Metabolism of Mandelate and Related Compounds by Bacterium NCIB 8250

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SUMMARY

Bacterium NCIB 8250 was grown on mandelate, benzyl alcohol, benzoyl-formate, benzaldehyde or benzoate and also on 2-hydroxy, 4-hydroxy, 3,4-dihydroxy and 4-hydroxy-3-methoxy derivatives of these compounds. Growth rates and yields of organism were measured for many of the substrates. The pathways of oxidation of mandelate and related compounds were investigated by the technique of simultaneous adaptation. Bacterium NCIB 8250 did not utilise D-mandelate; L-mandelate was oxidised via benzoyl-formate, benzaldehyde and benzoate to catechol. Benzyl alcohol was converted to catechol via benzaldehyde and benzoate. The 2-hydroxy-substituted compounds were oxidised to catechol by a parallel pathway. 4-Hydroxy-, 3,4-dihydroxy- and 4-hydroxy-3-methoxy-substituted compounds were all converted to 3,4-dihydroxybenzoate. Catechol and 3,4-dihydroxybenzoate underwent ring cleavage with the ultimate formation of β -oxoadipate.

INTRODUCTION

Mandelate can be utilized by some bacteria as sole source of carbon and energy for growth. The mechanism whereby *Pseudomonas putida* oxidizes this compound to intermediates of the tricarboxylic acid cycle has been thoroughly investigated by Stanier and his colleagues and is the original example of sequential induction (Stanier, 1947; Gunsalus, Stanier & Gunsalus, 1953; Gunsalus, Gunsalus & Stanier, 1953; Stanier, Gunsalus & Gunsalus, 1953; Hegeman, 1966*a, b, c*; Ornston & Stanier, 1966; Cánovas, Ornston & Stanier, 1967). Mandelate and a considerable number of related compounds also serve as growth substrates for bacterium NCIB 8250 (Fewson, 1967*a*). Bacterium NCIB 8250, which has generally been known as 'Vibrio 01', belongs to the Acinetobacter–Moraxella group of bacteria (Sebald & Véron, 1963; Véron, 1966; Fewson, 1967*b*). The members of this group have nutritional, and probably ecological, similarities to species of the genus *Pseudomonas*, but differ in important morphological respects and also possess a quite different DNA base composition (Cánovas *et al.* 1967) which suggests that the two groups are evolutionarily distinct. It therefore seemed of interest to investigate the way in which bacterium NCIB 8250 grows on a range of compounds related to mandelate and to compare the pathways with those operating in *P. putida*. It was also felt that the technique of simultaneous adaptation (Stanier, 1947) could be used to give information about the specificity of enzyme induction and activity. Thus it might be expected that if the organism were grown on as many of the likely intermediates and analogues as possible, and then

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exposed in the Warburg apparatus to a similar range of compounds, the resulting patterns of oxygen uptake would indicate the degree to which non-specificity of enzyme induction and activity contributed to the economy of the cell. Results of this type of experiment are reported in the present paper and suggest that relatively non-specific enzymes carry out the first stages in the oxidation of mandelate and a number of substituted derivatives. More specific enzymes are formed for the oxidation of benzoate and the substituted benzoates.

Some of the results given in this paper have been published in a preliminary form (Kennedy & Fewson, 1966).

METHODS

Organism. Bacterium NCIB 8250 was maintained, grown and harvested as described by Kennedy & Fewson (1968).

Mean generation times. Samples (8 ml.) of a 17 hr nutrient broth culture were inoculated into medium (800 ml.) contained in 1 l. flasks fitted with side arms to facilitate sampling. Cultures were grown under conditions of vigorous aeration at 30° in the apparatus described by Harvey, Fewson & Holms (1968). Growth was followed by taking samples (4 ml.) at appropriate times and measuring the extinction at 500 m μ in a Spectronic 20 colorimeter with standard tubes of internal diam. 11.7 mm. Growth rates were determined graphically by conventional means (Dawes, 1962).

Molar growth yields. The basal medium was $\text{KH}_2\text{PO}_4 + (\text{NH}_4)_2\text{SO}_4 + \text{MgSO}_4$ (Kennedy & Fewson, 1968) with various concentrations of carbon sources. Medium (50 ml.) contained in 250 ml. Erlenmeyer flasks plugged with cotton wool was inoculated (0.2 %, v/v) with a 17 hr nutrient broth culture. The cultures were incubated for 4 days at 30° on a rotary shaker (Mk V; L. H. Engineering Co., Bells Hill, Stoke Poges, Buckinghamshire) moving at about 180 oscillations/min. The extinctions of samples (4 ml.) were measured at 500 m μ as described above. Under these conditions the relationship between E_{500} and dry wt/ml. was constant up to $E_{500} = \text{approx. } 0.4$. Suspensions with extinctions greater than 0.3 were diluted with basal medium before reading. In this series of experiments, and for organisms grown in a range of media, a value of $E_{500} = 1.0$ was equivalent to 310 $\mu\text{g. dry wt/ml.}$ Controls were included for growth in the basal medium without added carbon source, but such growth was barely detectable. Preliminary experiments showed that the medium after growth did not contain material which absorbed at 500 m μ . Most compounds showed a constant molar growth yield ($\mu\text{g. dry wt formed}/\mu\text{mole substrate}$) up to at least 2 mM but a few substrates, e.g. benzaldehyde, gave lower yields at concentrations above about 1 mM; in all cases, therefore, the molar growth yield was calculated from the initial slope of the graph of yield of organism plotted against substrate concentration.

