Factors Influencing the Formation and Stability of D-Glucoside 3-Dehydrogenase Activity in Cultures of *Agrobacterium tumefaciens*

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SUMMARY

D-Glucoside 3-dehydrogenase specific activity in *Agrobacterium tumefaciens* was maximal towards the end of the exponential growth phase of batch cultures; over 90 % of the activity disappeared within the next 15 h. Manganese ions, although essential for growth of the organism, strongly repressed D-glucoside 3-dehydrogenase synthesis in sucrose medium but had little effect when the carbon source was methyl α -D-glucoside. D-Glucoside 3-dehydrogenase activity increased linearly with increasing specific growth rate in chemostat cultures limited by carbon, nitrogen, phosphate or manganese when methyl α -D-glucoside was the carbon source. High enzyme activity was found with sucrose as carbon source only when the growth medium was manganese-limited.

D-Glucoside 3-dehydrogenase activity disappeared from *A. tumefaciens* incubated in carbon- and nitrogen-free medium or in nitrogen-free medium containing succinate, but on continued incubation the activity returned and was then stable. The recovery of activity could be prevented by chloramphenicol or erythromycin. Bacteria containing the recovered dehydrogenase activity could not convert sucrose to 3-ketosucrose when oxygen acted as the terminal electron acceptor, but produced 3-ketosucrose at the normal rate in the presence of ferricyanide.

D-Glucoside 3-dehydrogenase activity disappeared irreversibly from bacteria incubated in nitrogen-free medium containing sucrose. Loss of activity followed first order kinetics in bacteria taken from nitrogen-, phosphate- or manganese-limited chemostat steady states; an accelerating rate of decay occurred in cells grown under carbon-limitation. 8-Hydroxyquinoline, chloramphenicol, erythromycin, 2,4-dinitrophenol and manganese ions could reduce the rate of decay.

INTRODUCTION

The D-glucoside 3-dehydrogenase (EC. 1.1.99.13) in *Agrobacterium tumefaciens* converts sucrose, maltose and other hexopyranosides to corresponding 3-keto compounds which accumulate in the supernatant medium during growth (Bernaerts & De Ley, 1958; Fukui & Hochster, 1963; Kurowski & Pirt, 1971; Tyler & Nakamura, 1971). The enzyme has been isolated and studied by Grebner, Kovach & Feingold (1966), Hayano & Fukui (1967) and Van Beeumen & De Ley (1968). Since the 3-keto sugars cannot conveniently be synthesized chemically, their production by the one-step enzymic route is of much interest.

The activity of the D-glucoside 3-dehydrogenase decays rapidly on cessation of growth (Fensom & Pirt, 1972; Kurowski, Fensom & Pirt, 1973) and this feature limits the useful life of the bacteria as biosynthetic agents. We have attempted to stabilize the enzyme activity in non-growing bacteria through an understanding of the mechanisms by which the activity is

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Table 1. *Chemical composition of media used for the growth of A. tumefaciens*

	Concentrations of substances (g/l) in media:			
	Carbon-limited	Nitrogen-limited	Phosphate-limited*	Manganese-limited
Methyl α -D-glucoside†	6.0	12.0	12.0	10.0
Urea	0.9	0.7	0.9	0.9
MgSO ₄ ·7H ₂ O	0.15	0.15	0.15	0.15
FeSO ₄ ·7H ₂ O	0.01	0.01	0.01	0.01
MnSO ₄ ·4H ₂ O	0.0022	0.0022	0.0022	0
CaCl ₂ ·2H ₂ O	0.025	0.025	0.025	0.025
Citric acid	0.17	0.17	0.17	0.17
KH ₂ PO ₄ ‡	1.07	1.07	0.095	1.07
Na ₂ HPO ₄ ·2H ₂ O‡	2.17	2.17	0	2.17
NaCl	0	0	1.42	0
KCl	0	0	0.55	0

* Phosphate-limited medium also contained the following (mg/l): ZnSO₄·7H₂O, 2.0; CuSO₄·5H₂O, 0.5; CoCl₂·6H₂O, 0.5; Na₂B₄O₇·10H₂O, 0.5; Na₂MoO₄·2H₂O, 0.5.

