- Chance, B. (1958). *J. biol. Chem.* **233**, 1223.
- Cleland, K. W. & Slater, E. C. (1953). *Biochem. J.* **53**, 574.
- Colpa-Boonstra, J. P. (1959). Ph.D. Thesis: University of Amsterdam.
- Colpa-Boonstra, J. P. & Holton, F. A. (1959). *Biochem. J.* **72**, 4P.
- Colpa-Boonstra, J. P. & Minnaert, K. (1959). *Biochim. biophys. Acta*, **33**, 527.
- Estabrook, R. W. (1957). *J. biol. Chem.* **227**, 1093.
- Fisher, R. A. (1950). *Statistical Methods for Research Workers*, 11th ed., p. 132. Edinburgh: Oliver and Boyd, Ltd.
- Hill, A. V. (1928). *Proc. Roy. Soc. B*, **104**, 40.
- Hill, R. (1954). *Nature, Lond.*, **174**, 501.
- Holton, F. A. (1955a). *Biochem. J.* **61**, 46.
- Holton, F. A. (1955b). In *Résumés des Communications*, 3rd int. Congr. Biochem., Brussels, p. 58.
- Holton, F. A. (1957). *Biochem. J.* **66**, 37P.
- Hodgman, C. D., Weast, R. C. & Selby, S. M. (1955). In *Handbook of Chemistry and Physics*, 37th ed., p. 1607. Cleveland, Ohio: Chemical Rubber Publishing Co.
- Hübscher, G., Kiese, M. & Nicolas, R. (1954). *Biochem. Z.* **325**, 223.
- Hüfner, G. (1897). *Ann. Phys. Lpz.*, **60**, 134.
- Keilin, D. & Hartree, E. F. (1947). *Biochem. J.* **41**, 500.
- Laki, K. (1935). *Hoppe-Seyl. Z.* **236**, 32.
- Mackler, B. & Green, D. E. (1956a). *Biochim. biophys. Acta*, **21**, 1.
- Mackler, B. & Green, D. E. (1956b). *Biochim. biophys. Acta*, **21**, 6.
- Margoliash, E. & Frohwirt, N. (1959). *Biochem. J.* **71**, 570.
- Okunuki, K., Hagihara, B., Sekuzu, I. & Horio, T. (1958). *Proceedings of the International Symposium on Enzyme Chemistry, Tokyo and Kyoto*, 1957, p. 265. London: Pergamon Press Ltd.
- Randall, M. & Failey, C. F. (1927). *Chem. Rev.* **4**, 271.
- Sekuzu, I. & Okunuki, K. (1956). *J. Biochem., Tokyo*, **43**, 107.
- Sidwell, A. E., Munch, R. H., Barron, E. S. G. & Hogness, T. R. (1938). *J. biol. Chem.* **123**, 335.
- Singer, T. P., Kearney, E. B. & Bernath, P. (1956). *J. biol. Chem.* **223**, 599.
- Slater, E. C. (1953). *Annu. Rev. Biochem.* **22**, 17.
- Slater, E. C. & Bonner, W. D. (1952). *Biochem. J.* **52**, 185.
- Smith, L. (1955). *J. biol. Chem.* **215**, 833.
- Stotz, E. H., Morrison, M. & Marinetti, G. (1956). In *Enzymes; Units of Biological Structure and Function*, p. 401. Ed. by Gaebler, O. H. New York: Academic Press Inc.
- Thorn, M. B. (1951). *Biochem. J.* **49**, 602.

Biochem. J. (1960) **76**, 189

The Isolation of Coproporphyrin III from *Mycobacterium tuberculosis avium*

By D. S. P. PATTERSON

Central Veterinary Laboratory, Ministry of Agriculture, Fisheries and Food, Weybridge, Surrey

(Received 31 December 1959)

After the demonstration that the addition of traces of certain metal ions to synthetic media had the effect of stimulating the growth of mycobacteria (Patterson, Wright & Patterson, 1958) it was observed during extraction of *Mycobacterium tuberculosis avium* with cold acetone that there was a difference in the pigment content of organisms grown on unsupplemented synthetic media and those grown on media containing added trace elements (Patterson, 1960). A pink pigment which fluoresced red in ultraviolet light was extracted in much greater quantity from the latter organisms and was thought to be a coproporphyrin, since the presence of free porphyrins in mycobacteria has been shown by Fink & Fischer (1925), and because Todd (1949) had isolated coproporphyrin III from *Mycobacterium karlinski*.

