Metabolism of *o*-Cresol by *Pseudomonas aeruginosa* strain T1

By D. W. RIBBONS*

*Department of Biochemistry, University of Hull and Milstead
Laboratory of Chemical Enzymology, Shell Research Ltd.,
Sittingbourne, Kent*

(Received 13 December 1965)

SUMMARY

There is evidence that oxidation of *o*-cresol by *Pseudomonas aeruginosa* proceeds through 3-methylcatechol and 2-hydroxy-6-oxohepta-2,4-dienoic acid. 3-Methylcatechol has been characterized as a metabolite in growing cultures, and is oxidized by cells or cell extracts obtained from cultures grown on *o*-cresol but not by those grown on glucose. The rates of dissimilation of 2-hydroxy-6-oxohepta-2,4-dienoic acid are in excess of its rates of formation from 3-methylcatechol by extracts of cells. The catechol 2,3-oxygenase is formed in response to a variety of inducers and will cleave the ring of catechol and 4-methylcatechol also. The low specificity of the 2,3-oxygenase and earlier enzymic activities (hydroxylases), to both inducers and substrates, is discussed.

INTRODUCTION

Some bacteria metabolize methyl substituted benzenoid compounds by oxidation of this side chain, eventually, to a carboxyl group. This is then either lost as carbon dioxide and replaced by a hydroxyl group or retained until fission of the 'benzoic acid' formed occurs. The best documented example is the oxidation of *p*-cresol to *p*-hydroxybenzoate by the pseudomonad of Dagley & Patel (1957). They further showed that 2,4- and 3,4-xyleneol were utilized by oxidation of the methyl group *para* to the phenolic function; this occurred also with the pseudomonad of Leibnitz, Behrens, Streigler & Gabert (1962). The oxidation of toluene by *Pseudomonas aeruginosa* also proceeds by oxidation of the methyl group (Kitagawa, 1956). On the other hand, Rogoff & Wender (1957) showed that 1-methyl- and 2-methylnaphthalene were metabolized without attack on the side chain and that 3-methyl- and 4-methylcatechol respectively appeared as products suggesting that the unsubstituted ring was cleaved first. 3-Methylcatechol has also been implicated as a bacterial metabolite of *m*-toluate (Ichihara, Adachi, Hosokawa & Takeda, 1962) and shown to undergo fission to 2-methylmuconate by a purified preparation of pyrocatechase from *Brevibacterium fuscum* (Nakagawa, Inoue & Takeda, 1963). This enzyme preparation will also oxidize 4-methylcatechol, and the u.v. characteristics of the product suggest that this is 3-methylmuconate. An alternative cleavage of 4-methylcatechol was suggested from the spectral data obtained by Dagley, Evans

* Present address: Milstead Laboratory of Chemical Enzymology, Shell Research Limited, Sittingbourne, Kent.

& Ribbons (1960), and this has been amply confirmed by Dagley, Chapman, Gibson & Wood (1964) who found that the 2-hydroxy-5-methylmuconic semialdehyde was the product. When, however, 4-methylcatechol was oxidized by the pseudomonad of Dagley & Patel (1957), grown on *p*-cresol, protocatechate was formed.

It was suggested in a previous report that *o*-cresol was not metabolized via salicylate and catechol since *Pseudomonas aeruginosa* strain T1, grown on *o*-cresol or on several other aromatic compounds, produced a non-specific catecho 2,3-oxygenase which cleaved the ring of 3-methylcatechol to yield keto-acids (Ribbons 1964). One mole of O₂ was consumed for each mole of 3-methylcatechol metabolized and CO₂ was not evolved. The details of the site of ring cleavage of several catechols, including 3-methylcatechol, have recently been elucidated and the subsequent route of degradation of the products of cleavage to pyruvate and other substances ascertained (Dagley *et al.* 1964). These observations are consistent with, but do not unequivocally establish 3-methylcatechol as an intermediary metabolite of *o*-cresol metabolism. Results reported here suggests that the reaction sequence, shown in Scheme 1, is responsible for the oxidation of *o*-cresol by *P. aeruginosa* T1.

Scheme 1

METHODS

Organisms. The strain used was *Pseudomonas aeruginosa* T1 and was kindly provided by Miss E. Pankhurst (Pankhurst, 1959). It was maintained on slopes of Lab-Lemco agar and subcultured every 6 weeks. Stock cultures were maintained at 4°. *Pseudomonas desmolyticum* (NCIB 8859) was maintained similarly.

