***cis-cis*-Muconate, the Product Inducer of Catechol 1,2-Oxygenase in
*Pseudomonas aeruginosa***

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In *Pseudomonas* species and *Moraxella calcoacetica*, *cis-cis*-muconate acts as the product inducer of catechol 1,2-oxygenase as well as the substrate inducer for the co-ordinate induction of the enzymes catalysing its conversion into β -oxoadipate enol lactone in *Pseudomonas putida* (Ornston, 1966) or into β -oxoadipoyl-CoA in *Moraxella calcoacetica* (Cánovas & Stanier, 1967; Cánovas, Ornston & Stanier, 1967). A similar enzyme pattern, however, can also be induced with catechol. Even though it is unlikely that two compounds as different in chemical and physical properties as a diphenol and an aliphatic unsaturated, dicarboxylic acid would have similar inducing ability (Ornston, 1966) [a suggestion that has been confirmed in a strain of *Pseudomonas aeruginosa* (Farr & Cain, 1968) in which catechol and *cis-cis*-muconate induce a catechol 2,3-oxygenase and a catechol 1,2-oxygenase respectively], the possibility remains that catechol and *cis-cis*-muconate might both be effective inducers of the 1,2-oxygenase in other species.

The problem was investigated by utilizing the ability of *Pseudomonas aeruginosa* to grow anaerobically with nitrate as the terminal electron acceptor (Stanier, Palleroni & Doudoroff, 1966). Under anaerobic conditions, the enzymic conversion of catechol into *cis-cis*-muconate (catalysed by the irreversible O_2 -requiring catechol 1,2-oxygenase) is prevented; it is then possible to determine the distinct inducing patterns of each compound.

Experimental. Strain 8b2 of a bacterium originally isolated from sewage by enrichment with benzoate as carbon source was identified as *Pseudomonas aeruginosa* by the criteria of Stanier *et al.* (1966). A mutant of this strain (PM) with increased permeability for *cis-cis*-muconate was obtained as described by Ornston (1966). For use in the anaerobic induction experiments, the permeability mutant was grown at 30° in 2l. conical flasks filled to the neck with a medium consisting of (g./l. of distilled water): glycerol, 10; K_2HPO_4 , 1; NH_4Cl , 1; $MgSO_4 \cdot 7H_2O$, 0.1; a trace-elements supplement. KNO_3 (10g./l.) was added as an electron acceptor and the pH adjusted to 7.0. Cells grown anaerobically in this medium were harvested

during exponential growth (25–30 hr.), washed twice with 0.1M-tris-HCl buffer, pH 7.2, and resuspended to approx. 0.7mg. dry wt./ml. in 50ml. of the mineral salts-nitrate medium, pH 7.0, contained in 1l. flasks. Each flask was fitted with an inlet and outlet tap, the latter fused into a deep side arm containing sufficient inducer and glycerol to give final concentrations 3mM and 0.5mM respectively when mixed with the flask contents. A control flask contained 0.5mM-glycerol only. The glycerol, which was added at this concentration to provide an initial source of energy for protein synthesis, did not significantly repress induction of the ring-fission enzymes in preliminary aerobic experiments.

The induction flasks, connected in sequence, were flushed with O_2 -free N_2 (containing less than 100p.p.m. of O_2 ; British Oxygen Co. Ltd.) that had also been bubbled through two deep solutions of freshly prepared alkaline pyrogallol (10%, w/v, in $N-NaOH$). After 30 min. flushing at 27°, the system was evacuated with a vacuum pump until the solutions no longer 'degassed'. This N_2 flush and evacuation cycle was repeated. Thereafter the N_2 was passed continuously through the flasks at a rate of approx. 200ml./min., maintaining a small positive pressure of N_2 during induction. After 15 min. temperature equilibration in a shaker bath, the side-arm contents were tipped into the flasks and incubation was continued at 27°. Samples (1ml.) of the flask contents were removed initially and at intervals thereafter for determinations of inducer concentration. During this operation the N_2 pressure was substantially increased. Aerobic inductions were carried out in the same medium (including the nitrate to maintain strict comparison) but in conical flasks loosely plugged with cotton wool and shaken rapidly (240 rev./min.) in a Gyro-tory incubator shaker (New Brunswick Scientific Co., New Brunswick, N.J., U.S.A.).

Inducing suspensions were harvested at times when approximately equivalent amounts (30%) of inducer had been consumed; this amount supported a doubling of the cell mass. Further protein synthesis was stopped by rapidly cooling the flasks to 0°. Subsequent centrifugation and washings were carried out as rapidly as possible with

Table 1. *Activities of the enzymes of the catechol pathway in extracts of Pseudomonas aeruginosa induced aerobically and anaerobically with catechol and cis-cis-muconate*

Expt. A (aerobic induction). Cell suspensions induced with catechol were harvested when 30% of the catechol had disappeared and catechol was being consumed at $2.3 \mu\text{moles/hr./mg. dry wt. of cells}$. Cells induced with *cis-cis*-muconate were harvested in two parts: (a) when 0.4% of the available *cis-cis*-muconate had disappeared and the substrate was being consumed at $0.01 \mu\text{mole/hr./mg. dry wt. of cells}$; (b) when 33% of the available *cis-cis*-muconate had disappeared, now at $0.4 \mu\text{mole/hr./mg. dry wt. of cells}$. The control flask was harvested at the same time as (b). Expt. B (anaerobic induction). Two separate experiments were performed, each with catechol and *cis-cis*-muconate as inducers. In (i) the cells were harvested when 15% of *cis-cis*-muconate had disappeared and was being consumed at $0.03 \mu\text{mole/hr./mg. dry wt. of cells}$. In (ii) the cells were harvested after 20% of *cis-cis*-muconate had disappeared, now at $0.05 \mu\text{mole/hr./mg. dry wt. of cells}$. In (i) and (ii) cells induced with catechol were harvested at the same time, but in neither case was there any disappearance of catechol. Cell suspensions from different anaerobic cultures were used in each experiment. Checks for possible contamination at the end of longer incubations yielded only the PM strain of *P. aeruginosa*.

