A Simple Chemical Method to Differentiate *Mycobacterium* from *Nocardia*

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SUMMARY

A simple, rapid, chemical method to differentiate *Mycobacterium* from *Nocardia* is described. After saponification of bacteria in 2.5% (w/v) KOH in methanol + benzene (1:1, v/v) at 37° for one day, crude mycolic acids of mycobacteria were precipitated by addition of an equal volume of ethanol to an ethereal solution of extracted lipids. Mycobacteria gave large amounts of white precipitates (about 10 mg/g wet whole bacteria) of m.p. between 45° and 70°, whereas nocardias gave no precipitate or very small amounts of precipitates (less than 2 mg/g whole bacteria) which did not melt below 150° or melted near 80°.

INTRODUCTION

Nocardias and rapidly growing mycobacteria are difficult to differentiate by their physiological and morphological characteristics alone (Uesaka, 1956; Gordon, 1966; Tsukamura, 1970). The GC content of DNA is the same in both genera (Yamaguchi, 1967) as are the chemical components of the bacterial walls (i.e. arabinose, galactose, alanine, glutamic acid and *meso*-diaminopimelic acid) which also show similar immunological cross-reactions (Cummins, 1962; Kwapinski, 1964). However, both genera have characteristic lipids, i.e. mycolic acids in mycobacteria and nocardic acids (nocardomycolic acids) in nocardias (Azuma, Yamamura & Fukushi, 1968; Kanetsuna, 1968; Amar-Nacasch & Vilkas, 1969; Acharya & Goldman, 1970; Guinand, Vacheron & Michel, 1970; Ionedá, Lederer & Rozanis, 1970). Although rapid identification of these lipids requires specialized techniques (Lanéelle, Asselineau & Castelnuovo, 1965; Etémadi, 1967; Lechevalier, Horan & Lechevalier, 1971) which may not be available in all laboratories, we have developed a simple method which differentiates mycobacteria from nocardias, based on the demonstration of mycolic acids in mycobacteria.

METHODS

Mycolic and nocardic acids. Mycolic acids were isolated from the cell wall of *Mycobacterium bovis* BCG (Kanetsuna, 1968). Nocardic acids were obtained from bound lipids in the cell wall of *Nocardia asteroides* 131, grown in Sauton medium for one week, as described by Guinand *et al.* (1970).

The solubility of both mycolic acids and nocardic acids in a mixture of ether and ethanol was determined by dissolving a sample (20 mg) of either mycolic acid or nocardic acid in a small volume of ether and adding ethanol to make a total volume of 2 ml. (The ratio of ether to ethanol in the mixture varied from 1:0 to 1:2, v/v.) After 2 h at 0 to 4°, the precipitates were collected by centrifuging at about 200 g for 5 min, dried and weighed.

Organisms and growth conditions. The mycobacteria and nocardias used are listed in Table 1. They were cultured, without shaking, on a heart infusion broth (Difco) containing 5% (w/v) glycerol with or without 1 µg malachite green/ml (medium I and II, respectively), or on a tryptose phosphate broth (Difco) containing 5% (w/v) glycerol (medium III) at 37° for several days as indicated in Table 1. Bacteria were killed in 2 to 3% formaldehyde, collected by centrifuging or filtering and washed with water.

Extraction of mycolic acids from bacteria. The procedure used was as follows. Bacteria (2 g wet wt) were saponified in a closed tube with 5 ml of 2.5% (w/v) KOH in methanol + benzene (1:1, v/v) for one day at 37° with occasional shaking. Three ml of 1 N-HCl were then added to the mixture followed by about 5 ml of ether and the whole mixed well. After centrifuging (200 g for 5 min) the clear ethereal layer was removed. Ether extraction was repeated three or four more times and the combined ether extracts were evaporated. The residue was extracted again with ether three times. The final ether extracts were evaporated to 1 ml, 1 ml ethanol was added and after 2 h at 0 to 4° the white precipitates were collected by centrifuging and dried. These were redissolved in 1 ml ether and 1 to 2 ml ethanol added. After several hours at 0 to 4° the precipitates were collected by centrifuging, dried, weighed and the m.p. determined. (When the precipitates turned yellowish as has been seen in some strains of mycobacteria, reprecipitation was repeated once more as described above.)

