The Utilization of some Halogenated Aromatic Acids by *Nocardia*

EFFECTS ON GROWTH AND ENZYME INDUCTION

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1. Halogen analogues of benzoate and *p*-nitrobenzoate did not support growth of *Nocardia erythropolis*. 2. These analogues, when present together with the parent compounds, inhibited growth of the organism. 3. The halogen analogues similarly inhibited oxidation of benzoate or *p*-nitrobenzoate by competent cells. 4. Fluoroacetate and 2-fluoro-4-nitrobenzoate caused comparable inhibition of growth on *p*-nitrobenzoate and both led to some citrate accumulation. 5. The induction of the *p*-nitrobenzoate-oxidation system was strongly inhibited by all the 2-halogeno-4-nitrobenzoates although the 2-fluoro and 2-chloro derivatives also acted as inducers. 6. Halogen analogues of benzoate also induced the benzoate-oxidation system.

During studies of the degradation of *p*-nitrobenzoate by *Nocardia erythropolis* (Cain, 1958), it was observed that *p*-nitrobenzoate-grown cells of this species could oxidize 2-fluoro-4-nitrobenzoate. Hughes (1953, 1955) and Behrman & Stanier (1957) found that the fluorobenzoates and 2-fluoro-nicotinate were extensively oxidized by pseudomonads previously grown with benzoate or nicotinate respectively, although the fluoro analogues would not support growth of the bacteria. The utilization of halogen-substituted nitrobenzoates by competent cells of *N. erythropolis* was therefore examined in more detail together with some comparative studies of the effects of similar analogues on benzoate metabolism by the same organism.

MATERIALS AND METHODS

Organism. The organism used was *N. erythropolis*, strain CA4, originally isolated from soil by enrichment with *p*-nitrobenzoate (Cain, 1958; Cartwright & Cain, 1959). It was subcultured on nutrient agar slopes from which transfer to a defined aromatic medium could be effected without difficulty.

Growth methods. Large batches of adapted cells were cultured in 10l. flasks containing 9l. of the defined medium; this consisted of: KH_2PO_4 , 0.1g./l.; K_2HPO_4 , 0.4g./l.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01g./l.; aromatic source, 1.0g./l.; Difco yeast extract, 0.05g./l.; trace-elements solution (Barnett & Ingram, 1955), 10ml./l. Where the aromatic compound did not contain nitrogen, $(\text{NH}_4)_2\text{SO}_4$ (0.5g./l.) was also added. The pH was adjusted to 7.2 with N-KOH. These large batches were inoculated with 500ml. cultures grown in the same medium for 36hr. in a New Brunswick Gyrotory

shaker. The 9l. batches were then incubated for 24–36hr. with forced aeration (5–7l. of air/min.) at 30° and harvested in a continuous centrifuge. Unadapted cells were cultivated similarly but with fumarate or glucose in place of the aromatic inducer.

Growth tests. Growth experiments with the halogen compounds were carried out in 250ml. conical flasks fitted with a side arm for nephelometric or spectrophotometric measurements and containing 100ml. of the following medium: phosphate buffer, pH 7.2, 1.25m-moles; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5mg.; substrate (neutralized to pH 7.0), 100–250 μmoles ; trace-elements solution, 0.1ml. The flasks were incubated with 1ml. of a washed suspension of cells (equivalent to about 50 μg . dry wt.) previously grown with either benzoate, *p*-nitrobenzoate or a non-aromatic substrate as required. Growth was measured turbidimetrically at 530m μ with a Unicam SP.1400 spectrophotometer or an EEL nephelometer during incubation at 30° in a New Brunswick Gyrotory shaker. In similar experiments to investigate the effects of the halogen compounds on utilization of benzoate, *p*-nitrobenzoate or succinate, the halogen compounds were also added over the range 20–400 μmoles /100ml. of medium. In some experiments, the dry weight of cells in a 50ml. sample of the medium taken at the end of the exponential phase was measured by collecting the cells on a tared Millipore filter, washing with several volumes of distilled water and drying to constant weight in an oven at 105°.

Freeze-dried cell preparations. Induced cells, grown as described, were washed twice with distilled water, frozen at –20° and then freeze-dried overnight. Such preparations were stored at –20° until required, when they were ground to a fine powder with a precooled pestle and mortar and suspended to 10 or 20mg. dry wt./ml. in distilled water.

