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Classification of *Mycobacterium avium* and related opportunist mycobacteria met in England and Wales

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Amongst the opportunist (syn. anonymous) mycobacteria responsible for human infections, the most important and widely distributed are those designated 'group III; Nonphotochromogens' by Runyon (1959) and 'dysgonic nonchromogens' (groups 4 and 5) by Marks & Richards (1962). In Britain, the dysgonic nonchromogens rank second to *M. kansasii* as a cause of overt opportunist infection, but surveys of skin sensitivity suggest that subclinical infections may be relatively common and responsible for many of the non-specific reactions met in tuberculin tests. Some dysgonic nonchromogens have been identified as *M. avium* by pathogenicity tests on fowls (Marks & Birn, 1963), but further experience has shown that the fowl test is not completely specific. Lack of suitable means to classify members of the group, which is well recognized to comprise a number of different entities, has hindered the study of their epidemiology. The present investigation has applied to the problem of their classification a combination of serotyping by agglutination with specific antisera (Schaefer, 1965), lipid analysis by the thin-layer chromatography of extracts (Marks & Szulga, 1965) and certain cultural and biochemical examinations chosen for their utility in subdividing the group. The majority of the strains were also tested for virulence in fowls, although the results were not considered decisive for the present classification. The material examined consisted of the dysgonic nonchromogens isolated from man in Wales since 1953 together with strains sent to the Reference Laboratory for identification from England since 1959.

METHODS

Strains were stored at 4° C., and were subcultured every 3 months on Löwenstein–Jensen medium at 37° C.

Serotyping

Subcultures were made in Dubos medium, plated when grown on oleic acid-albumin agar plates and examined for agglutinability by the methods described

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previously (Schaefer, 1965). Most of the strains were satisfactorily typed, but a number either failed to disperse adequately or agglutinated spontaneously during the test. With some of these a later subculture from egg proved satisfactory. Others yielded satisfactory suspensions but were not agglutinated by any of the absorbed sera then available; such strains have been designated 'unclassified'. Four strains agglutinated with two different sera, and with one strain two types of colony reacting differently were found. One of those reacting with two different sera appears in Table 2, but otherwise the serotype consistent with the remaining properties has been used in classification.

Lipid analysis

The dysgonic nature of the organisms studied made a change necessary in the method used previously to prepare extracts. Volumes of 10 ml. of Löwenstein–Jensen medium were inspissated in 120 ml. medical flat screw-capped bottles kept at an angle of 45° after which 15 ml. of Kirchner's medium, modified by the use of Tryptone (Oxoid) instead of asparagine and 0·1 % bovine serum albumin instead of 10 % serum, were added to each. Care was taken in dispensing to minimize dispersal of the egg medium over the walls of the bottle and subsequently to avoid detaching any of the solid into the liquid phase. The latter layer was inoculated and the bottles incubated vertically at 37° C until the cultures were well grown—usually 4 weeks. The bacilli were harvested by centrifuging the liquid layer after its removal by pipette. The deposits were transferred to tared sample tubes, dried over P_2O_5 in a partial vacuum of about 60 cm. Hg for 48 hr. at room temperature and their 'dry' weight obtained. They were then extracted overnight (it was found later that 24 hr. is preferable) at room temperature with a solvent made of ethyl ether 43 volumes, ethanol 43 and water 14, the tubes being closed with silicone rubber bungs. The solvent was used in the proportion of 16 μl. for each mg. dry weight of bacteria. If the bacterial mass fell short of 15 mg., it was made up to this weight with water and 0·24 ml. of solvent used; however, the rare sample below 10 mg. was discarded in favour of a fresh culture. The chromatography of the extracts on thin-layer silica gel and the subsequent spraying with orcinol-sulphuric acid followed the methods described by Marks & Szulga (1965). As in their case, solvent 1 for two-dimensional analysis was propanol-ammonia run for 10 cm. and solvent 2 was butanol–acetic acid–water run for 7·5 cm. Each was used also for uni-dimensional runs as was solvent 3, propanol–isopropyl ether–water run for 10 cm.

Cultural and biochemical methods

Morphology was examined in Ziehl–Neelsen stained films of cultures on egg medium of the Löwenstein–Jensen type, in most cases using the primary growth. To examine the rate of growth at different temperatures, a loopful of growth from egg was ground with a drop of sterile water and 1 mm. loopfuls of suspension subcultured on four 2 ml. slopes of egg medium in 7 ml. screw-capped bottles which were then incubated at 25, 37, 42 and 45° C. respectively. Growth is often very fine and readings were therefore made with a × 5 hand lens. Dubious growth

was recorded as negative. Similar slopes were inoculated directly from the primary growth for incubation at 37° C., one in continuous light, the other in darkness. When the growth on these was mature their pigmentation was inspected.

