The Metabolism of Halogen-Substituted Benzoic Acids by *Pseudomonas fluorescens*

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1. Washed suspensions of *Pseudomonas fluorescens*, grown with benzoate as sole carbon source, oxidize monohalogenobenzoates in the following descending order of effectiveness: benzoate, fluorobenzoates, chlorobenzoates, bromobenzoates, iodobenzoates. 2. Cells grown on asparagine oxidize benzoate after an adaptive period of 90–120 min. This adaptive period is increased by halogenobenzoates in the following approximate descending order of effectiveness: chlorobenzoates, fluorobenzoates (= bromobenzoates), iodobenzoates. This inhibition of adaptation by halogeno analogues depends on the concentration of benzoate and is thus apparently competitive. 3. Cells do not adapt to oxidize the halobenzoates when the halogeno analogues are inducers. However, the fluorobenzoates reduce the lag period taken to form the benzoate-oxidizing system. 4. The halogenobenzoates inhibit adaptation to citrate and nicotinate but not so effectively as benzoate itself. This is presumably a 'diauxic' effect. The analogues do not inhibit adaptation to catechol. 5. The halogenobenzoates are not used as sole carbon source for growth nor do they increase growth when cells grow with asparagine as the main carbon source. 6. It is suggested that this inability to use the analogues for growth is due partly to inability of the cells to liberate the halogen and to carry the oxidation to a stage at which carbon may be assimilated and partly to the inhibition of the induction of the oxidizing enzymes.

In a previous paper (Hughes, 1955) it was shown that washed suspensions of *Pseudomonas fluorescens* capable of oxidizing nicotinic acid could also oxidize some halogenonicotinic acids. This organism did not grow with nicotinic acid analogues as a sole carbon source although it grew readily upon nicotinic acid. Preliminary experiments suggested that the halogenonicotinic acids might inhibit adaptation to nicotinic acid but the experiments were not continued because of the short supply of the fluoro analogues. The present paper reports the results of a parallel series of experiments in which benzoic acid and halogenobenzoic acids were used. Although cells adapted to benzoic acid readily oxidized some of the halogenobenzoic acids, they did not grow when the analogues were the sole carbon source. In washed suspensions, the analogues of benzoic acid inhibited adaptation to benzoic acid when low concentrations of benzoic acid was added as inducing agent.

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EXPERIMENTAL

Preparation and handling of washed suspensions. *Pseudomonas fluorescens* strain KBI (Kogut & Podoski, 1953) was used in most of the experiments. Washed suspensions for manometric experiments were grown on the medium containing inorganic salts (Hughes, 1955), to which was added either 0.1% (w/v) of benzoate to produce adapted cells, or 0.1% (w/v) of asparagine to produce non-adapted cells. The pH of the media was adjusted to 7.0 by the addition of N-NaOH. For growth in a liquid medium, 200 ml. portions were shaken in Carrel culture flasks (18 cm. diam. x 10 cm. depth; Clayton, Hems, Robinson, Andrews & Hunwicke, 1944) at 25° for 22–24 hr.; the yield of cells was approx. 1–1.5 mg. dry wt./ml. In some cases cells were grown in a medium containing 2.5% of agar and distributed in 100 ml. batches in Roux bottles; the yield of cells under these conditions was approx. 0.5–1.0 mg. dry wt./ml. After growth the cells were collected by centrifugation, washed twice in 0.9% (w/v) NaCl and suspended in 0.9% (w/v) NaCl to contain 10–20 mg. dry wt./ml. Dry weights were estimated by drying a 1.0 ml. sample to constant weight at 110° and subtracting the dry wt. of an equal volume of saline. The suspension was diluted with an equal

quantity of 0.1M-sodium phosphate-potassium phosphate buffer, pH 7.0, immediately before use.

Manometric experiments. Oxygen uptake by washed suspensions was followed manometrically in the Warburg apparatus. In the experiments on oxidation, the cell suspension, usually 1.0 ml. containing about 6–10 mg. dry wt. of cells, was placed in the main compartment and the substrate added from the side arm after a period of equilibration at 30°. In the experiments on adaptation the cells were placed with the inducing agent in the main compartment and the benzoate serving as substrate was added from the side arm after the manometers had been shaken for 90–120 min. In all the manometric experiments, folded filter paper and KOH were added to the centre well to remove CO₂; the temperature of incubation was 30°. Rates of oxidation are given as μ moles of O₂/mg. dry wt. of cells/hr.

