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Biosynthesis and Utilization of Aromatic Compounds by *Mycobacterium smegmatis* with Particular Reference to the Origin of Salicylic Acid

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1. Although *Mycobacterium smegmatis* could utilize a number of aromatic compounds as sole sources of carbon for growth, it did not appear to be able to use salicylic acid for growth or to metabolize it to any great extent. 2. When *M. smegmatis* was grown on shikimic acid as sole source of carbon, salicylic acid, anthranilic acid and 3,4-dihydroxybenzoic acid were released into the medium. When it was grown on quinic acid these compounds, together with *p*-hydroxybenzoic acid, *p*-hydroxyphenylacetic acid and a number of unidentified compounds, were formed. When it was grown on glucose only small amounts of salicylic acid could be detected. 3. When a washed suspension of cells with a normal iron content was incubated with shikimic acid, only small amounts of aromatic compounds were formed in the medium. When the cells were iron-deficient, substantial amounts of salicylic acid, 3,4-dihydroxybenzoic acid and catechol were formed, together with several other compounds not definitely identified. 4. When washed suspensions of cells, whether iron-sufficient or iron-deficient, were incubated with tryptophan no evidence of formation of salicylic acid, anthranilic acid or phenolic compounds was obtained. Washed suspensions did not convert anthranilic acid into salicylic acid. 5. When cell-free extracts of *M. smegmatis* were incubated with shikimic acid, or shikimic acid 5-phosphate, traces of anthranilic acid were formed under certain conditions. No formation of salicylic acid or other phenolic compound was observed even when a number of combinations of cofactors and coenzymes were tried.

Salicylic acid has been isolated from the culture filtrates of *Mycobacterium smegmatis* and *Mycobacterium tuberculosis* grown on a simple synthetic medium, and its concentration in these filtrates has been shown to be greatly increased when the medium is iron-deficient (Ratledge & Winder, 1962). This accumulation is of particular interest because it is a component, and hence probably a precursor, of mycobactin T in *M. tuberculosis* (Snow, 1965*b*), and probably of a similar mycobactin in *M. smegmatis*. Mycobactins are growth factors for *Mycobacterium johnei* and are powerful iron-complexing compounds. It has been suggested that they act as iron-trapping agents (Snow, 1965*a*,*b*), as appears to be the case with other iron-containing microbial growth factors (sideramines). Mycobactins are produced in increased amounts by mycobacteria during iron deficiency (Dhople, Morrison & Hanks, 1965), and this may enable the organism to make use of any traces of iron available.

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A plausible explanation of the increased production of mycobactin T in iron deficiency is that it is consequent upon the accumulation of a precursor, salicylic acid. Hence an understanding of the mechanism of this accumulation may provide an insight into the regulation of mycobactin production.

In this paper the results of some investigations into the origin of salicylic acid in *M. smegmatis* are presented. In the course of these investigations various observations were made on the metabolism of other aromatic compounds and, since the metabolism of aromatic compounds in mycobacteria has been little investigated, some of these results are included. Brief reports of some parts of this work have appeared (Ratledge, 1964, 1966).

EXPERIMENTAL

Organism. The strain of *M. smegmatis* used was one of unknown origin which had been used for previous studies (Ratledge & Winder, 1962).

Media and growth of bacteria. Proskauer & Beck medium

Table 1. *Reactions, chromatographic behaviour and spectra of compounds isolated from media*

Compound	Fluorescence (at 253·7 mμ)	Reaction to chromatography sprays*								Absorption maxima in ethanol (mμ)	$10^3 \times R_F$ in solvents†							
		DPNA	DSA	$FeCl_3$	$FeCl_3$ + KOH	Ehrl.	B. & M.	Gibbs	Gibbs + borax		1	2	3	4	5	6	7	8
Salicylic acid	Strong blue	Orange–yellow	Pale yellow	Violet		None	None			222 & 295·5	83	71	90	—	—	—	—	—
Anthranilic acid	Strong purple–blue	Yellow	Yellow			Lemon yellow	Imperial purple	Turquoise	Blue	246 & 333	80	62	62	72	—	—	—	—
3,4-Dihydroxybenzoic acid	Dark purple		Pink	Intense dark green	Red–black turning crimson	None				251 & 291	—	—	45	—	82	—	—	—
p-Hydroxybenzoic acid	Dark, absorbing	Strong red				None				254 (280 in 0·1N-NaOH)	—	—	65	—	—	21	—	—
p-Hydroxyphenylacetic acid	None	Blue–purple	Pink–red	None		None	None			See Table 2	See Table 2							
Catechol	Very dark purple		Grey	Grey–blue				Pink–red	Purple turning yellow	276	—	—	—	—	—	—	78	62
Unknown D	White–blue		Red–brown					Blue–purple	Imperial purple	224 & 272 (& 334?)	—	—	—	—	25	—	—	10

* Sprays: DPNA, diazotized *p*-nitroaniline; DSA, diazotized sulphanilic acid; $FeCl_3$+KOH, ferric chloride followed by 0·5N-KOH; Ehrl., Ehrlich's reagent (*p*-dimethylaminobenzaldehyde in 3%, w/v, HCl); B. & M., Bratton & Marshall diazotization reagent (0·2% $NaNO_2$ in 0·1N-HCl) with *N*-(1-naphthyl)ethylenediamine dihydrochloride as coupling reagent; Gibbs, Gibbs' reagent (2,6-dichloroquinonechloroimide); Gibbs+borax, Gibbs' reagent followed with a saturated solution of sodium tetraborate.