The pigment present in *M. tuberculosis avium* is now shown to be identical with that present in the saprophyte *M. karlinski*.

MATERIALS AND METHODS

Organisms. *Mycobacterium tuberculosis avium* strain D4R was grown on synthetic media which were modifications of that used by Dorset (1934). In the first experiment, coproporphyrin was isolated from bulked extracts of the partially purified pigment obtained from 12 batches of organisms which had been grown for 33–49 days at 37° on 1 l. of the basic medium to which additions of cobalt (0.27 p.p.m.), copper (1.07 p.p.m.) and zinc (6.04 p.p.m.) had been made either separately or in combination. Organisms used in the second experiment were grown for 39–60 days on 1 l. batches of experimental media which differed from the medium of Dorset in iron content (2–64 p.p.m. instead of about 50 p.p.m.) and in zinc supplementation (2–64 p.p.m.). Coproporphyrin was isolated from bulked extracts from 70 cultures.

Extraction of live organisms with acetone. The organisms from 1 l. of medium were harvested by filtering through fine muslin. The filter cake was washed with 1 l. of water, and pressed dry. Pigments were extracted from the organisms with 250 ml. of acetone for 15 hr. at room

temperature, followed by further extractions with 150 and 100 ml. for shorter periods.

Extraction of heat-killed organisms. Larger bulks of heat-killed organisms obtained during the preparation of tuberculin were also extracted with acetone at room temperature. The pigment contained in the resulting aqueous acetone extract was partially purified by extraction with ether and transferred to approximately 2.3N-HCl, prepared by diluting the A.R. concentrated acid five times. This solution was neutralized with sodium acetate (indicator, Congo red), re-extracted with ether and the cycle of operations repeated. Finally, the pigment was obtained in approx. 2.3N-HCl and estimated as described below.

Catalase activity of mycobacterial suspensions. The method of Bonnischen, Chance & Theorell (1947), as described by Maehly & Chance (1954), was used for the assay of catalase activity of mycobacterial suspensions which were prepared by triturating the organisms with 0.01M-phosphate, pH 7.0. Activities were conveniently recorded as μ moles of H_2O_2 consumed/mg. dry wt./hr. at room temperature. Measurement was made at minute intervals during the first 5 min., i.e. during the linear portion of the assay curve.

Spectrophotometric measurements. These were made on a Hilger Uvispek quartz spectrophotometer. The wavelength scale was checked with a didymium glass filter over the range 550–600 $m\mu$, and the errors observed were of the same order of magnitude as the smallest wavelength differences easily discernible on the wavelength drum (i.e. not greater than 2.5 $m\mu$). The absorption spectrum of 0.005% K_2CrO_4 in 0.05N-KOH showed maxima at 272 and 372 $m\mu$, which is in close agreement with the data of Gillam & Stern (1954).

Coproporphyrin content of cell suspensions. A given volume of cell suspension (usually 50 ml.) was extracted with 200 ml. of acetone overnight, followed by 100 ml. of acetone (twice) for 3–4 hr. at room temperature. The aqueous acetone supernatant was collected by centrifuging after each extraction. Bulk extracts were acidified with acetic acid (to 5%, v/v) and the pigment was collected in three ether washes (150, 100 and 50 ml.). The combined ether extracts were then themselves extracted with 30 and 20 ml. of 2.3N-HCl. The HCl soln. so obtained was warmed to 75° to remove most organic solvents, then cooled and the volume adjusted to 100 ml. The extinction was measured at 402.5 $m\mu$. Coproporphyrin content was estimated from the specific absorption coefficient $E_{1\%}^{1\text{cm}}$ at 402.5 $m\mu$, 6520, in approx. 2.3N-HCl (see Results section).

RESULTS

Extraction and identification of coproporphyrin III

Cold acetone extracts of live *M. tuberculosis avium* were pigmented when obtained from organisms grown on synthetic media which had been supplemented with trace amounts of zinc and cobalt (Patterson, 1960). The extinctions of such extracts were particularly increased in the region 355–450 $m\mu$. A well-defined maximum was observed at about 395 $m\mu$ and a lesser peak was sometimes seen at about 370 $m\mu$. Some of these extracts

were pink to the naked eye and all showed a very strong red fluorescence in ultraviolet light. Coupled with the strong absorption at about 400 $m\mu$ this was indicative of the presence of porphyrins.

