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The role of mycobacteria and other bacterial adjuvants in modifying the hydrolase enzymes of phagocytes

by

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PART ONE

INTRODUCTION

AIMS OF INVESTIGATION

The main intention in this thesis is to report a study of the action of mycobacteria and anaerobic coryneform bacteria on macrophage function. This action was investigated in two ways. Firstly by studies of the digestion of mycobacteria and other bacteria by macrophages and secondly, the effects of such bacteria on the activities of the lysosomal enzymes responsible for this digestion were measured.

The mycobacteria and anaerobic coryneform bacteria are of biological and medical importance because there are many interesting features of their behaviour in the host. These features are described in turn below.

(i) EFFECTS ON IMMUNE RESPONSES

Many mycobacterial and coryneform bacteria have been used by earlier investigators for their adjuvant, specific and non-specific immune effects.

Mycobacterial adjuvants are known to stimulate the development of delayed-type hypersensitivity reactions to soluble protein antigens, and in recent years a microorganism designated Corynebacterium parvum has also been used as an adjuvant for stimulating the phagocytes of the reticulo-endothelial system (R.E.S.) (Halpern, Prevot, Biozzi, Stiffel, Mouton, Bouthillier and Fray, 1969; McCracken, McBride and Weir, 1971).

These and other immunological effects of the mycobacterial and coryneform bacteria are discussed below.

(a) IMMUNOLOGICAL EFFECTS OF ADJUVANT-ACTIVE BACTERIA

1. raising antibody levels
2. enhancing cell-mediated immunity (mycobacteria but not coryneforms)
3. anti-tumour effects.

1. raising antibody levels: The capacity of adjuvant-active bacteria to enhance the immune response was noted by various workers early in the development of immunology. Lewis and Loomis (1924) were among the first to note this phenomenon when they observed that the same amount of sheep red blood cells produced a higher titre of haemolysins in tuberculous than in normal guinea pigs. This observation was confirmed by Dienes (1927a, 1927b, 1928, 1929, 1936), who not only observed an increased production of antibodies to egg white, horse serum, and other antigens inoculated into the tuberculous lesion, but also made the interesting observation that two types of hypersensitivities were produced in these animals; one, an immediate or anaphylactic type, and the other, necrotic, delayed type. Couland (1935) found that an intense and persistent tuberculin sensitivity could be produced in rabbits with killed tubercle bacilli incorporated in paraffin. This observation was confirmed by Saenz (1935, 1937) who employed vaseline (petroleum) oil instead of paraffin. With these observations at hand, Freund started his classic experimentation on the

production not only of delayed sensitivity to tuberculin with killed tubercle bacilli but also on antibodies to antigens given in water-in-oil emulsions containing killed mycobacteria (Freund, J. et al., 1948; Freund and McDermott, 1942; Freund and Bonanto, 1946).

The powerful antibody-stimulating effect of Freund's adjuvant (light mineral oil, an emulsifier such as Aquaphor or Arlacel A, killed mycobacteria, and aqueous solution of antigen) has not been equalled by any other adjuvant.

Without mycobacteria (Freund's incomplete adjuvant) the adjuvant effect is less pronounced not only in intensity but also in duration. The tissue response to adjuvant without mycobacteria was minimal. This marked stimulation by mycobacteria of antibody responses to soluble protein antigens and viruses has been observed by various workers (Weigle et al., 1960; Coe and Salvin, 1964; Shepel and Klugerman, 1963). Whole mycobacteria are not needed for this effect since it has been found that lipids extracted from mycobacteria are also effective (Freund et al., 1948; Freund and Stone, 1959; White et al., 1955; White, 1959).

Most acid-fast organisms, including M. butyricum, M. tuberculosis (Freund, 1947; White, 1959), have been found active in stimulating the antibody response. Freund (1947) found that M. butyricum was slightly better and M. phlei slightly inferior to M. tuberculosis.

In recent years White, Coons and Connolly (1955) studied antibody production produced by wax fractions of Mycobacterium

tuberculosis in adjuvant emulsions on the production of antibody to egg albumin. All of these studies showed mycobacteria as being very good stimulators of antibody production.

2. DELAYED HYPERSENSITIVITY AND CELL-MEDIATED IMMUNITY

Delayed hypersensitivity has been defined as 'The hypersensitivity state mediated by primed lymphocytes (never antibody). The lesions, in which lymphocytes and macrophages are usually prominent, do not develop until about 24 hours following contact with antigen, e.g. by intradermal inoculation. The ability to react in this way can be transferred to another animal with cells from lymph node, spleen or bone marrow alone and is now known to be a manifestation of cell-mediated immunity' (Herbert and Wilkinson, 1971).

Mycobacteria when incorporated in Freund's complete adjuvant mixtures, exert a strong "directive effect" in directing the response to protein antigens incorporated into the mixture towards delayed sensitivity (Dienes, L. and Mallory, 1932). Wilkinson and White (1966) also discussed the effect of adjuvants and reported their observations on the "directive effect" produced by bentonite (aluminium silicate). Wilkinson and White produced evidence that mycobacteria and certain other adjuvants produce three events in the guinea pig which appear to be related, i.e. the development of macrophage-epithelioid granulomas, increased γ-2 globulin production, and delayed sensitivity to the protein accompanying the adjuvant. Reid and McKay (1967) have emphasized the association between granulomatous inflammation and the

development of delayed sensitivity. This association suggests that the macrophages of the granulomas contributes in some way to the development of delayed sensitivity and γ-2 globulin production.

Mycobacteria, unlike the anaerobic coryneforms, are well known for their striking ability to facilitate the induction of cell-mediated immunity when antigen is injected in water-in-oil emulsions with added mycobacteria. On the other hand, the anaerobic coryneforms have virtually no ability to facilitate the induction of cell-mediated immunity (O'Neill et al., 1973). A variety of anaerobic coryneforms in water-in-oil emulsion with added ovalbumin was used by O'Neill et al., (1973). Skin and corneal tests failed to show any increase in delayed-type hypersensitivity by guinea pigs receiving varying doses of anaerobic coryneforms (200 µg to 2.5 mg) with ovalbumin in water-in-oil emulsion over animals receiving ovalbumin in only water-in-oil emulsion.

3. ANTI-TUMOUR EFFECTS

A strain of coryneform organism referred to as C. parvum 936B was used originally by Halpern et al., (1964)in order to increase the clearance of carbon from the blood of mice. In the past few years, the same and other anaerobic coryneform bacteria have been used to inhibit tumour growth (Halpern et al., 1966; Smith and Woodruff, 1968; Fisher et al., 1970). There was no antigenic or taxonomic relationship between the organisms used.

Therefore, a study of the effects of these organisms on the

macrophage is likely to lead to new insights into defence, granuloma-formation and the nature of adjuvant action.

It has been known for years that the growth of transplanted tumours in rodents could be inhibited by prior treatment of the host with BCG and various other agents known to stimulate the RE activity, but there appeared to be no reports of such effects of organisms as C. parvum in the literature, but for one exception (Weiss, P., 1961). All this work had been done with allografted tumours.

Woodruff and Dunbar (1966) found that a single intravenous injection of 0.5 mg wet weight of a heat-killed suspension of C. parvum either two days before or 8-12 days after subcutaneous inoculation of 10^5 or 10^6 viable mammary carcinoma cells in "A" strain mice delayed the appearance of the tumour to an extent comparable to that achieved by reducing the number of cells injected to untreated mice by a factor of ten, though once the tumour had become palpable the rate of growth was the same in treated and untreated animals. With smaller inocula the appearance of a tumour was sometimes completely suppressed.

C. parvum was further found to be highly effective against both primary and secondary transplants of methylcholanthrene-induced fibrosarcomas in "A" (which lack the fifth component of complement, C5) and also in CBA mice (Woodruff and Dunbar, 1966).

BCG containing 10^6 viable organisms/ml was tested for its inhibition of A strain mammary tumour. When given in dosage of 0.1 or 0.25 ml it did not significantly inhibit the tumour development, nor did it affect the phagocytic index (K) in non-tumour bearing animals, measured four days after injection (Woodruff and Dunbar, 1966).

(ii) GRANULOMA FORMATION AND MACROPHAGE INFILTRATION IN VIVO

Granulomas are built up by emigration of circulating cells from the blood. Early granulomata appear to comprise largely monocytes which are of bone marrow origin (Ebert and Florey, 1939; Volkman and Gowans, 1965a, 1965b; Spector and Lykke, 1966).

The effects of whole mycobacterial cells and fractions extracted by chemical means in the production of chronic inflammation at the site of injection has long been observed. Mycolic acid induces persistent necrotic lesions at the site of injection (Gerstl, Tennent and Petzman, 1945). Delaunay, Asselineau and Lederer (1954) found that peptidoglycolipids of human tubercle bacilli, and glycolipids from bovine strains produce similar effects, with the presence of giant cells. Mycobacteria added to water-in-oil type emulsions form typical lymphoid nodules in the late stages of a granuloma formed after injecting this emulsion mixture.

Suter and White (1954) found that after injection of mycobacterial adjuvants into the foot-pads of guinea-pigs, swelling of the injected foot appears, followed by desquamation and ulceration. Hyperplasia occurred in the draining lymph nodes. On histological examination, a proliferation of macrophages, polymorphonuclear leucocytes and eosinophils was seen in the injected foot. White et al., (1955b) detected epithelioid macrophages in the foot-pad granulomas. Multinucleated giant cells were numerous. It is clear that the continuing existence of a granuloma is likely to represent the complex response to a wide variety of concurrent stimuli (White, 1974). In

the early stages, it is likely that macrophage chemotactic factors play an important part in recruiting cells from the circulation. Once the granuloma is established, the rate of entry of cells can be measured by transfusing monocytes labelled *in vivo* with tritiated thymidine to animals bearing granulomas of varying age. With this approach, a clear distinction becomes obvious between the different granulomata provoked by carrageenan and those provoked by *Bordetella pertussis* vaccine or complete Freund adjuvant (FCA) (White, 1974), containing mycobacteria. With a carrageenan granuloma the daily entry falls, after the first week or so, to a low level of around 10,000 -- 20,000 cells. This is a 'low turnover lesion' (Spector and Ryan, 1970). In high-turnover lesions, such as that provoked by local injection of *B. pertussis* vaccine or *M. tuberculosis* in mineral oil, the daily entry of monocytes remains high, i.e. around 250,000 cells, for months (Ryan and Spector, 1969). The high-turnover granuloma due to CFA is associated also with a significant entry of polymorphonuclear leucocytes.

Further experiments by Ryan and Spector (1970) have shown that when high-turnover granulomata produced by injecting *B. pertussis* have been established, destruction of the bone marrow with shielding of the reaction site is followed within 24 hours by virtual cessation of DNA synthesis in the macrophages of the reaction. Thus, even at 6 weeks, most of the macrophages of the granuloma were very recent arrivals from the bone marrow and their rate of proliferation was high. This presumably accounts for the parallelism which is constantly observed between the extent of macrophage immigration and proliferation.

Presumably, as suggested by Spector and Ryan (1970), the stimulus for immigration of macrophages is the systemic release of a chemotactic substance.

Presumably this is accompanied by a cytotoxic substance which damages the macrophages of the granuloma after injection. By keeping up the numbers of locally available macrophages, the systemic release of the cytotoxin is prevented. The system therefore reaches homeostasis, increased damage to macrophages causes chemotaxis of further macrophages and mitotic proliferation of the cells after emigration (White, 1974).

The anaerobic corynebacteria, of which C. parvum is the best-known example, are a group of micro-organisms which induce granuloma formation which is largely restricted to the site of injection (Pinckard et al., 1968). Mycobacteria on the other hand, can cause a systematised proliferation of macrophages in lymph-nodes throughout the body, spleen and lungs (White et al., 1955a; Suter and White, 1954), an effect which can be reproduced by the chloroform-soluble wax D fraction or peptidoglycolipid extracted from the bacilli (White, 1965).

(iii) EFFECTS ON PARTICLE CLEARANCE IN VIVO

The phagocytic index (K), based on the rate of clearance of intravenously injected colloidal carbon particles from the blood stream, was first determined by Biozzi et al.,(1954) four days after a single intravenous or intraperitoneal injection of a standard dose of

various C. parvum preparations, and after different doses of C. parvum (WEZ 174).

Recently in these laboratories, O'Neill, Henderson and White (1973) studied a wide range of different strains of anaerobic coryneforms and 'classical' propionibacteria for some of their macrocytostimulant effects (included under this term are the ability to increase the phagocytic uptake of carbon particles after intravenous injection of the latter into mice, and ability to stimulate an increase in lysosomal hydrolases and ability to exert directly a chemotactic stimulus on macrophages) and also their ability to increase humoral and cellular immunity when mixed with an immunogen.

Of the twenty-odd strains of anaerobic coryneforms tested by O'Neill et al., (1973), fifteen strains were able to produce an increase in phagocytic index in mice of at least 50 per cent. Microorganisms that were effective in these tests occurred in each of the four serological groups (Johnson and Cummins, 1972).

Among the many strains of bacteria studied by earlier investigators Mycobacteria were among the most active as far as RE stimulation is concerned. Stiffel, et al., (1963), showed that the administration of the avirulent strain M. tuberculosis (BCG) is followed by marked increase in the phagocytic index K, which lasts for about one month. Similar results were obtained using the saprophytic strain M. phlei, which is completely avirulent in mice. The substance responsible for RE stimulation is present in the cell wall of this mycobacterial strain.

The results of stimulation of RE macrophages (phagocytic indices K and α) produced by injection of BCG or C. parvum in inbred strains of mice and in random-bred Swiss mice showed that the degree of RE stimulation produced either by BCG or C. parvum is different in the various inbred strains of mice. For instance, the C_3H/Ajax mice showed a strong response to C. parvum and no response to BCG (Stiffel et al., 1970). C. parvum produces a maximal response in the A/He mice and a minimal one in the Balb/C.

Such intrastrain difference is probably under genetic control, and the ability to respond is transmitted at the F_1 hybrid as a dominant character (Stiffel et al., 1970).

Outbred Swiss mice presented a larger individual variation in the RES stimulation after injection of BCG.

(iv) MACROPHAGE CHEMOTACTIC EFFECTS IN VITRO

Leber (1891) and Metchnikoff (1892) observed that the migration of phagocytic cells into sites of bacterial infection is an essential factor in the inflammatory response. In some bacterial diseases, for example, tuberculosis is the type example, chronic granuloma formation is the characteristic lesion and the cell which migrates typically into the site of infection is the macrophage.

Symon et al., (1972) reported that whole organisms of M. tuberculosis contained a factor which was capable of reacting with guinea pig plasma to produce strong chemotactic activity for guinea

pig peritoneal macrophages. This activity of plasma was heat-labile and tests with several complement inhibitors - detailed by Symon et al., (1972) - indicated that it was probably mediated by a complement component. In the absence of plasma, M. tuberculosis showed little capacity to attract phagocytic cells. Tests to determine which fractions of M. tuberculosis were cytotaxigenic (Wilkinson, P.C., and McKay, I.C., 1972) indicated that the cytoplasmic fraction of M. tuberculosis was richer in activity than the cell wall. It further showed that removing the lipid from the bacterial cell did not remove the chemotactic activity.