Growth tests. Growth tests were carried out on the same media as used for bulk growth. Portions (10 ml.) were placed in 50 ml. conical flasks provided with test-tube-shaped (15 cm. long, 1.4 cm. internal diam.) side arms and growth was measured turbidimetrically (Hughes, 1955) in an Evans Electroselenium Ltd. nephelometer. The media and substances under test were mixed together and sterilized by autoclaving for 10 min. at 10 lb./in.². The inocula for the growth tests were grown for 24 hr. on the liquid media, washed once in 0.9% (w/v) NaCl and then resuspended in the original volume; one drop of the washed suspension was added to each of the test flasks, which were then incubated at 30°. In any single flask, measurement of growth was usually continued for 3 days after the first sign of growth

was detected because later cultures became slimy and nephelometric measurement unreliable.

Materials. Benzoate and the halogenobenzoates were commercial specimens. They were recrystallized from water after treating their hot aqueous solutions with charcoal to remove coloured impurities. Solutions for test were neutralized to pH 7.0 with N-NaOH.

RESULTS

Oxidation of halogenobenzoates by washed suspensions. Washed suspensions of cells which had been grown on the mineral medium containing benzoate as a sole source of carbon readily oxidized benzoate and the monohalogen-substituted benzoates (Table 1). In all cases the rate reached maximum within 5 min. after the addition of the substrate to the cells. Subsequently it was approximately linear (Fig. 1). The rate of oxidation of the analogues calculated from this approximately linear period was greatest for the fluorine-substituted analogues, where it was often 60–80% that of benzoate itself (Table 1), and was least for the iodine-substituted analogues, where it never ex-

Table 1. Rate of oxidation of benzoate and halogenobenzoates by washed suspensions

Cells were grown on liquid medium with benzoate as the sole source of carbon, collected, washed and their ability to oxidize the substrates was estimated as described in the text. The oxygen uptake without substrate has been subtracted. Each cup contained 9.2 mg. dry wt. of cells.

Substrate added (μ moles)	Rate of oxidation (μ moles of O ₂ /mg. dry wt./hr.)	Total oxygen uptake (μ moles/ μ mole of substrate)
Benzoate (2.0)	6.6	4.1
<i>o</i> -Fluorobenzoate (2.0)	3.0	3.0
<i>p</i> -Fluorobenzoate (2.0)	2.0	3.0
<i>m</i> -Fluorobenzoate (2.0)	2.1	3.3
Benzoate (10)	10.1	3.1
<i>o</i> -Fluorobenzoate (10)	5.9	1.9
<i>p</i> -Fluorobenzoate (10)	6.1	1.8
<i>m</i> -Fluorobenzoate (10)	6.1	1.9
Benzoate (2.0)	5.8	3.8
<i>m</i> -Fluorobenzoate (2.0)	3.2	2.2
<i>m</i> -Chlorobenzoate (2.0)	1.8	1.1
<i>o</i> -Bromobenzoate (2.0)	0.7	1.0
<i>m</i> -Iodobenzoate (2.0)	0.2	0.8
Benzoate (10)	11.2	—
<i>o</i> -Fluorobenzoate (10)	4.9	—
<i>o</i> -Chlorobenzoate (10)	0.5	—
<i>o</i> -Bromobenzoate (10)	0.2	—
<i>o</i> -Fluorobenzoate (10)	0.2	—

Fig. 1. Oxidation of benzoate and halogenobenzoates by washed suspensions of *P. fluorescens*. Cells were grown with benzoate as sole carbon source, collected, washed and their ability to oxidize the various substrates was estimated manometrically (see the text). Each cup contained 6.0 mg. dry wt. of cells. $\times$, Benzoate (upper curve, 10 μ moles; lower curve, 2 μ moles); $\bullet$, *o*-fluorobenzoate (10 μ moles); ∇ , *m*-fluorobenzoate (10 μ moles); $\blacksquare$, *o*-chlorobenzoate (10 μ moles); $\triangle$, *o*-bromobenzoate (10 μ moles); $\square$, *o*-iodobenzoate (10 μ moles); $\circ$, control with no added substrate.

ceeded 5% of that of benzoate (Table 1). In most experiments with the fluoro analogues the *ortho* and *para* isomers were oxidized at about the same rate or faster than the *meta* isomer. The slow rates of oxidation of the isomers of the bromo and iodo analogues made it difficult to estimate differences between them. The linear rate of oxidation of 2.0–5.0 μ -moles of benzoate continued for 10–20 min. and then fell off progressively until it reached that of the control without added substrate (Fig. 1). The total oxygen uptake was calculated by subtracting the value of the blank at the end of the linear period of gas uptake.

