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MYCOBACTERIAL PPD SENSITINS AND THE NON-SPECIFIC REACTOR PROBLEM*

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CONTENTS.

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CHAPTER 1

INTRODUCTION

(i) *General introduction and definition of terms*

The tuberculin test is probably the diagnostic test most widely applied by the veterinary profession. This test has been in use since the beginning of the century and has been used as the sole diagnostic tool in the eradication of bovine tuberculosis from many countries. Despite, or perhaps because of, its very widespread application international standardization with regard to tuberculin production methods, method of testing and interpretation of reactions has not yet been achieved. The tuberculin test has proved to be a very reliable diagnostic method, but as in all biological tests difficulties have been encountered. The greatest problem is the occurrence of so called non-specific reactors.

In tuberculin testing of cattle the aim is to find all cattle infected with *Mycobacterium bovis*. Any animal reacting to tuberculin while not infected with *M. bovis* is termed a non-specific reactor. Animals infected with *Mycobacterium tuberculosis* which react to the standard diagnostic tuberculin used in a particular country are therefore also regarded as non-specific reactors. In fact tuberculin prepared from *M. tuberculosis* has been used in many countries for the diagnosis of bovine tuberculosis so that reactions to this product in animals infected with human type tubercle bacilli are, strictly, specific reactions. In South Africa tuberculin is prepared from *M. bovis* and in this study any reaction due to any other cause than bovine tuberculosis will be regarded as non-specific. Other terms which will be used that require definition are:

Tuberculin (Koch) is a product containing tuberculoprotein prepared from a culture filtrate of *M. bovis*, *M. tuberculosis* or *Mycobacterium avium*, i.e. bovine, human or avian tuberculin.

Sensitin (Magnusson) is a product prepared from a culture filtrate of any organism, which will elicit a delayed hypersensitivity reaction in a suitably sensitized animal. Sensitins are usually prepared from mycobacteria and the tuberculins would be included in the wider definition of sensitins.

Tuberculin sensitivity—an animal is said to be tuberculin sensitive when an injection of tuberculin elicits a typical delayed hypersensitivity reaction. The use of the term " allergy " which is sometimes used in this respect, has been avoided.

Allergenic—the allergenic characteristics of an organism determine its ability to produce in a host animal a state of hypersensitivity to homologous and heterologous sensitins. Allergenic relationships of organisms are therefore referred to in much the same way as to antigenic relationships when antigenic characteristics of organisms are discussed.

Problem herd—a problem herd is a herd of cattle in which non-specific reactions occur so frequently that the interpretation of tuberculin tests in the herd is difficult. Most problem herds are TB free herds, but in some instances problems are encountered where bovine tuberculosis and non-specific sensitization occur in the same herd.

In South Africa due to shortage of staff and the overwhelming impact of other major epizootic diseases such as rinderpest and East Coast fever, eradication of bovine tuberculosis has not been undertaken on a large scale. Eradication on a national scale will probably soon be started. This study was, therefore, undertaken in an attempt to investigate some of the problems in tuberculin testing under South African conditions.

(ii) *Historical review*

In the history of tuberculosis the name of Robert Koch remains without parallel. He discovered the causative organism of tuberculosis in 1882, was the first to observe the peculiar delayed hypersensitivity reaction now known as a Koch's phenomenon and developed the first tuberculin. Although his tuberculin proved unsuccessful as a cure for tuberculosis, he recognized that it could be used as a diagnostic agent. Studies on tuberculin led him to believe that the active principle was of protein nature. He was, however, unable to prepare it in pure form as his tuberculin contained protein fractions of the broth culture from which it had been prepared (according to Svenkerud, 1955).

In an effort to develop a tuberculin free from protein impurities Kuhne (according to Svenkerud, 1955) developed in 1892 to 1894 a synthetic medium without proteins on which tubercle bacilli could be cultivated. Malm (according to Svenkerud, 1955) in 1894 also used a synthetic medium and was able to precipitate the active principle of tuberculin with alcohol; he concluded that it was of protein nature. Much valuable work has been done to define the chemical composition of tuberculin by Florence Siebert. She has shown the active principle to be of protein nature, but has also shown tuberculin prepared by the usual methods to contain other non-protein constituents. She was able to isolate three distinct proteins from tuberculin, and has reviewed her own and other work on the chemical composition of tuberculin (Siebert, 1950).

The three types of tuberculin still used are: Old Tuberculin (OT) which is heat concentrated and prepared from filtrates of broth cultures; heat concentrated synthetic medium tuberculin (HCSM) which is a heat concentrated product prepared from synthetic medium culture filtrates; purified protein derivative (PPD) tuberculin which is prepared by precipitation of the active principle from synthetic medium culture filtrates. The term OT is still sometimes used for all heat concentrated tuberculins.

After Koch's announcement of the discovery of tuberculin the possibility of using it for diagnostic purposes was investigated, and by 1892 Nocard had formulated a method of tuberculin testing (Calmette, 1923). In this test tuberculin was injected subcutaneously and the animal's temperature checked two hourly after the injection. A rise in temperature indicated a positive reaction. Various methods of testing local sensitivity including a number of skin tests, ophthalmic, intrapalpebral, vaginal and nasal mucosa tests were also evolved. These methods have been described by Calmette (1923). Of these tests the intradermal test, used for the first time by Moussu & Mantoux (1908) and Moussu (1908), has been universally adopted for testing cattle.

From 1925 to 1947 the double intradermal test described by Buxton (1925) was widely used in Britain. In this test a second injection of tuberculin is given after 48 hours at the same site as the first, and the result read after a further 24 hours. After a trial by the Ministry of Agriculture and Fisheries (1947) it was seen that the second injection was unnecessary. The single intradermal test then became the official method of tuberculin testing in Britain.

In 1913 Cobbett & Griffiths, according to Francis (1947), demonstrated the specificity of avian and mammalian tuberculins. Plum (1931, 1932) later used avian and mammalian tuberculins simultaneously in a comparative test, and was able to distinguish between cattle infected with bovine tuberculosis and cattle sensitized by *Mycobacterium paratuberculosis* and *M. avium*. Stenius (1938) also reported on

the use of the comparative test. In England after the Ministry of Agriculture and Fisheries (1942a) had taken over the production of tuberculin, it was found that an excessive number of non-specific reactions occurred with the new more potent PPD tuberculin. The mammalian-avian comparative test was therefore introduced by the Ministry of Agriculture and Fisheries (1942b). The test has been used exclusively in Britain ever since. It is also widely used in other countries (Nielsen & Plum, 1960; Meyn, 1960; Van Waveren, 1960; Gasse, 1960; Azevedo, 1960; Goor, 1960).

The conclusions agreed on by the 20 representatives of various countries and from F.A.O., O.I.E. and W.H.O. who attended the Second Symposium on the Eradication of Bovine Tuberculosis (Anon., 1960), included the following: " The intradermal technique has continued to be the most satisfactory method for the application of the tuberculin test and should not be replaced. A comparative test using mammalian and avian tuberculins is of great value in some countries for the differentiation of specific and non-specific reactions ".

There is, however, still no universally accepted method of tuberculin testing. Some countries still use OT, some HCSM and others PPD. The strength of the tuberculin varies from 50,000 TU per ml to 100,000 TU per ml. The injection site is either the neck or the caudal fold.

(iii) *Tuberculin and tuberculin testing in South Africa*

Until 1928 the subcutaneous tuberculin test was the method of tuberculin testing generally used in South Africa, but it was found to be impractical especially in ranch cattle (Viljoen, 1927). The double intradermal test was therefore introduced and proved to be reliable (W. S. Green, 1933). After the trials of the Ministry of Agriculture and Fisheries in England (1947) the single intradermal test was also used in South Africa.

Imported tuberculin was used in South Africa until 1936, when Robinson started production of HCSM at Onderstepoort. In 1947 he began the production of PPD using methods similar to those described by H. H. Green (1945, 1953). The PPD was made from the human strain T100 and later from strains PN, C, and DT. In 1950 the bovine strain AN5 was used by Robinson for PPD production, but bovine PPD was not issued until some years later (Robinson, 1965). Paterson (1948) showed that bovine PPD possessed only one third the activity of human PPD when tested on guinea pigs sensitized by *M. avium*. In Holland bovine PPD was also shown to be more specific than human PPD in both cattle and guinea pigs (Van Waveren, 1953, 1965). In 1954 a large scale field trial to compare bovine and human PPD was started by Lambrechts (1958). The result of this trial showed that the bovine PPD caused less non-specific reactions than the human PPD. From 1 January 1960 AN5 bovine PPD was therefore issued by Onderstepoort. At the same time a trial was begun to compare bovine PPD at strengths of 100,000 TU per ml and 70,000 TU per ml (Kleeberg, 1960a, 1961). The weaker tuberculin was found to cause less non-specific reactions but to be as effective in the detection of tuberculous animals. The strength of Onderstepoort bovine tuberculin was therefore reduced to 70,000 TU per ml from November 1961.

Avian PPD made from the Strain D4 has been produced at Onderstepoort for comparative testing for many years. The comparative test has, however, not proved popular with veterinarians in the field and only a few doses have been used annually for this purpose.

(iv) *The non-specific reactor problem*

(*a*) *Occurrence and cause of non-specific reactions*

The tuberculin test is an extremely accurate method of diagnosing bovine tuberculosis. When used in cattle populations, where the incidence of tuberculosis was comparatively high, various workers found the test to be between 94 and 98 per cent accurate (Anon., 1911; Rodgers, 1918; Ernest, 1920–21; W. S. Green, 1933; Dalling, 1948; Schaaf, 1955; Kleeberg, 1960a; Van Waveren, 1965), but even in these investigations a small percentage of false positive reactors were found. In cattle populations free from tuberculosis rare reactions do still occur. All reactions occurring in a TB free population must be false positive or non-specific reactions and would show no lesions at a post mortem examination. This is not a serious criticism of the test, as it should be remembered that only a small percentage of non-tuberculous cattle will react to mammalian tuberculin and that with well described methods most of these can be recognized as non-specific reactors. The occurrence of non-specific reactions assumes a position of greater importance when tuberculosis is eradicated from a country, province or individual herd. This situation is well demonstrated by the increasing number of reactors showing no gross lesions at post mortem examination in the United States of America as that country became progressively more free from bovine tuberculosis (Wilder, 1962).

The incidence of non-specific reactors to mammalian tuberculin varies from country to country and from place to place. Incidences as low as 0·25 per cent (Gow, 1948) and as high as 17 per cent (Herbert & Paterson, 1955; Paterson & Herbert, 1957) have been reported. Since the literature on non-specific reactions has been well reviewed (Paterson, 1956; Schaetz, 1956; Karlson, 1962), a complete review will not be given here. The mechanism and causes of non-specific sensitization will be briefly considered.

The injection of mycobacteria into a host causes the development of (1) delayed hypersensitivity, (2) a certain degree of protection against superinfection and (3) pathological changes in the host. These changes may represent distinct and separate processes, although in a natural infection they develop in parallel. Work done by Ribi, Perrine, List, Brown & Goode (1959) has helped to clarify our understanding of these processes. They developed a method by which mycobacterial cell walls could be isolated in a very pure form. It was then shown that the injection of the cell walls into rabbits induced a sensitivity to further injections of cell walls or to cell protoplasm (Ribi, Larson, List & Wicht, 1958; Larson, Ribi, Wicht & List, 1961; Ribi & Larson, 1964). Injections of protoplasm did not produce sensitivity to either cell walls or to protoplasm. It was also shown that disrupted killed cells produce a greater degree of immunity than whole killed cells (Larson, Ribi, Wicht, List & Goode, 1963; Ribi & Larson, 1964). Crowle (1961a, 1961b, 1963; Crowle & Hu, 1965) maintains that a polysaccharide fraction isolated from *M. tuberculosis* is capable of producing immunity to tuberculosis in mice and guinea pigs.

It is, therefore, tempting to postulate that sensitivity is produced by fractions of the cell walls, possibly lipids, or phosphatides, immunity by fractions found in disrupted cell material, possibly polysaccharide in nature, and the protein of the protoplasm corresponds to tuberculin. Viable virulent bacteria are required to produce progressive disease. Studies with fractions of mycobacteria have shown that a combination of lipid (or wax) fractions and protein fractions is most satisfactory for inducing tuberculin hypersensitivity (Raffel, 1948). It is possible

that Ribi's cell wall preparations still contained small amounts of protoplasm. Sensitivity can be induced independently of immunity (Raffel according to Crowle, 1958). The converse, i.e. immunity without hypersensitivity has not yet been produced. There is much clinical evidence indicating that hypersensitivity and immunity are very closely related if not the same process (Arnason & Waksman, 1963). Non-protein fractions, e.g. a lipopolysaccharide fraction (Hartson, 1964; Ross, 1964) and a carbohydrate fraction (Baer & Chaparas, 1964) have also been shown to be able to elicit delayed hypersensitivity reactions in tuberculous patients and BCG sensitized guinea pigs.

The genus *Mycobacterium* which includes a large number of pathogens, potential pathogens and saprophytes, is characterized by being acid-alcohol fast when stained by the Ziehl-Neelsen method. This is due to their peculiar lipid rich capsules. In view of the similar chemical composition of the capsules of different mycobacteria, it is not surprising that they are antigenically and allergenically also closely related. Many species apparently contain in their capsules fractions capable of sensitizing animals to mammalian tuberculin. These reactions to mammalian tuberculin are always smaller than the reaction to a similar dose of their homologous sensitin would be (H. H. Green, 1945; Magnusson, 1961; Magnusson, Engbaek & Bentzon, 1961; Edwards, Hopwood, Affronti & Palmer, 1961; Johnston & Smith, 1964; Edwards, Hopwood & Palmer, 1965). Although mycobacteria other than *M. bovis* are capable of causing sensitivity to mammalian tuberculin in cattle, they often fail to cause lesions. We therefore have the occurrence of no gross lesion reactors (NGL) or no visible lesion reactors (NVL).

(*b*) *Sensitization caused by* M. avium

Avian tubercle bacilli have frequently been isolated from cattle (Van Es, 1929; Van Es & Martin, 1930; McCarter, Hastings & Beach, 1937; Plum, 1938, 1952; Feldman & Karlson, 1939; Glover, 1941; Thordal Christensen, 1952; Burgisser & Schneider, 1957; Lesslie, 1959; Feldman, 1960; Cavrini & Gasparini, 1960; Robijns, 1960; Schaaf, J. & Beerwerth, 1960). Generally they cause no lesions or only small non-progressive lesions of the mesenteric lymph glands (Van Es, 1929; Feldman, 1960; Robijns, 1960). In rare cases lesions may be progressive and cases of metritis causing abortions (Plum, 1938; Burgisser & Schneider, 1957; Schmittdiel, 1964), mastitis (Thordal Christensen, 1952; Lesslie, 1959), pulmonary tuberculosis (Pearson & McGowan, 1958) and even generalized tuberculosis have been reported (Stuart & Marshall, 1952). Infections with *M. avium* can cause cattle to react non-specifically to mammalian tuberculin and therefore methods of comparative testing with avian and mammalian tuberculins were evolved (Plum, 1931, 1932). They are used in many countries (Nielsen & Plum, 1960; Meyn, 1960; Van Waveren, 1960; Gasse, 1960; Azevedo, 1960; Goor, 1960; Stableforth, 1960) for distinguishing non-specific reactors from tuberculous cattle. Results of comparative tests do not necessarily reflect a true incidence of *M. avium* sensitization as sensitization caused by a number of other mycobacteria, e.g. *M. paratuberculosis*, those belonging to the scotochromogenic group (Runyon Group II) and other para-avian strains could also be expected to cause greater sensitivity to avian than to mammalian tuberculin. The high incidence of reactions to avian tuberculin (Herbert & Paterson, 1955; Weber, 1955; Paterson & Herbert, 1957) does nevertheless emphasize the importance of this organism.

Attempts have been made to correlate the occurrence of reactions in cattle to avian tuberculin with the presence of infected poultry in contact with the cattle (Plum, 1931; Schalk, Roderick, Foust & Harschfield, 1935; Feldman & Moses,

1942; Fidler, 1946; Cavrini & Gasparini, 1960; Nassal, 1963; Zhurnakova, Malygin, Borisenkova & Bolotnikov, 1964; Vasilenko, 1964). In some cases this has not been possible (Feldman & Moses, 1942; Fidler, 1946) and other sources of infection have therefore been sought. Wild birds have been extensively investigated and the incidence of *M. avium* infection has been found to be fairly common. The literature on this subject has been well reviewed by McDiarmid (1962). Wilson & MacDonald (1965) have drawn attention to the increasing incidence of tuberculosis in wild birds and suggest that wild birds may be responsible for sensitizing cattle to avian tuberculin.

Recently it has been realized that organisms very closely related to *M. avium*—the so-called Battey strains—are responsible for a disease of man clinically indistinguishable from tuberculosis (Lewis, Dunbar, Lasche, Bond, Lerner, Wharton, Hardy & Davies, 1959). There is also strong epidemiological evidence that these organisms cause non-specific sensitization of humans to tuberculin (Palmer & Edwards, 1958, 1961). In South Africa Kleeberg, Stottmeier & Blokbergen (1965) have isolated avian-like strains from humans.

Baumann and other workers (Baumann, Krenn & Liebisch, 1955; Baumann, Kubin & Ritterhaus, 1957; Baumann & Ritterhaus, 1957) have isolated an organism from pigs which they believed differed from *M. avium*. They named the organism *Mycobacterium suis*. In South Africa strains which are indistinguishable from *M. avium* in cultural and allergenic characteristics are frequently isolated from pig gland lesions in piggeries in which there is no contact with poultry or poultry products.

The source of these infections is as yet unknown. It is, therefore, possible that cattle may also be infected by para-avian or avian strains from similar unknown sources as humans and pigs. Avian and avian-like organisms have been isolated from a number of sources. Kauker & Zettl (1964) isolated *M. avium*-like organisms from sawdust and caused lesions in pigs by feeding sawdust in their rations. W. L. Mallman, V. H. Mallman, Ray, McGavin & Ellis (1963) have isolated Group III organisms from NGL cattle, swine and soil, and Dormer, Martinaglia & Hobbs (1961) incriminated sawdust infected with avian-like mycobacteria as being responsible for non-specific reactions in cattle.

(*c*) *Sensitization caused by " skin lesions "*

The condition known as " skin lesions ", dermatitis nodosa, acid fast lymphangitis or " skin tuberculosis ", was first described by Perard & Ramon (1913) in France and by Traum (1916, 1919, 1923, 1929) in America. It has since been recorded in many countries (Andersson, 1938, 1939; Hole & Hulse, 1939; Wessels, 1948; Thomann, 1949; Sjollema, 1953; Frieling, 1953). The lesions are seen most commonly on the limbs and shoulders. They generally consist of a string of small abscesses following the course of one of the lymphatic vessels. Macroscopically and histopathologically they are similar to tuberculous lesions (Hole & Hulse, 1939), and acid fast bacilli can be demonstrated in sections or smears prepared from them. Although many strains of mycobacteria have been isolated from skin lesions by various workers, none of the isolates has been conclusively proved to be the etiological agent of skin lesions. In South Africa Lambrechts (1956) worked on the problem and isolated a number of strains from skin lesions. He was not able to reproduce the condition with his isolates. By injecting the strains into cattle in liquid paraffin adjuvant he was able to produce marked sensitivity to PPD produced from the homologous strains. Hedstrom (according to Paterson, 1956) has produced sensitivity to tuberculin by inoculation of lesion material.

Cattle sensitized by skin lesions generally give bigger reactions to mammalian tuberculin than to avian tuberculin (Marsh, Warren & Morrow, 1932; Andersson, 1939; Herbert & Paterson, 1955; Schaaf & Beerwerth, 1956; Paterson & Herbert, 1957), but the sensitivity wanes after the lesions become inactive and calcified. Herbert & Paterson (1955) and Paterson & Herbert (1957) found that in herds in which skin lesions occur, non-specific reactions are more common in young animals while lesions are more commonly seen in older cattle. This could be due to the sensitivity being highest in early infection, as in tuberculosis, and then waning, and the old, well encapsulated and calcified lesions of older animals being readily discernible.

The importance of skin lesions in causing skin sensitivity is difficult to assess, but it is considered by some authors to play a major role (Crawford, 1936). There is little recorded information on the occurrence or importance of skin lesions in South Africa. It is nevertheless common experience of veterinarians engaged in tuberculin testing that skin lesions are fairly common, and that in some herds the condition causes serious problems in the interpretation of the results of tuberculin tests. Lambrechts (1956) who worked extensively on the problem, believed that skin lesions play an important role in sensitizing cattle to tuberculin.

(*d*) *Sensitization caused by* M. paratuberculosis

Cattle suffering from Johne's disease are sensitive, at least at some stages during the course of the infection, to Johnin, avian tuberculin and to a lesser extent to mammalian tuberculin (Paterson, 1956). The difficulties caused by this organism in routine tuberculin testing (Goret, 1956; Herbert, Doyle & Paterson, 1959; Doyle, 1964) are such that Johne's disease vaccination cannot be undertaken on an extensive scale in countries where bovine tuberculosis is being actively combatted (Anon., 1956). In their allergenic properties *M. paratuberculosis* and *M. avium* are very closely related (Green, 1945), and avian tuberculin can be used for the diagnosis of Johne's disease (Hagan & Zeissig, 1929; Plum, 1932). The comparative test is, therefore, an efficient method of distinguishing this type of sensitization from bovine tuberculosis. Ingliss & Weipers (1963) have even shown that it is possible to recognize an infection of *M. bovis* superimposed on the sensitization caused by Johne's disease vaccination with the avian-mammalian comparative test.

Johne's disease is a serious problem in many countries. English workers (Taylor, 1949, 1952; Smith, 1954; Rankin, 1954; Withers, 1959) have shown that in addition to those animals clinically infected 15 to 17 per cent of apparently normal cattle harbour the infection. In these countries Johne's disease must, therefore, be reckoned as an important cause of non-specific reactions. In South Africa the incidence of the disease is extremely low. It is generally only seen in imported cattle and does not spread to local cattle. One large outbreak only has been recorded in locally bred cattle (Kleeberg, 1959). Sensitization caused by this organism is, therefore, of no practical importance.

(*e*) *Sensitization caused by* M. tuberculosis

Cattle are highly resistant to infection with *M. tuberculosis*, and when exposed to natural infection fail to develop any lesions or develop small regressive lesions only (Nielsen & Plum, 1940; Lesslie, 1960a; Karlson, 1962). Lesions can be produced by injecting large doses of organisms subcutaneously (Calmette, 1923). Infection with human tubercle bacilli causes marked reactivity to mammalian tuderculin (Lesslie, 1960a) but the sensitivity wanes and disappears especially if the infection source is removed (Nielsen & Plum, 1940; Lesslie, 1960a; Karlson, 1962).

Comparative testing with human and bovine tuberculin is of little practical value in distinguishing this type of sensitization as *M. bovis* and *M. tuberculosis* are very similar in allergenic make-up (Schliesser, 1954, 1958; Lauterbach, 1955). Fromm & Wiesmann (1953) claim that such a distinction can be made with monovalent tuberculins.

Human type sensitization would naturally be more common where the incidence of tuberculosis is high in man. Although tuberculosis is a major health problem in South Africa with about 67,000 new cases notified annually (Anon., 1965), only one case of cattle sensitization caused by *M. tuberculosis* has been described in the South African literature (Fourie, 1952). Highly suggestive cases have also been encountered by other workers. The paucity of recorded cases is probably due to a lack of intensive investigation rather than the rarity of the condition.

(*f*) *Sensitization caused by other mycobacteria*

Saprophytic mycobacteria occur commonly in soil and water and the digestive tract of herbivorous animals (Karlson, 1962; Bönicke & Juhasz, 1964; Singer & Rodda, 1965). They are also commonly isolated from post-mortem material. For this reason a number of workers have investigated their ability to cause non-specific reactions in cattle and other animals. In the past, results have generally proved inconclusive or negative (Hastings, Beach & Thompson, 1930; Frey & Hagan, 1931; Hagan, 1931; Müller, 1954). Canham (1944) was, however, able to sensitize cattle with large doses of the common saprophytes *Mycobacterium phlei* and *Mycobacterium smegmatis* suspended in liquid paraffin. It is now known that animals can be sensitized by many saprophytic mycobacteria, but that to produce a sensitivity of any note, it is necessary to use an adjuvant such as liquid paraffin.

The so-called atypical or anonymous mycobacteria capable of causing disease similar to tuberculosis in man (Runyon, 1965) have been investigated by Freerksen, Lauterbach, Rosenfeld & Wolter (1961). They were able to produce sensitivity in cattle to mammalian and avian tuberculin as well as to the homologous sensitins with large doses of *Mycobacterium kansasii* (Group I), Scotochromogens (Group II), Battey strains (Group III) and *Mycobacterium fortuitum* (Group IV) given *per os*. Photochromogens have been isolated from milk (Chapman & Bernard, 1963) and healed tuberculous lesions in cattle (Worthington & Kleeberg, 1964), and isolates resembling Group II, Group III and Group IV organisms have been made in this laboratory from cattle tissues. In a significant study by Morehouse, Singer, McDaniel, Howell, Sherman & Cassidy (1964) it was shown that of 151 strains of mycobacteria recovered from specimens of reactor cattle 65 were identified as *M. bovis*, 38 as Runyon Group III or *M. avium*, two as Runyon Group II and 46 as Runyon Group IV organisms. Furthermore it was shown histologically that granuloma's were present in a high percentage of the cases from which " atypical " mycobacteria were recovered. These findings provide convincing evidence that the " atypical " mycobacteria may be responsible for causing non-specific sensitization of cattle to mammalian tuberculin.

(*g*) *Sensitization caused by organisms other than mycobacteria*

Many organisms have been suggested as being responsible for non-specific reactions in cattle. The most convincing evidence concerns those organisms closely related to mycobacteria, especially the genus *Nocardia* (Awad, 1958, 1963; Affronti, 1959) and to a lesser extent *Actinobacillus* and *Actinomyces* (Feldman & Moses, 1942). *Brucella*, *Corynebacterium* and *Trichophyton* infections, non-specific infections

such as peritonitis, pleuritis, nephritis and abscessation, and infections with parasites like liver fluke, demodex and echinococcus have also been implicated. Recently Geurden & Devos (1964) have suggested that non-specific reactions may be caused by a combination of liver fluke and atypical mycobacteria. The literature on the subject has been reviewed by Schaetz (1956) and Paterson (1956). Results of experiments to induce sensitization with these agents have generally been inconclusive or negative and apart from the *Nocardia* it is unlikely that they are of any practical importance.

(h) Physiological causes of non-specific reactions

Non-specific reactions are known to occur more commonly in young animals than in older cattle (Herbert & Paterson, 1955; Paterson & Herbert, 1957). An increase in skin sensitivity has also been described during pregnancy (Schaaf, 1955) and in oxen (Schaetz, 1956; Kleeberg, 1960a). The increased sensitivity in these instances has been attributed to the influence of sex hormones (Schaetz, 1956).

(i) Recognition of non-specific reactions

For the veterinarian in the field, who must use the tuberculin test, the recognition of non-specific reactions is of paramount importance. Tuberculin syringes, callipers and testing method can be standardized and interpretation keys formulated, but the operator will still in many cases have to rely in his own judgement. The methods for recognizing non-specific reactions have been discussed previously (Worthington & Kleeberg, 1965). Although the tuberculin test, when used with discretion and good judgement, is a very reliable diagnostic tool, problems of interpretation often arise and mistakes are inevitably made. The aim of the research worker is to increase still further the specificity of the tuberculin test. Attempts have been made to produce a more specific product by fractionating tuberculin (Stockl & Mathois, 1960; Nemoto, Yugi, Hatakeyama, Ide & Takanami, 1961; Vakilzadeh, Vandiviere & Vandiviere, 1962; Kuttler & Baisden, 1962; Guld, Rhodes, Sorkin & Boyden, 1965). To date no definite breakthrough has been made along this line. Another line of investigation is to use highly diluted tuberculins either alone (Beerwerth, 1958; Bederke, 1960; Brühann, 1961) or comparatively with normal strength tuberculin (Kress, Mathois & Stockl, 1960). It was found that the diluted tuberculins caused fewer non-specific reactions than the usual strength tuberculins, but a number of workers are opposed to the use of diluted tuberculin on the grounds that there is an increased danger of missing cases of bovine tuberculosis (Freerksen & Lauterbach, 1960a, 1962; Kleeberg, 1961; Meyer, 1961; Seeleman & Rehm, 1962; Nottbohm & Funk, 1963; Funk, 1963). Investigations by Lesslie & Herbert (1965) indicated that there was no increased specificity of the tuberculin test in cattle when dilute tuberculins were used.