Media. Cultures were grown on minimal salt media made by mixing sterile solutions (a) and (b) [3:2 (v/v)]. Solution (a) adjusted to pH 7.0 with NaOH contained (g./l.): KH₂PO₄, 9.0; (NH₄)₂SO₄, 2.0; solution (b) contained the carbon source and a trace element mixture (2.5 ml./l.). The final concentration of the carbon source was 0.1% when phenol, glucose or benzoate (neutralized to pH 7.0 with NaOH) were used and 0.05% when the cresols were used. The trace element mixture contained (g./l.): MgO, 11.12; CaCO₃, 2.0; ZnO, 0.41; FeCl₃, 6H₂O, 5.4; MnCl₂, 4H₂O, 0.99; CuCl₂, 2H₂O, 0.17; CoCl₂, 6H₂O, 0.24; H₃BO₃, 0.062; and concentrated HCl (58 ml./l.). Cultures of the organism were routinely subcultured in the specific liquid media (12.5 ml. in 50 ml. conical flasks), containing one of the carbon sources, every 2–3 weeks. *Pseudomonas desmolyticum* was grown on media of the same compositions except that naphthalene was added as carbon source (0.5 g./l.).

Growth and harvest. Inocula for experimental purposes were taken from liquid cultures containing a specific carbon source. A 24-hr culture (12.5 ml.) was used to inoculate 250 ml. of similar media contained in a 1 l. flask. Cultures were harvested

after overnight growth (16–17 hr) by centrifugation at approximately 20,000*g* for 1–2 min. at room temperature in a Servall SS-1 bench centrifuge. The pellets were washed twice, resuspended in 0.067 M-phosphate buffer, pH 7.1, filtered through glass wool and diluted to give a bacteria density of approximately 1.5–3.0 mg. dry wt./ml. Larger quantities of pseudomonads were obtained from 15 l. cultures in 20 l. Pyrex bottles fitted with spargers (porosity 1) for forced aeration. These were harvested on an air-turbine-driven Sharples supercentrifuge. These organisms were normally washed with water and stored as a pellet at -14° until required for the preparation of extracts. All cultures were grown at 30° .

Dry weight of bacteria. The extinction values of bacterial suspensions were measured at 570 m μ with a Unicam SP 600 spectrophotometer. A standard curve relating bacterial dry weight (dried at 105° for 24 hr) to extinction was linear up to $E_{570}=0.6$ when the bacterial concentration was 325 μ g. dry wt./ml.

Cell-free extracts. Twice washed organisms were disrupted with an M.S.E. ultrasonic disintegrator (Model 60W) for 4 min. below 5° . Bacterial debris was removed by centrifugation at 20,000*g*, for 15–20 min. at 0° (crude extract) and high speed supernatants were obtained after centrifugation at 109,000*g* for 90 min. in an M.S.E. 50 ultracentrifuge.

Gas exchange. Oxygen consumption and carbon dioxide evolution were measured by the direct method in constant volume respirometers at 30° (Umbreit, Burris & Stauffer, 1957). Dissolved oxygen was measured with a Clark oxygen electrode (Yellow Springs Instrument Co., Inc., Yellow Springs, Ohio, U.S.A.).

Spectrophotometric determinations. U.v. and visible spectra were plotted with the Beckman DB or Unicam SP 800 recording spectrophotometers. Assays of enzymic activity were conducted at 30° in both instruments. Infra-red spectra were obtained with a Perkin-Elmer Infracord.

Chemical estimations. Catechols were detected and estimated by the Evans (1947) test. Keto-acids were detected by preparation of their acidic 2,4-dinitrophenylhydrazones, the extraction of these into ethyl acetate and then into sodium carbonate solution (Friedemann & Haugen, 1943).

Chromatography. Thin-layer plates were prepared with Silica gel G (Merck) and developed with the following solvents: (a) benzene + acetic acid + water (80 + 20 + satd); (b) ether + light petroleum, boiling range $60-80^{\circ}$ (7 + 3); (c) acetone + light petroleum, boiling range $60-80^{\circ}$ (2 + 5); (d) xylene + chloroform (1 + 1). *o*-Cresol and 3-methylcatechol were detected in chromatograms by coupling with diazotized *p*-nitroaniline.

Buffers. 0.067 M-Phosphate buffer was prepared from KH_2PO_4 (9 g./l.) and neutralized to the desired pH value with 5 N-NaOH solution.

