STUDIES ON THE METABOLISM OF MODEL COMPOUNDS RELATED TO SOIL HUMIC ACID

I. THE DECOMPOSITION OF *N*-(*o*-CARBOXYPHENYL)GLYCINE

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Summary

An *Achromobacter* sp. which has been isolated from Urrbrae loam adaptively oxidizes *N*-(*o*-carboxyphenyl)glycine, selected as a model compound since it contains an aromatic ring bonded directly to the nitrogen atom of an α-amino acid, as proposed for humic acid polymers. Simultaneous adaptation studies indicate that this compound is metabolized via anthranilate, 5-hydroxyanthranilate, and 2,5-dihydroxybenzoate. The rate of oxidation of *N*-(*o*-carboxyphenyl)glycine is inhibited by *N*-methylanthranilate and salicylate, the latter by inhibiting the conversion of anthranilate to 5-hydroxyanthranilate.

I. INTRODUCTION

Humic acid is generally accepted to be a complex material formed in the soil essentially by the oxidative polymerization of a variety of phenols derived directly or indirectly from plant, animal, and microbial sources (Davies, Coulson, and Lewis 1960; Flaig 1960*a*, 1960*b*; Scheffer and Ulrich 1960; Kononova 1961; Steelink and Green 1962; Swaby and Ladd 1962). Although humic acid preparations from different sources may be composed of different phenolic compounds bonded in random sequences within the polymer, it is probable that an underlying unity of structure exists within the humic acid complex.

In addition to phenolic compounds, humic acids contain varying amounts of nitrogenous material due mostly to the presence of α-amino acids (Bremner 1955; Carles and Decau 1960). Failure to detect peptide bonds in humic acid preparations has led to the suggestion that the amino acids are not present as proteins (Swaby and Ladd 1962); but are incorporated into humic acid during the oxidative polymerization of phenols by reactions analogous to those demonstrated for amines and ammonia (Mason 1955), i.e. reactions in which the nitrogen atom of the amino acid becomes directly bonded to the aromatic ring.

Humic acid is potentially a valuable source of nitrogen in the soil, but is highly resistant to biological decomposition. Whether or not this resistance is related to the cleavage of the *N*-phenyl amino acid linkage has been investigated using a model compound, *N*-(*o*-carboxyphenyl)glycine (OCPG).

To the author's knowledge studies on the oxidative metabolism of *N*-phenyl amino acids have not been previously reported, so no information is available whether preliminary attack involves the aromatic ring or the aliphatic side chain. However,

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the oxidation of anthranilic acid, a substance related to OCPG, has been studied using both animal and bacterial systems. At least three metabolic pathways have been indicated involving respectively 5-hydroxyanthranilic acid (Bray *et al.* 1948; Shirai and Uno 1951; Kotake, Shibata, and Toratani 1956; Hagihara and Okazaki 1956), catechol (Stanier and Hayaishi 1951), and salicylic acid (Ke, Gee, and Durham 1959). Hosokawa, Nakagawa, and Takeda (1961) and Ichihara *et al.* (1962) have shown that the oxidation of anthranilic acid to catechol involves an equivalent release of ammonia.

II. Materials and Methods

Isolates from Adelaide red-brown earth, previously enriched with OCPG, were made by plating soil dilutions on the chemically defined medium described below, containing OCPG as the sole source of available carbon. Utilization of OCPG was conveniently followed by observing the disappearance of a violet fluorescence due to OCPG when illuminated with ultraviolet light. Of a number of isolates, the predominant organism was identified as an *Achromobacter* sp. on the basis of the following tests (Skerman 1959): The organism was a short, straight, Gram-negative rod growing in nutrient broth as a uniformly turbid suspension with a fine viscid sediment. Cells from this medium were motile with peritrichous flagellae. Colonies on nutrient agar were viscid, creamy, opaque, smooth, glistening, convex, circular, and with an entire edge. On a nutrient agar stab, the organism gave abundant, viscid, creamy, filiform growth. The organism grew well at 25°C but not at all at 4°C or 37°C. It did not hydrolyse starch, liquefy gelatin, produce H_2S, utilize phenol or alginate, form indole from casein broth, ferment arginine, or produce acid from maltose, sucrose, lactose, glucose, galactose, xylose, mannitol, sorbitol, dulcitol, glycerol, or salicin. The organism was catalase-positive and grew in nitrate broth with vigorous gas evolution, nitrite being detected in younger (15 hr) cultures.

Stock cultures of the organism were maintained on agar slopes containing 0·05% OCPG, 0·42% K_2HPO_4, 0·36% KH_2PO_4, 0·2% NaCl, 0·2% NH_4Cl, and trace amounts of Fe^{2+}, Zn^{2+}, Cu^{2+}, Mn^{2+}, and MoO_4^{2-} ions in distilled water, pH 7·2.

It will be shown that the *Achromobacter* sp. oxidized OCPG and related aromatic compounds adaptively. Suspensions of adapted cells for respirometer studies were harvested after shaking for 3 days at 25°C in liquid media of the same mineral salts composition as above, supplemented with 0·1% yeast extract and 0·05% of the substrate under investigation. Yeast extract served as the sole carbon source for growth of unadapted cells.

Adapted cells, grown in the presence of increasing amounts of yeast extract, showed a decreasing specific activity and, in general, an increasing total activity towards the substrate to which they were adapted; e.g. cells grown in a medium containing 0·01% OCPG and 0·1% yeast extract oxidized OCPG at the rate of 34·0 μl oxygen/hr/mg dry weight cell suspension with a yield of cells of 170 mg dry weight per litre of medium. Cells grown in a medium containing 0·01% OCPG and 1·0% yeast extract oxidized OCPG at the rate of 9·2 μl oxygen/hr/mg dry weight cell suspension with a yield of cells of 900 mg dry weight per litre of medium.

The harvested cells were washed once with distilled water and suspended to a final concentration of approximately 12 mg dry weight per millilitre. Suspensions adapted to oxidize OCPG maintained their original activities after storage for at least 3 weeks at 4°C.