With 2 μ moles of benzoate as substrate, the total oxygen consumption was generally somewhat more than 50% of that needed for the conversion of the substrate into carbon dioxide and water (3.8–4.6 μ moles of oxygen/ μ mole compared with the theoretical value of 7.5 μ moles). With the halogeno analogues as substrates the duration of the linear period and the total oxygen consumption varied widely with various batches of cells but was always less than that with benzoate (Table 1); oxygen consumption was greatest with the fluoro compound and least with iodo compounds. Both rates and

total uptake of oxygen decreased in the following descending order: benzoate, fluorobenzoates, chlorobenzoates, bromobenzoates, iodobenzoates. The *ortho* and *para* isomers of the fluoro and chloro analogues were oxidized more readily than the *meta* isomers.

Effect of halogenobenzoates on the oxidation of benzoate. The fluorobenzoates, when present in concentrations five- to ten-fold that of benzoate, inhibited (10–20%) the oxidation of benzoate by suspensions of adapted cells (Fig. 2). The effect was most marked when both substances were added together or when the fluorobenzoate was added first (Fig. 2). There was no immediate inhibition if the analogue was added while benzoate oxidation was already proceeding, but later the rate of oxidation decreased and after about 10–20 min. became the same as in the control where the substances were added together (Fig. 2). The chloro analogues showed small and variable inhibitory effects whereas the bromo and iodo analogues did not inhibit under these conditions.

It seemed possible that the inhibition of the oxidation of benzoate as well as the lower consumption of oxygen when fluorobenzoates were sole substrates was due to the formation of fluoroacetate from the fluoro compounds with the subsequent inhibition of terminal respiration. It has been suggested that the formation of fluoroacetate from fluoronicotinate might account for similar effects on the oxidation of this compound (Hughes, 1955; Behrman & Stainer, 1957). It was found that the addition of fluoroacetate together with benzoate depressed neither the rate of oxygen uptake nor the total oxygen consumption (Fig. 3). Preincubation of cells with fluoroacetate in concentrations from 1 to 20 mM temporarily decreased the initial rate of oxygen uptake with benzoate as substrate, but this inhibition was overcome after 20–50 min. The total amount of oxygen was decreased by 6–10% but was always greater than that consumed during the oxidation of fluorobenzoate (Fig. 3). Preincubation with fluoroacetate did not markedly depress the endogenous respiration.

Effect of halogenobenzoates on the formation of induced enzyme in washed suspensions. Cells grown on the liquid medium, with asparagine as sole source of carbon, oxidized benzoate after a lag period of 90–120 min. (Fig. 4). The length of the lag period was not affected by 0.01 M-ammonium chloride, 0.01 or 0.05 M-phosphate or casein hydrolysate (1%, w/v). The concentration of benzoate between 0.01 M and 0.01 M likewise made no difference to the length of the lag period. In most of the following experiments a concentration of 0.01 M-benzoate was added to induce enzyme formation, and after incubation at 30° for 90–120 min. substrate (5 mM) was added to estimate the

Fig. 2. Effect of *p*-fluorobenzoate on the oxidation of benzoate. Cells grown with benzoate as sole carbon source were collected, washed and their oxidation of benzoate was followed manometrically. Each cup contained 10.0 mg. dry wt. of cells. ×, 1 mM-Benzoate alone; ●, 1 mM-benzoate and 1 mM-*p*-fluorobenzoate added together; ○, 1 mM-*p*-fluorobenzoate at 0 min. and 1 mM-benzoate at 30 min.; □, 1 mM-*p*-fluorobenzoate added to 1 mM-benzoate at 20 min.; ▲, no added substrate.

Fig. 3. Effect of monofluoroacetate on the oxidation of benzoate. Cells were grown with benzoate as sole carbon source, collected and washed and their rate of oxidation of benzoate was followed manometrically. Each cup contained 9.1mg. dry wt. of cells. $\circ$, No added substrate; $\times$, 1mM-benzoate alone; $\bullet$, 1mM-benzoate with 1mM-fluoroacetate added together; Δ , 1mM-fluoroacetate added at 0 min. and 10mM-benzoate added at 20 min.

