Accumulation of Iron-binding Phenolic Acids by Actinomycetales and Other Organisms Related to the Mycobacteria

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SUMMARY

After growth of representatives of eight genera in the order Actinomycetales and two others related taxonomically to Mycobacterium under iron-deficient conditions, the following compounds were found in the media: salicylic and phenylacetic acids, but no mycobactin, with *Thermoactinomyces vulgaris*; 2,3-dihydroxybenzoic acid with *Corynebacterium equi*, *Mycococcus* sp., *Nocardia opaca* and *Streptosporangium rubrum*; a conjugate of 2,3-dihydroxybenzoic acid and 3,4-dihydroxybenzoic acid were also found with *S. rubrum*. Unidentified compounds giving a positive reaction to FeCl_3 were found after growth of *Dactylosporangium thailandensis* and *Propionibacterium shermanii*. No iron-binding compounds were detected with *Actinoplanes phillippinensis*, *Micromonospora* sp. and *Streptomyces albus*.

INTRODUCTION

At least three types of hydroxybenzoic acid are excreted by bacteria under conditions of iron deficiency: (1) 2,3-dihydroxybenzoic acid and its conjugates with amino acids (Ito & Neilands, 1958; Dyer, Heding & Schaffer, 1964; Ratledge, 1964; Brot, Goodwin & Fales, 1966; Corbin & Bulen, 1969; Korth, 1970; O'Brien & Gibson, 1970; Pollack & Neilands, 1970); (2) 3,4-dihydroxybenzoic acid (Chao, Hawkins & Williams, 1967; Bakshi & Williams, 1969; Cagle & Williams, 1969); and (3) 2-hydroxybenzoic acid (salicylic acid) and 6-methylsalicylic acid (Ratledge & Winder, 1962, 1966; Dhople, Morrison & Hanks, 1965).

The acids of the first two groups are produced by a variety of bacteria but salicylic acid and 6-methylsalicylic have only been found among mycobacteria. This generalization is, however, based on the examination of only a few species. Indeed, the only member of the Actinomycetales, of which Mycobacterium is a member, to be examined before this work was *Streptomyces griseus*, which accumulated 2,3-dihydroxybenzoic acid when deficient in iron (Dyer *et al.* 1964).

Organisms belonging to the Actinomycetales and the related genera of *Corynebacterium* and *Propionibacterium* (Jensen, 1966; Lechevalier & Lechevalier, 1967) have been examined to see if the production of salicylic acid is restricted and, if so, whether it would provide a useful character for the identification of mycobacteria.

EXPERIMENTAL

Organisms. The following organisms were used: *Actinoplanes phillippinensis* ATCC 12427; *Corynebacterium equi* (obtained from Dr P. J. Brennan, Trinity College, Dublin University); *Dactylosporangium thailandensis* CUB 429 (from Mr T. Cross, curator of the Actinomycete Culture Collection, Bradford University); *Micromonospora* sp. CUB 26; *Mycococcus* sp.

ATCC 13556; *Nocardia opaca* NCIB 9409; *Propionibacterium shermanii* NCIB 5964; *Streptomyces albus* IMI 52766 (from Commonwealth Mycological Institute, Kew, Surrey); *Streptosporangium rubrum* CUB 159; *Thermoactinomyces vulgaris* CUB 76. All cultures were maintained on nutrient agar slopes except *C. equi*, which was maintained on slopes of Loeffler's serum. All organisms were subcultured monthly.

Medium and removal of trace metals. The basal medium which was used for cultivation of most of the organisms contained $(\text{NH}_4)_2\text{SO}_4$ (2 g.) and KH_2PO_4 (9 g.) in 1 l. distilled water made to pH 7.5 with NaOH. Trace metals were removed by autoclaving the medium with alumina (Donald, Passey & Swaby, 1952). After removal from the alumina, the medium was adjusted to pH 6.8 with 2 M- H_2SO_4 and dispensed either in 90 ml. amounts in 250 ml. conical flasks, 720 ml. in 2 l. conical flasks or 1440 ml. in 4 l. conical flasks and autoclaved for 15 min. at 121° . This medium was supplemented with glucose, from a 30% (w/v) solution previously treated with alumina to remove trace metals, and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ to 30 g./l. and 0.4 g./l. respectively.