RESULTS AND DISCUSSION

About 90% of mycolic acids from BCG were precipitated on adding 1 to 2 vol. of ethanol to 1 vol. of ethereal solution of mycolic acids. In ethanol + ether (1:2, v/v), about 75% of mycolic acids were precipitated. Nocardic acids from *Nocardia asteroides* remained dissolved in ethanol + ether (from 1:2, v/v to 2:1, v/v).

The yield of the precipitate of mycolic acids from BCG depends on the volume of ethanol + ether. Using 20 mg of mycolic acids, about 90% was precipitated in 1 or 2 ml of ether + ethanol (1:1, v/v), whereas only about 85 and 30% of mycolic acids were precipitated in 4 and 6 ml respectively. Large volumes of ether + ethanol must therefore be avoided.

The above procedures applied to various mycobacteria and nocardias showed that only mycobacteria gave large amounts of white precipitates (Table 1). Generally, about 10 mg of the precipitates were obtained from 1 g (wet wt) of mycobacteria with a m.p. between 45° and 70° which corresponds with published data (Asselineau, 1966). Since the rate of synthesis of mycolic acids parallels the growth of mycobacteria (Bennet & Asselineau, 1970), this amount of mycolic acid fraction is probably constant during growth. Furthermore, as the mycolic acid-arabinogalactan-mucopeptide complex may be an essential structure of all mycobacterial walls (Kanetsuna, 1968), the mycolic acid content in the cell wall may not be greatly influenced by culture conditions.

Several mycobacteria gave no precipitates of mycolic acids but all belonged to *Mycobacterium rhodochrous*; two of these strains (no. 549 and 1256) were, in fact, originally classified in the genus *Nocardia* (Gordon, 1966). In the present study, all strains of *M. rhodochrous* were non-acid-fast. Lanée et al. (1965) and Lechevalier et al. (1971) reported that *M. rhodochrous* does not contain mycolic acids but nocardic acids and that *M. rhodochrous* should be classified as *Nocardia*. We agree with their conclusion.

Species of *Nocardia* gave either no precipitate at all or just a small amount; i.e. less than 2 mg/g whole bacteria. However, the brownish precipitates of some nocardias did not melt below 150°, indicating that they were not mycolic acids. The precipitates of a few nocardias

Table 1. *Precipitates of lipids of mycobacteria and nocardias in ether + ethanol (1:1, v/v)*