Oxygen uptake by whole bacteria. The ability of cell suspensions of bacterium NCIB 8250 to oxidize a number of substrates was determined by conventional manometric techniques (Umbreit, Burris & Stauffer, 1957). Although it is not normal practice, respiration was measured in a medium potentially capable of supporting growth since it was anticipated that the patterns of enzyme induction would be of as much interest as the initial rates of oxygen uptake. In any case it is usually difficult to avoid some enzyme induction, even in so-called non-proliferating suspensions, unless inhibitors of protein synthesis are used. Suspensions of bacteria, washed once with ice-cold distilled water, were invariably used within 4 hr of harvesting. Each 15 ml. vessel

contained 0.1 ml. of 0.3 M-(NH₄)₂SO₄; 0.1 ml. of 0.03 M-MgSO₄·7H₂O; 0.1 ml. of 0.5 M-KH₂PO₄+K₂HPO₄ buffer (pH 7.0); 0.8 ml. of aqueous bacterial suspension (10 mg. wet wt bacteria/ml.). The reaction was started by tipping 0.5 ml. of 7.2 mM-substrate (dissolved in 0.1 M-KH₂PO₄+K₂HPO₄ and adjusted to pH 7.0 with 0.1 N-NaOH or 0.1 N-HCl) from the side arm. The centre well contained 0.2 ml. 20 % (w/v) KOH and pleated filter paper (Whatman no. 52). The total liquid volume was 2.0 ml. The reaction was run at 30° in air. Preliminary experiments showed that the rate of oxygen uptake was proportional to amount of bacteria at least up to the concentration used.

Chromatographic separations. Hydroxybenzoic acids were separated by two-dimensional chromatography on Whatman no. 1 paper. The first solvent (ascending for 15 hr) was benzene + acetic acid + water (2 + 2 + 1, by vol., upper phase; Bray, Thorpe & White, 1950) and the second solvent (ascending for 5 hr) was butan-1-ol + pyridine + water (14 + 3 + 3, by vol.; Smith, 1960). Compounds were detected on the dried papers by absorption or fluorescence under ultraviolet radiation, and by spraying with diazotized *p*-nitroaniline, diazotized sulphanilic acid or ammoniacal silver nitrate (Smith, 1960).

Analytical methods. Values for the wet weights of bacteria were obtained by centrifuging appropriate volumes of suspension in tared centrifuge tubes, washing the precipitate by suspension in water and again centrifuging; excess moisture was removed with tissue and the tubes weighed. Dry weights were determined by adding suspensions of washed bacteria (washed with ice-cold distilled water) to tared 20 ml. sample bottles, and drying to constant weight at 105°.

The nitrogen content of bacteria was determined by digesting samples (about 0.5 mg. wet wt) at 180° with 0.25 ml. of 1 % (w/v) selenium dioxide dissolved in 50 % (v/v) H₂SO₄. Ammonia assays were made on the resulting digests by the Nessler method (Paul, 1958); A.R. (NH₄)₂SO₄ was used as standard.

Chemicals. Dr P. G. Heytler (Central Research Department, E. I. du Pont de Nemours and Co., Wilmington, Del., U.S.A.) kindly gave a sample of carbonyl-cyanide-*p*-trifluoromethoxyphenylhydrazone. Actinomycin D was a gift from Merck, Sharp and Dohme Inc. (Rahway, N.J., U.S.A.). 3-Hydroxybenzoic acid was bought from British Drug Houses Ltd. (Poole, Dorset) and recrystallized from hot water. β -Oxadipic acid and puromycin dihydrochloride were bought from Sigma London Chemical Co. Ltd. (12 Lettice Street, London, S.W. 6). Chloramphenicol was from Parke, Davis and Co. Ltd. (Hounslow, Middlesex). The sources of other reagents are listed by Kennedy & Fewson (1968) or were the best quality which could be obtained commercially.

RESULTS

The growth parameters for batch cultures of bacterium NCIB 8250 grown on mandelate and related compounds are given in Tables 1 and 2. 4-Hydroxybenzoyl-formate, 3,4-dihydroxybenzoylformate, 4-hydroxy-3-methoxybenzoylformate and 3,4-dihydroxybenzyl alcohol were not available for testing. Growth rates with catechol and yields with catechol and 3,4-dihydroxybenzoate were not determined because of the brown colour produced in these media. Appreciable time lags (more than 10 min.) before the onset of exponential growth were observed with only a few substrates. Calculations of the length of these lags by conventional methods (e.g. Dawes, 1962)

Table 1. *Growth rates of bacterium NCIB 8250 with different substrates*

The basal medium contained $\text{KH}_2\text{PO}_4 + \text{MgSO}_4 + (\text{NH}_4)_2\text{SO}_4$. Concentrations of mandelate and the substituted mandelates refer to the L-isomers but all these compounds were added as double-strength D,L-mixtures. A 17 hr nutrient broth culture was used as inoculum (1.0 %, v/v) and growth at 30° was followed as described in Methods. Results are means of at least two experiments. Lags are described as present or absent since, as described in the text, their length tended to be difficult to estimate. n.g. = no growth.

Substrate	Substrate concentration (mM)				Lag
	0.3	0.5	1.0	1.5	
	Mean generation times (min.)				
D,L-Mandelate	95	87	73	71	—
Benzoylformate	.	82	83	84	—
Benzyl alcohol	.	51	52	56	—
Benzaldehyde	.	47	49	60	—
Benzoate	45	45	45	45	—
2-Hydroxy-D,L-mandelate	.	1893	1740	2280	+
2-Hydroxybenzoylformate	.	3696	2736	2352	+
2-Hydroxybenzyl alcohol	.	141	130	141	—
2-Hydroxybenzaldehyde	97	n.g.	n.g.	n.g.	—
2-Hydroxybenzoate	95	102	122	165	—
4-Hydroxy-D,L-mandelate	.	400	340	290	+
4-Hydroxybenzyl alcohol	.	83	76	76	—
4-Hydroxybenzaldehyde	.	87	99	112	—
4-Hydroxybenzoate	.	58	58	58	—
3,4-Dihydroxy-D,L-mandelate	.	+ *	+ *	+ *	+
3,4-Dihydroxybenzaldehyde	85	101	173	303	+
3,4-Dihydroxybenzoate	.	56	57	57	—
4-Hydroxy-3-methoxy-D,L-mandelate	.	+ *	+ *	+ *	+
4-Hydroxy-3-methoxybenzyl alcohol	.	230	220	220	+
4-Hydroxy-3-methoxybenzaldehyde	.	300	217	200	+
4-Hydroxy-3-methoxybenzoate	.	76	76	76	—
Succinate	.	45	45	45	—
Acetate	.	50	49	45	—

* These substrates supported growth but accurate estimates of mean generation times were not made.