On the other hand, tests of the chemotactic activities of the anaerobic Corynebacteria and Propionibacteria for macrophages, showed that several members of this group produce a chemotactic factor which attracts macrophages directly without the necessity for the additon of plasma (Wilkinson et al., 1973a). Organisms derived from cheese or milk ('classical' propionibacteria) had no chemotactic activity for macrophages.

O'Neill et al., (1973) have shown that many organisms in these groups other than C. parvum have macrophage-stimulating and adjuvant activities. Symon et al., (1972) reported that many strains of these organisms also produce a highly macrophage-specific chemotactic factor, and that anaerobic coryneforms also cause a burst of acid phosphatase production by macrophages in vitro and it was suggested that the macrophage-specificity of this chemotactic factor is related to its pattern of activation of lysosomal enzymes which differs from that of neutrophil cytotaxins. It should be noted that the majority of organisms from the serological groups 1-4 (Johnson and

Cummins, 1972) have a strong chemotactic effect on macrophages without a requirement for serum mediators such as complement (serum is required for M. tuberculosis).

THE EFFECTS ON MACROPHAGES WERE STUDIED AS FOLLOWS:

1. The ability of macrophages to degrade various strains of mycobacteria was studied, each organism being compared with Escherichia coli in respect of the ability of macrophages to break it down.

2. The effect of these organisms on activation of lysosomal hydrolases in macrophages was studied by injection of bacteria intravenously into chickens. Alveolar macrophages were harvested at various times thereafter and hydrolase levels were estimated at these times.

This investigation gave information about acid hydrolase stimulation in macrophages in vivo over a period of four days to 28 days. Also the short term effects of these bacteria on macrophage function were studied in vitro by incubation for a few hours of macrophages with the bacteria and estimation of hydrolase activity in these macrophages.

An attempt was also made to investigate the possible relationship between one measure of macrophage activation, namely stimulation of lysosomal hydrolases, and a different measure of macrophage stimulation, namely the production of macrophage chemotactic

factors by animals at various times after injection of adjuvant-active bacteria.

DISCOVERY OF THE PHAGOCYTIC SYSTEM AND ITS FUNCTIONS

Metchnikoff (1884) clearly outlined the role of macrophages in the economy of the mammal. He showed the major function of macrophages in the uptake of soluble and particulate agents and in their degradation within the cytoplasm. In recent years more detailed information has accumulated on the biological mechanisms and enzymology by which this process is carried out. The stimulus for many of these studies arose from the discovery of a class of cytoplasmic organelles by DeDuve and his colleagues in Louvain (DeDuve and Wattiaux, 1966). They demonstrated that lysosomes of rat liver were membrane-bounded organelles, heterogenous in size and ultrastructure, and containing a wide spectrum of hydrolytic enzymes with acid pH optima. It was noted that macrophages were rich in these organelles and that they were undoubtedly involved in the process of intracellular digestion.

It was at once established that lysosomes contained high levels of acid phosphatase. It was also noted that tissue macrophages or histocytes which were often present in inflamed lesions, reacted strongly for this hydrolase and a number of others (Cohn and Hirsch, 1960; Cohn and Wiener, 1963).

This work has been further developed by Woessner (1965) and Cohn, Z.A., and Benson, B. (1965), with information that indicates that the mononuclear phagocytes might be particularly a rich source of

lysosomes. To study this problem in more specific detail, quantitative biochemical assays were needed as well as large homogeneous populations of cells. Myrvik and his colleagues (1962) met this challenge through the use of peritoneal exudates and a new source of cells, the alveolar macrophage. Under normal conditions the number of alveolar macrophages present in lungs is small, but a vast enrichment can be obtained through stimulation using tubercle bacilli, heat-killed. The intravenous administration of dead BCG, leads to a widespread granulomatous response in the lungs and the macrophages which appear there can be collected by washing out the alveoli.

This source of cells is not only useful because of the large number of cells obtained but also because they contain high levels of acid hydrolases (Cohn, 1968).

Allison (1962) found that after BCG vaccination, the increase in resistance of rabbits to virulent infection with tuberculosis was paralleled by an increase in peritoneal macrophage acid phosphatase levels, and also to some extent by increases in other enzymes in the cells.

In experimental animals, infection with living organisms causes a rapid and prolonged increase in phagocytic activity. Biozzi et al., (1957) showed that infection of mice with virulent strains of Salmonella and Mycobacteria produced a marked stimulation of phagocytic activity within 24 hours of inoculation which was accompanied by a rapid increase in the size of the liver and spleen; the phase of stimulation was followed by a marked and rapid drop in phagocytic

activity as the animals became clinically ill and succumbed to the infection.

It has been shown that Mycobacteria and some of their constituents are potent stimulants of phagocytic activity. Living and killed virulent, avirulent and BCG strains of Myco. tuberculosis, Myco. phlei, Myco. fortuitum and the killed Myco. butyricum present in Freund's complete adjuvant are all effective stimulants (Biozzi et al., 1957; Bohme and Bouvier, 1960; DiCarlo et al., 1963a, 1963b).

A comparison has been made of the phagocytic and bactericidal abilities of macrophages washed out from guinea-pig lung and mouse peritoneum (Pavillard and Rowley, 1962). Cells from both sources were shown to be phagocytic but lung macrophages possessed relatively little bactericidal power. It is probable that individual cells within a large population of macrophages vary and that such variation may be reflected in their individual ability to eliminate bacteria. Mackaness (1960) has shown that this is highly probable in the case of rabbit peritoneal macrophages. The possibility that the degree of heterogeneity in the reticulo-endothelial system may differ from one anatomical region to another has also to be considered.

Another notable observation of phagocytosis, for example, is the inhalation of bacteria and their rapid disappearance from the lungs of experimental animals (Stillman, 1923; Robertson, 1941; Laurenzi, et al., 1964). This early clearance of bacteria is thought to be an important process in non-specific resistance to infection in the bronchopulmonary tree.

It has been shown that many strains of bacteria are rapidly killed in the peritoneum following phagocytosis by mouse macrophages (Whitby and Rowley, 1959). However, organisms such as tubercle bacilli can survive for prolonged periods of time within the phagocytic cells of susceptible and resistant hosts (Sabin and Doan, 1927; Tytler, 1930; Laurie, 1932). Sections from autopsy material from human and animals dead with tuberculosis, as well as tissue cultures infected with tubercle bacilli, contain phagocytic cells filled with bacilli (Canetti, 1946; Maximow, 1928).

For descriptive and functional purposes it is still sometimes profitable broadly to follow Metchnikoff in considering that the macrophages of the body may be grouped as 'fixed' macrophages lying along the walls of blood and lymph channels in direct contact with circulating blood or lymph, and 'free' macrophages situated extravascularly in the connective tissues, in solid organs, and in the central nervous system. It is, however, important to remember that both the 'fixed' and 'free' cells can be mobilized, and that these prefixes are used for descriptive convenience alone.

THE DISCOVERY OF THE ROLE OF ACID HYDROLASES IN INTRACELLULAR DIGESTION

Study of the living cell has in recent years established an increasingly complete catalogue of its working parts and identified these with their functions. The new information has come from a collaborative effort of, on the one hand, the cell anatomist, whose electron micrographs portray the internal structures of the cell in

almost molecular detail, and on the other, the biochemist, who studies the fractions of disrupted cells so that he can observe the activity of the cellular organelles and their molecular components in separation from one another.

This combined study of structure and function has shown, for example, that the organelles called mitochondria conduct the primary energy transformations of the cell and that the smaller organelles called ribosomes are the centres of enzyme manufacture. The latest addition to this list, the lysosome, was discovered and analysed in detail in 1955 by DeDuve et al.

The earliest study of lysosome-like organelles is that of Hetherington and Pierce (1930) who gave a vivid description of peri-nuclear inclusions (now termed lysososmes) that characterised the active phagocytic cell. They demonstrated that these organelles accumulated red granules from the vital stain neutral red and that they were areas of high acid phosphatase activity. However, the first detailed study of the lysosomes and their contents was carried out by DeDuve et al., (1955) who detected and assayed some twenty acid hydrolases. First identified in rat liver cells in 1955, lysosomes are now known to occur in many, possibly in all animal cells. It is important that they are particularly large and abundant in cells, such as the macrophages and the white blood cells, that are called on to perform important digestive tasks in clearance of foreign material.

The lysosomes of macrophage contain enzymes that, on liber-ation are capable of digesting the entire cell. DeDuve et al., (1955) postulated that the lysosome did not release its contents into the cell

cytoplasm but that its membrane fused with that of the phagocytic vacuole, so that the enzyme contents of the former were discharged into the latter with subsequent digestion of the ingested bacterium or degradation of the biologically active protein as the case might be, but without autodigestion of the cell.

Many of the enzymes in lysosomes have been identified. In addition to the above-mentioned acid phosphatase of Hetherington and Pierce (1930), lysozyme, lipases, cathepsins A, B, C and D; acid ribonuclease, β-glucuronidase, β-galactosidase, α-mannosidase, amino-peptidases, succinic dehydrogenase, neuraminidase, hyaluronidase, aryl sulphatase and non-specific esterases, to mention a few, have all been detected. Also in the case of lysosomes from macrophages, two proteases have been identified, one being a pepsin-like enzyme. Thus the macrophage is well equipped to deal with all the major classes of known chemical substances which might be introduced into the cell by the ingestion of bacteria.

The mode of action of these enzymes on bacterial protein or other macromolecules has become better understood in recent years, for example, lysozyme (muramidase). It was evident from earlier investigations by Meyer, et al., (1936) and Epstein and Chain (1940) that the characterization of the lysozyme substrate and the products formed by the action of this enzyme should throw a great deal of light on the problem of cell-wall structure. The demonstration that the isolated cell wall of Micrococcus lysodeikticus could be used as the 'substrate' for lysozyme (Salton, 1952) simplified the task of identification and eventual characterization of the digestion products.

Apart from the detection of reducing N-acetyl amino sugar groups and the decrease in viscosity on degradation of the soluble 'mucopolysaccharide' substrate (Epstein and Chain, 1940; Meyer and Hannel, 1946) the nature of the compounds produced by lysozyme action was not established until the question was re-examined by Salton (1956). Lysozyme splits a specific muramyl bond in the bacterial cell wall murein (Mandelstam and Strominger, 1961), the precise target sites on the cell wall have been characterised as shown in Figure 1.

The first biochemical study of the organisation of various lysosomal enzymes within the macrophage was performed by Cohn and Wiener (1963). In homogenates of rabbit peritoneal, and BCG induced alveolar macrophages, the acid hydrolases exhibited latency and were sedimentable. In this respect, the acid-hydrolase bearing particles resembled those described for rat liver. In adjunct, when a post-nuclear fraction was subjected to isopycnic centrifugation, the acid hydrolases all displayed a rather narrow density distribution quite distinct from that shown by the mitochondrial enzyme, cytochrome oxidase.

In the technique of centrifugal fractionation, cells are disrupted in a homogenizer and then spun in a centrifuge at successively higher speeds to field a number of fractions containing organelles of different types. When isolated in this manner, the organelles still maintain many of their properties, which can be explored by means of biochemical methods (DeDuve et al., 1955).

He reported that the acid-phosphatase activity was about a tenth of what he had come to expect from previous preparations that had been subjected to the more drastic homogenizing action of a Waring Blender. Neither methods of homogenizing or freeze-thawing completely lysed the lysozomes. Wattiaux et al., (1963) introduced a method by which after homogenization, 0.1% of Triton WRO1339 (a detergent) would cause complete lysis of lysosomes without interfering with the enzyme properties.

The numbers of enzymes known and studied as a result of improved techniques of processing tissues and exudates is already legion, and is steadily increasing. They may be divided into two major groups; hydrolases and desmolases. The hydrolases cleave substances of high-molecular weight into their constituents, i.e. proteins into peptides and then into amino acids; starch into glucose; fats into glycerine and fatty acids; nucleic acids into phosphoric acid, purines, or pyrimidines, and pentoses; and so on. The cleavage always takes place with the addition of water, and may therefore be called hydrolysis. Hydrolases can also reverse the cleavage and synthesize large molecules from their building blocks. The energies involved in the reactions catalyzed by hydrolases are generally relatively small.

Desmolases attack the simple building blocks themselves. They degrade glucose, for example, into carbon dioxide and water, add or remove hydrogen, perform decarboxylations and transaminations, and carry out countless other reactions which are non-hydrolytic.

The intracellular enzymes may be divided into lyoenzymes,

endoenzymes, and desmoenzymes. Lyoenzymes are easily extracted from the cell wall by lysosomal enzymes. Desmoenzymes are firmly bound to the various constituents of the cell (mitochondria, nuclei, etc.) that they can be separated only with aid of various reagents or by enzymatic attack on the cell particles.

A lysosomal localization of these enzymes in lymphoid tissues is suggested by the finding that several of them occur partly in latent, particle-bound form, in freshly prepared homogenates of spleen or thymus (DeDuve, 1959; Conchie and Hay, 1963; Levvy and Conchie, 1964; Rahman, 1964; Sachs et al., 1962). However, various investigators, using different techniques with these tissues, have obtained rather variable patterns for the intracellular distribution of individual acid hydrolases (Balousova, 1958; Roth and Eichel, 1959; Eichel and Roth, 1962; Roth, Bukovsky and Eichel, 1962; Conchie and Hay, 1963; Levvy and Conchie, 1964) and this fact has even led some workers to question the existence of lysosomes as a distinct group of cytoplasmic particles (Levvy and Conchie, 1964).

SOME FACTORS DETERMINING THE BACTERICIDAL ACTIVITY OF MACROPHAGES

Extensive studies of phagocytosis have shown that in typical experiments with intact macrophages some 95 per cent of ingested bacteria are destroyed in approximately 20 minutes, but the bactericidal activity of such cell preparations can vary enormously under the influence of a wide variety of factors. The animal species from which the macrophages were obtained and the tissue of origin both seem to be

of some importance. For example, there is good evidence that peritoneal macrophages from adult rats have greater bactericidal activity for Salmonella typhimurium than foetal macrophages from the same species (Reade, 1968) and that peritoneal macrophages have greater bactericidal activity than those obtained from the pulmonary alveolae (Pavillard and Rowley, 1962).

The qualitative and quantitative enzyme content of the macrophage must play some part in determining its bactericidal activity. Indeed, there is evidence suggesting that variations in resistance to bacterial infection are related to changes in lysosomal enzymes. Thus infections are commoner when the acid phosphatase activity of macrophages is reduced or lost (Auzinis and Rowley, 1962). However, it should not be assumed that bactericidal activity is always directly related to enzyme content. Alveolar macrophages generally have a higher acid phosphatase and lysozyme content than those from the peritoneum but their phagocytic activity is nevertheless poorer. A possible explanation for this apparent anomaly has been advanced by Leake and Myrvick (1966) who propose that the lysosomal enzymes of the alveolar macrophages do not discharge into the phagocytic vacuoles as their commitment to other cell processes renders them "unavailable".