† Solvents: 1, butan-1-ol–pyridine–aq. NH_3 (sp.gr. 0·880)–water (4:8:5:3, by vol.); 2, butan-1-ol–pyridine–aq. NH_3 (sp.gr. 0·880)–saturated NaCl soln. (4:8:5:3, by vol.); 3, ethanol–benzene–water–aq. NH_3 (sp.gr. 0·880) (8:4:2:1, by vol.); 4, 2-methylpropan-2-ol–butan-2-one–water (4:4:2, by vol.); 5, butan-1-ol–acetic acid–water (4:1:5, by vol.); 6, propan-2-ol–aq. NH_3 (sp.gr. 0·880)–water (8:1:1, by vol.); 7, 2-methylpropionic acid–aq. NH_3 (sp.gr. 0·880)–water (66:1:33, by vol.); 8, butan-1-ol–benzene–aq.NH_3 (sp.gr. 0·880)–ethanol (5:2:2:1, by vol.).

was prepared and depleted in trace metals as described by Ratledge & Winder (1962). Metals were added to the depleted medium to give the following concentrations (per ml.): in iron-supplemented medium, 2·3μg. of Fe^{3+} and 0·46μg. of Zn^{2+}; in iron-deficient medium, 0·43μg. of Fe^{3+} and 0·46μg. of Zn^{2+}. All culture glassware was cleaned as described by Ratledge & Winder (1962). Cultures were grown, normally without shaking, at 37° in 100ml. lots of medium contained in 250ml. conical flasks. When the cultures were grown with shaking this was done at about 200 rev./min. (amplitude 1in.) in a rotary incubator–shaker (model G25; New Brunswick Scientific Co., New Brunswick, N.J., U.S.A.).

Shikimic acid medium contained: shikimic acid, 0·94g., $(NH_4)_2SO_4$, 1·4g., KH_2PO_4, 4·7g., $MgSO_4,7H_2O$, 0·94g., and deionized water to make a final volume of 1l. after the pH had been adjusted to 7·0 with KOH. The medium was dispensed in 80ml. lots into 250ml. conical flasks and autoclaved at 10lb./in.^{2} for 10min. The flasks were inoculated with a suspension prepared from a young culture of the organism. After 7 days' static growth at 37° the flasks, including the uninoculated control, were placed on the shaker at 37° and shaken for 4 days.

Quinic acid medium contained: D-quinic acid, 4·0g., glycerol, 10ml., L-glutamine, 5·0g., KH_2PO_4, 5·0g., $MgSO_4,7H_2O$, 0·4g., and deionized water to make a final volume of 1l. after the pH had been adjusted to 7·0 with NaOH. The medium, in lots of 100ml., was sterilized in 250ml. conical flasks at 5lb./in.^{2} for 15min. The flasks were inoculated as before and were allowed to grow at 37° in a static condition. Cultures were harvested after 5 or 6 days' growth.

For testing aromatic compounds for ability to support growth the following basal medium was used: $(NH_4)_2SO_4$, 1·5g.; KH_2PO_4, 5·0g.; $MgSO_4,7H_2O$, 1·0g., and deionized water to make 1l. after the pH had been adjusted to 7·0 with NaOH. The aromatic compounds were added to this at concentrations varying from 0·1 to 2·0mg./ml., and glucose was added on occasions at concentrations from 0·1 to 10mg./ml. The media, which were in lots of 10 or 20ml. contained in test tubes or small conical flasks, were autoclaved at 5lb./in.^{2} for 15min. They were inoculated with surface growth by means of a loop and incubated under stationary conditions. Growth was assessed visually.

Washed cell suspensions. Cultures were grown for 3–5 days under stationary conditions on iron-supplemented or iron-deficient Proskauer & Beck medium (Ratledge & Winder, 1962). The cells were harvested by filtration, washed with deionized water on the filter and then twice by suspending in 0·1M-phosphate buffer, pH7·0, and centrifuging. The washed cells were then suspended in 0·1M-tris buffer, pH7·6, to give a dense suspension. Substrates were added to these suspensions and they were incubated at 37° with continuous shaking.

Shikimic acid was added to give a final concentration 38mM and tryptophan to give a concentration 33mM. Controls contained no substrate. Samples for analysis were made 2% with respect to $HClO_4$, centrifuged, and the supernatants decanted. The residues were mixed with water (about twice the volume of the supernatant), placed in a boiling-water bath for 30min., centrifuged and the supernatants added to the previous ones. The supernatants were extracted with ether and chromatographed as described below. The solvents used were propan-2-ol–aq. NH_3 (sp.gr. 0·88)–water (8:1:1, by vol.) and butan-1-ol–benzene–aq. NH_3 (sp.gr. 0·88)–ethanol (5:2:2:1, by vol.).

In another series of experiments, salicylic acid or anthranilic acid was added, each at a concentration of 1mM. Samples were removed at intervals for 8hr. and assayed for salicylic acid and anthranilic acid.