Fig. 4. Adaptation to benzoate and inhibition of adaptation by *m*-fluorobenzoate in washed suspensions. Cells were grown with asparagine as sole carbon source, collected, washed and incubated with the additions shown below to adapt the cells (see the text). Their ability to oxidize benzoate was followed manometrically after the addition of substrate benzoate (5mm) at 90 min., as indicated by the arrow. Each cup contained 8.0mg. dry wt. of cells. $\times$, 0.1mM-Benzoate; Δ , no addition; $\bullet$, 0.1mM-benzoate, 10mM-*m*-fluorobenzoate; $\blacktriangle$, 0.1mM-benzoate, 1mM-*m*-fluorobenzoate; $\circ$, 0.4mM-benzoate, 0.1mM-*m*-fluorobenzoate.

rate of oxidation. Usually the oxidation of the substrate began at about 5 min. after its addition, indicating that enzymic induction had taken place (Fig. 4). In the controls, where no inducing benzoate was added, the substrate was oxidized after incubating for a further 90–120 min. The length of the lag period before the oxidation of the substrate was increased by the addition of *m*-fluorobenzoate (0.01M to 0.1mM) at the same time as inducer (Fig. 4). The increase in the lag was the greatest in the highest concentration and the least in the lowest concentration. There was no significant increase when the analogue was added with the substrate (cf. Fig. 2). All the other halogenobenzoates behaved similarly to *m*-fluorobenzoate in increasing the length of time taken to induce enzyme formation. The descending order of their effectiveness was approximately: chlorobenzoates, fluorobenzoates (= bromobenzoates), iodobenzoates (Table 2). The *meta* and *para* isomers had similar inhibitory effects and both were more inhibitory than *o*-fluorobenzoate. To avoid any interference of the analogue with the oxidation of the substrate the conditions of the experiments were varied as follows. Cell suspensions (6ml., containing 20mg. dry wt. of cells), 0.01M-benzoate (0.5ml.) and analogues (0.5ml., 0.1M) were shaken at 30° in 50ml. Erlenmeyer flasks. Meanwhile 1ml. from a control flask containing benzoic acid but no analogue was shaken in the Warburg

Table 2. Effect of halogenobenzoates on induction by benzoate

Cells were grown on the solid asparagine medium, collected and washed as described. They were incubated in Warburg manometers with 0.1mM-benzoate as inducer and additions shown below. Each cup contained 6.5mg. dry wt. of cells. More benzoate (5mm) was added after 135 min. incubation and the time taken for the cells to oxidize benzoic acid at the maximal rate measured.

Addition (mM)	Time at which benzoate was oxidized (min.)
None	15
<i>p</i> -Fluorobenzoate	45
<i>p</i> -Chlorobenzoate	135
<i>p</i> -Bromobenzoate	45
<i>p</i> -Iodobenzoate	40
Control (with no inducer)	90

apparatus. When oxygen uptake began to increase, indicating that enzymic induction was virtually complete, the cells in the flasks were collected by centrifugation, washed once in 10ml. of 0.9% sodium chloride, resuspended in 2ml. of water and their ability to oxidize benzoate was tested in the Warburg apparatus. Under these conditions there was an immediate oxidation of substrate when the benzoate was present for the whole of the

induction period. The halogeno analogues inhibited the induction when added with the benzoate but there was no inhibition if the cells were incubated with the halogeno analogue alone, provided that this was removed by centrifugation and washing before the addition of substrate benzoate (Table 3). As judged by their effect on the length of the induction period, the halogenobenzoates thus inhibit the formation of the benzoate-oxidizing system induced by benzoate. The inhibition is competitive because increasing the concentration of the inducing benzoate decreases the effect of the inhibitor (Table 4). This is true, however, only when the inhibitor is added to the induction mixture at least 30 min. before testing for the formation of induced enzyme; if added after this period no inhibition was generally found.

Table 3. *Effect of prior incubation of washed suspensions with benzoate and halogenobenzoates*

For experimental details see Table 2.