Arylsulphatase was demonstrated by the method described by Marks & Trollope (1960) except that the substrate concentration was 0·001 M. The normal period of incubation used was 2 weeks, but a further week was allowed when growth was poor. The colour due to released phenolphthalein obtained by adding alkali was arbitrarily graded as +, ± or negative. Barely detectable tints were classed as negative.

The 2 ml. slopes of egg medium described above were also used for sensitivity tests, drugs being dispensed in them with twofold differences in concentration. A single minimal heating was used to solidify the medium. The test inoculum was a 2 mm. loopful (withdrawn edgewise) of a suspension prepared by emulsifying a loopful of growth from egg medium in 2 ml. of sterile water. The tests were read after 3 weeks' incubation. When severe but incomplete inhibition occurred in the last tube showing growth, an intermediate value was taken for the minimal inhibitory concentration (M.I.C.). Growth was assessed on the number of colonies and not their size.

Pathogenicity

The procedure for tests on fowls was finally standardized as the intravenous inoculation of 0·01 mg. moist weight of bacilli grown in Dubos medium. The fowls were previously shown to be negative reactors to 0·05 mg. of avian P.P.D. injected into the wattle; in most cases birds 6–10 weeks old were used. Before this method was evolved, however, a minority of the strains tested were inoculated either by a combination of the intramuscular and intraperitoneal routes (0·25 mg. by each) or similarly with the addition of 0·002 mg. bacilli intravenously. Wet weight was estimated by measuring the volume of centrifuged bacilli in a vaccine tube and taking 1 μl. as equivalent to 1 mg. or by matching the opacity of a Dubos culture with another estimated in the former manner. With 20 strains, the fowl test was supplemented by the intravenous inoculation of a rabbit with 0·01 mg bacilli. No strain was pathogenic for a rabbit which was harmless to a fowl, and some strains lethal to fowls produced only limited disease in rabbits. The rabbit test was not found helpful therefore and will not be considered further. The liver and spleen of fowls which died or were killed after 8–12 weeks were examined histologically and by culture. Lung and kidney were less useful and their examination was omitted in many cases.

Following the work of Scammon, Froman & Will (1964), a similar attempt was made to enhance the virulence of a number of strains, previously shown non-pathogenic for fowls, by growth at 42° C. Five strains were subcultured six times at weekly intervals in Dubos medium at 37° and 42° C. in parallel. Each of the final cultures was inoculated intravenously into three fowls using doses of 0·01, 0·1 and 1 mg. respectively, giving a total of 30 birds. Examination was as described above, survivors being killed after 12 weeks.

Table 1. *Properties of mycobacteria classified as* M. avium *and 'para-avian bacilli' isolated from man in England and Wales.*

(The number of strains is given for each property and the normal serotype and lipid pattern given in parentheses for each type.)

Proposed classification	Total no. strains	Normal serotype (Schaefer)	Normal lipid pattern	Virulence for fowl*			Sensitive to†		Arylsulphatase‡			Growth on egg at		Strains clinically significant
				+	±	−	Cycloserine	Ethionamide	+	±	−	42° C	45° C	
M. avium														
Type 1	13	8 (I)§	12 (A_1)	0	2	8	13	0	0	2	11	13	10	8
Type 2	24	21 (II)§	21 (A_2)	22	1	1	24	0	0	1	23	24	2	22
Para-avian bacilli														
Type 1	3	3 (IV)	3 (A_3)	0	1	1	3	3	2	1	0	3	3	1
Type 2	3	3 (VI)	3 (A_3)	1	0	1	3	3	0	1	2	3	3	3

* Five strains were not tested in fowls.

† Strains were defined as sensitive when the M.I.C. of cycloserine or ethionamide was 40 μg./ml. or less.

‡ Arylsulphatase activity is recorded as + when a deep red colour was obtained in the test and as ± with a pink colour.

§ Of the non-conforming strains, three had relatively non-specific serotypes and the rest were rough and could not be typed.