† Where stated in the text, sucrose or maltose was used as the carbon source in place of methyl α -D-glucoside. Sucrose-limited medium contained 5 g sucrose/l; nitrogen- and manganese-limited sucrose media contained 40 g sucrose/l. Maltose-limited medium contained 5 g maltose/l.

‡ The phosphate concentration was increased fivefold for shake-flask experiments.

lost. The present paper describes the production of D-glucoside 3-dehydrogenase in batch and chemostat cultures of *A. tumefaciens*, and the factors which influence the enzyme stability in non-growing cells.

METHODS

Organism. *Agrobacterium tumefaciens* strain 396 was obtained from the National Collection of Plant Pathogenic Bacteria (Ministry of Agriculture, Fisheries and Food, Harpenden, Hertfordshire). It was maintained on peptone-yeast agar (Difco) at 4 °C and subcultured monthly. Inocula for chemostat and shake-flask cultures were grown in media of identical composition to the experimental media.

Media. The compositions of carbon-, nitrogen-, phosphate-, and manganese-limited media used for growth of the organism are shown in Table 1. Medium was prepared in two parts: (i) methyl α -D-glucoside or sucrose, urea, chelated salts (pH adjusted to 7.0 with NaOH), 3 l; (ii) phosphates, 17 l. Parts (i) and (ii) were autoclaved at 121 °C for 25 and 55 min, respectively, and then mixed aseptically. Evaporation loss was compensated for by the addition of sterile distilled water. When maltose was used as the carbon source it was autoclaved separately from other components of the medium.

Culture apparatus and techniques. A 5 l chemostat, similar to the type described by Elsworth *et al.* (1956), was used for continuous-flow experiments. The culture volume was 1600 ± 50 ml. The pH was controlled at 7.00 ± 0.05 and the temperature at 28.00 ± 0.15 °C. Foaming was suppressed by the automatic addition (0.05 ml/h) of polypropylene glycol (PPG 2000; Shell). An unbaffled vortex aeration system with an 8-vaned disc impeller running at 1440 rev./min was employed. The air flow over the culture was 400 ml/min.

The culture was considered to be in a steady state when samples taken over a minimum of two mean residence times showed constancy in the observed parameters. The specific growth rate of steady-state chemostat cultures was assumed to be equal to the dilution rate, D (equal to F/V , where F is the sum of the nutrient and control reagent flow rates and V is the culture volume).

For batch cultures 250 or 1000 ml Erlenmeyer flasks containing one-tenth of their volume of medium were used. The flasks, which contained a stainless-steel wire coil to minimize clumping of the cells, were incubated at 28 °C and 200 rev./min on an orbital shaker with a 25 mm diameter stroke.

Bacterial growth was routinely determined by measuring the culture turbidity at 540 nm. Turbidity was directly related to the mass of water-washed bacteria dried overnight at 105 °C. Steady-state biomass concentrations were determined by direct dry weight measurement.

Non-growing bacterial suspensions. Bacteria from chemostat steady states or from the late exponential phase of batch cultures were separated from the growth medium by centrifugation (13 000 g; 4 °C; 10 min), washed by resuspension in 0.1 M-phosphate buffer pH 7.0 to the original volume, separated again, and finally resuspended in the appropriate starvation medium. Nitrogen-free starvation medium contained (g/l distilled water): KH_2PO_4 , 5.34; $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 10.86; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.15; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 2.50×10^{-2} ; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 1.03×10^{-2} ; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 2.20×10^{-3} ; citric acid, 0.17; sucrose, 40.0. Carbon- and nitrogen-free medium lacked sucrose. Bacterial suspensions (25 ml, containing 1 to 5 mg dry weight/ml, equivalent to 0.07 to 0.09 units of D-glucoside 3-dehydrogenase activity/ml) were shaken (200 rev./min) at 28 °C in 250 ml flasks baffled with a stainless-steel wire coil. Evaporation losses were monitored by regularly weighing the flasks, and were compensated for by adding distilled water. Large-scale suspensions (1.0 to 1.5 l) were contained in a 2 l vessel at 28 °C and were aerated through a sintered-glass sparger with water-saturated air (500 ml/min).