Manometric experiments. Oxygen uptake by washed cells and freeze-dried preparations was followed manometrically at 30° in the Warburg apparatus. Flasks contained: phosphate buffer, pH 7.2, 200 μmoles ; cell suspension (5–10mg. dry wt.), 0.5ml.; substrate (10 μmoles) and inhibitor (where appropriate) (0–50 μmoles), either in separate side arms or

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together in the same side arm; KOH (20%, w/v), 0.2 ml. in the centre well.

Halogen analogues as enzyme inducers. The ability of the halogen analogues to induce the enzymic mechanism for benzoate or *p*-nitrobenzoate oxidation was examined with unadapted cells. Cells grown with fumarate or malate were washed and incubated with casein hydrolysate (0.5 mg./ml.) and the halogen compounds (100 μ moles) in 0.1 M-phosphate buffer, pH 7.0 (total volume, 50 ml.). At intervals, 10 ml. samples were removed and centrifuged, the packed cells were washed and their ability to oxidize benzoate or *p*-nitrobenzoate (10 μ moles) was then examined immediately in the Warburg apparatus.

Determinations. Citrate and fluorocitrate were determined by the method of Taylor (1953) by using calibration curves prepared from the authentic substrates. Synthetic sodium fluorocitrate was kindly provided by Sir Rudolph Peters. Since fluorocitrate was not metabolized by any of the biological material used, citrate formation in incubations containing fluorocitrate as an inhibitor may be readily corrected for the original fluorocitrate content. Fluoride was determined by the method of Belcher, Leonard & West (1959).

RESULTS

Effects of halogen analogues on growth. *N. erythropolis* grew readily on mineral-salts medium where benzoate + ammonium sulphate or *p*-nitrobenzoate (2–5 mM) formed the sole source of carbon and nitrogen, but uniformly turbid growth was only achieved with rapidly shaken cultures. Stationary or slowly shaken cultures developed a surface pellicle or granular pellets of growth even in the presence of the non-ionic detergent Tween 80 (polyoxyethylene sorbitan mono-oleate). 2-Fluoro-, 2-chloro- or 2-bromo-4-nitrobenzoate or *o*-, *m*- or *p*-fluorobenzoate at concentrations over the range 0.05–5 mM did not support growth of this organism, nor did any of them increase the growth yield (measured as dry weight of cells) when added at 0.5 mM to cultures of the organism utilizing fumarate (2 mM) as carbon source.

The addition of either 2-fluoro-4-nitrobenzoate or 2-chloro-4-nitrobenzoate to cultures growing at the expense of *p*-nitrobenzoate diminished the growth (Table 1), but the 2-fluoro analogue was much the better inhibitor; the corresponding 2-bromo compound had no inhibitory effect. The inhibition of growth caused by lower concentrations of the analogues was partially reversed by increasing the concentration of *p*-nitrobenzoate; above 5 mM, however, *p*-nitrobenzoate itself was inhibitory to growth.

Ali, Callely & Hayes (1962) found that cultures of *Moraxella calcoacetica* (*Vibrio* O1, N.C.I.B. 8250) growing on benzoate in the presence of *p*-fluorobenzoate released F⁻ ions into the medium. Similar cultures of *N. erythropolis* growing on 5 mM-*p*-nitrobenzoate in the presence of either 1 mM-2-fluoro-4-nitrobenzoate or 1 mM-fluoroacetate showed

Table 1. *Inhibition of growth of N. erythropolis by halogen analogues*

Growth was in shaken cultures with 1 mM-*p*-nitrobenzoate available.

Inhibitor	Dry wt. of cells (mg./50 ml. of medium after 4 days)
None	2.10
2-Chloro-4-nitrobenzoate (1 mM)	2.05
2-Chloro-4-nitrobenzoate (2 mM)	2.10
2-Chloro-4-nitrobenzoate (4 mM)	1.50
2-Fluoro-4-nitrobenzoate (1 mM)	0.65
2-Fluoro-4-nitrobenzoate (2 mM)	0.27
2-Fluoro-4-nitrobenzoate (4 mM)	0.25

no such production of F⁻ ions even after 14 days. The effects of fluoroacetate on the growth of the *Nocardia* on *p*-nitrobenzoate were compared with those caused by added 2-fluoro-4-nitrobenzoate. Growth inhibition by fluoroacetate was apparent at 0.5 mM and virtually complete at 2 mM, and within this range of concentration the extent of inhibition closely paralleled that brought about by the same concentration of 2-fluoro-4-nitrobenzoate.

