with any of these preparations.

In these studies we were able to demonstrate for the first time the presence of hexulose phosphate isomerase (HPI), the second key enzyme of the RuMP cycle, in *Arthrobacter* P1 (Table 1). We previously observed (Levering et al. 1981 a)

that extracts of *Arthrobacter* P1 which contained no more than 10 mg protein/ml were virtually devoid of HPI activity. We now observed that in extracts with a protein content of more than 20 mg/ml HPI activity was detectable. Best results were obtained when the reaction was started by the addition of the cell-free extract (see Methods). In doing so these extracts were diluted 10—20-fold and this again resulted in a rapid loss of HPI activities. Attempts to stabilize HPI activity under these conditions by the addition of glycerol, bovine serum albumin or dithiothreitol were unsuccessful. Although cell-free extracts containing active HPI were frequently obtained, taking the precautions outlined above, the absolute levels of the enzyme observed showed a considerable variation in different samples prepared from cells grown under identical conditions. Formaldehyde-limited continuous cultures of *Arthrobacter* P1 ($D = 0.10$ h^{-1}) displayed HPI activities up to 200 nmol $\cdot$ min^{-1} $\cdot$ mg^{-1} of protein.

The activities of transketolase, an enzyme which plays a role in the regeneration of the formaldehyde-acceptor molecule, ribulose-5-phosphate (Levering et al. 1982), were comparably high in cells grown under either formaldehyde- or methylamine-limitation (Table 1). As previously reported for cells of *Arthrobacter* P1 grown on methylamine (Levering et al. 1981a), no activity of a formaldehyde dehydrogenase (NAD- or dye-dependent) was detectable following growth on formaldehyde, nor were these cells able to oxidize formate. The results presented in Table 1 add further strength to the view that in *Arthrobacter* P1 the synthesis of the four C_1-specific enzymes, the methylamine transport system, amine oxidase, HPS and HPI is not regulated coordinately.

Interestingly, the molar growth yield of *Arthrobacter* P1 on formaldehyde was found to be 11.5—12.0 g/mol, which is significantly higher than the Y_{mol} on methylamine (Table 1).

Addition of "heterotrophic" substrates to C_1 compound-limited continuous cultures of Arthrobacter P1

The availability of a method to grow *Arthrobacter* P1 on formaldehyde allowed a study of the effects of an addition of a "heterotrophic" substrate (acetate or glucose) to the culture vessels of C_1 substrate-limited ($S_R = 25$ mM) continuous cultures ($D = 0.10$ h^{-1}). During the ensuing period of transient state the absorbance of the culture, the concentrations of residual substrates in the culture fluid and the activities of some key enzymes in cell-free extracts were determined in samples taken from these cultures at appropriate time intervals. In the first experiments the effect of addition of acetate (25 mM final concentration) to a methylamine-limited culture was analyzed. The supply of fresh medium from the medium reservoir containing 25 mM methylamine continued at a dilution rate of 0.10 h^{-1}. As can be seen from Fig. 1A, addition of acetate (at $t = 0$ h) immediately resulted in an accumulation of methylamine in the supernatant, reaching a maximum concentration of 6 mM at $t = 4$ h. The absorbance of the culture decreased initially, but started to increase again from $t = 2$ h onwards. During the first 2 h after the addition of acetate to the culture, the concentration of this compound decreased according to the wash-out kinetics. Since from $t = 2$ h onwards a more rapid disappearance of acetate from the culture was observed this,