Table 2. *Molar growth yield of bacterium NCIB 8250 on various substrates*

The basal medium contained $\text{KH}_2\text{PO}_4 + \text{MgSO}_4 + (\text{NH}_4)_2\text{SO}_4$. A 17 hr nutrient broth culture was used as inoculum (0.2 %, v/v) and the bacteria were grown at 30° for 4 days. Results are the means of at least five experiments.

Substrate	Molar growth yield ($\mu\text{g. dry wt}/\mu\text{mole substrate}$)
D,L-Mandelate	31.3
L-Mandelate	62.0
D-Mandelate	0
Benzoylformate	60.7
Benzyl alcohol	71.2
Benzaldehyde	62.1
Benzoate	55.1
2-Hydroxybenzoate	53.7
4-Hydroxybenzoate	53.8
4-Hydroxy-3-methoxybenzoate	53.4

gave values up to 2000 min. with 4-hydroxy-3-methoxy-D,L-mandelate, but in fact most of this was a period of acceleration in growth. Some compounds gave signs of depressed growth rates at higher concentrations. In the most extreme case 2-hydroxybenzaldehyde supported growth only at less than 0.5 mM: this type of effect was to some extent dependent on inoculum size. Rates of growth on 3,4-dihydroxy-D,L-mandelate and 4-hydroxy-3-methoxy-D,L-mandelate were variable, but of the same order as the rate with 2-hydroxy-D,L-mandelate. Examination of the residual media after cessation of growth on D,L-mandelate or 4-hydroxy-D,L-mandelate showed an optical rotation corresponding to the presence of the D(-) isomers.

Fig. 1

Fig. 2

Fig. 1. Oxygen uptake by a washed suspension of bacterium NCIB 8250 prepared after growth on 4-hydroxy-3-methoxybenzaldehyde. The bacteria were grown, harvested, washed and the rates of oxygen uptake measured manometrically. Each vessel contained 8.0 mg. wet wt bacteria and 3.6 μ moles substrate. $\circ$, 4-Hydroxy-3-methoxybenzyl alcohol; $\triangle$, 3-hydroxybenzyl alcohol; $\square$, no added substrate; $\bullet$, 4-hydroxy-3-methoxybenzyl alcohol + 0.3 mM-puromycin; $\blacktriangle$, 3-hydroxybenzyl alcohol + 0.3 mM-puromycin.

Fig. 2. Oxygen uptake by a washed suspension of bacterium NCIB 8250 prepared after growth on 4-hydroxy-3-methoxybenzaldehyde. The bacteria were grown, harvested, washed and the rates of oxygen uptake measured manometrically. Each vessel contained 8.0 mg. wet wt bacteria and 3.6 μ moles substrate. $\circ$, Benzyl alcohol; $\triangle$, benzoate; $\square$, no added substrate; $\bullet$, benzyl alcohol + 0.3 mM-puromycin; $\blacktriangle$, benzoate + 0.3 mM-puromycin.

For the experiments with washed suspensions the organism was grown on each available compound in turn and harvested in the late exponential phase of growth. The bacteria were washed and their ability to oxidize several substrates tested manometrically in a basal medium similar to that used for growth (Tables 3, 4). In all cases bacteria exposed to the substrate on which they had been grown gave an immediate oxygen uptake showing that the appropriate enzymes had survived the preparation procedure. Care was taken to harvest organisms during the penultimate generation and to test the oxygen uptake within 4 hr of harvesting since organisms grown into the

Table 3. *Patterns of oxygen utilization obtained with various substrates when incubated with washed suspensions of bacterium NCIB 8250*

Bacterium NCIB 8250 was grown on each compound in turn, harvested, washed and the ability of the bacteria to oxidize the various challenge substrates estimated manometrically. Each vessel contained 8.0 mg. wet wt bacteria and 3.6 μ moles substrate. U, immediate and complete utilization of oxygen; L, lag followed by complete utilization; I-L-U, immediate incomplete oxygen uptake followed after an intermediate lag by complete oxygen utilization; n.t., not tested. The values in parentheses represent the initial rates of oxygen consumption (μ moles O_2 /hr/mg.N) with the endogenous rates subtracted.