Examination of growth media for aromatic compounds. The culture filtrate was concentrated to about one-tenth of its volume in a rotary vacuum evaporator, with a water-bath temperature of not more than 45°. The liquid was adjusted to pH2·5–3·5 by the addition of 10N-H_2SO_4 and was immediately extracted four or five times with its own volume of diethyl ether. The combined extracts were dried over anhydrous $MgSO_4$, filtered, and the filtrate reduced to a small volume in the rotary evaporator. Periodically aq. NH_3 soln. was introduced into the system to keep the material slightly alkaline. Chromatography of the concentrated extract was then carried out, usually as a band on Whatman 3MM or no. 17 paper. Subsequent chromatography was carried on Whatman no. 1 paper in an ascending direction and R_F values are quoted with respect to this paper and solvent direction unless stated otherwise.

A chromatogram was first viewed under u.v. light of

Table 2. *Spectra and R_F values of isolated material and authentic samples of* p-*hydroxyphenylacetic acid and* p-*hydroxyphenyl-lactic acid*

			Isolated material	*p*-Hydroxyphenyl-acetic acid	*p*-Hydroxyphenyl-lactic acid
Absorption maxima	in	0·1M-phosphate buffer, pH7·4	274–275mμ	276mμ	275mμ
	in	2ml. of 0·1M-buffer+0·8ml. of 6N-HCl	274mμ	274mμ	275mμ
	in	above solution+1·0ml. of 10N-NaOH	239mμ & 292mμ	240mμ & 294mμ	240mμ & 293mμ
R_F values	in	2-methylpropan-2-ol–butan-2-one–water (4:4:2, by vol.)*	0·56	0·58	0·60
	in	benzene–acetic acid–water (125:72:3, by vol.)†	0·80	0·80	0·90

* Run on Whatman no. 1 paper, ascending for 16hr.

† Run on Schleicher and Schull no. 2043 paper, ascending for 3hr.

wavelength 253·7 mμ; fluorescent areas were noted, and strips of the chromatogram were then sprayed with various reagents. Any prominent area was cut from the chromatogram and eluted with either redistilled aq. 95% ethanol or deionized water. When it was not intended to determine the spectrum of the material, ethanol–water (1:1, v/v) containing aq. NH_3 soln. (approx. 1·5%) was used as eluting solvent.

Spectrophotometry. The spectra were determined with a Beckman spectrophotometer, model DU, reading at intervals of not less than 5 mμ and at intervals of 2 mμ at any point of maximum or inflexion.

Chromatographic sprays. The composition of the sprays was as given by McGeer, Robertson & McGeer (1961), Dawson, Elliott, Elliott & Jones (1959), or Smith (1960).

Identification of compounds from the media. Salicylic acid, anthranilic acid, 3,4-dihydroxybenzoic acid, *p*-hydroxybenzoic acid, *p*-hydroxyphenylacetic acid and catechol were identified by chromatographic behaviour, reaction to various spray reagents and u.v. spectra. This information is detailed in Table 1. In each case the behaviour of the isolated material was the same as that of an authentic sample of the compound. Also listed in Table 1 are the properties of an unidentified material (unknown D).

The reactions and spectra of an isolated metabolite resembled *p*-hydroxyphenylacetic acid and *p*-hydroxyphenyl-lactic acid (Table 2). The two acids were resolved, however, by paper chromatography (Table 2). On this basis it was concluded that the isolated material was in fact *p*-hydroxyphenylacetic acid.

The smallest amount of anthranilic acid which could be detected on paper by its fluorescence under 253·7 mμ light was 0·02–0·04 μg., and by reaction to diazotized *p*-nitroaniline was 2·5 μg.

Assay of salicylic acid. Salicylic acid in culture supernatants was assayed as described by Ratledge & Winder (1962). In samples from the washed-cell experiments it was assayed as follows. To each 1 ml. sample was added 1 ml. of 0·5 N-$HClO_4$ and it was placed in a boiling-water bath for 20 min. The suspension was centrifuged and the supernatant was made up to 2 ml. with water. A sample (0·5 ml.) of this solution was mixed with 1 ml. of 1% (w/v) $Fe(NO_3)_3$ in 0·07 N-HNO_3 and the extinction at 530 mμ was read.

Assay of anthranilic acid. Samples were deproteinized as described above for salicylic acid. Anthranilic acid was determined in 0·5 ml. of the supernatant by using the modification of Eckert (1943) to the Bratton & Marshall (1939) diazotization reaction, *N*-(naphthyl) ethylenediamine dihydrochloride being used as coupling agent. The limit of sensitivity of this method was about 0·5 μg.

Use of cell-free extracts. Cells were grown, usually on Proskauer & Beck medium, and washed twice with 0·1 M-tris buffer, pH 8·0, and were formed into a paste by the addition of an equal volume of buffer. The material was treated with an MSE–Mullard 60 w ultrasonic disintegrator for 5–7 min. Occasionally, cells were freeze-dried, disrupted by grinding with Ballotini beads and suspended in buffer (Winder, Brennan & Ratledge, 1964). Disrupted material was immediately centrifuged at approx. 1° at 3000*g* for 15 min. On occasion, dialysis was carried out against a large volume of deionized water at 0° for 16 hr. The protein content of the extracts was determined by the biuret method (Gornall, Bardawill & David, 1949).