All respirometer measurements were performed with the Warburg apparatus at 30°C with air as the gas phase. Unless otherwise stated, compounds tested for

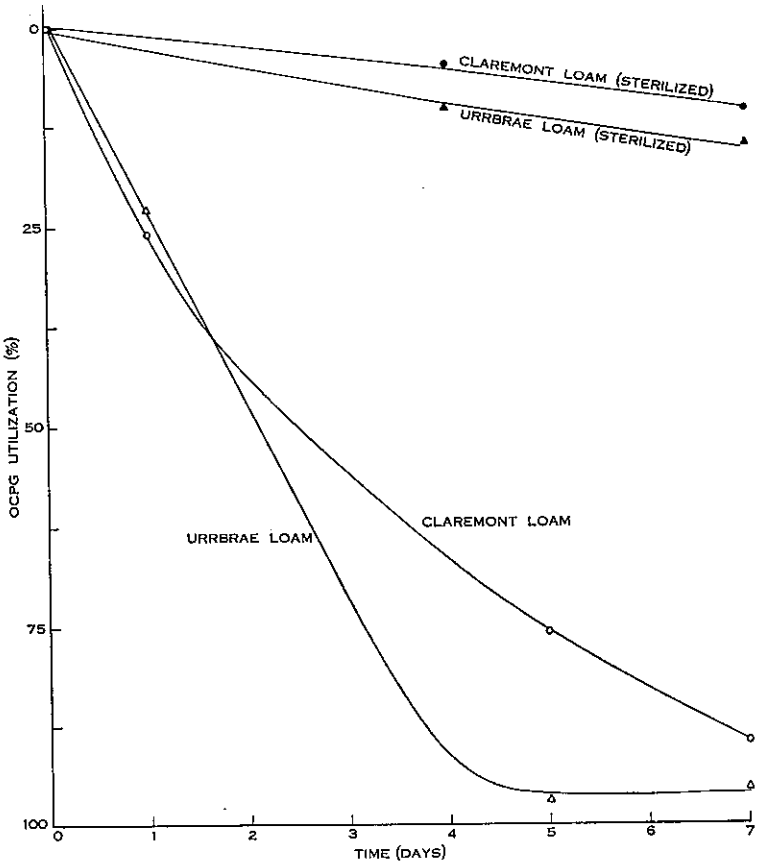


Fig. 1.—Decomposition of *N*-(*o*-carboxyphenyl)glycine (OCPG) by soil suspensions. 40 g soil incubated, with shaking, at 25°C with 50 ml 0·1% OCPG, pH 7·0.

their inhibitory or stimulatory effects on cell respiration were not pre-incubated with the bacterial suspension, but were added together with the substrate.

Estimations of the utilization of each aromatic substrate were made on aliquots of the deproteinized (1N H_2SO_4, final concentration) reaction mixture by absorbancy measurements in the ultraviolet region with a Shimadzu QR-50 spectrophotometer and cells of 1 cm light path or, where applicable, by fluorescence measurements with a Turner model 110 fluorimeter.

OCPG, *N*-acetylanthranilate, and *N*-formylanthranilate were prepared by reacting anthranilate with chloroacetate, acetic anhydride, and acetic–formic anhydride (Dalgliesh 1952), respectively. *N*-Phenylglycine was prepared from chloroacetate and aniline hydrochloride (de Mouilpied 1905).

Samples of 5-hydroxyanthranilate were donated by Professor E. Boyland, Royal Cancer Hospital, London, and by Dr. Y. Shibata, Wakayama Medical College, Japan. All other substrates and reagents were obtained commercially.

III. Results

Suspensions of two Adelaide soils, Urrbrae and Claremont loams, sampled at 3–6 in., readily decomposed added OCPG when incubated with shaking at 25°C at pH 7·0 (Fig. 1). The utilization of OCPG was essentially complete within 5–7 days.

Table 1

Oxidation of *N*-(*o*-carboxyphenyl)glycine (OCPG) and related compounds by adapted and unadapted suspensions of Achromobacter sp.

Cell suspensions (≡ 3·2 mg dry wt.) incubated at 30°C in 2·0 ml 0·05M potassium phosphate, pH 6·0, containing 2·0 μmoles of substrate. Substrate oxidation rates corrected for endogenous cell respiration

Substrate	Rates of Oxidation (μl O_2/30 min/mg dry wt. bacteria)			
	OCPG-adapted Cells	Anthranilate-adapted Cells	Salicylate-adapted Cells	Unadapted Cells
OCPG	18·6	1·0	2·8	0·3
N-Methylanthranilate	1·1	1·5	—	1·3
N-Formylanthranilate	28·8	30·7	1·3	1·3
Anthranilate	26·8	28·3	1·6	1·3
Catechol	1·8	1·5	1·0	0·3
o-Aminophenol	2·7	—	—	3·8
Salicylate	2·3	1·5	19·3	1·0
5-Hydroxyanthranilate	10·7	18·5	1·0	1·0
3-Hydroxyanthranilate	2·2	1·7	1·0	0·3
2,5-Dihydroxybenzoate	12·7	18·5	43·6	2·2
2,3-Dihydroxybenzoate	2·3	2·8	11·3	1·6
Endogenous	0·7	1·7	1·6	1·3

The rates of utilization of OCPG were very similar whether determined by absorbancy (330 mμ) or fluorescence (primary filter 360 mμ, secondary filter 415 mμ) measurements.

(*a*) *Adaptation Studies*

Suspensions of the soil isolate, *Achromobacter* sp., grown in the absence of OCPG, did not appreciably oxidize this compound within an experimental period of 4–6 hr. Adapted cells, however, readily oxidized OCPG, the total oxygen uptake varying with different batches between 50–70% of the theoretical for complete oxidation to carbon dioxide when a ten-fold range of substrate concentrations

(10^{-3}–10^{-2}M, final) was used. Ammonia was also released in yields of 30–70% of the theoretical based upon the nitrogen content of the substrate. Measurements of residual OCPG in the acidified reaction mixture by absorbancy measurements at 350 mμ showed that all had been utilized.