Preparation of extracts. Washed bacteria were resuspended in 0.1 M-phosphate buffer (pH 7.0) at 4 °C and a density of about 75 mg dry weight/ml. The bacteria were broken either by passage through a French pressure cell at 4 °C or by 2×1 min periods of sonication in a 100 J/s MSE ultrasonic disintegrator operating at maximum output. Supernatant fractions were recovered after centrifugation of homogenates for 30 min at 38 000 g and 4 °C.

Assay procedures. D-Glucoside 3-dehydrogenase activity was assayed in intact bacteria and in extracts by using 2,6-dichlorophenol indophenol (DCPIP) as an electron acceptor (Kurowski & Pirt, 1971). Activities obtained with the whole-cell and cell-free extract assays were consistently in agreement. 3-Ketosucrose was determined by the method of Fukui & Hayano (1969). Protein was measured by the method of Lowry *et al.* (1951) using crystallized bovine plasma albumin as the standard.

Respiratory activity. The respiratory activity of non-growing bacteria was determined by transferring portions (2.7 ml) of washed organisms to the chamber of a pre-calibrated Clark electrode (Rank Brothers, Bottisham, Cambridge) at 30 °C, and measuring the rate of oxygen consumption after the addition of 0.1 M-sucrose (0.3 ml).

RESULTS

Growth and D-glucoside 3-dehydrogenase activity in batch cultures

Figure 1 shows the time course of changes in biomass concentration and specific D-glucoside 3-dehydrogenase activity during batch growth of *A. tumefaciens* in manganese-limited sucrose medium. After a lag of 2 h the biomass concentration increased exponentially (maximum specific growth rate 0.28 h^{-1}). At bacterial densities above 1.30 g dry wt/l the growth rate progressively declined, until growth ceased at 2.50 g dry wt/l due to nitrogen-source exhaustion. During the phase of declining growth rate the culture was manganese-limited, since the addition of manganese ions, at 10^{-8} M or more, to the medium extended the period of exponential growth to give 2.35 g dry wt/l in a batch culture, and increased the steady-

Fig. 1. Time-course of changes in biomass concentration (○) and specific D-glucoside 3-dehydrogenase activity (●) during batch growth of *A. tumefaciens* in manganese-limited sucrose medium.

state biomass concentration of a chemostat culture (specific growth rate 0.12 h^{-1}) from 0.88 to 1.80 g dry wt/l; a solution containing 10^{-6} M concentrations of boron, cobalt, copper, molybdenum and zinc ions had no effect in either case. Specific D-glucoside 3-dehydrogenase activity increased, reaching a maximum near the end of exponential growth; over 90 % of the specific activity disappeared within the next 15 h.

Effect of specific growth rate, limiting nutrient and carbon source on D-glucoside 3-dehydrogenase activity

Steady-state specific D-glucoside 3-dehydrogenase activity increased linearly with increasing specific growth rate during carbon-, nitrogen-, phosphate- and manganese-limited chemostat growth of *A. tumefaciens* in methyl α -D-glucoside medium (Fig. 2). The lowest activities occurred during phosphate-limited growth and the highest at growth rates approaching the maximum in methyl α -D-glucoside-limited cultures. Manganese- or nitrogen-limitation enhanced the enzyme activity at low growth rates.

D-Glucoside 3-dehydrogenase production was also determined during chemostat growth in sucrose and maltose media (Table 2). Enzyme activity was not detectable in sucrose- and maltose-limited cultures and was very low in nitrogen-limited sucrose medium. However, high enzyme activity was found in sucrose medium when growth was limited by manganese.

D-Glucoside 3-dehydrogenase activity in batch cultures was also dependent upon the carbon source and the amount of manganese in the medium. With sucrose, the peak activity in nitrogen-limited medium was $15.9 \text{ } \mu\text{mol sucrose/g dry wt/min}$, compared with $70.2 \text{ } \mu\text{mol sucrose/g dry wt/min}$ in nitrogen-limited medium containing no added manganese. The omission of manganese ions from nitrogen-limited methyl α -D-glucoside medium had no significant effect on the peak enzyme activity ($76.9 \text{ } \mu\text{mol methyl } \alpha\text{-D-glucoside/g dry wt/min}$).