RESULTS

The present study is based on a series of 68 strains of dysgonic nonchromogenic opportunist mycobacteria isolated from different patients in England and Wales. In 54 cases the strain was defined as 'significant' on the criteria of repeated isolation or of culture from a biopsy or pus specimen, often supported by other evidence, whilst in 14 cases the strain was considered 'casual' or information was lacking. When all the properties studied were considered together, most of the strains could be assembled into four groups, of which three appear to merit recognition as species,

Table 2. *Properties of dysgonic nonchromogenic mycobacteria isolated from man in England and Wales and included in two provisional new species*

Group and strain no.	Serotype (Schaefer)	Lipid pattern	Virulence for fowl	Sensitive to*		Arylsulphatase†	Growth on egg at		Clinical significance
				Cycloserine	Ethionamide		42° C	45° C	
Provisional species 1									
18730	Boone	B	−	−	+	+	+	+	−
23774	VI	B	N.T.	−	+	±	+	+	+
24657	II + VI	B	−	−	+	+	+	+	+
25356	Rough	B	−	−	+	+	+	+	+
Provisional species 2									
10792	Unclassified	C	N.T.	+	+	−	−	−	+
18698	Unclassified	C	N.T.	+	+	±	−	−	+
27479	Unclassified	C	N.T.	+	+	±	−	−	+
31042	Unclassified	C	N.T.	+	+	−	−	−	−
31242	Boone	C	N.T.	+	+	−	−	−	+

N.T. = Not tested.

* Strains were defined as sensitive (+) when the M.I.C. of cycloserine or ethionamide was ⩽ 40 μg./ml.

† Arylsulphatase activity is recorded as + when a deep red colour was obtained in the test and as ± th a pink colour.

but 16 strains remain which are no doubt representatives of rarer species. Over half the strains have been assigned to the species *Mycobacterium avium* for which a new description is proposed below. Within this species, two types have been recognized in the present material, the most important properties of which are presented in Table 1. In the same table appear the properties of a group of six strains which appear to be closely related to *M. avium* and have therefore been called 'para-avian bacilli'. They are also divisible into two types. The two further groups have for convenience been called 'Provisional species 1 and 2'. Their chief properties are presented in Table 2 and those of the remaining, unclassified strains in Table 3. The results of each type of examination follow but formal descriptions of the groups proposed for recognition as entities will be deferred until the Discussion.

Morphology and cultural properties

All strains were acid- and alcohol-fast and nearly all exhibited short bacilli in films of growth on egg medium. However, seven *M. avium*, type 1 strains and two of Provisional species 1 presented long bacilli. The term 'dysgonic' applied to the

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organisms discussed is relative and refers to their effuse and rather slow growth on egg medium. This type of growth appears to be associated with microaerophilism in mycobacteria; all the strains tested were weak in catalase activity and grew deep in semi-solid medium. With the pigmentation test described above, all members of the series were buff-coloured except one. This was a type 2 para-avian bacillus considered nonchromogenic initially but later found to produce a weak and inconstant yellow pigment.

Table 3. *Properties of dysgonic nonchromogenic mycobacteria isolated from man in England and Wales and not yet assembled in groups*

Strain no.	Serotype (Schaefer)	Distinctive lipid pattern	Virulence for fowl	Sensitive* to: Cycloserine	Sensitive* to: Ethionamide	Arylsulphatase*	Growth on egg at: 42° C	Growth on egg at: 45° C	Clinical significance
3897	Boone	+	−	+	−	±	+	−	+
18587	Boone	−	N.T.*	+	−	−	+	−	+
35445	Davis	+	+†	+	−	±	+	+	+
21233	Lunning	+	±	−	−	±	+	−	+
25633	Watson	+	−	+	+	−	+	+	+
653	Yandle	+	−	+	−	−	+	−	+
14546	I	+	−	−	+	−	+	−	+
2142	I	−	N.T.	−	+	−	±	−	−
7468	III	+	N.T.	+	−	−	−	−	+
326	Unclassified	+	N.T.	+	+	±	−	−	−
13444	Unclassified	+	±	−	+	+	+	±	+
18332	Unclassified	+	N.T.	−	−	−	+	+	−
35336	Unclassified	+	N.T.	−	−	−	+	−	+
11961	Rough	−	—	+	−	±	+	±	+
29494	Rough	−	N.T.	+	+	−	±	−	+
37176	Rough	−	−	−	+	−	+	−	+

Non-distinctive lipid patterns presented only features common to most mycobacteria; the 'distincti patterns differed amongst themselves.

* See Table 2.

† Virulence was attenuated after 4 years' storage.