Several possible intermediates of OCPG oxidation were tested for their ability to be metabolized by cell suspensions, either unadapted or adapted to oxidize OCPG, anthranilate, or salicylate. The specific activities of the suspensions grown in various

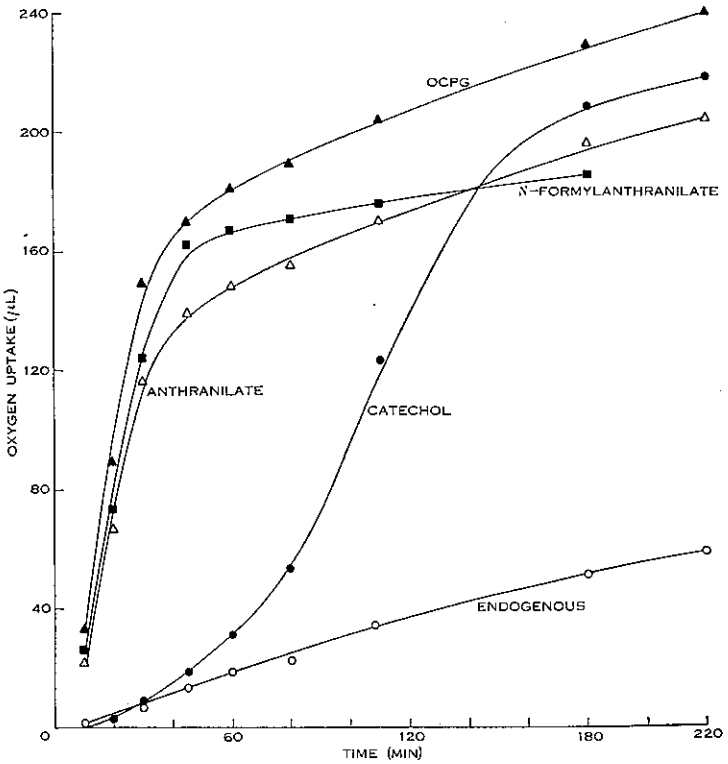


Fig. 2.—Oxidation of OCPG, anthranilate, *N*-formylanthranilate, and catechol by cells adapted to OCPG. Bacterial suspension (≡ 3·8 mg dry wt.) incubated with 2 μmoles substrate in 2·0 ml 0·05M potassium phosphate, pH 6·0, at 30°C.

ways are shown in Table 1. Cells grown in the presence of OCPG oxidized immediately OCPG, *N*-formylanthranilate, anthranilate, 5-hydroxyanthranilate, and 2,5-dihydroxybenzoate. A similar pattern of oxidation of the various substrates was obtained with cells grown in anthranilate, except for their inability to oxidize OCPG itself. Cells adapted to salicylate were simultaneously adapted to oxidize 2,5-dihydroxybenzoate only. None of the substrates was oxidized immediately by the unadapted suspensions.

A more detailed comparison of the metabolism of several of these substrates is shown in Figures 2–9.

(i) N-*Formylanthranilate.*—Cells adapted to OCPG or anthranilate converted *N*-formylanthranilate (and *N*-acetylanthranilate) to carbon dioxide and cell material. The rates of oxidation were similar to those of anthranilate itself (Figs. 2 and 3). By contrast, unadapted cells converted, with relatively low oxygen uptake (Fig. 4), the *N*-acylanthranilates to anthranilate, which accumulated in the reaction mixture. In each case the conversion was indicated by a gradually increasing violet fluorescence under ultraviolet light and was measured quantitatively by recording spectral

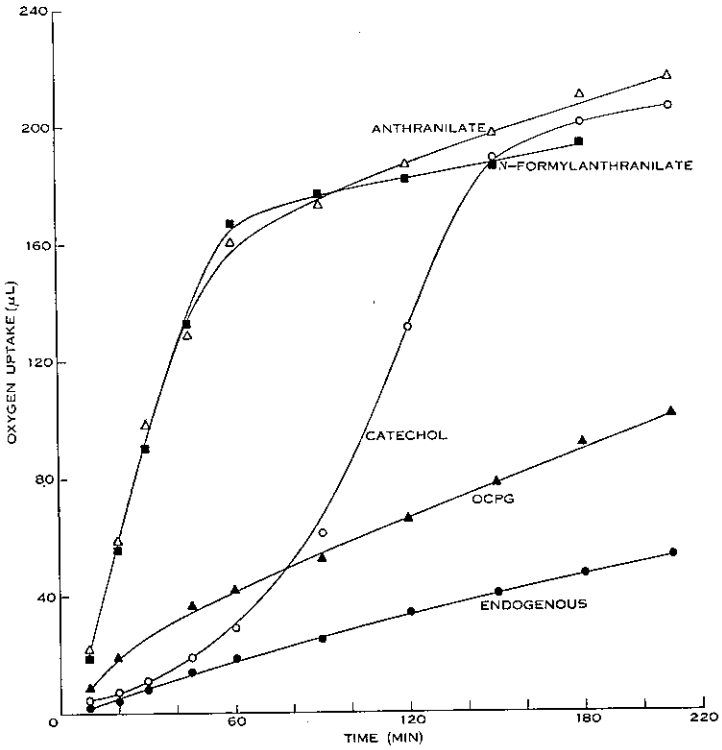


Fig. 3.—Oxidation of OCPG, anthranilate, *N*-formylanthranilate, and catechol by cells adapted to anthranilate. Bacterial suspension (≡ 3·1 mg dry wt.) incubated with 2 μmoles substrate in 2·0 ml 0·05M potassium phosphate, pH 6·0, at 30°C.

changes of aliquots of the acidified reaction mixture. This is shown for *N*-formylanthranilate in Figures 5 and 6. At pH 1, *N*-formylanthranilic acid exhibited peaks of maximal absorption at 248 and 300 mμ whereas anthranilic acid showed a minor absorption peak at 272 mμ and a band of low absorbancy at 310–330 mμ.

N-Formylanthranilate was slightly unstable in acid solution and, under the conditions used, approximately 4–5% of the original substrate decomposed non-enzymically. However, if a uniform procedure was maintained throughout, particularly in regard to the time period between acidification of the reaction mixture and final measurements in the spectrophotometer, the extent of non-enzymic hydrolysis

remained constant and contrasted clearly with the decomposition of *N*-formylanthranilate in the presence of the bacterial suspension. *N*-acetylanthranilate was relatively more stable at acid pH and such precautions were unnecessary to demonstrate its biological hydrolysis.