These extracts (0·5 ml.) were incorporated into the reaction mixture used by Srinivasan (1959) (total volume, 1 ml.), and were incubated at 37° for 2–5 hr., after which 0·5 ml. of 0·5 N-$HClO_4$ was added and the suspensions were centrifuged. The supernatants were extracted with 3 × 3 ml. of diethyl ether, the extracts were evaporated to a small volume and chromatographed. Evidence for the formation of aromatic compounds, in particular salicylic acid and anthranilic acid, was sought. In some cases the incubation mixture of Srinivasan (1959) was modified as outlined below.

Chemicals. All chemicals used were of the highest grade commercially available. Salicylic acid was recrystallized twice from water and exhibited only one spot when chromatographed in a number of solvent systems. Shikimic acid 5-phosphate was a generous gift from Dr B. Davis (Harvard Medical School, Boston, Mass., U.S.A.).

RESULTS

Attempts to demonstrate utilization of salicylic acid by M. smegmatis

Salicylic acid was added (10 μg., 100 μg. and 250 μg./ml.) to 3-day-old cultures growing with constant shaking in iron-supplemented Proskauer & Beck medium. Control cultures without the addition of salicylic acid were run simultaneously. Samples (5 ml.) were removed after 19, 49 and 91 hr. incubation and the content of salicylic acid was determined. In no case was there sufficient disappearance of the added salicylic acid to be certain that it was being metabolized by the cells. Similarly, when 1 mM-salicylic acid was incubated with a thick suspension of washed iron-supplemented cells (7·2 mg. of cell dry wt./ml.) as described earlier, the concentration of salicylic acid was unchanged after 8 hr. In this experiment chromatographic evidence for the formation of anthranilic acid from salicylic acid was also sought but none was obtained.

Attempts to grow M. smegmatis *on salicylic acid and other aromatic compounds*

Further evidence on whether *M. smegmatis* could metabolize salicylic acid was sought by attempting to grow it on this substance. The organism failed to grow during 3 weeks' incubation when inoculated (from Proskauer & Beck medium) into basal medium which contained salicylic acid (0·25, 0·5, or 1·0 mg./ml.) as the sole carbon source. When the medium contained 0·25 mg. of salicylic acid/ml. together with 0·1 mg. of glucose/ml., the slight growth obtained was not perceptibly better or worse than with glucose alone. Salicylic acid was inhibitory at 2 mg./ml. in a medium containing 10 mg. of glucose/ml.

To determine whether the organism would utilize salicylic acid after adaptation from growth on other aromatic compounds, various compounds were tried as sources of carbon for the growth of

M. smegmatis in the basal medium, either alone or accompanied by small amounts of glucose. The following compounds failed to support growth when used alone at 1mg./ml. and prevented it when used at 2mg./ml. with 10mg. of glucose/ml.: phenol, benzoic acid, catechol, resorcinol, anthranilic acid, 3-hydroxyanthranilic acid, cinnamic acid, 2-hydroxycinnamic acid, 4-hydroxycinnamic acid, kynurenine and menaphthone. The results for a number of other compounds, and for some of the above compounds at lower concentrations, are given in Table 3.

When growth occurred on any of the above media, a loopful from the surface was transferred into basal medium containing either 0·5mg. of salicylic acid/ml. as sole carbon source, or 1·0mg.

Table 3. *Growth of* Mycobacterium smegmatis *on various aromatic compounds*

Growth was assessed visually by using an arbitrary scale: −, no growth; ±, possible growth; +, poor growth; +++++, good growth.

Compound	Concn. of compound (mg./ml.)	Concn. of glucose (mg./ml.)	Growth index	
			After 5 days	After 13 days
None	—	10	+++	++++
None	—	0·5	+	++
Phenol	0·2	—	−	−
	0·2	10	+++	++++
Benzoic acid	0·2	—	++	
	0·2	10	+++	
Catechol	0·1	—	+	
	0·1	10	++	
Salicylic acid	0·1	—	−	
	0·1	10	++++	
2,3-Dihydroxybenzoic acid	0·5	—		−
	0·5	0·5		++
2,4-Dihydroxybenzoic acid	1·0	—	+	
	1·0	10	++	
2,5-Dihydroxybenzoic acid	2·0	—		−
	2·0	2·0		++
	0·5	—		−
	0·5	0·5		+++
2,6-Dihydroxybenzoic acid	1·0	—	++	
	1·0	10	++	
3,4-Dihydroxybenzoic acid	2·0	—		+++++
	1·0	—	++	
	1·0	10	+++	
4-Aminobenzoic acid	1·0	—	±	±
	1·0	10	+++	
Cinnamic acid	0·2	—	±	±
	0·2	10	+	+++
2-Hydroxycinnamic acid	0·2	—	−	−
	0·2	10	++	++++
4-Hydroxycinnamic acid	0·2	—	−	−
	0·2	10	+++	++++
Phenethyl alcohol	1·0	—	−	−
	1·0	10	+	+++
DL-Phenylalanine	1·0	—	−	−
	1·0	10	+++	++++
L-Tyrosine	1·0	—	−	−
	1·0	10	+++	++++
Naphthalene	1·0	—	+++	
	1·0	10	+++	
L-Tryptophan	0·5	—		+
	0·5	0·5		+++
5-Hydroxytryptophan	1·0	—		−
Kynurenic acid	1·0	—	+	
	1·0	10	++	
Shikimic acid	1·0	—	++	
	1·0	1·0	+++	

of salicylic acid/ml. with 1·0mg. of glucose/ml. In no case did definite growth occur on either of these salicylic acid-containing media. This indicated that salicylic acid at 0·5mg./ml. did not support growth and at 1·0mg./ml. inhibited growth on glucose even when the organism had been grown on the above aromatic compounds. After 13 days' incubation, cells from any tube which showed a suggestion of growth were transferred to a further medium which contained 0·2mg. of salicylic acid/ml. In no case was there definite growth on this further medium. Failure to grow was not due to the organisms' having been killed by their long sojourn in salicylic acid: inocula transferred simultaneously to Proskauer & Beck medium grew rapidly in all cases.