A further important factor in determining phagocytic digestion is the availability of the "target-site" on the ingested particle. This is best illustrated by reference to the lysozyme-sensitive muramyl bond present in all bacterial cell walls studied to date. When this linkage is exposed as in many gram-positive species, lysis with lysozyme readily occurs but where the cell surface is

protected by O-antigens, capsules or other macromolecular structures as in most gram-negative species, lysis rarely occurs in the native state (Strominger and Ghuysen, 1967). Removal of the "biological envelope" by treatment with chelating agents (Repaske, 1959), acid (Hirsch, 1960) or glycine (Ralston et al., 1961) widens the range of organisms attacked by lysozyme. These findings stress the importance of "steric hindrance" as a factor in determining bactericidal activity.

In some instances bactericidal activity can be stimulated by a wide variety of bacterial and non-bacterial substances. One of the best examples of this class is the increased bactericidal effect obtained with serum (Rowley, 1958).

Finally, there is some evidence that metabolic events in phagocytes may play a role in determining their bactericidal efficiency. Davis et al., (1968) and Rody et al., (1969) have shown that five patients suffering from chronic granulomatous disease of childhood had blood monocytes which were defective in their anti-staphylococcal bactericidal activity. There was no firm evidence to establish the precise nature of the cellular defect but it was believed to be metabolic in origin. This view was supported by the observation that polymorphonuclear activity was reduced when the rate of respiration, hexose monophosphate shunt activity (one of the most important alternative routes by which animal tissues oxidize glucose to carbon dioxide and water with the production of useful energy in the form of ATP) and formate oxidation were increased (Good, R.A. and Gray, B., 1971) and also when there was impaired reduction of tetrazolium dye (Baehner and Nathan, 1968). These authors suggested that all these activities could

be accounted for by their observation that the enzyme NADH (reduced form of NAD) oxidase was deficient.

These results offer an explanation as to how the peroxidase-linked bactericidal mechanisms of phagocytes may be disrupted by metabolic processes but do not explain the phagocytic activities of peroxidase-negative mononuclear cells. It should also be stressed that the precise relationship between the extra-lysosomal metabolic events and the intro-lysosomal digestive activities of monocytes has not yet been elucidated.

A number of other investigators have looked for a connection between the metabolic and phagocytic activities of the cell usually with negative results. Studies have been done on the metabolic requirements for the intracellular destruction of Salmonella enteritidis and Escherichia coli using fresh mouse peritoneal macrophages. Inhibition of glycolysis (with iodacetate and fluoride) of cytochrome oxidation (with cyanide), and lactate production (with oxamic acid) had no demonstrable effect on intracellular killing.

THE INTRACELLULAR DEGRADATION AND DISPOSAL OF INGESTED BACTERIA

The degradation of isotopically-labelled bacteria within polymorphonuclear leucocytes and macrophages has been studied in detail by Cohn (1963). Intracellular inactivation was followed by extensive degradation of bacterial lipids, nucleic-acids and proteins. The rate of this degradation varied with local conditions and factors as summarised previously. With ^{32}P-labelled E. coli, 80 per cent of the

labelled RNA and DNA and 50 per cent of the labelled lipid were recovered as acid soluble labelled products within 2 hours, the principal breakdown products being nucleotides and ^{32}P. The fate of bacterial proteins was examined with ^{14}C-labelled bacteria. Two hours after phagocytosis, more than 45 per cent of the ^{14}C was recovered in the acid soluble fraction, the labelled breakdown products in this case being peptides and amino-acids.

In Cohn's experiments, the intracellular digestion of bacteria was specifically inhibited by the homologous immune serum. In such circumstances, serum treatment reduced the rate of degradation of E. coli and Staphylococcus albus by 50-65 per cent as judged by the release of acid soluble ^{32}P from these organisms. Macrophages from rabbits immunized with heat-killed organisms were not more effective in the formation of acid soluble products than cells from normal rabbits (Cohn, 1963 a). These observations contrast with those of Donaldson et al., (1956) who found that peritoneal macrophages from immunized rabbits digested chicken erythrocytes more efficiently than control cells from non-immune animals. This apparent anomaly might be explained by the species differences in the macrophages used.

Ehrenreich and Cohn studied the intracellular fate of pure proteins ingested by pinocytosis (Ehrenreich and Cohn, 1967, 1969). Using ^{125}I-albumin, more than 50 per cent of the isotope escaped from the macrophages within 5 hours and was found in the surrounding media as free ^{125}I and ^{125}I-mono-iodotyrosine. Similar results were obtained with internally labelled leucine-^{3}H-haemoglobin but the rate of hydrolysis was slower. In this case 50 per cent of the intracellular

isotope had been released at 24 hours and was found in the medium as leucine-^{3}H. These studies suggest that the products of intra-lysosomal digestion are dipeptides or amino-acids. This finding is consistent with peptide-permeability studies of lysosomes in which it was found that peptides with a molecular weight of less than 230 daltons and a Stokes radius of less than 4 Å were not retained within lysosomes (Ehrenreich and Cohn, 1969). These findings also agree with those of Nakae et al., (1967) who noted that isolated liver lysosomes degraded protein to dipeptides and amino-acids.

A number of other interesting conclusions were also made from the above experiments. From the analysis of the degradation products, it appears that bacterial DNA is digested more slowly by bacterial protein, lipid or RNA and that heat-killed bacteria are more rapidly broken down than viable forms. The results also indicate that the breakdown products rapidly escape from the phagocyte into the surrounding pabulum.

Finally, the role of complement in phagocytosis has been the subject of a number of studies, all of which indicate that intra-cellular digestion is much more rapid in fresh than in complement-free-serum.

From the evidence of the foregoing studies it is clear that the intracellular digestion of ingested bacteria depends on a large number of variable factors that are difficult to assess and even more difficult to measure quantitatively. These factors include the nature and origin of the ingesting cell, the structure and properties

of the ingested particle, the presence of opsonins, the metabolic-state of the cell, its lysosomal enzyme content and activity, and many non-specific factors such as complement.

A BRIEF REVIEW OF SOME OF THE ENZYMES PROBABLY INVOLVED IN THE INTRACELLULAR DEGRADATION OF BACTERIA AND STUDIED IN THIS THESIS

1. Phospholipases: Lecithinase and Phosphatidases

Munder and his colleagues (1967, 1969) demonstrated that phagocytosis of silica particles by macrophages and microphages activated intracellular phospholipase A (PL-A) with breakdown of the diacylphospholipids (lecithin and cephalin) to give highly surface active and potentially cytotoxic lysophosphatides. These findings suggested that a known adjuvant such as M. avium might stimulate a similar increase of this enzyme (in the granulomatous lung) to account for the degradation of bacterial cell wall lipids in the granulomatous lung. This enzyme was assayed to study this possibility.

Phospholipase A catalyses the hydrolysis of phosphatidyl choline (lecithin) and other phosphatides by removing the fatty acid on the 2-position to yield lysolecithin or other lysophosphatides.

Lecithin + H_2O $\longrightarrow$ Lysolecithin + Fatty acid.

However, there is some doubt about the specificity of the enzyme for the 2-position and the degree of unsaturation required in the fatty acid side chain.

2. Carbohydrases

β-galactosidase: This enzyme, which is also known as lactase, is widely distributed in mammalian tissues (Findley et al., 1958). It hydrolyses β-D-galactosides, α-L-arabinosides and heptosides with a β-D-galactose configuration (Veibel, 1950). In this thesis, the distribution of this enzyme in lung exudates and whole lung preparations from immune and non-immune birds will be studied by the colorometric method of Cohn et al., (1952). The results obtained by this method will be compared with those obtained histochemically.

β-D-glucuronidase: This enzyme splits many -glucuronides according to the following reaction:

β-D-glucuronide + HOH $\longrightarrow$ alcohol + D-glucuronic acid

It also catalyses the transfer of β-glucuronyl residues to aliphatic alcohols and glycols. One of its well recognised physiological actions is the hydrolysis of conjugated glucuronides such as the steroid glucuronides of bilirubin. It may also be involved in the degradation of the oligosaccharide side chains of hyaluronic acid (Meyer, 1958). Some investigations relate increased β-glucuronidase activity to cellular proliferation and to neoplastic growth (Fishman, 1947; Fishman et al., 1951).

However, as carbohydrases act on the free non-reducing end of polysaccharides, their action on bacterial macromolecular complexes is probably very limited.

3. Lysozyme: Lysozyme is a low molecular weight enzyme which is

widely distributed in the tissues and body fluids of mammals. *In vitro* it specifically hydrolyses the β-N-acetylmuramic acid (1 → 4)-N-acetyl-glucosamine linkage found in all bacterial cell walls (see Fig 1). As it is present in large amounts in pulmonary alveolar macrophages (and to a lesser extent in the analogous peritoneal cells), it seems reasonable to suppose that it plays an important role in the dissolution of bacteria *in vivo*. Good evidence for this view comes from the inhibition of the intracellular lysis of *Micrococcus lysodeikticus* by methyl-lysozyme which specifically blocks the lysozyme system (Glynn, Brumfitt and Salton, 1966). Salton (1959) also demonstrated that gram-positive cell walls and isolated cell walls of *E. coli* were almost totally destroyed by this enzyme and that the cell wall products muramic acid, glucosamine, DAP or lysine, alanine and glutamic acid could be recovered from the soluble fraction.

From these and other experiments, there is now little doubt that lysozyme is important in the extra-and-intra-cellular degradation of the cell wall murein that is essential to structural rigidity and viability in bacteria. The approximate position of the backbone structure of gram-positive cell wall split by lysozyme and N-acetyl-glucosaminidase is shown in Fig 1.

Attention has also been drawn to the fact that not all bacteria containing the lysozyme-sensitive bond are destroyed by this enzyme. This appears to be due to protection by "steric hindrance". In the Mycobacteria and Coryneforms that are the subject of this thesis, the mechanism of lysozyme protection has still to be classifed and its

relationship to the adjuvant-active polymers of these genera elucidated. In this thesis an attempt will be made to correlate the lysozyme content of whole lung tissue with stimulation by these organisms.

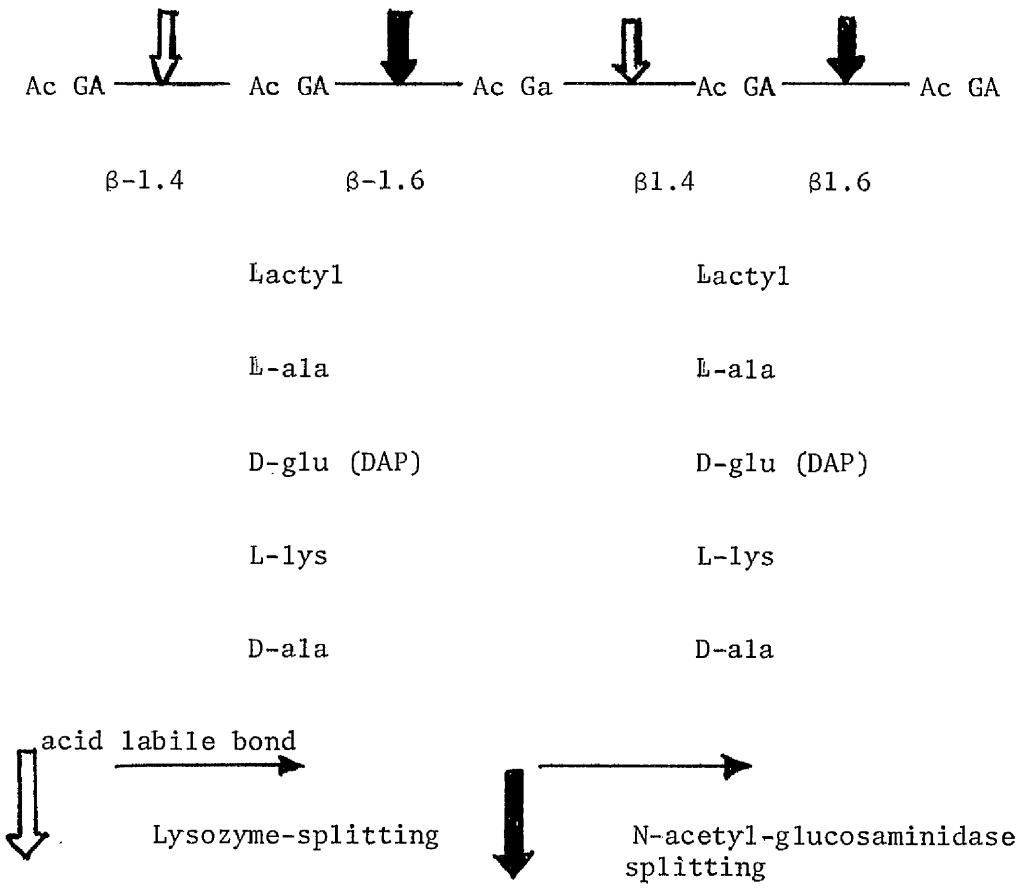
4. β-N-acetylglucosaminidase: In experiments with purified chitin, Berger and Weiser (1957) showed that the action of lysozyme was specifically directed against the β-N-acetylglucosamine bond in that disaccharide (Salton, 1959) and Salton and Ghuysen (1959) showed that cell walls were degraded by lysozyme and streptomyces F and that both could degrade mono- and tetra-saccharides of N-acetyl-glucosamine. These findings taken together imply a β-(1 → 4)-linkage for the glucosamine residues in bacterial cell walls. Perkins (1959, 1960) found that the simplest product of the action of lysozyme on the cell wall of Micrococcus lysodeikticus was a disaccharide and that this was identical to the disaccharide of Salton (1959). Another disaccharide of muramic acid and N-acetyl-glucosamine was also detected in the hydrolysis products (Salton, 1959; Perkins and Rogers, 1959). These findings led Salton to propose that the cell wall of M. lysodeikticus consisted of alternating units of muramic acid and N-acetyl-glucosamine and that some of the muramic acid residues carried peptide cross-links. The general structure proposed and the sites of action of both lysozyme and β-N-acetyl-glucosaminidase are shown in Fig 1.

5. α-mannosidase: This enzyme acts on the α-anomeric form of the appropriate mannoside. As this sugar is present in the cell wall polysaccharides of most gram-positive organisms including those of the genera Mycobacteria, Corynebacteria and Nocardia, attempts were made

to estimate its activity in the lysosomes of normal and stimulated animals.

6. Cathepsin D: Cathepsin D is a proteolytic lysosomal enzyme found in practically all animal species. The cathepsins were studied in detail by Anson (1938) but the characterisation of cathepsin D as a separate entity was first carried out by Press and his colleagues (1960). It is detected by its ability to degrade haemoglobin in acid conditions.

BACKBONE OF GRAM-POSITIVE ORGANISM'S CELL WALL



Taken from Otto Westphal and Otto Lüderitz (1963)

Fig. 1

PART TWO

MATERIALS AND METHODS

A. TERMS AND ABBREVIATIONS USED IN THIS THESIS

C.C test = carbon clearance test used to measure the phagocytic activity of the reticuloendothelial system. 16 mg colloidal carbon was used in experimental animals.

Mean = this is the arithmetic mean of the results.