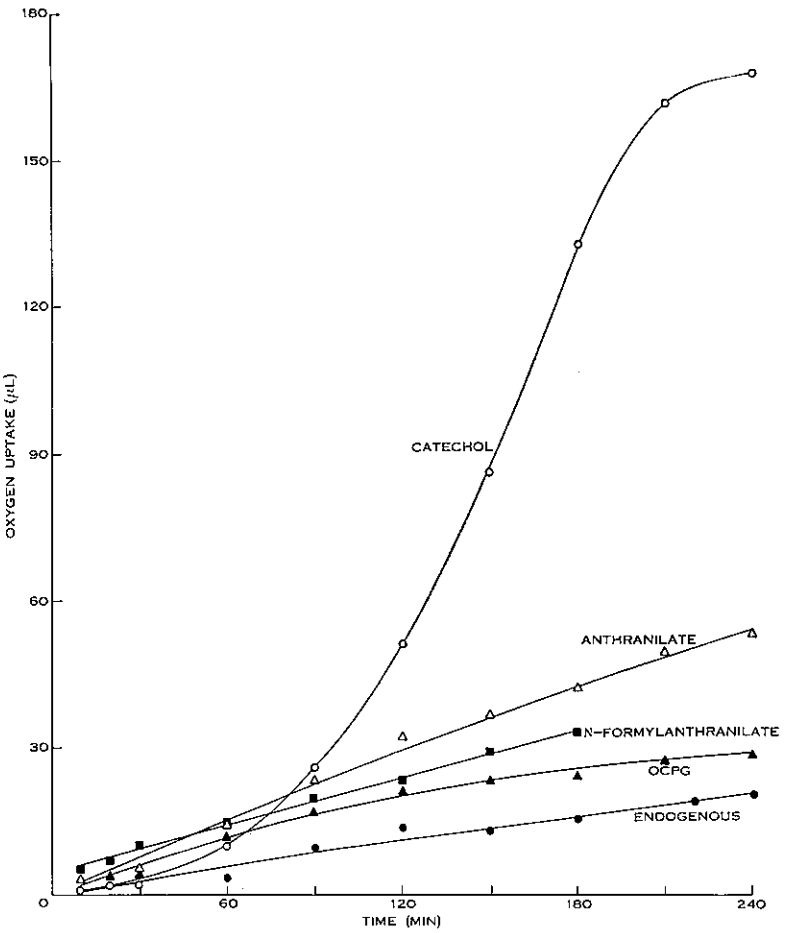


Fig. 4.—Oxidation of OCPG, anthranilate, *N*-formylanthranilate, and catechol by unadapted cells. Bacterial suspension (≡ 2·4 mg dry wt.) incubated with 2 μmoles substrate in 2·0 ml 0·05M potassium phosphate, pH 6·0, at 30°C.

(ii) *Anthranilate.*—Figure 2 shows that cells adapted to OCPG oxidized OCPG and anthranilate at similar rates and to the same extent. Oxygen consumption for each substrate ceased after similar incubation periods. Unadapted cells oxidized neither substrate (Fig. 4).

(iii) *Catechol.*—Adaptation of the organism to oxidize either OCPG or anthranilate did not result in its simultaneous adaptation to oxidize catechol. Both adapted (Figs. 2 and 3) and unadapted (Fig. 4) cells oxidized catechol after a lag period

(90–120 min) when oxidation occurred at similar rates (12–15 μl oxygen consumed/30 min/mg dry weight of cells) and to the same extent.

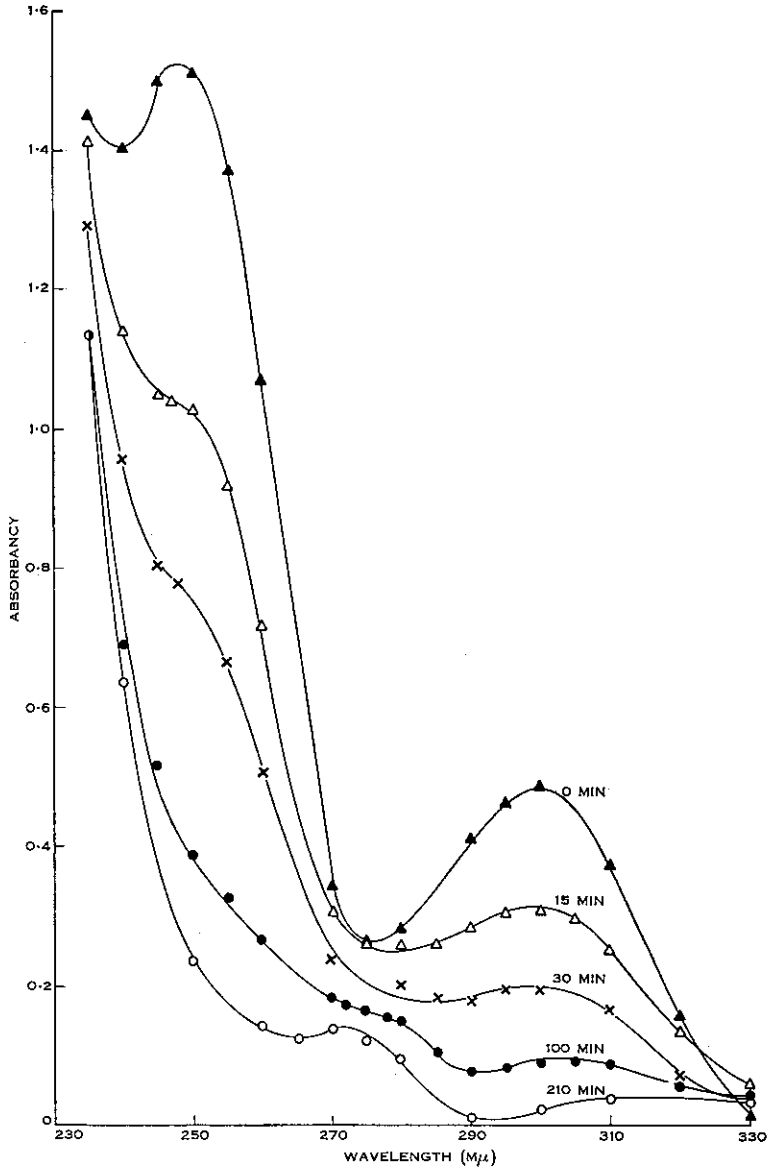


Fig. 5.—Conversion of *N*-formylanthranilate to anthranilate by unadapted cells. Bacterial suspension (≡ 5·7 mg dry wt.) incubated with 3 μmoles *N*-formylanthranilate in 2·0 ml 0·1M potassium phosphate, pH 6·0, at 30°C. At varying times, aliquots of the reaction mixture were acidified with H_2SO_4 (pH 1·0), centrifuged, and absorption spectra of the supernatants recorded.