The most reliable and practical method of recognizing non-specific reactors remains the avian-mammalian comparative test. This test has been used with success in many countries. Although attempts have been made to use other mycobacterial sensitins comparatively with mammalian tuberculin (Diernhofer, 1963; Rossi, 1964), avian tuberculin is the only one that has been proved of value and used internationally on a large scale.

(j) Economic importance of the non-specific reactor problem

A campaign for the eradication of bovine tuberculosis in South Africa has been considered for some time. According to the Report of the Departmental Committee on the Control of Bovine Tuberculosis (Diesel, 1956) it was estimated that it would

cost R23,000,000 and involve the testing of a cattle population of about 11,000,000. The same committee estimated that 3 per cent of the cattle population might be infected with bovine tuberculosis. The best estimate of the infection rate in South Africa can be made from records of the number of cattle tested in the interim scheme and for diagnostic purposes. The percentage of positive reactors in the years 1962–63 to 1964–65 (Mansvelt, 1966) tested in the country are given in Table 1.

TABLE 1.—*Incidence of positive reactions—cattle tested in interim scheme and for diagnostic purposes* 1962–63 *to* 1964–65

Year	Cattle tested	Per cent Positive
1962–63	51,602	3·2
1963–64	79,066	1·86
1964–65	58,897	1·08
TOTAL	189,565	2·0

In addition to those classed as positive an approximately equal number reacted suspiciously. For the three years approximately 2 per cent reacted positively and it can be assumed that about one third to one half of the suspicious reactors would react positively on retesting. The estimate of 3 per cent infected cattle in our cattle population, therefore, still seems to be reasonable. In a national eradication campaign 330,000 positive reactors may be encountered. If the test is 95 per cent accurate then 16,500 of these animals may be non-specific reactors. The figure is probably low as it is optimistic to think that the test will be 95 per cent accurate under the conditions of an eradication campaign.

Another estimate of the incidence of non-specific reactions was made from studies on 12 of the tuberculous herds in which chemotherapy trials with isoniazid were undertaken (Kleeberg & Worthinton, 1963; Kleeberg, 1966; Kleeberg, Nixon & Worthington 1966). At any one time these herds contained approximately 2,900 cattle. They were tested at intervals of 3 to 6 months for periods of up to four years. A total of 17,860 tests was done. Careful study of the records revealed the probable non-specific reactions. It was found that in eight herds non-specific reactions were rare and of little consequence, in three herds the occurrence of non-specific reactions caused some confusion in the interpretation of results, and in one herd the incidence of non-specific reactors was so high as to completely confuse the interpretation of tuberculin tests. Reactions of between 2·0 and 4·0 mm occurred in 3·7 per cent, and reactions of over 4·0 mm in 1·7 per cent of what were judged to be non-tuberculous cattle. It is obvious that many cases would have been classed as negative after considering the type of swelling, history of animal and other relevant factors. Nevertheless these figures indicate that substantially more than 16,500 non-specific reactors may be encountered in the entire cattle population.

The non-specific reactor problem tended to assume greater significance as the herds became progressively more free from tuberculosis. A similar position has been seen on a national scale in America (Wilder, 1962).

Although non-specific reactions may be rare in our cattle population when compared with countries like England, it is nevertheless obvious that compensation will have to be paid for many non-specific reactors. A very rough minimal estimate of 16,500 non-specific reactors was made for testing the 11,000,000 cattle in the country once only. In fact herds will have to be tested at least three times before becoming accredited as TB free and annual or two yearly retesting will have to be done on all clean stock. It is, therefore, obvious that non-specific reactions could cost the country vast sums before tuberculosis is finally eradicated. Even in cases where cattle showing non-specific reactions are not slaughtered, additional expenses will be incurred due to the necessity for retesting and isolating the animals. In a country where the main problem in a tuberculosis eradication campaign will probably be shortage of professional manpower, and where great distances will have to be travelled to undertake testing, additional retesting of non-specific reactors could have a significant influence on the economy of an eradication campaign. An improvement in the diagnostic accuracy of the tuberculin test could therefore have a profound influence on the cost of a national campaign.

(v) *Introduction to present study*

The present study was undertaken to investigate the causes of non-specific reactions in South African cattle and to study methods of recognizing them. Previous workers have concentrated mainly on bacteriological examination of tissues of cattle reacting non-specifically as a means of defining the cause of non-specific reactions. A number of causes of non-specific reactions have been found by this method but it is expensive and time-consuming, and in many cases doomed to failure as the causative organism may not be isolated. Confusion may also arise if contaminating mycobacteria are isolated. Palmer & Edwards (1961), Edwards *et. al.* (1961), and Edwards & Smith (1965) have shown that multiple comparative testing in humans yields valuable information concerning likely cause, geographic distribution and incidence of non-specific reactions. Thus a study of the sensitivity of non-specific reactor cattle to various mycobacterial sensitins could yield more information on the problem than bacteriological examination of biopsy and post-mortem material.

Before this type of examination could be undertaken in cattle, it was necessary to produce PPD sensitins from a variety of different mycobacteria, and to study their ability to produce hypersensitivity reactions in sensitized guinea pigs. In order to interpret the results of multiple comparative tests it had to be proved that the specificity of reactions to mycobacterial sensitins is influenced primarily by the sensitizing *Mycobacterium* rather than the host. There is strong evidence indicating that guinea pigs and humans sensitized by the same species of *Mycobacterium* show similar sensitivity patterns (Edwards *et al.*, 1961; Bjerkedal & Natvig, 1961). If the sensitivity profiles of guinea pigs and cattle sensitized by the same species are also similar, it is possible to apply deductions made in guinea pig experiments directly to cattle showing non-specific reactions. Tests were therefore undertaken in artificially sensitized cattle with the same sensitins used in guinea pigs.

A further aim was to learn whether avian tuberculin is the most suitable for comparative testing under South African conditions or whether one of the other sensitins would be more satisfactory. Comparative testing with avian tuberculin prepared from the strain D4 has not been enthusiastically accepted by the veterinary profession in South Africa. Reasons given for this are that both Johne's disease and avian tuberculosis in fowls are rare and that there is, therefore, no logical ground for the use of avian tuberculin. It has also been said that the avian tuberculin was inclined to cause excessive reactions in tuberculous cattle.

Finally an attempt was made to find the best interpretation standards and methods of recognizing non-specific reactions. Tuberculous and non-tuberculous cattle were tested with bovine tuberculin alone and with the comparative test and the results analysed statistically.

CHAPTER 2

BACTERIOLOGICAL STUDY OF THE STRAINS OF MYCOBACTERIA USED

(i) *Selection of strains*

In this investigation it was obvious that strains used for PPD production should be typical. All strains were therefore examined to define their cultural and biochemical characteristics and for the pathogenic species virulence tests were also done. Practical serological tests have not yet been developed for defining mycobacteria. An extensive study was, however, made of the allergenic properties of the strains used in these experiments (see Chapter 5).

By testing a variety of mycobacterial sensitins in non-specific reactors an attempt was made to find a suitable second tuberculin for routine comparative testing. As a preliminary step a number of PPD sensitins was produced from a wide variety of mycobacteria. A variety of strains, including world type strains and locally isolated strains, were screened for ability to produce pellicles on synthetic fluid medium and PPD's were produced from the suitable ones (see Chapter 3). In addition other strains from which PPD's had not been produced were also used for sensitizing guinea pigs. The following species of mycobacteria were included:

M. bovis: Only two *M. bovis* strains were used in the investigation. Our standard bovine tuberculin strain, AN5, which is a well-tried world strain, was used for PPD production. A virulent *M. bovis* strain, 9473, was used for guinea pig sensitization. Bovine tuberculin was included in the study as a reference standard with which the properties of other sensitins could be compared.

M. avium and para-avian strains: Despite the unpopularity of the avian-bovine comparative test there was good reason to investigate sensitivity to avian tuberculin thoroughly. Avian and avian-like strains have been isolated from pigs, cattle and humans in this laboratory despite the low incidence of avian tuberculosis in poultry in South Africa (Coetzee, 1966). Sensitivity to avian tuberculin has also been demonstrated in humans in South Africa (Kuper, 1958; Collins, 1965). Twenty locally isolated strains and some world strains were screened for pellicle production and PPD's produced from eight. Six PPD's were selected for further investigation in this study and four additional strains were used for guinea pig sensitization.

The atypical mycobacteria: PPD's were produced from representative strains of Groups I, II and III (see para-avian) mycobacteria and *M. fortuitum* and a number of additional strains from each group were used for guinea pig sensitization.

The saprophytic mycobacteria: The saprophytes *M. phlei* and *M. smegmatis* have a universal distribution and can occasionally cause tuberculoid mastitis in cattle (Stuart & Harvey, 1951; Lesslie, 1960b). Representative strains of *M. phlei* and *M. smegmatis* were used for PPD production, and additional strains included for guinea pig sensitization.

Unclassified mycobacteria isolated from bovine "skin lesions": This group of mycobacteria deserves special mention. The organisms studied form a homologus group and were generally isolated from bovine "skin lesions" by Lambrechts (1956) or Kleeberg (1957). Similar strains were also isolated from other sources. The characteristics of these strains will be discussed later. Although it is questionable

whether they are in fact the causal organisms of skin lesions, it was nevertheless thought necessary to make a study of their sensitizing properties. PPD's were produced from ten strains, six of which were included in this investigation.

(ii) *Source of strains*

Details of the name, species and source of the strains used in this study are given in Table 2.

TABLE 2.—*Source of strains studied*

Species	Strain	Source
M. bovis	AN5	Centraal Diergeneeskundig Instituut, Rotterdam, Holland
	9473*	Isolated Onderstepoort from a tuberculous cow
M. avium	D4	Central Veterinary Laboratory, Weybridge, England
	20485	Isolated Onderstepoort from a pig lymph node lesion
	Oosthuizen	Isolated Onderstepoort from a tuberculous fowl
M. avium-like	20315	Isolated Onderstepoort from a pig lymph node lesion
	2519	Isolated Onderstepoort from a pig lymph node lesion
	T 17 H	Isolated from man, received from Trudeau Laboratory, Saranac Lake, New York, U.S.A.
	20662*	Isolated Onderstepoort from a pig lymph node lesion
	15165*	Isolated Onderstepoort from a N.V.L. reactor cow
M. avium	Bredasdorp*	Isolated Onderstepoort from a tuberculous fowl
	OPH 1	Isolated Onderstepoort from a tuberculous fowl
M. kansasii	W 33	Isolated Onderstepoort from a cow
	Hunt	Isolated from man, received from Trudeau Laboratory, Saranac Lake, New York, U.S.A.
	P 8	Received from King George V Hospital, Durban. Originally from the American Type Culture Collection
	P 1*	Received from King George V Hospital, Durban. Originally from the American Type Culture Collection
	W 31*	Isolated Onderstepoort from a cow
Scotochromogen	Cole	Received from Trudeau Laboratory, Saranac Lake, New York, U.S.A.
	RS 7376	Received from Tuberculosis Research Institute Borstel, Borstel/Bad Oldesloe, Germany
	Hg 3*	Isolated Onderstepoort from a cow
M. fortuitum	Fortuitum	Received from Trudeau Laboratory, Saranac Lake, New York, U.S.A.
	A 1766	Received from King George V Hospital, Durban
	Minetti*	Received from Istituto Superiore Di Sanità, Roma
	394*	Received from King George V Hospital, Durban
	N 8075*	Received from King George V Hospital, Durban
M. phlei	8·56	Received from King George V Hospital, Durban. Originally from National Collection of Type Cultures, London
	Crottin	Received from Istituto Superiore Di Sanità, Roma
	Timothe*	Received from Trudeau Laboratory, Saranac Lake, New York, U.S.A.
	72–OR*	Isolated Onderstepoort from a cow

TABLE 2.—(continued)

Species	Strain	Source
M. smegmatis.....	Smegmatis O.P.	Laboratory culture, Onderstepoort
	Lacticola.....	Received from Trudeau Laboratory, Saranac Lake, New York, U.S.A.
	Butyricum*....	Received from Trudeau Laboratory, Saranac Lake, New York, U.S.A.
	Ranae*.....	Received from Trudeau Laboratory, Saranac Lake, New York, U.S.A.
	H 607*.....	Received from Trudeau Laboratory, Saranac Lake, New York, U.S.A.
Mycobacterium sp.	2–OR.....	Isolated from bovine "skin lesion", Onderstepoort TB Section
	13–OR.....	Isolated from bovine "skin lesion", Onderstepoort TB Section
	8–SO.....	Isolated from bovine "skin lesion", Onderstepoort TB Section
	9–OR.....	Isolated from bovine "skin lesion", Onderstepoort TB Section
	41–OR.....	Laboratory contaminant, Onderstepoort TB Section
	23–OR.....	Isolated from bovine mesenteric lymph gland, Onderstepoort TB Section

* No PPD's were prepared from these strains

(iii) *Methods*

Suspensions containing approximately 1·0 mg of organisms per ml were prepared from all strains and one loopful of suspension inoculated onto Löwenstein–Jensen slopes. The cultures were incubated at 31°C, 37°C, 45°C, 52°C, 35°C in continuous dark, and 35°C in continuous light. All cultures were checked every two days to determine growth rate, and those showing good growth in four days were regarded as rapid growers. Cultures taking more than seven days to show good growth were regarded as slow growers. Pigment formation was observed in the cultures incubated in light and dark.

Serial ten fold dilutions were also made of the suspensions of organisms to a concentration of 10^{-6}, and 10^{-7}. Löwenstein–Jensen media in Petri dishes were inoculated with four drops of these suspensions, sealed with wax before incubation and the morphology of single colonies studied under a stereoscopic microscope.

The following biochemical tests were performed: Niacin test according to the method described by Steenken (1961); catalase test as described by Steenken (1961); nitrate reduction test according to the method of Bönicke (1962); ability to break down the following amides—acetamide, benzamide, urea, nicotinamide, pyrazinamide and succinamide, the method used being that described by Bönicke (1962) but a reaction time of 15 hours was used; ability to utilize the following organic salt solutions—sodium acetate, sodium propionate, sodium succinate, sodium malate, sodium lactate, sodium citrate, sodium oxalate, sodium tartrate and sodium benzoate according to the method described by McMillen & Kushner (1959).

Virulence tests in laboratory animals were performed as follows: Suspensions of bacilli were prepared in normal saline solution. Guinea pigs were injected subcutaneously in the region of the precrural gland with 0·1 mg (wet weight) of organisms; rabbits were injected intravenously with 0·01 mg, and mice intravenously with 0·3 mg. Six mice were used for each strain tested. Two chickens 10 to 12 weeks old were injected with each strain tested, they received 1·0 mg and 0·1 mg intravenously. Guinea pigs and rabbits were killed after six weeks, mice after four weeks, and fowls after eight weeks. Virulence tests were done only on the strains for which they offer additional evidence on the type of the organism. Bovine strains were thus tested in guinea pigs, rabbits, mice and fowls. Avian strain were tested in guinea pigs and fowls, and *M. kansasii* strains were tested in guinea pigs and mice.

(iv) *Results*

(*a*) *The* M. phlei *strains*

The colony forms in the four strains were similar. All strains yielded only rough colonies varying in colour from yellow to orange. The colonies were irregular, dull, raised and sometimes veiled. All strains grew at 52°C, although Timothe did not grow as well at this temperature as the other strains. In all acetamidase, urease, nicotinamidase and pyrazinamidase were shown to be present, and the strains were capable of utilizing acetate, propionate, succinate, malate, lactate and citrate. They were all catalase positive, niacin negative and able to reduce nitrate (see Table 3).

(*b*) *Unclassified mycobacterial species*

The colony forms in the six strains tested showed notable variation. In strains 41-RO and 9-OR only smooth colony forms were seen, these were domed, regular and round in 9-OR and flat and spreading in 41-RO. In 23-OR and 13-OR only rough colonies were seen, these were usually irregular and veiled and often highly raised. In 2-OR and 8-SO both smooth and rough colonies were noted. The predominant colour was orange but in 8-SO, 13-OR and 2-OR a few cream variants were seen. The strains were shown to contain the enzymes urease, nicotinamidase and pyrazinamidase, nitrate reductase and catalase, but did not produce niacin. Some slight differences were noted in their ability to utilize organic salts. All strains could utilize acetate and propionate and all except 9-OR could utilize benzoate. None of the strains could utilize citrate, oxalate and tartrate, but reactions on succinate, malate and lactate were variable (see Table 3). In these experiments the strains appeared to be rapid growers. They have, however, been grown on Löwenstein-Jensen medium for many years and are well adapted to it. According to Kleeberg (1957) they were originally slow growers.

(*c*) *The* M. fortuitum *strains*

The five strains showed similar colony forms. All colonies were rough, " doughnut " forms were commonly seen, also irregular raised colonies, folded and spreading colonies. The colour was buff. The *M. fortuitum* strains were similar to the *M. phlei* strains in biochemical characteristics, but they were unpigmented and did not grow at temperatures above 37°C (see Table 3).

(*d*) *The* M. smegmatis *strains*

The colonies in all the strains were large, rough, folded and irregular, " doughnut " forms were commonly seen in the strain Butyricum, but more rarely in the other strains. Very rarely smooth, round, domed colonies were noted in all the strains. *M. smegmatis* was biochemically the most active species studied. All strains were able to break down the nine organic salts and all six amides, reduce nitrate, were catalase positive and niacin negative. The strains were non-pigmented and able to grow at temperatures up to 45°C (see Table 3).

(*e*) *The* M. kansasii *strains*

The five strains investigated were all strongly photochromogenic, slow growing, and did not grow at 45°C. Strains W33 and P8 yielded only rough colonies, P1 and Hunt only smooth colonies, and W31 a mixture of rough and smooth colonies. Smooth colonies were round, regular and domed as in W31 or round regular and mammillated as was usual in P1 and Hunt. The strains were able to break down acetate, propionate and sometimes lactate. Difficulty was experienced in performing these tests as the agar slopes were inclined to dry out despite sealing the tubes with wax and the results were not readily reproduced. Tests had to be repeated a number of times before an opinion could be formed on which salts could be utilized. The accuracy of the results could therefore be questioned. The strains all contained nicotinamidase and urease but none of the other amidases. They were able to reduce nitrate, and were catalase positive and niacin negative (see Table 3). All strains caused only local abscesses at the injection site in guinea pigs, but caused typical lung lesions in mice.

(*f*) *The* M. avium *and avian-like strains*

The majority of strains from this group showed similar colony forms. The predominant colonies were small, round and smooth with a number of large smooth colonies constantly present. In strain T17H a few rough colonies were seen, and in strains 2519, D4 and 15165 the predominant colonies were of the irregular, rough type. All avian strains utilized acetate and propionate and all except 2519 utilized lactate; the other organic salts were not utilized. Difficulties, similar to those experienced with *M. kansasii* and the other slow growing mycobacteria, were observed with these tests. Nicotinamidase and pyrazinamidase were present in all strains, but they contained none of the other amidases. All strains were catalase positive, niacin negative, and unable to reduce nitrate. All except T17H grew at temperatures up to 45°C (Table 3).

The results of virulence tests are given in Table 4. The true avian strains were highly virulent for fowls while T17H was avirulent. The strains 2519, 20662 and 20315 isolated from pigs were only very slightly virulent. All strains caused only local abscesses in guinea pigs with spread in most cases to the iliac lymph nodes. In no case was there any dissemination to the internal organs.

(*g*) *The* M. bovis *strains*

Only two old laboratory strains were used in the investigation. The predominant colony form was large, irregular and rough, which is in marked contrast to the small dysgonic colonies seen in newly isolated bovine strains. The strains produced urease, did not reduce nitrate or produce niacin, were catalase positive and utilized acetate and propionate. Organic salt utilization tests were not very satisfactory. The strains grew only at temperatures of 35°C to 37°C (see Table 3). Both strains were highly virulent for rabbits, guinea pigs and mice.

TABLE 3.—*Cultural and biochemical characteristics of mycobacterial strains*

Species	Strain	Pigmentation		Growth rate	Temp. range	Amides						Nitrate reduction	Catalase	Niacin	Organic salt utilization								
		Light	Dark			Acetamide	Benzamide	Urea	Nicotinamide	Pyrazinamide	Salicylamide				Acetate	Propionate	Succinate	Malate	Lactate	Citrate	Oxalate	Tartrate	Benzoate
M. phlei	8·56	0	0	fast	31–52° C	+	−	+	+	+	−	+	+	−	+	+	+	+	+	+	−	−	−
	Crottin	0	0	fast	31–52° C	+	−	+	+	+	−	+	+	−	+	+	+	+	+	+	−	−	−
	Timothe	0	0	fast	31–52° C	+	−	+	+	+	−	+	+	−	+	+	+	+	+	+	−	−	−
	72–OR	0	0	fast	31–52° C	+	−	+	+	+	−	+	+	−	+	+	+	+	+	+	−	−	−
Mycobacterium sp.	41–RO	0	0	fast	31–45° C	−	−	+	+	+	−	+	+	−	+	+	±	−	−	−	−	−	+
	8–SO	0	0	fast	31–45° C	−	−	+	+	+	−	+	+	−	+	+	±	±	+	−	−	−	+
	9–OR	0	0	fast	31–45° C	−	−	+	+	+	−	+	+	−	+	+	±	±	−	−	−	−	−
	2–OR	0	0	fast	31–45° C	−	−	+	+	+	−	+	+	−	+	+	+	+	+	−	−	−	+
	23–OR	0	0	fast	31–45° C	−	−	+	+	+	−	+	+	−	+	+	−	±	+	−	−	−	+
	13–OR	0	0	fast	31–45° C	−	−	+	+	+	−	+	+	−	+	+	−	−	±	−	−	−	+
M. fortuitum	Fortuitum	N.P.	N.P.	fast	31–37° C	+	−	+	−	+	−	+	+	−	+	+	+	+	+	+	−	−	−
	A 1766	N.P.	N.P.	fast	31–37° C	−	−	+	+	+	−	+	+	−	+	+	+	+	+	+	−	−	−
	Minetti	N.P.	N.P.	fast	31–37° C	+	−	+	+	+	−	+	+	−	+	+	+	+	+	+	−	−	−
	394	N.P.	N.P.	fast	31–37° C	+	−	+	+	+	−	+	+	−	+	+	+	+	+	+	−	−	−
	N 8075	N.P.	N.P.	fast	31–37° C	+	−	+	+	+	−	+	+	−	+	+	+	+	+	+	−	−	−
M. smegmatis	Lacticola	N.P.	N.P.	fast	31–45° C	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+
	Smegmatis OP.	N.P.	N.P.	fast	31–45° C	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+
	Ranae	N.P.	N.P.	fast	31–45° C	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+
	H 607	N.P.	N.P.	fast	31–45° C	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+
	Butyricum	N.P.	N.P.	fast	31–45° C	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+
M. kansasii	Hunt	0	N.P.	slow	31–37° C	−	−	+	+	−	−	+	+	−	+	+	−	−	±	−	−	−	−
	P 1	0	N.P.	slow	31–37° C	−	−	+	+	−	−	+	+	−	+	+	−	−	±	−	−	−	−
	P 8	0	N.P.	slow	31–37° C	−	−	+	+	−	−	+	+	−	+	+	−	−	−	−	−	−	−
	W 31	0	N.P.	slow	31–37° C	−	−	+	+	−	−	+	+	−	+	+	−	−	±	−	−	−	−
	W 33	0	N.P.	slow	31–37° C	−	−	+	+	−	−	+	+	−	+	+	−	−	−	−	−	−	−
M. avium and *M. avium*-like	20485	N.P.	N.P.	slow	31–45° C	−	−	−	+	+	−	−	+	−	+	+	−	−	+	−	−	−	−
	D 4	N.P.	N.P.	slow	31–45° C	−	−	−	+	+	−	−	+	−	+	+	−	−	+	−	−	−	−
	Oosthuizen	N.P.	N.P.	slow	31–45° C	−	−	−	+	+	−	−	+	−	−	+	−	−	+	−	−	−	−
	20315	N.P.	N.P.	slow	31–45° C	−	−	−	+	+	−	−	+	−	+	+	−	−	+	−	−	−	−
	2519	N.P.	N.P.	slow	31–45° C	−	−	−	+	+	−	−	+	−	+	+	−	−	−	−	−	−	−
	T 17 H	N.P.	N.P.	slow	31–37° C	−	−	−	+	+	−	−	+	−	+	+	−	−	+	−	−	−	−
	Bredasdorp	N.P.	N.P.	slow	31–45° C	−	−	−	+	+	−	−	+	−	+	+	−	−	+	−	−	−	−
	OP H1	N.P.	N.P.	slow	31–45° C	−	−	−	+	+	−	−	+	−	+	+	−	−	+	−	−	−	−
	20662	N.P.	N.P.	slow	31–45° C	−	−	−	+	+	−	−	+	−	+	+	−	−	+	−	−	−	−
	15165	N.P.	N.P.	slow	31–45° C	−	−	−	+	+	−	−	+	−	+	+	−	−	+	−	−	−	−
M. bovis	AN 5	N.P.	N.P.	slow	35–37° C	−	−	+	−	−	−	−	+	−	+	±	−	−	−	−	−	−	−
	9473	N.P.	N.P.	slow	35–37° C	−	−	+	−	−	−	−	+	−	+	±	−	−	−	−	−	−	−
Scotochromogens	Cole	0	0	slow	31–37° C	−	−	+	−	−	−	+	+	−	+	−	−	−	−	−	−	−	−
	RS 7376	0	0	slow	31–37° C	−	−	±	−	−	−	+	+	−	+	−	−	−	−	−	−	−	−
	Hg 3	0	0	fast	31–37° C	−	−	+	±	±	−	+	+	−	+	+	−	−	+	−	−	−	−

O — Orange pigmentation
N.P. — non pigmented

(h) The scotochromogens

The strain Hg 3 did not fit well into the group as it had a fast to intermediate growth rate. It was further able to utilize acetate, propionate and lactate while the other strains could only utilize acetate. The organic salt utilization tests were, however, again difficult to perform satisfactorily. The strain Hg 3 contained the amidases, nicotinamidase, pyrazinamidase and urease whereas the other two strains contained only urease. All strains were nitrate positive, catalase positive and niacin negative.

The colonies of strain Cole were generally rough with a few smooth round ones, whereas in RS7376 the predominant forms were smooth with only a few rough colonies. In Hg 3 all colonies were rough and irregular. All three strains were orange without unpigmented variants. The three strains grew at temperatures from 31°C to 37°C.

(v) *Discussion*

All strains were typical for the species they were selected to represent as can be seen from the data presented in Tables 3 and 4. A few comments are, however, necessary.

The colony forms were not always typical of those described for a certain species This is generally due to the fact that many strains were old laboratory strains which are well adapted to egg medium and have become eugonic and rough. For this reason all the *M. phlei* and *M. bovis* strains and some of the avian strains are now typically " rough ".

TABLE 4.—*Virulence test of* M. avium *and avian-like strains in fowls and guinea pigs*

Strain	Fowls		Guinea pigs
	1·0 mg	0·1 mg	0·1 mg
20485	++++	++++	+
D 4	+++	+++	+
Oosthuizen	+++	+	+
20315	++	—	+
2519	+	—	+
T 17H	—	—	+
Bredasdorp	++++	++++	+
OPH 1	++++	++++	+
20662	+	—	+
15165	++++	++++	+

++++ Died with typical lesions before being killed
+++ Advanced tuberculosis when killed
++ Moderate to few lesions when killed
+ Local abscess only (guinea pigs) or minimal lesions (fowls)
— No macroscopic lesions

The unclassified group of mycobacteria showed notable variations in colony morphology. These strains were originally named R for rough, S for smooth and O for orange by Kleeberg. He showed them to be capable of undergoing variations in both colony form and colour (Kleeberg, 1963). Strains 41-RO and 9-OR, which were originally picked from single rough colonies, have become completely smooth after a few years of sub-culturing and in 8-SO and 2-OR both smooth and rough colonies were seen. The strains remained predominantly orange during the same period with only a small proportion of non-pigmented colonies seen in the strains 8-SO, 13-OR and 2-OR.