Organism*	Source†	Medium‡	Days cultured	Dry wt of precipitate (mg/g wet bacteria)	M.p. of precipitate
<i>Mycobacterium avium</i> Jucho	C	II	35	13	51 to 54
<i>M. bovis</i> BCG	E	IV	30	10	52 to 55
<i>M. bovis</i> MR	C	II	35	11	46 to 49
<i>M. bovis</i> USHI 10	E	IV	30	13	48 to 50
<i>M. bovis</i> USHI 10	E	IV	30	13	53 to 55
<i>M. fortuitum</i>	E	IV	30	7	60 to 63
<i>M. phlei</i>	A	I	7	12	51 to 53
<i>M. rhodochrous</i> 549	B	III	4	0	—
<i>M. rhodochrous</i> 1256	B	III	3	0	—
<i>M. rhodochrous</i> 3406	B	III	3	0	—
<i>M. rhodochrous</i> 3407	B	III	3	0	—
<i>M. rhodochrous</i> 8036	B	III	4	0	—
<i>M. rhodochrous</i> NCTC 8139	B	III	3	0	—
<i>M. smegmatis</i> ATCC 607	A	I	5	9	64 to 65
<i>M. smegmatis</i> ATCC 607	A	III	5	9	64 to 65
<i>M. smegmatis</i> ATCC 14468	A	I	14	15	57 to 60
<i>M. tuberculosis</i> H37 Ra	A	I	7	10	59 to 62
<i>M. tuberculosis</i> H37 Ra	E	IV	30	8	56 to 58
<i>M. tuberculosis</i> AOYAMA B	E	IV	30	14	53 to 56
<i>M. sp.</i> RABINOWITCH	A	I	7	10	60 to 63
<i>M. sp.</i> 521	A	I	5	8	65 to 66
<i>M. sp.</i> 521	A	III	5	9	64 to 65
<i>M. sp.</i> P1	E	IV	30	12	52 to 54
<i>Nocardia asteroides</i> 10	B	II	11	1.6	77 to 79
<i>N. asteroides</i> 72	D	II	7	0	—
<i>N. asteroides</i> 73	D	II	7	0.6	80 to 82
<i>N. asteroides</i> 81	D	II	14	0	—
<i>N. asteroides</i> 93	B	III	13	0	—
<i>N. asteroides</i> 94	B	III	11	2.0	> 150
<i>N. asteroides</i> 123	D	II	14	—	—
<i>N. asteroides</i> 126	D	II	7	0	—
<i>N. asteroides</i> 127	D	II	7	0	—
<i>N. asteroides</i> 129	B	III	5	0	—
<i>N. asteroides</i> 131	E	IV	7	0	—
<i>N. asteroides</i> 132	B	III	6	0	—
<i>N. asteroides</i> 155	B	III	10	0.3	76 to 78
<i>N. asteroides</i> 156	B	III	4	0	—
<i>N. asteroides</i> 166	B	I	3	0.4	> 150
<i>N. asteroides</i> 166	B	III	3	1.7	> 150
<i>N. asteroides</i> 205	D	II	14	0	—
<i>N. brasiliensis</i> 145	D	II	7	0	—
<i>N. brasiliensis</i> 168	B	III	10	0	—
<i>N. brasiliensis</i> 197	D	II	7	0	—
<i>N. brasiliensis</i> 198	D	II	7	0	—
<i>N. brasiliensis</i> 199	D	II	7	0.7	80 to 82
<i>N. brasiliensis</i> 201	B	III	8	0	—
<i>N. brasiliensis</i> 851	D	II	7	0	—
<i>N. brasiliensis</i> 513	B	III	3	0	—
<i>N. brasiliensis</i> 1548	B	III	4	1.0	> 150
<i>N. caviae</i> 180	D	II	7	0	—
<i>N. sp.</i> 244	B	II	12	0	—

* Where a number is given following a species, this refers to the collections of Dr N. M. McClung (University of South Florida, U.S.A.) and Dr I. Uesaka (Chest Disease Research Institute, Kyoto University, Japan) who use the same reference number for their strains.

† A = strains kept in our laboratory. B = given by Dr J. A. Serrano, Universidad de Los Andes, Venezuela, originally received from Dr N. M. McClung, except for *N. brasiliensis* no. 1548 (from Dr Mackinnon, Uruguay), *N. brasiliensis* no. 513 (from Dr Lacaz, Brazil) and all strains of *M. rhodochrous* (from Dr Gordon, Rutgers University, U.S.A.). C = given by Dr M. Nishiura, Leprosy Research Laboratory, Kyoto University, Japan. D = given by Dr I. Uesaka. E = received as lyophilized organisms from Dr I. Azuma, Osaka University, Japan. *Nocardia asteroides* no. 131 was originally obtained from Dr I. Uesaka.

‡ Medium I = heart infusion broth (Difco) containing 5% (w/v) glycerol, and 0.0001% (w/v) malachite green. II = heart infusion broth (Difco) containing 5% (w/v) glycerol. III = tryptose phosphate broth (Difco) containing 5% (w/v) glycerol. IV = Sauton's medium.

had a m.p. near 80°. These might be saturated nocardic acids which are present in some strains of nocardia (Asselineau, 1966; I. Yano, personal communication). We propose, therefore, that to determine if a strain is a mycobacterium, the precipitate should be at least 5 mg/g wet whole bacteria and its m.p. be between 45° and 70°.

The differences in the results obtained from mycobacteria and nocardias are not caused by different culture media, since some mycobacteria and nocardias cultured in medium I gave similar results to the same bacteria cultured in medium III. A few strains of other genera were also examined. *Bacillus cereus* 569, *Escherichia coli* Hfr c6 and *Micrococcus lysodeikticus* which were cultured for 16 h at 37° in a Penassay broth (Difco) with continuous shaking did not give precipitates. *Streptomyces albus* G cultured at room temperature for 4 days in a heart infusion broth (Difco) with shaking gave no precipitates.

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