The effect of chloramphenicol on the oxidation of OCPG, anthranilate, and catechol by cells adapted to OCPG is shown in Figure 7. The oxidation rates of OCPG

and anthranilate were unaffected by concentrations of chloramphenicol which completely inhibited the adaptation of the cells to oxidize catechol.

(iv) *5-Hydroxyanthranilate.*—Since catechol did not appear to be an intermediate in the oxidation of OCPG or anthranilate, the possibility that the further metabolism of anthranilate might involve the introduction of a hydroxyl group before elimination of the amino group was investigated. Since the nature of the substituents in the benzene ring determines the position in which further substitution takes place and since the amino group is an ortho- and para-directing group, whereas the carboxyl group is a meta-directing group, the two isomers most likely to be

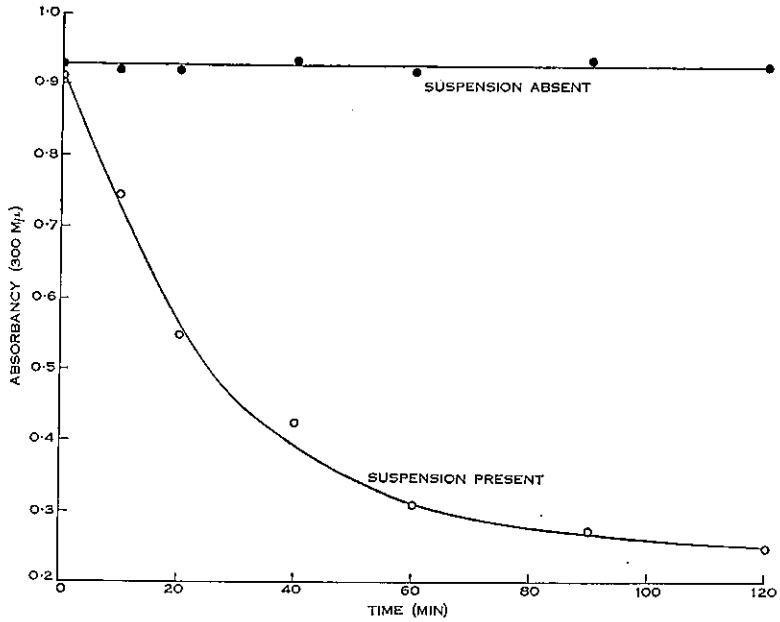


Fig. 6.—Decomposition of *N*-formylanthranilate by unadapted cells. Bacterial suspension (≡ 9·5 mg dry wt.) incubated with 9 μmoles *N*-formylanthranilate in 5·0 ml 0·04M potassium phosphate, pH 6·0, at 30°C. At varying times, aliquots of the reaction mixture were acidified with H_2SO_4 (pH 1·0), centrifuged, and absorbancy (300 mμ) measurements made on the supernatants.

involved are 3- and 5-hydroxyanthranilic acids. Figure 8 shows that cells adapted to OCPG and anthranilate were simultaneously adapted to oxidize 5-hydroxyanthranilate. 3-Hydroxyanthranilate was oxidized relatively slowly and, in general, maximal oxidation was observed only after a lag period.

(v) *2,5-Dihydroxybenzoate.*—Similar metabolic patterns were obtained when the corresponding isomers of dihydroxybenzoate were tested. Whereas cells adapted to OCPG and anthranilate became adapted to oxidize 2,5-dihydroxybenzoate, the 2,3-isomer was oxidized slowly after a lag period (Fig. 9). None of the four remaining isomers was oxidized. Similar results were obtained with cells adapted to salicylate.

(*b*) *Inhibition Studies*

The possible inhibitory effects of structurally similar compounds on the rates of oxidation of OCPG and its anticipated breakdown products was investigated using cells adapted to OCPG. Salicylate and *N*-methylanthranilate inhibited oxidation both of OCPG and anthranilate, but had little or no effect on the oxidation of 5-hydroxyanthranilate and 2,5-dihydroxybenzoate (Table 2). The degree of inhibition varied with different batches of cells under otherwise identical conditions.

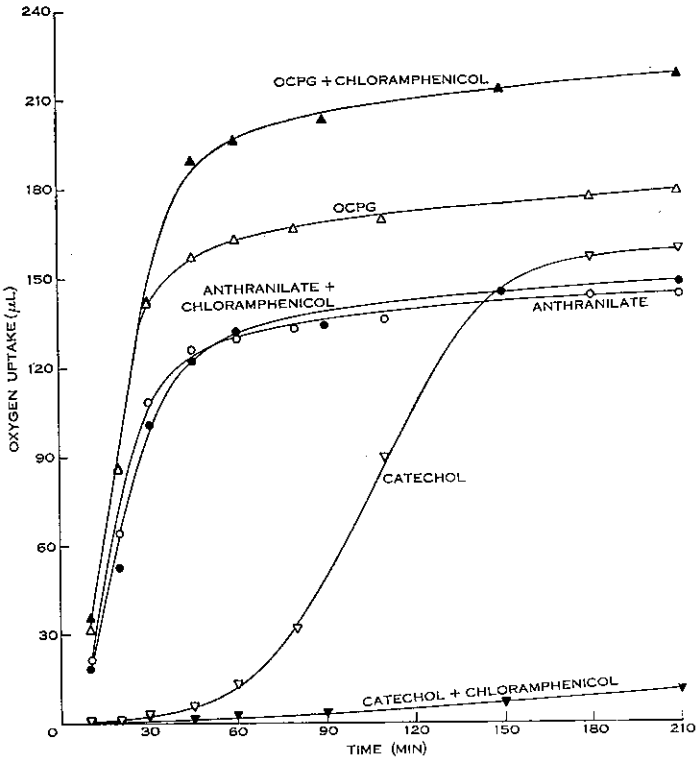


Fig. 7.—Effect of chloramphenicol on oxidation of OCPG, anthranilate, and catechol by cells adapted to OCPG. Bacterial suspension (≡ 3·0 mg dry wt.) incubated with 2 μmoles substrate and, where indicated, 0·1 mg chloramphenicol in 2·0 ml 0·05M potassium phosphate, pH 6·0, at 30°C.

However, in comparable experiments it was consistently demonstrated that the oxidation of OCPG was more sensitive to inhibition by salicylate than was that of anthranilate. *N*-methylanthranilate inhibited the oxidation of both substrates to a similar extent (Table 2).