Challenge substrate	Growth substrates									
	D,L-Mandelate	Benzoyl-formate	Benzyl alcohol	Benzaldehyde	Benzoate	D,L-mandelate	2-Hydroxy-benzoyl-formate	2-Hydroxy-benzyl alcohol	2-Hydroxy-benzaldehyde	2-Hydroxy-benzoate
D,L-Mandelate	U (175)	U (120)	L	L	L	U (108)	U (117)	L	L	L
Benzoylformate	U (172)	U (148)	L	L	L	U (76)	U (86)	L	L	L
Benzyl alcohol	U (43)	U (112)	U (186)	U (198)	L	n.t.	n.t.	U (194)	U (210)	L
Benzaldehyde	U (163)	U (150)	U (177)	U (212)	L	n.t.	n.t.	U (145)	U (175)	L
Benzoate	U (182)	U (134)	U (140)	U (188)	U (198)	U (40)	U (35)	U (145)	U (118)	L
2-Hydroxy-D,L-mandelate	I (24)-L-U	n.t.	n.t.	n.t.	n.t.	U (50)	U (100)	n.t.	n.t.	L
2-Hydroxybenzoylformate	I (6)-L-U	n.t.	n.t.	n.t.	n.t.	U (32)	U (65)	n.t.	n.t.	n.t.
2-Hydroxybenzyl alcohol	I (12)-L-U	I (29)-L-U	I (70)-L-U	I (55)-L-U	L	U (52)	U (88)	U (152)	U (116)	L
2-Hydroxybenzaldehyde	I (6)-L-U	I (11)-L-U	I (23)-L-U	I (31)-L-U	L	n.t.	n.t.	U (124)	U (81)	L
2-Hydroxybenzoate	L	L	L	L	L	U (43)	U (88)	U (124)	U (80)	L
4-Hydroxy-D,L-mandelate	I (19)-L-U	I (13)-L-U	L	L	L	I (23)-L-U	I (54)-L-U	L	L	L
4-Hydroxybenzyl alcohol	I (19)-L-U	I (20)-L-U	I (25)-L-U	I (25)-L-U	L	n.t.	n.t.	I (39)-L-U	I (33)-L-U	L
4-Hydroxybenzaldehyde	I (3)-L-U	I (5)-L-U	I (7)-L-U	I (9)-L-U	L	n.t.	n.t.	I (17)-L-U	I (12)-L-U	L
4-Hydroxybenzoate	L	L	L	L	L	n.t.	n.t.	L	L	L
3,4-Dihydroxy-D,L-mandelate	I (27)-L-U	I (28)-L-U	L	n.t.	L	n.t.	I (54)-L-U	L	n.t.	L
3,4-Dihydroxybenzaldehyde	I (7)-L-U	I (3)-L-U	I (9)-L-U	I (18)-L-U	L	n.t.	n.t.	I (91)-L-U	I (14)-L-U	L
3,4-Dihydroxybenzoate	L	L	L	L	L	n.t.	n.t.	L	L	L
4-Hydroxy-3-methoxy-D,L-mandelate	I (15)-L-U	I (3)-L-U	L	L	L	n.t.	I (42)-L-U	L	L	L
4-Hydroxy-3-methoxybenzyl alcohol	I (9)-L-U	I (11)-L-U	I (18)-L-U	I (15)-L-U	L	n.t.	n.t.	I (19)-L-U	I (28)-L-U	L
4-Hydroxy-3-methoxybenzaldehyde	I (4)-L-U	I (2)-L-U	I (19)-L-U	I (9)-L-U	L	n.t.	n.t.	I (15)-L-U	I (16)-L-U	L
4-Hydroxy-3-methoxybenzoate	L	L	L	L	L	n.t.	n.t.	L	L	L
Catechol	U (174)	U (150)	U (160)	U (200)	U (195)	U (153)	U (282)	U (170)	U (150)	U (205)
β -Oxoadipate	U (170)	U (100)	U (95)	U (105)	U (100)	U (59)	U (161)	U (160)	U (140)	U (110)

Table 4. *Patterns of oxygen utilization obtained with a number of challenge substrates when incubated with washed suspensions of bacterium NCIB 8250*

Bacterium NCIB 8250 was grown on each compound in turn, harvested, washed and the ability of the bacteria to oxidize various substrates estimated manometrically. Each vessel contained 8.0 mg. wet wt bacteria and 3.6 μ moles substrate. U, immediate and complete utilization of oxygen; L, lag followed by complete utilization; I-L-U, immediate incomplete oxygen uptake followed after an intermediate lag by complete oxygen utilization; n.t., not tested. The values in parentheses represent the initial rates of oxygen consumption (μ moles O_2 /hr/mg.N.) with the endogenous rates subtracted.