All the *M. phlei* strains contained the enzyme acetamidase, whereas Bönicke (1962) describes this as a variable characteristic. It is possible that the presence of this enzyme was more readily demonstrated due to the longer reaction time used in this investigation. The group of unclassified mycobacteria often appear culturally similar to *M. phlei*, but a number of clear differences were seen. They are unable to grow at 52°C, do not contain the enzyme acetamidase and show a different pattern of utilization of organic salts. The strains probably belong to a separate species. The *M. fortuitum* strains were biochemically similar to the *M. phlei* strains, but could be easily differentiated as they are non-pigmented and failed to grow at temperatures above 37°C. According to Gordon & Smith (1953) those organisms formerly described as *Mycobacterium lacticola*, *Mycobacterium butyricum* and *M. smegmatis* comprise a single species and there is further confusion over the naming of *Mycobacterium ranae* species, some of which are also *M. smegmatis*. The strains investigated, including old laboratory strains of the four types, proved identical and must be regarded as different strains of a single species, *M. smegmatis*.

The *M. avium* and avian-like organisms proved to be typical in their cultural and biochemical characteristics. The only notable exception was that T17H failed to grow at 45°C. This was to be expected as it is a true para-avian strain of human origin and these so-called Battey strains do not usually grow at 45°C. Strain T17H was avirulent for fowls, which is also typical of Battey strains. The strains 2519, 20662 and 20315 isolated from pigs were of very low virulence for fowls. They were cultured on Löwenstein-Jensen medium for one to two years before the virulence tests were done and may have become attenuated. It is therefore questionable whether they are true *M. avium* or para-avian strains. The *M. avium* strains were readily distinguishable from the other slow-growing strains as they were able to grow at 45°C, were urease negative, pyrazinamide positive and unable to reduce nitrate.

All the *M. kansasii* strains were typical. They could be distinguished from *M. tuberculosis* strains by their photochromogenicity, lack of niacin and their low virulence for guinea pigs, while still retaining their catalase activity. They could be distinguished from the other slow-growing strains by their amide reactions—urea and nicotinamide positive—and by the fact that they were nitrate reductase positive while *M. bovis* and *M. avium* are nitrate reductase negative. They were typically virulent for mice.

The *M. bovis* strains were fully virulent for rabbits, guinea pigs and mice, nitrate reductase and niacin negative, contained only urease of the amidases and would not grow at 31°C or 45°C.

The strain Hg 3 isolated from a bovine should not have been included in the scotochromogen group. It was able to utilize propionate and lactate which the other scotochromogens could not, and contained the enzymes nicotinamidase, pyrazinamidase and urease, while the other scotochromogens contained only urease. It was

originally thought to be a slow-growing strain, but the present investigation showed it to be fast-intermediate in growth rate. This excludes it from the scotochromogen group.

The utilization of organic salts proved to be a valuable differentiating test for the fast-growing strains, but the method was unsatisfactory for use with the slow-growing species. This test should, therefore, be used as a differential test in the rapidly growing strains only.

CHAPTER 3

PPD PRODUCTION

(i) *Method of PPD production*

The method used for PPD production was essentially similar to that used for production at Onderstepoort and described by Green (1945, 1953). Strains were inoculated onto Watson-Reid agar slopes, as used at the Central Veterinary Laboratory, Weybridge for the cultivation of *M. paratuberculosis* antigen (Hole, 1957), in 1 oz screw-capped McCartney bottles and incubated at 37°C. When a thin layer of actively-growing organisms was visible on the agar slope, sufficient Bureau of Animal Industries (BAI) synthetic medium (Green, 1953) with trace elements added as recommended by Dekker & Huitema (1958), was added to half fill the bottles. They are then reincubated until pellicles had formed. The pellicles were multiplied on small Erlenmeyer flasks of BAI medium and finally inoculated onto 2,000 ml "penicillin" flasks containing 500 ml of BAI medium. All flasks were incubated for 10 weeks at 37°C before being sterilized by flowing steam for three hours.

The cultures were filtered through two layers of S.S. No. L.S. filter paper and 40 per cent trichloracetic acid added to the filtrate to give a final concentration of 4 per cent. The supernatant was siphoned off, the precipitated protein centrifuged, washed in succession, twice in 1 per cent trichloracetic acid, twice in acetone and twice in ether, spread on petri dishes and dried in the incubator overnight. The powder was then weighed and stored in screwcapped McCartney bottles at 4°C for up to three years. The powder was reconstituted as required by dissolving in a diluting fluid prepared as follows:—

$\frac{M}{15}$ phosphate buffer pH 7·1	1,000 ml
Glycerol	100 ml
Sodium chloride	5 gm
Phenol	5 gm

When small amounts of PPD were required for testing guinea pigs, 20 to 30 mg amounts were weighed off and dissolved so as to give a concentration of 2 mg per ml. The solution was presumed to be equal to 100,000 sensitin units (SU) per ml. These solutions were then further diluted to contain 250 SU per ml. For testing cattle, relatively larger amounts were prepared by dissolving 0·55 gm of powder in 1,000 ml of diluting fluid. The solution was then filtered through Fords sterimat S.B. filter pads under sterile conditions, bottled in 1·2 ml amounts and stored at 4°C. The solutions were presumed to contain 25,000 SU per ml. These solutions were also used in some cases for testing guinea pigs. Some PPD's dissolved in the diluting fluid only after heating. Heating to 90°C did not affect the potency of the PPD's, a fact which is not surprising as all PPD is initially subjected to three hours

flowing steam during its preparation. It was not possible to standardize the PPD's by biological assay, as no standards exist for most of the PPD's used in this study. Avian and bovine PPD's standardized biologically against standard PPD's received from the Centraal Instituut voor Diergeneeskunde, Rotterdam, to contain 25,000 TU per ml and 70,000 TU per ml respectively, were used in the surveys on cattle described in Chapters 9 and 10.

(ii) *Correlation between PPD yield and changes of pH of the medium*

The BAI synthetic medium produced at Onderstepoort has an initial pH of about 6·6 to 6·8. The final pH of the medium after 10 weeks of growth, yield of PPD per flask and short comments on the growth of each strain are given in Table 5.

TABLE 5.—*PPD production, pH and yield*

Species	Strain	Growth	Final pH	Yield per flask
M. bovis	AN 5	good, sunk 8–10 weeks	8·0	0·35 gm
M. avium and *M. avium*-like	T17H	fair, sunk 8–10 weeks	8·8	0·13 gm
	D4	good, sunk 8–10 weeks	8·0	0·10 gm
	Oosthuizen	good, sunk early	5·8	0·04 gm
	20485	very rapid, sunk early	7·0	0·10 gm
	20315	good, no sinking	6·8	0·15 gm
	2519	good, no sinking	5·0	0·08 gm
M. kansasii	P8	good, no sinking	6·0	0·23 gm
	Hunt	good, no sinking	5·5	0·17 gm
	W33	slow, good, no sinking	6·4	0·26 gm
Scotochromogens	Cole	good, sunk early	8·5	0·10 gm
	RS7376	good, sunk early	8·2	0·06 gm
M. fortuitum	Fortuitum	good, sunk 8–10 weeks	8·6	0·24 gm
	A1766	good, no sinking	8·2	0·45 gm
M. phlei	M. phlei	good, no sinking	8·6	0·27 gm
	Crottin	good, no sinking	6·9	0·23 gm
M. smegmatis	Smegmatis (O.P.)*	—	—	—
	Lacticola	good, no sinking	7·8	0·58 gm
Mycobacterium sp.	2–OR	fair, no sinking	8·4	0·40 gm
	13–OR	good, no sinking	7·5	0·29 gm
	8–SO	fair, no sinking	6·0	0·03 gm
	9–OR	fair, no sinking	6·1	0·04 gm
	41–RO	good, no sinking	6·2	0·18 gm
	23–OR	good, no sinking	6·9	0·20 gm

*Small amount prepared no record of pH and yield

(iii) *Discussion*

The method of producing PPD's as described by H. H. Green was deviated from in one point. viz. the use of Watson-Reid medium for the production of pellicles. Considerable difficulty was experienced in producing suitable pellicles

from some strains, when egg medium or serum agar was used. A variety of different media were therefore tried. and Watson-Reid agar medium was found to be superior to any of the others.

The standardization of PPD's by using the weight of pure dry powder only, cannot be absolutely correct as differences in potencies of tuberculins containing similar amounts of protein are known to occur (Svenkerud, 1955). A method has been described by Lesslie & Herbert (1962) for determining biologically the concentrations at which two tuberculins should be compared. This method would, however, be laborious and time-consuming when a large number of different PPD's are to be compared, as was the case in this study. It can be seen from the results (see below) that the method of standardization was sufficiently accurate to allow good differentiation of various sensitizations in both guinea pigs and cattle.

A number of authors (Wong, 1937; Paterson, 1948; McIntosh & Konst, 1949; Svenkerud, 1955, Magnusson & Bentzon, 1958) have observed a correlation between the amount of tuberculin produced and the final pH of the medium. The strains that cause a high end pH generally produce the best yields of PPD. It can be seen from the results that the yields of PPD seem to be affected more by the species of *Mycobacterium* used than by individual strains within a species, e.g. the rapid growing strains produced better yields of PPD than the avian and scotochromogenic strains. Within each group, however, the best yields were generally obtained from strains producing an alkaline reaction in the BAI medium.

Chapter 4

Screening of PPD Sensitins in Guinea Pigs

(i) *Methods*

Sensitization and testing of guinea pigs: Strains used for sensitizing guinea pigs were cultivated on Löwenstein-Jensen medium. Young, well grown cultures were harvested and suspensions containing approximately 4 mg of organisms per ml prepared by grinding with sterile liquid paraffin in a mortar. The suspensions were then killed by heating in a waterbath to 85°C for 30 minutes. A volume of 0·25 ml of suspension was injected intramuscularly into the hind leg and the same quantity subcutaneously behind the neck of the guinea pigs. Three guinea pigs were sensitized with each strain and used for testing four weeks after the sensitizing injections. Guinea pigs sensitized in this manner retained their sensitivity for many months and could be retested repeatedly. A minimum period of four weeks was allowed between consecutive tests.

The hair was removed from the guinea pigs' sides by means of a commercial depilatory paste. At each test six different sensitins were used, three injections being made on each side. The injection sites were rotated so that each sensitin was injected in each of the three sites in every set of three guinea pigs. The test dose was 25 SU (0·1 ml of solution). The diameters of the reactions were measured after 24 hours with a Hauptner calliper. In asymmetrical reactions the mean of the greatest and the smallest diameters was used.

The experiments were so planned that for each group of sensitins to be studied a selection of homologous and heterologous species of mycobacteria was used for guinea pig sensitization. Only results from guinea pigs tested on the same day by the identical solutions of sensitin and injected with the same syringes and needles were used for calculating specificity differences (see below) and constructing sensitivity profiles. In this manner the number of experimental variables was reduced and reliable results could be obtained from a comparatively small number of guinea pigs.

Calculation of specificity differences: The mean reaction for each sensitin in each set of guinea pigs was calculated. Tables of specificity difference were then constructed according to the method described by Magnusson (1961), i.e. sensitin specificity difference (SPD) is taken as the difference in millimetres between homologous and heterologous reactions, or SPD = (Aa + Bb) − (Ab + Ba) where Aa is the reaction with sensitin A in guinea pigs sensitized by the homologous strain and Ab is the reaction with sensitin A in guinea pigs sensitized by a heterologous strain. Similarly Bb and Ba are the homologous and heterologous reactions to sensitin B.

Where guinea pigs were sensitized by strains from which no sensitins were available, a suitable sensitin belonging to the same species was used as the homologous sensitin. In this case SPD's were calculated for heterologous species only. SPD's can only be calculated for a number of strains belonging to the same species when sensitins have been prepared from each strain under investigation.

Construction of sensitivity profiles: For each species of *Mycobacterium* investigated a number of strains from which sensitins had not been prepared were also used for guinea pig sensitization. In these cases SPD's could not be calculated, and sensitivity profiles were therefore constructed according to the method described by Edwards *et al.* (1961). The mean reactions of a group of guinea pigs sensitized by a particular strain were plotted on graph paper against the sensitins used. The points were then joined by straight lines to form the profile. Guinea pigs sensitized by strains of the same species have similarly shaped profiles.

(ii) *Sensitivity patterns of* M. phlei *sensitization*

(*a*) *Experimental*

The two sensitins prepared from the *M. phlei* type strains 8·56 and Crottin were compared with four other sensitins AN5 (*M. bovis*), 20485 (*M. avium*), 2-OR (*M.* sp.) and Cole (scotochromogen). Three guinea pigs were sensitized with each of the homologous strains (for *M. bovis* strain 9473 was used as the sensitizing strain) as well as strains 72-OR (*M. phlei*) and Timothe (*M. phlei*). Each guinea pig was tested twice with an interval of one month between tests.

(*b*) *Results*

The means of the reactions to each sensitin are given in Table 6. The *SPD's* calculated from the means in Table 6 are given in Table 7. For the purpose of calculating SPD's for strains 72-OR and Timothe and strains of heterologous species, sensitin 8·56 was regarded as homologous. Sensitivity profiles of the four *M. phlei* strains are shown in Fig. 1.

TABLE 6.—*Mean ractions in mm to PPD sensitins prepared from strains of* M. phlei *and other mycobacteria in guinea pigs sensitized by homologous and heterologous strains*

Guinea pig sensitizations	No. of tests	Sensitin					
		AN5	20485	2–OR	Cole	Crottin	8·56
9473 (*M. bovis*)	6	**14·6**	9·1	5·5	6·9	4·8	5·5
20485 (*M. avium*)	6	6·7	**14·7**	7·0	9·8	7·4	8·0
2–OR (*Mycobacterium* sp.)	6	7·3	9·7	**14·0**	9·1	10·3	9·8
Cole (scotochromogen)	5	6·8	9·8	7·0	**13·1**	7·3	6·9
Crottin (*M. phlei*)	6	4·1	6·1	7·8	5·0	**12·6**	**12·8**
8·56 (*M. phlei*)	6	4·8	7·1	8·3	7·1	**13·6**	**14·4**
72–OR (*M. phlei*)	6	4·9	7·8	10·9	6·6	**13·4**	**14·1**
Timothe (*M. phlei*)	6	3·1	4·7	6·0	4·1	**12·2**	**12·5**

TABLE 7.—*Specificity differences of PPD sensitins prepared from strains of* M. phlei *and other mycobacteria*

Guinea pig sensitization	Sensitin							
	AN5	20485	2–OR	Cole	Crot-tin	8·56	8·56	8·56
9473 (*M. bovis*)	—	13·5	15·8	14·0	18·3	18·7	18·3	18·5
20485 (*M. avium*)	13·5	—	12·0	8·2	13·8	14·0	13·0	14·5
2–OR (*Mycobacterium* sp.)	15·8	12·0	—	11·0	8·5	10·3	7·4	10·7
Cole (cotochromogen)	14·0	8·2	11·0	—	13·4	13·5	12·0	14·6
Crottin (*M. phlei*)	18·3	13·8	8·5	13·4	—	0·6		
8·56 (*M. phlei*)	18·7	14·0	10·3	13·5	0·6	—		
72–OR (*M. phlei*)	18·3	13·0	7·4	13·7				
Timothe (*M. phlei*)	18·5	14·5	10·7	14·6				

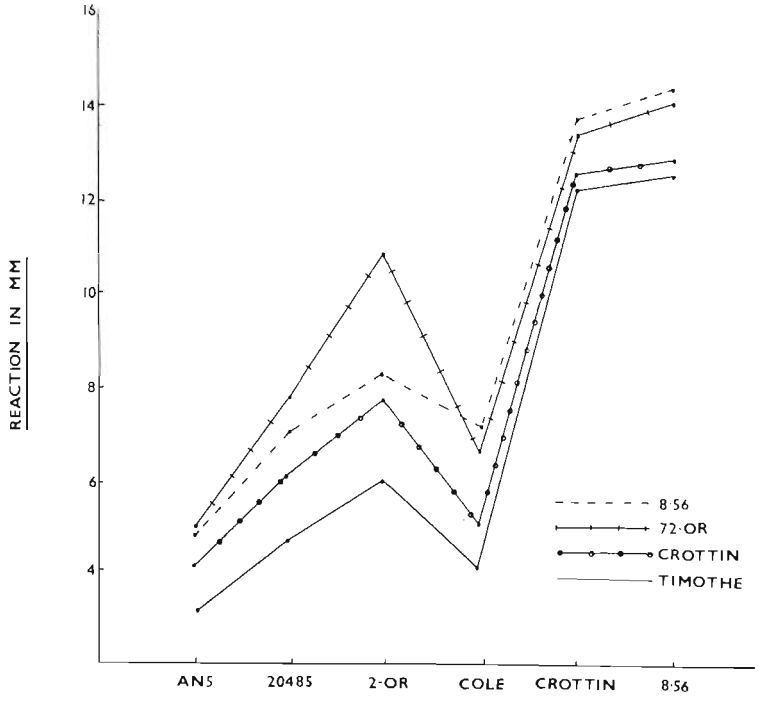

FIG. 1.—Sensitivity profiles of guinea pigs sensitized by four strains of *M. phlei*

(*c*) *Discussion*

It can be seen from the results that the four strains of *M. phlei* tested form a homologous group. The SPD between the two *M. phlei* sensitins was 0·6 indicating an almost identical allergenic make-up (Table 7), and the sensitivity profiles of the four *M. phlei* strains strikingly similar (Fig. 1). It can be seen that *M. phlei* is fairly closely related to the strain 2-OR (*Mycobacterium* sp.) as indicated by the comparatively low SPD's between the *M. phlei* strains and 2-OR and the fact that in all cases this sensitin gave the next highest reaction to the *M. phlei* sensitins in guinea pigs sensitized with *M. phlei* (see Fig. 1). This point was confirmed when a number of sensitins from strains similar to 2-OR was studied (see below).

In guinea pigs sensitized by *M. phlei* avian PPD caused higher reactions than bovine PPD, which caused minimal reactions. It can therefore be postulated that *M. phlei* is unlikely to sensitize cattle to bovine PPD under natural circumstances. Even if such sensitization should occur, it could be recognized as non-specific by using the avian-bovine comparative test.

(iii) *Sensitivity patterns of* M. fortuitum *sensitization*

(*a*) *Experimental*

The experimental design was similar to that used in studying the *M. phlei* sensitins. Two sensitins prepared from the *M. fortuitum* strains Fortuitum and A1766 were studied. They were compared with the sensitins 20485 (*M. avium*), AN5 (*M. bovis*), Lacticola (*M. smegmatis*) and W33 (*M. kansasii*). Three further *M. fortuitum* strains N8075, 394 and Minetti were also used for guinea pig sensitization. Each set of guinea pigs was tested twice at an interval of four weeks.

(*b*) *Results*

The mean reactions for each set of guinea pigs is given in Table 8, the SPD's in Table 9 and the sensitivity profiles of the four *M. fortuitum* strains in Fig. 2.

TABLE 8.—*Mean reaction in mm to PPD sensitins prepared from* M. fortuitum *and other mycobacteria in guinea pigs sensitized by homologous and heterologous strains*

Guinea pig sensitization	No. of tests	Sensitin					
		20485	AN5	Lacti-cola	W33	For-tuitum	A1766
20485 (*M. avium*)	6	**14·4**	9·0	8·6	10·5	8·9	9·6
9473 (*M. bovis*)	6	8·6	**14·8**	6·2	9·8	7·3	7·5
Lacticola (*M. smegmatis*)	6	7·5	5·2	**9·5**	6·4	6·9	7·3
W33 (*M. kansasii*)	5	11·6	10·0	9·3	**14·3**	9·8	9·1
Fortuitum (*M. fortuitum*)	4	9·8	7·1	9·3	10·0	**12·6**	**12·1**
A1766 (*M. fortuitum*)	5	7·8	6·2	8·6	8·4	**11·3**	**11·2**
Minetti (*M. fortuitum*)	6	8·4	6·9	8·4	9·0	**12·7**	**12·3**
394 (*M. fortuitum*)	6	9·4	7·6	8·8	10·5	**10·8**	**10·8**
N8075 (*M. fortuitum*)	6	8·4	7·4	8·4	10·0	**11·2**	**11·8**

TABLE 9.—*Specificity differences of PPD sensitins prepared from strains of M.* fortuitum *and other mycobacteria*

Guinea pig sensitization	Sensitin								
	20485	AN5	Lacti-cola	W33	For-tui-tum	A 1766	For-tui-tum	For-tui-tum	For-tui-tum
20485 (*M. avium*)	—	11·6	7·8	6·6	8·3	8·2	9·8	6·9	8·3
9473 (*M. bovis*)	11·6	—	12·9	9·3	13·0	12·3	13·3	10·7	11·3
Lacticola (*M. smegmatis*)	7·8	12·9	—	8·1	5·9	4·8	6·9	4·6	5·4
W33 (*M. kansasii*)	6·6	9·3	8·1	—	7·1	8·0	8·2	4·8	5·7
Fortuitum (*M. fortuitum*)	8·3	13·0	5·9	7·1	—	0·4			
A1766 (*M. fortuitum*)	8·2	12·3	4·8	8·0	0·4	—			
Minetti (*M. fortuitum*)	9·8	13·3	6·9	8·2					
394 (*M. fortuitum*)	6·9	10·7	4·6	4·8					
N8075 (*M. fortuitum*)	8·3	11·3	5·4	5·7					

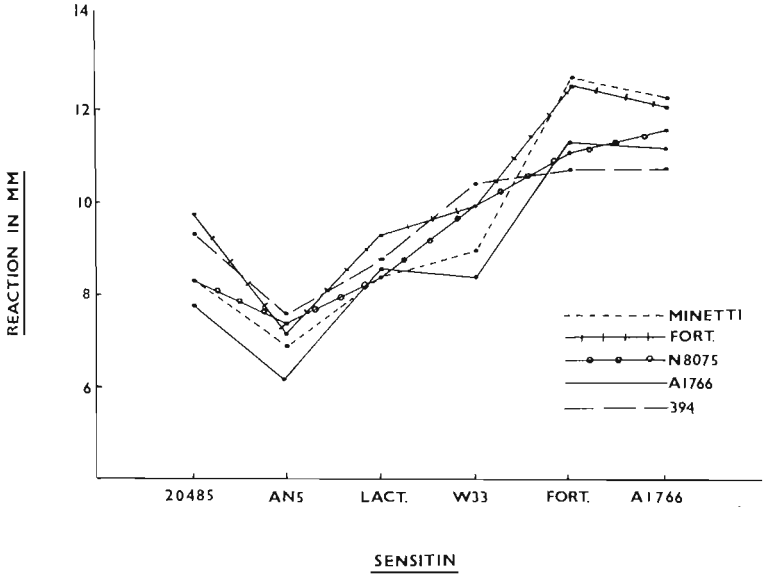

FIG. 2.—Sensitivity profiles of guinea pigs sensitized by four strains of *M. fortuitum*

(*c*) *Discussion*

The sensitivity produced in the guinea pigs by *M. smegmatis* and the five *M. fortuitum* strains was of a low order. As a result the SPD's were in some cases lower than would be expected. Species which appeared to have a similar allergenic structure to *M. fortuitum* were *M. smegmatis*, as can be seen by the low SPD's in Table 9, and *M. kansasii* which caused the greatest reactions of all the heterologous PPD's tested in the guinea pigs sensitized by *M. fortuitum* strains (see Fig. 2).

The sensitivity of guinea pigs sensitized by *M. fortuitum* species to *M. bovis* PPD was minimal. Sensitivity to *M. avium* PPD was distinctly higher. The comparative avian-bovine tuberculin test should therefore be adequate for recognizing non-specific reactions caused by *M. fortuitum* sensitization.

(iv) *Sensitivity patterns of* M. smegmatis *sensitization*

(*a*) *Experimental*

The experimental design was similar to those of the previously described groups. Two *M. smegmatis* sensitins prepared from strains Smegmatis OP and Lacticola were compared with sensitins AN5 (*M. bovis*), 20485 (*M. avium*), Cole (*scotochromogen*) and 8·56 (*M. phlei*). Guinea pigs were sensitized with the homologous strains and with three additional *M. smegmatis* strains—Butyricum, Ranae and H607. Each set of guinea pigs was tested twice at an interval of four weeks.

(*b*) *Results*

The mean reactions for each set of guinea pigs are given in Table 10, the SPD's in Table 11, and the sensitivity profiles of the five *M. smegmatis* strains in Fig. 3.

TABLE 10.—*Mean reaction in mm to PPD sensitins prepared from* M. smegmatis *and other mycobacteria in guinea pigs sensitized by homologous and heterologous strains*

Guinea pig sensitization	No. of tests	Sensitin					
		AN5	20485	Cole	8·56	Smeg. OP.	Lacti-cola
9473 (*M. bovis*)	6	**12·1**	9·0	8·5	6·8	7·7	7·4
20485 (*M. avium*)	6	7·3	**13·9**	10·8	8·8	8·4	7·7
Cole (scotochromogen)	6	6·9	9·8	**14·2**	7·5	7·6	7·7
8·56 (*M. phlei*)	6	4·5	6·4	7·6	**12·7**	8·2	7·7
Smegmatis OP (*M. smegmatis*)	6	6·4	7·4	7·2	7·4	**11·8**	11·2
Lacticola (*M. smegmatis*)	4	7·1	8·8	8·3	7·8	**13·1**	**12·3**
Butyricum (*M. smegmatis*)	6	5·4	7·3	6·7	8·3	**14·0**	**11·0**
Ranae (*M. smegmatis*)	6	5·5	6·2	6·7	6·4	**13·3**	**10·7**
R607 (*M. smegmatis*)	6	5·4	7·2	8·5	8·8	**12·9**	**11·4**

TABLE 11.—*Specificity differences of PPD sensitins prepared from strains of* M. smegmatis *and other mycobacteria*

Guinea pig sensitization	Sensitin								
	AN5	20485	Cole	8·56	Smeg. OP	Lacti-cola	Smeg. OP	Smeg. OP	Smeg. OP
9473 (*M. bovis*)	—	9·7	10·9	13·5	9·8	9·9	13·0	12·2	11·9
20485 (*M. avium*)	9·7	—	7·5	11·4	9·9	9·7	12·2	12·6	11·2
Cole (scotochromogen)	10·9	7·5	—	11·8	11·2	10·5	13·9	13·2	11·0
8·56 (*M. phlei*)	13·5	11·4	11·8	—	8·9	9·5	10·2	11·4	8·6
Smegmatis OP (*M. smegmatis*)	9·8	9·9	11·2	8·9	—	—0·2			
Lacticola (*M. smegmatis*)	9·9	9·7	10·5	9·5	—0·2	—			
Butyricum (*M. smegmatis*)	13·0	12·2	13·9	10·2					
Ranae (*M. smegmatis*)	12·2	12·6	13·2	11·4					
H607 (*M. smegmatis*)	11·9	11·2	11·0	8·6					

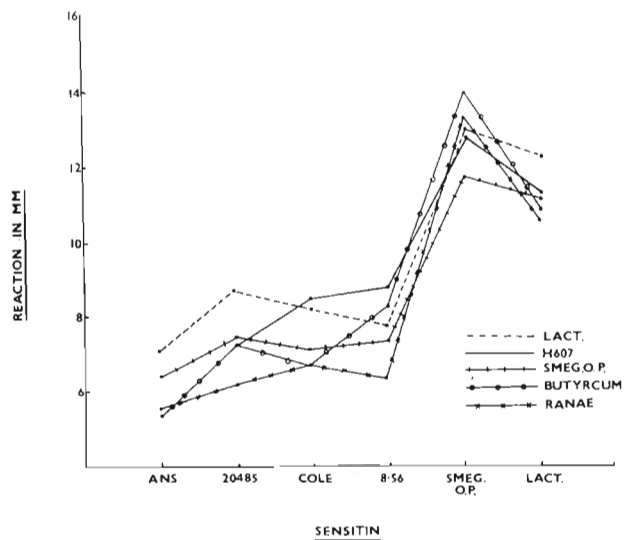

FIG. 3.—Sensitivity profiles of guinea pigs sensitized by five strains of *M. smegmatis*

(*c*) *Discussion*

The *M. smegmatis* strains were similar in allergenic characteristics as can be seen by the similar profiles of the five strains tested (Fig. 3). Guinea pigs sensitized by *M. smegmatis* strains showed minimal sensitivity to bovine PPD and slightly greater sensitivity to avian PPD (Table 10). There was little difference between the *M. smegmatis-M. bovis* and *M. smegmatis-M. avium* SPD's (Table 11). The avian-bovine comparative test should therefore be adequate to recognize non-specific reactions caused by *M. smegmatis*, although such sensitization is unlikely to be of much practical importance.

