pholipids in a complex mixture by successive chemical hydrolyses.

2. These extend its coverage to include phosphatidylglycerol, phosphatidylinositomannosides, the higher phosphoinositides and the alkyl ether phospholipids.

3. The recovery of individual plasmalogens is greatly improved by hydrolysing them to glyceryl-phosphoryl-base derivatives with a trichloroacetic acid–mercuric chloride reagent, which reduces the formation of cyclic acetals to a minimum.

4. Complete analyses are presented for the phospholipid distribution in human erythrocyte stroma and ox heart, liver and brain.

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The Accumulation of Salicylic Acid by Mycobacteria During Growth on an Iron-Deficient Medium

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Cultures of *Mycobacterium smegmatis* contain a number of fluorescent substances, most of which have not been identified. When growth of the organism takes place under conditions of iron deficiency the formation of a compound with a purple–blue fluorescence is particularly noticeable.

This paper describes the identification of this compound as salicylic acid, and gives the concentrations found in cultures of some species of *Mycobacterium* grown under various conditions.

Salicylic acid has been found previously in small amounts in the acetone-soluble lipid fraction of *M. tuberculosis*, where it is accompanied by phenylacetic acid (Stendal, 1934). Anisic acid, phthalic acid and phenethyl alcohol also have been isolated from lipid extracted from the same organism (Edens, Creighton & Anderson, 1944; Aebi, Asselineau & Lederer, 1953; Goris & Sebetay, 1946). There is no evidence about the origin or function of these simple aromatic substances in *M. tuberculosis*.

EXPERIMENTAL

Organisms. The strain of *Mycobacterium smegmatis* is of unknown origin (Winder & O'Hara, 1962). *Mycobacterium phlei* (N.C.T.C. 525) was obtained from the National Collection of Type Cultures.

Mycobacterium tuberculosis BCG was obtained from Dr O. Sievers, BCG Laboratory, Sahlgrens Hospital, Gothenburg S.V., Sweden.

Medium. The Proskauer and Beck medium used throughout contained: asparagine, 5·0 g.; magnesium citrate, 1·5 g.; KH_2PO_4, 5·0 g.; K_2SO_4, 0·5 g.; glycerol, 20·0 ml.; de-ionized water, 1 l. The pH was adjusted to 7·0 with 10N-NaOH for the growth of *M. smegmatis* and *M. phlei*, and to 6·8 for the growth of *M. tuberculosis*. The medium was depleted of trace metals by autoclaving with alumina, as described by Winder & O'Hara (1962). Metals were added to the depleted medium to give the following concentrations (per ml.): in normal medium, 2·3 μg. of Fe^{3+} and 0·46 μg. of Zn^{2+} ions; in iron-deficient medium, 0·43 μg. of Fe^{3+} and 0·46 μg. of Zn^{2+} ions; in zinc-deficient medium, 2·3 μg. of Fe^{3+} and 0·06 μg. of Zn^{2+} ions.

Chemicals for the medium were of analytical-reagent grade, except for L-asparagine, which was obtained from L. Light and Co. Ltd., Colnbrook, Bucks., and magnesium citrate 'true', which was obtained from British Drug Houses Ltd., Poole, Dorset. The alumina used was the chromatography grade from May and Baker Ltd., Dagenham, Essex. Iron was used as $FeCl_3,6H_2O$ and zinc as $ZnSO_4,7H_2O$.

Growth and harvesting. The culture glassware was cleaned by filling with 2% (w/v) KOH in ethanol, left overnight, rinsed with water, filled with HNO_3 (approx. 8N), left overnight, then rinsed six times with tap water and twice with de-ionized water. It was finally oven-sterilized.

Static cultures were grown at 37° as described by Winder & O'Hara (1962), except that after incubation for 24 hr. the flasks were swirled several times by hand. This decreased the variation in growth rate from flask to flask by facilitating uniform migration of bacteria to the surface. Shaken cultures were placed in a Gyrotory incubator–shaker (model G 25; New Brunswick Scientific Co. Inc., New Brunswick, N.J., U.S.A.) and shaken at 37° at 220 rev./min., amplitude 1 in. After the appropriate incubation period cells were removed from the medium by filtration through Whatman no. 1 paper.