Preincubation of the cell suspension for varying periods with salicylate did not affect the extent of inhibition when OCPG was added subsequently. Similarly, salicylate, added at various times to cell suspensions actively oxidizing OCPG, inhibited the reaction to the same extent.

Other compounds tested for their possible inhibition of OCPG oxidation included *N*-phenylglycine, *p*-aminobenzoate, catechol, hydroquinone, *m*- and *p*-hydroxybenzoate, the remaining isomers of gentisic acid, 4- and 5-aminosalicylate, *o*-aminophenol, phthalate, and *o*-nitrobenzoate. None of these inhibited oxidation of OCPG when present in equimolar concentrations; nor were they themselves oxidized immediately by cells adapted to OCPG.

Although salicylate markedly inhibited the rate of oxidation of OCPG, the final total oxygen uptake was greater than that due to oxidation of OCPG alone. Spectrophotometric and fluorimetric measurements on aliquots of the acidified

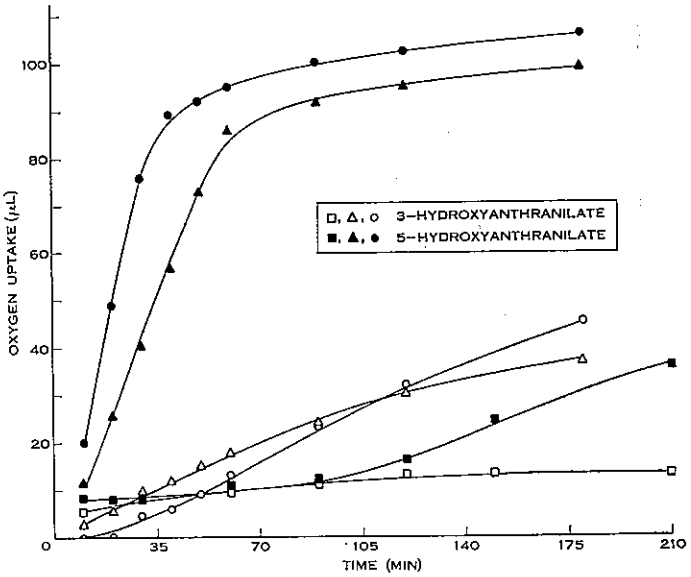


Fig. 8.—Oxidation of 3- and 5-hydroxyanthranilate by unadapted cells and by cells adapted to oxidize OCPG and anthranilate. 2 μmoles of each compound respectively incubated in 2·0 ml 0·05M potassium phosphate, pH 6·0, at 30°C with unadapted cells (≡ 3·2 mg dry wt.) and with cells adapted to oxidize OCPG (≡ 4·3 mg dry wt.) and anthranilate (≡ 4·0 mg dry wt.).

reaction mixture showed that, at the completion of gas uptake, both OCPG and salicylate had disappeared. In the same reaction period the oxygen uptake in vessels where cell suspensions had been incubated with salicylate alone were no greater than that of the endogenous cell suspension and measurements of residual salicylate showed that essentially none had been utilized.

The oxygen uptake in excess of that due to the oxidation of OCPG alone was proportional to the amount of salicylate added initially. Further, the effect was obtained whether the salicylate was added with the substrate to the cell suspension or added immediately after the gas uptake due to oxidation of OCPG had ceased (Fig. 10).

The initiation of salicylate oxidation by cells adapted to OCPG was also observed when OCPG was replaced by other substrates, e.g. 2,5-dihydroxybenzoate, pyruvate,

whose rates of oxidation were unaffected by salicylate (Table 3). Similarly, cells adapted to OCPG could be replaced by cells adapted to anthranilate. In this case salicylate was oxidized when present with anthranilate but not when alone or present with OCPG. OCPG was not oxidized by cells adapted to anthranilate.

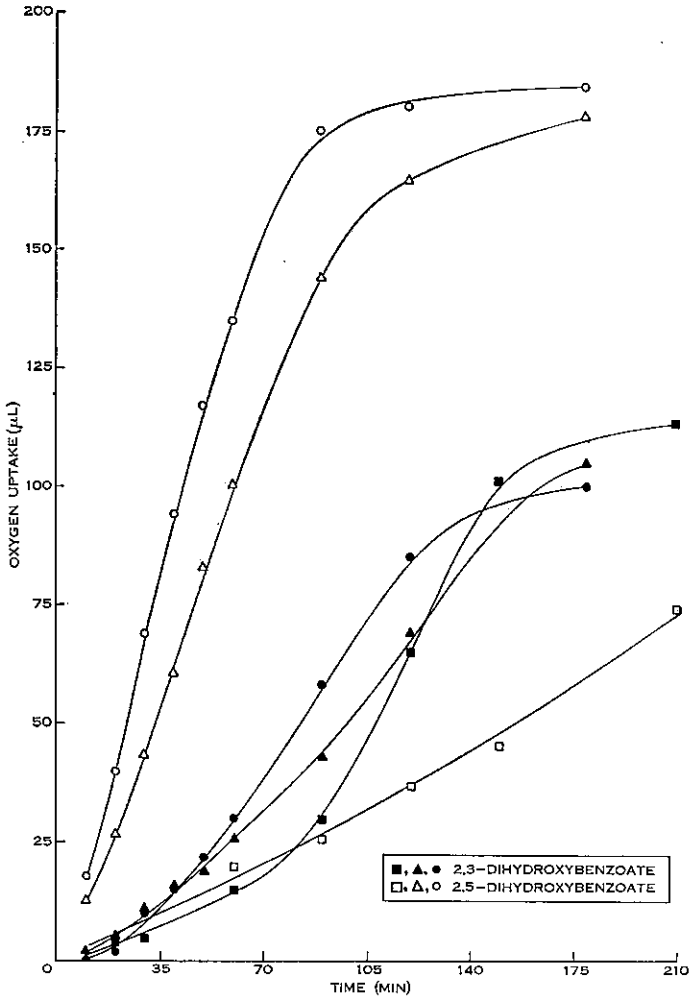
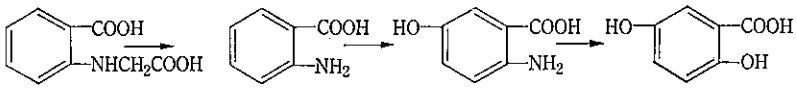


Fig. 9.—Oxidation of 2,3- and 2,5-dihydroxybenzoate by unadapted cells and by cells adapted to oxidize OCPG and anthranilate. 2 μmoles of each compound respectively incubated in 2·0 ml 0·05M potassium phosphate, pH 6·0, at 30°C with unadapted cells (≡ 3·2 mg dry wt.) and with cells adapted to oxidize OCPG (≡ 4·3 mg dry wt.) and anthranilate (≡ 4·0 mg dry wt.).