Effects of halogen analogues on the oxidation of benzoate and p-nitrobenzoate. Washed suspensions of competent cells rapidly oxidized *p*-nitrobenzoate or benzoate, but in both cases the presence of certain of the corresponding halogen analogues caused inhibition. Significant inhibition of benzoate (3.3 mM) oxidation was effected only by the fluoro analogues; *p*-chloro-, *p*-bromo- or *p*-iodo-benzoate had little effect at 16.7 mM in Warburg experiments, but *o*- or *p*-fluorobenzoate caused some 70–80% inhibition of oxygen uptake at this concentration (Table 2). *p*-Nitrobenzoate oxidation by the *Nocardia* was unaffected by 2-bromo-4-nitrobenzoate, and the corresponding 2-chloro compound produced only some 10% inhibition at 16.7 mM. In contrast, the 2-fluoro analogue was strongly inhibitory (Table 2). 2-Fluoro-4-nitrobenzoate also caused some 60% inhibition of the oxidation rate of *p*-nitrobenzoate by dried-cell preparations of *p*-nitrobenzoate-grown organisms, indicating that there were no appreciable permeability effects incurred by the use of intact cells.

Effect of fluoroacetate and fluorocitrate on substrate oxidation. The absence of F⁻ ion appearance, the inability of the fluoro aromatic compound to support growth and the comparable inhibition of growth on *p*-nitrobenzoate by 2-fluoro-4-nitrobenzoate and fluoroacetate suggested that 2-fluoro-4-nitrobenzoate might be converted into fluoroacetate and fluorocitrate and thus bring the tricarboxylic acid cycle to a halt by poisoning aconitase (Peters, 1952; Morrison & Peters, 1954).

Table 2. *Inhibition of benzoate and p-nitrobenzoate oxidation by N. erythropolis by their halogen analogues*

Washed suspensions equivalent to 8.4 mg. dry wt. from benzoate cultures and equivalent to 5.3 mg. dry wt. from *p*-nitrobenzoate cultures were used in Expts. I and II respectively. The substrate concentration was 3.3 mM. Oxidation rate was corrected for endogenous respiration and oxygen uptake in controls containing only the inhibitors; it was assumed that there were no additional effects when substrate and halogen analogue were being oxidized in each other's presence.

	Substrate	Inhibitor	Oxidation rate (μ l. of O_2 /hr.)	Inhibition (%)
Expt. I	Benzoate	None	250	—
		<i>o</i> -Fluorobenzoate (16.7 mM)	72	71
		<i>p</i> -Fluorobenzoate (0.33 mM)	225	10
		<i>p</i> -Fluorobenzoate (0.67 mM)	182	27
		<i>p</i> -Fluorobenzoate (1.67 mM)	126	50
		<i>p</i> -Fluorobenzoate (16.7 mM)	48	81
Expt. II	<i>p</i> -Nitrobenzoate	None	80	—
		2-Bromo-4-nitrobenzoate (16.7 mM)	81	0
		2-Chloro-4-nitrobenzoate (16.7 mM)	72	10
		2-Fluoro-4-nitrobenzoate (1.0 mM)	73	9
		2-Fluoro-4-nitrobenzoate (3.3 mM)	60	25
		2-Fluoro-4-nitrobenzoate (10 mM)	38	52
		2-Fluoro-4-nitrobenzoate (16.7 mM)	32	60

Fluoroacetate and fluorocitrate were thus examined for their effects on substrate oxidation.

In the presence of 3.3 mM-fluoroacetate added simultaneously, there was no uptake of oxygen above the endogenous value by washed suspensions oxidizing 3.3 mM-*p*-nitrobenzoate. Even at 0.33 mM, fluoroacetate caused a 70% inhibition of the oxidation rate with this substrate. Fluorocitrate was equally effective at 0.33 mM. Fluoroacetate and fluorocitrate were not themselves oxidized by washed suspensions. To rule out the possibility that permeability effects might be influencing the activity of these polar compounds, the experiments were repeated with dried preparations of *p*-nitrobenzoate-grown cells. Although the oxidation of *p*-nitrobenzoate was inhibited by both fluoroacetate and fluorocitrate to virtually the same extent, the kinetics of the inhibition were different. Fluorocitrate (0.33 mM) caused an immediate depression of some 70% in *p*-nitrobenzoate oxidation rate, whereas the effects of fluoroacetate were not fully manifested until some 30 min. after oxidation began (Fig. 1). Thereafter fluoroacetate stopped oxidation entirely. It is tempting to speculate that this lag before inhibition begins may represent the time required for fluoroacetate to be progressively converted into inhibitory concentrations of fluorocitrate, which actually halts cell respiration.