About two-thirds of the strains in the series were grown in semi-solid medium as described by Marks & Richards (1962). All proved to be microaerophilic on the criterion of growth 10 mm. or more deep. However, when incubation was continued for more than a week, some strains grew more densely near the surface.

All members of the series grew on egg medium at 25° and 37° C. and, except for the five strains of Provisional species 2 and two unclassified strains, also at 42° C. Growth at 45° C. was a feature of Provisional species 1, usual with strains of *M. avium*, type 1 but uncommon with *M. avium*, type 2.

With rare exceptions, growth from egg medium emulsified easily, the bacilli in thin films being well dispersed. Similarly, growth in liquid medium was almost always diffuse.

Biochemical properties

It has been found useful in classification to distinguish between strains which have an M.I.C. with sulphonamide*, cycloserine and ethionamide $\leqslant 40$ μg./ml. in Löwenstein–Jensen medium, here called *sensitive*, and those with an M.I.C. > 40 μg./ml., here called *resistant*. All the strains which have been classed as *M. avium* were sensitive to cycloserine and resistant to ethionamide on these criteria. Those classed as para-avian bacilli were sensitive to both cycloserine and ethionamide although the M.I.C. of ethionamide was fairly high and barely escaped a category of resistance. Strains placed in Provisional species 1 were resistant to cycloserine and sensitive to ethionamide and those in Provisional species 2 were sensitive to both drugs. Unclassified strains varied in their sensitivity pattern.

All the strains in Provisional species 1 and one thought to be related were sensitive to sulphonamide. Members of type 2 *M. avium* were almost all sensitive or gave borderline results. Otherwise only three strains were sensitive, one para-avian and two unclassified.

Arylsulphatase activity was absent in strains classified as *M. avium* except for three which were weakly positive. Two para-avian, type 1 strains were strongly positive and it is of interest that neither was clinically significant. Members of Provisional species 1 were arylsulphatase positive; those of species 2 were negative or weakly positive. Of the unclassified strains, one was strongly positive, five weakly positive and ten negative.

Details of the biochemical findings are presented in Tables 1, 2 and 3 except for sulphonamide sensitivity in which the M.I.C. is sometimes not clearly defined.

Serotyping

A total of 32 strains belonged to serotypes I and II which are held by Schaefer (1965) to be characteristic of organisms responsible for natural tuberculosis in birds. Of these, 29 are accommodated in the species *M. avium* as presently defined whilst one which gave a mixed serotype II and VI reaction is assigned to provisional species 1 and two of serotype I remain unclassified. The third most common serotype was VI which besides the mixed reaction noted above was found in six strains. These were distributed between the *M. avium*, para-avian and Provisional species 1 groups and the antigen therefore appears to make only a limited contribution to classification. This conclusion is supported by our finding antigen VI in two of three strains of *M. ulcerans* examined in a separate study. Serotype III was only met twice, in one case as a mixture with serotype II which was preferred for classification because of its link with the strain's lipid structure. Three strains assigned to the para-avian group (type 1) were serotype IV.

The remaining strains with a recognized serotype comprised five with Boone antigen and one each with Davis, Lunning, Watson and Yandle antigens ('Battey' serotypes—Runyon, 1959; Schaefer, 1965). One Boone strain was classified as *M. avium*, type 2 on its other properties and one each as Provisional species 1 and 2.

* 4-Sulphanilamido-5,6-dimethoxypyrimidine (Fanasil-Roche).

Two Boone strains and the other four named could not be assigned to any group. The Boone antigen thus appears to be even less useful in classification than VI.

Eight strains provided smooth suspensions but were not agglutinated by the set of sera then available; these appear in Tables 2 and 3 as 'unclassified'. Nine strains could not be tested because of rough suspensions and although in repeat attempts two of these gave acceptable suspensions, neither could be typed; all nine will be listed as 'rough'.