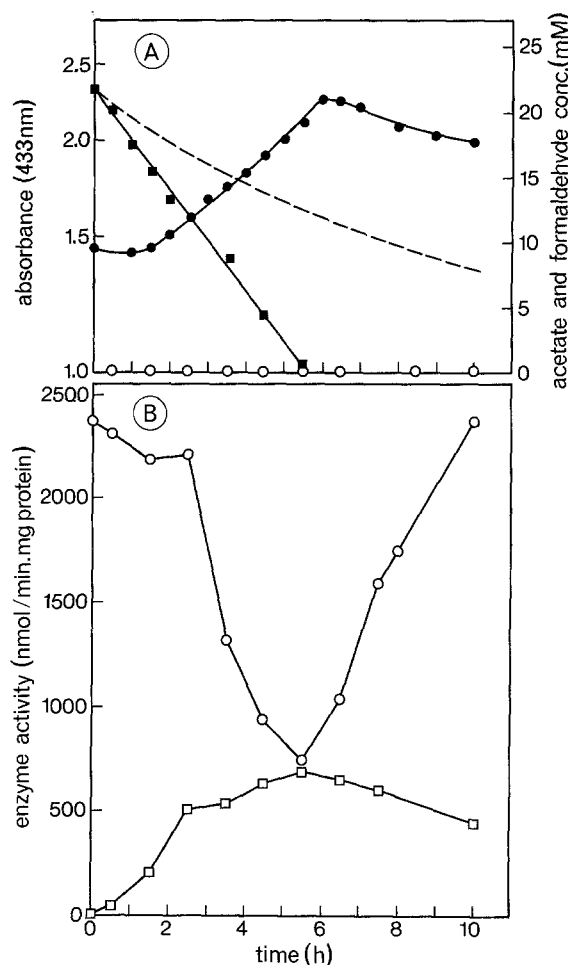**Fig. 2A, B.** Addition of acetate (25 mM) to a formaldehyde-limited ($S_R = 25$ mM) continuous culture of *Arthrobacter* P1 growing at $D = 0.10$ h^{-1}. **A** ●, absorbance; ○, formaldehyde concentration; ■, acetate concentration; ----, wash-out kinetics for acetate. **B** ○, hexulose phosphate synthase; □, isocitrate lyase

and the increasing absorbance of the culture, indicated the start of acetate utilization ($\mu_{max} = 0.31$ h^{-1}; Levering and Dijkhuizen 1985), enabling the organism to reach a specific growth rate above the imposed dilution rate (0.10 h^{-1}). After $t = 4$ h also the methylamine concentration in the supernatant diminished, indicating a simultaneous utilization of both compounds under these conditions. An analysis of the activities of some key enzymes of methylamine and acetate metabolism in the course of this experiment is presented in Fig. 1B. Both amine oxidase and HPS activities initially remained constant, but declined once acetate utilization started to about half the initial values. Following utilization of most of the acetate from the culture, both enzymes recovered and towards the end of this experiment resumed the original activities. Isocitrate lyase on the other hand, absent in methylamine-grown cells, was induced (or derepressed) upon addition of acetate and reached a maximum level of 600 nmol $\cdot$ min^{-1} $\cdot$ mg^{-1} protein, a figure normally found for cells growing on acetate (Levering and Dijkhuizen 1985).

When acetate was added to a formaldehyde-limited ($S_R = 25$ mM; $D = 0.1$ h^{-1}) culture of *Arthrobacter* P1 accumulation of formaldehyde in the culture was not observed (Fig. 2A). The absorbance of the culture remained
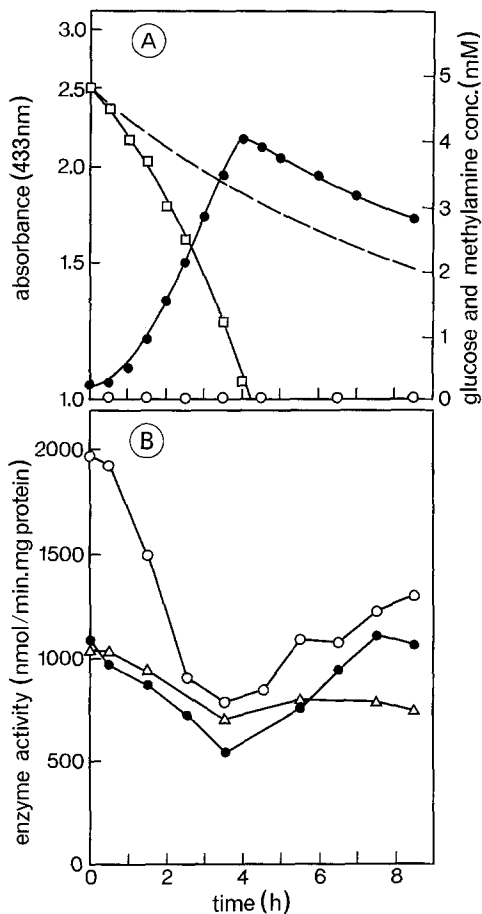
Fig. 3A, B. Addition of glucose (5 mM) to a methylamine-limited ($S_R = 25$ mM) continuous culture of *Arthrobacter* P1 at $D = 0.10\ h^{-1}$. **A** ●, absorbance; ○, methylamine concentration; □, glucose concentration; ————, wash-out kinetics for glucose. **B** ●, amine oxidase; ○, hexulose phosphate synthase; △, transketolase