The following nutrients were added to 0.1 g./l. from alumina-treated solutions as required by the organism: for *Actinoplanes phillippinensis*, peptone, yeast extract, malt extract, beef extract and tryptone; for *Dactylosporangium thailandensis* and *Micromonospora* sp., peptone and malt extract; for *Mycococcus* sp., peptone; and for *Streptosporangium rubrum* and *Thermoactinomyces vulgaris*, peptone and yeast extract.

For cultivation of *Corynebacterium equi* in liquid medium the medium of Brennan, Lehane & Thomas (1970) was used. Glucose was sterilized separately from the remainder of the medium. Both parts of the medium were autoclaved with alumina to remove trace metals and finally dispensed in 100 ml. amounts in 250 ml. conical flasks.

For cultivation of *Nocardia opaca* the medium contained glycerol (20 ml.), monosodium L-glutamate (0.24 g.), KH_2PO_4 (2.5 g.), NH_4NO_3 (1 g.), in distilled water to 1 l. and the pH was adjusted to 7.5 with NaOH. Trace metals were removed by alumina treatment and the pH was adjusted to 6.8 with 2 M- H_2SO_4 . The medium was finally dispensed in 100 ml. amounts in 250 ml. conical flasks; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ at 0.4 g./l. was included immediately prior to inoculation.

Propionibacterium shermanii was grown on medium which contained glucose (5 g.), yeast extract (5 g.), peptone (5 g.), sodium thioglycollate (1.0 g.) in distilled water to 1 l., pH adjusted to 7.5 with NaOH. After treatment with alumina the medium was adjusted to pH 6.8, dispensed in 1.5 l. amounts in 2 l. flasks and sterilized by autoclaving. The medium was supplemented with the following (mg./l.): calcium pantothenate (1.0), thiamine hydrochloride (1.0) and biotin (1.0). Each flask was fitted with a sintered glass disc to allow $\text{N}_2 + \text{CO}_2$ (95:5, v/v) to be introduced at about 20 ml./min. throughout incubation.

Streptomyces albus was grown on medium which contained KH_2PO_4 (5 g.), NH_4NO_3 (2 g.), L-asparagine (1 g.) in distilled water, pH adjusted to 7.5 with NaOH. After treatment with alumina the medium was adjusted to pH 6.8, dispensed in 180 ml. amounts in 500 ml. conical flasks and sterilized by autoclaving. It was supplemented with 20 ml./l. of 20% (w/v) alumina-treated glucose solution; yeast extract and peptone, both as solutions previously alumina-treated, were added to 0.1 g./l.

Metal ions added to media. The following metal ions were added to all media: $\text{ZnSO}_4 \cdot 6\text{H}_2\text{O}$, 0.46 μg . Zn/ml.; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.28 μg . Mn/ml.; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05 to 5 μg . Fe/ml. Growth was usually iron-deficient when the final concentration was 0.1 μg . Fe/ml. or less ('low-iron' medium) and iron-sufficient (or normal) at 2 to 5 μg . Fe/ml. ('high-iron' medium).

Inocula and growth. Half or 1% inocula were prepared from cultures grown on liquid media with 2 μg . Fe^{2+} /ml. With *Actinoplanes phillippinensis*, *Streptomyces albus* and *Thermo-*

actinomycetes vulgaris, where growth was either in the form of clumps or as a pellicle, inocula were prepared by shaking a few loopfuls of the clumps or pellicle with glass balls (undrilled, B.D.H. Chemicals Ltd, Poole, Dorset).