Oil/Arlacel = this term indicates a standard mixture of nine parts by weight of Drakeol 6VR (or Bayol 55) with one part of Arlacel A. The mixture was sterilized by Seitz filtration before use and stored at -20°C.

Saline = this is a 0.85 per cent weight per volume solution of sodium chloride in distilled water.

Titres = Titres are recorded as the reciprocals of the initial serum dilutions.

Abbreviations

A.B.C.30	antigen binding capacity (30 per cent)
Ab	antibody
Ag	antigen
C	centigrade
cm	centimetre
C.B.	citrate buffer
C.F.A.	complete Freund adjuvant
C.P.S.	cycles per second
B.D.H.	British Drug Houses, Poole, England
DNA	Deoxyribonucleic acid
H. and E.	Haematoxylin and Eosin

H.S.A.	human serum albumin
K	Phagocytic index
r.p.m.	revolutions per minute
DH	delayed hypersensitivity
gm	Gram
G	Relative centrifugal force X gravity
i/p	Intraperitoneal injection
i.v.	Intravenous injection
i.u.	International unit
mg	milligram
ml	millilitre
M.S.E.	Measuring and Scientific Co. Ltd., London, S.W.1.
NaOH	Sodium hydroxide
P.B.S.	Phosphate buffered saline
PL-A	Phospholipase A
N.C.S.	Normal chicken serum
LL	Lysolecithin
L.W.	Lung weight
N.D.	Not determined
µl	microlitre
oz	ounce
TCA	Trichloracetic acid
N.S.	Not significant
µg	microgram
v	volts
W	Body weight

mV	millivolt
Ω	Ohm
μV	microvolt
μA	microamp
W/O	Water-in-oil
Wt/v	Weight in volume
i.e.	That is to say
vol.	Volume
~	Approximately
μCi	microcurie
M	Molar
KI	potassium iodide solution
ppt.	precipitate
N.C.T.C.	National Collection of Type Cultures
orgs.	Organisms
u	unit

Greek Letters

α	alpha
β	beta
γ	gamma
λ	lambda

Symbols

>	greater than
<	less than
%	per cent
x	times

B. MATERIALS

Details of the materials used and their suppliers have usually been given with the first mention of each in the methods or results section. Other materials and equipment are listed below.

Adjuvants

Freund's Incomplete Adjuvant (a mixture of the mineral oil Drakeol 6 VR (or Bayol 55), 9 parts, with Arlacel A, 1 part) was prepared in the laboratory as described in the methods section.

Animals

Chickens

The chickens were randomly bred and obtained through Thorber Brothers Ltd., Darvel, Ayrshire, or through one of their agents at Lockwinnoch as six week old chickens. They were fed on 50 per cent intensive growers and 50 per cent layers pellets obtained from the British Oil and Cake Mills (Glasgow Branch). Both male and female chickens of the 606, 404 and 808 strains were used.

Mice

The mice used in the experiments were of the inbred strains, Balb C and Porton, male and female at 3-4 months old. They were all bred by the animal house of the Department of Bacteriology and Immunology.

Arlacel A

A supply of Arlacel was bought from Evans Medical Ltd., Speke, Liverpool. Arlacel A (mannide monooleate) is a product of Atlas Powder Co., Wilmington, Delaware, U.S.A.

Tween 80

A supply was obtained through Thomson Skinner and Hamilton, Ltd., 12 Cadogan Street, Glasgow. Tween 80 (Polysorbate 80) is also a product of Atlas Powder Co., U.S.A.

Mineral Oil

Drakeol 6 VR was obtained from the Pennsylvania Refining Company, Butler, Pennsylvania, U.S.A.

H.S.A.

The Human Serum Albumin used in these experiments was obtained from Behringwerke, Marburg-Lahn, W. Germany; dried and purified, and stored at $4^{o}C$. This was the soluble protein antigen employed principally in most of these studies.

CULTURE MEDIUM

Eagle's medium containing antibiotics (200 iu/ml penicillin, 100 µg/ml streptomycin and 5 iu/ml heparin) was the medium used in most of the tissue culture work reported in this thesis. It was prepared by the Virology Department of Glasgow Western Infirmary.

Hank's medium was used in some of the tissue culture preparations reported in this thesis. This medium was prepared by the Medium Department of the Department of Bacteriology and Immunology, Western Infirmary, Glasgow, and contained the preservatives listed under Eagle's medium.

Gey's solution - this medium was used for most of the lung and peritoneal washings in the experiments using mice. Penicillin (200 µg/ml) and Streptomycin (50 µg/ml) were used as preservatives as prepared and described by Paul (1959).

BACTERIAL CULTURES

M. Tuberculosis (Human type strain C), M. avium (strain D4ER)

Both these preparations were kindly supplied by the Ministry of Agriculture, Fisheries and Food, Weybridge, Surrey, England. They were supplied in a heat-killed and freeze-dried form.

M. fortuitum, M. butyricum, M. tuberculosis (ATCC H37Rv)

These preparations were kindly obtained from Dr. G.P. Kubica, Trudeau Institute Inc., Saranac Lake, N.Y. They were supplied in a heat-killed and freeze-dried form.

M. phlei

This organism was kindly supplied from Dr. I. McKay of this Department and was grown in 1% glucose broth, heat-killed and freeze-dried.

M. smegmatis (ATCC)

This preparation was kindly supplied by Dr. W.B. Redmond, Veterans Administration Hospital, Atlanta, Georgia. This culture was grown in 1% glucose glycerol broth, heat-killed and freeze-dried.

Escherichia coli (communis strain)

This organism was obtained from a clinical culture blood agar plate from the Departmental clinical laboratory, Western Infirmary, Glasgow. This culture was grown on nutrient agar plates, washed three times in sterile saline and heat-killed and freeze-dried.

Anaerobic coryneforms and 'classical' propionibacteria

Following page -

Table 1

Culture collection numbers and sources of strains of anaerobic coryneforms and 'classical' propionibacteria

Strain used	No. in culture collection	Source*	Alternative strain numbers			
			1	2	3	4
C. parvum	10390	1	10390	5936	-	643-C
C. parvum	10387	1	10387	5888	-	-
C. parvum	A	2	-	5888	-	-
C. parvum	B	2	-	5888	-	-
C. parvum	C	2	-	5888	-	-
C. parvum	0208	3	-	-	0208	2683
C. parvum	3085	4	-	-	-	3085
C. parvum	1383	4	-	-	0207	1383
C. parvum	6134	2	-	6134	-	not divulged
C. acnes	737	1	737	-	0389	-
C. acnes	6280	2	-	6280	-	-
C. diphtheroides	814	4	-	-	0200	2764
C. liquefaciens	2764	4	-	6295	0186	814
C. liquefaciens	6290	2	-	6290	-	3044-B
C. granulosum	6290	2	-	6290	-	3024-B
C. lymphophilum	6294	2	-	6294	-	-
C. anaerobium	578	4	-	-	0162	578
P. granulosum	0507	3	-	-	0507	-
P. avidum	0575	3	-	-	0575	-
P. avidum	0589	3	-	-	0589	-
P. avidum	4982	3	-	-	4982	-
P. freudenreichii	10470	1	10470	-	-	-
P. jensenii	5960	5	-	-	-	-
P. arabinosum	8901	5	-	-	-	-
P. rubrum	5958	5	-	-	-	-

*Source :

1. National Collection of Type Cultures, Colindale Avenue, London.
2. Burroughs Wellcome Laboratories, Beckenham, Kent.
3. Professor C. S. Cummins, Virginia Polytechnic and State University, Blacksburg, Virginia, U.S.A.
4. Professor Sebald, Pasteur Institute, Paris, France.
5. National Collection of Industrial Bacteria, Aberdeen, Scotland.

Distilled Water

Glass distilled water was used.

Carbon

Pelikan Ink Manufacturers, Gunther Wagner, Hanover, Germany, with a stock number C11/1431a; this was the same material as that used by Halpern et al., (1963). According to the manufacturer's letter to this Department of December 1968, the particles are homogenous in size and measure 200-500 Å in diameter. A 10% suspension was prepared for the carbon studies used in these experiments. The suspension was dialysed for 48 hours against physiological saline (0.15M NaCl) at 4°C to remove any toxic preservatives present. The suspension of carbon for injections was prepared by diluting in sterile saline containing 1% gelatin (Oxoid, Ltd.) as described by Biozzi et al., (1954), to give a concentration of carbon of 16 mg/ml. The suspension was stored at 4°C and warmed before animal injections.

Iodine 131 or 125

These isotopes were obtained from the Radiochemical Centre, Amersham, England. When needed, they were prepared in small samples of 1 μCi by the Western Infirmary Isotope Department.

Stains

Harris Haematoxylin and Eosin, Giemsa 'R66', Carbol Fuchsin, Fast Blue B, Acridine Orange, Fast Garnet, Oil Red O, Trypan Blue, Fluorescein (FK), and Neutral Red obtained from Gurr Ltd., London; Leishmann's stain from B.D.H., Poole, England.

C. METHODS

INOCULATION, COLLECTION OF BLOOD SAMPLES, SEPARATION AND PRESERVATION OF SERA

(a) Inoculation

Chickens

All bacterial saline suspensions were injected into the chicken intravenously by way of the basilic vein which is a tributary of the bronchial vein in the wing of the bird. All organisms suspended in water-in-oil emulsions were injected intravenously as for the saline suspensions.

(b) Collection of blood samples, Separation and Preservation of sera

Chickens

For the serum needed for estimating antibody levels, the chickens were bled in the same way as for injection and blood was collected from the basilic veins through a needle with a syringe into clean bijou bottles. About 1-2 ml of blood could be collected, proportionately more could be taken if needed. The blood samples were allowed to clot and to contract in a water bath at 37^{o}C for about one hour and then ringed with a sterile application stick to ensure complete clot contraction. The sera were centrifuged and stored in bijou bottles at -20^{o}C.

HISTOCHEMICAL EXAMINATION

For acid phosphatase, N-acetyl-β-D-glucosaminidase and lipids; the tissue was collected in formal calcium fixative containing 4% formaldehyde and 1% anhydrous calcium chloride and fixed for 24 hours at 4°C. Without washing, the tissue was blotted on filter paper and transferred to hypertonic gum sucrose medium (0.88M sucrose containing 1% gum acacia) for 24 hours at 4°C (Holt, 1958). A 5 μ section was then cut using a cold microtome as described by Adamstone and Taylor (1948). The sections were stained as described below and mounted in glycerine jelly on glass slides.

Acid phosphatase

Sections were stained according to Gomori (1952).

Principle: The substrate Na β-glycerophosphate is hydrolysed releasing phosphate groups which immediately combine with lead ions. Lead phosphate is insoluble in water and precipitates in the tissue at sites of enzyme activity. The colourless precipitate is converted to lead sulphide using hydrogen sulphide, which forms a brown or black stain.

N-acetyl-β-glucosaminidase

This enzyme was measured according to the method of Hayashia et al., (1964).

Principle: The substrate Naphthal-AS-B1-N-acetyl-β-D-blucuronide (7-bromo-3-hydroxy-2-naphth-0-anisidine) is hydrolysed releasing naphthol which combines with p-rosaniline at the site of enzyme activity. The reaction product appears red at the site of enzyme activity.

POST-COUPLING METHOD FOR BETA-GLUCURONIDASE*

(after Seligman, Tsou, Rutemburg and Cohen)

(Cold microtome, mounted sections, post-fixed in neutral formalin)

Preparation of Substrate Solution

30 mg 6-bromo-2-napthyl-β-D-glucopyruronoside (glucuronide) was dissolved in 5 ml absolute methanol and added to 20 ml phosphate-citrate buffer (pH 4.95) and 75 ml water.

Method

(1) The sections were incubated for 4-6 hours in the substrate solution at 37^{o}.

(2) After incubation, sections were rinsed in tap water.

(3) Sections were then immersed in a solution of Fast blue B in cold (4^{o}) 0.02M phsophate buffer (pH 7.5). A strength of 1 mg per ml is adequate.

(4) After 2 minutes the sections were removed and washed in two changes of cold distilled water.

(5) Sections were further rinsed in 0.1 per cent acetic acid.

(6) Sections were mounted in glycerine jelly.

Result

A blue or purple colour indicates sites of high β-glucuronidase activity. A red colour may indicate lower activity or the presence of lipid.

*A similar method can be used for β-galactosidase using 6-bromo-2-naphthyl-β-D-galactopyronoide (galactoside) as substrate.

FLUORESCENT ACRIDINE ORANGE TECHNIQUE (Bertalanffy)

This method gives good differentiation of RNA and DNA, although there is doubt as to its absolute specificity. It gives a brilliant red staining of RNA and is excellent for plasma cells and those cells actively synthesizing protein.

Solutions required

(1) N/15 Phosphate buffer pH 6.0.

(2) Acridine orange 0.01 per cent in phosphate buffer, pH 6.

(3) M/1 Calcium chloride differentiator (11.099 g to 100 ml distilled water).

Method

Smears were fixed in ether/alcohol for at least 30 minutes. Tissue sections fixed in an alcoholic fixative (formalin fixed tissue cannot be used) are brought to water.

(1) Hydrate by passing them through 80 per cent, 70 per cent and 50 per cent alcohol for 10 seconds each, and rinse in distilled water.

(2) Treat with 1 per cent acetic acid for 6 seconds, followed by rinsing in two changes of distilled water.

(3) Stain in 0.1 per cent acridine orange for 3 minutes.

(4) Wash in M/15 phosphate buffer, pH 6.0, for 1 minute.

(5) The sections were differentiated in M/10 calcium chloride for 30 seconds.

(6) Sections were mounted in a drop of pH 6.0 phosphate buffer, and sealed with clear finger-nail polish and examined under a fluorescence microscope.

Results: DNA fluoresces ..... Green

RNA fluoresces ..... Red

RAPID ACRIDINE ORANGE FLUORESCENT METHOD

Another method was used in staining (angry macrophage) cells. This method has been described by Riva and Turner (1962) as a 10 second staining method for unfixed cervical smears, which can be used in clinics. This method gives results comparable with the longer Bertalanffy method, but it has not been controlled histochemically. It provides an excellent rapid method for checking preparations or smears for the presence of angry macrophages or cells with an RNA rich cytoplasm.

Method (Riva and Turner, 1962, modified)

Staining solution: 0.025 per cent acridine orange in 2 per cent acetic acid, to which 0.01 per cent merthiolate has been added to prevent the growth of moulds and bacteria.

Technique:

(1) Agitate unfixed or fixed smears for 5 seconds in acridine orange solution.
(2) Differentiate in 2 per cent ethyl alcohol in physiological saline for 2 seconds.
(3) Rinse and then mount in physiological saline, and seal with clear finger-nail polish. This method causes rapid fading and preparations must be studied immediately (good for approximately ½ hour).

Results:

As described above for Bertalaniffy's method.

MEASUREMENT OF SERUM ANTIBODY LEVELS

The Farr test was used. This measures the antigen binding capacity of antisera to antigens. The test is carried out in two stages as described by White et al., (1970).