Citrate itself was rapidly oxidized by dried preparations, but the presence of 0.33 mM-fluoroacetate or 0.33 mM-fluorocitrate immediately decreased the citrate oxidation rate by 60 and 90% respectively. The oxidation of succinate by the same preparation, however, was unaffected by fluoroacetate below 5 mM.

Fig. 1. Effect of fluoroacetate and fluorocitrate on the oxidation of *p*-nitrobenzoate by a suspension of freeze-dried cells of *N. erythropolis*. Flasks contained: *p*-nitrobenzoate, 5 μ moles; fluoroacetate or fluorocitrate (where required), 1 μ mole; phosphate buffer, pH 7.2, 200 μ moles; cell suspension, equivalent to 7.2 mg. dry wt.; KOH (20%, w/v), 0.2 ml. in centre well (total volume, 3 ml.). Substrate and inhibitors were all put in the side arm and tipped simultaneously. Results are corrected for endogenous oxidation (in the presence of the appropriate inhibitor, where necessary). ●, No inhibitor; ○, + fluoroacetate; ▲, + fluorocitrate.

Table 3. *Citrate accumulation by N. erythropolis in the presence of fluoro compounds*

Incubations were performed aerobically in Warburg flasks at 30°. Flasks contained: substrate, 5 μ moles; inhibitor (where required), 2 μ moles; phosphate buffer, pH 7.0, 200 μ moles; freeze-dried *p*-nitrobenzoate-grown cells, 10mg. dry wt.; KOH (20%, w/v), 0.2 ml. in centre well (total volume, 3 ml.). Expts. I and II were performed with different batches of freeze-dried cells. Oxygen uptake was followed until substrate oxidation ceased and citrate analyses were then performed immediately. Results are corrected for the appropriate controls (no substrate) with and without inhibitor.

	Substrate	Inhibitor	Citrate formed (μ g.)	Oxygen uptake (μ moles)
Expt. I	<i>p</i> -Nitrobenzoate	None	0	26.7
	2-Fluoro-4-nitrobenzoate	None	33	16.9
	<i>p</i> -Nitrobenzoate	Fluoroacetate	58	17.8
	<i>p</i> -Nitrobenzoate	Fluorocitrate	96	22.5
	<i>p</i> -Nitrobenzoate	2-Fluoro-4-nitrobenzoate	12	6.9
Expt. II	<i>p</i> -Nitrobenzoate	None	0	—
	2-Fluoro-4-nitrobenzoate	None	23	—
	<i>p</i> -Nitrobenzoate	Fluoroacetate	57	—
	<i>p</i> -Nitrobenzoate	2-Fluoro-4-nitrobenzoate	12	—

Accumulation of citrate in incubation mixtures poisoned with fluoro compounds. A characteristic of fluoroacetate poisoning in both mammalian tissues (Peters, 1957; Pattison, 1959) and *Neurospora crassa* mutants (Strauss, 1956) is the pronounced accumulation of citrate, usually accompanied by a decrease in oxygen uptake. Evidence for citrate accumulation in 2-fluoro-4-nitrobenzoate- and fluoroacetate-poisoned *p*-nitrobenzoate incubations was therefore sought with the *Nocardia*; results of two such experiments are shown in Table 3, where the citrate accumulation is similar, on a tissue-weight basis, to that found in the tissues of fluoroacetate-poisoned mammals (Peters, 1952).

Effects of the halogen analogues on induced enzyme formation. When cells of *N. erythropolis*, grown with fumarate as the sole carbon source, were put in contact with benzoate (3.3 mM), there was a lag of some 65–70 min. before benzoate oxidation commenced. This lag period was shortened in the presence of the halogenobenzoates (3.3 mM) added at the same time (Fig. 2). *p*-Fluorobenzoate was the best accelerator, decreasing the lag time to 25–30 min.; the less effective *p*-chloro and *p*-bromo analogues decreased the lag time by 15–30 min.