(v) *Sensitivity patterns of* M. kansasii *sensitization*

(*a*) *Experimental*

In this case the experimental design was similar to that used in the previously described groups. Sensitins prepared from the *M. kansasii* strains P8, Hunt and W33 were compared with the following sensitins: AN5 (*M. bovis*), 20485 (*M. avium*) and Lacticola (*M. smegmatis*). Guinea pigs were sensitized with the homologous strains and with two additional *M. kansasii* strains W31 and P1. Each set of guinea pigs was tested twice at an interval of four weeks.

(*b*) *Results*

The results are given as before—mean reactions of the groups of guinea pigs in Table 12, SPD's in Table 13 and the sensitivity profiles of the five groups of guinea pigs sensitized with five strains of *M. kansasii* in Fig. 4.

TABLE 12.—*Mean reaction in mm to PPD sensitins prepared from* M. kansasii *and other mycobacteria in guinea pigs sensitized by homologous and heterologous strains*

Guinea pig sensitization	No. of tests	Sensitin					
		AN5	20485	Lacti-cola	Hunt	P8	W33
9473 (*M. bovis*)	5	**13·6**	9·7	7·3	9·1	10·2	10·5
20485 (*M. avium*)	5	8·8	**14·6**	7·9	8·9	12·0	12·9
Lacticola (*M. smegmatis*)	6	6·3	8·3	**12·4**	6·5	8·8	7·3
Hunt (*M. kansasii*)	6	8·3	10·5	8·3	**10·5**	**12·8**	**12·8**
P8 (*M. kansasii*)	6	6·8	9·0	7·0	**9·6**	**12·9**	**12·3**
W33 (*M. kansasii*)	6	8·2	10·6	7·6	**11·0**	**13·3**	**13·5**
W31 (*M. kansasii*)	6	9·9	11·3	8·1	**12·0**	**14·3**	**13·8**
P1 (*M. kansasii*)	6	6·8	9·6	9·5	**10·2**	**13·2**	**11·9**

TABLE 13.—*Specificity differences of PPD sensitins prepared from strains of* M. kansasii *and other mycobacteria*

Guinea pig sensitization	Sensitin							
	AN5	20485	Lacti-cola	Hunt	P8	W33	W33	W33
9473 (*M. bovis*)	—	9·7	12·4	6·7	9·5	8·4	7·0	8·2
20485 (*M. avium*)	9·7	—	10·8	5·7	6·5	4·6	4·2	4·0
Lacticola (*M. smegmatis*)	12·4	10·8	—	8·0	9·5	11·0	10·8	11·4
Hunt (*M. kansasii*)	6·7	5·7	8·0	—	1·0	0·2		
P8 (*M. kansasii*)	9·5	6·5	9·5	1·0	—	—0·7		
W33 (*M. kansasii*)	8·4	4·6	11·0	0·2	—0·7			
W31 (*M. kansasii*)	7·0	4·2	10·8					
P1 (*M. kansasii*)	8·2	4·0	11·4					

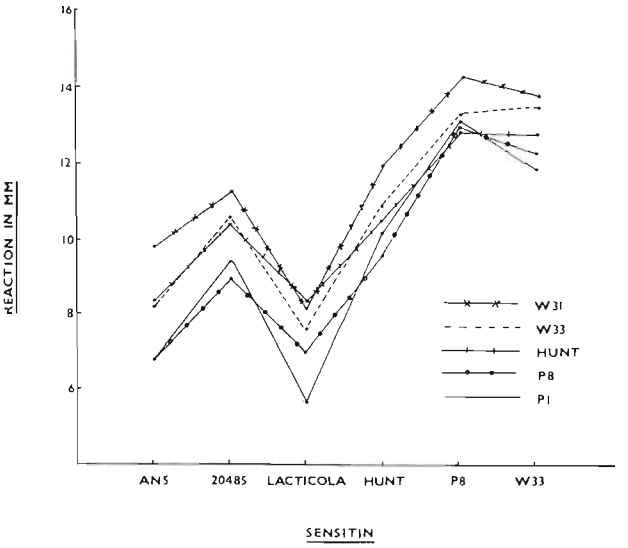

FIG. 4.—Sensitivity profiles of guinea pigs sensitized by five strains of *M. kansasii*

(*c*) *Discussion*

It can be seen that the five *M. kansasii* strains were similar in allergenic characteristics. The sensitin Hunt was obviously less potent than the other *M. kansasii* sensitins, so that the reactions to sensitin 20485 (*M. avium*) in guinea pigs sensitized by *M. kansasii* were almost as great as the reactions to sensitin Hunt (Table 12). Potency should, however, not be confused with specificity. In the SPD tables (Table 13) it can be clearly seen that the *M. kansasii* strains were similar in specificity while the avian strain, although fairly closely related, fell into a distinct group. Other workers have found *M. tuberculosis* to be closely related to *M. kansasii*. These studies indicated that *M. avium* is even closer to *M. kansasii* than *M. bovis*. The comparative avian-bovine test should therefore, be suitable for identifying cattle sensitized by *M. kansasii* as non-specific reactors.

The relationship of *M. kansasii* to *M. bovis* and *M. avium* was interesting. *M. kansasii* fell between the other two. In *M. kansasii* sensitized guinea pigs reactions to bovine and avian PPD's were both fairly large, although avian PPD usually caused slightly higher reactions than the bovine PPD. In *M. bovis* sensitized guinea pigs reactions to *M. kansasii* sensitins were fairly high, but *M. avium* sensitin caused smaller reactions. In *M. avium* sensitized guinea pigs the reactions to *M. kansasii* sensitins were fairly high, but reactions to bovine PPD were substantially lower (Table 12). This relationship was demonstrated consistently in other experiments (see Tables 16 and 22).

(vi) *Sensitivity patterns of scotochromogen sensitization*

(a) Experimental

Sensitins prepared from two scotochromogens, Cole and RS7376, were investigated. Sensitins prepared from strains AN5 (*M. bovis*), 20485 (*M. avium*), 8·56 (*M. phlei*) and W33 (*M. kansasii*) were used in comparative tests. A further scotochromogen strain Hg3 was also used for guinea pigs sensitization. All guinea pigs were tested twice with the six sensitins.

(b) Results

The mean reactions to each sensitin in each set of guinea pigs are given in Table 14 and the SPD's in Table 15. No SPD's are given for strain Hg3, as there was no homologous sensitin included in the investigation. The sensitivity profiles of the three scotochromogen strains are shown in Fig. 5.

TABLE 14.—*Mean reaction in mm to PPD sensitins prepared from scotochromogens and other mycobacteria in guinea pigs sensitized by homologous and heterologous strains*

Guinea pig sensitization	No. of tests	Sensitin					
		AN5	20485	8·56	W33	Cole	RS7376
9473 (*M. bovis*)	5	**13·8**	9·0	7·0	10·5	8·5	10·0
20485 (*M. avium*)	6	7·8	**13·3**	7·2	10·4	9·6	9·3
8·56 (*M. phlei*)	6	6·2	6·8	**12·9**	6·3	8·1	7·8
W33 (*M. kansasii*)	6	13·1	13·3	10·0	**15·5**	11·5	11·2
Cole (scotochromogen)	6	9·1	11·8	8·5	10·2	**14·3**	11·4
RS7376 (scotochromogen)	6	9·8	12·3	7·4	11·2	11·5	**14·1**
Hg3 (scotochromogen)	5	5·7	6·8	10·0	5·0	7·5	8·4

TABLE 15.—*Specificity differences of PPD sensitins prepared from scotochromogens and other mycobacteria*

Guinea pig sensitization	Sensitin					
	AN5	20485	8·56	W33	Cole	RS7376
9473 (*M. bovis*)	—	10·3	13·5	5·7	10·5	8·1
20485 (*M. avium*)	10·3	—	12·2	5·1	6·2	5·8
8·56 (*M. phlei*)	13·5	12·2	—	12·1	10·6	11·8
W33 (*M. kansasii*)	5·7	5·1	12·1	—	8·1	7·2
Cole (scotochromogen)	10·5	6·2	10·6	8·1	—	5·5
RS7376 (scotochromogen)	8·1	5·8	11·8	7·2	5·5	—

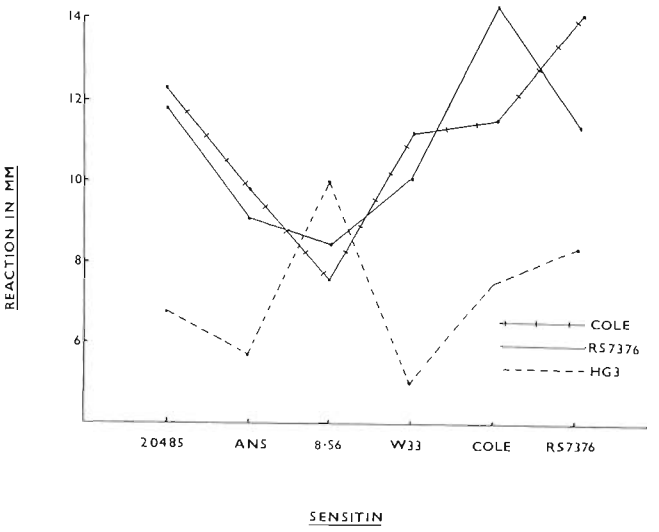

FIG. 5.—Sensitivity profiles of guinea pigs sensitized by three scotochromogen strains

(c) Discussion

The three scotochromogens studied do not belong to a group with homologous allergens. This is not surprising as Runyon, Runyon, Brisbay, Dietz, Raniga & Smith (1962) stated that it is evident that the Group II (scotochromogens) comprises more than one species.

Cultural and biochemical differences had already shown that strain Hg3 was probably not a true scotochromogen. This strain proved to be most closely related to *M. phlei* (Table 14). The strains Cole and RS7376 had a SPD of 5·5 (Table 15), which, although indicating a fairly close relationship, is sufficiently wide to consider them as different. The sensitivity profiles also show them to be distinct strains (Fig. 5). Strain Hg3 had a low sensitivity to all the sensitins with a slight peak to the *M. phlei* sensitin. The other two strains showed highest reactions to their homologous sensitins and fairly high reactions to the other scotochromogen sensitin and to the *M. avium* sensitin. Both Cole and RS7376 are closely related to *M. avium*—SPD's of 6·2 and 5·8 respectively—while the relationship to *M. bovis* sensitin is wider—10·5 and 8·1 respectively. The avian-bovine comparative test should therefore be adequate in differentiating sensitization caused by this organism from *M. bovis* sensitization.

(vii) *Sensitivity patterns of sensitization caused by an unclassified species of* Mycobacterium

(a) Experimental

In the experiment six sensitins prepared from the strains 41-RO, 9-OR, 13-OR, 8-SO, 2-OR and 23-OR were compared with each other, and with the sensitin 2-OR as a representative group sensitin they were also compared with sensitins 20485 (*M. avium*), AN5 (*M. bovis*), 8·56 (*M. phlei*), Lacticola (*M. smegmatis*) and W33

(*M. kansasii*). Three guinea pigs were sensitized with each of the above strains. Although a number of guinea pigs died intercurrently during the course of the investigation, there was probably no connection with any toxicity of the heat killed mycobacteria used for sensitization. Few intercurrent deaths occurred in previuos investigations when these strains were used.

For the purpose of comparing the group as a whole with other heterologous mycobacterial strains the guinea pigs were tested twice each with the sensitins 2-OR (*Mycobacterium* sp.), 20485 (*M. avium*), AN5 (*M. bovis*), 8·56 (*M. phlei*), Lacticola (*M. smegmatis*) and W33 (*M. kansasii*). The guinea pigs sensitized by the six strains of unclassified mycobacteria were also tested once with each of the six homologous PPD's.

(*b*) *Results*

The mean reactions of the various groups of guinea pigs to sensitin 2-OR and the five sensitins of heterologous species are given in Table 16 and the SPD's in Table 17. Sensitivity profiles of the guinea pigs sensitized with the six strains of unclassified mycobacteria are shown in Fig. 6.

TABLE 16.—*Mean reactions to PPD sensitins prepared from strain 2-OR* (Mycobacterium sp.) *and other mycobacteria in guinea pigs sensitized by homologous and heterologous strains*

Guinea pig sensitization	No. of tests	Sensitin					
		8·56	AN5	W33	20485	Lacticola	2–OR
8·56 (*M. phlei*)	5	**13·9**	8·5	10·3	8·7	10·3	10·0
9473 (*M. bovis*)	5	7·7	**15·1**	10·2	8·2	7·6	8·2
W33 (*M. kansasii*)	5	9·8	10·2	**14·7**	11·6	9·0	10·1
Mixed avian	5	8·7	8·1	10·0	**15·1**	8·2	7·5
Lacticola (*M. smegmatis*)	5	7·7	5·5	6·3	7·1	**11·7**	7·2
2–OR (*Mycobacterium* sp.)	6	9·9	5·8	5·9	8·0	8·1	**14·9**
8–SO (*Mycobacterium* sp.)	5	10·7	7·4	6·9	9·9	9·1	**13·7**
23–OR (*Mycobacterium* sp.)	4	10·1	5·1	5·8	8·0	7·1	**12·6**
13–OR (*Mycobacterium* sp.)	5	11·5	6·8	7·4	8·4	8·0	**14·9**
9–OR (*Mycobacterium* sp.)	3	11·2	8·2	10·5	11·2	9·5	**15·3**
41–RO (*Mycobacterium* sp.)	4	12·0	9·5	9·6	9·0	10·4	**15·4**

The mean reactions to the sensitins prepared from six strains of unclassified mycobacteria in six groups of guinea pigs sensitized by the same six strains are given in Table 18, and the SPD's are given in Table 19.

TABLE 17.—*Specificity differences of PPD sensitins prepared from strain* 2-*OR* (Mycobacterium *sp.*) *and other mycobacteria*

Guinea pig sensitization	Sensitin										
	8·56	AN5	W33	20485	Lacticola	2–OR	2–OR	2–OR	2–OR	2–OR	2–OR
8·56 (*M. phlei*)	—	12·8	8·5	11·6	7·6	8·9	6·9	6·4	7·3	8·0	7·3
9473 (*M. bovis*)	12·8	—	9·4	13·9	13·7	16·0	13·2	14·4	15·0	14·0	12·8
W33 (*M. kansasii*)	8·5	9·4	—	8·2	11·1	13·6	11·4	11·4	12·1	9·4	10·4
20485 (*M. avium*)	11·6	13·9	8·2	—	11·5	14·5	11·4	12·2	14·1	11·7	14·0
Lacticola (*M. smegmatis*)	7·6	13·7	11·1	11·5	—	11·3	9·1	10·0	11·4	10·3	9·5
2–OR (*Mycobacterium* sp.)	8·9	16·0	13·6	14·5	11·3	—					
8SO (*Mycobacterium* sp.)	6·9	13·2	11·4	11·4	9·1						
23–OR (*Mycobacterium* sp.)	6·4	14·4	11·4	12·2	10·0						
13–OR (*Mycobacterium* sp.)	7·3	15·0	12·1	14·1	11·4						
9–OR (*Mycobacterium* sp.)	8·0	14·0	9·4	11·7	10·3						
41–RO (*Mycobacterium* sp.)	7·3	12·8	10·4	14·0	9·5						

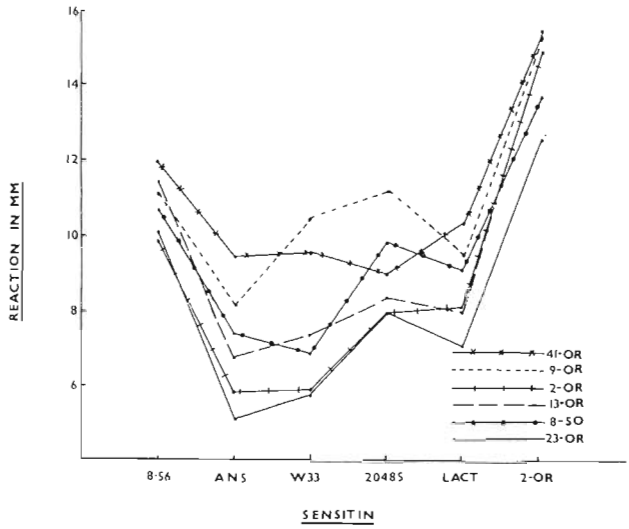

FIG. 6.—Sensitivity profiles of guinea pigs sensitized by six strains of an unclassified *Mycobacterium* sp

TABLE 18.—*Mean reactions of guinea pigs sensitized by six strains of unclassified mycobacteria to PPD sensitins prepared from homologous strains*

Guinea pig sensitization	No. of tests	Sensitin					
		41–RO	9–OR	13–OR	8–SO	2–OR	23–OR
41–RO (*Mycobacterium* sp.)..	3	16·0	12·5	14·3	14·0	13·2	14·2
9–OR (*Mycobacterium* sp.)...	3	14·3	13·3	14·5	14·3	13·5	14·7
13–OR (*Mycobacterium* sp.)..	3	15·2	12·0	15·0	13·2	13·8	14·5
8–SO (*Mycobacterium* sp.)....	3	15·5	15·8	15·2	15·7	14·5	14·7
2–OR (*Mycobacterium* sp.)...	3	14·3	14·3	14·0	14·7	14·0	13·8
23–OR (*Mycobacterium* sp.)..	3	14·3	13·3	14·7	13·7	13·7	14·0

TABLE 19.—*Specificity differences of six PPD sensitins prepared from unclassified mycobacteria*

Guinea pig sensitization	Sensitin					
	41–RO	9–OR	13–OR	8–SO	2–OR	23–OR
41–RO (*Mycobacterium* sp.).....	—	2·5	1·5	2·2	2·5	1·5
9–OR (*Mycobacterium* sp.).....	2·5	—	1·8	—1·1	—0·5	—0·7
13–OR (*Mycobacterium* sp.).....	1·5	1·8	—	2·3	1·2	—0·2
8–SO (*Mycobacterium* sp.).....	2·2	—1·1	2·3	—	0·5	1·3
2–OR (*Mycobacterium* sp.).....	2·5	—0·5	1·2	0·5	—	0·5
23–OR (*Mycobacterium* sp.).....	1·5	—0·7	—0·2	1·3	0·5	—

(*c*) *Discussion*

The sensitivity profiles of the unclassified mycobacteria were strikingly similar (Fig. 6); only two discrepancies were seen. In the guinea pigs sensitized by 9-OR the reactions to sensitin W33 (*M. kansasii*) were comparatively higher than in the other strains investigated. In strain 41-RO the sensitivity profile was basically similar to the other profiles, except that the sensitivity to sensitin 20485 (*M. avium*) was low (Fig. 6). The relationship of the six strains of unclassified mycobacteria to any other species was generally very similar. The SPD's were similar and fell into a comparatively narrow range. For example, all strains were fairly closely related to *M. phlei*, the SPD's ranging from 6·4 to 8·9, but they showed great differences from *M. bovis*, SPD's being from 12·8 to 16·0 (Table 17).

The close relationship between these unclassified mycobacteria and *M. phlei* mentioned earlier in this study was confirmed. The six unclassified strains studied caused only a low degree of sensitivity in guinea pigs to bovine PPD and a slightly higher sensitivity to avian PPD.

Specificity differences of the six strains indicate that they belong to a group with similar allergenic characteristics (Table 19). In view of the close allergenic, bacteriological and biochemical similarity of the strains studied, they probably comprise a single species. There is no convincing evidence that the species is pathogenic. Strains 8-SO, 9-OR and 13-OR did not produce typical skin lesions when injected into cattle (Kleeberg, 1957) and naturally occurring cases of " skin lesions " were often not sensitive to PPD's produced from them. It is therefore unlikely that this species is by itself the primary etiological agent of bovine skin lesions.

(viii) *Sensitivity patterns of* M. avium *and avian-like mycobacterial sensitization*

(*a*) *Experimental*

The following six PPD's were used: 20485, D4, Oosthuizen, 2519, 20315, and T17H. The selection included isolates from fowls, pigs and man, true avian strains virulent for fowls and strains non-virulent for fowls (see Chapters 2 and 3). In addition to these avian strains the following PPD's prepared from strains of mycobacteria belonging to heterologous species were used in comparative tests: 8·56 (*M. phlei*), AN5 (*M. bovis*), Cole (*scotochromogen*), Lacticola (*M. smegmatis*) and W33 (*M. kansasii*). Three guinea pigs were sensitized with each of the above-mentioned strains, and with four additional avian strains—20662, Bredasdorp, OPH1 and 15165. The guinea pigs sensitized by the avian strains were tested twice each with the six avian PPD's studied. All the guinea pigs sensitized were tested also with the five heterologous PPD's mentioned earlier and with PPD from avian strain 20485.

(*b*) *Results*

The mean reactions to the six avian PPD's in the 10 groups of guinea pigs sensitized by avian strains are given in Table 20 and the SPD's of the six sensitins are given in Table 21.

TABLE 20.—*Mean reactions to PPD sensitins prepared from* M. avium *and avian-like mycobacteria in guinea pigs sensitized by* 10 M. avium *and avian-like strains*

Guinea pig sensitization	No. of tests	Sensitin					
		20485	2519	20315	T17H	Oost-huizen	D4
20485 (*M. avium*)	6	13·8	12·5	11·7	11·3	12·1	13·6
2519 (*M. avium*-like)	5	12·8	12·4	11·8	11·8	11·7	13·1
20315 (*M. avium*-like)	6	12·2	11·8	11·8	11·2	11·4	12·2
T17H (*M. avium*-like)	6	12·9	12·5	13·6	14·0	13·1	12·5
Oosthuizen (*M. avium*)	6	13·7	12·8	12·1	10·6	12·2	12·9
D4 (*M. avium*)	6	12·8	12·3	12·3	12·4	12·4	12·9
20662 (*M. avium*-like)	6	12·1	11·4	11·4	10·3	11·7	10·8
Bredasdorp (*M. avium*)	5	13·5	12·6	10·9	11·9	11·5	13·0
OPH1 (*M. avium*)	6	13·5	12·7	12·6	12·0	12·8	13·3
15165 (*M. avium*)	6	13·1	12·9	12·0	11·8	12·6	13·3

TABLE 21.—*Specificity differences of six PPD sensitins prepared from strains of* M. avium *and avian-like mycobacteria*

Guinea pig sensitization	Sensitin					
	20485	2519	20315	T17H	Oost-huizen	D4
20485 (*M. avium*)	—	0·9	1·7	3·6	0·2	0·3
2519 (*M. avium*-like)	0·9	—	0·6	2·1	0·1	—0·1
20315 (*M. avium*-like)	1·7	0·6	—	1·0	0·5	0·2
T17H (*M. avium*-like)	3·6	2·1	1·0	—	2·5	2·0
Oosthuizen (*M. avium*)	0·2	0·1	0·5	2·5	—	—0·2
D4 (*M. avium*)	0·3	—0·1	0·2	2·0	—0·2	—

The mean reactions of all groups of guinea pigs to the PPD from the avian strain 20485 and to the five PPD sensitins prepared from other species of mycobacteria are given in Table 22 and the SPD's in Table 23. Sensitivity profiles of the guinea pigs sensitized by 10 strains of avian and avian-like mycobacteria are given in Fig. 7 and 8.

TABLE 22.—*Mean reactions of guinea pigs sensitized by* M. avium, *para-avian and other mycobacteria to PPD sensitins prepared from strain* 20485 (M. avium) *and other mycobacteria*

Guinea pig sensitization	No. of tests	Sensitin					
		8·56	AN5	Cole	Lacticola	W33	20485
8·56 (*M. phlei*)	3	**12·7**	3·7	3·7	4·5	4·8	5·0
9473 (*M. bovis*)	3	7·8	**14·5**	10·2	8·0	10·8	10·0
Cole (scotochromogen)	3	6·7	6·0	**15·2**	6·7	8·7	10·7
Lacticola (*M. smegmatis*)	3	8·2	5·3	6·7	**11·8**	5·2	6·0
W33 (*M. kansasii*)	3	10·3	10·7	10·0	10·0	**14·3**	11·3
20485 (*M. avium*)	3	8·8	7·8	11·3	9·0	10·8	**12·3**
D4 (*M. avium*)	3	9·8	8·8	11·7	8·7	11·2	**13·8**
Oosthuizen (*M. avium*)	3	9·8	8·2	11·5	9·3	10·5	**14·5**
20315 (*M. avium*-like)	3	9·0	7·8	11·5	9·0	11·8	**12·2**
2519 (*M. avium*-like)	3	9·3	8·5	11·0	9·5	11·3	**13·8**
T17H (*M. avium*-like)	3	9·0	9·3	11·3	10·5	12·2	**13·0**
20662 (*M. avium*-like)	3	8·0	8·3	10·7	8·8	9·7	**11·3**
Bredasdorp (*M. avium*)	2	8·5	9·5	11·3	9·5	10·3	**13·8**
OP H1 (*M. avium*)	3	8·5	8·8	11·2	7·5	11·7	**14·5**
15165 (*M. avium*)	3	8·7	8·5	11·7	9·3	11·3	**13·3**

TABLE 23.—*Specificity differences of* 20485 (M. avium) *PPD sensitin and five other mycobacterial PPD sensitins*

Guinea pig sensitization	Sensitin														
	8·56	AN5	Cole	Lacti-cola	W33	20485	20485	20485	20485	20485	20485	20485	20485	20485	20485
8·56 (*M. phlei*)	—	15·7	17·5	11·8	11·9	11·2	11·7	12·4	10·9	12·2	11·7	11·0	13·0	13·7	12·3
9473 (*M. bovis*)	15·7	—	13·5	13·0	7·3	9·0	9·5	10·8	8·9	9·8	8·5	7·8	9·8	10·5	9·1
Cole (scotochromogen)	17·5	13·5	—	13·6	10·8	5·5	6·6	7·5	5·2	7·3	6·2	5·1	7·0	7·8	6·1
Lacticola (*M. smegmatis*)	11·8	13·0	13·6	—	10·9	9·1	10·9	11·0	9·0	10·1	8·3	8·3	10·1	12·8	9·8
W33 (*M. kansasii*)	11·9	7·3	10·8	10·9	—	4·5	5·6	7·0	3·4	5·5	3·8	4·6	6·5	5·8	5·0
20485 (*M. avium*)	11·2	9·0	5·5	9·1	4·5	—									
D4 (*M. avium*)	11·7	9·5	6·6	10·9	5·6										
Oosthuizen (*M. avium*)	12·4	10·8	7·5	11·0	7·0										
20315 (*M. avium*-like)	10·9	8·9	5·2	9·0	3·4										
2519 (*M avium*-like)	12·2	9·8	7·3	10·1	5·5										
T17H (*M. avium*-like)	11·7	8·5	6·2	8·3	3·8										
20662 (*M. avium*-like)	11·0	7·8	5·1	8·3	4·6										
Bredasdorp (*M. avium*)	13·0	9·8	7·0	10·1	6·5										
OPH1 (*M. avium*)	13·7	10·5	7·8	12·8	5·8										
15165 (*M. avium*)	12·3	9·1	6·1	9·8	5·0										

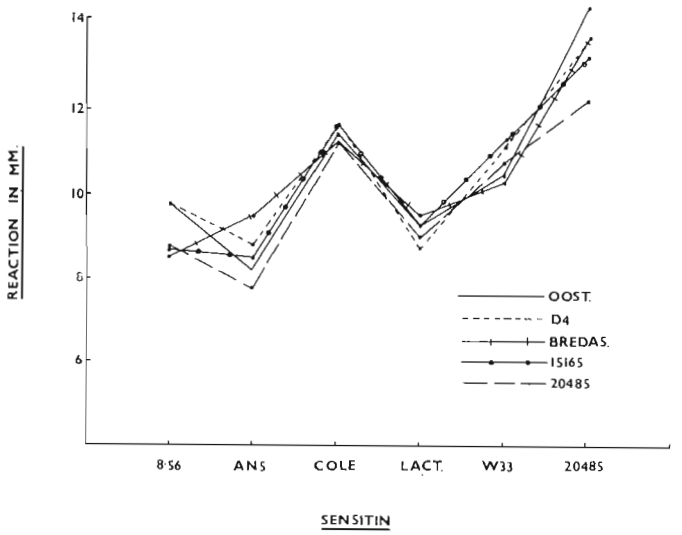

FIG. 7.—Sensitivity profiles of guinea pigs sensitized by five strains of *M. avium* and avian-like mycobacteria

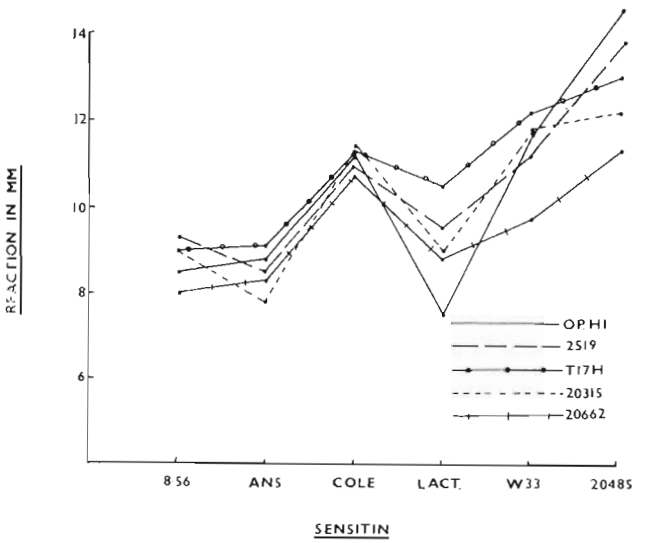

FIG. 8.—Sensitivity profiles of guinea pigs sensitized by five strains of *M. avium* and avian-like mycobacteria

(*c*) *Discussion*

It can be seen in Table 21 that all the avian and avian-like strains are similar in allergenic structure. The SPD's were always low, varying from —0·1 to 3·6 mm. The largest SPD's were those of strain T17H and the other strains indicating that there may be a slight difference between this strain and the avian strains. The strain T17H is a true Runyon Group III (Battey type) avian-like strain of human origin and, therefore, differs from the other strains. Magnusson *et al.* (1961) claimed that Battey strains can be differentiated from *M. avium* strains by similar methods to those used here. Karlson (1964) was, however, unable to demonstrate a significant difference in the sensitivity caused by avian and Battey strains. The difference seen in this study was slight and no definite conclusions can be drawn from this limited investigation. The relationship between all the avian and avian-like strains tested was, however, so close that it can be assumed that PPD prepared from any of the strains investigated would be efficient in differentiating non-specific reactions caused by a wide variety of avian and avian-like strains from bovine tuberculosis. In these experiments there was no difference in the specificity of the allergy produced by strains isolated from birds, pigs and cattle.