(a) Isotope labelling

H.S.A. was trace-labelled with radio iodide by the direct oxidation technique of Hunter and Greenwood (1962) by the use of chloramine T and thiosulphate free isotope (IBS-3 iodine or IMS03 iodine 125 from the Radiochemical Centre, Amersham, England). The level of activity used in the Farr test was 0.5 - 1.0 μCi per μg. Experience in the Department has shown that 35 - 40% of radioactive isotope could be attached to the protein.

After addition of sodium metabisulphite and potassium iodide, the free iodide ions were separated from the labelled protein by passing through a 30 cm G 25 Sephadex column, 1 cm in diameter, the radioactive portion being read in a spectrophotometer (M.S.E.) and the strength of the labelling calculated from the standard curve for the particular protein antigen used. The optical density will depend on the protein concentration. (H.S.A. has an optical density of 0.6 at 280 nm wavelength and a concentration of 1 mg / ml).

(b) Estimation of serum antibody levels (ABC 30)

Quantitative estimation of antibody as antigen binding capacity was carried out by a modification of the method of Farr (1958)

(see Campbell et al., 1964). The traced labelled H.S.A. was used at a concentration of 0.4 µg per ml. Tubes containing undiluted test serum and test serum diluted 1 in 5, 1 in 25, and 1 in 125 in normal chicken serum, in 0.1 ml volumes, were set up in duplicates. Subsequently, 0.5 ml of the trace-labelled antigen solution was added to each tube. After the tubes had been kept for two hours, in a refrigerator at 4°C, 0.4 ml ammonium sulphate solution (saturated at 4°C) was added to each. After precipitation had taken place overnight at 4°C, the deposit was spun in a refrigerated centrifuge (4°C) at 850 G for 15 minutes, and washed in 40% saturated ammonium sulphate. This washing procedure was repeated once more, and the final precipitate was suspended in 1 ml normal saline. Four tubes containing 0.1 ml normal chicken serum were included in the test. One pair was treated as were the other sera under test to determine the minimum precipitation of L-131 H.S.A. The resulting precipitate was centrifuged and washed in 1 ml saline. The washed precipitate from all tubes and the supernates from the normal chicken sera and TCA were counted in a Scintillation Counter (Nuclear Chicago Counter).

The radio-active counts of the precipitate were plotted against the serum dilutions on semi-log paper and the dilution of 0.1 ml serum equivalent to 30% precipitation determined (where 100% = total TCA precipitable protein minus the normal serum precipitate). From this the antigen binding capacity (ABC 30 in µg per ml) was calculated.

Preparation of bacterial suspensions in oil

The mineral oil Drakeol 6VR (or Bayol 55) and the emulsifier Arlacel were mixed together at 9 parts by volume of oil to one part of the emulsifier and, after addition of mycobacterium, the suspension was mixed for approximately 2-3 minutes with the MSE ultrasonic probe. A drop of this mixture could be put in water and the drop would remain intact for a few minutes without dispersing. The preparation is ready for injections at this stage.

TISSUE CULTURE METHODS

Collecting peritoneal macrophages

The chicken was killed by air embolism or cervical dislocation, and the mice by cervical dislocation. The ventral site of both was skinned so site of injection of culture medium could be best controlled. The method for the chicken is illustrated in Figure 3. For mice, the animal was laid on its back and 2-3 ml of Gey's medium were injected along the midanterior line. Care must be taken at this stage to avoid the puncture of the gut. When this happened, the animal and syringe plus needle were discarded. The abdomen was kneaded with a finger to detach adhering cells and leucocytes, and the fluid withdrawn by inserting a needle (size 21 gauge) to the left or right flank then pulled sideways to form a pocket of fluid. This prevented the blockage of needle with fat or intestine. The fluid was slowly aspirated and gently transferred to sterile universal (siliconized treated) bottles. The fluid from a number of mice was pooled together,

mixed thoroughly and then counted in a haemocytometer chamber after diluting 1 in 10 with white cell diluting fluid. A concentration of approximately 1 x 10^6 cells was used in each culturing test-tube. Mouse peritoneum cells will grow well in concentrations of 5-25% horse or foetal calf serum or even human serum. Chicken peritoneal macrophages however, did not grow well and would not tolerate the incorporating horse or calf serum and only grew well in homologous chicken serum.

The cell preparations were further distributed as outlined in various experiments for degradation and enzyme studies.

Stain for peritoneal or alveolar preparations

In these studies, Giemsa, Leishmann and Haematoxylin stains have all been successfully used to stain the cells. The fixatives used were Methanol or Carney. Rapid acridine orange has also been used to show the RNA and DNA portions of the cells when examined under the fluorescent microscope.

Histology

The organs studied (usually the liver, spleen, lungs and kidneys) were fixed in 10% formalin (formol saline). For histochemical study the tissues were fixed in formol calcium containing 4% formaldehyde and 1% anhydrous calcium chloride. For the routine histology studies the tissues were embedded in paraffin wax, and for the histochemistry, cold microtome sections were prepared. Sections 5 μ thick were

cut for histochemical study and the specific staining for the enzyme to be identified. Harris haematoxylin was counter-stained with eosin for the routine preparations. Sometimes methyl green pyronin (Pappenheim) was also used.

Preparation of cell walls

Mycobacteria were obtained as outlined in this thesis and treated for 2 weeks in 2% Tween saline solution (Tween 80); this was done to soften the freeze-dried preparations. The bacteria were ruptured using either a Braun mechanical disintegrater (E. Braun, Melsungen, W. Germany) according to the method described by Salton and Horne (1951), or by ultra-sonic rupture of Tween-Saline-treated cells.

Between 20-30 gm of the treated bacterial cells (wet weight) were suspended in ice-cold distilled water, placed in a metal homogeniser bottle with an equal volume of Ballotini beads (0.17-0.18 mm diameter) and disrupted at 4000 rpm in the Braun's disintegrater. The bottle was kept cooled with liquid carbon dioxide. Loss of acid-fastness was taken as a measure of the degree of cell rupture. Each preparation was observed with the electron-microscope to determine how well the cell walls were lysed.

The disintegrated bacterial material was then suspended in distilled water, filtered (using a sintered glass filter, porosity grade No. 2) to remove the glass beads and the cell fractions separated by centrifugation as described by Salton (1964). Unbroken cells were removed by spinning at 3-4000 X g for 10 minutes. The supernatant was

removed and centrifuged at 10,000 X g for 30 minutes to deposit the cell walls which were washed three or more times in distilled water.

Purified of cell walls

Purified cell walls free of cellular material were prepared by digesting the above crude cell wall fraction with Pronase (Calbiochem-Ltd., London) added to a concentration of 1 mg/ml in saline (0.15M NaCl) and incubated at pH 8.0 for 2 hours at 56°C. The cell walls were then washed three times in distilled water before use in experiments. 0.1% sodium azide was added as preservative.

EXTRACTION OF ENZYMES

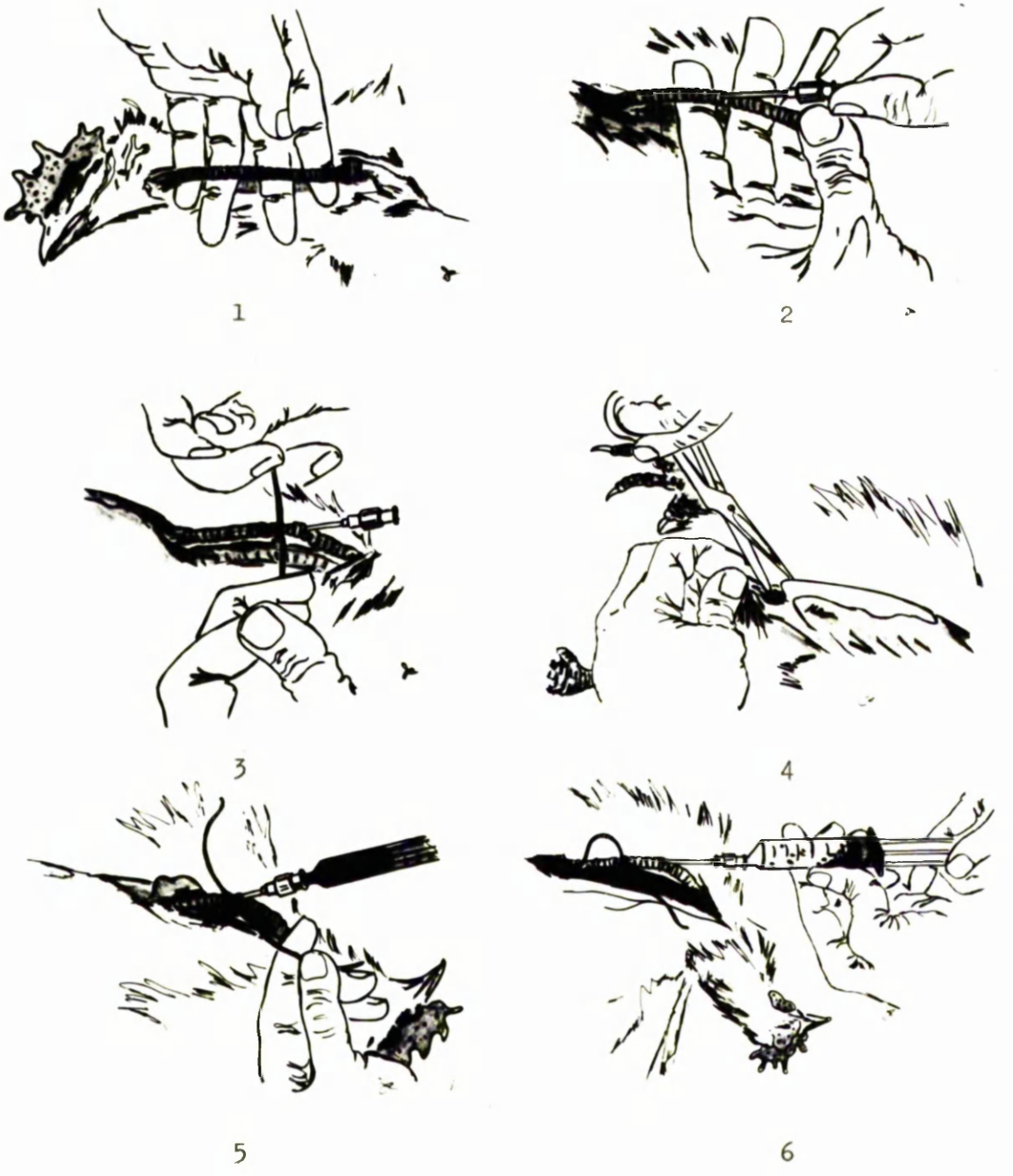
Whole lung homogenates:

The lungs were removed immediately after death, weighed and placed in chilled isotonic saline or 0.25M sucrose. A W/V (4 g lung, 25 ml PBS) preparation was made and homogenised, and filtered through surgical gauze. The preparation was frozen and thawed five times and to ensure lysosome lysis, Triton X-100 (0.1%) was added. The homogenate was allowed to stand on the bench for five minutes to ensure complete lysis of lysosomes. It was then centrifuged to clarify it before aliquots were removed for enzyme assays.

Lung wash exudates:

The lung exudates were collected as outlined and illustrated

Fig. 2



Collecting chicken alveolar macrophages

This diagram illustrates the steps in collecting alveolar macrophages, (1) exposing the trachea, (2) inserting special needle into trachea (this needle has had its bevel removed and two extra holes added to alleviate blockage), (3) needle secured in trachea with twine, (4) opening of the abdomen to allow inflation of lung when introducing fluid for washout, (5) injecting fluid into lung, withdraw and inject several times to free macrophages from alveoli, (6) withdrawal of fluid wash from lung.

Fig. 3.

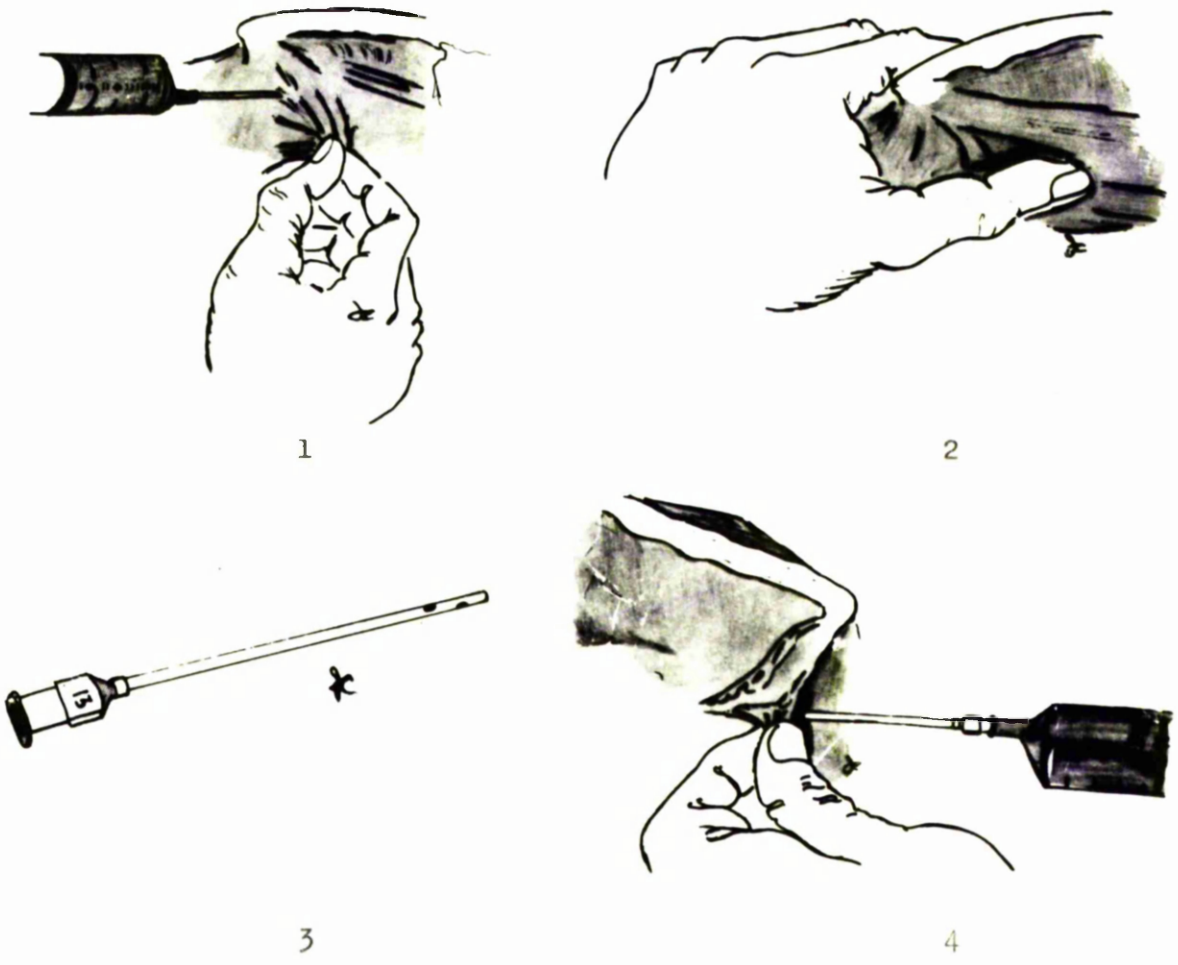


Diagram illustrating collecting peritoneal macrophages in the chicken. (1) injecting 50 ml culture media into peritoneal cavity, (2) kneading of abdomen to allow media to wash free macrophage from the gut, (3) special aspiration needle (with bevel removed and two added holes to alleviate blockage during aspiration of fat or gut), (4) aspirating peritoneal wash exudates with special needle.

under Methods Figure 2 for collecting lung macrophages. After collecting the exudate, counts were made to determine the number of cells per mm^3. Triton X-100 (0.1%) V/V was added to the cells and the mixture allowed to stand on the bench for 5 minutes as outlined under whole lung homogenate preparation. Aliquots were removed for enzyme assays.