Condition of growth. All cultures were at 30° except *Corynebacterium equi*, which was incubated at 37°, and *Thermoactinomyces vulgaris*, which was at 50°. Cultures were not usually shaken but *Micromonospora* sp. and *Streptosporangium rubrum* were also incubated in an orbital shaker.

Harvesting of cultures and determinations of bacterial dry weights. Cultures were centrifuged or, where growth was in pellicle-form, filtered through a sintered glass filter (no. 2 porosity); organisms were washed twice in distilled water before being dried over P₂O₅ under vacuum to constant weight.

Isolation of compounds from culture media. The medium was acidified with 5 M-H₂SO₄ to pH 3 and extracted three times with equal volumes of diethyl ether. The ether extract was dried over MgSO₄, filtered, evaporated in a rotary evaporator to 1.0 to 0.5 ml., and the concentrate was chromatographed on Whatman 3 MM or no. 17 paper using solvent system 6 (see below) in an ascending direction. Compounds were visualized under u.v. light (253.7 nm.) and the fluorescent areas noted. Strips of each chromatogram were sprayed with 2% FeCl₃ in 0.1 M-HCl. Areas with prominent fluorescence or giving a colour to the spray reagent were twice eluted by standing in ether-ethanol-water (3:1:1, by vol.) for 6 to 8 h. These extracts were then further investigated.

Paper chromatography on Whatman no. 1 paper was used in an ascending direction with the following solvent systems: 1, methanol+butan-1-ol+benzene+water (2:1:1:1, by vol.); 2, butan-1-ol+pyridine+aq. NH₃ (sp.gr. 0.880)+water (4:8:5:3, by vol.); 3, butan-2-one+2-methylpropan-2-ol+water (2:2:1, by vol.); 4, butan-1-ol+benzene+ethanol+aq. NH₃ (sp.gr. 0.880) (5:2:1:2, by vol.); 5, butan-1-ol+acetic acid+water (4:1:1, by vol.); 6, propan-2-ol+aq. NH₃ (sp.gr. 0.880)+water (8:1:1, by vol.).

Spray reagents. The composition of chromatography sprays were as given by Dawson, Elliot, Elliot & Jones (1959), Smith (1960) or McGeer, Robertson & McGeer (1961).

Mass spectra were determined in a high resolution mass spectrometer (model MS 902, A.E.I. Scientific Apparatus Ltd, Urmston, Manchester).

Absorption spectra were determined using a recording u.v. spectrophotometer (model SP800, Unicam Instruments Ltd, Cambridge) fitted with microcuvettes when necessary. Spectra were usually determined in ethanol, 0.1 M-NaOH and 0.1 M-HCl.

Identification of individual compounds isolated from culture filtrates. *Salicylic acid* (from *Thermoactinomyces vulgaris*) had *R_F* values coincident with authentic salicylic acid after paper chromatography in solvent systems 1, 2 and 3. Paper chromatography using ethanol+xylylene+toluene+aq. NH₃ (sp.gr. 0.880) (5:4:2:1, by vol.) distinguished the isolated material (*R_F* 0.27) from 6-methylsalicylic acid (*R_F* 0.34) (obtained as a gift from Dr G. A. Snow, I.C.I. Ltd, Alderley Park, Macclesfield). Identification of the isolated material was confirmed by reactions to a number of reagents, u.v. and mass spectrometry. Mass measurement of the parent ion of the isolated material gave 138.03169: the expected value for C₇H₆O₃ was 138.03194.