RESULTS

Figure 1 demonstrates the ability of whole organisms to oxidize a variety of aromatic compounds after growth on glucose or *o*-cresol. Catechol, 3-methylcatechol and 4-methylcatechol were all oxidized without lag after growth on *o*-cresol. Periods of adaptation to these substrates were apparent when the bacteria were grown on glucose. The total oxygen consumption during oxidation of 3-methylcatechol by whole organisms was generally greater than that observed during the oxidation of

catechol or 4-methylcatechol, by 0.5–1.0 mole oxygen per mole substrate. The reasons for this are not known. This observation is not a peculiarity of *o*-cresol grown organisms where 3-methylcatechol is a metabolite, but may be shown with organisms grown on phenol or *p*-cresol (amongst many other growth substrates) where 3-methylcatechol is unlikely to be an intermediate.

Fig. 1. Each Warburg flask contained 1.7 ml. of 0.067 M-phosphate buffer, (pH 7.0) and 1 ml. of cell suspension containing: (a) equiv. 1.71 mg. dry wt. bacteria, and (b) equiv. 3.02 mg. dry wt. bacteria; 0.1 ml. of a 25 mM substrate solution, or water was tipped in from the side-arm; the centre well contained 0.2 ml. of 20 % (w/v) KOH.

Experiments with whole organisms quickly led to the conclusion that enzymes induced by growth with specific aromatic compounds were also able to metabolize a variety of other, though related, aromatic structures. Thus it is shown in Table 1 that it was immaterial which of the four aromatic substances (*o*-, *m*- or *p*-cresol or phenol) was the inducer during growth; the induced organisms oxidized all the aromatic substances except benzoate without a lag period. On the other hand, organisms previously grown on glucose metabolized the aromatic substances only after a lag period, albeit short in some instances. Organisms obtained after growth on benzoate rapidly oxidized the catechols without lag; the response to *o*- and *m*-cresol was difficult to evaluate as very short periods of adaptation (3–10 min.) occurred; *p*-cresol and phenol oxidations by these organisms were, however, preceded by a distinct period of induction. The patterns of oxidation of aromatic substances by the enzymes induced in washed suspensions of bacteria harvested from glucose media, by exposure of these to aromatic substances, were similar to those shown in Table 1. A distinction therefore between possible pathways of *o*-cresol metabolism, via salicylate and catechol or via 3-methylcatechol, was impossible on the basis of differences in oxygen consumption. The inability of

salicylate and 2,3-dihydroxybenzoate to increase the rates of oxygen consumption beyond the endogenous rate may have been due to permeability barriers. Therefore direct demonstration of the formation of 3-methylcatechol from *o*-cresol was sought by the isolation of 3-methylcatechol.

A substance containing the *o*-dihydroxy groups (positive reactions in the Evans, 1947, test) frequently appears and later disappears in cultures containing *o*-cresol as sole source of organic carbon. Media which were not supplemented with Fe produced larger amounts of this catechol compound. This material was isolated and identified by the following procedures.

Table 1. *Oxidation of substrates by Pseudomonas aeruginosa with or without a lag period, after induction by growth*

Compounds tested as substrates	Growth substrates (inducers)					
	<i>o</i> -Cresol	<i>m</i> -Cresol	<i>p</i> -Cresol	Phenol	Benzoate	Glucose
<i>o</i> -Cresol	+	+	+	+	?	—
<i>m</i> -Cresol	+	+	+	+	?	—
<i>p</i> -Cresol	+	+	+	+	—	—
Phenol	+	+	+	+	—	—
Benzoate	—	—	—	—	+	—
Glucose	NT	NT	NT	NT	NT	+
Catechol	+	+	+	+	+	—
3-Methylcatechol	+	+	+	+	+	—
4-Methylcatechol	+	+	+	+	+	—

+, substrate oxidized without a lag period.

—, substrate oxidized with a lag period.

?, not evaluated; induction of enzymes to these substrates is often too rapid to demonstrate significant lag periods.

NT, not tested.