After concentrating the acetone extract and acidifying with acetic acid, an ethereal extract was obtained from which a purple pigment could readily be transferred to 2.3N-HCl. A repetition of this partial purification procedure was carried out after neutralizing the HCl with sodium acetate (indicator, Congo red). The resulting 2.3N-HCl soln. showed maximum absorption at 402.5 $m\mu$. Dilution of the pigment solution with water to 0.15N with respect to HCl had the effect of moving the absorption maximum from 402.5 to 401 $m\mu$. This is in agreement with the data of Jope & O'Brien (1945) for coproporphyrin.

Since the amount of the pigment that could be extracted from *M. tuberculosis avium* organisms was dependent upon a number of factors, including the age of the culture and the trace-element content of the medium (D. S. P. Patterson, unpublished work), it was important to identify this fluorescent material. Pooled HCl extracts obtained from experimental *M. tuberculosis avium* cultures supplemented with trace elements were subjected to the procedure used by Todd (1949) for the extraction and isolation of coproporphyrin from *M. karlinski*. In two experiments dark reddish purple needles of coproporphyrin tetramethyl ester were obtained. Properties of the two products and the free coproporphyrin are set out in Tables 1 and 2. Agreement with published data for coproporphyrin is generally very good. However, although the value obtained for the extinction coefficient was only about 90% of the values quoted by Jope & O'Brien (1945) for recrystallized natural or synthetic coproporphyrin III, it was identical with that of Todd (1949) for coproporphyrin obtained from a mycobacterial source. The evidence indicates that the free porphyrin and not its metal complex was extracted from the organisms. The quality of the material obtained is strictly comparable with that of Todd's pigment isolated as the tetramethyl ester. Although the double melting point is characteristic of the III isomer (Gray & Holt, 1947), Jope & O'Brien (1945) showed that up to 15% of the I isomer may remain undetected in samples of naturally occurring coproporphyrin, which, on the basis of melting point alone, is thought to be the III isomer. Consequently the twice-recrystallized ester was examined by the paper-chromatographic method of Granick & Mauzerall (1958). Chromatograms of about 0.5 μ g. of ester revealed only one fast-moving constituent corresponding with coproporphyrin III tetramethyl ester.

Table 1. *Coproporphyrin III*, isolated as the tetramethyl ester from mycobacteria

	<i>M. tuberculosis avium</i>		<i>M. karlinski</i> (Todd, 1949)
	Expt. 1	Expt. 2	
Approx. wt. of organisms (g.)	213 (dried)	1437 (dried)	4800 (wet)
Vol. of 2.3N-HCl extract	110 ml.	8.5 l.	—
Extinction at 402.5 m μ	20.06	5.05	—
Wt. of coproporphyrin ester twice recrystallized (mg.)	Approx. 0.8	25.2	16.0
M.p. (uncorrected):			
first	151°	153.5°	150°
second, after cooling	172–5°	179–81°	172–4°
Purity by copper titration (%) (Oliver & Rawlinson, 1951)	—	93.8	—
Free porphyrin:			
$E_{401\text{ m}\mu}/E_{547.5\text{ m}\mu}$ in 0.15N-HCl	28.3	30.9	30.5
$E_{1\text{ cm}}^{1\%}$ at 401 m μ in 0.15N-HCl	—	7400	7400
$E_{1\text{ cm}}^{1\%}$ at 402.5 m μ in approx. 2.3N-HCl	—	6520	—