Fig. 2. The relationship between specific growth rate and the steady-state specific D-glucoside 3-dehydrogenase activity during (a) carbon-limited, (b) nitrogen-limited, (c) phosphate-limited and (d) manganese-limited chemostat growth of *A. tumefaciens* in methyl α -D-glucoside media. The activity was assayed in intact bacteria. Each point represents the mean of a minimum of three enzyme determinations; bars indicate extreme values.

Table 2. Steady-state D-glucoside 3-dehydrogenase activities during chemostat growth of *A. tumefaciens* in sucrose and maltose media

Specific growth rate (h^{-1})	Specific D-glucoside 3-dehydrogenase activity ($\mu\text{mol substrate}^*/\text{g dry wt/min}$)			
	Growth-limiting nutrient:			
	Sucrose	Nitrogen†	Manganese†	Maltose
0.04	—	<0.5	—	—
0.05	<0.5	—	26.8	<0.5
0.09	—	0.9	—	—
0.10	<0.5	—	54.1	—
0.14	<0.5	—	—	<0.5
0.19	<0.5	1.2	—	—
0.20	—	—	63.4	—
0.24	<0.5	—	—	<0.5
0.26	—	3.7	—	—

* The enzyme substrate in the assay system was the carbon source used for growth. The rate of reaction with sucrose was 88 % of that with maltose.

† Sucrose as carbon source.

Reversible disappearance of D-glucoside 3-dehydrogenase activity from non-growing bacteria

Organisms from carbon-, phosphate-, nitrogen- and manganese-limited chemostat steady states were incubated at 28 °C for up to 600 h in carbon- and nitrogen-free medium (Fig. 3). Cell lysis, as shown by release of protein, during the incubation period was negligible. In bacteria from carbon-limited (Fig. 3a) and phosphate-limited (Fig. 3b) steady states, up to

Fig. 3. Time-course of changes in D-glucoside 3-dehydrogenase activity in *A. tumefaciens* transferred from chemostat steady states to carbon- and nitrogen-free medium. The organism was grown in (a) carbon-limited, (b) phosphate-limited, (c) nitrogen-limited and (d) manganese-limited methyl α -D-glucoside medium at specific growth rates of ($\circ$) 0.05, ($\triangle$) 0.10, ($\blacktriangle$) 0.15, ($\blacksquare$) 0.19, ($\bullet$) 0.20 and ($\square$) 0.22 h^{-1} . The dehydrogenase was assayed in intact cells; the broken line in (a) indicates the results obtained when activity was measured in extracts.

90 % of the D-glucoside 3-dehydrogenase activity was lost in 6 h, the rate of disappearance being approximately exponential; on continued incubation, however, the activity recovered and was then fairly stable for several weeks. The rates of disappearance and percentage losses of activity in cells from nitrogen-limited (Fig. 3c) and manganese-limited (Fig. 3d) steady states were considerably less than the corresponding values for carbon- or phosphate-limited growth. The recovered activity was again stable. Except with manganese limitation, the rate of loss of activity was not markedly influenced by the previous growth rate; recovery of activity commenced sooner in cells from steady states at low growth rates. The recovered activity was sometimes higher than the initial value; either the additional activity represented that lost during the manipulative period (30 min), or the enzyme at zero time was not all in an active form. The D-glucoside 3-dehydrogenase was routinely measured in whole cells; a similar loss and recovery of activity was observed when the enzyme was assayed in extracts prepared from cell samples taken over the incubation period (Fig. 3a).

D-Glucoside 3-dehydrogenase activity was determined by measuring the rate of reduction

Table 3. Effect of ferricyanide on the production of 3-ketosucrose by *A. tumefaciens*

Time (h)	D-Glucoside 3-dehydrogenase activity (units/ml)	Relative rate of 3-ketosucrose accumulation (μmol 3-ketosucrose/min/ unit D-glucoside 3-dehydrogenase activity)	
		Oxygen	Ferricyanide
0	0.088	0.68	2.68
22	0.009	0.18	2.57
66	0.072	0.06	2.58

Bacteria from a methyl α -D-glucoside-limited chemostat steady state (specific growth rate 0.16 h^{-1}) were incubated in carbon- and nitrogen-free medium and portions were supplied with sucrose (40 g/l) after 0, 22 and 66 h. The production of 3-ketosucrose was measured with oxygen or ferricyanide (50 mM) as the electron acceptor. The initial rate of 3-ketosucrose accumulation was related to the D-glucoside 3-dehydrogenase activity (determined by DCPIP reduction) at the time of sucrose addition.