Isolation and identification of salicylic acid. Two litres of cell-free medium that had supported growth of *M. smegmatis* for 4 days under conditions of iron deficiency were treated with 40 g. of activated charcoal (British Drug Houses Ltd.), which had been washed previously with aqueous 50% (v/v) ethanol containing 1% of ammonia. The adsorbed materials were eluted with 100 ml. of aqueous 50% ethanol–1% ammonia. The eluate was evaporated to dryness at 30–40° under reduced pressure and the residue suspended in 10 ml. of water and then shaken with 2 × 20 ml. of ethylene dichloride. The ethylene dichloride was extracted with 2 × 5 ml. of 10% Na_2CO_3,H_2O solution and this extract acidified and extracted with 4 × 5 ml. of ether. The ethereal layer, dried over anhydrous Na_2SO_4, was evaporated to a small volume and all of this was chromatographed, as a band, on Whatman 3 MM paper with butan-1-ol–benzene–aqueous NH_3–ethanol (5:2:2:1, by vol.) as solvent. The developed paper, viewed under u.v. light (λ 366 mμ), showed the presence of a purple–blue fluorescent band corresponding in R_F (0·45) and fluorescence to that given by authentic salicylic acid. This band was cut from the paper and eluted with 5 × 8 ml. of redistilled ether. The ether extract, after drying over anhydrous Na_2SO_4, was allowed to evaporate slowly. A minute amount of colourless crystals was produced; these were purified by sublimation *in vacuo* and had m.p. 153–154° (uncorr.) in a Kofler block. The mixed m.p. with an authentic sample of salicylic acid (m.p. 153–154°; uncorr.) was 153–154° (uncorr.).

When the isolated material was subjected to chromatography with pentan-1-ol–ethanol–aqueous NH_3 (15:3:1, by vol.), butan-1-ol–pyridine–aqueous NH_3–water saturated with NaCl (4:8:5:3), ethanol–benzene–water–aqueous NH_3 (8:4:2:1) or 2-methylpropan-2-ol–methanol–water (4:5:1), it gave R_F values (0·25, 0·82, 0·90 and 0·63 respectively) identical with those of authentic salicylic acid. The u.v. spectra of the isolated salicylic acid in water and in 0·05N-NaOH were the same as those of authentic material, the spectra being determined with a Beckman spectrophotometer model DU2, reading at intervals of 2 mμ. Light-absorption maxima in water for both materials were 230 and 295 mμ, and in 0·05N-NaOH were 228 and 296 mμ.

Estimation of salicylic acid. (*a*) In culture media. The method of estimation was based on that of Brodie, Udenfriend & Coburn (1944). The cell-free medium from each culture flask (originally 100 ml.) was made 2% with respect to perchloric acid. Precipitated protein was removed by centrifuging after the solution had been held at 4° for 60 min. Each batch of medium was extracted three times by vigorous shaking for 5 min. with 30 ml. of redistilled ethylene dichloride. The ethylene dichloride extract was extracted with 10 ml. of 10% (w/v) Na_2CO_3,H_2O solution.

To 1 ml. of the carbonate extract 0·4 ml. of 3N-HNO_3 and 0·25 ml. of 1% (w/v) $Fe(NO_3)_3,9H_2O$ in 0·07N-HNO_3 were added. After shaking, 3N-HNO_3 was added dropwise (one or two drops usually were needed) until the solution became slightly acid, as indicated by the disappearance of the $Fe(OH)_3$ precipitate. The volume was made up to 4·25 ml. with water, and the extinction at 530 mμ was read (with a Unicam SP. 600 spectrophotometer) against a blank that contained 1 ml. of 10% Na_2CO_3,H_2O solution, $Fe(NO_3)_3$ reagent and which was acidified with HNO_3. The amount of salicylic acid was calculated from a standard curve prepared with known amounts of the acid.

This procedure gave a quantitative extraction of salicylic acid from Proskauer and Beck medium at least over the range 0·5–15 μg./ml. The method was sufficiently sensitive to determine salicylic acid at concentrations down to 0·15 μg./ml. of culture medium. Smaller concentrations could be estimated by using an increased amount of the carbonate extract for the final colour-development procedure.

