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Evidence for the *in vivo* Regulation of Glucose 6-Phosphate Dehydrogenase Activity in *Hydrogenomonas eutropha H 16* from Measurements of the Intracellular Concentrations of Metabolic Intermediates

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Abstract. The inhibiton of fructose utilization by whole cells of *Hydrogenomonas eutropha H 16*, following the addition of hydrogen to the gas phase, has been explained as an inhibition of glucose 6-phosphate dehydrogenase (Blackkolb and Schlegel, 1968a, b).

The intracellular concentrations of glucose 6-phosphate, 6-phosphogluconate, three inhibitors of the enzyme (NADH, ATP and phospho*enol*pyruvate) and some related metabolites were measured in cells incubated in the presence and absence of hydrogen.

Inhibiton of glucose 6-phosphate dehydrogenase was confirmed by an increase in the glucose 6-phosphate pool and a decrease in the 6-phosphogluconate concentration. The regulatory control is apparently due to a threefold increase in the NADH concentration while the concentrations of the other two inhibitors fell slightly. When the measured intracellular concentrations of intermediates were used in the *in vitro* assay of glucose 6-phosphate dehydrogenase activity, an almost total inhibition of the dehydrogenase was observed, therefore further regulatory factors must be considered.

Key words: Intracellular Concentrations — Pool Size (PEP, ATP, ADP, AMP, NAD, NADH, G-6-P, 6-PG, F-6-P) — Glucose 6-Phosphate Dehydrogenase — Enzyme Regulation.

Axiomatically, the flux of metabolites *in vivo* is adjusted to the needs of the cell by altering the rate of enzyme synthesis, by enzyme modification and by modulation of enzyme activity. Many data exist describing the kinetic behaviour of regulatory enzymes and their sensitivity to metabolites and coenzymes *in vitro*, but evidence for the importance of most of these properties *in vivo* is still lacking.

Hydrogenomonas eutropha H 16 is a chemolithoautotrophic hydrogen bacterium which inducibly degrades fructose by the Entner-Doudoroff

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pathway (Gottschalk *et al.*, 1964). The synthesis of the enzymes of fructose degradation is repressed by the presence of hydrogen (Gottschalk, 1965) and the activity of the induced enzyme system is inhibited by the presence of hydrogen (Blackkolb and Schlegel, 1968a). On physiological evidence, Blackkolb and Schlegel (1968a) located this inhibition primarily as a function of glucose 6-phosphate dehydrogenase. The purified enzyme is subject to competitive inhibition by NADH at the $NAD(P)^+$ binding site (Blackkolb and Schlegel, 1968b) and to inhibition by ATP (Blackkolb and Schlegel, 1968b) and phospho*enol*pyruvate (Tunail and Schlegel, 1972) which both increase the sigmoidity of the glucose 6-phosphate saturation curves. Blackkolb and Schlegel (1968b) argue that, as hydrogen serves as an additional hydrogen donor and energy source, NADH and ATP may be expected to be direct mediators of the effect exerted by hydrogen.

To test the hypothesis that glucose 6-phosphate dehydrogenase suffers inhibition on the addition of hydrogen to the gas phase, the intracellular concentrations of substrate (glucose 6-phosphate) and product (6-phosphogluconate) were followed when cell suspensions were successively exposed to atmospheres of air and a hydrogen + oxygen mixture (Knallgas). Simultaneously, the concentrations of inhibitors (NADH, ATP and phospho*enol*pyruvate) and other metabolites (ADP, AMP, NAD^+ and fructose 6-phosphate) were measured to estimate their role in control. A preliminary report of part of this work has been published (Cook and Bowien, 1973).

