sulphate which is 4-chloro-2-hydroxyphenylsulphuric acid.

3. Similarly, 4-chlororesorcinol forms 78% of 4-chloro-3-hydroxyphenylglucuronide, characterized as 4-chloro-3-methoxyphenylglucuronamide. Some 24% is excreted as ethereal sulphate.

4. The orientation of conjugation in these two compounds is discussed, and it appears that conjugation takes place at the hydroxyl group *para* to the chloro group.

5. (+)-Adrenaline forms 20% of glucuronide but practically no ethereal sulphate. The results suggest that (+)-adrenaline is conjugated on one of its phenolic hydroxyl groups.

6. With protocatechuic acid, some 30% is conjugated and the rest is excreted unchanged.

7. Protocatechuic aldehyde forms two glucuronides, is more highly conjugated than the acid and is largely transferred to protocatechuic acid and its conjugates.

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Occurrence of Cytochrome and Coproporphyrin in Mycobacteria

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Yaoi & Tamiya (1928) and Fujita & Kodama (1934) observed a four-banded spectrum in tubercle bacilli, the bands corresponding closely to those of reduced cytochrome in yeast or heart muscle. Frei, Riedmüller & Almasy (1934) confirmed the description in the case of *Mycobacterium tuberculosis hominis*, but observed a three-banded spectrum lacking component c in strains of *Mycobact. avium* and *Mycobact. tuberculosis bovis*. In this laboratory it has been noticed that the absorption bands visible in suspensions of acid-fast saprophytes vary in different species, some of which exhibit a distinct narrow band at 624 mμ. This band could be attributed to the presence of cytochrome a_2; alternatively, it could be due to free porphyrin, the other bands of which are obscured by the cytochrome spectrum.

Fischer & Fink (1925) extracted from heat-killed tubercle bacilli a pigment that was identified spectroscopically as a metal complex of coproporphyrin (bands centred at 522·6 and 560·4 mμ.). The spectrum of free coproporphyrin was not visible. The same authors, working with extracts of crude tuberculin, observed intense absorption bands attributed to protoporphyrin but 'only a very weak coproporphyrin spectrum'. Since the culture media were not known to be free from porphyrin initially, Fischer & Fink (1925) did not regard their results as conclusive evidence of porphyrin formation by tubercle bacilli. The occurrence of porphyrin in acid-fast bacteria was demonstrated unequivocally by Dhéré, Glücksmann & Rapetti (1933), who observed the fluorescence spectrum of coproporphyrin in cells of *Mycobact. smegmatis*, *Mycobact. ranae*, *Mycobact. tuberculosis hominis* and *Mycobact. tuberculosis bovis* (strain B.C.G.).

The work described in this paper, originally undertaken with the object of elucidating the atypical spectra of certain mycobacteria, led to the isolation of coproporphyrin III.

METHODS

Organisms. The following were used: *Mycobact. smegmatis* (no. 523), *Mycobact. phlei* (no. 525), *Mycobact.* sp. *Karlinski* (no. 2071), *Mycobact. ranae* (no. 2891) and *Mycobact. stercoris* (no. 3820). The numbers quoted are the catalogue numbers of the National Collection of Type Cultures, Lister Institute, London. The organisms were cultivated at 38° on meat infusion broth containing 2% (w/v) peptone and 5% (v/v) glycerol. Cultures were harvested on the seventh or eighth day, and washed thrice with distilled water (by centrifuga-

Table 1. *Absorption bands observable in suspensions of mycobacteria*

(Thickness of suspension, 1 cm. Temp., 15–18°. Presence or absence of each band is denoted by + or − respectively.)

	Absorption bands					
Band no.	1	2	3	4	5	6
Centre of band (mμ.)	624	598	570	564	552	526
Organism						
Mycobact. phlei (glycerinated broth)	−	+	−	−	−	−
Mycobact. phlei (non-glycerinated broth)	−	+	−	+	+	+
Mycobact. ranae	−	+	−	+	+	+
Mycobact. Karlinski	+	+	+	+	+	+
Mycobact. smegmatis	+	+	+	+	+	+
Mycobact. stercoris	+	+	+	+	+	+

tion) before use in experiments which required intact organisms. Cultures used for extraction of porphyrin were harvested at the same age by decanting the broth. Bulked cultures were then washed thoroughly with several changes of water, transferred to a linen bag and pressed into a cake.