(*b*) In cells. For the liberation of salicylic acid from the cells, the procedure of Ekstrand & Sjögren (1945) was followed. The harvested cells, after being dried *in vacuo* over P_2O_5, were weighed and then mixed with 10 parts (w/v) of N-NaOH and autoclaved for 45 min. at 20 lb./in.^{2} The solutions were acidified with N-HCl to pH 1–3 and extracted with 3 × 20 ml. of redistilled ethylene dichloride; it was necessary to centrifuge to separate the two layers. The extracts were shaken with 5 ml. of 10% (w/v) Na_2CO_3,H_2O and any material that was precipitated was filtered off before the salicylic acid was determined in the aqueous solution.

The existence of compounds, other than salicylic acid, in the carbonate extracts from cells and medium that might react with the $Fe(NO_3)_3$ reagent was investigated by chromatography. The carbonate extract (2 ml.) was acidified with 5N-H_2SO_4 to pH 1–3 and extracted with 2 × 4 ml. of redistilled ether. The extract was evaporated to dryness and the residue was dissolved in six drops of ethanol and all this was chromatographed with 2-methylpropan-2-ol–methanol–water (4:5:1, by vol.) as solvent. The developed paper was sprayed with 1% (w/v) of $Fe(NO_3)_3,9H_2O$ in 0·07N-HNO_3. In no case was any material found, apart from salicylic acid, that reacted with the spray reagent.

Examination of the culture medium for the presence of phenols and phenolic acids. Cell-free medium (200 ml.) at pH 7, and not deproteinized, was extracted by vigorous shaking with ether (2 × 50 ml.). The extracted medium was acidified with H_2SO_4 to pH 3–4 and re-extracted with ether (2 × 50 ml.), and this ether extract then extracted first

with 10 ml. of 10% (w/v) Na_2CO_3 solution and then with 10 ml. of N-NaOH. Both alkaline extracts were acidified with H_2SO_4 and re-extracted with ether (3 × 5 ml.).

The three separate ether extracts from each type of culture medium were evaporated to about 0·5 ml. Duplicate samples (approx. 0·2 ml.) from each extract were chromatographed with pentan-1-ol saturated with water as solvent. The developed papers were examined first under u.v. light and then one was sprayed with 1% $Fe(NO_3)_3$ solution and the other with 0·1 N-$AgNO_2$–aqueous 5 N-NH_3 (1:1) followed by heating at 95° for 5 min.

RESULTS

Quantities of salicylic acid formed by M. smegmatis. The quantities of salicylic acid extracted from 100 ml. of culture medium that had supported the growth of *M. smegmatis* in static culture are shown in Table 1. The results of the four experiments showed good agreement. Incubated but uninoculated medium did not contain salicylic acid.

Both the bacteria grown under normal conditions and those grown under conditions of zinc deficiency released only small amounts of salicylic acid into the medium, the zinc-deficient bacteria releasing slightly more per unit dry weight than the normal bacteria. The iron-deficient bacteria released very much larger amounts of salicylic acid than either of the other two cultures.

Table 2 shows the quantities of salicylic acid that were extracted from 100 ml. of medium in which the bacteria had been grown with continuous shaking, and also the amounts that were extracted from the harvested cells. The results show that the greater part of the salicylic acid formed was released into the medium during growth. Up to the fourth day at least the concentrations reached in the medium were higher in shaken than in stationary cultures both with normal and iron-deficient bacteria, even though the respective cell yields from stationary and shaken cultures were about the same. Again the shaken iron-deficient cultures gave very much higher yields of salicylic acid than the shaken normal cultures.