The halogen-substituted nitrobenzoates, in similar experiments, inhibited induction of the enzymes responsible for *p*-nitrobenzoate oxidation. To ensure that there was no possible interference by the analogue with oxidation of *p*-nitrobenzoate, the Hughes (1965) technique was used for confirming the results with the *p*-nitrobenzoate system. In this method, the cell suspension of fumarate-grown *Nocardia*, *p*-nitrobenzoate (25 μ moles), casein hydrolysate, halogen analogue and phosphate buffer were shaken at 30° in 100 ml. flasks, and induction in a pilot vessel with *p*-nitrobenzoate but no analogue was followed manometrically in the

Fig. 2. Effects of halogenobenzoates on the induction of the benzoate-oxidation system in washed suspensions of fumarate-grown *N. erythropolis*. Warburg flasks contained: phosphate buffer, pH 7.2, 200 μ moles; benzoate, 10 μ moles, and analogue (where required), 10 μ moles, both in the side arm; washed-cell suspension (from a culture grown with fumarate), 9.6 mg. dry wt.; KOH (20%, w/v), 0.2 ml. in centre well (total volume, 3 ml.). Oxygen uptakes were corrected for the corresponding controls (no substrate). ●, Benzoate only; ○, benzoate + *p*-bromobenzoate; ▽, benzoate + *p*-chlorobenzoate; ▲, benzoate + *p*-fluorobenzoate.

Warburg respirometer. When the increase in oxygen uptake in this vessel indicated that induction was nearly complete, a portion of the cells from each flask was harvested and washed, resuspended in buffer and its ability to oxidize *p*-nitrobenzoate (10 μ moles) tested manometrically; samples were taken similarly at intervals thereafter. There was a pronounced inhibition of the induction process by the halogenonitrobenzoates in this experiment (Table 4), the 2-fluoro analogue being particularly

Table 4. *Effect of halogen analogues on induction of the p-nitrobenzoate-oxidation system in fumarate-grown N. erythropolis*

Induction was carried out by incubating (on a Gyrotory shaker incubator, 210 rev./min.) washed suspensions of fumarate-grown cells (21.0 mg. dry wt.) in 100 ml. flasks containing: phosphate buffer, pH 7.0, 2 m-moles; casein hydrolysate, 12.5 mg.; the additions shown, 25 μ moles (total volume, 25 ml.). Incubation was at 30°. At intervals, 5 ml. samples were withdrawn, and the cells were centrifuged down, washed once and resuspended to 5 ml. in water. Oxygen uptake with *p*-nitrobenzoate as substrate was examined manometrically, with control flasks (no substrate) for endogenous respiration run at the same time.

Additions to induction mixture	Induction period (hr.)	Activity of cells (μ l. of O ₂ /hr.)		
		0	2.5	5.0
None		0	0	0
<i>p</i> -Nitrobenzoate		0	112	136
<i>p</i> -Nitrobenzoate + 2-bromo-4-nitrobenzoate		0	30	36
<i>p</i> -Nitrobenzoate + 2-chloro-4-nitrobenzoate		0	16	20
<i>p</i> -Nitrobenzoate + 2-fluoro-4-nitrobenzoate		0	0	4

effective. The inhibition appeared to be competitive because an increase in the *p*-nitrobenzoate/halogen analogue concentration ratio from 1.0 to above 4.0 invariably led to a decrease in the time required to induce high activities of the *p*-nitrobenzoate-oxidation system.

Halogen analogues as enzyme inducers in washed suspensions. The competitive effects on induction produced by the halogen analogues of *p*-nitrobenzoate suggested that the analogues themselves might be capable of acting as enzyme inducers. In the first experiments, washed fumarate-grown cells were incubated in phosphate buffer, pH 7.0, with the halogenonitrobenzoate (2 mM) as inducer, together with fumarate (1 mM) and casein hydrolysate (0.5 mg./ml.) as energy and nitrogen sources respectively. Controls containing no inducer were run concurrently. Samples were withdrawn at 30 min. intervals, centrifuged, the packed cells were washed and resuspended in water and their ability to oxidize *p*-nitrobenzoate (5 μ moles) was examined manometrically. Fumarate, however, completely repressed the formation of any inducible enzyme. When the experiments were repeated, but with fumarate omitted, the halogenonitrobenzoates were able to induce the *p*-nitrobenzoate-oxidizing system in 3–4 hr.; *p*-nitrobenzoate itself required only 60–80 min. to elicit the same activity. The inductive response was confirmed by including D-chloramphenicol in some incubation mixtures, resulting in inhibition of enzyme induction by the halogen analogues; the biologically inactive L-chloramphenicol used in control experiments had no such effect (Fig. 3).