It will be noted in Table 1 that eight strains of *M. avium* lacked the characteristic serotype. The anomalies were as follows:

M. avium, type 1 (normal serotype I). Four strains were rough, one serotype VI.

M. avium, type 2 (normal serotype II). One strain was rough, one serotype VI and one Boone.

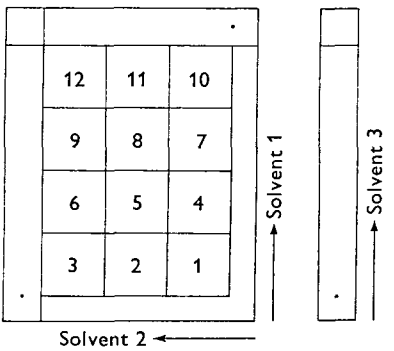

Fig. 1

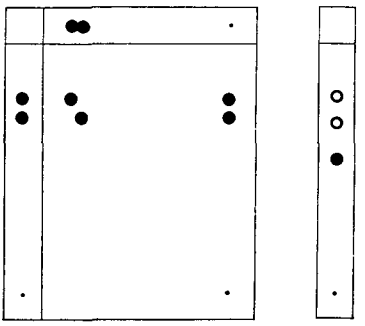

Fig. 2

Fig. 1. Diagram for reference in the text to positions of lipid spots. The numbered areas represent 2·5 cm. squares. The use of the solvents is described in the section on Methods. Samples for analysis were applied at the sites indicated by dots.

Fig. 2. Unidimensional and two-dimensional chromatographs of the characteristic lipids presented by strains of *M. avium*, type 1. The solvents and their direction of flow are as shown in Fig. 1.

Lipid structure

For ease of reference a diagram is given in Fig. 1 of a two-dimensional chromatogram divided into numbered 2·5 cm. squares. The directions are indicated of the flow of solvent 1 (propanol-ammonia) which was run for 10 cm. and of solvent 2 (butanol–acetic acid–water), run for 7·5 cm. All results refer to thin-layer chromatography on silica gel read after a spray with orcinol–sulphuric acid followed by heating. For clarity, non-specific lipids and substances derived from the medium have not been represented in the figures. With the two-dimensional runs the former almost all occur in squares 1, 2, 4 and 5 and the latter, which are faint, occur particularly at the lower margins of squares 7 and 8.

In Fig. 2 are presented the lipid patterns characteristic of extracts of *M. avium*, type 1, the unidimensional and two-dimensional results with solvents 1 and 2 appearing together, whilst alongside, the unidimensional 10 cm. run with solvent 3 (propanol–propyl ether–water) is depicted. This combination will be designated lipid pattern A_1. Its characteristic feature is the pair of spots which in the two-

dimensional run approximately straddle the lower margin of square 12 and always bear the same relative position to one another. In unidimensional runs in solvent 1 these spots are usually golden-brown but their colour in the two-dimensional run is often bluish grey. The difference appears to be due to a fall below a critical lipid concentration owing to splitting of the spots in the latter run, parts remaining immobile in solvent 2 as shown. The same effect on colour may be produced by over-spraying. Spots of moderate or high density are represented in the figures as solid circles, weak or inconstant spots as outlines; colour is not indicated. With solvent 3 the feature of pattern A_1 is a set of three rather weak brownish spots with R_F values of 0·55, 0·7 and 0·8 approximately. Unhelpful spots are again omitted from the illustration.

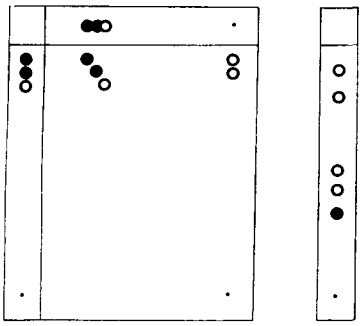

Fig. 3

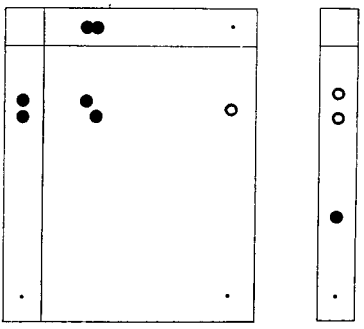

Fig. 4

Fig. 3. Chromatrographs in the style of Fig. 2 of the lipids characteristic of *M. avium*, type 2.

Fig. 4. Chromatographs in the style of Fig. 2 of the lipids characteristic of mycobacteria classed as 'Para-avian bacilli'.

The lipid pattern characteristic of *M. avium*, type 2 and designated A_2 is shown in Fig. 3. In the two-dimensional run it presents a triad of spots on or adjoining the right-hand margin of square 12. With a non-specific elongated pink spot above which is not shown they form an arc which often continues to the upper left corner of the square. The lowest of the three spots shown is the weakest and is occasionally missing, but the upper two are stronger than the pair in pattern A_1 and almost always golden-brown besides occupying a different position. The middle spot is always much the densest of the three. One or two spots are usual on the right-hand margin of square 10 but they are weaker and less constant than those seen in pattern A_1. With solvent 3 there is a characteristic dense golden-brown spot at R_F 0·33 and usually two weak spots at R_F 0·43 and 0·5. These spots appear to correspond to the triad in square 12. Two faint brown spots also occur at R_F 0·8 and 0·9.