Spectroscopic examination of bacteria. The moist bacterial mass obtained by centrifuging was packed into an optical cell (internal width, 1 cm.) and illuminated by a 250 W. Philips projection lamp adequately housed. Protection against heat was afforded by a hollow glass cube (side, 3 cm.) filled with water and put between the light source and the optical cell. A small condenser lens was used to concentrate light on the cell. The spectra were observed with a direct vision spectroscope (Schmidt & Haensch) of small dispersion equipped with a wavelength scale. The density of the suspension was adjusted by dilution with water until the definition of the bands was optimal.

Spectrophotometric measurements. Porphyrin solutions were examined in a Beckman quartz spectrophotometer, DU model. The absorption curve of coproporphyrin tetramethyl ester was determined in $CHCl_3$ solution. The ester was hydrolysed by standing in conc. HCl and the absorption curve of the free porphyrin determined in 0·15N-HCl (see Jope & O'Brien, 1945). The extinction coefficients of the free porphyrin ($E^{1\%}_{1\,cm.}$) were measured at 401 and at 548 mμ., the respective slit widths being 0·04 mm. (≡0·5 mμ. at λ = 400 mμ.) and 0·03 mm. (≡0·9 mμ. at λ = 550 mμ.).

RESULTS

Absorption spectrum of acid-fast saprophytes

The absorption bands seen in thick suspensions of five saprophytic mycobacteria are listed in Table 1. *Mycobact. Karlinski* and *Mycobact. stercoris* consistently exhibit a sharply defined narrow band (1) at 624 mμ. This band is not present in all cultures of *Mycobact. smegmatis*, and it is absent from the spectra of *Mycobact. phlei* and *Mycobact. ranae*.

Band 2, which is regularly present, extends from 592 to 603 mμ. Stretching from 551 to 568 mμ. there is a zone of absorption which contains two dense bands centred at 552 mμ. (5) and at 564 mμ. (4) respectively. A fainter band (3), centred approximately at 570 mμ., is seen only in *Mycobact. Karlinski* and *Mycobact. stercoris*. Band 6, situated at 520–535 mμ. is partially obscured by end absorption. Bands 2, 4 and 5 were sharply defined in preparations which had been stored at −40°.

When *Mycobact. phlei* is cultivated in glycerol broth it is so deeply pigmented with lipochromes that only one absorption band (2) can be identified; but when the organism is grown on plain broth it is possible to define bands 2, 4, 5 and 6. These results indicate that bands 2, 4 and 5 occupy the positions of the α-bands of cytochromes *a*, *b* and *c*, while band 6 probably includes the β-bands of the *b* and *c* components (Keilin & Hartree, 1939). The intensity of the blue end absorption made it difficult to search for the γ-bands of the cytochromes.

Behaviour of the absorption bands

Effect of aeration. When oxygen was bubbled through suspensions of *Mycobact. Karlinski* and *Mycobact. stercoris*, bands 2, 4, 5 and 6 disappeared, only to reappear after a few minutes of anaerobiosis. Bands 1 and 3 were unaffected, which suggests that they do not belong to a cytochrome complex.

The same phenomena were observed with *Mycobact. smegmatis*, but prolonged aeration was necessary in order to oxidize the cytochromes. Filtered, moist air was passed for 18 hr. through a sterile suspension kept at 37° in a thermostat. Spontaneous reduction at room temperature was slow, but the reduction time was diminished significantly by addition of lactate or malate (Table 2).

Table 2. *Reduction of cytochrome* (Mycobacterium smegmatis)

(Sterile suspension previously aerated for 18 hr. Reduction time assessed by visual observation of intensity of absorption bands.)

Substrate (neutral Na salt, 0·1M)	Reduction time at 18° (min.)
None	30–40
DL-Lactate	14
L-Malate	15

Oxidation and reduction. The cytochrome bands (2, 4, 5 and 6) disappeared on addition of potassium ferricyanide (two drops of saturated solution/2 ml. bacterial suspension) in all species and reappeared after treatment with a small quantity of solid sodium dithionite ($Na_2S_2O_4$). Bands 1 and 3 (where present) were unaffected by these reagents; nor did these bands change after addition of ammonium sulphide in the absence of ferricyanide (see Fujita & Kodama, 1934).