Challenge substrate	Growth substrates									
	4-Hydroxy-D,L-mandelate	4-Hydroxy-benzyl alcohol	4-Hydroxy-benzaldehyde	4-Hydroxy-D,L-mandelate	3,4-Di-hydroxy-benzaldehyde	3,4-Di-hydroxy-benzoate	4-Hydroxy-D,L-mandelate	4-Hydroxy-3-methoxy-benzyl alcohol	4-Hydroxy-3-methoxy-benzaldehyde	4-Hydroxy-3-methoxy-benzoate
D,L-Mandelate	I (48)-L-U	L	L	n.t.	L	L	I (38)-L-U	L	L	L
Benzoylformate	I (48)-L-U	L	L	n.t.	L	L	n.t.	L	L	L
Benzyl alcohol	I (82)-L-U	I (128)-L-U	I (128)-L-U	n.t.	I (101)-L-U	L	I (25)-L-U	I (117)-L-U	I (115)-L-U	L
Benzaldehyde	I (40)-L-U	I (34)-L-U	I (43)-L-U	n.t.	I (34)-L-U	L	I (48)-L-U	I (48)-L-U	I (43)-L-U	L
Benzoate	L	L	L	n.t.	L	L	L	L	L	L
2-Hydroxy-D,L-mandelate	I (40)-L-U	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	L
2-Hydroxybenzoylformate	I (20)-L-U	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
2-Hydroxybenzyl alcohol	I (60)-L-U	I (128)-L-U	I (128)-L-U	n.t.	I (107)-L-U	L	n.t.	I (73)-L-U	I (95)-L-U	L
2-Hydroxybenzaldehyde	I (17)-L-U	I (34)-L-U	I (43)-L-U	n.t.	I (34)-L-U	L	n.t.	I (39)-L-U	I (33)-L-U	L
2-Hydroxybenzoate	L	L	L	n.t.	L	L	n.t.	L	L	L
4-Hydroxy-D,L-mandelate	U (187)	L	L	n.t.	L	L	I (31)-L-U	L	L	L
4-Hydroxybenzyl alcohol	U (187)	U (185)	U (210)	n.t.	I (60)-L-U	L	n.t.	I (125)-L-U	I (30)-L-U	L
4-Hydroxybenzaldehyde	U (152)	U (185)	U (210)	n.t.	I (19)-L-U	L	n.t.	I (108)-L-U	I (13)-L-U	L
4-Hydroxybenzoate	U (187)	U (185)	U (210)	n.t.	L	L	n.t.	L	L	L
3,4-Dihydroxy-D,L-mandelate	U (93)	n.t.	n.t.	U (34)	n.t.	n.t.	U (46)	n.t.	n.t.	n.t.
3,4-Dihydroxybenzaldehyde	U (126)	U (147)	U (220)	U (34)	U (160)	L	U (130)	U (116)	U (185)	L
3,4-Dihydroxybenzoate	U (203)	U (147)	U (255)	U (99)	U (160)	U (174)	U (194)	U (194)	U (54)	U (172)
4-Hydroxy-3-methoxy-D,L-mandelate	I (14)-L-U	L	L	n.t.	n.t.	n.t.	U (46)	L	L	n.t.
4-Hydroxy-3-methoxybenzyl alcohol	I (31)-L-U	I (62)-L-U	I (100)-L-U	I (13)-L-U	L	L	U (130)	U (116)	U (120)	L
4-Hydroxy-3-methoxybenzaldehyde	I (14)-L-U	I (23)-L-U	I (25)-L-U	I (7)-L-U	L	L	U (114)	U (100)	U (120)	L
4-Hydroxy-3-methoxybenzoate	L	L	L	L	L	L	U (96)	U (100)	U (148)	U (185)
Catechol	L	L	L	n.t.	L	L	n.t.	L	L	L
β -Oxoadipate	U (95)	U (130)	U (140)	n.t.	U (180)	U (110)	n.t.	U (155)	U (200)	U (105)

stationary phase or stored (even at 4°) showed less reproducible results; the benzoate-oxidizing system especially appeared to be lost quite rapidly.

The types of oxygen uptake recorded in Tables 3 and 4 and Fig. 1 and 2 show three patterns. First, as exemplified by 4-hydroxy-3-methoxybenzyl alcohol in Fig. 1, an immediate and rapid uptake which came to completion at about 70 % of the oxygen consumption required for complete oxidation of substrate. Presumably the balance of 30 % represented oxidative assimilation (Clifton, 1946) since the presence of 0.5 mM-2,4-dinitrophenol increased the stoichiometry of oxygen uptake to 92 %. Another uncoupling agent, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone (Heytler & Prichard, 1962), had no effect at concentrations from 1 mM to 0.1 μ M; higher concentrations merely decreased the rate of oxidation. Presumably this compound did not penetrate the bacteria. The second pattern (as shown by benzoate in Fig. 2) was a lag followed by an increasing rate of oxygen uptake which again came to completion at about 70 % of theoretical. The lag varied from a few minutes to several hours and presumably corresponded to *de novo* protein synthesis since the induced oxygen uptake was abolished by 0.3 mM-puromycin (Fig. 2). Chloramphenicol (20 μ M) had the same effect, but actinomycin D (0.1 to 1 μ g./ml.) was inactive, again probably because of failure to reach the active site. 2,4-Dinitrophenol (0.5 mM) increased the length of the lag. The third pattern of oxygen consumption was an immediate but limited uptake followed after an intermediate lag by complete oxygen utilization (e.g. benzyl alcohol in Fig. 2). The plateau corresponded to either 1 μ atom or 1 μ mole oxygen/ μ mole substrate and was always the amount of oxygen required for oxidation of the substrate to the benzoate level. The second part of the curve was inhibited by puromycin (Fig. 2) or chloramphenicol. The length of the plateau varied with the substrate and was generally longest for bacteria grown with a hydroxy-substituted compound and then exposed to the non-substituted analogue. Results with a few substrates (e.g. benzaldehyde, 2-hydroxybenzaldehyde) were sometimes more difficult to interpret because they distilled out of the side-arm before tipping. In all cases, however, the use of chloramphenicol allowed an unambiguous assignment of pattern of oxygen utilization, although the apparent stoichiometry was low. Values for oxygen uptake were corrected for the small amount of respiration in the absence of added substrate (about 6 μ moles O₂/mg. N/hr). The substrate(s) for the endogenous respiration is not known: preliminary experiments (Fewson, unpublished work) have failed to detect glycogen or poly- β -hydroxybutyrate.

The results given in Tables 3 and 4 show that bacterium NCIB 8250 grown on mandelate, benzoylformate or their analogues oxidized any compound which was more reduced than benzoate to at least the level of the corresponding benzoate. The oxygen uptake with D,L-mandelate or any substituted D,L-mandelate was only about half that for other substrates with the same number of carbon atoms. That this was due to the inactivity of the D-isomer was confirmed by the fact that L-mandelate was completely oxidized, whereas D-mandelate supported no oxygen uptake above the endogenous level. Bacteria grown with benzyl alcohol, benzaldehyde or their analogues oxidized any benzyl alcohol or benzaldehyde, but not mandelate or benzoylformate, to at least the benzoate level. Oxidation beyond the benzoate stage proceeded without a lag only when the challenge substrate had the same substituents on the benzene ring as had the growth substrate: the exceptions were that bacterium NCIB 8250 grown on any 2-hydroxy-substituted substrate totally oxidized the non-substituted compounds

and the organisms grown on 4-hydroxy- and 4-hydroxy-3-methoxy-substituted compounds immediately and totally oxidized the corresponding 3,4-dihydroxy analogues. Oxidation of the various benzoates by non-adapted bacteria proceeded only after a lag. All the bacteria grown with aromatic compounds immediately oxidized β -oxoadipate. Bacteria grown with succinate did not possess the enzymes required for the oxidation of the aromatic compounds or β -oxoadipate since none of these substances was oxidized until after a period of protein synthesis. In other experiments it was found that acetate was oxidized only after a very short lag by all batches of bacteria except those grown with acetate itself, which gave immediate oxidation. This lag was not changed by puromycin or chloramphenicol but was extended in the presence of 2,4-dinitrophenol, which may suggest that it was due to an ATP requirement for acetate activation or entry.