Fig. 4A, B. Addition of glucose (5 mM) to a formaldehyde-limited ($S_R = 25$ mM) continuous culture of *Arthrobacter* P1 at $D = 0.10\ h^{-1}$. **A** ●, absorbance; ○, formaldehyde concentration; □, glucose concentration; ————, wash-out kinetics for glucose. **B** ○, hexulose phosphate synthase; △, transketolase

constant at first and increased after 1 h, concomitant with the start of acetate utilization. From this moment on both substrates were metabolized simultaneously. The absorbance of the culture slowly decreased once the acetate had disappeared from the culture (at $t = 6$ h). Whereas isocitrate lyase activity steadily increased until $t = 5.5$ h, the level of HPS, which was 2,300 nmol · min^{-1} · mg^{-1} protein at zero time, decreased to approximately 30% at $t = 5.5$ h (Fig. 2B). From this moment on, however, the activity of HPS rapidly recovered whereas the activity of isocitrate lyase slowly decreased. Amine oxidase activity was not detected in this experiment.

Unlike the situation observed following addition of acetate, methylamine did not accumulate in the supernatant of a methylamine-limited chemostat culture ($S_R = 25$ mM; $D = 0.10$ h^{-1}) after addition of glucose to a final concentration of 5 mM (Fig. 3A). The organism almost immediately started to utilize the added glucose (μ_{max} 0.58 h^{-1}; Levering et al. 1981a) simultaneously with methylamine, as indicated by the increasing absorbance of the culture and a decrease of the glucose concentration in the medium. This decrease was much faster than could be accounted for by wash-out of glucose caused by the continuous supply of glucose-free medium (Fig. 3A). At $t = 4$ h glucose was no longer detectable in the culture fluid and the absorbance of the culture, which by then had reached twice the initial value,
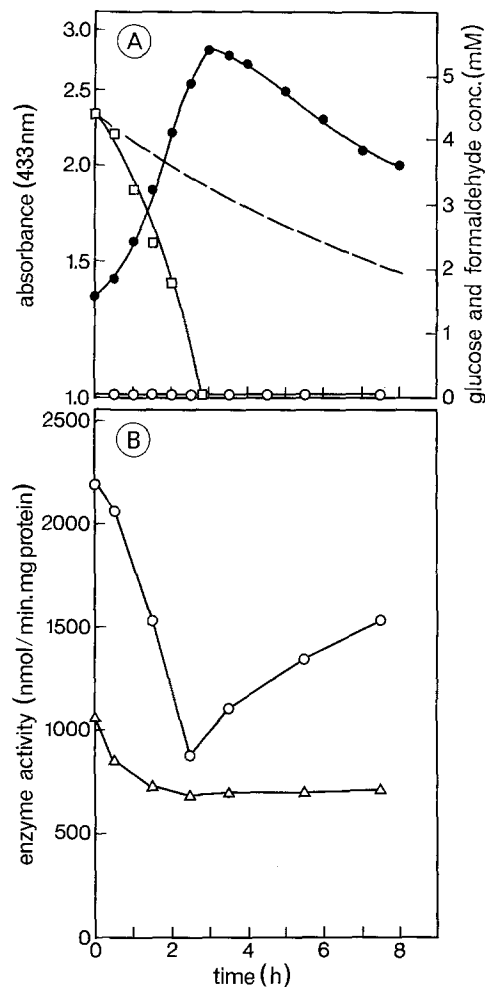
slowly began to decrease following wash-out kinetics. Upon addition of glucose an almost immediate decrease in the levels of amine oxidase, HPS and transketolase was observed and at $t = 3.5$ h all three enzymes showed minimum activities: approximately 40% of the initial value for HPS, 50% for amine oxidase and 65% for transketolase (Fig. 3B). After the glucose concentration had dropped to zero level, the activity of amine oxidase rapidly recovered to its initial level, whereas HPS and transketolase activities increased more slowly.