Quantities of salicylic acid formed by M. phlei *and* M. tuberculosis. Table 3 shows the quantities of salicylic acid extracted from the cells and from 100 ml. of culture media of *M. phlei* and *M. tuberculosis* BCG grown in normal, in iron-deficient and in zinc-deficient media held stationary throughout the period of growth, and harvested at the times shown. The concentrations of salicylic acid reached in the normal and in the zinc-deficient culture media were similar to those attained in the corresponding unshaken cultures of *M. smegmatis*. Again iron-deficient cultures gave substantially the greatest yields of salicylic acid/g. of cells dry wt. However, neither *M. phlei* nor *M. tuberculosis* accumulated as high a concentration of salicylic acid as did *M. smegmatis* in iron-deficiency. Again, particularly when larger amounts of salicylic acid had been formed, the greater part of this was released into the medium.

Absence of phenols and other phenolic acids in the culture medium. It was decided to determine whether the accumulation of salicylic acid had been brought about by a prior accumulation of either catechol or gentisic acid (2,5-dihydroxybenzoic acid). Both of these materials have been shown

Table 1. *Salicylic acid in culture media after surface growth of* Mycobacterium smegmatis *under normal, iron-deficient and zinc-deficient conditions*

Normal medium: 2·3 μg. of iron and 0·46 μg. of zinc/ml. Medium A (iron-deficient medium): 0·43 μg. of iron and 0·46 μg. of zinc/ml. Medium B (zinc-deficient medium): 2·3 μg. of iron and 0·06 μg. of zinc/ml. Means are given ± S.E.M., with the numbers of determinations in parentheses.

Age of culture (days)	Growth medium	Yield of salicylic acid (μg./100 ml. of medium)	Cell yield (mg. dry wt./100 ml. of medium)	Salicylic acid/unit dry wt. (μg./mg. of cells dry wt.)
2	Normal	16·7 ± 3·3 (3)	167 ± 22 (3)	0·100
	A	32·5 ± 4·5 (4)	88 ± 9·3 (3)	0·369
	B	21·3 ± 1·4 (3)	85 ± 2·3 (3)	0·250
3	Normal	48·0 ± 2·7 (3)	369 ± 45 (4)	0·130
	A	465 ± 24 (3)	211 ± 2 (4)	2·200
	B	37·0 ± 6·3 (3)	138 ± 8 (4)	0·268
4	Normal	59·8 ± 8·1 (4)	733 ± 44 (4)	0·082
	A	999 ± 34 (4)	247 ± 35 (4)	4·050
	B	37·3 ± 5·8 (4)	291 ± 18 (4)	0·128
5	Normal	56	780	0·072
	A	1680	400	4·200
	B	58	370	0·157
6	Normal	52	—	—
	A	2280	—	—
	B	34	—	—

Table 2. *Salicylic acid in the cells and media after growth of* Mycobacterium smegmatis *in shaken culture under normal and iron-deficient conditions*

Bacteria were grown in stationary culture for the first day and thereafter shaken on a rotary shaker. Normal medium: 2·3 μg. of iron and 0·46 μg. of zinc/ml. Medium A (iron-deficient medium): 0·43 μg. of iron and 0·46 μg. of zinc/ml. Salicylic acid was extracted from 100 ml. of culture medium and from the amount of cells yielded by 100 ml. of medium.

Age of culture (days)	Growth medium	Cell yield (mg. dry wt./ 100 ml. of medium)	Salicylic acid extracted (μg.)		Total yield of salicyclic acid from medium and cells (μg./g. of cells dry wt.)
			From medium	From cells	
1	Normal	<10	<5	—	—
	A	<10	<5	—	—
2	Normal	156	60	17	494
	A	60	157	8	2740
3	Normal	398	127	26	384
	A	294	1260	112	4670
4	Normal	604	123	24	244
	A	254	1420	82	5920
8	A	205	931	35	4720

Table 3. *Salicylic acid in the cells and culture media after surface growth of* Mycobacterium phlei *and* Mycobacterium tuberculosis *BCG under normal, iron-deficient and zinc-deficient conditions*

Normal medium: 2·3 μg. of iron and 0·46 μg. of zinc/ml. Medium A (iron-deficient medium): 0·43 μg. of iron and 0·46 μg. of zinc/ml. Medium B (zinc-deficient medium): 2·3 μg. of iron and 0·06 μg. of zinc/ml. Salicylic acid was extracted from 100 ml. of culture medium and from the cells yielded by 100 ml. of medium.