A 20 l. culture of *Pseudomonas aeruginosa* T1 containing approximately 160 mg. of the catechol was clarified at 60,000*g* and concentrated to approximately 500 ml. under reduced pressure, between 30 and 37°. The concentrate (pH 6.9) was extracted three times with an equal volume of ether. The ether extracts were dried over sodium sulphate and distilled to dryness, yielding a crystalline material. Thin-layer chromatography revealed two distinct zones which had the R_f values of *o*-cresol and 3-methylcatechol in four solvents (see Experimental). The *o*-cresol residue was removed by steam distillation and the dark brown residue dried under vacuum. After recrystallization from water it had a m.p. 66–68° which was not depressed when mixed with an authentic specimen of 3-methylcatechol. The product analysed C, 67.4; H, 4.73; (calculated for $C_7H_8O_2$, C, 67.8; H, 4.84). In addition, its ultraviolet and infrared spectra (Fig. 2 and 3), and the spectrum of the product of reaction in the Evans test, were in accord with authentic samples of 3-methylcatechol and unlike data obtained for catechol itself.

Extracts of organisms induced to metabolize *o*-cresol rapidly oxidized catechol, 3-methylcatechol and 4-methylcatechol with the consumption of 1 mole O_2 /mole substrate (Table 2; the extra oxygen consumption by whole bacteria exposed to 3-methylcatechol was not observed with extracts). For catechol and 4-methylcatechol, yellow keto acids appeared with λ_{max} 375 and 379 m μ respectively, and later disappeared without further oxygen consumption or carbon dioxide evolution.

Fig. 2. Ultraviolet spectra of *o*-cresol metabolite and 3-methylcatechol in ethanol.

Fig. 3. Infrared spectra of *o*-cresol metabolite and 3-methylcatechol. Mulls in liquid paraffin B.P. were pressed between KBr discs and scanned with a Perkin-Elmer Infra-red.

However, no coloured product appeared during 3-methylcatechol dissimilation by extracts. The presence of a catechol 2,3-oxygenase in these extracts makes the possibility of cleavage of 3-methylcatechol between positions 1 and 2, to form a colourless muconic acid, unlikely. The following experiments support this view. Cell-free extracts from *Pseudomonas desmolyticum* grown on naphthalene contain catechol 2,3-oxygenase which will oxidize catechol, and the two methyl substituted catechols with the intermediary formation of the yellow keto acids in all three cases. The product from 3-methylcatechol, 2-hydroxy-6-oxohepta-2,4-dienoic acid, which

Table 2. *Gaseous exchange during metabolism of catechol, 3-methylcatechol and 4-methylcatechol by cell suspensions and cell free extracts*

Preparation used	Substrate (μ moles)		Oxygen consumed (μ moles)	Carbon dioxide evolved (μ moles)
Cell suspension*	None		2.5	—
	Catechol	2.5	8.2	—
	3-Methylcatechol	2.5	11.8	—
	4-Methylcatechol	2.5	7.9	—
Cell free extract*	None		0.2	—
	Catechol	10	9.8	0.25
	3-Methylcatechol	10	9.5	0.1
	4-Methylcatechol	10	9.6	0.34
Cell free extract†	None		0	—
	Catechol	0.5	0.49	—
		1.0	1.0	—
	3-Methylcatechol	0.5	0.49	—
		1.0	0.99	—
	4-Methylcatechol	0.5	0.5	—
		0.75	0.75	—

— Not measured; * Oxygen consumption measured manometrically; † oxygen consumption measured with an O_2 electrode.

absorbs maximally at 386 $m\mu$ at pH values greater than 7.2, is then metabolized slowly to acetate and to 4-hydroxy-2-oxovalerate (Dagley *et al.* 1964). Extracts of *Pseudomonas aeruginosa* T1, however, rapidly affected the latter reaction (Fig. 4). The product of 3-methylcatechol cleavage by extracts of an orcinol-grown pseudomonad was also degraded rapidly by extracts of *P. aeruginosa* T1. The failure to observe 2-hydroxy-6-oxohepta-2,4-dienoic acid as an intermediate of 3-methylcatechol oxidation is therefore not surprising since kinetic studies have shown that *P. aeruginosa* T1 extracts are able to degrade the intermediate at rates faster than it is formed (Table 3).

DISCUSSION

The pathway of metabolism of *o*-cresol by *Pseudomonas aeruginosa* T1 most probably involves hydroxylation of the nucleus, ring-fission of the 3-methylcatechol and subsequent fissions of the carbon chain by the pattern recently disclosed by the experiments of Dagley *et al.* (1964). It is worth examining the criteria used to establish that 3-methylcatechol is an intermediate: (1) whole bacteria oxidize this substrate without lag after growth on *o*-cresol but not after growth on glucose; (2) extracts of these bacteria oxidize 3-methylcatechol and further metabolize the products by established mechanisms; (3) 3-methylcatechol has been isolated as a

transient metabolite in *o*-cresol culture filtrates. Conclusions often drawn from the data supplied by methods (1) and (2) would establish 3-methylcatechol as a metabolite *o*-cresol. However, both catechol and 4-methylcatechol are metabolized by the *o*-cresol induced bacteria and extracts derived from them—facts which cast doubt on the validity of the conclusion that 3-methylcatechol is a metabolite of *o*-cresol.