Suda, Naono, Kato, Inoue & Yugari (1959), Hughes (1965) and Calley & Jones (1965) have shown that the chlorobenzoates and fluorobenzoates would act as inducers of the enzyme system

Fig. 3. Induction of the *p*-nitrobenzoate-oxidation system of *N. erythropolis* by halogen analogues. Warburg flasks contained: phosphate buffer, pH 7.2, 200 μ moles; *p*-nitrobenzoate, 10 μ moles; cell suspension, equivalent to 8.8 mg. dry wt.; KOH (20%, w/v), 0.2 ml. in centre well (total volume, 3 ml.). Oxygen uptakes were corrected for endogenous respiration, measured under identical conditions. Cells were induced (4 hr.) in the presence of: ▽, no inducer; Δ, 2-fluoro-4-nitrobenzoate (2 μ moles/ml.); ○, 2-chloro-4-nitrobenzoate (2 μ moles/ml.); ●, 2-fluoro-4-nitrobenzoate (2 μ moles/ml.) + D-chloramphenicol (80 μ g./ml.); ◐, 2-fluoro-4-nitrobenzoate (2 μ moles/ml.) + L-chloramphenicol (200 μ g./ml.).

Fig. 4. Induction of the benzoate oxidation system of *N. erythropolis* by the halogenobenzoates. Flask contents were as given in Fig. 3, but with benzoate ($10\mu\text{moles}$) replacing *p*-nitrobenzoate, and cell suspension equivalent to 15mg. dry wt. Cells were induced (4hr.) in the presence of: ●, benzoate ($2\mu\text{moles/ml.}$); ○, *o*-fluorobenzoate ($2\mu\text{moles/ml.}$); ■, *p*-fluorobenzoate ($2\mu\text{moles/ml.}$); ▲, *p*-chlorobenzoate ($2\mu\text{moles/ml.}$); △, *p*-bromobenzoate ($2\mu\text{moles/ml.}$); ▼, *p*-iodobenzoate ($2\mu\text{moles/ml.}$) and no inducer. Oxygen uptakes were corrected for endogenous respiration of controls from the same inductions.

oxidizing benzoate in *Micrococcus ureae*, *Pseudomonas fluorescens* and *Moraxella calcoacetica* respectively and that the *o*-fluoro isomer was the most effective inducer. This also appeared to be true for *N. erythropolis*; in experiments similar to those described above, the fluorobenzoates and *p*-chlorobenzoate induced the benzoate-oxidizing system within 90min., and of the isomers tested *o*-fluorobenzoate was the most efficient (Fig. 4). There was negligible induction by *p*-bromobenzoate and none by *p*-iodobenzoate up to 8hr. after exposure of fumarate-grown cells to these compounds.

DISCUSSION

The inability of halogenonitrobenzoates to support growth of *N. erythropolis* where they provide the sole carbon and nitrogen sources, although they are extensively oxidized by competent cells (Cain, Tranter & Darrah, 1968), could be due to two principal factors: (i) the oxidation of the analogues progresses to a stage where the products are not assimilated; (ii) the analogues

Table 5. Some physical properties of the halogen atom in halogenoacetic acids

Nature of X	Atomic radius of X (Å)	Internuclear distance of X-C (Å)	Bond energy of X-C (kcal./mole)
H	0.29	1.14	87.3
F	0.64	1.45	107.0
Cl	0.99	1.74	66.5
Br	1.14	1.90	54.0

inhibit both the activity and the induction of enzymes responsible for metabolism of the (non-halogenated) parent compound. Either or both are possible and the experimental evidence in this and the following paper (Cain *et al.* 1968) shows that, with the halogenonitrobenzoates, both factors seem to be operating.

The fluoro analogue has been examined in more detail because it was by far the most extensively oxidized and had the greatest inhibitory effect on substrate oxidation and on enzyme induction by *p*-nitrobenzoate, as well as serving as an excellent inducer of the enzyme system oxidizing *p*-nitrobenzoate. The reasons for this ability lie in the small size of the fluorine atom and the short internuclear distance of the C-F bond, which is comparable with that of the C-H bond (Table 5). The spatial similarity of C-F and C-H bonds means that fluoro analogues can mimic their parent compounds in many metabolic reactions (Hughes, 1955; Behrman & Stanier, 1957; Birnie, Kroeger & Heidelberger, 1963; Richmond, 1963), whereas the corresponding chloro and bromo analogues, because of the much greater size of the individual halogen atom and the C-Cl and C-Br bonds, are much less effective substitutes. Enzymes that can incorporate fluoro compounds into their active centres are unable to deal with the analogues containing the bulkier halogen atoms.