Further evidence of the close allergenic relationship of the 10 avian and para-avian strains investigated was provided by the experiments in which sensitin 20485 was compared with five sensitins prepared from other mycobacteria. Only minor and probably insignificant differences were seen in the sensitivity profiles of the 10 avian strains (Fig. 7 and 8). The SPD's (Table 23) of the 10 avian strains and any of the heterologous strains always fell into a fairly confined range, e.g. with sensitin 8·56 (*M. phlei*) they varied from 11·0 to 13·7, with AN5 (*M. bovis*) from 7·8 to 10·8 etc., thus providing additional evidence of the close relationship between the avian and avian-like strains.

The avian group of mycobacteria proved to be most closely related to *M. kansasii* and the scotochromogens. Groups of guinea pigs sensitized by six avian strains were more sensitive to the sensitin prepared from the scotochromogen strain than to that prepared from the *M. kansasii* strain. In guinea pigs sensitized by another four avian strains the position was reversed with the *M. kansasii* sensitin causing the greater reactions. Differences were, however, small in all cases. The mean reactions of 28 guinea pigs sensitized by avian strains are given in Table 24.

The SPD's also show the relationship between *M. avium* and *M. kansasii*, and *M. avium* and the scotochromogen. Here the closer relationship appears to be between W33 (*M. kansasii*) and the avian strains, SPD's from 3·4 to 7·0, with a slightly higher difference between the avian strains and Cole (*scotochromogen*), SPD's 5·1 to 7·8 (Table 23). In all cases the sensitivity to bovine PPD was distinctly lower than to avian PPD.

TABLE 24.—*Mean reactions of guinea pigs sensitized by* M. avium *and avian-like strains to six mycobacterial PPD sensitins*

No. of guinea pigs	No. of tests	20485 (*M. avium*)	8·56 (*M. phlei*)	AN5 (*M. bovis*)	Cole (scotochromogen)	Lacticola (*M. smegmatis*)	W33 (*M. kansasii*)
28.....	28	13·2	9·0	8·5	11·3	9·1	11·2

TABLE 25.—*Mean reactions of guinea pigs sensitized by* 10 *strains of* M. avium *and avian-like mycobacteria to PPD sensitins prepared from* M. avium *and avian-like strains*

No. of guinea pigs	No. of tests	20485 (*M. avium*)	2519 (*M. avium*-like	20315 (*M. avium*-like	T17H (*M. avium*-like	Oost. Av. (*M. avium*)	D4 (*M. avium*)
30.....	58*	13·0	12·4	12·0	11·7	12·2	12·7

*Two intercurrent deaths

The most potent PPD's were those prepared from strains 20485 and D4 as can be seen from the mean reactions of two tests in 30 guinea pigs sensitized with avian strains (Table 25). There was little difference in the specificity of the two sensitins, and it therefore seems that they should be equal for use in comparative testing. In a previous investigation with a different batch of D4 tuberculin it was found that the particular batch of 20485 PPD was both more potent and more specific (Worthington, 1963) and strain 20485 was therefore used for routine production of avian PPD. The switch to a new strain had the added advantage that it stimulated the interest of field veterinarians in comparative testing. There is now little reason to believe that either strain is superior to the other. Both strains grow well on synthetic medium and form pellicles easily, and yields of PPD from the two strains are similar.

(ix) *General Discussion*

The study proves that the sensitivity caused by mycobacteria when they infect an animal is highly specific for each species. In all cases there is a degree of cross sensitivity so that reactions will be elicited by heterologous mycobacterial sensitins. The size of reactions produced by heterologous sensitins varies according to the allergenic structure of the sensitizing strain and the species from which the sensitin is produced. In some cases the relationship is so close that fairly large reactions are elicited by heterologous sensitins, e.g. sensitins produced from *M. kansasii* or from scotochromogens in guinea pigs sensitized by *M. avium*. The sensitivity is, however, so specific that it can be used as a means of grouping mycobacteria, and as an aid to other methods of species classification.

The methods used to standardize the strength of the PPD's could probably be improved by comparing sensitins biologically, as has been done by Lesslie & Herbert (1962), but in investigations on large numbers of sensitins this is not practical. Specific type strains should later be established and standard potency PPD solutions prepared so that more accurate experiments can be undertaken. The simple standardization method used in this study did not always give PPD solutions of similar potency, e.g. in previous studies it was seen that PPD's prepared from the strains Lacticola and Smegmatis O.P. were of equal potency while in this study Lacticola PPD was weaker; also W33 sensitin was previously found to be more potent than P8 sensitin, while in the present study the position was reversed; 20485 sensitin was previously found to be more potent than D4 sensitin, while in the present study they were approximately equal. The specificity did not vary. One actually wishes to study specificity as potency can be adjusted to suit any particular requirement. Magnusson's (1961) method of studying specificity differences is particularly suited to the present study as differences in potency do not greatly affect the SPD.

The selection of PPD's for investigations in cattle was not based on their potency in the guinea pig experiments. The specificity of sensitins prepared from different strains of any species of *Mycobacterium* was similar. The differences in specificity of the sensitins prepared from scotochromogens were probably due to the fact that the strains used represent different species of mycobacteria. The choice of PPD used for each species was more or less arbitrary and influenced mainly by such practical considerations as the quantity of each PPD available and whether the strain generally produced good pellicles and yields of PPD. The following strains were selected: AN5 was used as the *M. bovis* strain in all experiments as it is our standard strain for bovine PPD production; 20485 was used as our *M. avium* strain (in previous experiments this strain had appeared superior to D4 and it was introduced as our standard PPD strain); W33 was used as our *M. kansasii* strain (PPD from this strain was available in fair quantities and it had the additional advantage of being a strain isolated from local cattle); Lacticola, Fortuitum, 8·56, Cole and 2-OR were used as *M. smegmatis*, *M. fortuitum*, *M. phlei*, scotochromogen and *Mycobacterium* sp. strains respectively, because they were available in adequate quantities. From the studies in guinea pigs there appeared to be no reason to believe that the substitution of any other PPD prepared from a strain of the same species for any of the PPD's used would have materially influenced subsequent results.

The avian-bovine comparative tuberculin test has been criticised in South Africa. The main question is whether any other suitable PPD could be found for comparative testing or whether avian PPD is adequate. Data of reactions to AN5 (bovine) and to 20485 (avian) tuberculin in guinea pigs sensitized by various strains of mycobacteria were, therefore, extracted from all the results in the investigations described in this chapter. The results are given in Table 26.

TABLE 26.—*Mean reactions of guinea pigs sensitized by various mycobacteria to PPD sensitins prepared from strains AN5* (M. bovis) *and* 20485 (M. avium)

Sensitizing Mycobacterium	No. of tests	AN5			20485		
		$\bar{X}$	S	$\hat{O}x$	$\bar{X}$	S	$\hat{O}x$
M. bovis	36	14·0	2·85	0·47	9·0	1·99	0·33
M. avium	61	8·2	1·77	0·27	13·8	2·29	0·29
M. kansasii	49	9·2	2·84	0·41	10·9	2·43	0·35
Scotochromogen	20	7·4	2·67	0·60	10·6	3·10	0·69
M. fortuitum	27	7·1	1·91	0·37	8·7	2·10	0·40
M. phlei	44	5·0	2·22	0·34	6·6	2·31	0·35
M. smegmatis	48	5·8	2·19	0·32	7·3	2·21	0·32
Mycobacterium sp.	33	7·0	2·57	0·45	9·1	2·52	0·44

$\bar{X}$ — mean reaction S — standard deviation $\hat{O}x$ — standard error of the mean

It can be seen that the avian PPD caused higher reactions than bovine PPD in guinea pigs sensitized by all strains except *M. bovis*. On the assumption that the distribution of reactions was approximately normal, Student's T test for significance was applied. The differences between the mean reactions to the two tuberculins in all cases were statistically significant at the 1 per cent level. In practice, when comparative testing is undertaken in cattle, equal reactions to avian and bovine PPD or even slightly higher reactions to bovine PPD than to avian PPD are classified as negative (this point will be discussed in more detail in Chapter 8). The reasons for this are obvious. In the case of sensitization to *M. bovis* the reactions to bovine PPD are distinctly larger than to avian PPD and should therefore be readily recognizable. In the case of sensitization caused by another *Mycobacterium* the sensitivity to the two products more closely approximates each other and it is quite conceivable that some species of mycobacteria might cause greater sensitivity to bovine PPD than to avian PPD. The difference will not be as clear-cut as in an animal infected with bovine tuberculosis. For sensitization caused by species of mycobacteria similar to those used in this investigation, a comparative test with bovine and avian tuberculins should be satisfactory for differentiating specific and non-specific reactions.

It is perhaps not acceptable to draw conclusions about what will happen in cattle from experiments on guinea pigs. For this reason it is necessary to show that the delayed hypersensitivity developed by cattle is as specific as that seen in guinea pigs. This point is discussed in the next chapter.

CHAPTER 5

INVESTIGATIONS ON EXPERIMENTALLY SENSITIZED CATTLE

(i) *Introduction*

It is well known that different species of animals show varying sensitivity to tuberculin. Thus, when testing humans, a dose of 5 TU is usually sufficient to elicit good reactions in tuberculous patients, while in cattle 5,000 to 10,000 TU are generally used. There is, however, no reason to believe that specificity is affected by species differences. The necessity of proving that the allergenic reelationships of various mycobacterial sensitin systems are similar in guinea pigs and in cattle was mentioned previously. This has been shown in guinea pigs and humans by Bjerkedahl & Natvig (1961) and Edwards *et al.* (1961).

(ii) *Materials and methods*

Cattle, varying in age from six months to adult, of various breeds and both sexes were used in the experiment. Only animals negative to the comparative avian-bovine tuberculin tests were used. They were divided into groups and injected with 10 mg (wet weight) of heat killed mycobacteria of six different species, suspended in 2·5 ml of liquid paraffin. The injections were usually made subcutaneously in the dewlap, but intradermally in the case of cattle sensitized with *M. bovis* and *M. avium*, which were later retained for the routine work of standardization of tuberculin. Sensitization in the group of cattle sensitized with Lacticola (*M. smegmatis*) was at first unsatisfactory; a second group was therefore sensitized with a dose of 15 mg of organisms. The planning of the experiment was influenced by the availability of experimental animals and housing facilities. Details of the sensitization are given in Table 27.

TABLE 27.—*Sensitization of cattle to various strains of mycobacteria*

Sensitizing strain	Dose	Route	No. of cattle
9473 (*M. bovis*)	10 mg	Intradermal	6
OPH1 & Bredasdorp (*M. avium*)	10 mg	Intradermal	4
W33 (*M. kansasii*)	10 mg	Subcutaneous	3
Cole (scotochromogen)	10 mg	Subcutaneous	3
8·56 (*M. phlei*)	10 mg	Subcutaneous	5
Lacticola (*M. smegmatis*) 1	10 mg	Subcutaneous	4
Lacticola (*M. smegmatis*) 2	15 mg	Subcutaneous	5

Six weeks after sensitization the animals were tested intradermally with 2,500 SU of the following sensitins: Lacticola (*M. smegmatis*), W33 (*M. kansasii*), 20485 (*M. avium*), AN5 (*M. bovis*), Cole (*scotochromogen*) and 8·56 (*M. phlei*). The sensitins were injected in three sites on each side of the neck and the order of injection rotated in each animal. The skinfold measurements at the time of injection and 72 hours later were measured with a Hauptner semi-automatic calliper and the skinfold increases recorded. The tests were repeated after a further six weeks. Sensitivity profiles were then constructed for each differently sensitized group. The sensitivity profiles were compared with profiles of similarly sensitized groups of guinea pigs. The guinea pig profiles were constructed by extracting data from the results given in the previous chapter. The reactions in all guinea pigs, sensitized by a particular species (a number of strains may be included) and tested by the same sensitins as employed in the investigations in cattle, were used.

(iii) *Results*

The mean reactions of each group of cattle to the six sensitins are given in Table 28 and the mean reactions of all guinea pigs sensitized by similar species of mycobacteria and tested with the same six sensitins are given in Table 29.

TABLE 28.—*Mean reactions of groups of cattle sensitized by six species of mycobacteria to PPD sensitins prepared from the same six strains*

Sensitizing strain	No. of tests	Sensitin					
		Lacticola	W33	20485	AN5	Cole	8·56
Lacticola (*M. smegmatis*) 1	8	**3·0**	2·0	2·5	1·0	3·7	0·7
Lacticola (*M. smegmatis*) 2	10	**5·4**	1·1	1·4	0·7	3·4	1·6
W33 (*M. kansasii*)	6	3·2	**8·4**	5·1	3·2	5·5	2·5
20485 (*M. avium*)	7	5·0	2·0	**6·9**	1·9	3·5	0·4
9473 (*M. bovis*)	12	2·8	5·3	2·7	**9·2**	3·7	0·9
Cole (scotochromogen)	6	1·1	2·8	4·0	1·7	**9·0**	0·8
8·56 (*M. phlei*)	10	2·2	1·7	1·3	0·9	3·8	7·**0**

TABLE 29.—*Mean reactions of groups of guinea pigs sensitized by six species of mycobacteria to PPD sensitins prepared from six similar strains*

Sensitization	Sensitin					
	Lacticola (*M. smegmatis*)	W33 (*M. kansasii*)	20485 (*M. avium*)	AN5 (*M. bovis*)	Cole (scotochromogen)	8·56 (*M. phlei*)
M. smegmatis	**11·3**	6·5	7·3	5·8	7·3	7·8
M. kansasii	7·9	**13·6**	10·9	9·2	11·0	10·0
M. avium	8·7	11·0	**13·8**	8·2	10·8	8·6
M. bovis	6·9	10·3	9·0	**14·0**	8·3	6·6
Scotochromogen	7·3	9·7	10·6	7·4	**14·1**	7·5
M. phlei	7·9	7·4	6·6	5·0	6·2	**13·3**

The mean reactions given in Table 29 represent a total of 1,225 reactions in guinea pigs. Individual means given in the table represent 9 to 61 reactions.

The sensitivity profiles of the cattle, sensitized by the six species of mycobacteria together with the sensitivity profiles of similarly sensitized guinea pigs, are given in Fig. 9.

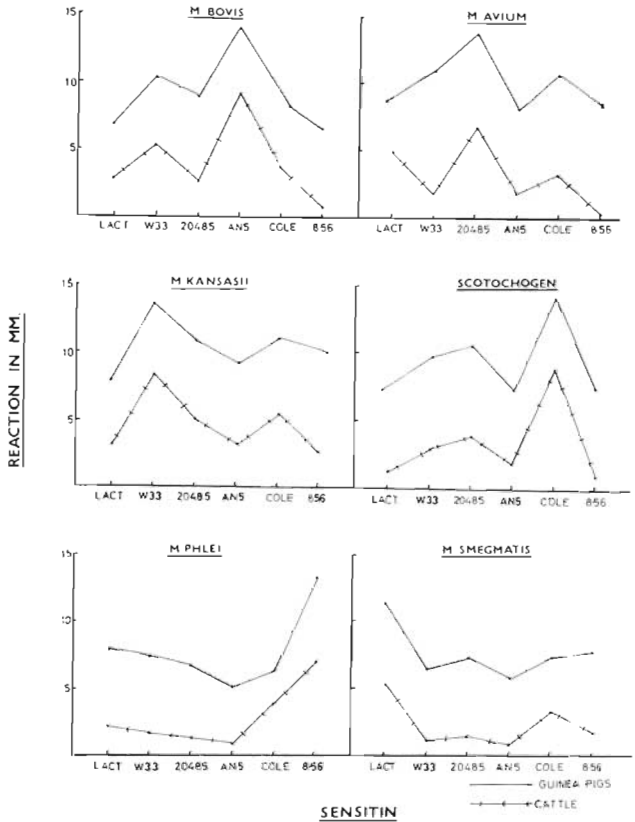

FIG. 9.—Sensitivity profiles of guinea pigs and cattle sensitized by six different mycobacterial species

(iv) *Discussion*

It was seen that the host species, at least in the case of guinea pigs and cattle, does not greatly affect the specificity of the tuberculin reaction. The sensitivity profiles of cattle and guinea pigs for each species of *Mycobacterium* studied are remarkably parallel (Fig. 9). The cattle sensitized with *M. smegmatis* were most sensitive to the scotochromogen sensitin (Table 28). This result was in such marked contrast to the other results that it was assumed that it must have been due to experimental error. The cultures used for sensitization could have been contaminated with a scotochromogen or the animals could perhaps have become naturally sensitized by a similar strain. A further group of cattle sensitized by a higher dose of strain Lacticola (*M. smegmatis*) showed a more typical sensitivity pattern similar to that seen in guinea pigs sensitized by *M. smegmatis* (Fig. 9). The results of the first experiment with cattle sensitized to *M. smegmatis* were therefore ignored. In every case in the cattle just as in the guinea pigs the homologous sensitin caused distinctly larger reactions than the other six heterologous sensitins. A few irregularities in the similarity of the profiles were seen, e.g. in the case of the cattle sensitized by *M. avium* the reactions to the sensitin prepared from strain Lacticola (*M. smegmatis*) were comparatively higher than those in similarly sensitized guinea pigs, and in cattle sensitized by *M. phlei* the reactions to the scotochromogen sensitin were higher than expected. Since the number of cattle used in the experiments was comparatively small, it was to be expected that minor discrepancies would be observed. In general, however, the sensitivity profiles were so similar that it can be assumed that the specificity of delayed hypersensitivity systems is similar in the two species. There is therefore no reason why all the preliminary screening of sensitins to be used in cattle should not be done in guinea pigs. Knowledge obtained in guinea pig experiments can confidently be utilized to interpret results of multiple comparative tests on cattle.

It is also of interest to note that in cattle as well as in guinea pigs the sensitivity to avian tuberculin was greater than the sensitivity to bovine tuberculin in all cases except where they were sensitized with *M. bovis*. By means of the Mann-Whitney U test for significance it was seen that there was a significant difference (at the 5 per cent level) between the reactions to bovine and avian tuberculin in the case of cattle sensitized by *M. avium*, *M. bovis* and the scotochromogenic strain Cole. In cattle sensitized by *M. phlei*, *M. smegmatis* and *M. kansasii* the difference was not significant.

CHAPTER 6

FIELD INVESTIGATIONS WITH PPD SENSITINS IN PROBLEM HERDS

(i) *Introduction*

In order to establish the common causes of non-specific reactors in South African cattle, multiple comparative tests were used. This approach was successful in defining non-specific reactor problems in humans (Edwards *et al.*, 1961; Edwards & Smith, 1965; Edwards, *et al.*, 1965). Large numbers of cattle could not be submitted to the test at random, because tuberculin testing is under statutory control and reactors must be branded. Many farmers are, therefore, unwilling to allow

testing of their animals. Also farmers were unlikely to agree to large numbers of milking cows being injected six times each. The approach decided on was that " problem herds " should be sought. Many of these herds are accredited TB free herds in which non-specific reactors occur sporadically. After routine tests in these herds a small number of suspected non-specific reactors could be selected and submitted to carefully planned tests in which the injection sites were varied and tests read by the veterinarian concerned without prior knowledge of which sensitins were used. In this way it was hoped to obtain significant data from a small number of tests as each small group of cattle tested represents the type of sensitization to be found in that particular herd. Farmers owning this type of herd were usually willing to co-operate as they wished to see the problem resolved.

(ii) *Materials and methods*

The investigation of sensitivity patterns in cattle in problem herds was undertaken in conjunction with the Division of Veterinary Field Services. A circular letter was sent to all state veterinarians explaining the purpose of the investigation and the method of study. Suitable herds were selected on the basis of their testing history by the state veterinarians concerned. After a routine tuberculin test on a herd, between six and 24 suspected non-specific reactors were selected for further investigation. These animals were then retested with six PPD sensitins. The sensitins contained $\pm$ 0·5 mg of dried PPD powder per ml (25,000 sensitin units per ml) and were given code numbers. They were injected in 0·1 ml amounts (2,500 S.U.) in three sites on each side of the neck, the sensitins being rotated in the different sites according to charts supplied. The skinfold measurements before and 72 hours after injection were recorded and the results sent to this laboratory where they were decoded and analysed.

The test method was apparently followed accurately by all participating veterinarians except in three herds where the injection sites were not varied. One of the difficulties encountered was that field veterinarians were sometimes unable, or neglected, to carry out the multiple comparative test within the recommended time of four to six weeks after the routine test at which the non-specific reactors were found. The time lapse was in some cases as long as six months with the result that some of the animals had lost their sensitivity and failed to react to any of the sensitins. For this reason only animals showing skinfold increases of 2 mm or more to at least one of the sensitins were included in the final analysis. In the first 18 herds the following sensitins were used: 2-OR (*Mycobacterium* sp.), Lacticola (*M. smegmatis*), W33 (*M. kansasii*), 20485 (*M. avium*), AN5 (*M. bovis*) and Fortuitum (*M. fortuitum*). It then became apparent that sensitivity to the sensitins 2-OR and Lacticola was generally of a very low level. The sensitin Cole (scotochromogen) was therefore substituted for 2-OR but unfortunately only three herds were offered for investigation with it.

(iii) *Results*

The 21 herds selected for the investigation were widely distributed over the eastern and northern parts of the country (see Fig. 10). Unfortunately no herds were tested in the Western Province where " skin lesions " and other non-specific reactor problems are frequently reported. A total of 205 animals was tested of which 143 had reactions of 2 mm or more to at least one of the sensitins.

In two herds the general sensitization pattern was of such a low level as to be of no importance, a further two herds were infected with bovine tuberculosis, another showed a type of sensitization of undefined aetiology, and in 16 herds sensitization was apparently caused by *M. avium* or a closely related organism.

Herds infected with bovine tuberculosis: In the herd We. there was a history of reactions of an atypical nature at previous tuberculin tests. Post mortem examination of one such case had failed to reveal any lesions, and cultures from pooled lymph glands sent to this laboratory failed to yield any mycobacteria. The results of the multiple comparative test are given in Table 30. Subsequently cow No. 141 and bull No. 303 were sent to Onderstepoort where typical lesions of bovine tuberculosis were found at autopsy. The diagnosis was further confirmed by isolation and identification of *M. bovis.*

In herd G. there was a history of tuberculosis, but the interpretation of reactions at previous tests was lenient. Tuberculous animals with low sensitivity were not removed and the disease had, therefore, continued to smoulder in the herd. The results of the multiple comparative test are given in Table 31. Cow No. 78 was subsequently slaughtered and typical lesions found at autopsy. The diagnosis was further confirmed by isolation and identification of *M. bovis.*

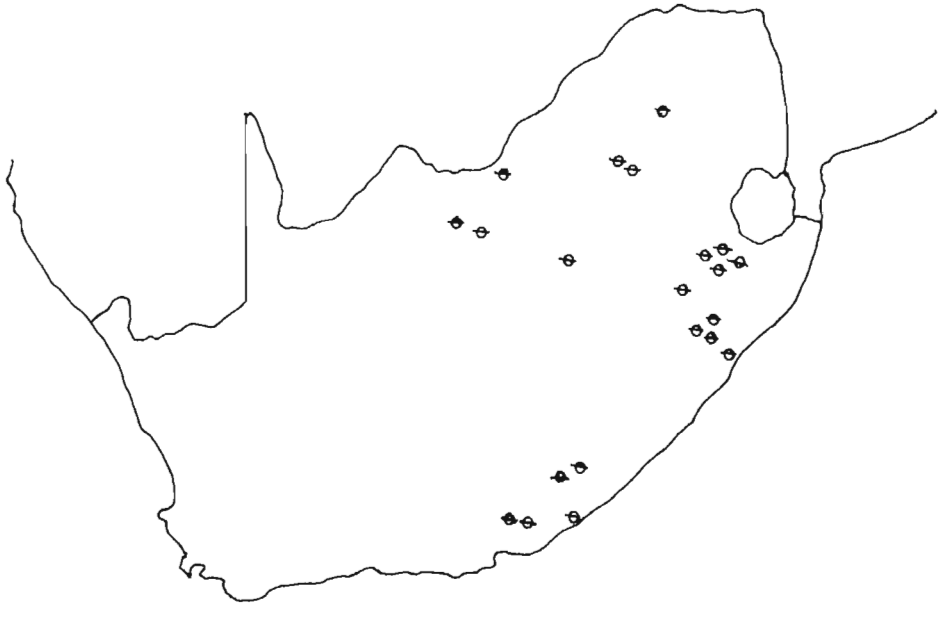

FIG. 10.—Geographical distribution of herds investigated

TABLE 30.—*Skinfold increases (mm) in herd We. at multiple comparative test*

No. of animal	Sensitin					
	2–OR (*Mycobacterium* sp.)	Lacticola (*M. smegmatis*)	W33 (*M. kansasii*)	20485 (*M. avium*)	AN5 (*M. bovis*)	Fortuitum (*M. fortuitum*)
C321	0	0·5	0·7	4·1	3·8	1·7
C303	0·5	0	3·9	5·4	5·7	1·0
C141	1·3	0·5	9·5	4·3	16·6	3·3
C272	0	0·5	3·5	5·1	6·0	1·7
C283	0	0·3	2·3	1·8	5·2	0
C210	0·3	0	2·6	0·6	5·5	0·4
C303	2·8	2·7	8·0	2·3	16·4	2·5
C317	0·3	0·6	4·2	0·4	6·2	6·7
C274	0·4	1·3	2·0	0·6	3·3	0·6
B10	0	0	4·0	6·6	7·7	0·3
BRU	0·6	0·2	10·3	4·9	13·0	1·6
C115	0·2	0	4·1	2·7	3·9	0·3
TOTAL	6·4	6·6	55·1	38·8	93·3	20·1
MEAN	0·5	0·6	4·6	3·2	7·8	1·7

TABLE 31.—*Skinfold increases (mm) in herd G at multiple comparative test*

No. of animal	Sensitin					
	2–OR (*Mycobacterium* sp.)	Lacticola (*M. smegmatis*)	W33 (*M. kansasii*)	20485 (*M. avium*)	AN5 (*M. bovis*)	Fortuitum (*M. fortuitum*)
7	0·8	0·1	0·4	1·1	3·8	0
56	0	0·6	0·1	0·3	3·6	0·6
68	0·7	1·1	4·0	2·4	4·3	0·2
78	0·5	0·3	2·7	10·6	14·0	2·1
120	0·4	0·9	2·0	2·0	5·8	0·7
149	0	0	0·6	3·7	1·5	0·4
184	0·5	0	0·2	0·1	2·0	0
222	2·3	0·5	4·6	7·7	8·6	1·2
TOTAL	5·2	3·5	14·6	27·9	43·6	5·2
MEAN	0·7	0·4	1·8	3·5	5·5	0·7

Herd showing non-specific sensitivity of unknown aetiology: This herd (Vi) was previously infected with tuberculosis, but the disease was eliminated by isoniazid treatment and gradual removal of reactors. The cattle selected for these tests were from that part of the herd in which no case of tuberculsosis had been detected in three years of constant control and testing. Reactions occurred most frequently in calves, young heifers and oxen and were generally hard and circumscribed. The results of the multiple comparative test are given in Table 32.