2,3-Dihydroxybenzoic acid (from *Corynebacterium equi*, *Mycococcus* sp., *Nocardia opaca* and *Streptosporangium rubrum*). The *R_F* values of the isolated materials after chromatography using solvent systems 1, 4 and 5 were always within an *R_F* of 0.03 of the authentic sample. Extinction maxima of the isolated compounds and reactions to various spray reagents were the same as those obtained with an authentic sample (see Ratledge, 1967). Mass spectra of the four isolated samples of 2,3-dihydroxybenzoic acid and an authentic

sample showed similar fragmentation patterns. Mass measurement of each parent ion gave 154.02575 for the material from *C. equi*; 154.02665 for that from *Myococcus* sp.; 154.02499 for that from *N. opaca*; and 154.02575 for that from *S. rubrum*. For $C_7H_6O_4$ the expected value of the parent ion was 154.02660.

Conjugate of 2,3-dihydroxybenzoic acid (from *Streptosporangium rubrum*) had a blue fluorescence under u.v. light and turned blue when sprayed with $FeCl_3$. Both these properties were similar to those of 2,3-dihydroxybenzoic acid. However, it had an R_F value of 0.75 after chromatography on Whatman 3MM paper using solvent system 1, whereas 2,3-dihydroxybenzoic acid had an R_F value of 0.66. The extinction maxima of the isolated compound in 0.1 M citric acid-NaOH buffer at pH 6.0 were at 232 nm., 250 nm. (shoulder) and 309 nm. (for authentic 2,3-dihydroxybenzoic acid the maxima were at 243 and 307 nm.) and in 0.1 M-tris HCl buffer, pH 8.0, were at 230 nm., 256 nm. (slight shoulder only) and 329 nm. (for authentic 2,3-dihydroxybenzoic acid the maxima did not shift and remained at 241 and 308 nm.). The shift observed for the isolated material is similar to that observed for 2,3-dihydroxybenzoylserine (Luke, 1970). A dry sample of the isolated material was hydrolysed in 6 M-HCl (0.5 ml.) at 140° in a sealed glass tube for 16 h. The cooled solution was adjusted to a value between pH 1 and 3 with 10 M-NaOH and extracted with ether (3 × 1 ml.). The ether extract was evaporated to near dryness and after chromatography in solvent system 6 2,3-dihydroxybenzoic acid was identified by its fluorescence, R_F value and reactions to a number of reagents ($FeCl_3$, Arnow's reagent, diazotized sulphanilic acid and diazotized *p*-nitroaniline).

Phenylacetic acid (from *Thermoactinomyces vulgaris*) had no fluorescence under u.v. light and was detected by 0.1 % bromocresol green after chromatography in solvent system 2. It was identified by its u.v. and mass spectra: the isolated material gave a parent ion of mass 136.05249; the expected value for $C_8H_8O_2$ was 136.05242. Mass spectra of *o*-, *m*- and *p*-toluic acids (which are isomeric with phenylacetate) were not the same as the isolated material. The methyl ester of isolated material was analysed by g.l.c. and gave the same retention time as authentic methyl phenylacetate.

3,4-Dihydroxybenzoic acid (from *Streptosporangium rubrum*) was identified by its fluorescence, reaction to various reagents, chromatographic mobility and u.v. spectra (see Ratledge & Winter, 1966).

Extraction of Thermoactinomyces vulgaris for mycobactin. Bacteria were dried to a constant weight over P_2O_5 , extracted with ethanol (White & Snow, 1968) and the extract + $FeCl_3$ was further extracted into chloroform which was then taken to dryness. The residue was dissolved in 0.5 ml. methanol and its extinction at 450 nm. was determined. It was separated by t.l.c. on silica-gel G using cyclohexane + 2-methylpropan-2-ol + propan-1,2-diol (40:10:1, by vol.) (White & Snow, 1968). Authentic mycobactin s (prepared by extraction of *Mycobacterium smegmatis*; see Snow, 1965) was chromatographed simultaneously. The isolated material was viewed under u.v. light and also sprayed with 25 mM NH_4VO_3 + 6M-HCl (1:1, v/v) (White & Snow, 1968).