Effect of oxygen in presence of cyanide. Bands 1 and 3 of *Mycobact. Karlinski* and *Mycobact. smegmatis* were unchanged when oxygen was bubbled through suspensions containing 0·002M-hydrogen cyanide. Bands 2, 4, 5 and 6 persisted in the presence of oxygen and cyanide, behaving like typical cytochrome bands. The experiment shows that band 1 at 624 mμ. is not due to cytochrome a_3 (see Negelein & Gerischer, 1934; Keilin, 1934; Fujita & Kodama, 1934).

Preparation of pyridine haemochromogen. In suspensions of *Mycobact. ranae*, *Mycobact. Karlinski*, *Mycobact. smegmatis* and *Mycobact. stercoris* the cytochrome spectrum was replaced by that of pyridine haemochromogen (dense band at 548–562 mμ. and weaker one at 525 mμ.) following treatment with 2N-potassium hydroxide, a few drops of 10% (w/v) aqueous pyridine solution and solid $Na_2S_2O_4$, added successively. Bands 1 and 3 were unaltered.

Extraction with acetone. When a heavy suspension of *Mycobact. Karlinski* was extracted with acetone, bands 1 and 3 disappeared from the spectrum, although bands 2 and 4 remained visible in moistened acetone powder. After dilution with water the acetone extract was acidified with acetic acid and shaken with ether. The ether layer was faint purplish red, gave a brilliant red fluoresence under a Wood lamp and showed a porphyrin spectrum with bands at 624, 570, 528 and 500 mμ.

These experiments show that the anomalous features of the absorption spectrum encountered in some species of mycobacteria are due to superposition of a porphyrin spectrum upon that of a cytochrome complex containing *a*, *b* and *c* components. Attempts to resolve band 2 into *a* and a_3 components by the use of carbon monoxide (Keilin & Hartree, 1939) were inconclusive.

Isolation of coproporphyrin from Mycobacterium Karlinski

Extraction. Since the band at 624 mμ. was most intense in *Mycobact. Karlinski*, this organism was chosen for an attempt to isolate the porphyrin. Each batch of washed organisms, pressed into a cake weighing about 500 g., was stirred into 500 ml. of cold acetone for 15 min. and filtered on a Büchner funnel. Extraction and filtration were repeated, once with 500 ml. and twice with 250 ml. of acetone, and the several filtrates combined.*

The acetone extract was diluted with 2 volumes of water, acidified with glacial acetic acid (3% of the total volume) and extracted four times with ether. The combined ethereal extracts were shaken with 1·4N-hydrochloric acid, the ether separated, and the porphyrin stored in the acid solution until sufficient had been collected. Prior to storage residual ether was removed *in vacuo* at room temperature. The original acetone extract contained substantial amounts of lipids, which, being ether soluble, were largely removed during the transference of the porphyrin to the acid.

Preliminary purification. In order to remove persistent traces of colourless lipid, the hydrochloric acid solution, representing extraction of 4·8 kg. of bacterial press cake, was neutralized to congo red with solid sodium acetate, acidified with 0·05 volume of glacial acetic acid and the porphyrin transferred to ether. Transference between ether and hydrochloric acid was repeated four times in the usual way until the whole of the porphyrin was concentrated into 150 ml. of 1·4N-hydrochloric acid. Again residual ether was removed *in vacuo* at room temperature.

Sodium hydroxide (30%) was added slowly to bring the solution to pH 3·3 when the porphyrin flocculated. After standing overnight it was separated by centrifugation, dried *in vacuo* over concentrated sulphuric acid, dissolved in 15 ml. of methanol previously saturated with dry hydrogen chloride gas and kept in the ice box for 24 hr. The solution of methyl ester was poured into 150 ml. of chilled water, and the solution extracted repeatedly with small volumes of chloroform. The chloroform solution was washed twice with water, once with 2N-ammonium hydroxide, and then three times with water. After filtration through a paper moistened with chloroform, the solution was evaporated to dryness *in vacuo*.