Hence benzoic acid, catechol, 2,4-dihydroxybenzoic acid, 2,6-dihydroxybenzoic acid, 3,4-dihydroxybenzoic acid, naphthalene, tryptophan, kynurenic acid and shikimic acid can act as sole sources of carbon for the growth of *M. smegmatis*, and there is no good evidence that salicylic acid can be metabolized by the organism.

Compounds accumulated in culture media filtrates

M. smegmatis grew on both quinic acid medium and shikimic acid medium, though growth was much slower and poorer than on Proskauer & Beck medium. As the analyses of the accumulated materials in both media were similar (see below) more experiments were carried out with quinic acid because of its lower cost. *M. smegmatis* was able to grow on quinic acid as sole carbon source but at a much reduced rate. Aromatic compounds, including salicylic acid, were detected in this medium. However, to achieve better growth it was found beneficial to add glycerol and glutamine to the quinic acid. When growth took place on a medium which did not contain either quinic acid or shikimic acid, i.e. on Proskauer & Beck medium, no phenolic acid other than salicylic acid was detected in the medium filtrate (Ratledge & Winder, 1962).

Compounds isolated from shikimic acid medium. The compounds identified in this medium were: salicylic acid, anthranilic acid and 3,4-dihydroxybenzoic acid. One or two unidentified compounds were also evident but neither these nor the characterized acids were observed upon examination of uninoculated shikimic acid medium. The main difference noticed between the compounds which accumulated in the shikimic acid medium and the quinic acid medium was a much larger proportion of 3,4-dihydroxybenzoic acid in the former medium.

Compounds isolated from quinic acid medium. Analyses of several separate batches of quinic acid medium after it had supported growth gave consistent results for the content of accumulated aromatic materials. The following compounds, found in each batch of quinic acid medium, were identified by chromatography and u.v. spectra: salicylic acid, anthranilic acid, *p*-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid and *p*-hydroxyphenylacetic acid. Of these, salicylic acid, anthranilic acid and 3,4-dihydroxybenzoic acid were the main components and occurred in roughly equal amounts. There had been no attempt to exclude trace metals from the medium and so it was presumed that growth took place in the presence of adequate amounts of iron.

A large number of other compounds were encountered during analysis of the quinic acid culture filtrates and although it was not possible to identify them a considerable amount of information was amassed about several of them and one of the most prominent of these unknowns is detailed below.

Unknown A. This compound exhibited a bright sky-blue fluorescence and after paper chromatography gave the following reactions: with ninhydrin a strong purple colour developed which was similar to the usual colour produced when ninhydrin is made to react with an amino acid. The reaction of the material to Ehrlich's reaction was was not well-defined, but some slight yellowing may have occurred. No reactions were given with ferric chloride, diazotized *p*-nitroaniline or Bratton & Marshall (1939) diazotization reagent. The compound was stable to acid hydrolysis (6N-hydrochloric acid at 100° for 4hr.) but was partly destroyed by alkaline hydrolysis (10N-sodium hydroxide at 100° for 2hr.), though no definite product could be located after subsequent paper chromatography. Electrophoresis of this compound indicated that it was negatively charged at pH8·9, but bore no net charge at pH4·8. Its spectra in water, in dilute acid, and in dilute alkali are given in Fig. 1 together with the spectrum of the material after it had been held for 72hr. in 1N-sodium hydroxide. The following R_F values were obtained: butan-1-ol–acetic acid–water (4:1:5, by vol.), 0·09; butan-1-ol–pyridine–aq. ammonia (sp.gr. 0·880)–water (4:8:5:3, by vol.); 0·65; propan-2-ol–aq. ammonia (sp.gr. 0·880)–water (20:1:2, by vol.), 0·00; benzene–acetic acid–water (125:72:3, by vol.), streak, mid-point 0·33; sodium formate–water–100% (v/v) formic acid (10:200:1, w/v/v), 0·50.