Enzyme	Specific activity (mμmoles/min./mg. of protein) in extracts of cells incubated with					
	Catechol		<i>cis-cis</i> -Muconate		No inducer	
			(a)	(b)		
Expt. A (aerobic induction)						
Catechol 1,2-oxygenase	405		59	64		5
Muconate-lactonizing enzyme	72		3	40		1
Muconolactone isomerase + β-oxoadipate enol lactone hydrolase	84		12	79		7
Expt. B (anaerobic induction)	(i)	(ii)	(i)	(ii)	(i)	(ii)
Catechol 1,2-oxygenase	0	1.5	7	20	0	1
Muconate-lactonizing enzyme	0.5	0.7	2	13	0	0
Muconolactone isomerase + β-oxoadipate enol lactone hydrolase	0	0	8	40	0	0

pre-cooled equipment and buffer. Extracts were prepared within 30 min. by exposing the harvested cells for 10 min. to the output of the MSE 60w Ultrasonic Disintegrator at 20 kcy./sec. and centrifuging off the debris at 2500g for 15 min. Enzyme assays were carried out at 30° in a Unicam SP.800 recording spectrophotometer fitted with the SP.840 Expansion Unit and a Honeywell Elektronik 15 recorder to increase the extinction scale tenfold when low enzyme activities were being measured. Catechol 1,2-oxygenase (EC 1.13.1.1) and *cis-cis*-muconate-lactonizing enzyme (EC 5.5.1.1) activities were determined by the methods of Hegeman (1966). An overall measure of the activity of (+)-muconolactone isomerase and β -oxoadipate enol lactone hydrolase was obtained by following the disappearance of (+)-muconolactone at 230m μ (Sistrom & Stanier, 1954).

Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) and catechol by the method of Evans (1947). *cis-cis*-Muconate was determined spectrophotometrically at 260m μ (ϵ 17200), and growth was measured turbidimetrically at 450m μ .

Results. After aerobic growth in benzoate or *cis-cis*-muconate (10mM) medium, the permeability mutant oxidized *cis-cis*-muconate at 20 $\mu\text{l. of}$

O₂/hr./mg. dry wt. of organism at 30°, whereas the corresponding values for the wild-type varied between zero activity and 3 $\mu\text{l. of O}_2$ /hr./mg. dry wt. The mutant still exhibited some restriction in its ability to assimilate *cis-cis*-muconate, because catechol was much more rapidly consumed in aerobic experiments by benzoate-grown cells and consistently induced some five- to eight-fold more catechol 1,2-oxygenase (Table 1, Expt. A). In the PM strain almost full induction of catechol 1,2-oxygenase was present after 8.5 hr. aerobic exposure to *cis-cis*-muconate [Table 1, Expt. A(a)], whereas activities of the other enzymes of the co-ordinate block, for which *cis-cis*-muconate acts as a substrate inducer, were at this time very low. Some 18 hr. later, however [Table 1, Expt. A(b)], these enzyme activities were comparable with those found in catechol-induced cells. Cánovas & Johnson (1968) have also isolated mutants of *Moraxella calcoacetica* in which catechol 1,2-oxygenase showed a much greater response than did the enzymes of the muconate co-ordinate block to induction by *cis-cis*-muconate, but Ornston (1966) found evidence to suggest that *cis-cis*-muconate was a less efficient inducer of the oxygenase compared with the muconate block of enzymes in *Pseudomonas putida*. In our strain of *Pseudomonas aeruginosa*,

which obviously resembles *Moraxella*, the uptake of less than 0.1 μ mole of *cis-cis*-muconate/ml. at 8.5 hr. effected the induction of nearly the maximum amounts of catechol 1,2-oxygenase possible with this inducer.

Because the conversion of *cis-cis*-muconate into succinate involves no O₂-requiring stage and *Pseudomonas aeruginosa* can ferment succinate anaerobically in the presence of nitrate, *cis-cis*-muconate should be utilized under anaerobic conditions. Suspensions of cells grown anaerobically with glycerol and incubated with *cis-cis*-muconate under N₂ consumed this substrate more slowly than they did aerobically; the rate of uptake reached a maximum after about 50 hr. incubation.

The results (Table 1, Expt. B) showed that anaerobiosis almost completely prevented induction by catechol of the enzymes of the catechol pathway in *Pseudomonas aeruginosa*. *cis-cis*-Muconate, in similar conditions, induced all three enzymes that it likewise induced under aerobic conditions, though the actual amounts of enzyme were somewhat less than those found in aerobic incubations. This result clearly establishes that catechol itself has no inducing capacity for any of the enzymes of the catechol pathway in this organism and that

cis-cis-muconate is the product inducer of catechol 1,2-oxygenase. The inducing properties of catechol observed aerobically must therefore be due to endogenous formation of *cis-cis*-muconate by the very small amounts of enzyme present in uninduced cells.

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