Table 5. *Oxidation of substrates which do not support growth by washed cell suspensions of bacterium NCIB 8250*

Bacterium NCIB 8250 was grown on D,L-mandelate as sole source of carbon, harvested and washed. The ability of these bacteria to oxidize various substrates was estimated manometrically. Each vessel contained 8.0 mg. wet wt bacteria and 3.6 μ moles substrate.

- (a) *Compounds which gave an immediate oxygen uptake corresponding to oxidation beyond the benzoate level:* 4-fluoro-D,L-mandelate, 4-fluorobenzyl alcohol, 2-fluorobenzaldehyde, 4-fluorobenzaldehyde, 2-fluorobenzoate, 3-fluorobenzoate, 4-fluorobenzoate.
- (b) *Compounds which gave an immediate but limited oxygen uptake which corresponded stoichiometrically to oxidation to the corresponding benzoate:* 4-bromo-D,L-mandelate, 4-chloro-D,L-mandelate, 3-hydroxy-D,L-mandelate, 3-hydroxy-4-methoxy-D,L-mandelate, 3-chlorobenzyl alcohol, cinnamyl alcohol, 3,4-dimethoxybenzyl alcohol, 3-hydroxybenzyl alcohol, 3-methoxybenzyl alcohol, 4-methoxybenzyl alcohol, 3-chlorobenzaldehyde, 4-chlorobenzaldehyde, cinnamaldehyde, 3,4-dichlorobenzaldehyde, 2,4-dihydroxybenzaldehyde, 2,5-dihydroxybenzaldehyde, 3-hydroxybenzaldehyde, 2-hydroxy-3-methoxybenzaldehyde, 3-hydroxy-4-methoxybenzaldehyde, 3-hydroxy-5-methoxybenzaldehyde, 3-methoxybenzaldehyde, 4-methoxybenzaldehyde, 3-nitrobenzaldehyde, 4-nitrobenzaldehyde.
- (c) *Compounds which gave no detectable oxygen utilization above the control:* 2,3,4,5,6-pentafluorobenzyl alcohol, 2-carboxybenzaldehyde, 2,6-dichlorobenzaldehyde, 2,4-dimethoxybenzaldehyde, 2-methoxybenzaldehyde, 2-nitrobenzaldehyde, 2,3,4,5,6-pentafluorobenzaldehyde, 3-aminobenzoate, 4-aminobenzoate, 2-bromobenzoate, 3-bromobenzoate, 4-bromobenzoate, 2-carboxybenzoate, 3-carboxybenzoate, 4-carboxybenzoate, 2-carboxy-3-nitrobenzoate, 2-carboxy-4-nitrobenzoate, 2-chlorobenzoate, 3-chlorobenzoate, 4-chlorobenzoate, 2,4-dichlorobenzoate, 2,6-dichlorobenzoate, 2,3-dihydroxybenzoate, 2,4-dihydroxybenzoate, 2,5-dihydroxybenzoate, 2,6-dihydroxybenzoate, 3,5-dihydroxybenzoate, 3,4-dimethoxybenzoate, 3,5-dinitrobenzoate, 2-hydroxy-4-aminobenzoate, 2-hydroxy-3-nitrobenzoate, 2-hydroxy-5-nitrobenzoate, 4-hydroxy-3-aminobenzoate, 3-hydroxybenzoate, 2-iodobenzoate, 3-iodobenzoate, 4-iodobenzoate, 2-mercaptobenzoate, 2-methoxybenzoate, 3-methoxybenzoate, 4-methoxybenzoate, 2-methylbenzoate, 3-methylbenzoate, 4-methylbenzoate, 2-nitrobenzoate, 3-nitrobenzoate, 4-nitrobenzoate, 3,4,5-trihydroxybenzoate, atrolactate, atropate, *p*-benzoquinone, cinnamate, 2-coumarate, 3-coumarate, 4-coumarate, cyclohexanecarboxylate, cyclohexanol, homocatechol, 2-hydroxycyclohexanone, phenoxyacetate, 2-phenylethanol, 3-phenylpropionate, phenylsuccinate, resorcinol, styrene glycol.

The experiments described in Tables 3 and 4 were all done with substrates which support growth and these results were therefore extended by using as 'challenge' substrates several compounds which do not give growth (Fewson, 1967*a*). Table 5 shows that bacterium NCIB 8250 grown with mandelate oxidized a number of fluorine-substituted compounds beyond the benzoate level. These bacteria metabolized a number of other substituted mandelates, benzyl alcohols and benzaldehydes with an oxygen uptake corresponding to the formation of the appropriate benzoate. The

exceptions were pentafluorobenzyl alcohol and a few benzaldehydes with large substituents at the 2-position which were not oxidized at an appreciable rate. None of the compounds tested showed an induced oxygen uptake, even after prolonged incubation. Similar results were obtained with bacteria grown on 2-hydroxybenzyl alcohol except that in this case none of the substituted mandelates was oxidised. Growth with 3,4-dihydroxybenzaldehyde or 4-hydroxy-3-methoxybenzyl alcohol gave the same results as were obtained by growth with 2-hydroxybenzyl alcohol, with the exception of the fluoro-compounds which were oxidized only to the fluorobenzoate level.