Supplementary iron additions to quinic acid and glucose media

No attempt had been made in the studies reported above to remove traces of iron from the shikimic acid and quinic acid media. However, on

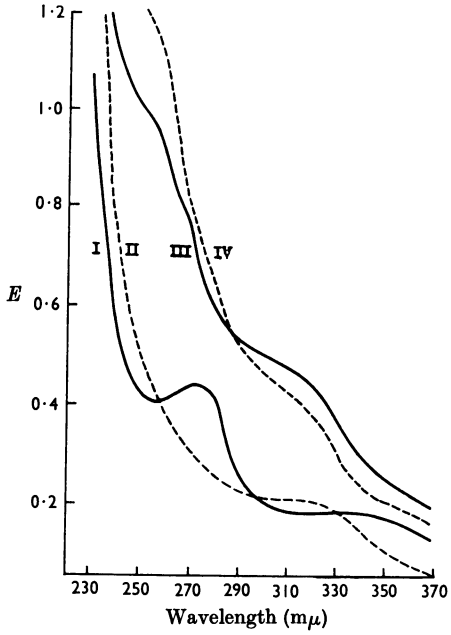

Fig. 1. Spectra of the unknown compound A: I, in water; II, in acid (1·5 ml. of aq. solution + 1·5 ml. of 1·33 N-HCl); III, in alkali (1·5 ml. of aq. solution + 1·5 ml. of 0·67 N-NaOH); IV, in alkali after 72 hr. standing (1·5 ml. of aq. solution + 1·5 ml. of 2 N-NaOH).

the basis of experience with Proskauer & Beck medium (Winder & Denneny, 1959), it seemed possible that iron deficiency might have occurred in these media. To determine whether addition of supplementary iron had any effect, an experiment was conducted in which Fe^{2+} ions at 2 μg./ml. were added to the quinic acid medium. On the basis of visual examination of chromatograms, the total amount of aromatic materials accumulating in the medium after 6 days' growth appeared to be slightly decreased when iron was added. Salicylic acid and 3,4-dihydroxybenzoic acid were the main aromatic acids present in this iron-supplemented medium.

Likewise the effect of the addition of supplementary iron to a medium containing 0·4% glucose in place of quinic acid was investigated. To half of this batch of medium Fe^{2+} ions were added at 2 μg./ml. Chromatography of extracts of these media after 6 days' growth showed that the amount of aromatic compounds formed was less than in the quinic acid medium, only salicylic acid being clearly identifiable. The addition of the supplementary iron had no obvious effect.

Products formed by washed cells

Shikimic acid, tryptophan and anthranilic acid were incubated with washed cells as described earlier and samples were taken and examined by chromatography for the presence of aromatic compounds.

With shikimic acid as substrate, and with iron-sufficient cells (17 mg. of cell dry wt./ml.), only one sample, at 18 hr., was taken. Small amounts of anthranilic acid, 3,4-dihydroxybenzoic acid and *p*-hydroxybenzoic acid were found, together with a trace of salicylic acid.

When iron-deficient cells (32 mg. of cell dry wt./ml.) were used with the same substrate, only salicylic acid and 3,4-dihydroxybenzoic acid were identified in the sample taken at 8 hr. In the sample taken after 19 hr. substantial amounts of salicylic acid, 3,4-dihydroxybenzoic acid and catechol were found, together with a compound which was possibly 2,3-dihydroxybenzoic acid. The properties of this compound (unknown B) are given in Table 4. Several unidentified materials were also present, and the properties of the best characterized of these (unknown C) are also given in Table 4. No trace of anthranilic acid, indole or tryptophan was found in either of these samples.

The control samples from washed cell suspensions of iron-sufficient and iron-deficient cells, used at the same concentrations as with shikimic acid, did not contain any recognizable aromatic compounds.

With tryptophan as substrate, and with iron-supplemented cells (17 mg. of cell dry wt./ml.), much tryptophan still remained in the sample taken after 7 hr. (as shown with Ehrlich's reagent oversprayed with 12 N-hydrochloric acid), but none could be found in the sample taken after 18 hr. Other compounds which reacted to spraying with Ehrlich's reagent, two of which resembled indole and kynurenine respectively, were present particularly in the 7 hr. sample, and a very complex mixture of unidentified fluorescent compounds was present in the 18 hr. sample. No indication of the presence of anthranilic acid, salicylic acid or other phenolic acid was obtained.

When iron-deficient cells (32 mg. of cell dry wt./ml.) were incubated with tryptophan the two samples (at 8 hr. and 19 hr.) appeared similar to each other. Both samples still contained tryptophan and again no anthranilic acid or phenolic acid was detected. Indole (or a closely related compound) was present in both samples, judging by R_F values and reaction to Ehrlich's reagent. A compound, unknown D, very similar to kynurenine was again found in the earlier of the two samples. Some of the properties of this material are given in Table 1. It also reacted with ninhydrin, turning purple; and on exposure to cyanogen bromide followed by a spray of *p*-aminobenzoic acid it became pink.

There was no evidence that anthranilic acid was metabolized at a significant rate either by iron-

Table 4. *Comparison of properties of two materials formed from shikimic acid by iron-deficient* Mycobacterium smegmatis *with 2,3-dihydroxybenzoic acid and 2,5-dihydroxybenzoic acid*

Property	Unknown B	Unknown C	2,3-Dihydroxy-benzoic acid	2,5-Dihydroxy-benzoic acid
Fluorescence	Light blue	Purple	Light blue	Light blue
R_F in butan-1-ol–benzene–aq. NH_3–ethanol (5:2:2:1, by vol.)	0·12–0·30	*	0·13	0·18
R_F in 2-methylpropionic acid–aq. NH_3–water (66:1:33, by vol.)	0·55	0·60	0·57	0·53
Colour with $FeCl_3$	Grey–blue	None	Grey–blue	Grey–blue
Colour with Gibbs' reagent	Pink	*	Pink	Brown
Colour with Gibbs' reagent + borax	Blue	*	Mauve	Brown
Colour with diazotized *p*-nitroaniline	Faint pink	Faint yellow–purple	Pink	*
$\lambda_{max.}$ in ethanol (mμ)	260, 296	262, 296	247, 317	234, 322
$\lambda_{max.}$ in water (mμ)	244, 294	256, 292	240, 307	235, 320
$\lambda_{max.}$ in acid (mμ)	248, 252, 300	260, 292	248, 314	238, 329
$\lambda_{max.}$ in alkali (mμ)	306	262, 290	330	256