The oxidation of salicylate in the presence of OCPG by cells adapted to OCPG was abolished by chloramphenicol in concentrations which did not inhibit OCPG oxidation (Table 3).

IV. Discussion

The results of simultaneous adaptation experiments indicate that an *Achromobacter* sp. oxidizes OCPG via anthranilate, 5-hydroxyanthranilate, and 2,5-dihydroxybenzoate:



The mechanism for the initial conversion to anthranilate is unknown. A pathway involving an initial decarboxylation of OCPG to *N*-methylanthranilate is eliminated on the basis of the following evidence:

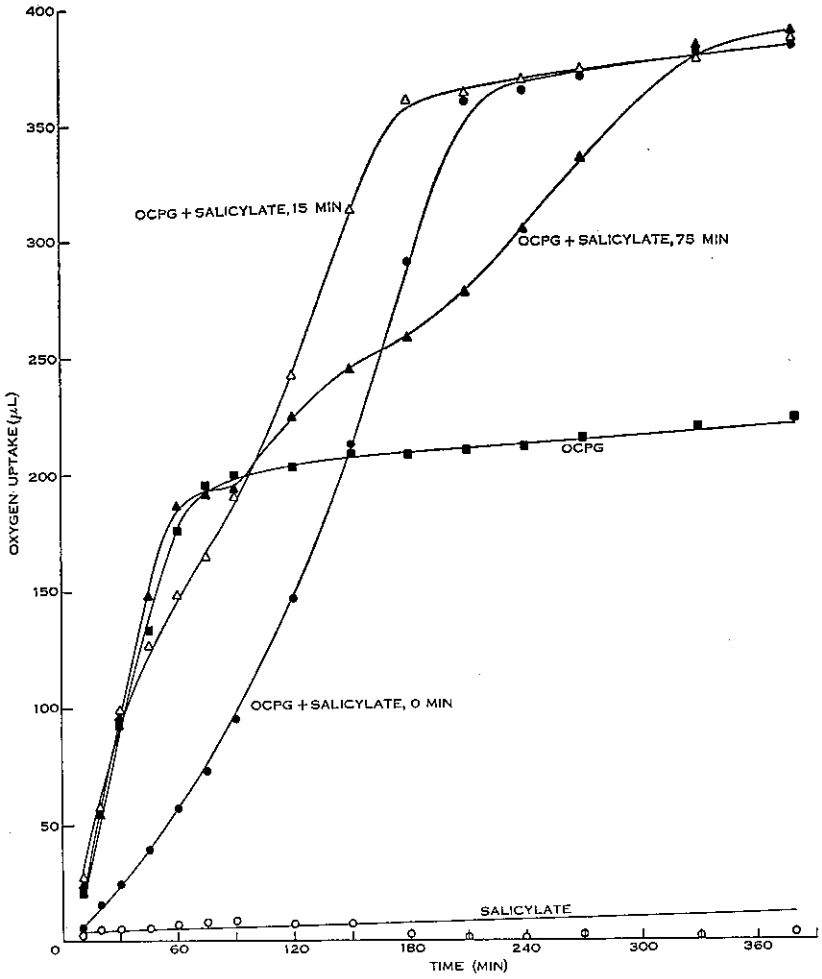


Fig. 10.—Oxidation of salicylate in the presence of OCPG by cells adapted to oxidize OCPG. Bacterial suspension (≡ 3·5 mg dry wt.) incubated with 2·0 μmoles OCPG in 2·0 ml 0·05M potassium phosphate, pH 6·0, at 30°C. Salicylate (1·0 μmole) added to the incubation mixture at varying times as indicated.

(1) *N*-Methylanthranilate is not oxidized by cells adapted to OCPG or anthranilate.

(2) Suspensions of *Achromobacter* sp. do not adapt to *N*-methylanthranilate when grown in its presence.

(3) *N*-Methylanthranilate inhibits the oxidation of both OCPG and anthranilate.

(4) Cells adapted to OCPG and which aerobically readily oxidize OCPG do not decarboxylate OCPG anaerobically.

TABLE 2

INHIBITION OF OXIDATION OF OCPG AND RELATED COMPOUNDS BY SALICYLATE AND *N*-METHYLANTHRANILATE

Cell suspensions (≡ 3·5 mg dry wt.) incubated at 30°C in 2·0 ml 0·05M potassium phosphate, pH 6·0. Percentage inhibition calculated from corrected linear oxidation rates over 30 min

Substrate	Substrate Concn. (M)	Inhibitor Concentration (M)		Inhibition (%)
		Salicylate	*N*-Methylanthranilate	
OCPG	0·001	0·001		70
Anthranilate				56
5-Hydroxyanthranilate				0
2,5-Dihydroxybenzoate				6
OCPG	0·0008		0·0008	33
Anthranilate				20
5-Hydroxyanthranilate				4
2,5-Dihydroxybenzoate				0
OCPG	0·001	0·001		58
		0·0025		72
		0·0075		74
		0·015		78
Anthranilate	0·001	0·001		0
		0·0025		15
		0·0075		48
		0·015		65
OCPG	0·0008		0·0008	31
			0·004	55
			0·02	75
Anthranilate	0·0008		0·0008	19
			0·004	64
			0·02	83

However, it is possible that OCPG is oxidatively decarboxylated to *N*-hydroxymethylanthranilate which is further oxidized to *N*-formylanthranilate. On hydrolysis, formate and anthranilate would be formed. *N*-Hydroxymethylanthranilate is unstable and exists as its lactone; its ability to be oxidized by OCPG-adapted cells has not been tested. *N*-Formylanthranilate is readily oxidized by cells adapted to

OCPG and anthranilate. However, the initial hydrolysis is not an adaptive reaction since unadapted cells readily break down *N*-formylanthranilate to anthranilate which accumulates in the reaction mixture. Thus although *N*-formylanthranilate may still be an intermediate of OCPG oxidation, evidence based on simultaneous adaptation experiments is inadmissible in this case.