The results obtained with a formaldehyde-limited chemostat culture of *Arthrobacter* P1 to which a pulse of glucose (5 mM) was given, were essentially similar as observed for cells grown under methylamine-limitation. Upon addition of glucose, this compound was rapidly metabolized and exhausted from the medium between $t = 2.5$ h and $t = 3$ h, resulting in a doubling of the absorbance of the culture (Fig. 4A). Meanwhile no accumulation of formaldehyde in the supernatant was observed although in this phase the activity of HPS, again, decreased rapidly. This enzyme reached a minimum activity at $t = 2.5$ h and, after glucose depletion, steadily increased. Transketolase activity on the other hand, also decreased till
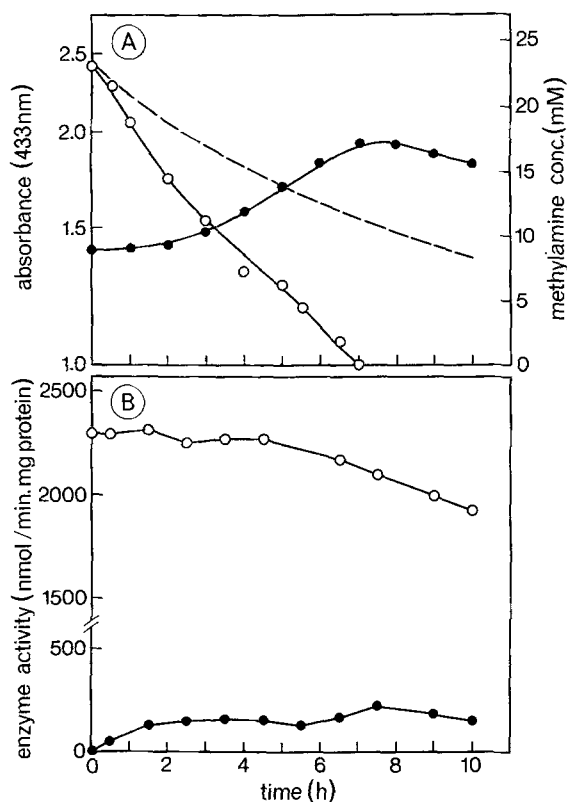
Fig. 5A, B. Addition of methylamine (25 mM) to a formaldehyde-limited ($S_R = 25$ mM) continuous culture of *Arthrobacter* P1 at $D = 0.10$ h^{-1}. **A** ●, absorbance; ○, methylamine concentration; ————, wash-out kinetics for methylamine. **B** ●, amine oxidase; ○, hexulose phosphate synthase

$t = 2.5$ h, but thereafter remained approximately constant (Fig. 4 B).

Addition of methylamine to a formaldehyde-limited continuous culture of Arthrobacter P1

For comparison experiments were performed in which methylamine (25 mM) was added to a formaldehyde-limited ($S_R = 25$ mM; $D = 0.10$ h^{-1}) continuous culture of *Arthrobacter* P1 (Fig. 5). No accumulation of formaldehyde occurred under these conditions and the data show (Fig. 5A) that at $t = 1$ h both substrates were metabolized simultaneously. From this time onwards this resulted in a steady increase in absorbance of the culture (μ_{max} methylamine = 0.28 h^{-1}; Levering et al. 1981a) until the methylamine was depleted ($t = 7$ h). Addition of methylamine resulted in the immediate synthesis of amine oxidase (and the methylamine transport system; data not shown) but the maximum specific activity attained (225 nmol · min^{-1} · mg^{-1} protein; Fig. 5B) was considerably lower than that observed in methylamine-limited cultures (Table 1). Interestingly, for most of the experiment the specific activity of HPS remained constant and only slowly decreased after the disappearance of methylamine from the medium.

Discussion

In general, the presence of formaldehyde in concentrations of 50 mg/l completely prevents the growth of bacteria

(Walker 1964). Methylotrophic bacteria do not represent an exception in this respect (Attwood and Quayle 1984). It is therefore virtually impossible to study growth in the presence of formaldehyde as the sole carbon- and energy source by using batch cultivation techniques. However, attempts to grow methylotrophs under formaldehyde-limitation employing continuous cultures are more likely to be successful (Attwood and Quayle 1984; Goldberg et al. 1976; Hazeu et al. 1983). When using this approach we were indeed able to establish stable and relatively dense cultures of *Arthrobacter* P1 in which the residual formaldehyde concentrations were undetectable low (up to dilution rates of 0.20 h^{-1}). The maximum growth rate of the organism on formaldehyde was not further investigated but may be considerably higher. Rather unexpectedly, the molar growth yield of *Arthrobacter* P1 on formaldehyde turned out to be approximately 30% higher than the value observed with methylamine (Table 1). An analysis of the enzymes of methylamine and formaldehyde metabolism in *Arthrobacter* P1 revealed that in either of these cultures formaldehyde is utilized in an identical way. The methylamine transport system and amine oxidase, however, are absent in formaldehyde-grown cells whereas catalase activity is much lower (Table 1). Since the latter two enzymes catalyze reactions that neither yield nor consume reducing equivalents, the clear difference between the growth yields of cells grown on methylamine or formaldehyde has (mainly) to be ascribed to the expenditure of energy in the uptake of methylamine from the medium into the cells. We previously demonstrated that the methylamine transport system in *Arthrobacter* P1 is energy-dependent and employs the membrane potential as the driving force (Dijkhuizen et al. 1982). In contrast, no convincing evidence for the presence of a formaldehyde transport system in this organism has been obtained sofar and it is likely that this small, uncharged molecule enters the cell simply via diffusion.