Another feature that makes fluoro derivatives useful 'markers' is the remarkable stability of the C-F bond. There are several instances known of biological dechlorination by bacteria (Evans & Smith, 1954; Hirsch & Alexander, 1960; Kester, 1961; Alexander & Aleem, 1961; Davies & Evans, 1962; McRae & Alexander, 1965), by fungi (Munnecke & Solberg, 1958) and by insects such as the housefly (Lipke & Kearns, 1960) and locusts (Gessner & Smith, 1960), all of which involve fission of the C-Cl bond in the substrate. In contrast, direct evidence for biological defluorination is very sparse. Ali *et al.* (1962) recorded the presence of F⁻ ions in cultures of *Moraxella calcoacetica* (*Vibrio* O1) containing benzoate and *p*-fluorobenzoate, and the phenylalanine-hydroxylating system described by Kaufman (1961) defluorinated fluoro aromatic

compounds. Pseudomonads capable of utilizing fluoroacetate as sole carbon source have, however, been isolated by Kelly (1965) and Goldman (1965); in these instances the organically bound fluorine was released as F^- ions.

Many authors have suggested that fluoro aromatic and fluoro heterocyclic compounds might be degraded by micro-organisms to fluoroacetate (Behrman & Stanier, 1957; Hughes, 1965), which poisons the tricarboxylic acid cycle by conversion into fluorocitrate, the ultimate toxic agent (see review by Pattison, 1959). This would certainly account for the inability of the fluoro aromatic compound to support growth of the organism in most instances, and also implies that the C-F bond of the fluoro aromatic compound remains intact throughout its metabolism. No F^- ion was released from 2-fluoro-4-nitrobenzoate or fluoroacetate by *N. erythropolis*, and both these compounds brought about comparable inhibitions in growth rate of the organism on *p*-nitrobenzoate. In addition, both 2-fluoro-4-nitrobenzoate and fluoroacetate resulted in some citrate accumulation from *p*-nitrobenzoate, the classic symptom of fluoroacetate poisoning. An even stronger indication that 2-fluoro-4-nitrobenzoate may be converted into fluoroacetate, and thus maintain the integrity of the C-F bond, was the significant citrate accumulation in incubations where 2-fluoro-4-nitrobenzoate was the sole carbon source (Table 3).

In addition to inhibitory effects on growth resulting from its probable oxidation to fluoroacetate, 2-fluoro-4-nitrobenzoate and the other halogen analogues inhibited the induction of enzymes responsible for *p*-nitrobenzoate oxidation. The competitive nature of this inhibition makes it likely that the inhibitory effects here are due to the analogue itself competing at the enzyme site and not one of its (aliphatic) metabolic products. Unadapted cells oxidized neither *p*-nitrobenzoate nor its halogen analogues, whereas the *p*-nitrobenzoate-oxidation system could be induced either by the halogenonitrobenzoates or by *p*-nitrobenzoate itself; so it is reasonable to assume that all these compounds are oxidized by the same enzyme. Similar results were found with the benzoate system for *Moraxella* (*Vibrio* O1) by Calley & Jones (1965). Hughes (1965), however, was able to elicit an inductive response for benzoate in *Pseudomonas fluorescens* KB1 only with benzoate itself, and yet another situation was found by Bernheim (1953) in *Mycobacterium tuberculosis* where *o*- or *p*-fluorobenzoate acted as inducer of the benzoate system though neither was itself oxidized by the enzymes.

Adaptation to *p*-nitrobenzoate in *N. erythropolis* was effected by contact with this substrate for about 60 min., but exposure to 2-fluoro- or 2-chloro-4-nitrobenzoate did not induce the *p*-nitro-

benzoate oxidation system for nearly 4 hr. Modern ideas of enzyme induction involve the inducer as antagonizing an enzyme suppressor, thus permitting expression of the enzymic activity. It is reasonable to suppose that chemically distinct, though structurally similar, molecules will show differences in their suppressor-antagonizing abilities and that analogues might be less efficient in this respect than the parent compound.

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