Chromatographic purification. The crude ester, dissolved in a mixture of benzene (15 ml.) and light petroleum (5 ml.), was adsorbed on a calcium carbonate column (1·5 × 15 cm.) according to the method of Grinstein, Schwartz & Watson (1945). A broad purple band of coproporphyrin methyl

* The completeness of acetone extraction was demonstrated by subsequent treatment of the dry powder from which acetone had been removed *in vacuo*. A sample was extracted with acetic acid and ether, and the ether shaken with a small volume of 2% (w/v) hydrochloric acid. Porphyrin could not be detected in the acid layer either spectroscopically or by fluorescence in ultraviolet light. The success of acetone extraction may depend on the solubility of porphyrin in acetone-soluble lipids of the bacteria.

ester, which migrated slowly down the column, was eluted with the original solvent mixture. The ester was evaporated to dryness *in vacuo* at room temperature, crystallized from anhydrous ether and the crystals dried thoroughly *in vacuo* over concentrated sulphuric acid and paraffin wax.

Since a small amount of pigment remained on the upper and middle zones of the calcium carbonate, the column was cut and both zones extracted with chloroform. The upper zone yielded a quantity of coproporphyrin too small to permit isolation. The middle zone released a brownish red pigment (single absorption band at 645 mμ.) which may be identical with a decomposition product of coproporphyrin described by Rimington (1939). The crystalline coproporphyrin ester was taken up in benzene and chromatography repeated by the method of Gray & Holt (1948). The eluate was evaporated to dryness *in vacuo* at room temperature, and the coproporphyrin ester twice recrystallized from anhydrous ether as before. Yield, 16 mg.

Properties of the crystalline ester. Dark red crystals, assuming the rosette pattern of coproporphyrin III tetramethyl ester (Rimington, 1939). Sharp m.p. at 150°; after cooling, remelt at 172–174°. HCl number, 1·5. Absorption curve (chloroform solution) in the visible region conformed to a 'step-ladder' type with the chief band maxima at (I) 623·5, (II) 567, (III) 534 and (IV) 498·5 mμ. Relative intensities, IV > III > II > I.

Properties of the free porphyrin. Solutions in hydrochloric acid and in ether had the characteristic violet colour of coproporphyrin and gave a strong red fluorescence in ultraviolet light. HCl number, 0·1. Absorption curve in 0·15N-hydrochloric acid showed three bands with maxima at (I) 591, (II) 548 and (III) 401 mμ. Relative intensities, III > II > I. In 0·15N-hydrochloric acid, $E^{1\%}_{1\,cm.} = 7400$ at 401 mμ. and 243 at 548 mμ.

These data show that the crystalline material is a fairly pure specimen of coproporphyrin III tetramethyl ester. The extinction coefficients are about 9% lower than the maximum values quoted by Jope & O'Brien (1945) for recrystallized specimens of coproporphyrin methyl ester, both natural and synthetic. The melting points are within the range recorded for natural specimens.

Coproporphyrin content of mycobacteria

The coproporphyrin content of *Mycobact. Karlinski* was determined by extracting 22 g. of bacterial cake with acetone, transferring the pigment quantitatively through ether to 0·15N-hydrochloric acid and measuring log I_0/I at 548 mμ. Two transfers between ether and 5% (w/v) hydrochloric acid preceded the final concentration in 0·15N-hydrochloric acid. The organism contained 5–8 mg. of coproporphyrin/kg. of bacteria, depending on the batch.

Protoporphyrin was not encountered at any stage during the isolation. If metal complexes or uroporphyrin were present, these, too, escaped detection.

In the course of this work it was found that the acetone powder contains an unstable haem pigment (possibly derived from cytochrome) which is incompletely removed by acetone-hydrochloric acid. This pigment has a single absorption band at 545–555 mμ. and is readily converted into a pyridine haemochromogen.

Other species. The methods employed failed to extract coproporphyrin from *Mycobact. phlei* and *Mycobact. ranae.* Small quantities were obtained from *Mycobact. smegmatis*; *Mycobact. stercoris* contains considerably more than *Mycobact. smegmatis*, though less than *Mycobact. Karlinski.* These findings were consistent with spectroscopic observations on the intact organisms.

Origin of coproporphyrin

Each 400 ml. nutrient broth was extracted with acetic acid-ether and hydrochloric acid before inoculation to ensure that it was free from coproporphyrin. Spectroscopic and fluorescence tests did not reveal the presence of porphyrin. Tests on the broth for haematin by the conversion to pyridine haemochromogen were also negative.