Fig. 4. Degradation of 2-hydroxy-6-oxohepta-2,4-dienoic acid by extracts of *P. aeruginosa* T1. The product of 3-methylcatechol cleavage was generated with extracts of naphthalene grown *P. desmolyticum*. The cuvette contained; 0.067 M-phosphate buffer, pH 6.9, (2.5 ml.); extract from *P. desmolyticum* (20 μl., 300 μg. protein); 10 μl. of 25 mM solution of 3-methylcatechol were added at the first arrow. The extract of *P. aeruginosa* T1 was added at the second arrow (10 μl., 120 μg. protein). Temperature 30°.

Table 3. Rates of formation and dissimulation of the products of ring cleavage of catechol, 3-methylcatechol and 4-methylcatechol by extracts of *Pseudomonas aeruginosa* T1

Substrate	Rate of O ₂ consumption μmoles/min./ml. of extract		Rate of dissimulation of ring cleavage product μmoles/min./ml. of extract	
	Manometric	O ₂ electrode		
Catechol	14	14.6	6.35	10.8
3-Methylcatechol	14	14.1	76.5	89
4-Methylcatechol	—	48.8	—	5.84

— Not measured.

Demonstration of the presence of enzyme activities in induced whole bacteria and extracts of them cannot be regarded as strong evidence for a metabolic route. Indeed Stanier (1950) clearly emphasized that the induced enzymes involved in the catabolism of these compounds must show high specificity for substrate and inducer, if the technique of sequential induction is to be used validly.

The isolation of 3-methylcatechol as an intermediary metabolite of *o*-cresol cultures of *Pseudomonas aeruginosa* T1 provides more satisfactory evidence for its role as the substrate of ring fission. It is possible that catechol may arise from 3-methylcatechol by oxidation to 2,3-dihydroxybenzoate and decarboxylation to catechol, which may then undergo ring cleavage. However, 2,3-dihydroxybenzoate is not oxidized by whole organisms or extracts and catechol is not formed anaerobically from this substrate, though enzymes capable of oxidizing 2,3-dihydroxybenzoate are easily obtained from other bacteria (Ribbons, 1965, unpublished). The existence of a metabolic route under these circumstances of poor specificity of the enzyme should, then, be confirmed by the detection of metabolites and their subsequent disappearance, e.g. by methods like those used by Bassham & Calvin (1957).

The low specificity suggested for the enzymes metabolizing catechol, the methylcatechols and the products derived from them is also exhibited by the inducer. The specificity of enzymes formed in response to any of the cresols or phenol further suggests that the hydroxylase does not discriminate these four substrates and does not metabolize benzoate. Benzoate however induces enzymes for its own catabolism to catechol which are quite distinct from those hydroxylating the cresols or phenol, although a common route for catechol oxidation is evident (Scheme 2).

Scheme 2

The route by which *m*-cresol is metabolized has not yet been determined, but 4-methylcatechol has been detected chromatographically as a metabolite of *p*-cresol.

Although these enzymes have not been studied in any detail the results so far obtained suggest that multi-enzyme systems are elaborated in response to a variety of structures and these multi-enzyme systems show low specificity. When more than one chemical structure is available for metabolism, the existence of such inducible non-specific multi-enzyme systems provides an economy of information

storage and retrieval, as well as of protein synthesis. Reaction sequences of this sort do not occur generally; but the biosynthesis of valine and of isoleucine are catalysed by the same enzyme sequence (Umbarger & Davis, 1962) and provide an analogy to the present catabolic scheme. More recently Gunsalus, Chapman & Kuo (1965) have studied reactions involved in bicyclic mono-terpenoid catabolism by pseudomonads and diphtheroids. They concluded that the enzymes catalysing reaction sequences to common intermediates are non-specific to both substrate and inducer. Low specificity in some instances would allow the exact order of three reactions in a sequence to be transposed without appreciable loss of efficiency. The multi-enzyme system involved in the degradation of the catechols has not been as extensively studied. Further purification of the enzymes involved may show more subtle differences in their control by catabolite repression or as isoenzymes. It is possible that the economic provision of a sequence of enzymes that will tackle a variety of similar structures is more widespread. Thus studies of the microbial metabolism of polynuclear hydrocarbons, naphthalene, phenanthrene and anthracene and methyl- or halogen-substituted naphthalenes have shown that end ring attack occurs with formation of 1,2-diols, cleavage between positions 1 and 8a, loss of a C_3 unit, presumably as pyruvate, and oxidation to a substituted salicylate and thence to the catechol containing one ring less (Evans, Fernley & Griffiths, 1965; Treccanni, 1963). This reaction sequence may also be catalysed by a non-specific multi-enzyme system that can be induced by a variety of similar structures. In the case of phenanthrene the first and second rings may be manipulated by the same enzymes.

