Repression of Inducible Tartrate Dehydratases in *Pseudomonas* Strains

BY R. F. ROSENBERGER AND M. SHILO

*Laboratory of Microbiological Chemistry, Department of Biochemistry,
Hebrew University-Hadassah Medical School, Jerusalem, Israel*

(Received 1 October 1962)

SUMMARY

The effect of various growth substrates on the synthesis of three inducible enzymes, the stereo-specific tartrate dehydratases, has been followed in strains of *Pseudomonas*. Four organic acids (succinate, malate, acetate, *meso*-tartrate) inhibited induction of some or of all of the tartrate dehydratases, while three (pyruvate, *l*-tartrate, and *d*-tartrate) did not inhibit. Whenever inhibition occurred, synthesis of *d*- and *l*-tartrate dehydratase was repressed much more strongly than synthesis of *meso*-tartrate dehydratase. Pre-induction of the bacteria did not prevent subsequent repression and a degree of correlation was found between the growth rate supported by substrates and the extent of repression. The significance of the different degrees of repression given by substrates feeding into closely related sites of the Krebs cycle and of the differences in repression exerted on enzymes acting on closely related compounds is discussed.

INTRODUCTION

Several structurally unrelated metabolizable substrates have been shown to inhibit the synthesis of certain enzymes in bacteria (Monod, 1942; Mandelstam, 1961; Magasanik, 1961). To explain the inhibition, it has been suggested that these substrates give rise to metabolic intermediates which act as enzyme repressors. Thus the effect, which has been known for many years as diauxie or the 'glucose effect', has recently been termed catabolite repression (Mandelstam, 1961; Magasanik, 1961). While little is known about the nature or the specificity of the substances responsible for catabolite repression (Engelsberg, Watson & Hoffe, 1961; Magasanik, 1961; Mandelstam, 1962), studies on the relation between substrate metabolism and concentration of constitutive enzymes in *Escherichia coli* and yeasts have indicated that compounds other than the specific repressors considered responsible for the induction phenomenon are involved (Brown & Monod, 1961; Mandelstam, 1962; McQuillan & Halvorson, 1962).

In the experiments to be reported, aspects of catabolite repression have been studied in strains of *Pseudomonas* which metabolize inductively two or all three of the optical isomers of tartaric acid. Induction to tartrate utilization in these strains involves the synthesis of dehydratases which catalyse the conversion of tartrate isomers to oxaloacetate. Three tartrate dehydratases, each specific for one of the optical isomers, are known; these are induced only by their particular substrates (Shilo, 1957; Shilo & Stanier, 1957). Catabolite repression of tartrate dehydratase synthesis by organic acids has been reported (Rosenberger & Shilo, 1961). In the

present work attempts have been made to study the specificity of repression against the three dehydratases and to determine, by the use of substrates which enter metabolism at identical or closely related points of the Krebs cycle, which metabolic steps are critical for the accumulation of enzyme repressors. Substrates such as the three tartrate isomers, succinate, malate, acetate and pyruvate share all or nearly all of their catabolic pathways and differences in the degree of repression should be traceable to differences in the rates of the small number of metabolic steps not common to each or to specific unshared intermediates.

METHODS

Organisms. The strains of *Pseudomonas* used were described by Shilo & Stanier (1957).

Media and growth conditions. The basal medium used contained per l. distilled H_2O : $(NH_4)_2SO_4$, 1 g.; KH_2PO_4 , 1.5 g.; Na_2HPO_4 , 0.25 g.; $MgSO_4 \cdot 7H_2O$, 50 mg.; $(NH_4)_2SO_4 \cdot FeSO_4 \cdot 6H_2O$, 5 mg.; Yeast extract (Difco), 25 mg.; pH 6. Carbon sources were added to a final concentration of 0.3% (w/v) or as indicated in the text. Carbon source and basal medium were sterilized together by autoclaving (20 min. at 121°) except in the case of sodium pyruvate which was sterilized by filtration. Cultures were grown in Erlenmeyer flasks on a rotary shaker at 30° .

Enzyme assays. For *d*-tartrate dehydratase estimations organisms were washed once in 50 mM-tris (2-amino-2 hydroxymethylpropane-1,3-diol) buffer (pH 8.7), re-suspended in the same buffer and lysed by the addition of 4 μ mole ethylenediaminetetra-acetic acid (EDTA) and 20 μ g. lysozyme/ml. (Repaske, 1958). The lysates were incubated for 10 min. at 30° with 12.5 mM-*d*-tartrate, 4 mM-EDTA and 50 mM-tris buffer (pH 8.7). In the presence of dehydratase and EDTA, oxaloacetate accumulated without further enzymic breakdown (Shilo, 1957). The reaction was stopped by the addition of trichloroacetic acid, and, after centrifugation to remove precipitated proteins, the keto acids formed were estimated by the method of Friedman & Haugen (1943).