Fig. 4. The effect of chloramphenicol on the recovery of D-glucoside 3-dehydrogenase activity in *A. tumefaciens* incubated in carbon- and nitrogen-free medium. The suspension was divided into two equal portions at 10 h. Chloramphenicol was added to one portion to give a concentration of $200\text{ }\mu\text{g/ml}$ (●); an equal volume of water was added to the control (○). The organism was grown (specific growth rate 0.19 h^{-1}) under steady-state conditions in a chemostat with phosphate-limited methyl α -D-glucoside medium.

of DCPIP. However, as it was possible that the recovered activity was not that of the D-glucoside 3-dehydrogenase, but a non-specific reduction of the dye, the enzyme activity measured by DCPIP reduction was checked by direct measurement of the initial rates of product (3-ketosucrose) formation (Table 3). The ability of carbon- and nitrogen-starved bacteria to accumulate 3-ketosucrose with oxygen as the electron acceptor declined irreversibly with time. However, with ferricyanide as the electron acceptor (Fensom, Kurowski & Pirt, 1974) the rate of 3-ketosucrose accumulation per unit DCPIP activity was unchanged over 66 h. This indicated that the activity measured with DCPIP in bacteria incubated in the absence of carbon and nitrogen for long periods was specifically that of the D-glucoside 3-dehydrogenase, and that the loss with time of the ability to accumulate 3-ketosucrose with oxygen as the electron acceptor was probably a consequence of an instability of one or more components of the electron-transport pathway.

The initial loss of D-glucoside 3-dehydrogenase activity in bacteria from a phosphate-

Fig. 5. Time-course of the disappearance of D-glucoside 3-dehydrogenase activity from *A. tumefaciens* transferred from chemostat steady states to nitrogen-free medium containing sucrose (40 g/l). The organism was grown in (a) nitrogen-limited, (b) manganese-limited, (c) phosphate-limited and (d) carbon-limited methyl α -D-glucoside medium at specific growth rates of (O) 0.05, (Δ) 0.10, ($\blacktriangle$) 0.15, ($\blacksquare$) 0.19, ($\bullet$) 0.20 and ($\square$) 0.22 h^{-1} .

limited steady state (specific growth rate 0.19 h^{-1}) was not affected by chloramphenicol (200 $\mu\text{g/ml}$). However, the addition of chloramphenicol at zero time or at the point of minimum enzyme activity (Fig. 4) strongly inhibited the recovery process. Analogous results were obtained with erythromycin (200 $\mu\text{g/ml}$).

Although the loss and recovery of D-glucoside 3-dehydrogenase activity was usually examined in carbon- and nitrogen-free medium, it could also occur in nitrogen-free medium containing succinate. When bacteria from a methyl α -D-glucoside-limited steady state (specific growth rate 0.17 h^{-1}) were incubated in nitrogen-free medium containing 20 g succinate/l, 65 % of the initial dehydrogenase activity was lost within 5 h. However, on continued incubation the activity recovered, reaching its original value after 70 h. In the presence of D-fructose, D-glucose, methyl α -D-glucoside or sucrose (all at 20 g/l) no recovery of the lost activity occurred even after 250 h incubation.

Fig. 6. The effect of (■) 8-hydroxyquinoline (5×10^{-3} M), (○) Mn^{2+} (10^{-3} M), (△) erythromycin (200 μ g/ml) and (□) 2,4-dinitrophenol (10^{-3} M) on the rate of disappearance of D-glucoside 3-dehydrogenase activity from *A. tumefaciens* transferred from a chemostat steady state to 0.1 M-phosphate buffer (pH 7.0) containing sucrose (40 g/l). No addition was made to the control (●). The organism was grown in phosphate-limited methyl α -D-glucoside medium at a specific growth rate of 0.18 h^{-1} .

