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# PRELIMINARY TAXONOMIC STUDIES ON THE LEPROSY BACILLUS

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**Summary.—Antigens extracted from leprosy bacilli obtained from infected human and armadillo tissues have been examined by immunodiffusion analysis with serum samples from lepromatous patients and with immune sera raised in rabbits. Using the best combinations of serum and antigen extracts, 12 antigenic constituents were found in the leprosy bacilli. Six of these were antigens common to all mycobacteria and nocardiae, 4 were specific to the leprosy bacillus and the position of 2 could not be determined. Groups ii and iii antigens (*i.e.* those associated with the slow growing and fast growing subgenera of mycobacteria) were not found in the leprosy bacillus, suggesting some relationship with *M. vaccae* and similar strains, in which these antigens are also missing. Lymphocyte transformation tests performed on lymph node cells of mice infected or immunized with leprosy bacilli also showed the leprosy bacillus to have a closer relationship with *M. vaccae* than with other mycobacteria.**

ALTHOUGH the leprosy bacillus was the first acid-fast bacterium to be described (Hansen, 1874), it remains an enigma in many respects. Claims for its culture outside of living cells remain unsubstantiated and mechanisms underlying the pathology of the disease it causes remain only partially understood (Godal *et al.*, 1974). There has even been doubt as to which genus of bacteria it should be allocated (Fisher and Barksdale, 1973). However, it has been shown to possess mycobacterial mycolic acid, a definitive characteristic of mycobacteria (Etemadi and Convit, 1974).

The recognized members of the genus *Mycobacterium* fall into 2 subgenera, those commonly called "slow growers" and those called "fast growers". The difference between these subgenera is much greater than it sounds; there are phenetic (Tsukamura and Tsukamura, 1967), metabolic (LeCam-Sagniez and Sagniez, 1973) and antigenic differences (Stanford, 1973) between them. This division is probably very ancient since each contains a number of well defined distinct species. A few species, although showing obvious affinities with the fast growing subgenus, have proved difficult to allocate antigenically.

Although the apparent long replication time of the leprosy bacillus favours allocation to the slow growing subgenus, other characteristics make this unlikely. The studies of Godal *et al.* (1974) using lymphocyte transformation and the study by Paul, Stanford and Carswell (1975) on the skin test reactions of leprosy patients and their contacts, produced some evidence indicating a relationship with certain fast growing species. The present investigation employing sera from leprosy patients and immune sera raised in rabbits is an attempt by means of immunodiffusion analysis to determine the exact taxonomic status of the leprosy bacillus.

## MATERIALS AND METHODS

*The bacteria.*—Small samples of human lepromatous tissues were kindly supplied by Dr S. Goodwin from Addis Ababa, by Dr Grace Warren from Hong Kong and by one of us (R.J.W.R.) from Malaya. Subcutaneous nodules from heavily infected 9 banded armadillos and hepatic tissue from a heavily infected 8 banded armadillo were prepared by 2 of us (G.P.W. and J.C.).

Each tissue sample was treated individually in the following way. It was cut into thin strips and homogenized in an M.S.E. Atomix. This material was filtered through a gauze and both the filtrate and residue were saved. The filtrate was centrifuged at 15,000 *g* for half an hour and the supernatant was added to the filter residue. The centrifuged deposit was resuspended in 20 ml M/15 phosphate buffer pH 6·8, and this was shaken in a separating funnel with an equal volume of Whitemor oil as previously described (Stanford, 1973*a*) for the separation of organisms from tissue antigens. The clean organisms, of which there were about 4–5 mg from the human tissues and 10–15 mg from the armadillo tissues, were suspended in 5 ml of M/15 phosphate buffer, pH 6·8 and treated for 15 min in the M.S.E. 100 watt ultrasonic disintegrator.

The filter residues and centrifuged supernates were each shaken and, in the cases of human tissue from Addis Ababa and the tissue from 9 banded armadillos, ground with a pestle and mortar to release most of the bacilli from the tissues. This was achieved in the other tissue samples by using a simple "stomach-like" device prepared by sealing the tissue suspensions in 2 heavy gauge polyethylene bags, one inside the other, and pummelling them for 15 min by hand. In each case the suspensions were allowed to settle for about 20 min and the supernates carefully pipetted off. Each was then centrifuged at 15,000 *g* for half an hour to deposit all the bacilli. Most of the supernates were removed and concentrated to 1/10 volume by pervaporation. The deposits were suspended in about 3 ml of supernate and treated in the M.S.E. ultrasonic disintegrator as described above.