Materials and Methods

Growth Conditions and Experiments in Cell Suspensions. Hydrogenomonas eutropha H 16 (ATCC 17699, Wilde, 1962) was grown heterotrophically under air with 30 mM fructose as carbon source and the mineral medium and growth conditions described by Schlegel *et al.* (1961a). Cells were harvested (6000 *g*, 30 min, 4° C) in the exponential growth phase, washed twice in 0.067 M phosphate buffer pH 7.0, and resuspended to a cell density of 1.5—2 mg dry wt./ml in complete or nitrogen-free mineral medium. Cell suspensions (55 ml) were gassed by a magnetically driven stirring bar (3 cm, 900 rev./min) in a stoppered 200 ml Erlenmeyer flask at 30° C. The gas mixture [250 ml/min, air or Knallgas (20% O_2 + 80% H_2)] was led through tubes in the stopper. Samples (1.0 ml) were taken with a syringe through a short needle in a stopper in the side of the flask. The sample was quenched immediately, either in 0.25 ml of 0.25 M NaOH at 85° C, extracted at 85° C for 10 min and stored on ice, or quenched in 2.0 ml of 0.85 M perchloric acid at room temperature (20—24° C), extracted for 40 min at room temperature and stored on ice. The time delay between starting to take a sample and quenching was approx. 5 sec. Cell debris was removed from the samples by centrifugation (40000 *g*, 20 min, 4° C) and the supernatant fluid decanted into chilled tubes. Portions of the acidified extracts were neutralized (pH 7.2) with 5.5 M KOH and the clarified supernatant solutions stored at — 30° C. Portions (0.8 ml) of the alkaline extracts were neutralized (30 µl of 1 M KH_2PO_4), NADH oxidized to NAD^+ (50 µl of 10 mM pyruvate containing

12 µg lactate dehydrogenase, room temperature, 30 min), the mixture deproteinized on ice with 0.5 ml of 1.3 M perchloric acid, and the sample treated subsequently as an acid extract.

After the addition of fructose to the cell suspension, a steady state (*i.e.* constant pool sizes) was attained within 30 min. In replicate experiments, different pool sizes were observed (*e.g.* ATP 3—5 mM) but the patterns of changes were similar if the gassing was adequate.

Determinations of Intracellular Concentrations of Metabolites. Quantitative estimates of metabolic intermediates were obtained by the fluorimetric detection of NAD(P)H in $NAD(P)^+$-coupled enzymic reactions in Eppendorf Fluorimeters (Eppendorf Gerätebau, Hamburg, W.-Germany) as described by Estabrook and Maitra (1962), Maitra and Estabrook (1964) and Williamson and Corkey (1969). These intermediates were considered to be uniformly distributed in the cell water; samples of cell suspensions were filtered and analyses of the filtrates showed that less than 5% of the metabolites were outside the cell. The cell water enclosed by the membrane was measured by the method of Reid and Frank (1966) using [^{14}C] glucose, which does not penetrate the membrane of this organism (Gottschalk, 1964). A value of 1.14 µl cell water/mg dry wt. was observed in cells lacking the storage material poly-β-hydroxybutyrate; when cells then accumulated the storage material no increase in cell water was observed. Bacterial dry weight was measured turbidimetrically, by reference to a standard curve, at a wavelength of 436 nm in 1 cm pathlength cuvettes in a Zeiss PMQ II Spectrophotometer (Carl Zeiss, Oberkochen/Württ., W.-Germany). One turbidity unit ($E_{436} = 1.0$) was observed to be equivalent to 176 µg bacterial dry wt./ml when measured in dilutions of turbidity less than 0.3.

Measurement of Glucose 6-Phosphate Dehydrogenase Activity. Cell-free extracts containing glucose 6-phosphate dehydrogenase with a specific activity of 200 µmole/mg protein were obtained from organisms grown in fructose-salts medium, as described by Blackkolb and Schlegel (1968b). The enzyme assay was that of Blackkolb and Schlegel (1968b) but with the buffer added at a higher concentration to compensate for dilution on addition of the intermediates under test. Assays of enzyme activity were done at 30°C in 1 cm pathlength cuvettes at 340 nm in a Zeiss PMQ II Spectrophotometer linked to a Servogor recorder (Metrawatt AG, Nürnberg, W.-Germany). Reactions were started by the addition of extract.

Materials. Enzymes and biochemicals were obtained from Boehringer Mannheim GmbH, W.-Germany. Chemicals were purchased from E. Merck, Darmstadt, W.-Germany. The Radiochemical Centre, Amersham, Bucks., UK, provided the [1-^{14}C] glucose.