Table 2. *Light absorption of naturally occurring coproporphyrin III*

Solvent	Source	Absorption maxima (m μ)					Relative intensities
		I	II	III	IV	V	
Ether	Crude extract from <i>M. tuberculosis avium</i>	625	570	528	498	400	V > IV > III > II > I
	<i>M. karlinski</i> (Todd, 1949)	624	570	528	500	—	—
	<i>Corynebacterium diphtheriae</i> (Gray & Holt, 1947)	622.5	574.1	528.9	496.8	—	—
	Coproporphyrin (Schumm, 1927)	623.6	568.4	526.3	495	—	—
	Zn-coproporphyrin from corynebacteria and myco- bacteria (Coulter & Stone, 1938)	574	538	407.9	—	—	—
Approx. 2.3N-HCl	Partially purified porphyrin from <i>M. tuberculosis avium</i>	590	547.5	402.5	—	—	—
	Porphyrin via ester	590	547.5	402.5	—	—	—
25% HCl	Coproporphyrin (Schumm, 1927)	593.6	550.2	405.8	—	—	—
Pyridine	Purified porphyrin from <i>M. tuberculosis avium</i>	622.5	567.5	532.5	497.5	—	—
	Coproporphyrin (Schumm, 1927)	622.5	567.5	531.5	499.5	—	—
Chloroform	Twice-recrystallized ester { (1)	622.5	568.5	532.5	498	400	—
	from <i>M. tuberculosis avium</i> { (2)	622.5	568.5	532.5	499	402	—
	Twice-recrystallized ester from <i>M. karlinski</i> (Todd, 1949)	623.5	567	534	498.5	—	—
0.15N-HCl	Free-porphyrin via ester { (1)	590	547.5	400.5	—	—	—
	from <i>M. tuberculosis avium</i> { (2)	590	547.5	401	—	—	—
	{ (3)	590	547.5	401	—	—	—
	{ (4)	591.3	547.5	401	—	—	—
	Free-porphyrin via ester <i>M. karlinski</i> (Todd, 1949)	591	548	401	—	—	—
Acetic acid	Twice-recrystallized ester { (1)	560	528	—	—	—	I > II
	from <i>M. tuberculosis avium</i> { (2)	560	523	—	—	—	—
	Cu-ester complex, <i>M. tuberculosis avium</i>	562.5	527.5	399.5	—	—	III > I > II

*Extraction of coproporphyrin from
steam-killed organisms*

The porphyrin pigment described above was isolated entirely from live bacilli. In view of the possibility that coproporphyrin would resist auto-claving and because large quantities of steam-killed mycobacteria are disposed of during the production of tuberculin it was considered that this might constitute a useful natural source of coproporphyrin. Two large batches of steam-killed organisms were extracted with acetone. The first batch, 5.9 kg. (wet wt.; or 744 g. dry wt. of avian-type organisms), yielded 0.59 mg. of partially purified coproporphyrin and the second, 8.3 kg. (wet wt. of human-type organisms), 0.48 mg.

Examination of culture filtrates for porphyrins

The extraction of coproporphyrin from the mycobacterial cell with acetone is, according to Todd (1949), dependent upon its solubility in the acetone-soluble lipids of the bacillus. Possibly it is for the same reason that porphyrins were not detected in the culture filtrate of *M. tuberculosis avium*, being preferentially soluble in cellular lipids.

The spectra of culture filtrates revealed no absorption maximum in the region of 400 m μ and no red fluorescence was seen in ultraviolet light. Further, filtrates having high extinctions at 402.5 m μ failed to yield coproporphyrin when extracted with ether acidified with acetic acid.

*Relationship between free coproporphyrin
and catalase content*

The inverse relationship between coproporphyrin production by *Corynebacterium diphtheriae* and its cellular-cytochrome content is well known (Pappenheimer, 1947). Possibly a similar connexion occurs in mycobacteria, but in the present experiments this aspect of porphyrin metabolism was not investigated. However, the activity of another porphyrin-containing enzyme, catalase, was observed in 12 mycobacterial suspensions of known coproporphyrin content. Increased amounts of coproporphyrin were found to be associated

with a low catalase activity and a low coproporphyrin content was found in cells having high catalase activity. The relationship was significant ($P < 0.05$) (Fig. 1). This could mean either that at high concentrations coproporphyrin itself was acting as an inhibitor of catalase activity or that the free porphyrin was being formed in preference to combining with the apoenzyme of catalase. The former possibility was excluded experimentally. Table 3 shows the results of typical experiments in which the activity of 0.5 ml. of a freshly prepared aqueous solution of bovine catalase (25 μ g./ml. of bovine-liver catalase; L. Light and Co. Ltd.) was not inhibited significantly by coproporphyrin III or its tetramethyl ester at concentrations comparable with those found experimentally in mycobacterial suspensions.

Since the activity of the catalase solution in the presence of added porphyrin was always measured a few minutes after that of the original enzyme dilution, the small decrease in activity which is observed may be accounted for by the demonstrable instability of the enzyme in dilute solution. Thus, 40 min. after its preparation, the diluted catalase had lost about two-thirds of its activity (see Table 3).

Fig. 1. Relationship between free coproporphyrin III content (μ g./g. dry wt.) and catalase activity (μ moles of H_2O_2 /mg. dry wt./hr.) in *M. tuberculosis avium* (strain D4R) suspensions.

Table 3. *Effect of coproporphyrin III and its tetramethyl ester on activity of bovine-liver catalase*

Each reaction flask contained 500 μ moles of phosphate, 456 μ moles of H_2O_2 and 12.5 μ g. of enzyme. Total volume was 52.5 ml.; pH 7.0. Experiments were carried out at room temperature.