Meso-tartrate dehydratase was assayed as above, but with the substitution of 20 mM-phosphate buffer (pH 7.2) for tris buffer and 12.5 mM-*meso*-tartrate for *d*-tartrate. For both enzymes under the above conditions, keto-acid accumulation proceeded at a linear rate for at least 15 min., and was proportional to the enzyme concentration. One dehydratase unit has been taken as the amount forming 1 μ mole keto acid/hour.

Oxygen uptake with various substrates. Ability to oxidize substrates with or without lag was measured by the conventional Warburg technique at 30° .

Estimation of bacterial dry weight and protein. Bacterial dry weights were determined by turbidity measurements in a Klett-Summerson colorimeter by comparison with a calibration curve. Protein was estimated by the method of Lowry, *et al.* (1951) with bovine serum albumin as standard.

Estimation of d-tartrate was by the method of Matchett *et al.* (1944). When the kinetics of *d*-tartrate utilization by washed suspensions were followed, samples were boiled for 2 min. to stop further breakdown, solids removed by centrifugation and assays performed on the supernatant fluid.

RESULTS

Repression of tartrate dehydratase synthesis by organic acids

Organisms were grown with the substrate under test as sole source of carbon and energy and, when the logarithmic phase of growth was reached, one of the tartrate isomers was added as inducer. Cultures had a density of 0.1–0.2 mg. bacterial dry weight/ml. at the time of inducer addition and induction was followed for about two generations. When induction occurred under the above conditions, plots of the amounts of *d*- or *meso*-tartrate dehydratase formed against total bacterial protein had the form of a straight line. The slope of this line, the differential rate of synthesis, was used to compare the rates of inductive enzyme formation. As no quantitative assay methods for *l*-tartrate dehydratase are available, adaptation to *l*-tartrate utilization was determined manometrically. Organisms were harvested approximately two generations after inducer addition, and their ability to oxidize *l*-tartrate without lag determined.

Table 1. *Rate of d-tartrate dehydratase synthesis on inducer addition to Pseudomonas strains growing with various organic acids*

Pseudo- monas strain	Differential rate of enzyme synthesis* during growth on following substrates plus inducer†						
	<i>d</i> -tartrate	<i>l</i> -tartrate	<i>meso</i> - tartrate	Succinate	<i>dl</i> -malate	Acetate	Pyruvate
MD9	28.3	25.9	< 2	< 2	< 2	n.g.	42.7
LML	26.9	32.2	< 2	< 2	< 2	< 2	35.1
MD	32.6	n.g.	< 2	< 2	< 2	< 2	41.7
12	16.5	11.6	n.g.	< 2	< 2	< 2	6.4

* *d*-tartrate dehydratase units formed/mg. total protein synthesized; † 0.3 % (w/v) substrate + 0.1 % (w/v) inducer; n.g., no growth with substrate as sole carbon source.

Table 2. *Rate of meso-tartrate dehydratase synthesis on inducer addition to Pseudomonas strains growing with various organic acids*

Pseudo- monas strain	Differential rate of enzyme synthesis* during growth on following substrates plus inducer†					
	<i>meso</i> - tartrate	<i>d</i> -tartrate	<i>l</i> -tartrate	succinate	<i>dl</i> -malate	acetate
MD9	147	81	117	19	29	n.g.
MD	114	121	n.g.	54	53	46
LML	83	196	135	17	74	28

* *meso*-tartrate dehydratase units formed/mg. total protein synthesized; † 0.3 % (w/v) substrate + 0.1 % (w/v) inducer; n.g. = no growth with substrate as sole carbon source.

The results obtained with four strains and seven organic acids are summarized in Tables 1, 2 and 3. Induction of *d*- or *l*-tartrate dehydratase was completely inhibited by intermediates of the Krebs cycle and by *meso*-tartrate. With both these dehydratases, pyruvate and the non-inducing optically active tartrate isomer allowed synthesis to proceed at rates comparable to those with inducer

alone. *Meso*-tartrate dehydratase synthesis was not inhibited by the other tartrate isomers and was in no case completely repressed. Pyruvate stood out from the other Krebs cycle intermediates tested, since in its presence synthesis of any of the three inducible dehydratases proceeded at rates equal to those with inducer alone.