Organism	Age of culture (days)	Growth medium	Cell yield (mg. dry wt./ 100 ml. of medium)	Salicylic acid extracted (μg.)		Total yield of salicylic acid from medium and cells (μg./g. of cells dry wt.)
				From medium	From cells	
M. phlei	10	Normal	508	29	15	87
		A	142	156	15	1200
		B	58	13	5	310
	13	Normal	633	104	17	191
		A	220	594	9	2740
		B	120	45	19	534
M. tuberculosis BCG	18	Normal	741	94	41	182
		A	142	194	61	1800
		B	116	15	28	370
	23	Normal	669	105	28	199
		A	174	270	45	1810
		B	167	41	13	324

not only to be intermediates of salicylic acid metabolism by other micro-organisms, but also, in some cases, to depend on iron for their subsequent metabolism (see Discussion). In no case has a dependence on iron been reported for the first step of salicylic acid metabolism.

Analysis of the culture media for the presence of phenols, in particular catechol, and dihydroxybenzoic acids, in particular gentisic acid, was carried out on cultures of *M. smegmatis* that had been grown for 4 days under normal, iron-deficient and zinc-deficient conditions and which had been held stationary throughout growth.

Examination did not reveal the presence of any material that corresponded to either a phenol or dihydroxybenzoic acid. Salicylic acid only was detected in the ether extracts of acidified culture medium.

DISCUSSION

The increased accumulation of salicylic acid that takes place in iron-deficient cultures of *M. smegmatis* must be presumed to be due to a requirement for iron for one of the next steps in the utilization of salicylic acid or of a proximate precursor, whereas it is not required or a lower concentration will suffice for the steps leading to its formation.

Although mycobacteria oxidize benzoic acid, protocatechuic acid and catechol (Fitzgerald, Bernheim & Fitzgerald, 1949; Wagner, 1951;

Sistrom & Stanier, 1953), they do not appear to metabolize salicylic acid. Several workers have found that salicylic acid can neither act as a growth source for these organisms nor be oxidized by them, even after growth on such substrates as benzoic acid or catechol (Lehmann, 1946; Fitzgerald & Bernheim, 1947, 1948; Wagner, 1951; Gale, 1952). Preliminary attempts to show metabolism of salicylic acid by *M. smegmatis*, though not conclusive, support these reports (C. Ratledge & F. G. Winder, unpublished work).

No trace of gentisic acid or catechol could be found on examination of the culture media, a finding which in the light of what is already known about the metabolism of salicylic acid by other micro-organisms would support the idea that salicylic acid is not metabolized by mycobacteria. Of the three different pathways for salicylic acid oxidation in other micro-organisms, the product before ring cleavage occurs would appear to be either gentisic acid (Yano & Arima, 1958; Henderson, 1961) or catechol arising either by direct oxidation (Walker & Evans, 1952; Roof, Lannon & Turner, 1953) or through the intermediary formation of 2,3-dihydroxybenzoic acid (Shepherd & Villanueva, 1959). A dependence on iron has been demonstrated with enzymes responsible for ring cleavage of gentisic acid and catechol (Hayaishi, Katagiri & Rothberg, 1957; Sugiyama, Yano, Tanaka, Komagata & Arima, 1958; Dagley, Evans & Ribbons, 1960), but not for the immediate metabolism of salicylic acid. Thus Henderson (1960) found an accumulation of catechol, together with decreased utilization of salicylic acid, in iron-deficient fungi incubated with salicylic acid. Therefore the apparent absence of gentisic acid, catechol and other phenols and phenolic acids from the iron-deficient culture medium of *M. smegmatis* makes it difficult to hold that salicylic acid is normally metabolized by these cultures unless a new iron-dependent enzyme for the direct ring cleavage of salicylic acid is assumed. Such an enzyme would be analogous to one of the known ring-splitting enzymes among which pyrocatechase, homogentisicase and, in certain cases, gentisicase, protocatechuic acid 4,5-oxygenase and catechol 2,3-oxygenase are known to be iron-dependent (Suda, Hashimoto, Matsuoka & Kamahora, 1951; Suda & Takeda, 1950; Dagley *et al.* 1960).