Thus, there were 3 preparations of antigens from each tissue sample: (1) An extract of oil-passaged, tissue-antigen free organisms for use in production of antisera as previously described (Stanford and Beck, 1968); (2) An extract of organisms still contaminated with tissue antigen; and (3) a concentrate of the soluble tissue extracts.

Antigens 2 and 3 were reserved for use in the immunodiffusion plates.

*The antisera.*—Two individual serum samples known to contain easily demonstrable antimycobacterial antibodies, obtained from Malaysian patients with lepromatous disease were provided by one of us (R.J.W.R.). A pool of sera from 25 patients with lepromatous leprosy in Addis Ababa was also provided (by G.K.) This was

TABLE I.—*Details of Immunization Schedules used for the Preparation of Immune Sera in Rabbits*

| Rabbit no. | Primary immunization with 6 injections at weekly intervals of | Booster injection given after (months) | Blood taken |
|---|---|---|---|
| 107<br>108 | Leprosy bacilli from human lesions (Addis Ababa) | 6 | 2 weeks after primary course and 4 weeks after booster |
| 130<br>131 | Leprosy bacilli from human lesions (Malaya) | 1 and 2 | 2 weeks after primary course and after each boost |
| 172 | Leprosy bacilli from human lesions (Malaya) | 15 | 1 month after primary course and after boost |
| 229<br>230 | Leprosy bacilli from human lesions (Hong Kong) | 16, 20 and 25 with bacilli from 9 banded armadillo | 1 month after primary course and after each boost |
| 259<br>260 | Leprosy bacilli from 9 banded armadillo | 2 and 7 | 2 and 4 weeks after primary course and after each boost |
| 285<br>286 | Leprosy bacilli from 9 banded armadillo Dose: 0·1 ml. ID. | 3 | 2 months after primary course and 3 months after boost |
| 301<br>302 | Leprosy bacilli from 8 banded armadillo | | 2 and 5 weeks after |

With the exception of rabbits 285 and 286 which received 0·1 ml intradermally all injections were of 1 ml, and were given by the intramuscular route.

a sample of that referred to as serum pool I in the work of Kronvall *et al.* (1975).

Thirteen rabbits were used to raise antisera. They were immunized according to the schedule shown in Table I. (Details of these are provided because none was particularly successful and some other technique appears to be necessary to obtain antisera equivalent in quality to those readily raised to other mycobacteria.)

*Other reagents.*—Antigens were prepared from the liver of a healthy 9 banded armadillo by the same methods described for infected tissues, and these were used as control antigens. Antigenic preparations of cultured organisms of many mycobacterial species were available from previous or concurrent studies, as also were antigens prepared from strains of 13 other genera. Rabbit antisera to almost all mycobacterial species were similarly available.

*Immunodiffusion tests.*—The methods of carrying out the tests and analysing the results were as previously described (Stanford and Beck, 1968; Stanford, 1973*b*). Each of the sera obtained from patients or produced in rabbits was tested with each of the antigenic preparations of leprosy bacilli. The rabbit antisera were improved by concentration. This was achieved by salting out the globulins and reconstituting in $\frac{1}{4}$–$\frac{1}{3}$ volume.

Antigens of all the species listed in Table III were tested with one of the concentrated rabbit antisera to leprosy bacilli and the best of the leprosy antigen extracts was tested with antisera to some other species.

*Lymphocyte transformation.*—Lymphocyte transformation tests were carried out as previously described (Rook, 1975) using mouse lymph node cells. The mice had received either immunizing ($10^6$ organisms) or infecting ($10^4$ organisms) doses of the leprosy bacillus into their hind footpads.

## RESULTS

None of the 13 rabbit antisera to the leprosy bacillus were as good in quality as those raised to most mycobacterial species. Prior to their concentration, the sera produced 3 precipitates at best with leprosy extract or other antigens. Following concentration, the serum pooled between rabbits 259 and 260 produced most precipitates.

The antigen preparations of leprosy bacilli extracted from armadillo tissues were qualitatively better than those extracted from human tissues, even when the latter were pooled and concentrated by pervaporation. The reaction between the best serum concentrate and the best antigen extract produced 13 immunoprecipitates. Two of these precipitates were also produced when the control extract of normal armadillo liver was tested with the serum and these are omitted from further consideration.