Additions to induction mixture	Time (min.)	Rate of oxidation after adding substrate (μ moles of O_2 /mg. dry wt./hr.)		
		...5	70	100
None		0.2	0.2	5.0
Benzoate		2.6	7.8	3.0
1 mM-Benzoate; 0.01 M- <i>o</i> -fluorobenzoate		0.2	0.5	5.0
1 mM-Benzoate; 0.01 M- <i>o</i> -chlorobenzoate		0.2	0.2	4.3
1 mM-Benzoate; 0.01 M- <i>o</i> -bromobenzoate		0.2	0.2	5.0
1 mM-Benzoate; 0.01 M- <i>o</i> -iodobenzoate		0.2	0.4	5.3

Table 4. *Effect of concentration of inducing benzoate on the inhibition of induction by o-fluorobenzoate*

Washed suspensions containing 8 mg. dry wt. were incubated in Warburg cups for 170 min. with the additions shown below; substrate benzoate (5 mM) was added and the time taken for the cells to achieve the maximal rate of oxidation measured.

Additions	Time to achieve maximal rate of oxidation (min.)
None	65
0.01 M-Benzoate	15
0.01 M-Benzoate; 0.05 M- <i>o</i> -fluorobenzoate	15
1 mM-Benzoate	15
1 mM-Benzoate; 0.05 M- <i>o</i> -fluorobenzoate	65
0.1 mM-Benzoate	15
0.1 mM-Benzoate; 0.05 M- <i>o</i> -fluorobenzoate	105

Effect of halogenobenzoates as enzymic inducers in washed suspensions. In nine out of eleven batches of cells, the length of the lag period before the oxidation of benzoate was decreased by replacing benzoate normally used as inducer by the fluorobenzoates (Fig. 5). None of the other halogenobenzoates showed this effect in any of the batches of cells tested. Of the three fluoro isomers, *o*-fluorobenzoate was the most effective inducer. Its effect, however, differed from that of benzoate itself in that the substrate benzoate was never oxidized immediately it was added. In other words, enzymic induction was never complete as it was when

Fig. 5. *Effect of o-fluorobenzoate as inducer for benzoate oxidation.* Cells were incubated with: x, 0.1 mM-benzoate; ●, 0.1 mM-*o*-fluorobenzoate; ○, no inducer; Δ, 5 mM-*o*-fluorobenzoate. 5 mM-Benzoate was added from the side arm as indicated by the arrow to all except the 5 mM-*o*-fluorobenzoate. Each cup contained 6.4 mg. dry wt. of cells.

Table 5. *Effect of fluorobenzoates as inducers of the benzoate-oxidizing system*

Washed cells (6 mg. dry wt.) were incubated for 20 min. with the additions shown below, centrifuged, washed and their ability to oxidize benzoate (5 mM) was measured manometrically.

Addition (0.01 M)	Time (min.)	Rate of oxidation (μ moles/mg. dry wt./hr.)		
		...10	40	110
None	0.8	0.8	3.2	
<i>o</i> -Fluorobenzoate	0.8	2.4	7.0	
<i>m</i> -Fluorobenzoate	0.8	1.6	10.1	
<i>p</i> -Fluorobenzoate	0.8	1.6	7.5*	
Benzoate	1.2	4.4	7.0*	

* Maximal rate of 12.2 μ moles/mg. dry wt./hr. was found at about 80 min.

benzoate was used as inducer and the process was only completed upon the addition of the substrate (Table 5). In no experiment did the cells adapt to oxidize the halogenobenzoates when these were used both as inducers and substrates and the incubation was continued up to 3 hr. Unless the cells are completely adapted to oxidize benzoate therefore they cannot oxidize the halogeno analogues. This is in contrast with the effect of 5-fluoro-nicotinic acid, which is able to induce systems oxidizing both itself and nicotinic acid (Hughes, 1955).

Effect of o-fluorobenzoate on adaptation to substances other than benzoate. Washed suspensions of strain KBI when grown upon asparagine adapted to oxidize citric acid and nicotinic acid. In adaptation to citrate it is not certain whether a new oxidizing system is formed or whether adaptation makes the substrate accessible to an existing oxidizing system (Kogut & Podoski, 1953). It is likely that at least one of the enzymes oxidizing nicotinic acid has to be formed adaptively (Hughes, 1955). The addition of o-fluorobenzoate (0.01M–5mM) together with

the inducing substrate (citrate, 0.1–1mM, or nicotinic acid, 0.1mM) inhibited adaptation to these substrates.