The absence of activities of the methylamine transport system and amine oxidase in formaldehyde-grown cells provides clear evidence that synthesis of these enzymes is not controlled by C_1 compounds in general but specifically induced in *Arthrobacter* P1 when the organism is incubated in the presence of methylamine (and probably ethylamine; Levering et al. 1984). The synthesis of the RuMP cycle enzymes HPS (and HPI) is apparently induced by formaldehyde, present in the medium reservoir or following its intracellular production from methylamine.

Over the years it has become clear that glucose may be a strong source of catabolite repression in some organisms, but that organic acids may act as such in other organisms (Harder and Dijkhuizen 1982). Therefore the effects of both glucose and acetate were subsequently studied by adding a pulse of these substrates to the C_1 compound-limited cultures. Only in one of these experiments, namely when acetate was added to a methylamine-limited culture, an accumulation of the C_1 substrate in the culture fluid was observed in time (Fig. 1). Previously we showed that acetate is a strong inhibitor of both the methylamine transport system and amine oxidase in *Arthrobacter* P1 (Dijkhuizen et al. 1982; Levering and Dijkhuizen 1985). The presence of these "heterotrophic" substrates caused, once they were being utilized, a strong reduction in the specific activities of HPS. However, it can be calculated from the data obtained that, at the moment glucose or acetate concentrations reached zero levels and HPS activities reached a minimum

in these experiments, the total capacity of each of these cultures to fix formaldehyde via HPS is still more than five times higher than actually required at this dilution rate and these cell densities.

The observed strong reduction in HPS specific activities in these experiments strongly suggest that the synthesis of this RuMP cycle enzyme is controlled via catabolite repression exerted by the metabolism of the "heterotrophic" substrates. If such a control mechanism is involved, resulting in a complete switch-off of HPS synthesis, one would expect a dilution of existing HPS protein over newly formed cells to occur, accompanied by loss of activity according to the wash-out kinetics. A closer analysis of the data in Figs. 1 — 4 revealed that this indeed may be the case. In each of these experiments the specific activities of HPS decreased not only in proportion to the observed increase in the absorbance of the cultures but in most cases even clearly faster. No decrease in the specific activity of HPS occurred following addition of methylamine, the oxidation of which results in formaldehyde production, to a formaldehyde-limited continuous culture (Fig. 5).

In one of these experiments, addition of acetate to a methylamine-limited continuous culture, it was also observed that synthesis of HPS clearly started to recover before the depletion of acetate (Fig. 1). This may be due to the fact that in the same phase utilization of the methylamine that had accumulated in the culture fluid started again, by virtue of a sufficiently decreased acetate concentration (Levering and Dijkhuizen 1985), thus resulting in an enhanced rate of generation of formaldehyde, the presumptive inducing signal for HPS synthesis. It thus appears that in *Arthrobacter* P1 the synthesis of HPS is regulated both by induction and catabolite repression, but that under certain growth conditions induction by formaldehyde may be the overriding control mechanism. In this respect the question is relevant, of course, what would have happened to the activity of HPS and hence to the consumption rate of formaldehyde by the organism in time when higher concentrations of glucose or acetate had been added to formaldehyde- (or methylamine-)-limited cultures of *Arthrobacter* P1. But since this also would have resulted in synthesis of increasing amounts of cell material and ultimately in limitations other than carbon, such an approach was considered too complex. To circumvent these problems it was decided to device a somewhat different experimental approach for a more detailed study of the relative importance of induction and repression mechanisms in the regulation of HPS synthesis in *Arthrobacter* P1 (see Levering et al. 1986, accompanying paper).

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