RESULTS AND DISCUSSION

Table 1 summarizes the growth of the various organisms studied and the compounds which were identified after extraction of media. Although 2,3-dihydroxybenzoic acid was identified after acid hydrolysis of the conjugate isolated from the 'low-iron' medium of *Streptosporangium rubrum*, the attempt to characterize the other component by preparation of a dansyl derivative (Boulton & Bush, 1964) was unsuccessful. Both *Actinoplanes phillip-*

Table 1. Growth of organisms and compounds isolated from the media

Organism	Growth period (days)	Conditions of growth	Bacterial yield (mg./l.) with iron added (μ g./ml.) at					Compounds identified from 'low-iron' medium
			0	0.05	0.1	2	5	
<i>Actinoplanes philippinensis</i>	14	Static	nil	123	—	143	—	None
<i>Corynebacterium equi</i>	14	Static and shake	297	—	—	673	—	2,3-Dihydroxybenzoic acid
<i>Dactylosporangium thailandensis</i>	14	Static	1150	—	—	2370	—	See Table 2
<i>Micromonospora</i> sp.	7	Static	nil	165	—	198	—	None
<i>Mycrococcus</i> sp.	16	Static	152	171	203	1585	—	2,3-Dihydroxybenzoic acid
<i>Nocardia opaca</i>	7	Static	84	—	—	1020	—	2,3-Dihydroxybenzoic acid
<i>Propionibacterium shermanii</i>	14	Static	—	—	53	101	—	See Table 2
<i>Streptomyces albus</i>	12	Static and shake	—	—	353	805	—	None
<i>Streptosporangium rubrum</i>	5	Shake	—	486	—	—	852	2,3-Dihydroxybenzoic acid + a conjugate of it
<i>Thermoactinomyces vulgaris</i>	13	Static	—	59	—	—	239	3,4-Dihydroxybenzoic acid
	11	Static	—	—	430	—	*	Salicylic acid and phenylacetic acid

* Yield was not calculated but greater pellicle formation in 'high-iron' medium than in 'low-iron' medium was noted.

Table 2. Unknown compounds isolated from 'low-iron' medium of *Dactylosporangium thailandensis* and *Propionibacterium shermanii*

Compound*	Fluorescence (at 253.7 nm.)	Colour with FeCl ₃ spray	Extinction maxima (nm.) in				R _F values in solvent system†			
			Ethanol	0.1 M-HCl	M-NaOH		1	4	5	6
DT 1	Blue	Blue-violet	278	245, 275 (shoulders)	240, 286		0.72	—	—	0.50
DT 2	Blue	Blue-violet	239, 333	240, 333	255, 341		—	—	—	0.30
DT 3	Indistinct	Violet	No clearly defined spectra				—	0.20	—	—
DT 4	Yellowish	Pink	298	298	273		—	—	—	0.9
PS 1	Greenish	Red-brown	272	265	264		—	0.34	0.86	—
PS 2	Yellowish	Orange	273	264	252 (shoulder)		—	0.26	0.81	0.59
Authentic 2,3-dihydroxybenzoic acid	Sky blue	Blue	243 (shoulder), 305	246, 313	233 (shoulder), 326		0.45	0.13	0.83	0.48
Salicylic acid	Deep blue	Blue-violet	233, 298	237, 305	225, 298		—	0.46	—	0.74

* DT indicates isolated from *Dactylosporangium thailandensis*, PS from *Propionibacterium shermanii*; conditions of growth as given in Table 1.
† For details see Experimental.

pinesis and *Micromonospora* sp. showed only a small difference in yield between 'low-iron' and 'high-iron' conditions but did not grow when iron was omitted. Apparently the iron requirement of each organism was satisfied by a small concentration (cf. Chesters & Rolinson, 1951). The components isolated from all 'low-iron' media were not detected in high-iron' conditions.

Several compounds reacting with FeCl_3 were isolated from 'low-iron' media of *Dactylosporangium thailandensis* and *Propionibacterium shermanii*, but were not present in sufficient amount to permit identification (Table 2). Compound DT I was probably an *o*-dihydric phenolic acid as, in addition to the properties given in Table 2, it became yellow-red with Arnow's reagent, a reagent specific for this group of compounds.