TECHNIQUE FOR PREPARING MACROPHAGE CULTURES FOR HEAT-KILLED BACTERIAL DEGRADATION STUDIES

The majority of *in vitro* studies reported in this thesis used a technique described by Jenkins and Bernacerraf (1960) for bactericidal studies. A known number of phagocytes was mixed with a suspension of bacteria in the ratio of 100 macrophages to 10 bacteria (Jenkins and Benacerraf, 1960; Jenkins, 1963).

In this study, the ratios of bacteria to macrophages were approximately the same as those reported by Jenkins. Glass slides were placed in empty petri dishes and these were flooded with the macrophage bacterial suspension. The plates were incubated at 37°C for 1 hour, with occasional agitation. The slides were then removed carefully from the petri dishes, washed gently with sterile saline to remove all non-adhered macrophages and free bacteria. Individual slides were coated with a thin film of Formvar as described by Suter (1952). The preparations were placed in individual siliconised (4 x ½ inch) culture tubes. 25% chicken serum in Eagle's medium (saturated with 5% CO_2) was

added and the tubes immediately stoppered and returned to the 37°C incubator.

Individual slides were removed at timed intervals, fixed in absolute ethanol and stained by Gram, Leishman, or Wright's stain. Counts were made of 100 phagocytic cells and the number of these cells containing stainable organisms recorded. Therefore, the rate of degradation could be assessed by the number of cells showing stained bacilli at any given time.

TECHNIQUE FOR PREPARING MACROPHAGE CULTURES FOR VIABLE E. coli DEGRADATION STUDIES

In the limited E. coli experiments viable organisms were exposed to chicken lung phagocytic cells. The ratio of bacteria to macrophages was again approximately 10 to 100. A series of siliconized culture tubes (size 4 x ½) were set up containing 0.5 ml of the phagocyte-bacterial suspension in 25% chicken serum Eagle's medium (Saturated with 5% CO_2), and stoppered immediately. The tubes were incubated at 37°C. Individual tubes were studied at 10 minute intervals to provide information on bacterial degradation over a 1 hour period. One aliquot from each culture tube was used for degradation studies by counts of stainable organisms per 100 phagocytic cells. A second aliquot of cells was lysed in H_20 and viability counts made by inoculation onto blood agar plates.

TECHNIQUE FOR FLUORESCEIN-LABELLING OF CELL WALLS FROM VARIOUS MYCOBACTERIA

The method used is one designed by Dr. Ian McKay of this Department. Cell walls of the following organisms were labelled as was an inert carrier-Sephadex (grade 25).

Cell walls:	M. fortuitum, M. avium, M. phlei, M. smegmatis and M. tuberculosis H37Rv.
Inert material:	Superfine Sephadex G25.

To one volume of the above materials (wet) was added 1 vol H_2O, and one vol of saturated $NaHCO_3$ (freshly prepared). To each 10 ml of the resulting suspension was added 1 ml aminofluorescein (crude) 20 mg/ml in very dilute alkali, and 1 ml cyanuric chloride, recrystallized from CCl_4 and dissolved in anhydrous dioxan, 10 mg/ml. The mixture was allowed to react for 45 minutes and the labelled products were sedimented by centrifugation and washed repeatedly with $NaHCO_3$ solution and water.

Note:

Cellulose preparation only:

Cellulose powder was broken up by sonication for 2.5 hours with the Daw soniprobe at full power. The final particle size was approximately 10-25 μ. This was labelled by the above method but with the omission of the 1 vol H_2O, as the wet packed suspension was rather dilute when judged by its low viscosity.

SLIDE CHAMBER PREPARATIONS (macrophages and bacteria for degradation studies)

The method developed was described by Schrek and Rabinowitz (1962). A sterile cover glass, 50 x 75 mm was covered with the top of a petri dish to prevent contamination and evaporation during the preparation of the slide. In the centre of the cover slip was placed a metal plate, 40 x 40 x 0.85 mm, with a central hole 26 mm in diameter. A thin rectangle about 12 x 15 mm was drawn with white petrolatum on the cover glass inside the whole of the metal plate; 0.2 ml of the cellular suspension was placed inside the petrolatum ring, which prevented excessive spreading or movement of the fluid. After 5% CO_2 enrichment for 5 minutes in a sealed wet box, the preparation was incubated for one hour to allow the phagocytosis of organisms and to allow cells to adhere to the glass. After incubation the preparation was washed with Eagle's medium without disturbing the petrolatum. After replacing the medium with CO_2 enriched medium containing 25% homologous serum, another cover glass, 40 x 50 mm, was gently lowered on the drop of suspension. The drop filled the petrolatum ring and the space between the two cover slips. The preparation was sealed with freshly melted yellow wax (beeswax) and incubated at $37^{o}C$.

The cells in the preparation settled rapidly on the lower cover glass. It was therefore necessary to invert the slide chamber when used with the phase-microscope, and for making viable counts. Since the culture preparation was fluorescent-labelled the microscope was equipped as outlined under fluorescence studies.

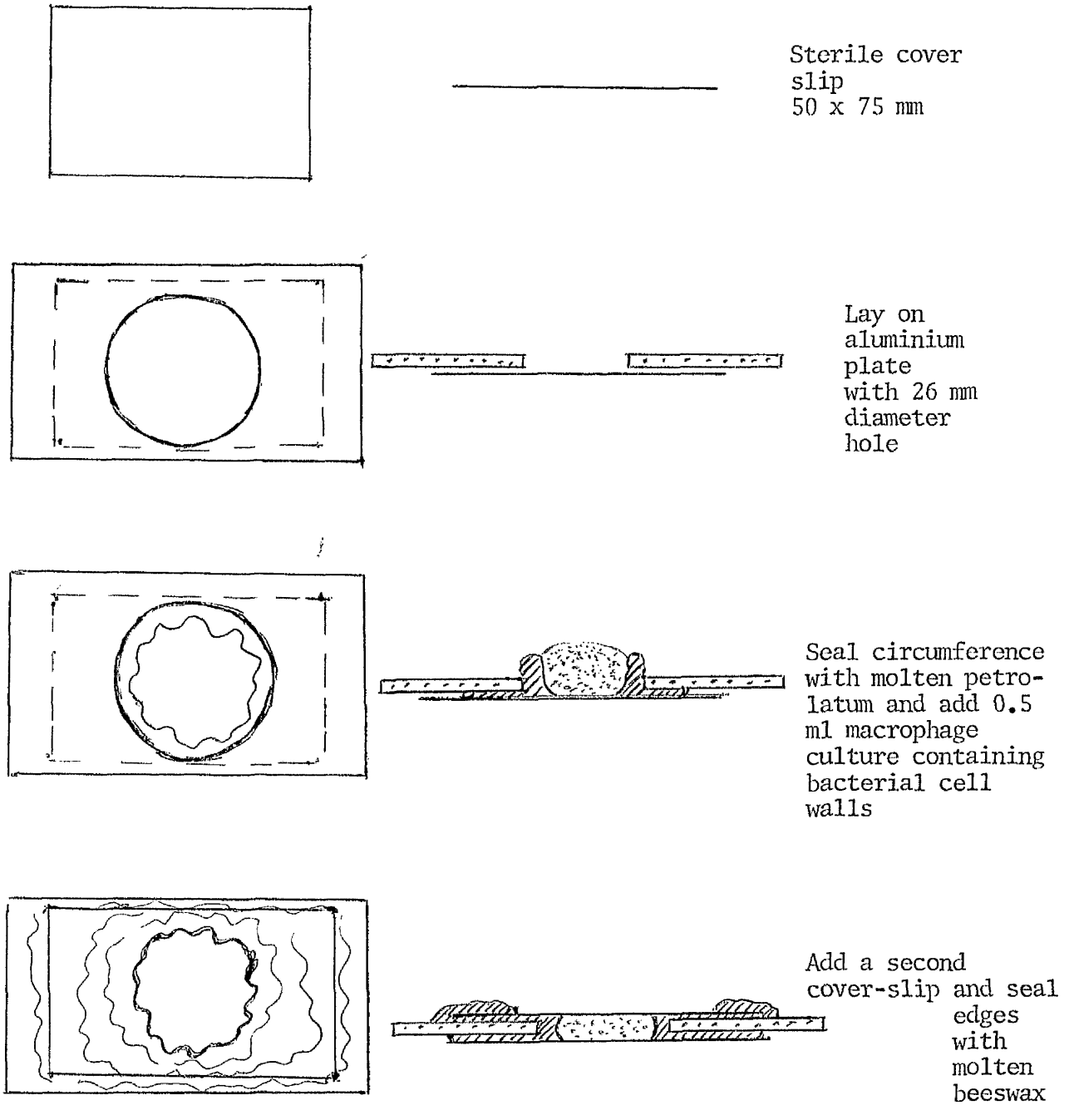


Fig. 4 Preparation of macrophage cultures for digestion studies, after the method of Schrek and Rabinowitz (1962)

CONDITIONS FOR FLUORESCENCE MICROSCOPE STUDIES

(a) Fluorescence (flow of light through Leitz Ortholux microscope) using Incident illumination. Mercury vapour HBO 200 lamp light source. Primary filters (known as excitation filters) 4 mm BG 38, KP 490, interference filter, plus KP 490 built in Ploem illuminator (position 3). Secondary or barrier filters, K 510.

(b) For phase contrast

source (tungsten lamp)
↓
phase condenser
↓
specimen
↓
phase objective

Note: (phase studies can be used at the same time as fluorescent, through Ploem illuminator)

(c) Degradation measurements of fluorescence

(Also use stabilized light source XBO 100-Xeon lamp for measurements of fluorescence for degradation studies).

For measurements:

Source
↓
primary filters
↓
specimen
↓
Barrier filter

eyepiece

photomultiplier tube through variable sized diaphragm
Output to voltmeter
(voltage $\propto$ light intensity)

Preparation of ^{131}I labelled Mycobacterial cell walls for studies of the rate of digestion using whole body counts in chickens

This method was developed by Dr. I McKay of this Department. Cell walls of M. tuberculosis (human strain C) and M. avium strain D4ER were prepared by sonication. These organisms (freeze-dried), had been treated by soaking for 2-3 weeks in 2% Tween saline broth in order to soften the cell walls. This treatment did not affect the composition of the cell wall as confirmed by electromicroscope observations. The lysed cell walls were cleansed of cellular material by treating with Pronase as outlined, in methods of preparing cell walls.

Labelling with m-aminophenol to facilitate attachment of Iodine

10 ml cell wall suspension (6 mg/ml dry weight in water)
8 ml saturated $NaHCO_3$
1 ml m-aminophenol (1 mg/ml in water)
1 ml cyanuric chloride (1 mg/ml in anhydrous dioxan) (freshly made up)

The above reagents were mixed at room temperature in the order shown and allowed to react for 1.5 hours with occasional agitation. The cell walls were sedimented by centrifugation, resuspended in water and sedimented once again. The same process was carried out with Whatman microgranular cellulose powder (mean particle size 10-25 μm).

Attachment of radiolabel

Each 60 mg sample of cell wall or cellulose with aminophenol attached was suspended in 5 ml of 0.1M phosphate buffer pH 7.5 with

gentle sonication. To each sample was added 33 μCi ^{131}I as Iodide in the same buffer (0.5 ml) and 2.5 mg chloramine T in 0.5 ml buffer. The samples were mixed and left at room temperature for 15 minutes before the reaction was stopped by adding 2.5 mg sodium metabisulphite in 0.5 ml buffer. The suspensions were diluted to 25 ml with water, and sedimented by centrifugation (10,000 g for 30 minutes), resuspended in dilute KI solution, again sedimented and resuspended in 10.0 ml saline for injection.

From the readings obtained by counting the emissions from 1 chicken dose of each injection mixture, it would seem that the three preparations were equally well labelled; the injection mixtures were counted on day 3 in the same whole-body counter as was used for the chickens, and with the samples placed in about the same position at the centre of the chickens. The counts per 10s were:

			Mean-background
M. tuberculosis			
Strain C	161847,	163617	162208
M. avium	151342,	152403	151324
Cellulose	155695,	157267	155957
Background	527,	520	

TEST FOR ADJUVANT ACTIVITY OF PHOSPHOLIPASE A (BEES VENOM)*

METHOD OF PREPARATION

These experiments were carried out on 6 week old chickens. Periodic blood samples were collected from a vein in the wing for antibody studies. In three gorups of chickens the injections were as follows: (all injections were i.v.)

Group	
I	W.O.W. 100 µg H.S.A.
II	W.O.W. 20 µg phospholipase A + 100 µg H.S.A.
III	W.O.W. 200 µg phospholipase A + 100 µg H.S.A.

One bird from each group was killed (blood sample collected before death) at day 6, 12 and 49, for antibody titres levels.

Note: Some of the experiments for phospholipase A adjuvant activity studies were modified with various doses of PLA.

METHOD OF ASSAY FOR PHOSPHOLIPASE A

(A) FIRST EXPERIMENT

Working preparation	ml incubated/test
1. 200 mg egg lecithin/20 ml H_2O	2 ml
2. NaCl 1.0M	1 ml
3. Na deoxycholate 0.01M	1 ml
4. $CaCl_2$ 0.01M	1 ml
5. H_2O (glass distilled)	2 ml
6. Tissue homogenate	1 ml
Total	8 ml

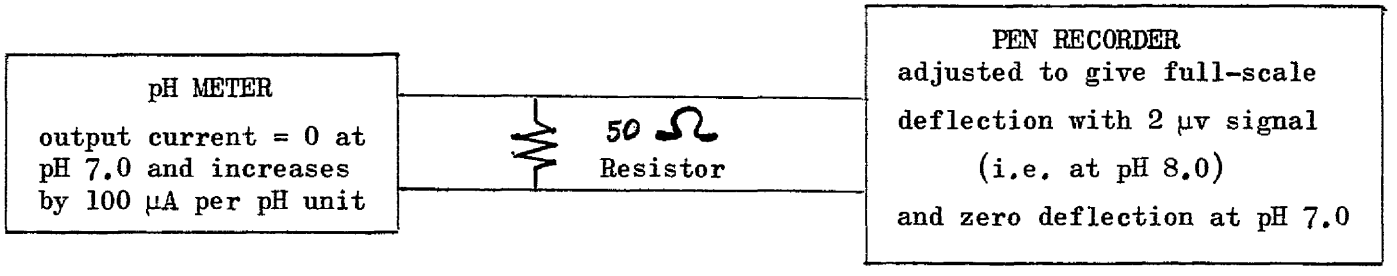
A mixture of materials 1-5 above was adjusted to pH 8.0 at room temperature. The tissue was added and immediately the universal container was sealed (this bottle was fitted with a hole to insert the pH meter electrode through, with a tight fitting seal). This was done in order to maintain a stable atmosphere which can be controlled by adding a little Nitrogen gas.