Herds showing sensitivity to avian tuberculin: In the remaining 16 herds the sensitivity pattern was similar. The highest reactions occurred to avian PPD followed by the reactions to the scotochromogen (in the few herds tested) and *M. kansasii* sensitins. Of 160 animals tested in these herds 09 had reactions of 2 mm or more to at least one of the sensitins. The avian PPD caused the highest reaction in 77 cases, the *M. kansasii* PPD in 17, the *M. fortuitum* PPD in five, the *M. bovis* PPD in four and the *M. smegmatis* PPD and scotochromogen PPD (used in 17 reactors only) in one each. In two cases equally high reactions occurred to the *M. avium* and *M. kansasii* sensitins, in one case to the *Mycobacterium* sp. and *M. kansasii* sensitins and in one case to the *M. fortuitum* and *M. kansasii* sensitins.

The means of the reactions to each sensitin in the 16 herds are given in Table 33, and the frequency distributions of the reactions to the six sensitins are shown in histogram form in Fig. 11. No distribution is shown for the scotochromogen sensitin as too few tests were done with it.

Sensitivity profiles constructed from the mean reactions to the seven sensitins in the 109 reactor cattle and from reactions in guinea pigs are shown in Fig. 12. The mean reactions to the sensitins in guinea pigs represent 46 to 61 reactions and are taken from the results given in Table 29.

TABLE 32.—*Skinfold increases (mm) in herd Vi at multiple comparative test*

No. of animal	Sensitin					
	2–OR (*Mycobacterium* sp.)	Lacticola (*M. smegmatis*)	W33 (*M. kansasii*)	20485 (*M. avium*)	AN5 (*M. bovis*)	Fortuitum (*M. fortuitum*)
505	3·0	2·6	2·5	2·5	6·7	2·0
63–0	0·5	0·2	1·3	2·0	0·1	0·6
415–0	1·1	0·2	4·5	1·2	3·8	1·3
236	1·5	0·3	2·0	3·2	1·5	0·7
52	1·4	2·4	1·0	5·4	2·8	1·0
501–3	1·8	2·5	1·9	1·8	5·8	2·1
99–1	0·9	1·0	0	2·0	0	1·6
504	1·5	1·0	1·4	6·0	3·5	0·8
490	1·9	2·2	5·2	2·7	2·2	2·2
272–3	0·7	0	2·8	2·7	8·0	1·0
62–2	0	0	1·3	3·5	1·3	0·2
209–3	0·6	2·3	1·3	1·3	4·4	1·0
22	1·0	0	3·1	3·8	3·0	0·7
294	2·7	1·0	4·0	5·5	2·1	0·7
TOTAL	18·6	15·7	32·3	43·6	45·2	15·9
MEAN	1·3	1·1	2·3	3·1	3·2	1·1

TABLE 33.—*Mean skinfold increase (mm) at multiple comparative test in 16 herds*

Area of origin	No. Tested	No. Reactors	Sensitin						
			2–OR (*Mycobacterium* sp.	Lacticola (*M. smegmatis*)	W33 (*M. kansasii*)	20485 (*M. avium*)	AN5 (*M. bovis*)	Fortuitum (*M. fortuitum*)	Cole (scotochromogen)
Eastern Cape	9	7	2·1	2·3	3·4	3·2	2·5	2·6	—
Natal	6	6	3·6	1·0	3·7	7·6	2·2	1·3	—
Eastern Cape	4	4	1·5	1·5	3·0	4·6	2·5	2·2	—
Natal	7	7	1·0	0·9	2·0	4·4	1·3	1·0	—
Transvaal	9	8	1·3	1·0	3·4	4·3	2·2	0·8	—
Natal	25	14	0·8	0·4	2·0	3·0	1·4	1·0	—
N.W. Cape	7	5	0·2	0·2	0·8	3·9	1·0	0·4	—
N.W. Cape	12	6	0·6	0·6	1·1	3·7	0·8	1·1	—
N.W. Cape	14	8	0·5	0·8	2·4	4·1	2·8	1·9	—
Transvaal	8	7	2·0	1·7	4·3	5·7	2·9	2·8	—
Eastern Cape	9	6	1·5	1·6	2·7	3·0	1·4	2·1	—
Natal	11	6	0·7	0·9	2·1	2·1	1·4	0·8	—
Natal	8	8	0·3	0·4	1·3	3·7	1·6	0·9	—
Natal	6	5	—	1·3	2·3	3·1	1·4	1·8	2·6
Eastern Cape	16	7	—	0·7	2·3	3·6	1·7	1·3	2·6
Eastern Cape	9	5	—	1·2	2·8	3·6	1·7	3·4	3·1
MEAN			1·17	0·97	2·45	3·92	1·81	1·52	2·76
STANDARD DEVIATION			1·27	0·96	1·74	2·14	1·40	1·32	—

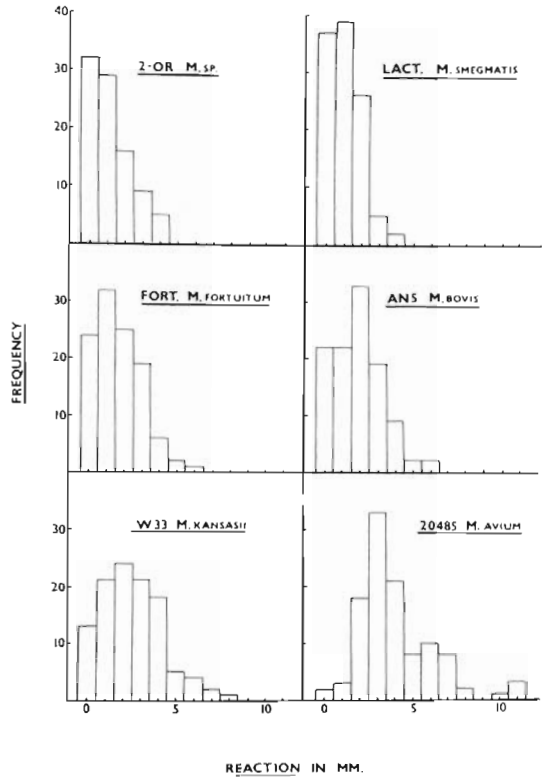

FIG. 11.—Frequency distributions of reactions to six PPD sensitins in non-specific reactor cattle

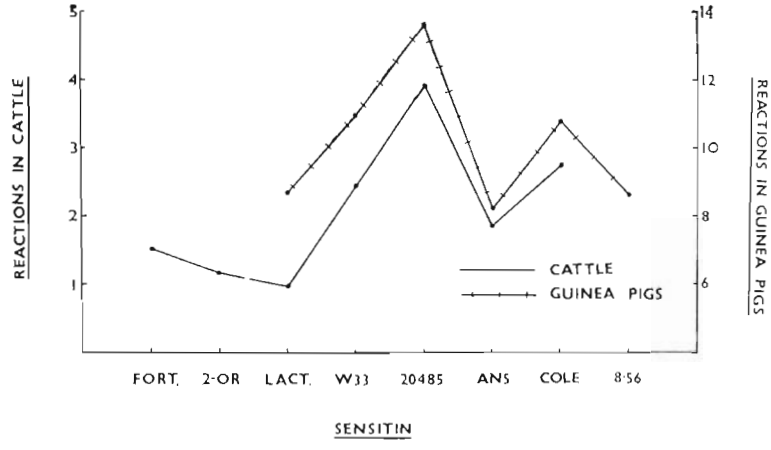

FIG. 12.—Comparison of sensitivity profiles in naturally sensitized cattle and guinea pigs sensitized by *M. avium*

(iv) *Discussion*

The inclusion of two herds infected with *M. bovis* in this study was fortunate, because it was possible to show that bovine tuberculosis could be readily recognized by the method and with the sensitins employed. In both herds there were cases where the sensitization was possibly not due to bovine tuberculosis, e.g. Herd G., No. 149 and Herd We., No. C321. The inclusion of doubtful cases did not influence the overall picture of an infection caused by *M. bovis*. Of considerable interest is the fact that the degree of sensitivity to the *M. kansasii* sensitin (W33) in herd We. was second only to the sensitivity to bovine tuberculin. This was expected as a similar picture was seen in guinea pigs sensitized by *M. bovis*. In herd G. the reactions to *M. kansasii* sensitin (W33) were lower than the reactions to the *M. avium* sensitin (20485). This unexpected result can only be explained in terms of some procedural error such as a leaking syringe delivering the incorrect dose.

The sensitivity pattern in the other herds was in marked contrast to that seen in the tuberculous herds. In one herd (Vi.) some animals were more sensitive to avian tuberculin than to bovine tuberculin, while in others the sensitivity was reversed. Sensitivity in this herd may have been caused by a mixture of sensitizing agents or it may be due to another unknown organism. No definite conclusions can be made from the results in a single herd.

In the other 16 herds a very uniform pattern was seen. The greatest degree of sensitivity was demonstrated to the *M. avium* sensitin followed in descending order by the sensitivity to the scotochromogen, *M. kansasii*, *M. bovis* and *M. fortuitum* sensitins. Sensitivity to the *M. smegmatis* and the *Mycobacterium* sp. sensitins was of a very low order. Reactions to Lacticola (*M. smegmatis*) and 2-OR (*Mycobacterium* sp.) sensitins were rare and, if they did occur, small as can be seen from the histograms in Fig. 11. The size of reactions to the sensitins Fortuitum (*M. fortuitum*), AN5 (*M. bovis*), and W33 (*M. kansasii*) were progressively greater, and finally it can be seen that the reactions to 20485 (*M. avium*) sensitin were distinctly larger with reactions of less than 2 mm occurring rarely. This sensitivity pattern was exactly analagous to that seen in guinea pigs sensitized with *M. avium*. The sensitivity profiles (Fig. 12) were remarkably parallel and almost 75 per cent of all the reacting cattle were most sensitive to avian tuberculin. The difference between the means of the reactions to the *M. avium* and *M. bovis* sensitins was significant at the 1 per cent level. The difference between the means of the reactions to the *M. avium* and *M. kansasii* sensitins was not significant. The number of cattle tested with scotochromogen sensitin was too small to be considered statistically.

Bacteriological confirmation of the type of sensitization was also provided in one of these herds. Lymph glands from a no-visible-lesion-reactor cow were sent to this laboratory where *M. avium* (virulent for chickens) was isolated. In five herds in which comparative avian-bovine tests have subsequently been performed on the whole herd it was seen that the comparative test greatly facilitated the interpretation of reactions. It seems reasonable to assume that the main cause of sensitization in these herds was *M. avium* or closely related mycobacteria. The avian-bovine comparative test should therefore greatly facilitate the interpretation of reactions in many South African herds in which non-specific reactions occur. It is difficult to understand from what source cattle become infected with avian tuberculosis. In many instances there is no contact with poultry and in any case the disease is rare in South African poultry. It is known that about 70 to 75 per cent of tuberculous lesions in lymph nodes of pigs in South Africa are caused by avian or avian-like mycobacteria from a completely unknown source (Worthington, 1966). Cattle and pigs may therefore be infected from a similar unknown source.

CHAPTER 7

THE SINGLE INTRADERMAL TUBERCULIN TEST WITH BOVINE PPD TUBERCULIN: STUDIES IN TUBERCULOUS AND PROBLEM HERDS

(i) *Introduction*

In the previous chapter it was shown that cattle with non-specific reactions were usually more sensitive to avian tuberculin than to any of the other sensitins used in the investigation. The avian-bovine comparative tuberculin test should, therefore, be the most logical tool in the hands of the veterinarian for distinguishing non-specific reactors from tuberculous cattle. In practice little comparative testing is done in South Africa. Because the test with bovine tuberculin alone has been used successfully for many years and the comparative test is more time-consuming, it is possible that the bulk of testing will continue to be done with this method and comparative testing used mainly in problem herds. Large scale field investigations were undertaken to compare the two methods of testing in naturally infected tuberculous and non-specifically sensitized cattle.

In this chapter the sensitivity to bovine tuberculin only is considered. The comparative test is discussed in the next chapter. The accuracy of the presently accepted formulae for interpretation of tuberculin tests was also investigated.

Multiple tuberculin testing in humans with 100 TU and 10 TU of tuberculin simultaneously causes a significant decrease in the size of the reactions to 10 TU of tuberculin when compared to the reactions in people tested with 10 TU of tuberculin only (Rosenthal & Libby, 1960). Meyn & Schliesser (1962) have claimed that simultaneous injections of tuberculin may similarly depress the size of reactions in guinea pigs and in cattle. Freerksen & Lauterbach (1960b) on the other hand could find no such depression in sensitivity in cattle and claim that " In cattle simultaneous tests with heterologic as well as with homologic tuberculins may be made without hesitation. The reactions have no influence on each other ". The present investigation also provided an opportunity to study this point.

The problem of non-specific sensitization occurring simultaneously with bovine tuberculosis and the influence of the age of the animals and animal husbandry methods on the problem were also studied.

(ii) *Materials and methods*

All cattle were tested with Onderstepoort bovine tuberculin prepared from the strain AN5 and containing 70,000 TU per ml. In some herds Onderstepoort avian tuberculin prepared from the strain 20485 and containing 25,000 TU per ml was used comparatively. Doses of 0·1 ml of tuberculin were injected intradermally into a small clipped area in the middle of the neck when bovine tuberculin was used alone and in the comparative test in two sites about six inches apart with the avian tuberculin in the front site. The skinfold thickness was measured at the time of injection and 72 hours later. The reaction was recorded as the increase in skinfold measurement in mm.

Tuberculous cattle: The reactions in 1,011 tuberculous cattle of a total of 3,464 cattle in 16 heavily infected herds were used to represent a population of reactions in tuberculous cattle. The herds were mainly commercial dairy herds and the cattle mainly Friesians. The presence of bovine tuberculosis was proved in each herd by post mortem and laboratory examination of at least one or more reactors. All the herds except one were used in an investigation on the control of bovine tuberculosis by means of isoniazid (Kleeberg, 1960b; Kleeberg & Worthington,

1963; Kleeberg, 1966; Kleeberg *et al.*, 1966). All animals were tested before treatment started, two months later and thereafter every six months for up to four years. The positive reactors under treatment gradually became less sensitive to bovine tuberculin and finally, if they were cured, reacted completely negatively. The test records were of great value, because, by following the records of each animal over a number of tests, it was possible to decide which animals were tuberculous with a far higher degree of accuracy than is possible from a single test. After a careful consideration of the test records, all reactions at the test before treatment started in cattle considered to be tuberculous were included in the study. Seven of the herds were tested by the comparative test and nine with bovine tuberculin only. Only the reactions to bovine tuberculin were considered in this part of the study. The total incidence of infection in the herds studied was 29·2 per cent. Details of infection in the individual herds are given in Table 34.

TABLE 34.—*Incidence of bovine tuberculosis in* 16 *infected herds*

Herd	Breed	Type of Farm	No. of cattle	No. of TB cattle	Percentage tuberculous
Cull	Friesian	Commercial Dairy	300	57	19·00
Mic	Jersey	Commercial Dairy	43	17	39·53
Gu	Friesian	Commercial Dairy	336	68	20·24
Gr. II	Friesian	Commercial Dairy	289	23	7·96
We	Brown Swiss	Stud	152	21	13·82
Scha	Jersey & Friesian	Commercial Dairy	165	58	35·15
Sch	Friesian	Commercial Dairy	147	65	44·22
Gr. I	Friesian	Commercial Dairy	272	133	48·90
Ho	Friesian	Stud & Dairy	175	75	42·86
Ja	Friesian	Commercial Dairy	355	65	18·31
Br	Friesian	Commercial Dairy	81	20	24·69
Gr. C	Friesian	Stud & Dairy	160	58	36·25
Wo	Friesian	Commercial Dairy	476	123	25·84
Wa	Friesian	Commercial Dairy	58	22	37·93
Wy	Friesian	Commercial Dairy	45	28	62·22
Ve	Friesian	Commercial Dairy	410	178	43·41
TOTAL			3,464	1,011	29·19

Non-tuberculous cattle: In order to obtain a suitable sample of non-specific reactors, figures were extracted mainly from the records of the Division of Veterinary Field Services. All the herds used had been tested with bovine tuberculin only. Herds were selected which, according to their history, appeared to be free from bovine tuberculosis. The reactions in the herds were generally small, hard and circumscribed. At each retest in these herds a number of new reactors was found, but at the same time a number of previous reactors had become negative. In herds where the incidence of non-specific reactors was low, all reactors from a number of consecutive tests were included. In cases where the incidence of non-specific reactors was high, reactions occurring at one test only were used. Results from one INH treated tuberculous herd were also included. In this case the spread of tuberculosis was completely halted by chemotherapy, and complete test records of six-monthly tests for a period of four years were available for each cow. Reactions are included only from the clean part of the herd in which no new case of tuberculosis

was detected during this time. The incidence of non-specific reactors in this herd (Schm.) was very high. A total of 600 non-specific reactions (8·9 per cent) occurring in 6,739 tests in nine herds was taken to represent a sample of non-specific reactors.

The data collected in the tuberculous and non-tuberculous herds were organized separately into frequency distribution tables with class intervals of 1 mm (0·5 to 1·4, 1·5 to 2·4 etc.) and the mean reactions in each group calculated from frequency tables by a standard method (Van Heerden, 1964). Histograms were than constructed for each group. In the case of the tuberculous cattle the reactions were distributed over a wide range and class intervals of 3 mm were therefore used, whereas in the non-specific reactors the range was narrower and class intervals of 1 mm were used. Less-than-cumulative frequencies expressed as percentages were then calculated for the two distributions and less-than-cumulative frequency curves with class intervals of 1 mm constructed.

The results from the tuberculous herds were also divided into two parts consisting of 588 cattle tested with bovine tuberculin alone, and 423 cattle tested by the comparative test. Mean reactions and cumulative frequencies were calculated as before and the Kolmogorov-Smirnov test (Siegel, 1956) applied to determine whether there was a significant difference between the two distributions.

In two tuberculous herds (Wo. & Cull.) many non-specific reactions occurred. The two herds were both well managed commercial dairy herds of Friesian cattle, which bred their own replacement stock. The frequency distributions of reactions to bovine tuberculin in these herds were investigated individually. By studying the test records of each animal it was possible to decide which animals were tuberculous and which non-specific reactors. Histograms were constructed to show the distribution of reactions in all the reacting animals, the tuberculous animals and the non-specific reactors. In one of these herds (Cull.) the animals were accurately identified according to date of birth. The distribution of reactions in animals one to five years old and over five years was studied. Histograms were constructed from the two frequency distributions.

(iii) *Results*

The frequency distributions of the reactions in tuberculous and non-tuberculous cattle and the less-than-cumulative frequency distributions are given in Appendix Tables 1 and 2. The distributions are shown in histogram form in Fig. 13. The mean reaction in the tuberculous animals was 13·92 mm and in the non-tuberculous cattle 4·18 mm. Both distributions are positively skewed thus precluding the use of the normal distribution theory in further analysis of the data. The cumulative frequency distributions of the two populations are shown as cumulative frequency curves in Fig. 14. From the cumulative frequency curves it was possible to read off the percentage of cattle in each sample which had reactions below any given limit. Thus if any limit is selected as the size of reaction above which an animal will be declared positive and below which it will be classed as negative, one can read off from the graph the percentage of tuberculous cattle that had reactions of less than this limit, i.e. in which the diagnosis would have been missed in this sample. Similarly one can read off the percentage of non-tuberculous cattle which would have had reactions below this limit and therefore by subtraction the number above this limit, i.e. the number which would have been incorrectly diagnosed as positive. The percentage of tuberculous cattle missed and the percentage of non-tuberculous cattle that would be made positive at different levels are shown in Table 35.

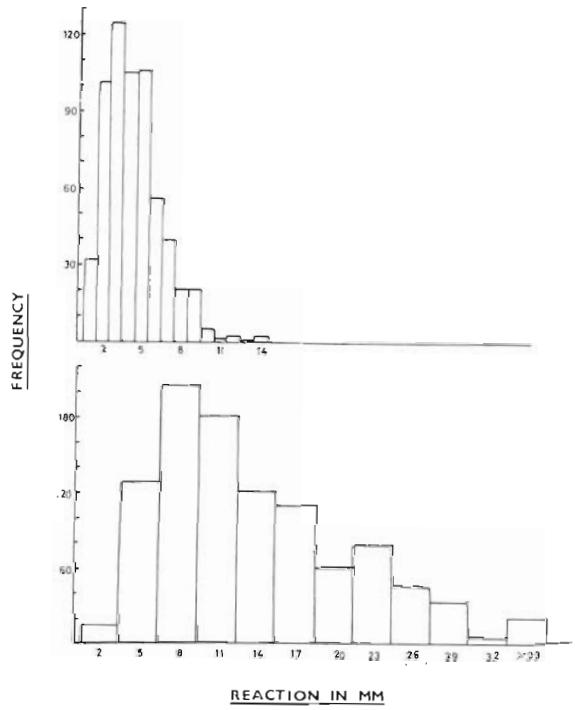

FIG. 13.—Frequency distribution of reactions to 7,000 TU of bovine PPD in 600 non-tuberculous (above) and 1,011 tuberculous cattle (below)

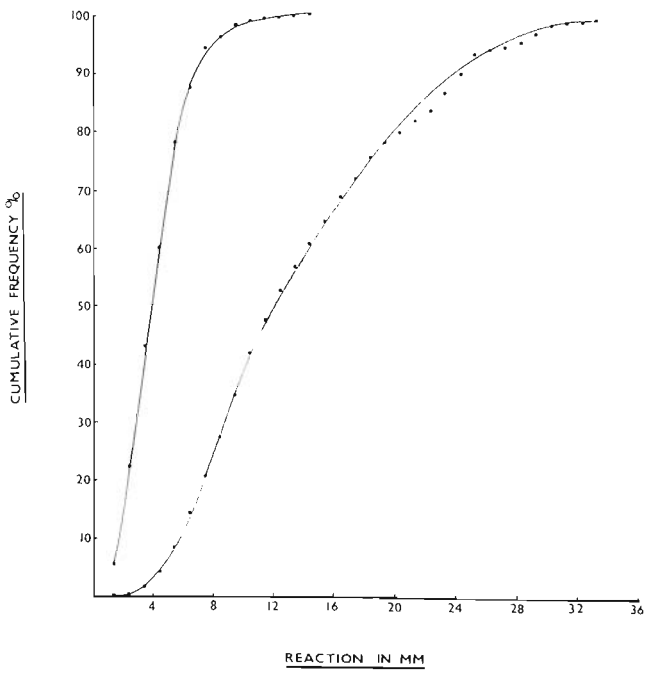

FIG. 14.—Less-than-cumulative frequency curves of reactions to 7,000 TU of bovine PPD in non-tuberculous (left) and tuberculous cattle (right)

TABLE 35.—*Diagnostic accuracy of tuberculin test using* 7,000 *TU of bovine PPD alone*

Reaction to bovine tuberculin	Percentage of tuberculous animals with reactions below the limit given in the first column	Percentage of non-specific reactors with reactions above the limit given in the first column
2·0 mm	0·05	87·0
3·0 mm	0·7	67·0
4·0 mm	2·9	47·0
5·0 mm	6·5	28·0
6·0 mm	11·5	16·0
7·0 mm	18·0	8·0
8·0 mm	24·5	4·5

The mean reaction to bovine tuberculin in the tuberculous cattle tested by the comparative test was 14·3 mm and in those tested with bovine tuberculin alone 13·4 mm. The cumulative frequencies of the reactions in the two groups are given in Appendix Table 3. By applying the Kolmogorov-Smirnov test to the two samples it was found that there was no significant difference in the distributions of the reactions in the two samples (see Appendix Table 3).

The distributions of reactions in the two herds Wo. and Cull. are shown in Fig. 15 and 16. In both herds there were more small reactions of 1 mm to about 6 mm than would be expected in a typically tuberculous population. The distribution of the reactions in animals classed as tuberculous after considering their subsequent records, more closely approximated the distribution seen in tuberculous herds (Fig. 13). The distribution of reactions in the animals judged to be non-tuberculous was similar to the distribution of reactions in non-specific reactor cattle (Fig. 13).