The sera from leprosy patients were similarly examined and the results for these are shown in Table II. The best serum was one of those (No. 2) from a Malaysian patient. Using this serum and the armadillo leprosy extract, 12 lines of precipitation were observed; no precipitates formed with the control armadillo antigen.

The results of tests between the 2 best antisera to leprosy bacilli and antigenic extracts of all the other species are shown in Table III. In no case could more than 6 antigens be demonstrated. Whichever antisera to other mycobacterial species were employed, no more than 6 antigens could be demonstrated in the extracts of leprosy bacilli (Fig. 1).

TABLE II.—*Numbers of Precipitates Formed between Various Preparations of Leprosy Bacilli and Sera from Patients with Lepromatous Leprosy*

| Origin of antigen | No. of precipitates formed with sera from lepromatous patients | | |
|---|---|---|---|
| | Serum 2 | Serum 7 | Serum pool I |
| Leprosy bacilli from human tissues | 6 | 3 | 4 |
| Leprosy bacilli from 9 banded armadillo | 12 | 6 | 7 |
| Leprosy bacilli from 8 banded armadillo | 10 | 5 | 6 |
| Normal tissue extract of 9 banded armadillo | 0 | 0 | 0 |

TABLE III.—*Numbers of Precipitates Formed between All the Antigenic Preparations used and the Two Best Antisera*

| Origin of antigen | No. of precipitates formed with the best antisera: Serum 2 | Rabbits 259/260 conc. |
|---|---|---|
| Leprosy bacilli from: | | |
| human tissue | 6 | 6 (+3)* |
| 9 banded armadillo | 12 | 11 (+2)* |
| 8 banded armadillo | 10 | 11 (+2)* |
| *M. tuberculosis (BCG)* | 6 | 6 |
| *M. chelonei abscessus* | 6 | 5 |
| *M. avium avium* | 6 | 6 |
| *M. fortuitum* | 5 | 5 |
| *M. vaccae* | 6 | 6 |
| *M. smegmatis* | 6 | 4 |
| *M. kansasii* | 6 | 6 |
| *M. nonchromogenicum* | 4 | 6 |
| Other mycobacteria | 4–6 | 4–6 |
| *N. asteroides* | 3 | 3 |
| *N. brasiliensis* | 4 | 4 |
| *N. caviae* | 3 | 3 |
| *N. uniformis* | 3 | 3 |
| *Gordona rubra* | 5 | 5–6 |
| *G. bronchialis* | 5 | 5–6 |
| "Rhodochrous" strains | 3 | 4–5 |
| Corynebacteria: 6 species | 3 | 3 |
| *Cellulomonas fimi* | 2 | 3 |
| *Listeria monocytogenes* | 1 | 2 |
| *Kurthia zopfii* | 1 | 3 |
| *Bacterionema matruchotii* | 1 | 3 |
| *Bifidobacterium bifidum* | 1 | 3 |
| *Propionibacterium freudenreichii* | 1 | 3 |
| *Leptotrichia buccalis* | 1 | 3 |
| *Brevibacterium ammoniogenes* | 3 | 3 |
| *B. linens* | 1 | 3 |
| *Arthrobacter globiformis* | 2 | 2 |

* Numbers shown in parentheses are of precipitates formed between the rabbit antiserum and extracts of non-infected human and armadillo tissues.

Although the quality of antigenic extracts of leprosy bacilli from human tissues was inferior, this material was analysed for antigens missing from the armadillo leprosy extract. None could be found with patient sera and 3 extra precipitates formed with the rabbit antisera could also be demonstrated when normal human serum was used as antigen.

Lymph node cells from mice which had been infected 18–24 months previously with $10^4$ leprosy bacilli in each hind footpad were assayed for lymphocyte transformation responses to 19 mycobacterial species. These animals were well advanced in the plateau phase of the disease. The lymph node cells gave abnormal responses *in vitro*, very similar to those described previously in the late anergic phases of *Mycobacterium ulcerans* (Rook, 1975) or *Mycobacterium lepraemurium* infections (unpublished observations) in the mouse. That is to say, lymph node cells from infected mice underwent mitosis spontaneously *in vitro*, in the absence of added antigen. This spontaneous mitosis was inhibited by the addition of antigens prepared from the leprosy bacillus, or from a number of fast growing species—*Mycobacterium vaccae*, *M. gilvum*, *M. nonchromogenicum* and *M. duvalii*—but not by any of the antigens prepared from slow-growing species. The earlier studies with *M. ulcerans* infected mice suggested that such responses do reflect antigenic specificity. Subsequently, in order to avoid using mice actually suffering from leprosy infection, BALB/c mice were injected in the footpad