The fatty acids liberated were titrated with 0.0806M NaOH back to pH 8.0 after allowing one hour reaction at room temperature. This acidimetric assay utilized a Pye Unicam (Pye Model No. 291) expanding scale pH meter equipped with Pye Unicam (KOH) Eo Value (Eo7) electrodes. The meter was coupled by decade resistances (50 Ω) to a Unicam AR 25 linear recorder. By varying the coupling resistance, the recorded pH span could be altered to 0.5 or 1.0 pH full scale deflection.

pH for phospholipase A assay

For the change in pH in all experiments, except experiment 42, the figure quoted is the average rate of change in pH within the range pH 8.0 - 7.9. (The liberation of fatty acids lowers the pH, and back titration with NaOH to original pH 8.0 to determine the amount (LL) of fatty acids liberated over a set period of time).

Fig. 5 SCHEMATIC DIAGRAM OF CONDITIONS EMPLOYED FOR ASSAY OF PHOSPHOLIPASE A



Method for adjuvant activity of phospholipase A (PLA)

(B) Second experiment

12 to 16 week old hens were divided into four groups as follows, and injected in the left breast muscle with 0.5 ml of W/O emulsion containing the active ingredients shown, mixed together in the aqueous phase (except M. avium):

Group	No. of hens	Ag and adjuvant dose per bird
1	4	40 µg H.S.A.
2	6	40 µg H.S.A. + 10 µg PLA (in aqueous phase)
3	6	40 µg H.S.A. + 400 µg PLA (in aqueous phase)
4	4	40 µg H.S.A. + 5 mg M. avium D4ER (in oil phase)

All emulsions were made up of the basic components listed below:

0.5 ml emulsion — 0.25 ml aq. phase (HSA + PLA in saline)

0.25 ml oil phase — 0.2 ml Drakeol 6 VR; 0.05 ml Arlacel A; Mycobacterium if any

*BEE VENOM PHOSPHOLIPASE A

Assayed in terms of activity. The solution at a concentration of 320 µg/ml had activity of 400 µ moles per minute per ml at 25°C; i.e. 400 i.u. (bee venom) per ml, thus the dose received by each chicken was 50 i.u. and 500 i.u.

ACID PHOSPHATASE

PRINCIPLE: Phosphatases hydrolyse phenyl phosphate at the appropriate pH to phenol and inorganic phosphate. The phenol liberated is measured using Folin & Ciocalteau's reagent.

SOURCE: Gutman, A.B., Gutman, E.B. (1940) J. Biol. Chem. 136, 201. Varley, 4th Ed. p.461.

REAGENTS

1. Substrate. Disodium phenyl phosphate 0.01M 2.18 g/litre (store in fridge).
2. Citrate buffer pH 4.9 at room temperature.

   Citric acid 42 g)
   N NaOH 376 ml) to 1 litre

   Check pH and adjust if necessary with NaOH or HCl. Store in fridge.
3. Buffer substrate. Equal volumes buffer and substrate mixed before use.
4. Folin and Ciocalteau phenol reagent. Before use, dilute 1 volume with 2 volumes of water. Avoid contamination from deposits by rinsing with deionised water at the neck of the bottle.
5. Sodium carbonate 20 g / 100 ml
6. Stock phenol 1 mg / ml
7. Working phenol - 10 µg / ml. Dilute stock phenol 1 in 100 (1 ml = 1 mg phenol).

METHOD

1. 4 ml of buffer substrate (or 2 ml buffer + 2 ml substrate) Add 0.2 ml test sample. Incubate 60 min. at 37°C.

2. Add 1.8 ml dilute Folin reagent to "test" to stop reaction. Mix and centrifuge.

3. To a "control" tube add 4 ml buffer substrate, 0.2 ml serum, followed immediately by 1.8 ml Folin reagent. Mix and centrifuge.

4. To 3 ml supernatant and to a reagent blank (0.9 ml dil. Folin reagent and 2.1 ml water) add 1 ml sodium carbonate. Incubate at 37°C for 10 min.

5. Read on UNICAM SP 1800 at 680 nm (red photocell) against reagent blank.

6. Calculate OD (Test-Control) and read result off calibration curve.

CALCULATION. 1 King Armstrong (Gutman) unit for acid phosphatase is equivalent to the liberation of 1 mg phenol (from 100 ml serum) in 60 min. (pH 4.9 and 37°C).

$$\text{Phosphatase activity} = \frac{\text{g phenol}}{} \times \frac{6}{3} \times \frac{100}{0.2} \text{ K.A. units/100 ml}$$

$$= \mu\text{g phenol K.A. units/100 ml}$$

CALIBRATION CURVE. Set up tubes as follows:

K.A. units/100 ml	5	10	15	30
ml dil. phenol	0.5	1.0	1.5	2.0
ml water	1.6	1.1	0.6	0.1

To each tube add 0.9 ml dil. Folin reagent, 1 ml Na_2CO_3, incubate and read as before against reagent blank.

LACTIC DEHYDROGENASE

PRINCIPLE: Pyruvate is converted to lactate by reduced nicotinamide adenine dinucleotide (NADH) in the presence of lactic dehydrogenase.

NADH absorbs light at 340 nm, whereas NAD does not. The decrease in optical density at 340 mu is therefore a measure of the consumption of $NADH_2$.

SOURCE: Wroblewski, F., La Due, J., Proc. Soc. Exptl. Biol. Med. (1955) 90, 210 (modified). Varley, H. Practical Clinical Biochemistry 4th Ed. 1967, p. 278.

REAGENTS

1. Phosphate buffer 0.1M pH 7.4 $\pm$ 0.05 at 25°C.

   13.97 g anhydrous dipotassium hydrogen phosphate K_2HPO_4)
   2.69 g anhydrous dihydrogen potassium phsophate K_2PO_4) to 1 litre
   pH must be as accurate as possible

2. Reduced nicotinamide-adenide dinucleotide (NADH) 2.5 mg/ml
   Add 2 ml phosphate buffer to 5 mgNADH (Sigma disodium salt Grade III)
   The solution can be kept deep frozen for 48 hours.

3. Sodium pyruvate 2.5 mg/ml 0.125 g sodium pyruvate/50 ml to be made up freshly once weekly.

METHOD

1. Incubate in water bath set at 25°C and read in UNICAM SP 1800 using deuterium lamp. Set to fixed wavelength setting at 340 nm.

The spectrophotometer was connected to a UNICAM AT 25 linear recorder with expansion x 2 and chart speed 30 mm/min.

2. To test (and blanks) was added: 2.8 ml phosphate buffer
   0.1 ml test preparation
   (mixed well)

3. To tests only; add 0.1 ml NADH and mix. Leave for 10 min in water bath at 25°C to allow temperature equilibration and reduction of endogenous keto acids.

4. Using water or reagent mixture for blank, 0.1 ml sodium pyruvate was added and mixed. 0.1 ml sodium pyruvate was then added to tests and mixed immediately (in cuvette) in UNICAM SP 1800, and chart roll on external recorder was started and recorded for at least 5 min.

5. Measure temperature of solution in cuvettes (to 0.2°).

6. Draw the best straight line through the first few points on the recorder chart, extend it over 5 min. (15 divisions at a chart speed of 30 mm/min. Activity should be linear over the whole five minutes, but at high levels the NADH is used up too rapidly and only the first points will be linear, so the steepest line is taken. If the recording is not linear over 5 min. at normal levels, check temperature, pH, reagents, etc.

CALCULATION

$$\text{Reading of 100 on chart recorder} = \frac{2.0}{\text{expan. factor}} \quad \text{OD}$$

i.e. R (chart reading) = $\frac{R}{100} \times \frac{2.0}{2}$ OD (for expan. factor of 2)

E (extinction) = $\frac{R}{100}$

1 Iu of enzyme activity acts on 1 µM substrate/min under specific conditions.

1 µM DPNH/ml solution has extinction 6.22 (using 1 cm light path).

Here volume solution is 3.1 ml, containing 0.1 ml test preparation.

Therefore L.D.H. activity = $\frac{\Delta E/5 \text{ min}}{5} \times \frac{1}{6.22} \times \frac{3.1}{1} \times \frac{1000}{0.1}$

= $\Delta E/5$ min. x 1000 Iu/litre

or $\Delta R/5$ min. x 10 Iu/litre

NOTES

1. At high levels of activity, measure ΔR over 2 min, multiplying answer x 5/2.
2. If the OD of the test at the beginning is less than 0.45, use a fresh vial of DPNH.
3. Temperature of reaction is critical, and must be measured each time and noted on the chart. If the temperature is not exactly 25.0°C, multiply answer by temperature factor below, extrapolating between points.

T°C	22	23	24	25	26	27	28
Factor	1.26	1.17	1.08	1.00	0.93	0.86	0.80

Conditions and manipulations after incubation for assaying the various mammalian glycosidases - Tables 2 and 3.

Table 2 : INCUBATION CONDITIONS FOR ENZYME ASSAYS CARRIED OUT AT 37°C or 25°C (LDH)* for 1-2 hrs

Enzyme	Assay sample vol used (ml) during incubation	Substrate Added	ml/test	Buffer Added	ml/ test	pH	Reference
Acid phosphatase	0.2	Disodium Phenyl Phosphate	2.0	Citrate	2.0	4.9	Gutman and Gutman (1940) J. Biol. Chem. 136, 201
Cathepsin D	0.2	Denatured Hemoglobin 2.5%	2.0	Citrate 0.04M	2.0	3.2	Anson (1938) J. Gen. Physiol. 22, 79
α-mannosidase	0.2	p-Nitrophenyl-α-monophosphate (6 mM)	0.15	Citrate 0.05M	1.25	5.0	Conchie and Hay (1963) Biochem. J. 87, 354-361
β-N-acetylglucosaminidase	0.2	p-Nitrophenyl-N-Acetyl-β-glucosaminide 0.01M	0.15	Acetate 0.05M	1.25	5.0	Conchie and Hay (1963) Biochem. J. 87, 354-361
β-glucuronidase	0.2	p-Nitrophenyl-β-D-glucuronide 0.01M	0.15	Citrate 0.05M	1.25	5.0	Levvy, G.A. (1952) Biochem. J. 52, 464
β-galactosidase	0.2	p-Nitrophenyl-β-D-galactoside 0.01M	0.15	Citrate 0.05M	1.25	5.0	Cohn et al., (1952) J.Biol.Chem. 195, 607-14
Lactic acid dehydrogenase	0.2	DPNH (reduced diphosphopyridine nucleotide) 2.5 mg/ml	0.1	Phosphate 0.10M	2.8	7.4±	(Modified) Wroblenski, F. (1955) J.Proc.Soc.Expl.Biol.Med. 90, 210

Table 3 : ANALYTICAL PROCEDURES FOR ENZYME ASSAYS

Enzyme	Manipulations (after incubation)	Analysis
Acid phosphatase	Add 1.8 ml Folin and Ciocalteau phenol reagent and centrifuge. To 3 ml of supernatant add 1 ml 20% Na_2CO_3, and dilute to 10 ml with water, incubate 10 min.	Colorimetric for phenol at 680 nm
Cathepsin D	Nothing more (read every 5 min for a 30 min period)	Colorimetric change during haemoglobin digestion at 660 nm
α-mannosidase	Add 2 ml 10% TCA, centrifuge, add 1 ml of glycine-NaCl-Na_2CO_3 mixture, pH 10.7 to supernatant and mix. Incubate at 37°C for 5-10 min.	Colorimetric for nitrophenol (420 nm) in glycine-sodium carbonate buffer, pH 10.8 (0.078M in glycine)
N-acetyl-β-D-glucosaminidase		Do
β-D-galactosidase		Do
β-D-glucuronidase		Colorimetric for nitrophenol (420 nm or 540 nm)
Lactic acid Dehydrogenase	To test only add 0.1 ml DPNH and mix, leave for 10 min in water bath at 25° or at room temperature (to allow temperature equilibration and reduction of endogenous keto acids)	Colorimetric at 340 nm. Add 0.1 ml sodium pyruvate (2.5 mg/ml) mixture to test mixture. Record on external recorder for at least 5 min. Measure temperature of solution in cuvettes (to 0.2°)

PART THREE

EXPERIMENTAL RESULTS

A. INTRACELLULAR DEGRADATION OF ESCHERICHIA COLI BY STIMULATED AND NON-STIMULATED CHICKEN ALVEOLAR AND PERITONEAL MACROPHAGES: *in vitro* STUDIES

PURPOSE:

To determine the rate of digestion of heat killed *E. coli* by normal and stimulated chicken alveolar and peritoneal macrophages *in vitro*.

In these experiments, lung and peritoneal exudates were collected from 5-6 week old chickens. One group had been stimulated with 6 mg of heat-killed *Mycobacterium avium* (it had been previously found in other experiments that 6 mg dry weight was the optimal dose for stimulatory purposes) intravenously. The second group, a control group, received intravenous injections of saline and peritoneal exudates were collected as described in Methods. After collecting the cells from the lung and peritoneum, the numbers were adjusted to 10^6 cells per ml, and mixed with a culture *E. coli* or with a heat-killed preparation of *E. coli* in ratio of 10 bacteria per 100 macrophages.

The mixtures dispersed in 0.5 ml aliquots on glass slides (25 x 75 mm) were incubated in petri dishes at 37°C for 1 hour with occasional agitation to allow phagocytosis to take place. After incubation, the free bacteria and macrophages that had not adhered to the slide were washed from the slides. The slides were then flooded with Eagle's medium containing 25% (v/v) chicken serum saturated with 5% CO_2. Degradation studies were done over the following 180 minutes, incubating the preparations at 37°C.

The number of macrophages per 100, containing stainable E. coli were counted. The rate of degradation was measured by the number of stainable organisms at any given time.

The results of the above experiments are shown graphically in Figures 6 and 7. Figure 6 shows the degradation of heat-killed E. coli organisms by normal and stimulated alveolar and peritoneal macrophages of the chicken. A marked difference is seen in the degradation ability of the two groups of normal and stimulated macrophages, regardless of whether the cells were derived from alveolar or peritoneal exudates. It is also clear that although lung macrophages possess digestive abilities, the peritoneal macrophages have superior degrading abilities. These findings were observed with both normal and stimulated cells.

Figure 7 shows the degradation rate of live E. coli organisms by normal and stimulated alveolar and peritoneal macrophages of the chicken. The viable E. coli studies confirm the findings for heat-killed E. coli (Fig 6): that normal and stimulated peritoneal macrophages have a greater capacity for degradation of E. coli than alveolar macrophages.

CONCLUSIONS

1. Peritoneal macrophages from normal chickens degrade E. coli more rapidly than alveolar macrophages.
2. Peritoneal macrophages from M. avium stimulated chickens also degrade E. coli more rapidly than alveolar macrophages.