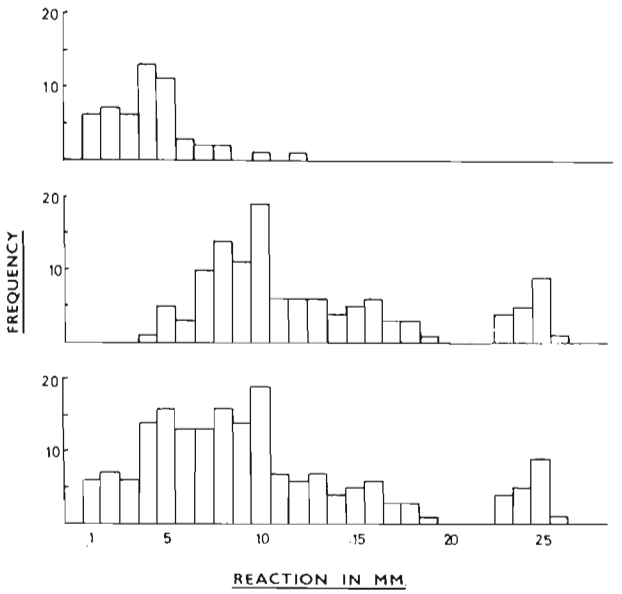

FIG. 15.—Frequency distribution of reactions in herd Wo. to 7,000 TU of bovine PPD—non-specific reactors (above), tuberculous animals (middle) and all reactors (below)

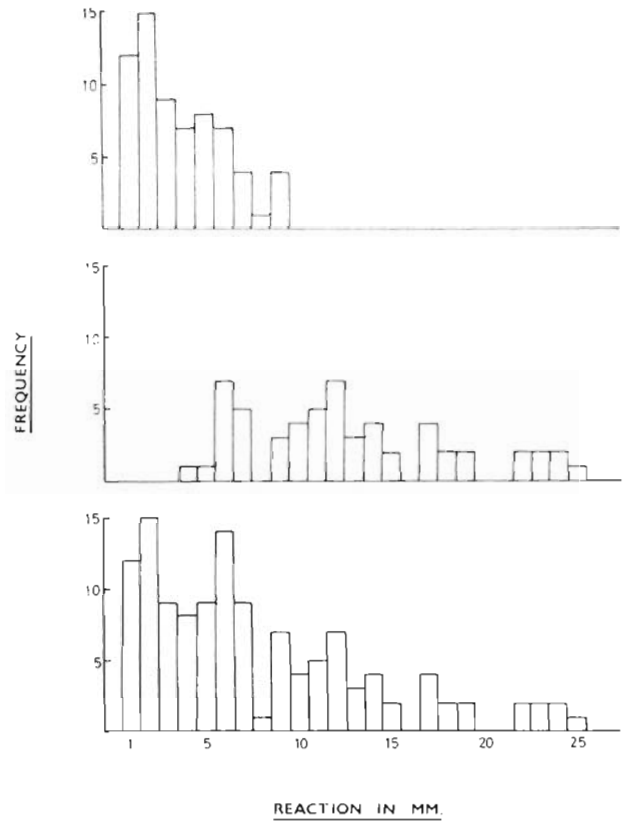

FIG. 16.—Frequency distribution of reactions in herd Cull. to 7,000 TU of bovine PPD—non-specific reactors (above) tuberculous animals (middle) and all reactors (below)

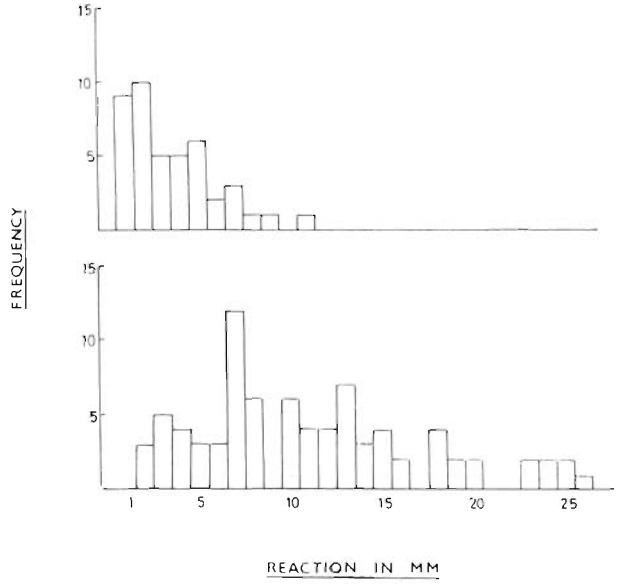

FIG. 17.—Frequency distributions of reactions to 7,000 TU of bovine PPD in herd Cull. in animals 1 to 5 years old (above) and over 5 years old (below)

In herd Cull. the distribution of the reactions in the animals one to five years old and in the animals over five years old (Fig. 17) clearly demonstrated that the distribution of reactions in the young animals closely resembled the distribution of the reactions in non-specific reactor cattle and the distribution of reactions in the old cattle resembled the distribution of reactions in tuberculous cattle (Fig. 13).

(iv) *Discussion*

A criticism that can be levelled at the method of investigation is that the procedure used to classify animals as tuberculous or non-tuberculous was based on the tuberculin test. The test was, however, used on a herd basis, and only animals from herds in which there was bacteriological proof of infection with *M. bovis* were included. Furthermore the animals were judged to be tuberculous on the results of a number of consecutive tests, which is a far more accurate method of diagnosis than a single test. The samples were large and a few wrongly diagnosed cases should not affect the final results to any great extent. To further check interpretation formulae post-mortem examination of large numbers of reactors should be done at the beginning of an eradication campaign.

The frequency distribution of the reactions in tuberculous cattle was found to be distinctly positively skewed. In tuberculous humans an approximately normal distribution is found (Edwards & Smith, 1965). It is possible that the positive skewness is a result of the high dose of tuberculin used in testing cattle in comparison with the dose used in man. In a previous study the distribution of reactions in tuberculous cattle appeared to be bimodal (Worthington & Kleeberg, 1965). The data presented in this work included the reactions in the 500 tuberculous cattle used in the previous study together with the reactions in a further 511 tuberculous cattle. In this larger sample there was only a very slight tendency to a bimodal distribution. It was mentioned previously that the distribution of reactions in the very high range might not be strictly accurate, b cause many very large reactions were merely recorded as 30 mm or 30 + mm, this being the limit of the calliper in general use. As the normal skinfold is usually around 6 mm, an excessive number of reactions would be recorded in the range 23 to 25 mm and give a false impression of a bimodal distribution. It now appears that this must have been the case and that the distribution is not bimodal.

In the non-tuberculous cattle the distribution was also positively skewed. Here, however, it is possible that we are not dealing with the true frequency distribution. When the comparative test is used and there are definite reactions to avian tuberculin, the operator is inclined to measure reactions to both avian and bovine PPD with the result that many very small reactions to bovine PPD are read which are generally ignored when bovine tuberculin is used alone. The distribution was different when the comparative test was used (see Fig. 19) with most of the reactions recorded in the 1 mm class and progressively fewer in each subsequent class. A similar distribution was found by Herbert & Paterson (1955) and Paterson & Herbert (1957) with the comparative test. In any consideration of the tuberculin test with bovine tuberculin only it is, however, preferable to base the conclusions on the type of distribution found in practice with this test rather than on a theoretically more correct distribution which will not be attained unless the comparative test is used.

The important point is that the typical frequency distributions of reactions in tuberculous cattle and non-specific reactors can be recognized. This knowledge can help in deciding whether a herd is tuberculous or not. Reduced to simple terms, the reactions in a typically tuberculous herd would be expected to be on an average around 14 mm with also some very high reactions. In a group of cattle with non-specific sensitivity the mean reaction is generally low, but a few reactions which would normally be classed as positive may be encountered. The reactions in tuberculous cattle are usually painful, diffusely oedematous, and hot and in non-specifically sensitized cattle they are circumscribed and hard, but many exceptions will be found to this rule.

The tuberculin test is never completely accurate. The diagnostic accuracy of the test may be improved if interpretation is done very strictly in tuberculous herds and more leniently in herds free from tuberculosis. This is especially true when dealing with large herds, as is often the case in South Africa. To decide whether a herd is tuberculous or not one must consider the outcome of previous autopsies, abattoir examinations of carcasses from the herd, results of routine examinations of bulk milk samples, clinical examinations in the herd, type of herd and the distribution of reactions in the herd. In addition test slaughter of one or more reactors may be possible. Kleeberg (1960a) suggested that the formula shown in Table 36 should be used in herds in which tuberculosis has been established, and the formula shown in Table 37 in herds in which bovine tuberculosis has not yet been established.

TABLE 36.—*Interpretation formula recommended by Kleeberg* (1960*a*) *for tuberculous herds*

Skin-fold increase	Pain, consistence of swelling, other changes	Interpretation of reaction
Below 2 mm.....	Not tender, hard, circumscribed, no other changes.....	Negative
2–4 mm.....	Almost painless, circumscribed or slightly diffuse, no other changes	Suspicious
3–4 mm.....	Almost painless to painful, diffuse, oedematous, with or without other changes	Positive
4 mm or more.....	Painless or tender, circumscribed or diffuse, with or without other changes	Positive

TABLE 37.—*Interpretation formula recommended by Kleeberg* (1960*a*) *for herds in which tuberculosis has not been established*

Skin-fold increase	Pain, consistence of swelling, other changes	Interpretation of reaction
0–3 mm.....	Hard, circumscribed, painless, no other changes.....	Negative
3–6 mm.....	Hard or slightly oedematous, circumscribed or slightly diffuse, painless or almost painless, no other changes	Suspicious
6 mm or more.....	Hard or oedematous, circumscribed or diffuse, tender or painless, without or with other changes	Positive

In the sample of tuberculous cattle 2·9 per cent of the animals had reactions of less than 4·0 mm and only 0·05 per cent of the animals had reactions of less than 2·0 mm as read from the less-than-cumulative frequency curve (see Table 35). In actual fact there were only 25 reactions (2·5 per cent) of less than 4·0 mm. At the two subsequent retests on these animals it was seen that the sensitivity had increased in 10 cases so that reactions of between 7·0 mm and 24·0 mm were recorded, and in 14 cases the reactions had increased to between 4·0 mm and 6·5 mm. In one case the reaction remained below 4·0 mm, but the animal was considered tuberculous, because the sensitivity to bovine tuberculin was always distinctly greater than to avian tuberculin. This increase in sensitivity was seen despite the fact that isoniazid treatment generally causes a reduction in skin sensitivity. The majority of reactions of less than 4·0 mm therefore appears to occur in animals which have been recently infected and which have not yet developed a high degree of skin sensitivity. On retesting it should therefore be possible to identify virtually all tuberculous animals with the present South African interpretation standards.

In the case of the non-tuberculous animals reacting to bovine tuberculin 47 per cent had reactions of more than 4·0 mm and 11·5 per cent of more than 6·0 mm. If the interpretation formula suggested by Kleeberg (1960a) for herds in which tuberculosis has not been proved had been used in these herds, 11·5 per cent of the non-specific reactors would have been made positive. The non-specific reactors constituted

8·9 per cent of the cattle tested so that approximaetly 1 per cent of all the negative cattle would have been classed as positive. The non-specific reactor rate would, however, almost certainly be lower than 8·9 per cent in herds selected at random. The operator must also use his own judgement in making a diagnosis based on the tuberculin test. After considering all the relevant facts he should be allowed to class a positive reaction as suspicious or *vice versa* and a suspicious reaction as negative or *vice versa*, but not a positive reaction as negative or a negative reaction as positive. In problem herds a number of positive reactors can be made suspicious and the animals subjected to a retest, preferably with the comparative test. The interpretation standard suggested by Kleeberg (1960a) should therefore prove satisfactory and the test when used with discretion and judgement should be accurate, but a small percentage of non-tuberculous animals will be classed as positive.

In humans the reaction to 10 TU of human tuberculin is significantly lower when given simultaneously with 100 TU of human tuberculin than when used alone (Rosenthal & Libby, 1960). It has, therefore, been suggested that the simultaneous injection of avian tuberculin in cattle may have a depressive effect on the reaction to bovine tuberculin. In this series the animals in which the comparative test was used showed slightly higher mean reactions to bovine tuberculin than those tested with bovine tuberculin alone (14·3 mm to 13·4 mm). The difference in the distributions of the reactions in the two groups was not statistically significant. There is therefore no reason to believe that the simultaneous injection of 2,500 TU of avian tuberculin will significantly affect the sensitivity to 7,000 TU of bovine tuberculin.

A possible complication in the interpretation of reactions is the occurrence of bovine tuberculosis and non-specific sensitization in the same herd. This problem was studied in two herds. The distribution of all the reactions occurring in these herds was in fact a distribution of reactions in a population of non-specific reactors superimposed on a distribution of reactions in a population of tuberculous cattle (see Fig. 15 and 16). When the herd is large enough to give a reasonable idea of the frequency distribution of reactions in the herd, the two factors can be recognized. Interpretation of reactions can then be made cautiously and recourse made to retesting with the comparative test.

In both herds it was striking that non-specific reactions were more common in the young stock, and bovine tuberculosis more common in the older cows. In herd Wo. there was no method of accurately determining the age of the animals, but in the herd Cull. the animals were all numbered according to their date of birth and it was therefore simple to study the frequency distribution of the reactions in various age groups (Fig. 17). Quite obviously the majority of the young animals reacting to tuberculin were non-specific reactors and the majority of the old animals reacting were tuberculous. This position is caused by practical farm management methods. The heifers are run on the veld and have little close contact with tuberculous cows. During this time they may come into contact with other mycobacteria, probably most commonly of the avian group. When they have calved they are brought into close contact with tuberculous animals in the milking stables and are often infected with bovine tuberculosis.

CHAPTER 8

THE AVIAN-BOVINE COMPARATIVE TEST: STUDIES IN TUBERCULOUS AND PROBLEM HERDS

(i) *Introduction*

The comparative test with mammalian and avian tuberculin used simultaneously in the same animal has been applied with success in many countries (Azevedo, 1960; Gasse, 1960; Goor, 1960; Meyn, 1960; Nielsen & Plum, 1960; Stableforth, 1960; Van Waveren, 1960), but this test has not been popular with South African veterinarians. Reasons generally given are that there are no logical grounds for the use of the test as avian tuberculosis is rare in South African poultry and Johne's disease almost non-existent. The avian tuberculin prepared at Onderstepoort from the strain D4 was also blamed for causing too large reactions in tuberculous cattle and for not covering a sufficiently wide range of non-specific sensitization.

In a previous chapter it was shown that the most common cause of sensitization in non-tuberculous herds in South Africa is probably *M. avium* or closely related avian-like strains. These findings indicated that the avian-bovine comparative test could probably be used to advantage in this country.

The locally isolated strain, 20485, is now being used instead of D4 for the production of avian tuberculin. Our bovine tuberculin is produced from the strain AN5 which is also the strain used in Holland. Onderstepoort avian tuberculin is issued in the same strength (25,000 TU per ml) as avian tuberculin used in other countries, but our bovine tuberculin is issued at a strength of 70,000 TU per ml while the European common market countries use mammalian tuberculin at a strength of 50,000 TU per ml and England, the United States, Australia and New Zealand use tuberculin containing 100,000 TU per ml. Our interpretation standards for tuberculin testing have been derived arbitrarily as were the standards in other countries. In view of our different tuberculins, different animals, husbandry methods and climatic conditions it was necessary to thoroughly investigate the comparative test with Onderstepoort tuberculins under local conditions. Suitable interpretation standards could then be derived from this study.

(ii) *Materials and methods*

The method of testing was exactly the same as described in the previous chapter. The comparative test was used in all cases.

Tuberculous cattle: The sample of tuberculous cattle consisted of the 423 cattle tested by the comparative test which were included in the investigations described in the previous chapter plus another small group of 20 tuberculous cattle in an isoniazid treated herd. The cattle were all from herds within 100 miles of Onderstepoort, 93 per cent were Friesians, 6 per cent Jerseys and 1 per cent Brown Swiss.

Non-tuberculous cattle: In order to obtain a large sample of non-specific reactors, herds were selected in which this type of reaction occurred more frequently than usual. Only cattle from herds considered to be free from tuberculosis were used. A herd was considered to be free from tuberculosis when a consideration of its

history indicated that there was no infection, when reactions were generally small, hard and circumscribed, when reacting animals showed a tendency to convert to negative on retesting, and when reactions to avian tuberculin were generally larger than to bovine tuberculin.

The animals used came mainly from three sources:—

(1) Herds in which isoniazid treatment had been used. In this case only herds in which the spread of the disease had been completely arrested were used. Animals which were previously positive reactors were not included. Records of six-monthly tests of each animal for a period of 3 to 4 years were available. Only animals from the clean part of the herd in which no new cases of tuberculosis had been detected during this time were included.

(2) To increase the number in the sample we included cattle which had been tested by members of the Veterinary Field Services. Only herds judged to be free from tuberculosis by the veterinarian performing the test, and where a study of the available records confirmed this opinion were included.

(3) Herds which were specifically investigated by the author because of their history of containing non-specific reactors.

The reactions in 940 non-tuberculous animals from some 3,500 cattle in 22 herds were included in this study. The sample again included mainly Friesian cattle, mainly from the Transvaal Highveld region. Some herds widely distributed over the whole country and of varying breeds were, however, also included.

The reactions (skinfold increase) to bovine tuberculin, to avian tuberculin and the difference between the reactions to bovine and avian tuberculin were recorded. Frequency distribution tables of reactions to the two tuberculins and the differences between the reactions to the two tuberculins were constructed for tuberculous cattle and non-specific reactor cattle. According to the range of the reactions class intervals of 1 or 3 mm were chosen and histograms drawn. The mean reactions were calculated for each distribution from the grouped data by standard statistical methods (Van Heerden, 1964). Less-than-cumulative frequencies were calculated for the distributions of the differences between reactions to bovine and avian tuberculin for the tuberculous and the non-tuberculous cattle. Cumulative frequencies were then expressed as percentages and plotted on a large scale on graph paper as less-than-cumulative frequency curves with class intervals of 1 mm. Similarly less-than-cumulative frequency curves were constructed for all the reactions to bovine tuberculin in the tuberculous cattle and for the reactions of 1 mm or more to bovine tuberculin in the non-tuberculous cattle.

(iii) *Results*

In the 1,700 cattle tested in tuberculous herds, 443 or 26·1 per cent were considered to be tuberculous. The mean reaction to the bovine tuberculin was 14·0 mm and the mean reaction to the avian tuberculin was 4·5 mm. The distribution of the reactions to the two tuberculins are shown in histogram form in Fig. 18. Details of frequency distributions in individual herds are given in Appendix Tables 4 and 5. Both distributions show a marked positive skewness precluding the use of normal distribution theory for further analysis of the data. It is obvious that the reactions to bovine tuberculin were generally much greater than the reactions to avian tuberculin.

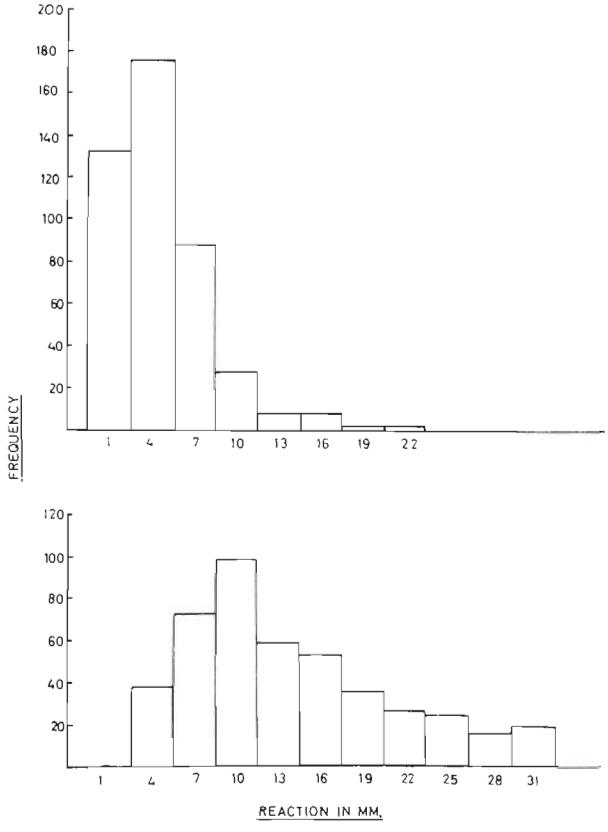

FIG. 18.—Frequency distribution of reactions to avian (above) and bovine (below) tuberculin in 443 tuberculous cattle

In the 3,500 cattle tested in non-infected herds, 940 or 26·9 per cent reacted to one or both of the tuberculins. Details of frequency distributions in individual herds are given in Appendix Tables 6 and 7. The mean reaction to bovine tuberculin in the reactor cattle was 2·3 mm and the mean reaction to avian tuberculin was 3·7 mm. In contrast to the position in the tuberculous herds it can be seen in Fig. 19 that the reactions to bovine tuberculin were generally smaller than the reactions to avian tuberculin. Both distributions again show a definite positive skewness. The range of the reactions to both avian and bovine tuberculin was much smaller in the non-tuberculous cattle than in the tuberculous animals.

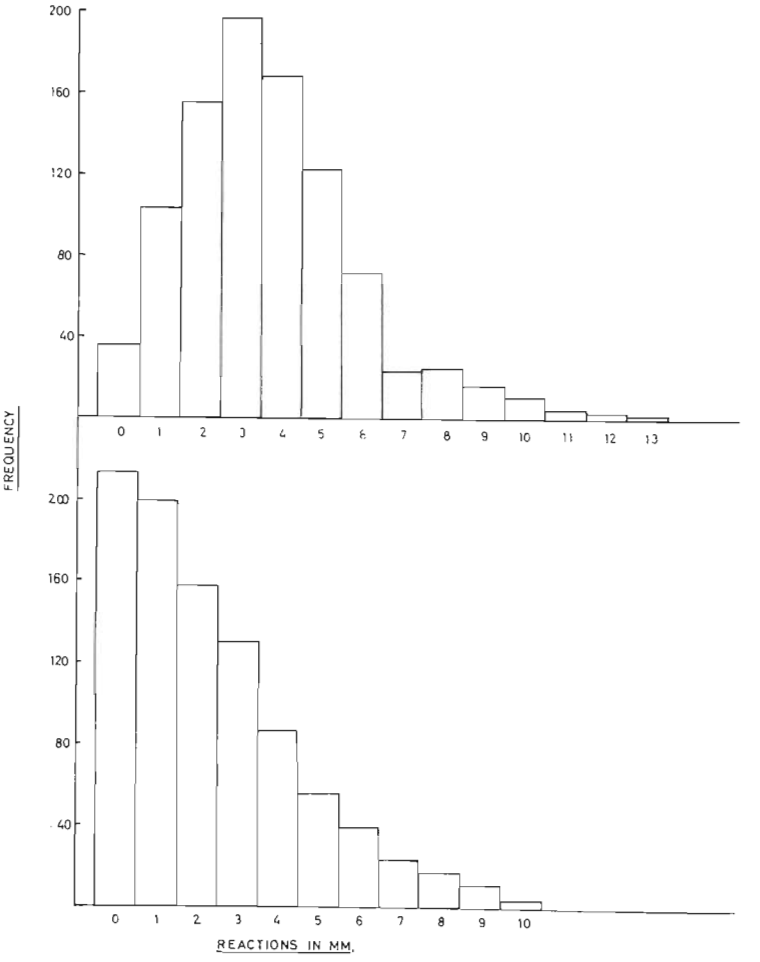

FIG. 19.—Frequency distribution of reactions to avian (above) and bovine (below) tuberculin in 940 non-specific reactors

The frequency distribution of differences between the reactions to bovine and avian tuberculin (bovine reaction—avian reaction) in the tuberculous cattle and in the non-tuberculous reactor cattle are given in Appendix Tables 8 and 9; less-than-cumulative frequencies expressed as percentages are also given in these tables. The distributions are shown in histogram form in Fig. 20. The reactions to bovine tuberculin were greater than the reactions to avian tuberculin in almost all the tuberculous animals. Alternatively, most of the non-tuberculous animals were more sensitive to avian tuberculin than to bovine tuberculin. The mean of the difference between the reactions was 9·66 mm in the tuberculous cattle and — 1·22 mm in the non-tuberculous cattle. The distribution in the case of non-specific reactors appeared to be approximately normal, but in the tuberculous animals there was a marked positive skewness. The less-than-cumulative frequency curves for the two distributions are shown in Fig. 21. The percentage of tuberculous and non-tuberculous cattle in the samples in which the difference between the bovine reaction and the avian reaction was less than any given limit was read off from the graph as in the previous chapter. These results for various differences in the two reactions are given in Table 38.

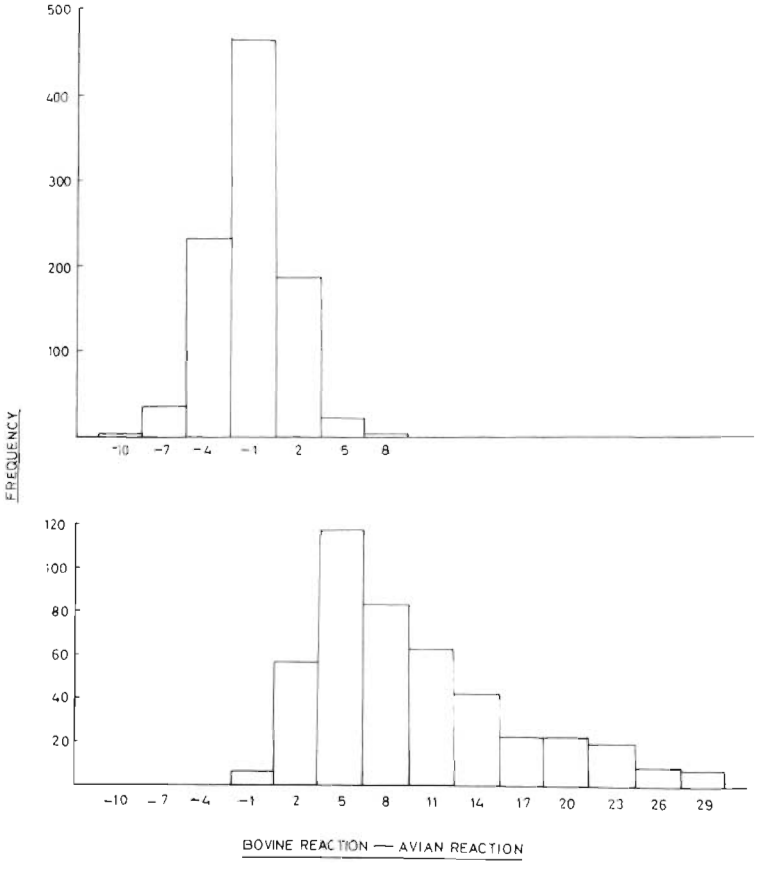

FIG. 20.—Frequency distribution of the difference bovine reaction–avian reaction in 940 non-specific reactors (above) and 443 tuberculous cattle (below)

TABLE 38.—*Diagnostic accuracy of the comparative test*

Difference bovine reaction–avian reaction	Percentage tuberculous animals in which the difference was less than the limit given in column 1	Percentage non-specific reactors in which the difference was greater than the limit given in column 1
−1·0 mm	0·0	41·5
0 mm	0·7	27·0
1 mm	2·5	14·0
2 mm	6·0	8·0
3 mm	12·0	4·0
4 mm	19·0	1·5
5 mm	28·0	0·5

Less-than-cumulative frequency curves of all the reactions to bovine tuberculin in the tuberculous and the non-specific reactor cattle are shown in Fig. 22. From these curves the percentage of tuberculous cattle that would be classed as negative and the percentage of non-specific reactors that would be classed as positive at any level were also determined. These figures are given in Table 39.