If salicylic acid were not metabolized, this would imply that there is some precursor of salicylic acid whose metabolism to products other than salicylic acid is dependent on the presence of iron. The number of possible precursors of salicylic acid is limited in that it does not appear to be an intermediate in the bacterial metabolism of major aromatic cellular components such as phenylalanine, tyrosine or tryptophan (Evans, 1956), or of *p*-aminobenzoic acid (Sloane, Samuels & Mayer, 1954). Salicylic acid arises in the metabolism of naphthalene (Treccani, Walker & Wiltshire, 1954; Klausmeier & Strawinski, 1957) and of *o*-methoxybenzoic acid (Farmer, Henderson & Russell, 1959). It has been suggested also that it arises in the oxidation of benzoic acid by *Pseudomonas convexa* (Bhat, Ramakrishnan & Bhat, 1959), though direct oxidative decarboxylation to catechol in a single step seems to be the usual route of metabolism (Evans, 1956). These possible precursors have not been found in mycobacteria, though they may be present in low concentrations, but compounds related to them such as phenylacetic acid (Stendal, 1934), phenethyl alcohol (Goris & Sebetay, 1946), phthalic acid (Aebi *et al.* 1953) and naphthaquinones (Noll, Rüegg, Gloor, Ryser & Isler, 1960), which likewise might be metabolized to salicylic acid, have been isolated. However, nothing is known about the iron requirements for the metabolism of these substances.

There remains the possibility that salicylic acid is synthesized directly from a non-aromatic precursor such as shikimic acid by steps analogous to those involved in anthranilic acid formation (Srinivasan & Weiss, 1961), but with the introduction of a hydroxyl group instead of an amino group, and that this reaction is accentuated during iron deficiency possibly by a requirement for iron by a competing pathway.

SUMMARY

1. Salicylic acid has been isolated from and identified in the culture medium of *Mycobacterium smegmatis* grown on Proskauer and Beck medium that originally had contained no aromatic compounds.

2. During surface growth of *M. smegmatis*, *M. tuberculosis* BCG and *M. phlei* on full medium (2·3 μg. of iron and 0·46 μg. of zinc/ml.), salicylic acid accumulated in the medium at 100–160 μg./g. of cells dry wt. Whereas only slightly more salicylic acid accumulated during growth on zinc-deficient medium (0·06 μg. of zinc/ml.), with iron-deficient medium (0·43 μg. of iron/ml.) 10–50 times the normal concentration accumulated, being greatest with *M. smegmatis* at 4·2 mg./g. of cells dry wt.

3. The accumulation of salicylic acid was slightly increased when cultures of *M. smegmatis* were shaken continuously.

4. In all cases the amount of the acid found on hydrolysis of cells was small, the majority being released into the medium during growth.

5. The presence of other phenolic acids or phenols was not revealed on examination of the culture media of *M. smegmatis*.

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Lipid Metabolism in the Normal and Vitamin B_{12}-Deficient Chick Embryo

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From time to time reports have appeared in the literature (reviewed by Arnstein, 1955, 1958) suggesting that vitamin B_{12} might play some part in the metabolism of lipids by animals. Since it has been established that vitamin B_{12} functions in the synthesis of labile methyl groups from more highly oxidized one-carbon-atom compounds (Arnstein, 1958), in the utilization of the α-carbon of glycine for the synthesis of ethanolamine (Stekol, Weiss & Weiss, 1952) and in the conversion of formate into serine (Arnstein, 1958), an association between vitamin B_{12} and phospholipid synthesis is self-evident. These facts led to a study in this Laboratory of the effect of vitamin B_{12} on the lipid metabolism of the chick embryo. The chick embryo seemed to present a suitable organism in which to study this relationship in view of the intensive lipid metabolism that occurs during the last week of incubation. In addition, Ferguson Rigdon & Couch (1955) had already reported histological evidence for the occurrence of fatty livers in the vitamin B_{12}-deficient chick embryo. Preliminary experiments (Moore & Doran, 1961) did not support these findings, nor did they provide any evidence for a decreased phospholipid synthesis in the livers of vitamin B_{12}-deficient chick embryos. However,