Irreversible disappearance of D-glucoside 3-dehydrogenase activity from non-growing bacteria

Bacteria from nitrogen-, manganese-, phosphate, and carbon-limited chemostat steady states were incubated at 28°C in nitrogen-free medium containing 40 g sucrose/l (Fig. 5). The major effect of sucrose was to reduce the rate of enzyme decay, but the lost activity was not subsequently recovered despite prolonged incubation. In organisms from nitrogen-limited steady states, loss of activity started immediately and was exponential (half-life 15.5 h) for at least 40 h (Fig. 5a). After an initial period of stability lasting about 4 h, activity was also lost exponentially (half-life 20.5 h) from bacteria grown under manganese-limitation (Fig. 5b). In both cases the rate of loss of activity was independent of the previous specific growth rate of the organisms. In bacteria from phosphate-limited steady states (Fig. 5c) the initial period of stability lasted 3 to 9 h; the activity then decayed at two distinct rates: the half-life of the enzyme was 10 to 23 h during the first decay phase, and 22 to 26 h during the second. D-Glucoside 3-dehydrogenase activity in bacteria from carbon-limited steady states (Fig. 5d) was stable for up to 8 h and then decayed at an accelerating rate, which was most rapid in cells grown at high specific rates.

Stabilization of D-glucoside 3-dehydrogenase activity in nitrogen-starved bacteria

The effect of metabolic inhibitors and metal ions on loss of D-glucoside 3-dehydrogenase activity was determined. Bacteria (2.0 mg dry wt/ml) from a phosphate-limited steady state (specific growth rate 0.18 h^{-1}) were incubated in 0.1 M-phosphate buffer pH 7.0 containing sucrose (40 g/l) and the test compound (Fig. 6). 8-Hydroxyquinoline stabilized D-glucoside 3-dehydrogenase activity; erythromycin and 2,4-dinitrophenol also reduced the rate of decay, especially in the initial stages. Manganese ions accelerated the loss of activity during

the initial 3 h, but thereafter reduced the rate of decay. Chloramphenicol (500 $\mu\text{g/ml}$) had no stabilizing effect on D-glucoside 3-dehydrogenase activity in cells from the phosphate-limited steady state, but it increased the half-life of the enzyme from 15.5 to 26.5 h in cells grown at 0.07 or 0.17 h^{-1} under nitrogen-limitation.

Although it stabilized D-glucoside 3-dehydrogenase activity (Fig. 6), 8-hydroxyquinoline destroyed the ability of bacteria to produce 3-ketosucrose when oxygen acted as the terminal electron acceptor. However, treated bacteria produced 3-ketosucrose at the normal rate in the presence of ferricyanide. This suggested that 8-hydroxyquinoline inactivated one or more components of the electron-transport pathway required for the formation of 3-ketosucrose in respiring cells. A rapid loss of respiratory activity from cells in nitrogen-free medium containing 8-hydroxyquinoline ($5 \times 10^{-3} \text{ M}$) was confirmed by oxygen uptake studies using a Clark electrode.

DISCUSSION

Manganese ions strongly repressed D-glucoside 3-dehydrogenase synthesis in sucrose medium (Table 2), but had little effect when the carbon source was methyl α -D-glucoside (Fig. 2). D-Glucoside 3-dehydrogenase is thus not essential for the catabolism of sucrose by *A. tumefaciens*. The repressive effect of manganese ions could reflect inhibition of D-glucoside 3-dehydrogenase synthesis by a metabolic intermediate whose concentration is manganese-dependent during growth on sucrose but relatively independent of manganese concentration when methyl α -D-glucoside is the carbon and energy source. The addition of succinate (10^{-2} M) to an exponentially-growing culture of *A. tumefaciens* reduced the differential rate of D-glucoside 3-dehydrogenase synthesis by over 80 % (Kurowski, Fensom and Pirt, unpublished results) which shows that some metabolites may repress D-glucoside 3-dehydrogenase synthesis. The linear increase in specific D-glucoside 3-dehydrogenase activity with growth rate in methyl α -D-glucoside medium suggests a corresponding decline in the degree of repression with increasing growth rate. Repression of β -galactosidase synthesis in *Escherichia coli* is at a minimum during carbon-limited growth, where the rate of catabolism is restricted relative to that of anabolism (Mandelstam, 1962; Clark & Marr, 1964). However, limitation of growth by methyl α -D-glucoside did not lead to markedly higher D-glucoside 3-dehydrogenase activities. During nitrogen-, phosphate- and manganese-limited growth, greater repression may have been balanced by the presence of excess inducer (methyl α -D-glucoside) in the supernatant medium. The rate of synthesis of many enzymes probably depends on the relative intensities of the opposing controls of induction and repression under the particular growth environment (Clarke & Lilly, 1969).