TABLE 3

OXIDATION OF SALICYLATE IN THE PRESENCE OF OCPG AND OTHER COMPOUNDS

Cell suspension (≡ 3·5 mg dry wt.) incubated at 30°C in 2·0 ml 0·05M potassium phosphate, pH 6·0, containing where indicated OCPG, anthranilate, 2,5-dihydroxybenzoate, salicylate (each 2·0 μmoles), pyruvate (6·0 μmoles), or chloramphenicol (0·5 mg)

Suspension	Substrate	Rate of Oxygen Uptake (μl/30 min)	Oxygen Uptake (μl)		Residual Salicylate† (%)
			Total	Net*	
OCPG-adapted cells	OCPG	75	184	—	—
	OCPG + salicylate	20	378	194	0
	Pyruvate	28	111	—	—
	Pyruvate + salicylate	29	312	201	0
	2,5-Dihydroxybenzoate	33	179	—	—
	2,5-Dihydroxybenzoate + salicylate	36	363	184	0
	Salicylate	0	−1	−1	90
OCPG-adapted cells	Anthranilate	76	177	—	—
	Anthranilate + salicylate	50	477	300	4
	Salicylate	1	6	6	100
Anthranilate-adapted cells	OCPG	1	7	—	—
	OCPG + salicylate	0	0	−7	96
	Anthranilate	52	160	—	—
	Anthranilate + salicylate	85	471	311	5
	Salicylate	0	−10	−10	90
OCPG-adapted cells	OCPG	61	192	—	—
	OCPG + salicylate	20	450	258	25
	OCPG + chloramphenicol	75	254	—	—
	OCPG + salicylate + chloramphenicol	22	261	7	84
	Salicylate	0	−5	−5	96
	Salicylate + chloramphenicol	0	−10	−10	84

* Oxygen uptake due to salicylate after correction for oxygen uptake due to co-substrate.

† Expressed as a percentage of the initially added salicylate.

As an alternative pathway, OCPG may be oxidatively deaminated to anthranilate and glyoxylate. Glyoxylate is not present in extracts of cells actively oxidizing OCPG and is itself oxidized immediately by both adapted and unadapted suspensions. Measurements of either oxygen uptake or glyoxylate concentration show that the specific activity of cells adapted to OCPG towards glyoxylate is at least 4–5 times greater than that of unadapted cells.

Stanier and Hayaishi (1951) have shown that a *Pseudomonas* sp. oxidizes anthranilate via catechol and that salicylate has no role in this conversion. By contrast Ke, Gee, and Durham (1959), using a *Flavobacterium* sp., have implicated salicylate (but not catechol) as an intermediate. Neither of these compounds is an intermediate of anthranilate oxidation by the *Achromobacter* sp. presently studied, although this organism can be adapted to metabolize both catechol and salicylate under the appropriate conditions. The *Achromobacter* sp. appears to oxidize anthranilate via 5-hydroxyanthranilate, a pathway which may also operate in animal tissues (Bray *et al.* 1948; Shirai and Uno 1951; Hagihara and Okazaki 1956; Kotake, Shibata, and Toratani 1956) and illustrates further the diversity of mechanisms by which different bacteria may metabolize one substrate.

With the exception of 3-hydroxyanthranilate (which is metabolized slowly after a lag by cells adapted to OCPG) and of 5-hydroxyanthranilate, none of the remaining isomers has been available for testing. However, all isomers formed by their deamination, viz. isomers of dihydroxybenzoate, have been tested and of these, only one, 2,5-dihydroxybenzoate, corresponding to 5-hydroxyanthranilate, is oxidized immediately by adapted cells. The further decomposition of 2,5-dihydroxybenzoate to maleylpyruvate by cell-free extracts of *Achromobacter* sp. has been reported elsewhere (Ladd 1962).

Salicylate is a powerful competitive inhibitor of the oxidation of OCPG and anthranilate by *Achromobacter* sp. but has no effect under the same conditions on the oxidation of 5-hydroxyanthranilate or 2,5-dihydroxybenzoate. Since adaptation studies have shown that salicylate-adapted cells oxidize salicylate via 2,5-dihydroxybenzoate, it seems probable that the inhibitory effect of salicylate is due to competition with anthranilate for the active site on the surface of the enzyme which catalyses the hydroxylation of the aromatic ring at position 5.

Washed cell suspensions of *Achromobacter* sp., previously grown in the absence of salicylate, are unable to oxidize salicylate when added alone, within an experimental period of 6–8 hr. However, when salicylate is added together with a second substrate, which alone is readily oxidized by the cell suspensions, oxidation of both substrates takes place. The second substrate may be aliphatic or aromatic and may be a compound (e.g. OCPG) whose rate of oxidation is actually depressed by the presence of salicylate. It is of interest that just as the oxidation of salicylate is initiated by, for example, the oxidation of anthranilate by cells adapted to anthranilate, so too is the oxidation of anthranilate initiated by the oxidation of salicylate by cells adapted to salicylate. Further, salicylate may be replaced by other substrates to which the cells are potentially able to adapt, e.g. 2,3-dihydroxybenzoate but not *N*-methylanthranilate. The obvious explanation for the effect is that the oxidation of one substrate causes a decrease in the time required for the cells to adapt to the second substrate and since it is not confined to any specific pair of substrates, the effect is essentially a quantitative one, i.e. the oxidation of one substrate provides energy or carbon products or both necessary for enzyme synthesis at a more rapid rate than the oxidation of endogenous material. This explanation is supported by the experiment with chloramphenicol (Table 3) which prevents the oxidation of the substrate to which adaptation is required without inhibiting the oxidation of the substrate to which the cells are already adapted.

The ease of decomposition of OCPG by soils and by the *Achromobacter* sp. isolated from them suggests that the resistance of humic acid to biological attack is not due essentially to an inability of organisms to attack compounds containing an aromatic ring bonded directly to the α-amino N atom of an amino acid, although preliminary changes to either moiety may be necessary before the *N*-phenyl bond is cleaved. This ease of decomposition of the model compound may be altered by the nature of the amino acid moiety or by the nature of other substituents in the aromatic ring. The *Achromobacter* sp. cannot be adapted to oxidize *N*-phenylglycine, *N*-(*p*-hydroxyphenyl)glycine, or *N*-(*p*-nitrophenyl)glycine. This is not surprising since bacteria tend to be highly specific in the range of aromatic substrates they can attack. The final solution of this problem must depend upon greater knowledge of the nature of the monomeric units in humic acid itself.

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