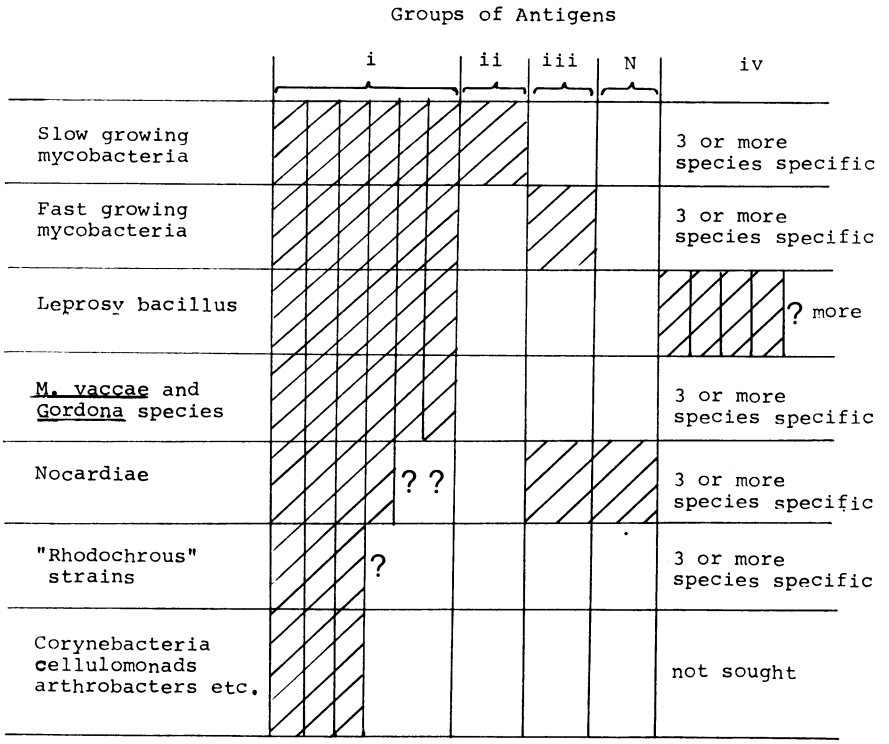


FIG. 1.—Diagram showing the antigenic relationships between the leprosy bacillus, other species of mycobacteria and other genera. The antigens of Groups i and iii are shared vertically where this is indicated by hatching.

with $10^6$ organisms from human tissue. This is considered to be an immunizing rather than an infecting dose. After 5 months and 6 months, lymphocyte transformation tests were performed using cells from the injected animals, and from control animals living in the same cage. At 5 months, the injected animals showed increased responses not only to the leprosy bacillus and *Mycobacterium vaccae* but also to all the environmental organisms to which the control animals responded. This phenomenon, which is due either to an adjuvant effect or perhaps to weak cross-reactions, is seen routinely after the injection of any mycobacterial species and in humans after BCG. However, the effect is transient and 6 months after injection only the responses to the leprosy bacillus and *M. vaccae* were greater than those in the control mice (Fig. 2).

## DISCUSSION

It has long been known that patients with lepromatous leprosy have readily demonstrable circulating antibodies to other mycobacteria. Recently Axelson and colleagues (1974) detected 14 antibodies to BCG in such sera using Crossed immunoelectrophoresis, and Kronvall *et al.* (1975) detected 7 antibodies against leprosy bacilli from armadillo tissue by the same technique. Using extracts of a very crude kind, Abe (1970) demonstrated both common mycobacterial antigens and a leprosy specific antigen of protein nature using rabbit antisera. Navalkar's (1971) studies and the studies of Kwapinski, de Almeida and Kwapinski (1972) employing rabbit antisera and freeze-press or ultrasonic extracts of leprosy bacilli from human lesions, confirm the presence of antigens in common with other myco-