Salicylic acid had previously only been found in culture filtrates of *Mycobacterium smegmatis* and *M. tuberculosis*. It is always associated with an intracellular iron-chelating compound, mycobactin, of which salicylic acid forms a part (Snow, 1970). The occurrence of mycobactin may be useful as an aid to the identification of mycobacteria (Snow, 1970; Snow & White, 1969) but this would not be so if *Thermoactinomyces vulgaris* produced a similar compound. Because of the occurrence of salicylic acid in medium of the latter organism, the occurrence of mycobactin was sought most carefully. About 650 mg. of dry *T. vulgaris*, obtained from 1.5 l. of 'low-iron' medium, was extracted for mycobactin. No material was found which chelated with iron to give a coloured product, or had an extinction maxima at 450 nm. After thin-layer chromatography of the entire material which was soluble in methanol, no fluorescent area or area reacting to NH_4VO_3 spray reagent could be seen.

The occurrence of salicylic acid in culture filtrates of *Thermoactinomyces vulgaris* as well as in *Mycobacterium smegmatis* and *M. tuberculosis* is probably fortuitous as the two different genera have little taxonomic or evolutionary connexion (Cross, 1968).

The role of salicylic acid in biosynthesis in the two genera is undoubtedly different. With *Mycobacterium smegmatis* salicylic acid is taken up from the medium and used in the biosynthesis of mycobactin (Ratledge & Hall, 1970). Such a relationship cannot occur in *T. vulgaris* as this organism lacks mycobactin. The formation of salicylic acid in this latter organism parallels that of 2,3-dihydroxybenzoic acid in other organisms (such as *Escherichia coli* and *Klebsiella aerogenes*) where there is no intracellular counterpart to the extracellular iron chelator. The function of all these iron-chelating compounds could be the same, namely to transport iron into the cell (cf. Peters & Warren, 1968; Wang & Newton, 1969; Downer, Davis & Byers, 1970). Their increased accumulation under iron-deficient growth conditions is consistent with this view.

The production of salicylic acid by bacteria is not common. The compound was not found in *Mycococcus* sp., which is currently classified (Breed, Murray & Smith, 1957) as a genus of the family Mycobacteriaceae, the only other member of which is *Mycobacterium* itself. Nor was it found in *Corynebacterium equi* or *Nocardia opaca*, two species from genera close to *Mycobacterium* (Lechevalier & Lechevalier, 1967). In both these species, and in *Mycococcus* sp. and *Streptosporangium rubrum*, 2,3-dihydroxybenzoic acid was the iron-binding phenolic acid produced. The distribution of this compound may therefore be as widespread among the Actinomycetales as it is among the Eubacteriales (see Introduction).

The apparent absence of an iron-binding phenolic acid in the culture media of some of the organisms studied here may be due to an insufficiently sensitive means of detection. However, these compounds might be replaced functionally by ferrioxamines (see Neilands, 1966) which have been isolated from several genera of the Actinomycetales: from species of *Micromonospora*, *Nocardia*, *Streptomyces* and *Streptosporangium* (Zahner, Bachmann,

Hutter & Nuesch, 1962). Although 2,3-dihydroxybenzoic acid occurs in two of these genera (i.e. in *Nocardia* and *Streptosporangium*) and this compound has also been isolated from *Streptomyces* (Dyer *et al.* 1964), it is not possible to say whether ferrioxamines are simultaneously formed along with phenolic acids. The absence of any iron-binding phenolic acid in *Actinoplanes phillippinensis* and *Micromonospora* sp. could perhaps be attributed to prior formation of a ferrioxamine. Further investigations may show whether the functions of the ferrioxamines and the phenolic acids are the same, and, if so, whether their formations are mutually exclusive.