Expt. no.	Addition	Enzyme	Activity (initial velocity, μ moles of H_2O_2 /hr.)
1	1 μ g. of coproporphyrin ester	Freshly diluted	1024
		Freshly diluted	944
		40 min. after dilution	315
2	1 μ g. of coproporphyrin	Freshly diluted	1339
		Freshly diluted	1160
3	2 μ g. of coproporphyrin	Freshly diluted	1460
		Freshly diluted	1281

DISCUSSION

The isolation of coproporphyrin III from cells of *M. tuberculosis avium* grown on synthetic media containing asparagine as the sole source of nitrogen and glucose and glycerol as carbon sources demonstrates the ability of this organism to synthesize the porphyrin nucleus. The organisms used for the isolation of relatively large amounts of coproporphyrin had been grown on media containing trace-element supplements, but the fact that coproporphyrin was also extracted from organisms grown on a basic unsupplemented synthetic medium (Patterson, 1960), although in small amount, shows that this is not an abnormal constituent of the cell.

Metals such as zinc and cobalt are therefore able to stimulate porphyrin synthesis, although this is dependent upon a sufficient concentration of iron in the medium (D. S. P. Patterson, unpublished work). Mycobacteria are now cultured on media supplemented with trace elements for the production of tuberculin (Paterson *et al.* 1958) and although the large bulk of steam-killed organisms which are discarded during the process constitutes a natural source of coproporphyrin, only about 0.6 mg. was obtained from 744 g. (dry wt.) of *M. tuberculosis avium* organisms.

The catalase activities of *M. tuberculosis avium* suspensions and their coproporphyrin contents were shown to be inversely related. Taken with the demonstration that free coproporphyrin III or its tetramethyl ester did not significantly inhibit the activity of purified bovine catalase, this means that trace elements such as zinc and cobalt stimulate the production of free coproporphyrin although they decrease levels of activity of the porphyrin-containing enzyme catalase. This effect could be explained in terms of an inhibition of haem synthesis by these metal ions. This would result in a reduced rate of formation of the prosthetic group of catalase and increased amounts of free porphyrins, mainly as coproporphyrin.

SUMMARY

1. Cold acetone extracts of *Mycobacterium tuberculosis avium* that had been cultivated on synthetic media containing trace-element supplements were often heavily pigmented. The pigment was isolated and identified as free coproporphyrin III.

2. Of the 12 mycobacterial-cell suspensions examined, those with a high coproporphyrin content exhibited a low catalase activity, whereas those low in coproporphyrin had most enzyme activity. This result is discussed.

The permission of the Director, Central Veterinary Laboratory, to publish this paper is gratefully acknowledged.

REFERENCES

- Bonnischen, R. K., Chance B. & Theorell, H. (1947). *Acta chem. scand.* **1**, 685.
Coulter, C. B. & Stone, F. M. (1938). *Proc. Soc. exp. Biol., N.Y.*, **38**, 423.
Dorset, M. (1934). *J. Amer. vet. med. Ass.* **84**, 439.
Fink, H. & Fischer, H. (1925). *Hoppe-Seyl. Z.* **150**, 243.
Gillam, A. & Stern, E. S. (1954). *An Introduction to Electronic Absorption Spectroscopy in Organic Chemistry*. London: E. J. Arnold and Son Ltd.
Granick, S. & Mauzerall, D. (1958). *J. biol. Chem.* **232**, 1141.
Gray, C. H. & Holt, L. B. (1947). *J. biol. Chem.* **169**, 235.
Jope, E. M. & O'Brien, J. R. P. (1945). *Biochem. J.* **39**, 239.
Maehly, A. C. & Chance, B. (1954). In *Methods in Biochemical Analysis*, vol. 1. Ed. by Glick, D. New York: Interscience Publishers Inc.
Oliver, I. T. & Rawlinson, W. A. (1951). *Biochem. J.* **49**, 157.
Pappenheimer, A. M., jun. (1947). *J. biol. Chem.* **167**, 251.
Paterson, A. B., Wright, E. C. & Patterson, D. S. P. (1958). *Tubercle, Lond.*, **39**, 275.
Patterson, D. S. P. (1960). *Nature, Lond.*, **185**, 57.
Schumm, O. (1927). Cited in *Porphyrins—their biological and chemical importance*. By A. Vannotti. London (1954): Hilger and Watts Ltd.
Todd, C. M. (1949). *Biochem. J.* **45**, 386.