When *A. tumefaciens* was incubated in carbon- and nitrogen-free medium (Fig. 3) or in nitrogen-free medium containing succinate, D-glucoside 3-dehydrogenase activity disappeared, but subsequently returned and was then highly stable. Preliminary studies using polyacrylamide gel electrophoresis indicated that the enzyme protein may be present at all times, even when no enzyme activity is detectable. We conclude that the dehydrogenase is either reversibly inhibited or reversibly inactivated, possibly by an enzyme-mediated process (Holzer, 1969). The kinetics of loss and recovery of activity were sometimes dependent on the limiting nutrient in the previous growth phase, which could indicate that the levels and distributions of enzymes and metabolites are important. Chloramphenicol did not affect the initial rate of loss of activity. Therefore, if the loss of activity is an enzyme-catalysed process, it is probable that the inactivating enzyme is present in growing bacteria, or is formed without appreciable lag after growth ceases and is not subject to rapid turnover. The complete inhibition of the recovery of activity by chloramphenicol (Fig. 4) could mean either that it

inhibits the synthesis of a specific protein required in the reactivation process, or that in the absence of general protein metabolism the loss of an inhibitory metabolite does not occur.

D-Glucoside 3-dehydrogenase activity was lost irreversibly from cells of *A. tumefaciens* starved of nitrogen in the presence of sucrose (Fig. 5). Except in cells from carbon-limited steady states, loss of activity followed first order kinetics. First order decay probably implies that once a given protein molecule is synthesized its chance of degradation is random (Schimke, 1970). Decay of enzyme activities in mammalian systems usually follows first order kinetics, for example all enzymes examined in the rat liver decay exponentially to basal levels (Schimke, 1970), but exponential decay in micro-organisms (e.g. Cole & Hinshelwood, 1947) is less common.

The loss of D-glucoside 3-dehydrogenase activity from bacteria starved of nitrogen in the presence of sucrose was accompanied by disappearance of the enzyme band from polyacrylamide gels (Kurowski, Fensom & Pirt, unpublished results). It is therefore possible that the loss of activity was due to degradation of the D-glucoside 3-dehydrogenase protein, but it is also possible that the disappearance of the enzyme band on polyacrylamide gels could result from relatively minor changes in the protein which affect its electrophoretic mobility. Tryptophan pyrrolase in rat liver (Schimke, Sweeney & Berlin, 1965) and isocitrate lyase in *Chlorella pyrenoidosa* (John, Thurston & Syrett, 1970) are enzymes which can be inactivated by degradation. If the D-glucoside 3-dehydrogenase is degraded, then the simplest explanation is proteolytic attack. A physical dissociation of the protein into inactive subunits seems unlikely; the stability of the recovered activity in bacteria incubated in carbon- and nitrogen-free medium indicates that the enzyme is not inherently labile. General protein turnover in *A. tumefaciens* starved of nitrogen in the presence of sucrose occurs at an initial rate of 3.9 %/h but declines to a very low level after 6 h (Fensom & Pirt, 1975). Thus, if the D-glucoside 3-dehydrogenase is degraded proteolytically, the process must continue beyond the point when general protein turnover has ceased.

Some studies (e.g. Schlessinger & Ben-Hamida, 1966; Willetts, 1967; Prouty & Goldberg, 1972) have shown that inhibitors of protein synthesis and uncouplers of ATP production limit protein breakdown; however, in KB cells, cycloheximide does not affect protein turnover (Feldman & Yagil, 1969). Loss of D-glucoside 3-dehydrogenase activity in the presence of substrate was not prevented by inhibitors of protein or ATP synthesis, although chloramphenicol, erythromycin and 2,4-dinitrophenol could reduce the rate of inactivation. Such compounds could stabilize the enzyme by reducing the activity of the degradative system itself, possibly through causing an accumulation of tRNA charged with amino acids (Goldberg, 1971). The mechanism by which the metal-chelating agent, 8-hydroxyquinoline, stabilized the dehydrogenase activity is not known; it could involve inhibition of metal-containing enzymes similar to carboxypeptidase.

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