Table 3. *Induction to l-tartrate oxidation after inducer addition to Pseudomonas strains growing with various organic acids*

Pseudomonas strain	O ₂ uptake* (0–20 min.; substrate <i>l</i> -tartrate) of washed organisms harvested after growth on following substrates plus inducer†			
	<i>d</i> -tartrate	<i>meso</i> -tartrate	succinate	pyruvate
MD9	76	4	16	70
LML	68	10	15	61
12	80	n.g.	16	64

* μ l. O₂/mg. dry wt., bacteria corrected for endogenous respiration; † 0.3 % (w/v) substrate + 0.1 (w/v) inducer; n.g. = no. growth of strain with substrate as sole carbon source.

Table 4. *Meso-tartrate dehydratase synthesis in washed suspensions of Pseudomonas strain MD9*

Time	Treatment and substrates added	Rate of <i>meso</i> -tartrate dehydratase synthesis (units/mg. protein/hr.) in washed organisms* previously grown on succinate
0–30 min.	succinate (20 mM)	—
30–120 min.	<i>meso</i> -tartrate (6.6 mM) succinate (20 mM)	3.4
120–180 min.	Organisms centrifuged, re-suspended in buffer + 6.6 mM <i>meso</i> -tartrate	10.6

* Organisms washed once in 22 mM phosphate buffer (pH 7.2), resuspended in same buffer.

Repression of meso-tartrate dehydratase synthesis in washed suspensions

Synthesis of *meso*-tartrate dehydratase was inhibited to a rather variable extent during growth on Krebs cycle intermediates, but was never completely repressed (Table 2). Since catabolite repression of certain enzymes has been shown to be more severe in washed suspensions than in growing cultures (Mandelstam, 1961), the effect of succinate on induction by *meso*-tartrate in washed suspensions was examined (Table 4). While succinate decreased the rate of dehydratase formation, induction did take place. *Meso*-tartrate dehydratase thus appeared to be more resistant to repression by metabolic intermediates than most other catabolic enzymes (Magasanik, 1961).

Effect of meso-tartrate on induction by gluconate

As growth on *meso*-tartrate inhibited induction of the *d*- or *l*-tartrate dehydratases, it seemed of interest to determine its effect on a third inducible system.

Organisms of *Pseudomonas* strain MD9 grown on *meso*-tartrate oxidized gluconate only after a pronounced lag, showing the inducible nature of this system. Addition of gluconate to organisms growing on *meso*-tartrate yielded organisms fully induced to gluconate oxidation. The tartrate isomer thus did not repress induction by gluconate.

Influence of inducer and substrate concentrations on repression

Pseudomonas strain md₉ was grown on various concentrations of *meso*- or *d*-tartrate and graded concentrations of *d*- or *meso*-tartrate were added as inducer. With *meso*-tartrate as substrate and the *d*-tartrate as inducer, the highest inducer/substrate ratio tried (6:1) did not induce. In the reverse case, with *d*-tartrate as substrate and *meso*-tartrate as inducer, the lowest inducer/substrate ratio tried (1:6) yielded induced organisms (Fig. 1).

Fig. 1. Effect of differing ratios of inducer to substrate on induction in strain md₉. Cells were grown on $8.8 \times 10^{-3} \text{ M}$ *meso*-tartrate with subsequent addition of $2 \times 10^{-3} \text{ M}$ *d*-tartrate (○—○) or on $2 \times 10^{-3} \text{ M}$ *d*-tartrate with subsequent addition of $8.8 \times 10^{-3} \text{ M}$ *meso*-tartrate (●—●). Approximately 2 generations after inducer addition cells were harvested and ability to oxidize *meso*- or *d*-tartrate without lag examined by the conventional Warburg technique.

Effect of organic acids on inducer entry

Some of the organic acids which repressed *d*-tartrate dehydratase induction are structurally related to the inducer and it seemed possible that they inhibited by competing with the inducer for its entry-site into the cell. To test this possibility, the rate of *d*-tartrate metabolism of resting suspensions of organisms fully induced to *d*-tartrate was measured in the presence and absence of *dl*-malate and *meso*-tartrate. *d*-Tartrate alone was utilized at a rate of $5.08 \mu\text{mole/hr./mg. dry weight}$ of organisms, while in the presence of threefold greater concentrations of *dl*-malate or *meso*-tartrate the rates were 4.22 and $3.54 \mu\text{mole/hr./mg. dry weight}$ respectively. These inhibitions (17–30 %) indicate that competition at the site of entry must be relatively slight.

Repression in pre-induced organisms

Cohn & Horibata (1959) reported that glucose completely repressed β -galactosidase induction in uninduced *Escherichia coli*, but that organisms actively forming β -galactosidase continued to do so at an unchanged rate for many generations in the presence of glucose plus inducer.