Results

Intracellular Metabolite Concentrations

When cells of *Hydrogenomonas eutropha H 16*, which had been grown in fructose-salts medium, were resuspended under non-growing conditions in the presence of fructose and with an atmosphere of air, the turbidity rose (Fig. 1a) due to the synthesis and accumulation of poly-β-hydroxybutyrate (Schlegel *et al.*, 1961b). When the gas atmosphere was changed from air to Knallgas, the rate of accumulation of poly-β-hydroxybutyrate decreased (Fig. 1a). Simultaneously, the pool of glucose 6-phosphate spiked upwards and that of 6-phosphogluconate spiked downwards

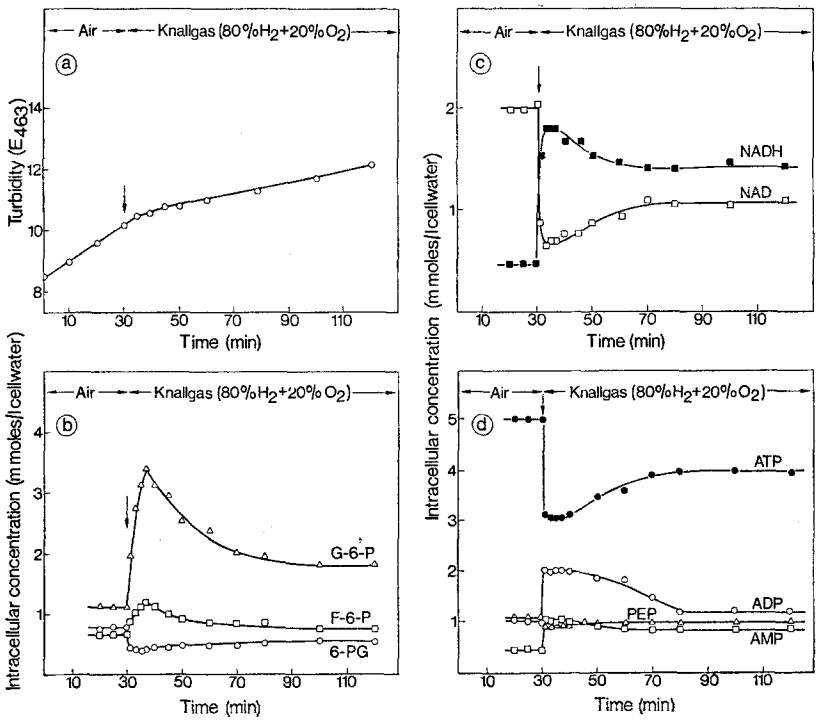

Fig. 1 a—d. The effect of a change in the gas atmosphere from air to Knallgas (80% H_2 + 20% O_2) on the pool sizes of metabolic intermediates in non-growing cells of *Hydrogenomonas eutropha H 16*. Cells grown in fructose-salts medium were harvested, washed and resuspended in nitrogen-free mineral medium. Fructose, to a final concentration of 40 mM, was added to the aerated cell suspension. Samples were taken at intervals for turbidity measurements and for pool size estimations as detailed in the Methods section. At the time indicated (↓), the gas atmosphere was changed from air to Knallgas

(Fig. 1 b). This behaviour of the substrate and product of glucose 6-phosphate dehydrogenase proved that the enzyme was inhibited *in vivo*.

The transition time between the two steady states was of the order of 20—25 min for all intermediates, and each pool approached the new steady state by overshoot (Fig. 1 b—d).

Glucose 6-phosphate dehydrogenase has three characterized inhibitors, NADH, ATP and phospho*enol*pyruvate. Of these, NADH increased threefold in concentration (Fig. 1 c; from 0.5 mM to 1.4 mM at a constant total pyridine nucleotide pool) while both ATP and phospho*enol*pyruvate showed small decreases in concentration (Fig. 1 d).

The total pool of adenine nucleotides showed little change (a loss of 6%) on the transition and the energy charge fell from 0.86 to 0.78, also by overshoot (not shown).