I thank Miss Margaret Lowson and Mr T. G. Gajdatsy for technical assistance, and Dr G. W. Gray for help with the I.R. spectra.

REFERENCES

- BASSHAM, J. A. & CALVIN, M. (1957). *The Path of Carbon in Photosynthesis*. Englewood Cliffs, N. J. U.S.A.: Prentice-Hall.
- DAGLEY, S. & PATEL, M. D. (1957). Oxidation of *p*-cresol and related compounds by a *Pseudomonas*. *Biochem. J.* **66**, 227.
- DAGLEY, S., EVANS, W. C. & RIBBONS, D. W. (1960). New pathways in the oxidative metabolism of aromatic compounds by micro-organisms. *Nature, Lond.* **188**, 560.
- DAGLEY, S., CHAPMAN, P. J., GIBSON, D. T. & WOOD, J. M. (1964). Degradation of the benzene nucleus by bacteria. *Nature, Lond.* **202**, 775.
- EVANS, W. C. (1947). Oxidation of phenol and benzoic acid by some soil bacteria. *Biochem. J.* **41**, 373.
- EVANS, W. C., FERNLEY, H. N. & GRIFFITHS, E. (1965). Oxidative metabolism of phenanthrene and anthracene by soil pseudomonads. *Biochem. J.* **95**, 819.
- FRIEDEMANN, T. E. & HAUGEN, G. (1943). Pyruvic acid. II. The determination of keto acids in blood and urine. *J. biol. Chem.* **147**, 415.
- GUNSALUS, I. C., CHAPMAN, P. J. & KUO, W. J. F. (1965). Control of catabolic specificity and metabolism. *Biochem. Biophys. Res. Comm.* **18**, 924.
- ICHIHARA, A., ADACHI, K., HOSOKAWA, K. & TAKEDA, Y. (1962). The enzymatic hydroxylation of aromatic carboxylic acids; substrate specificities of anthranilate and benzoate oxidases. *J. biol. Chem.* **237**, 2296.
- KITAGAWA, M. (1956). Studies on the oxidation mechanism of methyl group. *J. Biochem.* **43**, 553.
- LEIBNITZ, E., BEHRENS, U., STREIGLER, G. & GABERT, A. (1962). Zur mikrobiellen Oxydation der *p*-ständigen Methylgruppe in Xylenolen. *Z. allg. Mikrobiol.* **2**, 81.

- NAKAGAWA, H., INOUE, H. & TAKEDA, Y. (1963). Characteristics of catechol oxygenase from *Brevibacterium fuscum*. *J. Biochem.* **54**, 65.
- PANKHURST, E. S. (1959). The biological oxidation of spent gas liquor. *J. appl. Bact.* **22**, 202.
- RIBBONS, D. W. (1964). Bacterial metabolism of *o*-cresol. *Proc. 6th int. Congr. Biochem.*
- ROGOFF, M. H. & WENDER, I. (1957). Methyl-naphthalene oxidations by pseudomonads. *J. Bact.* **77**, 783.
- STANIER, R. Y. (1950). Problems of bacterial oxidative metabolism. *Bact. Rev.* **14**, 179.
- TRECCANNI, V. (1963). The bacterial metabolism of hydrocarbons. *Prog. indust. Microbiol.* **3**, 3.
- UMBARGER, E. & DAVIS, B. D. (1962). Pathways of amino acid biosynthesis. In *The Bacteria*. Ed. by I. C. Gunsalus & R. Y. Stanier, Vol. 3, p. 167. New York: Academic Press.
- UMBREIT, W. W., BURRIS, R. H. & STAUFFER, J. F. (1957). *Manometric Techniques*, 3rd edn. Minneapolis, Minn., U.S.A.: Burgess Publishing Co.