* Not determined.

supplemented cells (7·2mg. of cell dry wt./ml.) or by iron-deficient cells (6·6mg. of cell dry wt./ml.): samples taken during the first 8hr. had the same concentration of anthranilic acid as at the start. No salicylic acid formation resulted from its presence.

Experiments with cell-free extracts

Because it was thought that salicylic acid might be formed from shikimic acid by steps analogous to those in the synthesis of anthranilic acid, but lacking the introduction of an amino group at some point, attempts were made to determine whether cell-free extracts of *M. smegmatis* could synthesize anthranilic acid from shikimic acid and, if so, whether they could also form salicylic acid. Many combinations of substrates and cofactors were tried. However, no synthesis of salicylic acid was demonstrated, and only occasionally was there evidence for the formation of anthranilic acid. In one experiment the extract was prepared by ultrasonic treatment of iron-deficient cells and was dialysed. It contained 29mg. of protein/ml. Incubation with the mixture used by Srinivasan (1959) was carried out for 4hr. The products of the reaction were chromatographed and a small amount of anthranilic acid was found, just sufficient to give a faint yellow reaction with diazotized sulphanilic acid. No other fluorescent spot or area which reacted with the reagent was observed. This spot was not given by extracts from control tubes containing no shikimic acid.

In another experiment the extract was prepared by grinding iron-deficient cells and contained 18·6mg. of protein/ml. The incubation mixture of Srinivasan (which normally contains glutamine, shikimic acid and ATP) was modified by using various combinations of glutamine, shikimic acid, shikimic acid 5-phosphate, 2-oxoglutaric acid, oxaloacetic acid, pyruvic acid and ATP, and incubation was for 2hr. Chromatography of the products, followed by examination under u.v. light and reaction to diazotized *p*-nitroaniline, again showed traces of anthranilic acid in all tubes containing shikimic acid or shikimic acid 5-phosphate, but more particularly when ATP and shikimic acid were used together.

These two experiments were the only ones to give any positive result for the synthesis of aromatic materials. Subsequent assay mixtures were modified to include phosphoenolpyruvic acid (Morgan, Gibson & Gibson, 1962), other phosphorylated compounds (e.g. glucose 6-phosphate, ribose 5-phosphate etc.), sodium fluoride (Gibson, Gibson, Doy & Morgan, 1962) and numerous cofactors such as CoA, pyridoxal phosphate and boiled cell extract. Other amino acids examined as donors of nitrogen besides glutamine were: asparagine, serine, phenylalanine, glycine and *N*-carboxyglycine. Incubation times up to 5hr. were sometimes used, and extracts were also examined from cells which had grown on shikimic acid or quinic acid but they still did not produce the required reactions.

Anthranilic acid (5μmoles/ml.) was also incubated with cell-free extracts in the presence of various combinations of cofactors (ATP, NAD^+, NADH, $NADP^+$, FAD, CoA, Mn^{2+}, Mg^{2+}, glutathione, pyridoxal phosphate, glutamine, yeast extract, phosphoenolpyruvic acid and boiled

enzyme) to see whether salicylic acid was formed from it. No trace of salicylic acid could be found on chromatography of the ether-soluble materials from the incubation mixture. Assays for anthranilic acid to see whether any disappeared were not carried out, but no compounds reacting with 2,4-dinitrophenylhydrazine (which would have suggested ring cleavage) were observed.

DISCUSSION

The findings in this paper support previous conclusions (Ratledge & Winder, 1962) that the utilization of salicylic acid by *M. smegmatis* is very slight, even though a number of other aromatic compounds can be used as sole sources of carbon for growth. It is unlikely that this failure is due to an inability of salicylic acid to enter the cells: *p*-aminosalicylic acid and benzoyl-*p*-aminosalicylic acid are taken up by cells of *M. tuberculosis* (Barclay, 1955; Zeyer *et al.* 1960); salicylic acid affects the respiration of some mycobacteria, which suggests penetration (Bernheim, 1941); salicylic acid escapes from *M. smegmatis* into the medium (Ratledge & Winder, 1962).

Hence, as suggested by Ratledge & Winder (1962), the explanation of the accumulation of salicylic acid in iron deficiency is to be sought in a requirement for iron for an alternative pathway of metabolism of a salicylic acid precursor, since the accumulation cannot be attributed to a requirement for iron for the metabolism of salicylic acid itself.