Effects of pre-induction were tested in *Pseudomonas* strain MD9 growing on *d*- or *l*-tartrate. Cultures were transferred to medium containing the same isomer together with another organic acid in equimolar proportions. Transfers to fresh medium containing the same two substrates were made approximately every five generations to ensure the continued presence of high concentrations of unchanged substrates. After 15–23 generations, organisms were harvested and tested for ability to oxidize *d*- or *l*-tartrate without lag. Pre-induction did not prevent repression of the *d*- or *l*-tartrate oxidizing system by *meso*-tartrate or succinate (Table 5). Organisms grown on a mixture of *d*- and *l*-tartrate were induced for the oxidation of both isomers, irrespective of the state of adaptation of the inoculum.

Table 5. *Repression of tartrate dehydratase synthesis in pre-induced Pseudomonas strain MD9*

Growth substrate of inoculum*	Substrates in cultures (each 10^{-2} M)	No. of generations before harvest	O ₂ uptake on addition of substrate 0–20 min. (μ l./mg. dry weight organism)			
			<i>d</i> -tartrate	<i>meso</i> -tartrate	<i>l</i> -tartrate	succinate
<i>d</i> -tartrate	<i>d</i> -tartrate + <i>meso</i> -tartrate	20	4	118	n.t.	n.t.
<i>d</i> -tartrate	<i>d</i> -tartrate + succinate	23	3	n.t.	n.t.	86
<i>d</i> -tartrate	<i>d</i> -tartrate + <i>l</i> -tartrate	15	71	n.t.	60	n.t.
<i>l</i> -tartrate	<i>l</i> -tartrate + <i>meso</i> -tartrate	15	n.t.	79	6	n.t.
<i>l</i> -tartrate	<i>l</i> -tartrate + <i>d</i> -tartrate	15	68	n.t.	53	n.t.

* Substrate conc. 0.3 % (w/v); n.t. = not tested.

Table 6. *Average generation times of Pseudomonas strains MD9, md and lml during growth on organic acids*

Pseudomonas strain	Substrate			
	succinate	<i>meso</i> -tartrate	<i>d</i> -tartrate	pyruvate
	generation time (min.)			
MD9	50	50	65	75
MD	54	53	85	72
LML	58	59	65	93

Correlation between growth rate in batch culture and repression

Neidhardt & Magasanik (1957) and Mandelstam (1962) showed that the degree of enzyme repression by various substrates tended to be related to the maximal growth rate supported by them. To determine whether a similar correlation exists in organisms which form tartrate dehydratases, the growth rate of three *Pseudomonas* strains in batch culture was compared (Table 7). In all cases growth was slower with pyruvate or *d*-tartrate than with succinate or *meso*-tartrate, though with strain lml the difference was no greater than 10%. These results are in keeping with those of the above mentioned authors, who found that carbon sources which supported a low growth rate repressed less effectively.

DISCUSSION

One of the aims of the present work was to compare the degree of repression of the three tartrate dehydratases in the same strain and under the same growth conditions and thus to evaluate the specificity of the inhibition. When repression occurred in any of the four strains examined, inhibition of the *d*- and *l*-tartrate dehydratases was always much greater than inhibition of the *meso*-tartrate enzyme. This was most strikingly shown during growth on *meso*-tartrate itself, where the *meso*-tartrate dehydratase was formed at high rates while induction of the enzymes attacking *d*- or *l*-tartrate was completely repressed. Similarly, Krebs cycle intermediates completely prevented induction by *d*- or *l*-tartrate, while *meso*-tartrate induced formation of its dehydratase, although at a decreased rate. It may be noted that growth on *meso*-tartrate inhibited induction by the other two tartrate isomers, but did not prevent induction to gluconate oxidation.

The literature contains few reports of catabolite repression specific for particular enzymes. Neidhardt & Magasanik (1957) described differential repression of histidase and inositol dehydrogenase in a strain of *Aerobacter* growing with glucose and histidine as sole nitrogen source. Both of these inducible enzymes were normally repressed by glucose, but under the above conditions histidase was formed while induction of inositol dehydrogenase remained inhibited. From these results the authors concluded that two different repressors acted on the synthesis of the enzymes (Magasanik, 1961). In the case of the tartrate dehydratases, however, differential repression could be equally well explained by different inhibitors or by a widely varying sensitivity to the same repressor.