The para-avian bacilli of both types provided a lipid pattern A_3, which is presented in Fig. 4. With solvent 1 and 2 this pattern resembles A_1 although the R_F of the two main spots in solvent 2 is about 0·1 less, so that they are nearer to the lower right angle of square 12. With solvent 3 there is a dense brown spot of R_F 0·3

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and two weaker spots of R_F 0·7 and 0·8 so that here the resemblance is more to pattern A_2.

Although the question did not arise in the main study, it is convenient to mention here that when three examples of SmT–SmD variation in *M. avium* were examined (Moehring & Solotorovsky, 1965), the SmD variants all exhibited the same lipid patterns as their parent strains. The cultures were kindly provided by Professor S. R. Pattyn, two strains being classified by us as *M. avium*, type 1, the other as *M. avium*, type 2.

When extracts of different strains in Provisional species 1 were examined in parallel, their similarity was obvious. Unfortunately, minor variations in technique appeared to affect the picture considerably, apparently owing to the low concentration of the lipids of interest. Because of this factor, unidimensional patterns

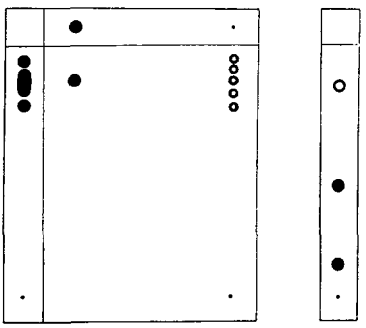

Fig. 5

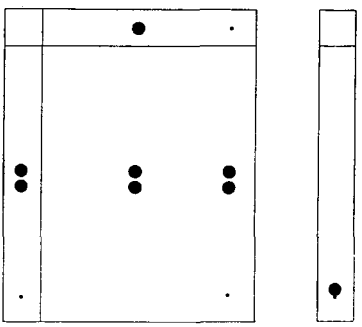

Fig. 6

Fig. 5. Chromatographs in the style of Fig. 2 of the lipids characteristic of mycobacteria classed as 'Provisional species 1'.

Fig. 6. Chromatographs in the style of Fig. 2 of the lipids characteristic of mycobacteria classed as 'Provisional species 2'

were more consistent than two-dimensional. In Fig. 5, the pattern provided by the best extracts is presented. It has been designated B in Table 2. There are five weak bluish spots on the right margin of square 10 and a single spot near the centre of square 12. In the unidimensional runs some of these six spots fuse. With solvent 3 there is a weak bluish spot of R_F 0·13 and two weak brownish spots of R_F 0·45 and 0·85.

The lipid pattern characteristic of Provisional species 2 is presented in Fig. 6. It consists of a pair of spots having R_F values of 0·5 and 0·45 in solvent 1. Each splits into a stationary and a mobile component (R_F 0·5) in solvent 2. The spots have a yellow or greenish tinge in unidimensional runs but this colour is less distinct with runs in two dimensions. In solvent 3, a single spot is produced of R_F 0·05. With two strains (10792 and 18698), migration rates were 0·05 to 0·1 R_F greater in all three solvents and the faster of the two spots in solvent 1 was considerably denser than its fellow.

Amongst the ungrouped strains in Table 3, those with serotypes Watson, Davis and Lunning presented a rather similar pattern on lipid chromatography. Its chief

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feature was a very prominent golden-brown spot at or near the lower right angle of square 12. In solvent 3, the R_F of this spot was 0·05, 0·1 and 0·2 respectively.

Virulence tests

The fowl tests recorded in the tables were carried out over a period of some years during which the dose and route of inoculation varied. The procedure which evolved from this experience was the intravenous inoculation of 0·01 mg. (moist weight) of bacilli grown in Dubos medium. This method was used for 30 strains. In 21 earlier tests a dose was used of 0·25 mg. of bacilli from Dubos medium intraperitoneally and 0·25 mg. intramuscularly. This was supplemented in nine cases by 0·002 mg. intravenously. In only six of the negative fowl tests had an intravenous dose been omitted, the strains being four of *M. avium*, type 1, one of Provisional species 1 (no. 24657) and one unclassified (no. 11961).