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REFERENCES

- BAKSHI, Y. K. & WILLIAMS, R. P. (1969). Biosynthesis of protocatechuic acid by non-proliferating cells of *Bacillus anthracis*. *Bacteriological Proceedings*, p. 145.
- BOULTON, A. A. & BUSH, I. E. (1964). Qualitative and quantitative analysis of amino acids as their 'dansyl' derivatives. *Biochemical Journal* **92**, 11 P.
- BREED, R. S., MURRAY, E. G. D. & SMITH, N. R. (1957). *Bergey's Manual of Determinative Bacteriology*, 7th edn. London: Baillière, Tindall and Cox.
- BRENNAN, P. J., LEHANE, D. P. & THOMAS, D. W. (1970). Acylglucoses of the corynebacteria and mycobacteria. *European Journal of Biochemistry* **13**, 117-123.
- BROT, N., GOODWIN, J. & FALES, H. (1966). *In vivo* and *in vitro* formation of 2,3-dihydroxybenzoylserine by *Escherichia coli* K12. *Biochemical and Biophysical Research Communications* **25**, 454-461.
- CAGLE, C. & WILLIAMS, R. P. (1969). Production of protocatechuic acid by *Bacillus cereus*. *Bacteriological Proceedings*, p. 146.
- CHAO, K. C., HAWKINS, D. & WILLIAMS, R. P. (1967). Production of protocatechuic acid by non-proliferating suspensions of *Bacillus anthracis*. *Bacteriological Proceedings*, p. 31.
- CHESTERS, C. G. C. & ROLINSON, G. N. (1951). Trace elements and streptomycin production. *Journal of General Microbiology* **5**, 559-565.
- CORBIN, J. L. & BULEN, W. A. (1969). The isolation and identification of 2,3-dihydroxybenzoic acid and 2-N,6-N-Di-(2,3-dihydroxybenzoyl)-L-lysine formed by iron-deficient *Azotobacter vinelandii*. *Biochemistry* **8**, 757-762.
- CROSS, T. (1968). Thermophilic actinomycetes. *Journal of Applied Bacteriology* **31**, 36-53.
- DAWSON, R. M. C., ELLIOT, D. C., ELLIOT, W. H. & JONES, K. M. (1959). *Data for Biochemical Research*. Oxford University Press.
- DHOPLE, A. M., MORRISON, N. E. & HANKS, J. H. (1965). Effect of iron limitation on the production of salicylic acid and bound hydroxylamine by *Mycobacterium phlei*. *Bacteriological Proceedings*, p. 56.
- DONALD, C., PASSEY, B. J. & SWABY, R. J. (1952). A comparison of methods for removing trace metals from microbiological media. *Journal of General Microbiology* **7**, 211-220.
- DOWNER, D. N., DAVIS, W. B. & BYERS, B. R. (1970). Repression of phenolic acid-synthesizing enzymes and its relation to iron uptake in *Bacillus subtilis*. *Journal of Bacteriology* **101**, 181-187.
- DYER, J. R., HEDING, H. & SCHAEFFER, C. P. (1964). Phenolic metabolite of 'low-iron fermentation' of *Streptomyces griseus*. Characterization of 2,3-dihydroxybenzoic acid. *Journal of Organic Chemistry* **29**, 2802-2803.
- ITO, T. & NEILANDS, J. B. (1958). Products of 'low-iron fermentation' with *Bacillus subtilis*: isolation, characterization and synthesis of 2,3-dihydroxybenzoylglycine. *Journal of the American Chemical Society* **80**, 4645-4647.
- JENSEN, H. L. (1966). Some introductory remarks on the coryneform bacteria. *Journal of Applied Bacteriology* **29**, 13-16.
- KORTH, H. (1970). Über das Vorkommen von 2,3-Dihydroxybenzoesäure und ihrer Aminosäurederivate in Kulturmedien von *Klebsiella oxytoca*. *Archiv für Mikrobiologie* **70**, 297-302.
- LECHEVALIER, H. A. & LECHEVALIER, M. P. (1967). Biology of actinomycetes. *Annual Review of Microbiology* **21**, 71-100.