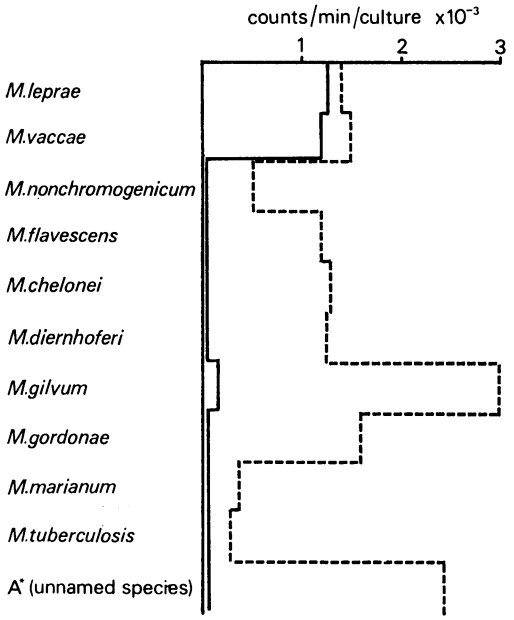


FIG. 2.—Lymphocyte transformation tests performed 5 months (– – –) and 6 months (———) after immunization of BALB/c mice with $10^6$ leprosy bacilli. Results are expressed as the increment in ct/min per culture minus the response to the same antigen in control cultures. Thus, only the responses attributable to the injection of leprosy bacilli are shown.

bacteria and nocardiae. Despite these studies, the precise generic position of the leprosy bacillus and its relationship with other species remain elusive.

Our present study demonstrates that the leprosy bacillus, whether obtained from human or armadillo tissues, possesses all the antigens known to be shared by all the species of mycobacteria that can be shown by the method used (Group i antigens in Fig. 1). It has not been possible to demonstrate those antigens limited to slow growing (Group ii in Fig. 1) or fast-growing (Group iii in Fig. 1) mycobacterial species in the extracts of the leprosy bacillus, or antibodies to them in the leprosy sera or rabbit serum 259/260. However, the leprosy bacillus does not appear to be unique in this deficiency since the same has been observed in the readily culturable species *M. vaccae* and its close relatives.

By the same criteria, absence of Group iii antigens and of the nocardial generic antigens (N in Fig. 1) would seem to exclude the leprosy bacillus from *Nocardia*. Species of the newly described genus *Gordona* (Tsukamura, 1971) possess the Group i antigens of mycobacteria, but they do not contain mycolic acids of the mycobacterial type (Goodfellow, personal communication) and in view of this are not considered further. The "Rhodochrous" strains and the various genera of "coryneform" organisms possess only a few of the Group i antigens (3 or 4 at the most), making it most improbable that the leprosy bacillus is at all closely related to any of these. Thus, the evidence provided by immunodiffusion indicates that the closest relatives of the leprosy bacillus might be sought among *M. vaccae* and similar organisms. Just why the antisera raised in rabbits were of poor quality is uncertain. The amount of organism injected in each case was within the limits of that employed successfully with many other species. However, the number of solidly staining leprosy bacilli in the inocula was only between 5 and 30%; much less than in inocula of other species. Evidence based on studies of delayed hypersensitivity in leprosy patients and their contacts either by lymphocyte transformation (Godal *et al.*, 1974) or by multiple skin testing (Paul *et al.*, 1975), indicates a closer relationship with fast growing mycobacteria than with the slow growing species. Additionally the study of Paul *et al.* (1975) suggested an especially close relationship between the leprosy bacillus, *M. nonchromogenicum* and *M. vaccae*. A small subsequent skin test study has again indicated this relationship with *M. vaccae*.

Further evidence for an antigenic relationship between *M. vaccae* and the leprosy bacillus has emerged from the preliminary lymphocyte transformation studies in mice infected or sensitized with the leprosy bacillus.

The ability of pyridine to remove the acid fastness from leprosy bacilli but not

from some other mycobacterial species, a characteristic stressed by Fisher and Barksdale (1973), might also be used to help establish relationships with other organisms. Preliminary studies have shown that the acid fastness of some, but not all, strains of *M. vaccae* is very sensitive to pyridine.

The term *M. vaccae* and its close relatives is used in this study to encompass *M. vaccae* itself and those strains referred to as the "*M. parafortuitum* complex" in the work of Saito *et al.* (1975). All these strains grow rapidly on many culture media and are biochemically very active. They are common in the environment and, with the possible exception of bovine mastitis, have not been incriminated as pathogens. Their taxonomy still leaves much to be desired and nothing has been published on their antigenic constitution. Numerous species names have been proposed for variants within the group, largely determined on the basis of numerical analysis of phenetic characters. The possibility of their relationship with the leprosy bacillus should add impetus to their more detailed study.

In conclusion our studies indicate that the leprosy bacillus is a *Mycobacterium*, but that it does not belong to either of the major subgenera, as determined serologically, and that its closest relatives might be sought amongst *M. vaccae* and similar organisms.