The inhibition of the adaptation to citrate by fluorobenzoate was not reversed by the addition of more citrate (0.01M). This is in contrast with the inhibition of adaptation to benzoate which is reversed by the addition of more substrate. No inhibition was found when the o-fluorobenzoate was preincubated with the cells and removed before addition of substrate by centrifugation, as described above. Benzoate (0.1–5mM) also inhibits adaptation to citric acid and nicotinic acid because the cells adapt to benzoate preferentially. This preferential adaptation is similar to the 'diauxic effect' (Monod, 1942). o-Fluorobenzoate (0.01M) inhibited neither adaptation to, nor the oxidation of, catechol. Cells adapted to oxidize catechol did not oxidize o-fluorobenzoate.

Effect of benzoate and halogenobenzoates on the growth of Pseudomonas fluorescens. At concentrations of benzoate of 0.01–0.02M the growth of strain KBI reached a maximum in 24 hr. At higher concentrations (0.02–0.05M) the time taken for maximum growth was anything up to 4 days. Con-

Table 6. *Effect of o-fluorobenzoic acid on the growth of Pseudomonas fluorescens with benzoate as carbon source*

Conditions and measurement of growth were as described in the text. A nephelometer reading of 100 indicates a dry wt. of approx. 0.5–0.7 mg./ml.

Substances added		Growth (nephelometer readings)			
Concn. of benzoate (mM)	Concn. of o-fluorobenzoate (mM)	16hr.	24hr.	40hr.	76hr.
60	0	0	0	0	0
50	0	0	0	6	98
40	0	0	0	16	82
30	0	2	17	92	84
30	30	0	2	14	86
30	20	0	0	46	74
30	10	0	0	42	94
20	0	34	98	98	82
20	30	0	0	16	74
20	20	0	0	40	84
20	10	6	36	94	84
10	0	28	84	74	56
10	30	2	2	16	28
10	20	4	23	46	44
10	10	16	64	60	48
5	0	36	38	28	26
5	30	2	0	7	26
5	20	5	12	20	22
5	10	6	14	20	14
2	0	19	64	18	0
2	30	0	0	0	0
2	20	0	0	0	6
2	10	4	6	7	8

Table 7. *Effect of o-halogenobenzoates on growth with asparagine*

Growth media and measurements of growth were as described in the text. A nephelometer reading of 100 indicates a dry wt. of approx. 0.5–0.7 mg./ml.

Addition	Concn. of addition (mM)	Growth (nephelometer readings)		
		24hr.	40hr.	3 days
None		36	36	36
Benzoate	50	12	84	102
	30	68	100	84
	20	120	100	90
	10	90	100	90
o-Fluorobenzoate	50	8	27	28
	30	20	27	36
	20	30	33	36
	10	32	33	36
o-Chlorobenzoate	50	20	27	28
	30	28	30	36
	20	28	36	34
	10	30	36	36
o-Bromobenzoate	50	10	28	28
	30	26	30	36
	20	28	30	36
	10	30	36	36
o-Iodobenzoate	50	16	30	30
	30	20	27	36
	20	34	36	36
	10	36	36	36

centrations of benzoate over 0.05M inhibited growth (Table 6). Below 0.01M-benzoate the final yield was limited by the available carbon source since more growth was obtained when asparagine was also present (Table 7). None of the halogenobenzoates supported growth when added as the sole carbon source nor did they increase the final yield when added to a medium containing asparagine (Tables 6 and 7). The addition of fluorobenzoate to a medium containing benzoate as carbon source reduced growth (Table 6). When the total concentration of benzoate and analogue was above the optimum concentration of benzoate for growth, the inhibitory effect of the analogue was additive but less than that expected if benzoate itself had been added. When the total concentration of analogue and benzoate was below the optimum concentration of benzoate the analogues inhibited growth. This suggests that they could act as competitors of benzoate (Table 6).

Twenty soil samples, ranging in type from a light sandy loam to a rich humus from beneath conifers, were incubated at 25° in the mineral medium (about 0.2g./10ml.) containing either benzoate or the halogeno analogues (0.05%). In all cases strains of pigment-forming *Pseudomonas* were isolated from the benzoate enrichment cultures. These grew with benzoate as their sole carbon source but not with halogenobenzoates. No such organisms were isolated from the enrichment cultures containing the halogenobenzoates.