Four of the seven substrates tested in the present work were effective repressors of induction by *d*- or *l*-tartrate, while three (pyruvate, *d*-tartrate, *l*-tartrate) allowed induction to proceed at rates equivalent to those with inducer alone. It has been known for several years that some substrates repress enzyme induction much more strongly than others and Neidhardt (1960), Mandelstam (1961, 1962) and Magasanik (1961) have related these differences to differences in the relative rates of substrate metabolism and total cell synthesis. The compounds tested in the present work all enter cell metabolism via the Krebs cycle and at sites in the cycle close to each other. Thus, *meso*-tartrate and *l*-tartrate are both converted to oxaloacetate in a single enzymic step, and therefore give rise to identical metabolic intermediates, yet they have very different effects on induction by

d-tartrate. It would appear therefore that the only metabolic differences which make for stronger or weaker repression are the rates of the first steps in substrate metabolism. Thus *d*- and *l*-tartrate are ineffective as repressors because of relatively slow rates of conversion to oxaloacetate and pyruvate, due to relatively slow rates of entry or carboxylation to oxaloacetate. The suggestion that the initial steps of pyruvate, *d*-tartrate and *l*-tartrate metabolism are slower than those of the other compounds tested is in keeping with the somewhat slower growth rates supported by these three substrates. It is more directly supported by the finding that the amounts of *d*-tartrate dehydratase during growth on inducer alone were three to five times lower than those of *meso*-tartrate dehydratase. Such low amounts of *d*-tartrate dehydratase (and by analogy of *l*-tartrate dehydratase) during growth with inducer as sole carbon source may in fact be a corollary of the sensitivity of this enzyme to repression by its own catabolic products. Higher dehydratase concentrations would lead to repressor pools of the size formed during growth on *meso*-tartrate or Krebs cycle intermediates, and these would be accompanied by inhibition of enzyme synthesis and return to a low dehydratase content.

REFERENCES

- BROWN, D. D. & MONOD, J. (1961). Carbon source repression of β galactosidase in *Escherichia coli*. *Fed. Proc.* **20**, 222.
- COHN, M. & HORIBATA, K. (1959). Physiology of the inhibition by glucose of the induced synthesis of the β galactosidase enzyme system in *Escherichia coli*. *J. Bact.* **78**, 624.
- ENGELSBURG, E., WATSON, J. A. & HOFFE, P. A. (1961). The glucose effect and the relationship between glucose permease, acid phosphatase and glucose resistance. *Cold. Spr. Harb. Symp. quant. Biol.* **26**, 261.
- FRIEDMAN, T. E. & HAUGEN, G. E. (1943). Pyruvic acid. II. The determination of keto acids in blood and urine. *J. Biol. Chem.* **147**, 415.
- LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, A. J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265.
- MACQUILLAN, A. M. & HALVORSON, H. O. (1962). Metabolic control of β galactosidase synthesis in yeasts. *J. Bact.* **84**, 23.
- MAGASANIK, B. (1961). Catabolite repression. *Cold. Spr. Harb. Symp. quant. Biol.* **26**, 249.
- MANDELSTAM, J. (1961). Induction and repression of β -galactosidase in non-growing *Escherichia coli*. *Biochem. J.* **79**, 489.
- MANDELSTAM, J. (1962). The repression of constitutive β -galactosidase in *Escherichia coli* by glucose and other carbon sources. *Biochem. J.* **82**, 489.
- MATCHETT, J. R., LEGAULT, R. R., NIMMO, C. C. & NOTTER, G. K. (1944). Tartrates from grape wastes. *Industr. Engng Chem. (Industr.)* **36**, 851.
- MONOD, J. (1942). *Recherches sur la croissance des cultures bactérienne*. Paris: Hermann et Cie.
- NEIDHARDT, F. C. & MAGASANIK, B. (1957). Reversal of the glucose inhibition of histidase biosynthesis in *Aerobacter aerogenes*. *J. Bact.* **73**, 253.
- NEIDHARDT, F. C. (1960). Mutants of *Aerobacter aerogenes* lacking glucose repression. *J. Bact.* **80**, 536.
- REPASKE, R. (1958). Lysis of gram negative organisms and the role of versene. *Biochim. biophys. Acta*, **30**, 225.
- ROSENBERGER, R. F. & SHILO, M. (1961). Diauxie in tartrate utilizing strains of *Pseudomonas* and its control by oxaloacetate. *Biochem. biophys. Res. Comm.* **4**, 414.
- SHILO, M. (1957). The enzymic conversion of the tartaric acids to oxaloacetic acid. *J. gen. Microbiol.* **16**, 472.
- SHILO, M. & STANIER, R. Y. (1957). The utilization of the tartaric acids by *Pseudomonas*. *J. gen. Microbiol.* **16**, 482.