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## REFERENCES

ABE, M. (1970) Studies on the Antigenic Specificity of *Mycobacterium leprae*. Demonstration of Soluble Antigens in Leprosy Nodules by Immunodiffusion. *Int. J. Leprosy*, **38**, 113.

AXELSON, N. H., HARBOE, M., CLOSS, O., & GODAL, T. (1974) BCG Antibody Profiles in Tuberculoid and Lepromatous Leprosy. *Infect. Immun.*, **9**, 952.

ETEMADI, A. H. & CONVIT, J. (1974). Mycolic Acids from "Non-cultivable" Mycobacteria. *Infect. Immun.*, **10**, 236.

FISHER, C. A. & BARKSDALE, L. (1973) Cytochemical Reactions of Human Leprosy Bacilli and Mycobacteria: Ultrastructural Implications. *J. Bact.*, **113**, 1389.

GODAL, T., MYRVANG, B., STANFORD, J. L. & SAMUEL, D. R. (1974) Recent Advances in the Immunology of Leprosy with Special Reference to New Approaches in Immunoprophylaxis. *Bull. Inst. Pasteur*, **72**, 273.

HANSEN, G. A. (1874) Undersøgelser angäende spedalskhedens årsager tildels udførte sammen med vorstander Hartwig. *Norsk. Mag. Laegevidensk*, **4**, 1.

KRONVALL, G., BJUNE, G., STANFORD, J. L., MENZEL, S. & SAMUEL, D. R. (1975) Mycobacterial Antigens in Antibody Responses of Leprosy Patients. In the press.

KWAPINSKI, J. B. G., DE ALMEIDA, J. O. & KWAPINSKI, E. H. (1972) Immunological Analysis of *Mycobacterium leprae* using Cross Reactions with Cytoplasmic Antigens of the Actinomycetales. *Can. J. Microbiol.*, **18**, 391.

LECAM-SAGNIEZ, M. & SAGNIEZ, G. (1973) Metabolisme des glucides chez *Mycobacterium phlei* et *Mycobacterium tuberculosis*. *Ann. Soc. belge med. Trop.*, **53**, 347.

NAVALKAR, R. G. (1971) Immunologic Analysis of *Mycobacterium leprae* Antigens by Means of Diffusion-in-gel Methods. *Int. J. Leprosy*, **39**, 105.

PAUL, R. C., STANFORD, J. L. & CARSWELL, J W. (1975) Multiple Skin Testing in Leprosy. *J. Hyg., Camb.*, **75**.

ROOK, G. A. W. (1975) The Immunological Consequences of Antigen Overload in Experimental Mycobacterial Infections of Mice. *Clin. & exp. Immunol.*, **19**, 167.

SAITO, H., GORDON, R. E., JUHLIN, I., KAPPLER, W., KWAPINSKI, J. B. G., MCDURMONT, C., PATTYN, S. R., RUNYON, E. H., STANFORD, J. L., TARNOK, I., TASAKA, H., TSUKAMURA, M. & WEISZFEILER, J. (1975) A Co-operative Numerical Analysis of Rapidly Growing Mycobacteria—the Second Report. In the press.

STANFORD, J. L. (1973*a*) An Immunodiffusion Analysis of Strains of *Mycobacterium ulcerans* isolated in Australia, Malaya, Mexico, Uganda and Zaire. *J. med. Microbiol.*, **6**, 405.

STANFORD, J. L. (1973*b*) Immunodiffusion Analysis —a Rational Basis for the Taxonomy of Mycobacteria. *Ann. Soc. belge med. Trop.*, **53**, 321.

STANFORD, J. L. & BECK, A. (1968) An Antigenic Analysis of the Mycobacteria: *Mycobacterium fortuitum*, *Mycobacterium kansaii*, *Mycobacterium phlei*, *Mycobacterium smegmatis* and *Mycobacterium tuberculosis*. *J. Path. Bact.*, **95**, 131.

TSUKAMURA, M. & TSUKAMURA, S. (1967) A Practical System for Identification of *Mycobacterium tuberculosis*, *Mycobacterium bovis*, *Mycobacterium kansaii* and *Mycobacterium fortuitum*. *Scand. J. resp. Dis.*, **48**, 58.

TSUKAMURA, M. (1971) Proposal of a New Genus, *Gordona* for Slightly Acid-fast Organisms Occurring in Sputa of Patients with Pulmonary Disease and in Soil. *J. gen. Microbiol.* **68**, 15.