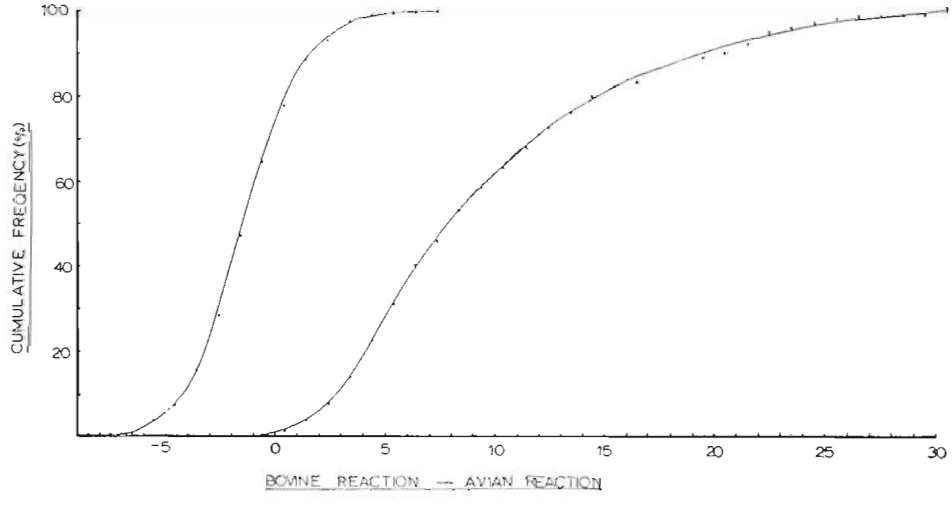

FIG. 21.—Less-than-cumulative frequency curves of difference, bovine reaction–avian reaction, in non-specific reactors (left) and tuberculous cattle (right)

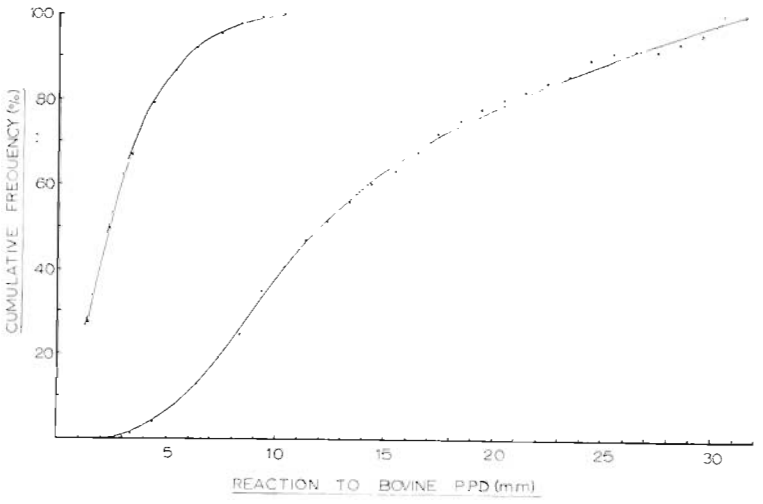

FIG. 22.—Less-than-cumulative frequency curves of reactions to bovine tuberculin in non-specific reactors (left) and tuberculous cattle (right)

TABLE 39.—*Diagnostic accuracy of the tuberculin test using bovine tuberculin alone*

Reaction to bovine tuberculin	Percentage tuberculous animals with reactions lower than limit given in column 1	Percentage non-specific reactors with reactions greater than limit given in column 1
2 mm	0	57·5
3 mm	1·0	39·5
4 mm	2·5	25·0
5 mm	6·0	16·0
6 mm	11·0	10·0
7 mm	17·5	5·5
8 mm	22·5	3·0

(iv) *Discussion*

A big percentage of non-specific reactors in South Africa was found to be more sensitive to avian tuberculin than to other mycobacterial sensitins (see Chapter on field investigations with PPD sensitins in problem herds). The frequency distributions of the reactions are, therefore, similar to what was expected with the tuberculous cattle, being markedly more sensitive to bovine PPD and the non-tuberculous cattle more sensitive to avian PPD. What is, however, of great importance is to derive optimal interpretation standards for the comparative test with Onderstepoort PPD's in naturally sensitized South African cattle. An ideal test would detect 100 per cent of tuberculous cattle and class 100 per cent of non-specific reactors as negative. This ideal cannot be achieved at present. For epidemiological reasons

it is preferable to have an interpretation formula erring slightly to the strict side. In practice a herd is never declared negative as a result of a single test, three negative tests being required in the herd before it is granted accreditation. Theoretically, if the first test is 95 per cent accurate, 5 per cent of tuberculous animals will be missed at the first test; 95 per cent of these should be detected at the next test so that only 0·75 per cent will be undetected after two tests and 0·01 per cent after three tests. A triple test which detects 95 per cent of infected animals at each test is, therefore, a very accurate test. A method which still further increases the accuracy of the tuberculin test is the classification of herds as tuberculous and non-tuberculous and very strict interpretation in tuberculous herds and more lenient interpretation in non-tuberculous herds. This method is used for the interpretation of comparative tests in England (Ritchie, 1953) and for the interpretation of tests with bovine tuberculin only in South Africa (Kleeberg, 1960a). This procedure should be adopted when laying down interpretation standards for the comparative test.

After careful scrutiny of the data given in Table 38, the following formula was considered to be the most satisfactory:—

Tuberculous herds

Avian reaction equal to or larger than bovine reaction—negative for TB.

Bovine reaction up to 2 mm larger than avian reaction—suspicious for TB.

Bovine reaction more than 2 mm larger than avian reaction—positive for TB.

The reaction to bovine tuberculin must, however, be 4 mm or greater before an animal can be declared positive.

Non-tuberculous herds

Bovine reaction up to 2 mm larger than avian reaction—negative for TB.

Bovine reaction 2–4 mm larger than avian reaction—suspicious for TB.

Bovine reaction more than 4 mm larger than avian reaction—positive for TB.

It can be seen from Table 38 that, if this key had been used, 94 per cent of tuberculous animals in our sample would have been classed as positive and 99 per cent as positive and suspicious. If the second formula had been used in the non-tuberculous cattle only, 1·5 per cent of non-specific reactor cattle would have been classed as positive. Furthermore the operator should be allowed to use his discretion in classing positive reactors as suspicious or *vice versa*, so that when the whole herd history and other relevant factors are considered, the accuracy should be still further increased. In this respect the type of reaction, i.e. whether oedematous, painful, hot and diffuse or whether hard and circumscribed should be particularly considered. A problem in using the comparative test is the classification of reactions in animals highly sensitive to both avian and bovine tuberculin. These cattle might have been sensitized by *M. avium* or another species of *Mycobacterium* and later infected with bovine tuberculosis. These cases should, therefore, be regarded as highly suspicious in tuberculous herds.

The principal finding of this study is that the comparative test is a more accurate diagnostic method than a test with bovine tuberculin alone. It was shown in the previous chapter that there was no significant difference in the distribution of reactions to bovine tuberculin in tuberculous cattle tested with bovine tuberculin alone or by the comparative test. Whether there is a significant difference in non-specific reactors is not known. It can, however, be seen from the results given in Tables 38 and 39 that the comparative test is able to differentiate more accurately between tuberculous cattle and non-specific reactors than a test with bovine tuberculin alone. In the comparative test the most definitive limit for interpretation is a 2 mm difference between reactions to bovine and to avian tuberculin. In this case 6 per cent of tuberculous cattle are missed and 8 per cent of non-tuberculous cattle are declared positive. With bovine tuberculin alone the most definitive limit is a reaction of 6 mm. In this case 11 per cent of the tuberculous cattle would have been missed and 10 per cent of the non-tuberculous cattle declared positive. At the moment a limit of 6 mm is used in non-tuberculous herds and 4 mm in tuberculous herds (Kleeberg 1960a). The 4 mm limit would have missed only 2·5 per cent of tuberculous cattle in this sample but would have made 25 per cent of non-specific reactors positive.

In South Africa the bulk of tuberculin testing is done with bovine tuberculin only. This method has given satisfactory results, but when large numbers of non-specific reactors are encountered, confusion arises. In known problem herds and on retesting suspicious reactors the comparative test should always be used. The interpretation of tests as described in this article is based to a large extent on a consideration of the herd history and test results. In small herds and individual animals the operator will have less information available; in this case the comparative test is again superior. In general, when the comparative test is used, non-specific reactors can be more readily recognized and, therefore retesting, test slaughter and unnecessary slaughter can often be avoided. Great economic benefit could, therefore, be derived from a more general use of the comparative test.

CHAPTER 9

SUMMARY

A review of the literature on the history of the tuberculin test and causes of non-specific reactions in cattle is given. The economic importance of the non-specific reactor problem in South Africa is discussed.

The cultural and biochemical characteristics and in some instances the virulence for laboratory animals of the 42 strains used in the investigation are given. The strains used were typical of the species they represented in these characteristics, except for the scotochromogen Hg 3.

Details are given of the preparation of PPD sensitins from one strain of *M. bovis*, three strains of *M. kansasii*, two strains of *M. fortuitum*, two strains of *M. phlei*, two strains of *M. smegmatis*, six *M. avium* and avian-like strains, two strains of scotochromogenic mycobacteria, and six strains of unclassified mycobacteria probably belonging to a single species.

The allergenic characteristics of the 42 strains of Mycobacteria and the twenty four sensitins were studied in guinea pigs. Specificity differences of the sensitins and sensitivity profiles of the strains are given. The sensitivity caused by various species of mycobacteria was found to be species specific, although varying degrees of cross sensitivity do occur between different species. Guinea pigs and cattle sensitized by all the species investigated except *M. bovis* were more sensitive to avian PPD than to bovine PPD.

It was shown that the specificity of the sensitivity caused by different species of mycobacteria is similar in calves and guinea pigs.

Multiple comparative tests in naturally sensitized non-specific reactor cattle showed most of these animals to be more sensitive to avian tuberculin than to any of the other sensitins used in the investigation. The sensitivity profiles in these animals were similar to sensitivity profiles in guinea pigs sensitized by *M. avium*. This indicates that the most common cause of non-specific reactors in these herds was *M. avium* or avian-like mycobacteria.

Field trials with Onderstepoort PPD tuberculins showed the comparative test to be more accurate than a test with bovine tuberculin alone in differentiating non-specific reactors and tuberculous animals. The simulataneous injection of avian and bovine tuberculin did not influence the sensitivity to the latter. Results are given of investigations in two herds where both non-specific reactors and tuberculous animals occurred. Non-specific reactions were more common in young and tuberculosis more common in older cattle.

The most suitable interpretation standards for the comparative test and the test with bovine tuberculin alone were investigated. Suggested interpretation keys are given.

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APPENDIX TABLE 1.—*Frequency distribution of reactions to* 7,000 *TU of bovine PPD in tuberculous cattle in* 16 *herds*

Skinfold increase	Herds															Total		Cumulative frequency Per cent
	Ve.*	Cul.*	Mic.*	Gu.*	C. Gr.*	We.*	Scha.*	C. Gr.	Sch.	Ho.	Ja.	Br.	G. Gr.	Wo.	Wa.	Wy.			
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		0
2	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	15	0·10
3	2	0	1	0	0	0	2	1	2	1	3	0	2	0	0	0	14		1·48
4	2	1	0	2	0	2	4	1	3	1	5	2	2	1	0	0	26		4·06
5	5	1	0	2	1	5	3	1	7	2	2	1	9	5	0	0	44	129	8·41
6	7	7	0	2	0	1	3	2	6	8	6	2	8	3	3	1	59		14·24
7	6	5	1	3	3	3	4	2	8	8	2	1	7	10	2	1	66		20·77
8	10	0	0	5	1	2	4	3	5	6	1	1	9	14	2	2	65	206	27·20
9	13	3	3	8	4	3	7	3	2	3	4	4	3	12	1	2	75		34·62
10	8	4	2	3	4	2	6	1	6	4	5	3	5	19	4	2	74		41·94
11	10	5	3	4	1	2	2	1	6	3	2	1	5	6	2	2	55	181	47·38
12	3	7	2	3	0	0	4	6	4	5	0	0	7	6	3	2	52		52·52
13	5	3	2	5	1	0	4	2	4	3	1	0	3	6	2	1	43		56·78
14	7	4	1	3	1	0	1	1	1	3	3	0	7	4	2	3	41	122	60·83
15	3	2	1	3	1	1	2	2	4	3	4	0	5	5	0	2	38		64·59
16	9	0	1	1	2	0	6	1	4	3	0	1	6	6	0	1	41		68·64
17	9	4	0	4	1	0	1	0	1	2	1	0	5	3	0	2	33	110	71·90
18	9	2	0	2	1	0	0	3	0	1	3	0	8	3	1	3	36		75·47
19	8	2	0	1	1	0	0	1	0	3	2	0	6	1	0	1	26		78·04
20	7	0	0	2	1	0	0	3	1	1	1	0	1	0	0	1	18	61	79·82
21	7	0	0	1	0	0	0	3	1	0	1	0	5	0	0	0	17		81·50
22	6	2	0	1	0	0	0	1	0	1	1	0	5	0	0	0	17		83·19
23	2	2	0	2	1	0	1	8	0	1	5	1	3	4	0	0	30	79	86·15
24	8	2	1	2	1	0	0	3	0	2	2	0	5	5	0	1	32		89·31
25	5	1	0	2	2	0	0	5	0	2	8	1	1	9	0	0	36		92·88
26	0	0	0	0	0	0	2	0	0	1	3	0	0	1	0	0	7	46	93·57
27	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	3	3		93·86
28	6	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	8		94·66
29	9	0	0	0	0	0	0	1	0	0	0	0	2	0	0	0	12	34	95·85
30	11	0	0	0	0	0	0	0	0	1	0	0	2	0	0	0	14		97·23
31	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	2		97·43
32	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6	97·43
33	0	0	0	0	1	0	0	0	0	3	0	0	0	0	0	0	4		97·82
Over 33	0	0	0	5	0	0	2	0	1	3	0	0	11	0	0	0	22	22	100·00
TOTAL	178	57	18	68	23	21	58	58	65	75	65	19	133	123	22	28	1,011		

*Herds tested by comparative test

APPENDIX TABLE 2.—*Frequency distribution of reactions to* 7,000 *TU of bovine PPD in non-tuberculous cattle in* 9 *herds*

Skinfold increase	Herd									Total	Cumulative frequency per cent
	Nat. E.	Schm.	Sm.	I.E.	C.K.	Nel.	Rey.	Meu.	Hu.		
1	5	8	0	1	10	3	5	0	0	32	5·3
2	6	24	0	6	15	5	8	20	17	101	22·3
3	8	39	2	6	11	6	7	24	22	125	43·0
4	13	37	9	6	9	2	7	13	9	105	60·1
5	12	25	8	9	13	3	5	14	17	106	78·2
6	9	25	2	0	3	2	2	8	5	56	87·5
7	7	13	0	1	3	4	4	7	1	40	94·2
8	3	0	0	1	0	0	2	5	1	12	96·2
9	2	1	0	0	0	2	0	6	1	12	98·2
10	2	0	0	0	0	0	0	2	1	5	99·0
11	1	0	0	0	0	0	0	0	0	1	99·2
12	1	0	0	0	0	0	1	0	0	2	99·5
13	0	0	0	1	0	0	0	0	0	1	99·7
14	0	0	0	1	0	0	0	0	1	2	100·0
TOTAL	69	172	21	32	64	27	41	99	75	600	

APPENDIX TABLE 3.—*Cumulative frequencies of reactions to 7,000 TU of bovine PPD in cattle tested by the comparative test and with bovine PPD alone*

Class mark	Comparative test			Non-comparative test			Difference between proportions i.e. column 4 to column 7
	Frequency	Cumulative frequency	Cumulative frequency (proportion)	Frequency	Cumulative frequency	Cumulative frequency (proportion)	
1	0	0	0·0000	0	0	0·0000	0·0000
2	0	0	0·0000	1	1	0·0010	0·0017
3	5	5	0·0118	9	10	0·0170	0·0052
4	11	16	0·0378	15	25	0·0425	0·0047
5	17	33	0·0780	27	52	0·0884	0·0104
6	20	53	0·1253	39	91	0·1548	0·0295
7	25	78	0·1849	41	132	0·2245	0·0401
8	22	100	0·2364	43	175	0·2976	0·0612
9	41	141	0·3333	34	209	0·3554	0·0221
10	25	166	0·3924	49	258	0·4388	0·0464
11	27	193	0·4563	28	286	0·4864	0·0301
12	19	212	0·5012	33	319	0·5425	0·0413
13	20	232	0·5485	23	342	0·5816	0·0331
14	17	249	0·5889	24	366	0·6224	0·0335
15	13	262	0·6194	25	391	0·6650	0·0456
16	19	281	0·6643	22	413	0·7024	0·0381
17	19	300	0·7092	14	427	0·7262	0·0170
18	14	314	0·7423	22	449	0·7636	0·0213
19	12	326	0·7707	14	463	0·7874	0·0167
20	10	336	0·7943	8	471	0·8010	0·0067
21	8	344	0·8132	9	480	0·8163	0·0031
22	9	353	0·8345	8	488	0·8299	0·0046
23	8	361	0·8534	22	510	0·8673	0·0139
24	14	375	0·8865	18	528	0·8980	0·0095
25	10	385	0·9102	26	554	0·9422	0·0320
26	2	387	0·9149	5	559	0·9507	0·0358
27	0	387	0·9149	3	562	0·9558	0·0409
28	7	394	0·9314	1	563	0·9575	0·0261
29	9	403	0·9527	3	566	0·9626	0·0099
30	11	414	0·9787	3	569	0·9677	0·0110
31	1	415	0·9811	1	570	0·9694	0·0117
32	0	415	0·9811	0	570	0·9694	0·0117
33	1	416	0·9835	3	573	0·9745	0·0090
Over 33	7	423	0·1000	15	588	1·0000	1·0000

The largest difference between the cumulative frequencies was 0·0612 (class mark 8 mm)

Critical value of D in the Kolmogorov Smironov two sample test at the 10 per cent level of significance—

$$= 1{\cdot}22\sqrt{\frac{n1 + n2}{n1n2}}$$

$$= 1{\cdot}22\sqrt{\frac{423+588}{423\times 588}}$$

$$= 0{\cdot}0772$$

As 0·0612 is smaller than the critical value of D the difference is not significant at the 10 per cent level

APPENDIX TABLE 4.—*Frequency distribution of reactions to bovine tuberculin in* 443 *tuberculous cattle in eight infected herds*

Class mark	Herd								Total		Cumulative frequency per cent
	1	2	3	4	5	6	7	8			
0	0	0	0	0	0	0	0	0	0		0
1	0	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0		0
3	2	0	0	0	0	1	0	2	5		1·1
4	4	2	2	0	1	0	1	2	12	38	3·8
5	3	2	5	1	1	0	2	5	19		8·1
6	3	2	1	0	7	0	1	7	21		12·9
7	4	3	3	3	5	1	2	6	27	73	19·0
8	4	5	2	1	0	0	3	10	25		24·6
9	7	8	3	4	3	3	4	13	45		34·8
10	6	3	2	0	4	2	1	8	26	99	40·6
11	2	4	2	1	5	3	1	10	28		47·0
12	4	3	0	0	7	2	1	3	20		51·5
13	4	5	0	1	3	2	1	5	21	59	56·2
14	1	3	0	1	4	1	1	7	18		60·3
15	2	3	1	1	2	1	1	3	14		63·4
16	6	1	0	2	0	1	1	9	20	53	68·0
17	1	4	0	1	4	0	0	9	19		72·2
18	0	2	0	1	2	0	0	9	14		75·4
19	0	1	0	1	2	0	0	8	12	36	78·1
20	0	2	0	1	0	0	0	7	10		80·4
21	0	1	0	0	0	0	0	7	8		82·1
22	0	1	0	1	2	0	0	6	10	26	84·4
23	1	2	0	1	2	0	0	2	8		86·2
24	0	2	0	2	2	1	0	8	15		89·6
25	0	2	0	0	1	0	0	5	8	25	91·4
26	2	0	0	0	0	0	0	0	2		91·9
27	0	0	0	0	0	0	0	0	0		91·9
28	0	1	0	0	0	0	0	6	7	16	93·5
29	0	0	0	0	0	0	0	9	9		95·5
30	2	6	0	0	0	0	0	11	19		99·8
31	0	0	0	0	0	0	0	1	1	20	100·0
32	0	0	0	0	0	0	0	0	0		
TOTAL	58	68	21	23	57	18	20	178	443		

APPENDIX TABLE 5.—*Frequency distribution of reactions to avian tuberculin in* 443 *tuberculous cattle in eight infected herd*

Class mark	Herd								Total		Cumulative frequency per cent
	1	2	3	4	5	6	7	8			
0	2	4	5	—	4	2	1	25	43		9·7
1	4	3	5	2	7	—	1	15	37	133	18·1
2	8	10	2	2	7	1	3	20	53		30·0
3	15	6	5	4	7	1	7	26	71		46·1
4	14	8	4	2	7	4	4	22	65	176	60·7
5	8	10	—	3	4	1	2	12	40		69·8
6	4	6	—	1	3	3	1	24	42		79·2
7	2	6	—	6	7	3	1	8	33	88	86·7
8	—	3	—	3	2	1	—	4	13		89·6
9	—	4	—	—	1	1	—	7	13		92·6
10	—	1	—	—	2	—	—	7	10	28	94·8
11	—	—	—	—	3	—	—	2	5		95·9
12	—	1	—	—	1	1	—	1	4		96·8
13	—	1	—	—	—	—	—	2	3	8	97·5
14	—	—	—	—	—	—	—	1	1		97·7
15	—	1	—	—	—	—	—	2	3		98·4
16	—	3	—	—	—	—	—	—	3	8	99·1
17	1	—	—	—	1	—	—	—	2		99·6
18	—	—	—	—	—	—	—	—	0		99·6
19	—	—	—	—	—	—	—	—	0	1	99·6
20	—	—	—	—	1	—	—	—	1		99·8
21	—	—	—	—	—	—	—	—	0		99·8
22	—	1	—	—	—	—	—	—	1	1	100·0
23	—	—	—	—	—	—	—	—	0		100·0
TOTAL	58	68	21	23	57	18	20	178	443		

APPENDIX TABLE 6.—*Frequency distribution of reactions to bovine tuberculin in* 940 *non-specific reactors in* 22 *non-infected herds*

Class mark	Herd																					Total	Cumulative frequency per cent*
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	
0	22	4	1	13	58	4	5	—	4	35	13	7	6	4	—	7	7	4	10	7	—	4	215	
1	13	2	3	3	41	1	5	1	10	43	19	12	17	3	2	6	2	1	10	1	2	3	200	27·5
2	7	9	6	2	19	—	2	1	14	32	17	9	8	4	2	1	1	8	8	3	3	2	158	49·3
3	4	6	5	3	23	4	4	—	9	21	15	1	5	3	1	8	2	1	7	4	2	2	130	67·3
4	3	1	7	4	8	2	2	2	12	9	17	3	3	4	1	2	1	—	3	2	—	1	87	79·3
5	1	3	6	2	7	—	1	—	7	7	9	1	3	—	3	1	—	2	—	3	—	—	56	87·0
6	4	5	4	1	4	3	—	—	4	1	4	—	3	1	2	1	—	—	—	2	—	—	39	92·4
7	3	1	1	—	2	3	—	1	1	3	6	1	1	—	—	—	—	—	—	—	—	—	23	95·6
8	—	1	1	4	1	3	—	—	1	1	4	—	—	—	—	1	—	—	—	—	—	—	17	97·9
9	—	—	—	—	2	1	—	1	—	—	4	—	1	—	—	—	—	1	—	1	—	—	11	99·5
10	—	—	—	—	2	—	—	—	—	—	2	—	—	—	—	—	—	—	—	—	—	—	4	100·0
TOTAL	57	32	34	32	167	21	19	6	62	152	110	34	47	19	11	27	13	17	38	23	7	12	940	

*Cumulative frequency of reactions of 1 mm or more

APPENDIX TABLE 7.—*Frequency distribution of reactions to avian tuberculin in* 940 *non-specific reactors in* 22 *non-infected herds*

Class mark	Herd																					Total	Cumulative frequency per cent
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	
0	2	2	1	2	—	—	—	—	7	2	5	1	1	1	—	3	2	—	3	—	2	2	36	3·8
1	9	3	1	2	8	1	2	—	8	11	15	5	3	1	1	8	6	7	4	2	1	6	104	14·9
2	14	8	5	4	18	—	5	—	9	17	18	12	9	5	3	6	3	2	12	4	—	2	156	31·5
3	7	5	8	4	38	3	5	3	11	32	22	5	17	3	—	8	2	7	8	6	2	2	198	52·6
4	9	4	8	9	34	3	3	—	15	35	20	3	8	2	3	2	—	—	6	4	1	—	169	70·5
5	7	5	5	6	24	3	2	1	7	25	14	7	4	5	3	—	—	—	1	3	1	—	123	80·6
6	6	3	4	—	17	4	—	—	3	17	6	1	3	2	1	—	—	—	3	2	—	—	72	91·3
7	0	1	—	2	12	2	1	1	1	2	—	—	1	—	—	—	—	—	—	—	—	—	23	93·7
8	3	—	1	1	7	3	—	—	1	4	2	—	—	—	—	—	—	1	1	1	—	—	25	96·3
9	—	1	1	1	4	—	1	—	—	3	4	—	—	—	—	—	—	—	—	1	—	—	16	98·1
10	—	—	—	1	2	—	—	—	—	3	3	—	1	—	—	—	—	—	—	—	—	—	10	99·2
11	—	—	—	—	1	—	—	1	—	1	1	—	—	—	—	—	—	—	—	—	—	—	4	99·6
12	—	—	—	—	1	2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	3	99·9
13	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	100·0
TOTAL	57	32	34	32	167	21	19	6	62	152	110	34	47	19	11	27	13	17	38	23	7	12	940	

APPENDIX TABLE 8.—*Difference bovine reaction—avian reaction in 443 tuberculous cattle in eight infected herds*

Class mark	Herds								Total		Cumulative frequency per cent
	1	2	3	4	5	6	7	8			
−2	0	0	0	0	0	0	0	0	0		
−1	0	0	0	0	0	0	0	1	1	6	9·2
0	0	0	1	1	1	1	0	1	5		1·4
1	2	0	0	2	2	0	1	4	11		3·8
2	4	4	1	2	2	0	0	4	17	56	7·7
3	5	1	2	0	4	4	3	9	28		14·0
4	4	9	3	1	7	1	1	13	39		22·8
5	10	7	3	1	5	2	3	6	37	117	31·2
6	5	5	3	1	5	0	6	16	41		40·4
7	3	5	2	2	4	1	2	6	25		46·1
8	4	8	4	2	6	1	0	8	33	82	53·5
9	6	3	0	0	2	6	0	7	24		58·9
10	3	2	1	2	4	1	2	6	21		63·7
11	4	5	0	0	2	0	1	10	22	62	68·6
12	2	3	0	0	5	1	1	7	19		72·9
13	0	4	0	3	4	0	0	6	17		76·5
14	1	2	1	1	1	0	0	9	15	42	80·1
15	0	0	0	1	1	0	0	8	10		82·4
16	0	0	0	1	0	0	0	5	6		83·8
17	0	2	0	0	0	0	0	4	6	22	85·1
18	0	1	0	1	1	0	0	7	10		87·4
19	1	1	0	0	0	0	0	6	8		84·2
20	1	1	0	0	0	0	0	3	5	22	90·3
21	0	1	0	1	0	0	0	7	9		92·3
22	1	0	0	0	1	0	0	8	10		94·6
23	0	1	0	0	0	0	0	3	4	19	95·5
24	0	0	0	1	0	0	0	4	5		96·6
25	0	1	0	0	0	0	0	4	5		97·7
26	0	0	0	0	0	0	0	3	3	8	98·4
27	0	0	0	0	0	0	0	0	0		98·4
28	0	0	0	0	0	0	0	1	1		98·7
29	0	0	0	0	0	0	0	1	1	7	98·9
30	2	2	0	0	0	0	0	1	5		100·0
TOTAL	58	68	21	23	57	18	20	178	443		

APPENDIX TABLE 9.—*Frequency distribution of difference bovine reaction—avian reaction in* 940 *non-tuberculous cattle in* 22 *non-infected herds*

Class Mark	Herd																						Total		Cumulative frequency per cent
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22			
−11	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2		0·2
−10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0·2
−9	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1		0·3
−8	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1		0·4
−7	0	0	0	0	4	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	7	33	1·2
−6	1	0	0	0	14	1	1	0	0	5	1	0	0	0	0	0	0	0	1	1	0	0	25		3·8
−5	4	0	1	5	12	0	1	0	0	9	0	0	2	0	0	0	0	0	0	0	0	0	34		7·5
−4	3	0	1	5	21	4	1	0	2	21	8	1	2	1	0	1	0	0	2	2	0	0	75	231	15·4
−3	9	5	0	4	34	3	0	0	7	26	9	4	8	2	0	3	0	2	4	1	0	1	122		28·4
−2	14	3	4	5	39	0	6	0	8	38	10	9	17	6	0	3	2	1	6	5	1	1	178		47·3
−1	13	4	10	4	22	2	6	2	10	23	15	9	7	2	3	8	2	3	9	7	2	0	163	465	64·7
0	7	7	5	3	9	2	1	2	7	13	17	8	8	4	4	7	6	2	7	1	1	3	124		77·9
1	2	7	7	2	4	2	3	2	7	12	23	0	3	2	4	2	1	7	5	3	2	6	106		89·2
2	2	3	3	2	4	2	0	0	5	1	8	2	0	1	0	0	2	1	3	2	1	0	42	185	93·6
3	1	2	2	1	2	4	0	0	9	1	10	0	0	1	0	2	0	0	1	0	0	1	37		97·6
4	0	1	1	0	0	0	0	0	5	1	5	0	0	0	0	1	0	1	0	0	0	0	15		99·2
5	1	0	0	0	0	0	0	0	1	0	3	0	0	0	0	0	0	0	0	0	0	0	5	21	99·7
6	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1		99·8
7	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	2		100·0
8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	
9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		
TOTAL	57	32	34	32	166	21	19	6	62	152	110	34	47	19	11	27	13	17	38	23	7	12	940		