Table 6. *Stoichiometry of oxygen uptake by washed cell suspensions of bacterium NCIB 8250 when incubated with 3-hydroxy-substituted compounds*

Bacterium NCIB 8250 was grown with some of the aromatic compounds listed in Tables 3 and 4, harvested and washed. The final oxygen uptake obtained when the bacteria were incubated with 3-hydroxy-substituted compounds was measured manometrically. Each vessel contained 8.0 mg. wet wt bacteria and 3.6 μ moles substrate. Mean values $\pm$ S.E.M. are given with the number of determinations in parentheses. Values are corrected for the endogenous oxygen uptake.

Substrate	μ moles O_2/μ mole substrate
3-Hydroxy-D,L-mandelate	0.49 ± 0.04 (4)
3-Hydroxybenzyl alcohol	0.98 ± 0.02 (12)
3-Hydroxybenzaldehyde	0.51 ± 0.02 (12)
3-Hydroxybenzoate	-0.03 ± 0.01 (17)

The partial oxidation of the 3-hydroxy-substituted compounds was examined in more detail. An example of the type of oxygen uptake observed is shown in Fig. 1. The stoichiometry of the reaction is given in Table 6 and corresponds closely to the oxidation of 3-hydroxy-L-mandelate, 3-hydroxybenzyl alcohol and 3-hydroxybenzaldehyde to 3-hydroxybenzoate. The formation of 3-hydroxybenzoate was confirmed as follows. Bacterium NCIB 8250 was grown into late exponential phase in 2 l. basal medium containing 10 mM-D,L-mandelate as carbon source. The bacteria were harvested by centrifugation, washed once with sterile basal medium and then resuspended in 2 l. sterile basal medium containing 2 g. 3-hydroxy-D,L-mandelate. The flask was stirred vigorously at 30° for 12 hr, 40 ml. 5 N-HCl then added and the bacteria removed by centrifugation. The supernatant fluid was extracted five times with 200 ml. quantities of ether. The aqueous residue had an ultraviolet absorption spectrum corresponding to 3-hydroxymandelic acid (extinction maximum at 274 $m\mu$ with a shoulder at 278 $m\mu$, minimum at 240 $m\mu$ in 0.1 N-HCl; extinction maximum at 293 $m\mu$ and minimum at 264 $m\mu$ in 0.1 N-NaOH). The extinction at 274 $m\mu$ in 0.1 N-HCl (extinction coefficient of $1.82 \cdot 10^6 \text{ cm}^2 \text{ mole}^{-1}$) corresponded to exactly half the amount of 3-hydroxy-D,L-mandelate originally present. The solution was laevorotary and presumably contained the residual 3-hydroxy-D-mandelate. The bulked ether extracts were washed twice with 100 ml. quantities of 0.1 N-HCl and evaporated to dryness at room temperature. The residue was dissolved in water and had an ultraviolet absorption spectrum corresponding to that of 3-hydroxybenzoate (absorption maxima: 296 $m\mu$ in 0.1 N-HCl, 314 $m\mu$ in 0.1 N-NaOH) except for a shoulder extending to about 350 $m\mu$. Chromatographic examination as described in Methods revealed the presence of small amounts (less than 5%) of 2,3-dihydroxybenzoic acid and 2,5-dihydroxybenzoic acid. These were removed by five recrystallizations from hot water. The final material (240 mg.)

Fig. 3. The metabolism of mandelate and related compounds by bacterium NCIB 8250.

had an ultraviolet absorption spectrum identical with that of 3-hydroxybenzoic acid and m.p. 202–204°, unchanged by admixture with an authentic sample of 3-hydroxybenzoic acid.

DISCUSSION

The results reported in this paper suggest that bacterium NCIB 8250 oxidizes mandelate and related compounds by the converging pathway shown in Fig. 3. 4-Hydroxybenzoylformate, 3,4-dihydroxybenzoylformate and 4-hydroxy-3-methoxybenzoylformate are included by analogy with benzoylformate and 2-hydroxybenzoylformate. The inducible enzymes required for the ring cleavage of catechol and 3,4-dihydroxybenzoate have been found in this organism (Kennedy & Fewson, 1968). The aliphatic products presumably undergo rearrangement to give, ultimately, β -oxoadipate (e.g. Cánovas *et al.* 1967) which is oxidized by bacteria grown with aromatic compounds (Tables 3 and 4). Bacterium NCIB 8250 differs in this respect from *Moraxella calcoacetica* (strain 73) which is impermeable to β -oxoadipate (Cánovas *et al.* 1967). The system of oxidation of the non-substituted and 4-hydroxy-substituted compounds is the same as that in *Pseudomonas putida* (Stanier, 1947; Gunter, 1953) except that bacterium NCIB 8250 does not appear to possess the enzyme mandelate racemase (EC 5.1.2.2) since the organism can neither oxidize nor grow on D-mandelate. Stanier (1950) suggested that *P. putida* oxidized benzyl alcohol through benzaldehyde and benzoate, but no subsequent work appears to have been done on this branch of the pathway. There seems no reason to doubt that benzyl alcohol is oxidized in this way by bacterium NCIB 8250, although some workers (Claus & Walker, 1964; Hegeman, 1966*a*), working with a *Pseudomonas* species, have suggested that it is metabolized through some other unspecified pathway. The formal possibility that at least some proportion of mandelate might be metabolized by decarboxylation to benzyl alcohol does not appear to have been ruled out by workers using *P. putida*. A strong argument against this route in bacterium NCIB 8250 is the fact that benzyl alcohol supported a higher molar growth yield than did mandelate (Table 2): this probably means that the oxidation of benzyl alcohol to benzaldehyde yields biologically useful energy, whereas the oxidation of L-mandelate to benzaldehyde does not.