The ability of *Mycobact. Karlinski* to synthesize coproporphyrin was demonstrated by cultivating the organism in a synthetic medium composed of a basal salt mixture (Edson & Hunter, 1943) to which were added lactate (1%), glycerol (0·5%) and sodium citrate (0·001M; final concentrations). The porphyrin bands at 624 and 570 mμ., accompanied by those of the cytochrome spectrum, were seen in the bacteria, and acetone extracted the usual quantity of coproporphyrin.

DISCUSSION

Since the mycobacteria are strictly aerobic, exhibition of the four absorption bands of a typical cytochrome is to be expected. Indeed, Fujita & Kodama (1934) have classified the tubercle organism, along with *Bacillus subtilis* and other aerobic bacteria which possess *a*, *b*, *c* and *d* bands. The observations described in this paper show that *Mycobact. phlei* and *Mycobact. ranae* conform to group I of Fujita & Kodama's classification, whereas *Mycobact. Karlinski*, *Mycobact. smegmatis* and *Mycobact. stercoris* present a composite spectrum containing bands at 624 and 570 mμ. in addition to those of the typical cytochrome components.

A tentative hypothesis that the band at 624 mμ. represented cytochrome a_2 was disproved by experiments, all of which indicate that neither this band nor the band at 570 mμ. can be due to cytochrome components. *A priori* it was unlikely that the

organisms would contain cytochrome a_2, because this pigment has been found only in association with cytochromes a_1 and b_1 (Fujita & Kodama, 1934; Keilin, 1934; Keilin & Harpley, 1941), which are absent from the mycobacteria in question.

Successful extraction of coproporphyrin has explained the origin of the anomalous bands. In consequence of an unusually high intracellular concentration of coproporphyrin two porphyrin bands are visible, although the remainder are probably obscured by the intensity of the *c* and *d* cytochrome bands and by end absorption. The positions of the two visible porphyrin bands indicate that coproporphyrin may be present as neutral salt or as a natural ester, but not as a metal complex.

Coproporphyrin III has been isolated in the form of a tetramethyl ester, the properties of which suggest that it may be contaminated by a small amount of coproporphyrin I. It was suspected that a small quantity of coproporphyrin I (unidentified) had separated on the top of the column during chromatographic purification of the bacterial extract. Although coproporphyrin is known to occur in a variety of bacteria, yeasts and fungi (see Vannotti, 1937), isolation of characterized methyl esters has seldom been reported. Crystalline coproporphyrin esters were obtained by Fischer & Fink (1925) from *Saccharomyces anamensis* and by Kench & Wilkinson (1945), who obtained both natural isomers from brewer's yeast.

The presence of porphyrin in culture filtrates from *Corynebacterium diphtheriae* has attracted attention ever since Coulter & Stone (1931) demonstrated proportionality between toxin production and porphyrin concentration. Pappenheimer (1947) and Pappenheimer & Hendee (1947) have shown that the relationship can depend on the iron content of the medium, and have suggested a connexion between porphyrin and cytochrome *b*, the chief haem pigment observable spectroscopically in suspensions of *C. diphtheriae*. Gray & Holt (1948) have isolated and characterized the porphyrin as coproporphyrin III, which is accompanied by smaller amounts of uroporphyrin I in culture filtrates.

The significance of a substantial production of coproporphyrin by some mycobacteria, and not by others, is not clear. The possibility of a connexion with cytochrome requires exploration. Preliminary experiments with *Mycobact. Karlinski*, grown on synthetic media, indicate that specific organic nutrients are involved in coproporphyrin formation.

SUMMARY

1. The cytochrome spectrum of certain mycobacteria showing *a*, *b* and *c* components is complicated by two absorption bands, one at 624 mμ. and the other at 570 mμ. It has been shown that these bands are due to coproporphyrin.

2. Coproporphyrin III tetramethyl ester (16 mg.) have been isolated from *Mycobacterium Karlinski* (4·8 kg.) by an acetone extraction method. Coproporphyrin is synthesized by the organism.

3. The occurrence of coproporphyrin in other saprophytic mycobacteria has also been studied.

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