Several aromatic substances have to be considered as possible precursors of salicylic acid. Anthranilic acid and tryptophan are obvious choices, but we found no evidence that *M. smegmatis* converts either into salicylic acid. Where anthranilic acid oxidation has been studied in sufficient detail to determine the mechanism it is converted directly into catechol without intermediate formation of salicylic acid (Kobayashi *et al.* 1964). Naphthalene can support the growth of *M. smegmatis* and hence is metabolized by the organism. It is converted into salicylic acid by other micro-organisms (Fernley & Evans, 1958; Trecanni, 1963) and, while it itself is unlikely to arise in *M. smegmatis*, naphthaquinones are present in mycobacteria in large amounts (Asselineau & Lederer, 1964). However, some of the steps that could link naphthaquinones to salicylic acid are likely to be iron-dependent and consequently this route is unlikely to account for the accumulation of the latter acid in iron deficiency. Benzoic acid is another possible precursor of salicylic acid (Bhat, Ramakrishnan & Bhat, 1959; Voets, 1963). However, the oxidation of benzoic acid appears usually to be directly to catechol without salicylic acid as an intermediate (Taniuchi *et al.* 1964) and this direct route seems to operate in mycobacteria (Gale, 1952), but it is conceivable that in iron deficiency an intermediate, such as the postulated epoxide (Taniuchi *et al.* 1964), normally bound to the enzyme might escape and be transformed into salicylic acid. Benzoic acid has not been found in mycobacteria, but compounds which might be degraded to it (phenylalanine, phenethyl alcohol and phenylacetic acid) have been identified (Asselineau & Lederer, 1964). Snow (1965*b*) suggested that 6-methylsalicylic acid may be a precursor of salicylic acid. However, although methylsalicylic acid is metabolized by *M. smegmatis* it does not seem to give rise to salicylic acid (F. G. Winder & E. Johnson, unpublished work).

The lack of evidence for an aromatic precursor of salicylic acid led to the suggestion that it may be synthesized from a non-aromatic precursor by steps analogous to those involved in anthranilic acid formation but without the introduction of the amino group (Ratledge & Winder, 1962). The fact that growth of *M. smegmatis* on shikimic acid or quinic acid, or its incubation with these acids as a washed cell suspension, led to increased production of salicylic acid together with other aromatic compounds suggests that it arises by the shikimic acid pathway, presumably via chorismic acid (Gibson, 1964). If iron is required for one of the other pathways from chorismic acid, the accumulation of salicylic acid in iron-deficiency could be accounted for. There is no definite indication about iron-dependence of these pathways: Srinivasan & Rivera (1963) found that Fe^{2+} or Mg^{2+} ions are required in the conversion of shikimic acid 5-phosphate into anthranilic acid by extracts of *Escherichia coli*, but F. Gibson (personal communication) found that Fe^{2+} ions act as an inhibitor of anthranilic acid synthesis by extracts of *Aerobacter aerogenes*. Unfortunately, cell-free extracts of *M. smegmatis*, which might help to settle this question, appear to have a low capacity for aromatic synthesis, producing only traces of anthranilic acid with no evidence of any phenolic compounds.

Although salicylic acid is not appreciably metabolized by *M. smegmatis*, our results do not exclude its conversion into mycobactin; condensation of salicylic acid with serine to form *N*-salicyloylserine, followed by ring closure to form the 2-(*o*-hydroxyphenyl)-2-oxazoline-4-carboxylic acid moiety of the mycobactin, may be involved. Contrary to our previous report (Ratledge & Winder, 1962) 6-methylsalicylic acid, not salicylic acid, is produced by *Mycobacterium phlei* N.C.T.C. 525 (F. G. Winder, unpublished work; G. A. Snow, personal communication; A. M. Dhople & N. E. Morrison, personal communication), though some other strains of *M. phlei* produce salicylic acid

(A. M. Dhople & N. E. Morrison, personal communication). Methylsalicylic acid is incorporated into mycobactin P, the mycobactin produced by at least some strains of *M. phlei*, and Snow (1965*a*) has suggested that it originates from acetic acid, which is its precursor in fungi. If this is the case, then the mechanism by which iron deficiency brings about its accumulation is probably quite different from that operating with salicylic acid, even though in both cases the production of the corresponding mycobactin may be consequent upon the accumulation of the acid.

Of the other identified compounds from the media, it is most likely that anthranilic acid and *p*-hydroxybenzoic acid originate from shikimic acid by way of chorismic acid (Gibson & Gibson, 1964). It has been suggested that 3,4-dihydroxybenzoic acid is formed from the last intermediate lacking nitrogen in the pathway from shikimic acid to anthranilic acid (Pittard, Gibson & Doy, 1962). Its accumulation in iron deficiency is probably due to inhibition of its oxidation, which is probably carried out by a Fe^{2+} ion-dependent oxygenase, and the same probably applies to the accumulation of catechol (Mehler, 1962; Mason, 1965). Catechol could have arisen from a number of sources. *p*-Hydroxyphenylacetic acid probably arose from *p*-hydroxyphenylpyruvic acid, an intermediate in the tyrosine pathway from shikimic acid (Schwink & Adams, 1959).

Of the three unknown compounds which are partly described in this paper, unknown A appears to be an aromatic compound with an unsubstituted amino group on a side chain. Unknown B appears from spectral evidence to be a dihydroxyphenolic acid with one hydroxyl group in the 2-position. It does not, however, correspond either to any of the four dihydroxybenzoic acids or to 2,3-dihydroxybenzoylglycine (Ito & Neilands, 1958). Little can be said about unknown C except that no shifts of absorption maxima were noted in either acid or alkali.

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