The oxidation of mandelates by bacterium NCIB 8250 grown on the benzoylformates, and the oxidation of the benzyl alcohols by organisms grown on mandelates, benzoylformates or benzaldehydes (Tables 3 and 4) suggests that the enzymes are controlled in coordinate groups. The regulons can apparently be de-repressed by the products as well as by the substrates of the first enzymes of the branches of the pathway. The control of these enzymes is considered more fully elsewhere (Kennedy & Fewson, 1968). Recent work has indicated that during mandelate oxidation by *Pseudomonas putida* also, coordinate groups of enzymes rather than individual enzymes, are induced sequentially (Stanier, Hegeman & Ornston, 1964; Hegeman, 1966*a-c*; Cánovas *et al.* 1967).

The patterns of oxygen uptake (Tables 3 to 5; Fig. 1, 2) suggest that the oxidation of compounds to the corresponding benzoates is carried out by enzymes which are, at least qualitatively, fairly non-specific both in their activity and induction. This is similar to the more restricted situation in *Pseudomonas putida* where the same series of enzymes catalyse the oxidation of mandelate and 4-hydroxymandelate to benzoate and 4-hydroxybenzoate respectively (e.g. Gunter, 1953). Substitution of the aromatic ring does lead to quantitative differences between the analogues as shown by the

variation in rates reported in Tables 3 and 4. This has been confirmed by studies with cell-free preparations (Kennedy & Fewson, 1968). In some cases substitution can completely block activity, for instance a large substituent in the 2-position (e.g. nitro, methoxy or carboxy) prevents oxidation of the benzaldehydes (Table 5). The quantitative differences in growth rates observed with the various substrates (Table 1) are frequently reflected in the rates of oxygen uptake with washed suspensions (Tables 3, 4). Presumably, however, growth rate is a function not only of enzyme activity but also of factors such as induction and permeability. Toxicity is clearly important in some cases.

Metabolism of the benzoates is brought about by specific enzymes which are generally produced only by growth on a homologous substrate. The exception to this appears to be benzoate oxidase which is induced under conditions where only 2-hydroxybenzoate hydroxylase would be expected (Kennedy & Fewson, 1968). This gave rise to the 'double simultaneous adaptation' of cells grown on 2-hydroxy-substituted compounds towards their non-substituted analogues (Table 3). The evidence presented in this paper points to the fact that the enzymes metabolising the various benzoates are much more specific than the enzymes higher up the pathway. This is not surprising since the benzoate enzymes all carry out reactions centred on the aromatic ring itself. Nevertheless, it must be borne in mind that much of the evidence for the benzoate specificity was obtained from manometric experiments in which some types of reaction, e.g. hydration, would not be detected. It is still possible, however, to use the data to make a number of deductions about the specificity of these enzymes. This can be illustrated by the benzoate oxidizing system. Cells possessing benzoate oxidase oxidized only benzoate and the monofluorobenzoates. This presumably reflects the similarities of the van der Waal's radii of the hydrogen and fluorine atoms (Pauling, 1960) since other substitutions, e.g. chloro or bromo, gave compounds which were inactive as substrates. Ali, Callely & Hayes (1962) have also shown that bacterium NCIB 8250 grown on benzoate can oxidize 3- and 4-fluorobenzoate. It must be noted that cells possessing benzoate oxidase did oxidize 3-hydroxybenzoate to the 2,3-dihydroxy and 2,5-dihydroxy derivatives to a very slight extent. Ichihara, Adachi, Hosokawa & Takeda (1962) found that benzoate oxidase from *Pseudomonas aeruginosa* and *Micrococcus ureae* (strain *Et*) had slight activity towards 3-hydroxybenzoate but did not determine the product of the reaction.

Cartwright & Smith (1967), in a paper published after the experimental work described here had been completed, described experiments on the demethylation of 4-hydroxy-3-methoxybenzoate by a *Pseudomonas* species isolated from coal tar. This organism was sequentially adapted to 4-hydroxybenzoate by growth on 4-hydroxy-3-methoxybenzoate so that these workers could not rule out the possibility that 3,4-dihydroxybenzoate arose by demethoxylation followed by hydroxylation, although other evidence strongly suggested a straightforward demethylation. No such ambiguity exists in bacterium NCIB 8250 since cells grown on 4-hydroxy-3-methoxybenzoate oxidized 4-hydroxybenzoate only after a lag (Table 4).

Attention has been drawn in recent years to the presence in micro-organisms of multi-enzyme sequences induced by, and active on, a range of related substrates. Examples of this are the enzymes involved in the degradation of camphor and related compounds (Gunsalus, Chapman & Kuo, 1965), perillyl alcohol (Ballal, Bhattacharyya & Rangachari, 1966) as well as mandelate and 4-hydroxymandelate (Gunter,

1953; Stevenson & Mandelstam, 1965). The observations recorded in this paper have extended these findings to a quite different organism and to a larger number of compounds. In bacterium NCIB 8250 there is a considerable degree of economy of protein synthesis since just four enzymes mediate the conversion of almost twenty compounds to five benzoates. These key intermediates are subjected to a series of manipulations resulting in the convergent formation of catechol or 3,4-dihydroxybenzoate which then undergo ring cleavage with the subsequent degradation of the aliphatic products. This type of non-specificity has obvious advantages, especially in soil where a particular environment may well contain, for instance, a number of different analogues of benzaldehyde all formed by the degradation of lignin (see, for example, Stevenson, 1964). The possible ecological significance of partial degradations, such as the oxidation of 3-hydroxymandelate to 3-hydroxybenzoate should not be overlooked. Not only might this type of transformation be capable of providing energy, even if not serving as a carbon supply, but the product might be acted upon by other organisms.

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