Six strains were tested for enhancement of virulence after their culture from the spleen of fowls which in five cases were judged negative histologically in the initial test and in the sixth case as showing limited lesions indicative of attenuated virulence. Each was re-inoculated intravenously into two fowls in a dose of 0·01 and 0·1 mg. respectively. Three strains were classified as *M. avium*, type 1, two as para-avian bacilli, type 1 and one as Provisional species 1 (serotype Boone). No increase of virulence was observed.

Five strains which showed no pathogenicity for fowls in their initial tests were re-tested after six parallel subcultures in Dubos medium at 37° and 42° C. using an intravenous dose of 0·01, 0·1 and 1 mg. for each of three fowls for each of the ten final cultures. The strains used for the experiment were one of *M. avium*, type 1, the only avirulent member of *M. avium*, type 2, one of para-avian bacilli, type 1, one of Provisional species 1 (serotype Boone) and one unclassified strain (serotype Watson). A certain degree of enhancement of virulence by growth at 42° C. as compared with growth at 37° C. was seen with the last two of the strains listed but only at the dose level of 1 mg. The pathogenicity thus elicited was of an order far below that of typical strains of *M. avium*, type 2 which are lethal with 0·01 mg. intravenously. Nevertheless, the results agree with those reported by Scammon *et al.* (1964).

In Table 1, a strain of *M. avium*, type 1 is recorded for convenience as being partially virulent. The first isolate from the patient was in fact lethal for a fowl, although not as destructive as a normal type 2 strain, but another obtained after 5 years' continued excretion of the organism proved to be avirulent although unchanged in its cultural properties.

DISCUSSION

It is widely recognized that the opportunist mycobacteria classed as dysgonic nonchromogens include diverse organisms despite their superficial similarity. Pathogenicity for the fowl is commonly used to distinguish *M. avium* amongst them, but even this limited contribution to the problem can mislead. The fowl test fails to identify certain members of the species which appear to have lost their

virulence for the natural host owing to prolonged residence in man and it occasionally misdiagnoses as *M. avium* the most pathogenic of the remaining dysgonic nonchromogens, which can be shown to be clearly distinct on other grounds. Serotyping has so far proved to be a promising means of classification but except where support is obtained by association with other discriminating properties or epidemiological evidence, the validity of any single character in classification must always remain in doubt. In fact, our findings suggest that certain antigens are so widely distributed that their contribution is of limited value. Moreover, a number of strains do not provide suitable suspensions for serotyping and a few appear to possess a mixture of the recognized antigens. The chief object of the present investigation was to determine to what extent the techniques of serotyping and lipid analysis could support one another as a basis for classification of the dysgonic nonchromogens. In the event, even these two means supplemented by certain biochemical, cultural and pathogenicity tests did not prove wholly successful, as almost a quarter of the strains in the series examined still await classification. However, considerable progress has been made which is embodied in the descriptions which follow of the entities recognized in our material. In the course of the work it became clear that the specificity of fowl tests would be improved by standardization of route and dose and the intravenous inoculation of 0·01 mg. moist weight of bacilli grown in Dubos medium is now recommended. Nevertheless, on the basis of this study it would appear that the fowl test is no guide to the clinical significance of a strain and plays only a subsidiary role in its classification.

The preliminary definition of the subject-matter of the present investigation and descriptions of the groupings identified in it now follow.

The 'dysgonic nonchromogenic mycobacteria'

Organisms in this category give on egg medium an effuse growth which takes at least a week to mature. Its colour is normally buff, but certain strains on continued incubation with ample aeration show some yellow pigmentation. The bacilli grown on egg are strongly acid- and alcohol-fast and most often short; they usually disperse well in suspensions and films. Growth extends at least 10 mm. below the surface in semi-solid medium and catalase activity is weak or absent. All strains in the present series grew at 25° and 37° C., almost all at 42° C. and many at 45° C. The majority were tested against the common anti-tuberculous drugs and, in comparison with normal *M. tuberculosis*, all were highly resistant to PAS and isoniazid and almost all to streptomycin, viomycin, kanamycin and thiosemicarbazone. Sensitivity to cycloserine, ethionamide and sulphonamide varied and proved useful in classification.

In the following descriptions of entities recognized within the dysgonic nonchromogens, the general properties of the latter will not be reported again except to note exceptional behaviour.