Growth of *Hydrogenomonas eutropha* was also inhibited by a change in the gas phase from air to Knallgas. A similar pattern of alterations in pool sizes and energy charge was seen, with the additional effect that the total pool of adenine nucleotides fell by 49% during the transition (not shown).

Inhibition of Glucose 6-Phosphate Dehydrogenase

Glucose 6-phosphate dehydrogenase is sensitive to ATP, phosphoenolpyruvate and NADH. At the concentrations measured under steady state conditions each metabolite (free ATP, phosphoenolpyruvate or NADH; Table 1) was highly inhibitory to the enzyme (Blackkolb and Schlegel, 1968b). It was, however, not possible to predict the extent of cumulative inhibition in the presence of all three inhibitory compounds. Therefore, assays of enzyme activity were done with the various combinations of the inhibitors and substrates to simulate the *in vivo* conditions (Table 1); NAD^+ was present in these assays, but had little effect on the rates (not tabulated) except to alleviate NADH inhibition by 15—20% when the latter alone was added. The measured activities confirmed the idea that there was strong inhibition of the dehydrogenase under both simulated steady state conditions (air or Knallgas as gas phase) and that there was no simple relationship between the presence of single and multiple inhibitors except that the presence of another inhibitor always intensified the effect.

Although the activity of the dehydrogenase was lower in the presence of the pool-sizes representing a Knallgas atmosphere, the very strong inhibitions observed prompted us to compare the *in vitro* and *in vivo* rates of the enzyme. The initial rate of poly-β-hydroxybutyrate synthesis in Fig. 1a (110 nmol monomer/min · ml; the increase in turbidity was considered to be entirely due to poly-β-hydroxybutyrate synthesis) was far in excess of the estimated activity of the glucose 6-phosphate dehydrogenase (14 nmol glucose 6-phosphate/min · ml). The figure of 14 nmol/min · ml was calculated by considering the initial turbidity ($E_{436} = 8.5$) equivalent to 1.4 mg dry wt./ml *i.e.* 0.72 mg protein/ml or 0.172 units/ml of glucose 6-phosphate dehydrogenase [specific activity of 0.24 units/ml; Blackkolb and Schlegel (1968b)] which operates at only 8% activity. Assays were then done at the pH-value observed in the cytoplasm of fructose-grown organisms of *Hydrogonomonas eutropha H 16* (pH 7.2; Ahrens, 1970). This did alleviate the effect due to single and even pairs of effectors, but the overall picture of very severe inhibition

Table 1. The effects of metabolites on glucose 6-phosphate dehydrogenase from *Hydrogenomonas eutropha H 16*

The *in vitro* assays of glucose 6-phosphate dehydrogenase activity was done using the indicated intracellular concentrations of metabolites which were measured in *Hydrogenomonas* cells under steady state conditions in the presence of fructose and gassed with air or Knallgas (80% H_2 + 20% O_2) (data from Fig. 1). Reaction rates are given as a percentage of the enzyme activity under the standard conditions given in the "Method" section.

Metabolites	Gas phase					
	Air			Knallgas		
	Concentration mM	Enzyme activity, % of standard conditions		Concentration mM	Enzyme activity, % of standard conditions	
		pH 7.6	pH 7.2		pH 7.6	pH 7.2
Glucose 6-phosphate	1.2	75	94	1.8	89	94
ATP (free)[a,b]	0.21	21	49	0.14	70	57
Phospho*enol*-pyruvate	1.08	17	33	0.97	37	no value
NADH	0.45	50	67	1.4	< 2	4
ATP + Phospho*enol*pyruvate	0.21 ± 1.08	8	15	0.14 + 0.97	35	26
ATP + NADH	0.12 + 0.35	20	34	0.14 + 1.4	< 2	4
Phospho*enol*-pyruvate + NADH	1.08 + 0.45	8	9	0.97 + 1.4	< 2	< 2
ATP + NADH + Phospho*enol*-pyruvate	0.21 + 0.45 + 1.08	8	9	0.14 + 1.4 + 0.97	< 2	< 2

[a] Only free ATP inhibits (Blackkolb and Schlegel, 1968b); the concentration is an adaption from Table 2 of Blair (1970) using the total ATP pool and energy charge derived from Fig. 1.