- LUKE, R. H. J. (1970). Studies on the biosynthesis of 2,3-dihydroxybenzoic acid and related compounds in *Escherichia coli* K12. Ph.D Thesis, The Australian National University, Canberra, Australia.
- MCGEER, E. G., ROBERTSON, M. C. & MCGEER, P. L. (1961). Chromatographic characteristics of some aromatic compounds of biological interest. *Canadian Journal of Biochemistry and Physiology* **96**, 605–627.
- NEILANDS, J. B. (1966). Naturally occurring non-porphyrin iron compounds. *Structure and Bonding* **1**, 59–108.
- O'BRIEN, I. G. & GIBSON, F. (1970). The structure of enterochelin and related 2,3-dihydroxy-*N*-benzoyl serine conjugates from *Escherichia coli*. *Biochimica et biophysica acta* **215**, 393–402.
- PETERS, W. J. & WARREN, R. A. J. (1968). Phenolic acids and iron transport in *Bacillus subtilis*. *Biochimica et biophysica acta* **165**, 225–232.
- POLLACK, J. R. & NEILANDS, J. B. (1970). Enterobactin, an iron transport compound from *Salmonella typhimurium*. *Biochemical and Biophysical Research Communications* **38**, 989–992.
- RATLEDGE, C. (1964). Relationship between the products of aromatic biosynthesis in *Mycobacterium smegmatis* and *Aerobacter aerogenes*. *Nature, London* **203**, 428–429.
- RATLEDGE, C. (1967). The production of an *N*-acylantranilic acid from shikimic acid and the effect of iron deficiency on the biosynthesis of other aromatic compounds by *Aerobacter aerogenes*. *Biochimica et biophysica acta* **141**, 55–63.
- RATLEDGE, C. & HALL, M. J. (1970). Uptake of salicylic acid into mycobactins by growing cells of *Mycobacterium smegmatis*. *FEBS Letters* **10**, 309–312.
- RATLEDGE, C. & WINDER, F. G. (1962). The accumulation of salicylic acid by mycobacteria during growth on an iron-deficient medium. *Biochemical Journal* **84**, 501–506.
- RATLEDGE, C. & WINDER, F. G. (1966). Biosynthesis and utilization of aromatic compounds by *Mycobacterium smegmatis* with particular reference to the origin of salicylic acid. *Biochemical Journal* **101**, 274–283.
- SMITH, I. (1960). *Chromatographic and Electrophoretic Techniques*. Vol. 1: *Chromatography*. London: Heinemann.
- SNOW, G. A. (1965). Isolation and structure of mycobactin T, a growth factor from *Mycobacterium tuberculosis*. *Biochemical Journal* **97**, 166–175.
- SNOW, G. A. (1970). Mycobactins: iron-chelating growth factors from mycobacteria. *Bacteriological Reviews* **34**, 99–125.
- SNOW, G. A. & WHITE, A. J. (1969). Chemical and biological properties of mycobactins isolated from various mycobacteria. *Biochemical Journal* **115**, 1031–1045.
- WANG, C. C. & NEWTON, A. (1969). Iron transport in *Escherichia coli*: roles of energy-dependent uptake and 2,3-dihydroxybenzoylserine. *Journal of Bacteriology* **98**, 1142–1150.
- WHITE, A. J. & SNOW, G. A. (1968). Methods for the separation and identification of mycobactins from various species of mycobacteria. *Biochemical Journal* **108**, 593–597.
- ZAHNER, H., BACHMANN, E., HUTTER, R. & NUESCH, J. (1962). Sideramine, iron-containing growth factors from micro-organisms. *Pathologica et microbiologia* **25**, 708–726.