Mycobacterium avium

All members are sensitive to cycloserine and resistant to ethionamide on the criteria given above and lack arylsulphatase activity, with a few weakly positive

exceptions. The great majority of strains disclose recognizable lipid constitutions on the chromatography of extracts on silica gel. Two characteristic lipid patterns have been met which appear to be related since there is a general similarity and intermediate forms occur occasionally.

Most strains belong to Schaefer serotypes I or II. In the present series, a few strains were rough and unsuitable for serotyping and three, qualifying for the species on their other properties, belonged to the VI or Boone serotypes. The latter two antigens are widely distributed, however, and appear to be relatively non-specific.

In the present series of 68 dysgonic nonchromogens, 43 could be selected as possible members of *M. avium* simply on cycloserine, ethionamide and arylsulphatase tests. Six of these were then excluded primarily for having neither an appropriate serotype nor lipid structure. None of the 43 had strong arylsulphatase activity but three of the 37 strains accepted in the species were weakly positive compared with three of the six excluded. Even weak activity thus weighs somewhat against admission. Our experience suggests that *M. avium*, especially type 1 strains, can lose virulence for birds on residence in man and from the latter source are sometimes more accurately identified by drug and arylsulphatase tests than by the inoculation of fowls. Additional aids such as serotyping and lipid analysis improve the advantage of *in vitro* methods.

M. avium can be divided into two types on the basis of serotype and lipid structure. The validity of these divisions is supported by the incidence of certain other properties. Both types are known to cause natural tuberculosis in birds.

M. avium, *type* 1

Members are normally of Schaefer serotype I and exhibit the lipid pattern designated A_1. Only one of ten strains of this type isolated from man was highly pathogenic for a fowl and even then the usual florid picture of tuberculosis was muted; moreover a later isolate from the same patient was avirulent although culturally similar to the first. Most members of the type grow at 45° C. and about half of them exhibit long bacilli when grown on egg, an unusual property amongst dysgonic nonchromogens. Members are resistant to sulphonamide.

M. avium, *type* 2

Members are normally of Schaefer serotype II and exhibit the lipid pattern A_2. They are almost always pathogenic for fowls. Growth is always obtained at 42° C. but strains isolated from man seldom grow at 45° C. Most members are sensitive to sulphonamide or give a borderline result.

Para-avian bacilli

This name has been given to a group of organisms which appear to be closely related to *M. avium* but are excluded from the species by their greater sensitivity to ethionamide and sometimes by strong arylsulphatase activity. They exhibit a lipid pattern designated A_3 on chromatography of extracts which although apparently related to patterns A_1 and A_2, is distinct from either. The strains met so far are

divisible into types 1 and 2, the former of Schaefer serotype IV and the latter of serotype VI. All three para-avian type 2 strains met were isolated from cases of cervical adenitis.

Provisional new species 1

Four strains in the series were resistant to cycloserine, sensitive to ethionamide and sulphonamide, and arylsulphatase positive; they grew at 45° C. and were nonpathogenic for fowls (one not tested, however). This combination of properties appears to be distinctive and although serotyping has not been helpful, lipid analysis supports the recognition of the group as an entity. Three strains gave identical lipid patterns and that of the fourth (23774), although aberrant, appeared to be related. One further strain (13444) conformed to Provisional species 1 except in respect of lipid pattern and a moderate virulence for fowls. It has been placed for the present in the unclassified group.

Provisional new species 2

Only seven strains in the series failed to grow at 42° C. and of these five appeared to be related. These were sensitive to both cycloserine and ethionamide and either arylsulphatase negative or weakly positive. Serotyping did not assist classification; four of the strains failed to react with any of the sera available. However, the patterns observed on lipid chromatography were distinctive and supported recognition of the group as an entity.

SUMMARY

A broad division of the opportunist mycobacteria has been defined under the name of 'dysgonic nonchromogens'. The classification was attempted of 68 strains isolated from man in England and Wales, of which 54 at least appeared to be clinically significant. The means used were chiefly drug sensitivity, arylsulphatase activity, specific agglutination, lipid analysis and pathogenicity tests on fowls. On the results, a new definition is proposed for the species *M. avium*, extending beyond the boundaries of pathogenicity for birds, and a scheme put forward for its division into two types. In addition, two provisional new species and a group of 'para-avian' bacilli have been recognized and defined. The remaining 16 strains included six with 'Battey' serotypes but otherwise could not be classified. The need for standardization of fowl tests has been noted together with their diminished importance in the field of classification with the emergence of new *in vitro* methods of examination.

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