[b] In this and the following enzyme activity measurements the concentration of glucose 6-phosphate was 1.2 or 1.8 mM as appropriate.

in the presence of all inhibitors was unchanged (Table 1, bottom line), an observation repeated at the pH optimum (8.2) of the enzyme.

Discussion

Enzyme inhibition *in vivo* has been clearly established (Fig. 1 b). The behaviour of the pools of substrate and product follows the predicted theoretical pattern (Webb, 1963), fulfills Krebs (1957) principle that the

concentration of the substrate of a regulated enzyme should change in the opposite direction to the change in the rate of flow through the reaction, and may be compared to the evidence indicating that ribulose-1,5-diphosphate is the CO_2 acceptor and 3-phosphoglycerate is the observed product of the Calvin cycle of CO_2 fixation (Wilson and Calvin, 1955).

The transition time between the 2 steady states (20 to 30 min) is very long compared to the relaxation time of enzymes (3—300 msec for dehydrogenases; Gutfreund, 1971) and the transient time between steady states in the redox chain (15—30 sec; Chance and Williams, 1956), and presumably reflects the very large number of interactions in integreted cell metabolism, different facets of which may be seen in Fig. 1.

The transient accumulation of fructose 6-phosphate is interpreted as reflecting equilibrium in the phosphoglucose isomerase reaction, and adenylate kinase is presumed to catalyze the alterations in the adenine nucleotide pools. The loss of adenine nucleotide from cells during the transition phase under growth conditions resembles similar patterns seen on cessation of growth (Chapman *et al.*, 1971; Bächi and Ettlinger, 1973) and possibly indicates the presence of AMP nucleosidase (Schramm, 1973) in this organism.

The evaluation of data derived from pool-size measurements has been critically reviewed (Sols and Marco, 1970; Rolleston, 1972; see also Reeves and Sols, 1973) and questioned (Atkinson, 1969). The problems of interpretations are highlighted in this work, where the observed level of glucose 6-phosphate dehydrogenase activity, measured as the rate of poly-β-hydroxybutyrate synthesis, is 8-fold higher than the *in vitro* activity of the enzyme under simulated intracellular conditions. The use of standard enzyme kinetics for this calculation appears to be generally valid as the glucose 6-phosphate concentration of 1.5 ± 0.3 mM is some 33-fold in excess of the enzyme concentration (46 μM), although other ligands, notably ATP (Table 1), are present at lower concentrations and larger proportions will be enzyme bound. The intracellular enzyme concentration was estimated from the specific activities of the crude cell extract and the purified protein [(0.24 and 22.4 units/mg protein respectively; Blackkolb and Schlegel (1968b)], a representative molecular weight [105000; Bonsignore and de Flora (1972)] and a cell water of 2.2 μl/mg protein. The measurement of enzyme activity depends on the accuracy of the pool-size measurements, and Table 1 (especially the first 4 lines, comparing the 2 steady states) indicates the very considerable changes in enzyme activity to be expected from small variations in metabolite concentrations. Error in either the measurement of the quantities of metabolites present or of cell water would markedly alter the results, and the effect of compartition in the bacterial cell (*e.g.* Davis *et al.*, 1969; MacNab *et al.*, 1973) has an unknown effect on the distri-

bution of metabolites in cell water. Should the assumptions described above be correct, an as yet unidentified activator of this catabolic enzyme could explain the discrepancy between observed *in vivo* and *in vitro* rates. Further, the data emphasize the caution that must be observed when extrapolating from enzyme kinetics to the roles of enzyme effectors inside the cell.

Note Added in Proof. The glucose 6-phosphate dehydrogenase of rat liver is inhibited by NADPH, and a recent report by Eggleston and Krebs [Biochem. J. **138**, 425—435 (1974)] shows that oxidized glutathione (0.2 mM) totally relieves this effect in liver homogenates. Oxidized glutathione (0.2—0.6 mM) did not relieve inhibition of glucose-6-phosphate dehydrogenase by NADH in extracts of *Hydrogenomonas eutropha H 16.*

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