DISCUSSION

Strain KBI of *Pseudomonas fluorescens* oxidizes to some extent all of the monohalogenobenzoic acids but cannot utilize them for growth when present as a sole carbon source. Similar results were found with halogenonicotinic acids (Hughes, 1955) and more recently with a *Vibrio* sp. (Ali, Callely & Hayes, 1962). The inability of the cells to grow on the halogeno analogues appears to be due to two main causes: (a) the oxidation of the analogues is not carried to a stage at which the carbon may be assimilated; (b) the analogues inhibit the induction of the enzymes responsible for the oxidation and assimilation of the purest compound.

Because cells are unable to oxidize benzoic acid or the halogenobenzoic acids unless they have been induced to form the oxidizing system by benzoic acid itself, it is reasonable to assume that the same enzyme oxidizes both the benzoic acid and the analogues. The oxidation of benzoic acid has been shown to lead to the formation of catechol followed by ring fission to form β -oxoadipic acid, which in its turn yields acetic acid and succinic acid. These latter products may then either be oxidized further

to carbon dioxide and water or may be assimilated (Evans, Smith, Linstead & Elvidge, 1951). The oxygen uptake in the experiments with benzoic acid alone was too low for complete oxidation (3.8–4.6 μ moles instead of 7.5 μ moles) and suggests that assimilation took place. The oxygen uptake with fluorobenzoates as substrates (2.0–3.0 μ moles/ μ mole of substrate) suggests that these are not oxidized beyond the formation of corresponding halogenated β -oxadipic acid. Likewise it is unlikely that the other halogenated benzoic acids are oxidized beyond the formation of halogenophenols (cf. Ichihara, Adachi, Hosokawa & Takeda, 1962).

Evans & Smith (1954), in studies on the oxidation of the chlorophenoxyacetic acid herbicides, isolated from soil an organism capable of oxidizing 4-chlorophenol. The oxidation was accompanied by the simultaneous splitting of the ring and the liberation of Cl⁻ ions. No halide ion has been detected during the oxidation of the halogenobenzoic acids in the present work. However, liberation of free fluoride by *p*-fluorobenzoate by the *Vibrio* mentioned above has been demonstrated (Ali *et al.* 1962). The inability to oxidize the analogues further and to utilize them for growth may be due to the inability of *P. fluorescens* to liberate the organic halogen as halide ion. It is also noteworthy that the organism isolated by Evans & Smith (1954) grew with *p*-chlorophenoxyacetic acid as sole carbon source and formed some at least of the enzymes adaptively. The authors did not report experiments on the effect of *p*-chlorophenoxyacetic acid on the metabolism of phenoxyacetic acid and it is not certain whether the enzymes concerned in the oxidation of the halogeno analogues are the same as those oxidizing the parent compound. As judged by the isolation of corresponding chlorophenols, the route of oxidation followed was the same. It would be interesting to know whether an enzyme responsible for the liberation of free halide is formed adaptively or whether this can take place non-enzymically as in animal tissues (Bray, Thorpe & Vallance, 1952). The occurrence of organisms in the soil capable of growing on halogenobenzoates is rare as judged by the failure to isolate such organisms from elective cultures of many differing types of soil.

The inhibition of enzymic induction by benzoate by the analogues is competitive and is possibly concerned with the induction process rather than the inhibitions caused by inhibitors of energy-yielding reactions and amino acid analogues (Halvorson & Spiegelman, 1953). The inhibition is likely to occur at some site concerned with the early stages of the induction process and before the oxidizing system is synthesized (Pollock, 1953). It is not known for certain whether a permease system for benzoate uptake is necessary but an

inhibition of this or of its formation by the halogeno analogues could explain the present results. Alternatively the inhibition may take place by competitive inhibition of the de-repression by benzoate. In *Mycobacterium tuberculosis* some halogen analogues which themselves are not oxidized can induce the formation of both the benzoic acid- and catechol-oxidizing system (Bernheim, 1953). In this case some of the fluoro analogues are more effective inducers than is benzoate. That the fluoro analogues behave more effectively than the parent compound than do the other halogeno analogues is paralleled by other halogeno analogues of biological activity (Peters, 1952; Hughes, 1955; Azouz, Parke & Williams, 1952). In higher animals this is often due to the longer persistence of the analogue compared with the parent compound and this is possibly the explanation of the effects of the fluorobenzoates in the mycobacterium. In the pseudomonad no fluoro analogue tested was as an effective inducer as was benzoate. In fact induction appeared not to be completed until the substrate concentration of benzoate was added.

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