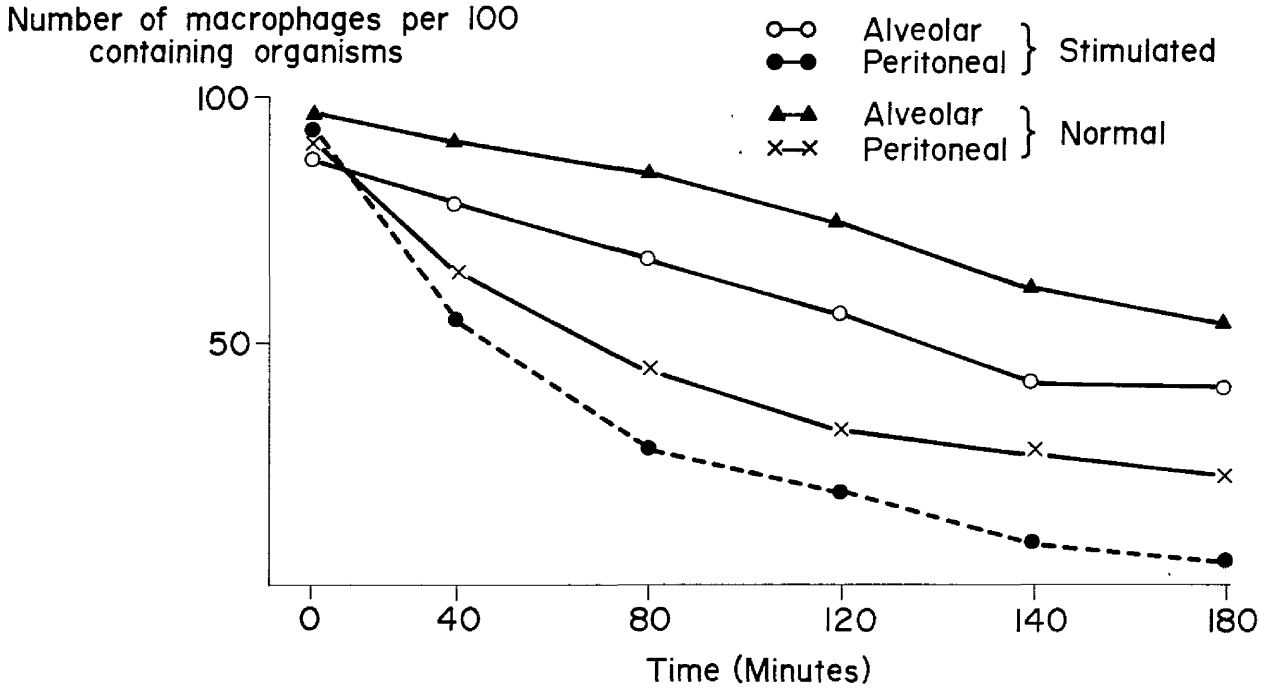


Fig 6: DEGRADATION OF ESCHERICHIA COLI (HEAT KILLED) BY NORMAL AND STIMULATED CHICKEN MACROPHAGES

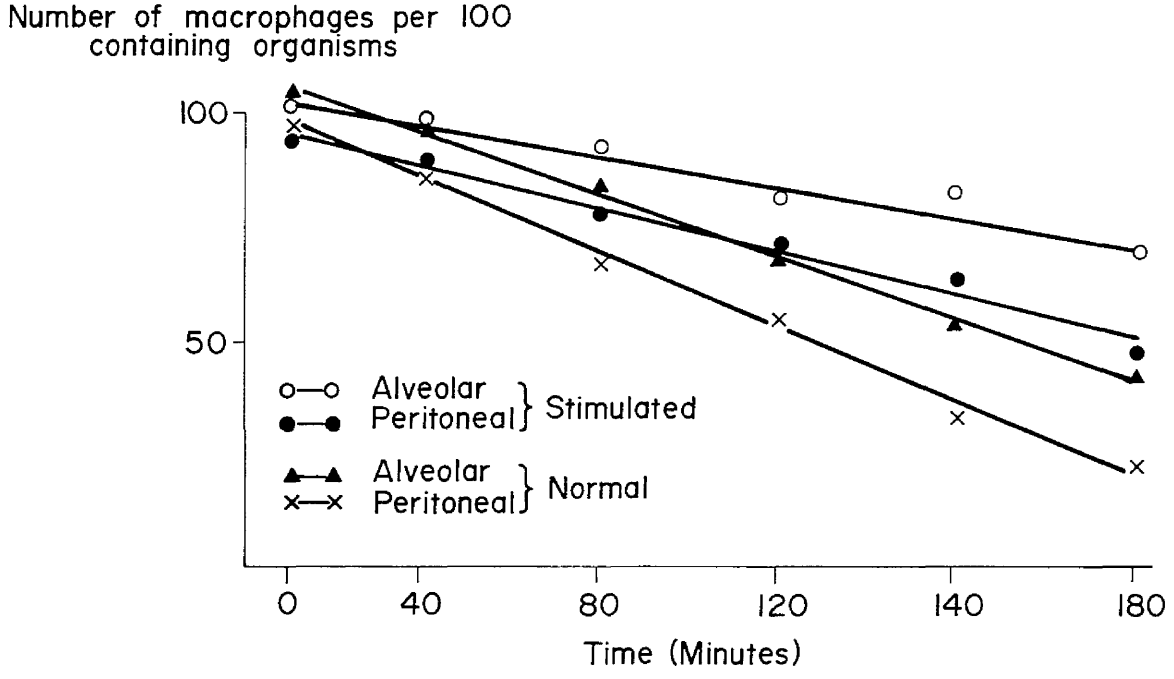


Fig 7: DEGRADATION OF E.COLI (VIABLE) BY NORMAL AND STIMULATED CHICKEN ALVEOLAR AND PERITONEAL MACROPHAGE

B. (i) INTRACELLULAR DEGRADATION OF VARIOUS ACID-FAST ORGANISMS BY ALVEOLAR MACROPHAGES OF NORMAL AND *M. avium* STIMULATED CHICKENS

(ii) SPECIES DIFFERENCES IN DEGRADATION RATE DEMONSTRATED WITH NORMAL CHICKEN AND NORMAL MOUSE MACROPHAGES

These experiments were designed to determine the difference in digestion time between human pathogenic and seprophytic heat-killed acid-fast organisms by macrophages from normal and *M. avium* stimulated chicken lung washings. Species differences in degradation between normal chicken and mouse lung macrophages will be studied.

In these experiments macrophage cultures were incubated with various heat-killed acid-fast organisms. The preparation of these cultures is described in the methods section.

These preparations were set up using flying cover slips (7 x 22 mm) and the method used for the comparison of intracellular bacterial digestion was a modification of the method of Suter (1952), developed in his study of multiplication of mycobacterial organisms intracellularly.

This experiment made use of the ability of *Mycobacteria* to retain their acid-fast property whether viable or dead, provided the cell wall structure had not been altered. In this investigation, the numbers of both acid-fast and non-acid-fast mycobacteria ingested by macrophages were recorded to establish the number of organisms with degraded cell wall structure.

Initial counts of ingested bacteria were made after 1 hour of incubation at 37°C and recorded as the determination for day 0. Ingested organisms were similarly enumerated on consecutive days for a 21 day period.

The experimental designs of part (i) and (ii) are described below:

(i) Cultured alveolar macrophages from normal and M. avium stimulated chickens. The following mycobacteria were used in this series of studies:

Saprophytic	M. fortuitum
	M. phlei
	M. smegmatis
Pathogens	M. avium
	M. tuberculosis (strain C)
	M. tuberculosis (H37Rv)

(ii) Cultured alveolar macrophages from normal mice and normal chickens; the following mycobacteria were used in this series:

Saprophytic	M. phlei
	M. fortuitum
Pathogens	M. avium
	M. tuberculosis (H37Rv)

RESULTS Part (i)

Out of counts of 100 macrophages the percentage containing stainable organisms is tabulated in Figures 8 - 13.

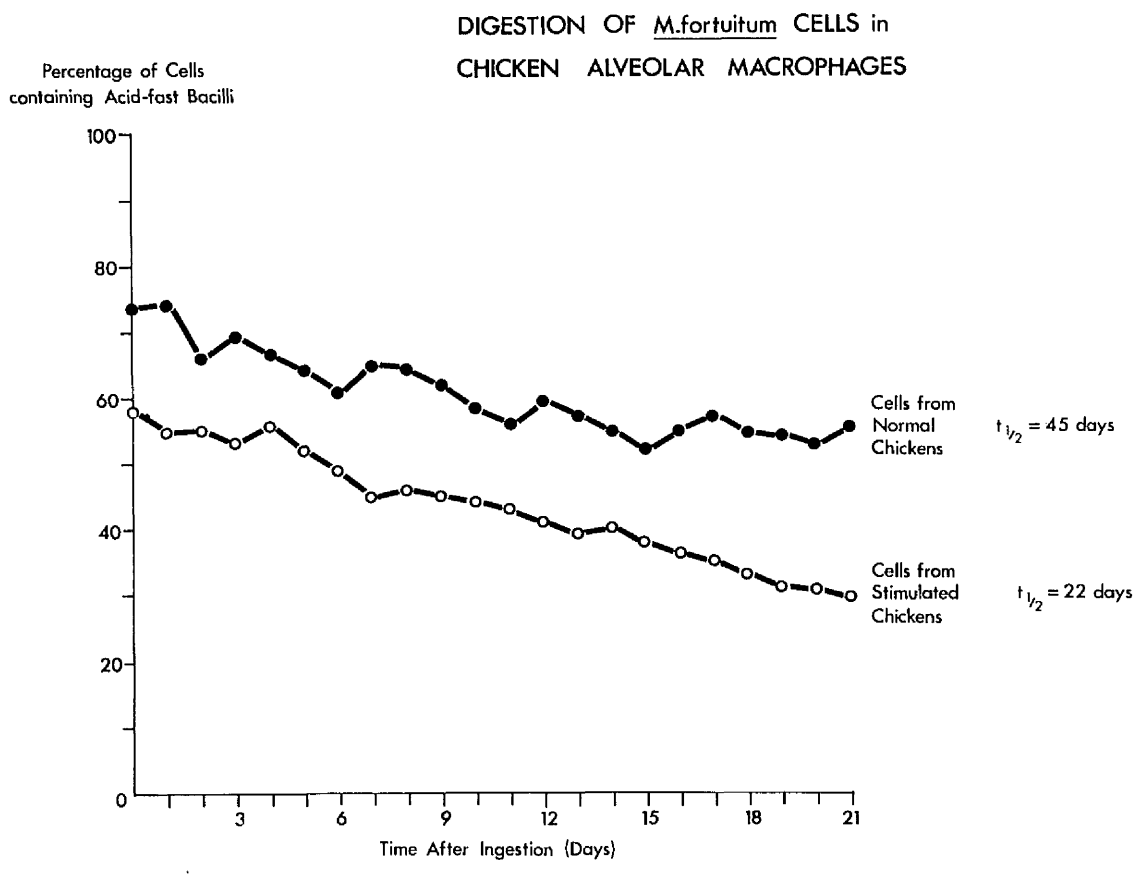


Fig. 8: Comparison between alveolar macrophages of normal and M. fortuitum stimulated chickens in respect of the rate at which they digest heat-killed bacilli in vitro. (it should be noted that the normal cells phagocytosed 4-10% more organisms at day 0). Each point represents the mean of 3 tests.

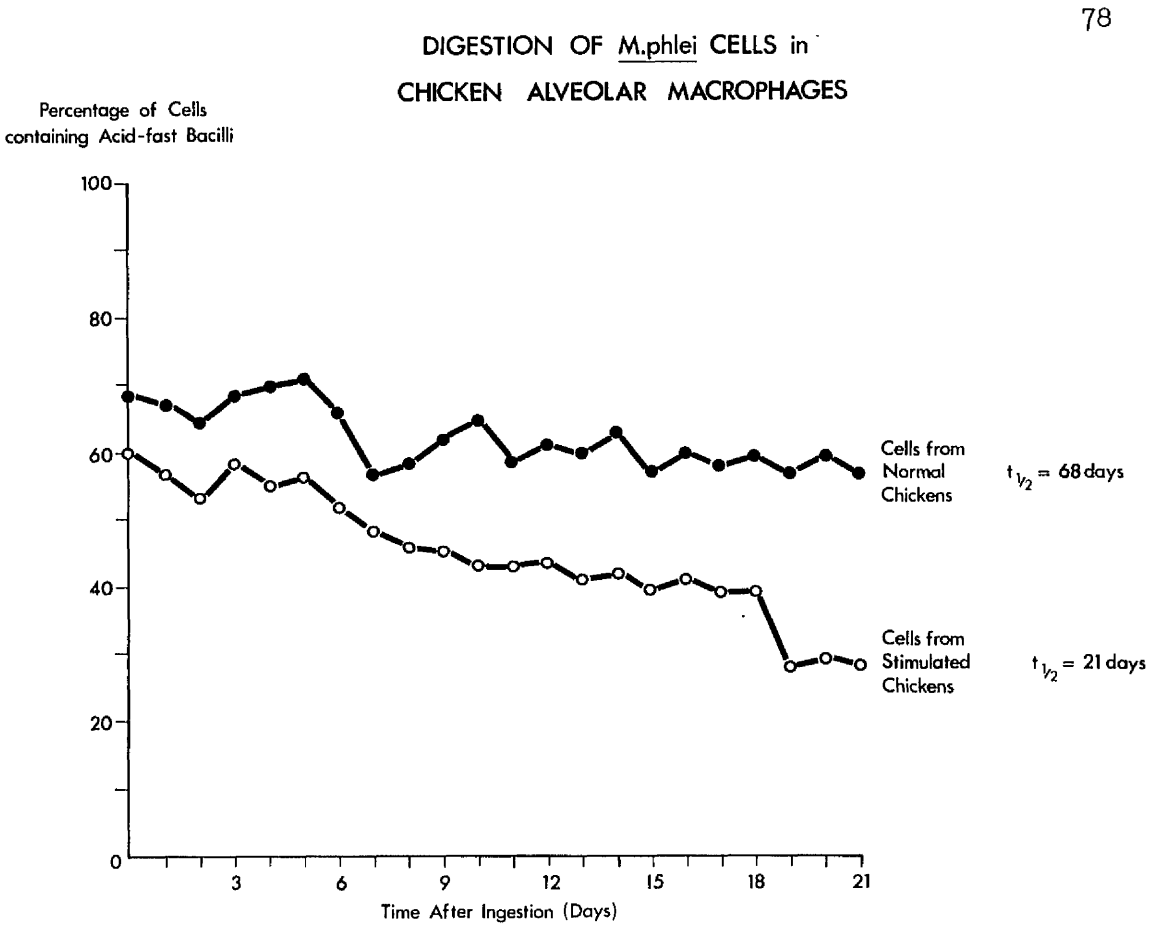


Fig 9: Comparison between alveolar macrophages of normal and M. phlei stimulated chickens in respect of the rate at which they digest heat-killed bacilli in vitro. (It should be noted that the normal cells phagocytosed 4-10% more organisms at day 0). Each point represents the mean of 3 tests.

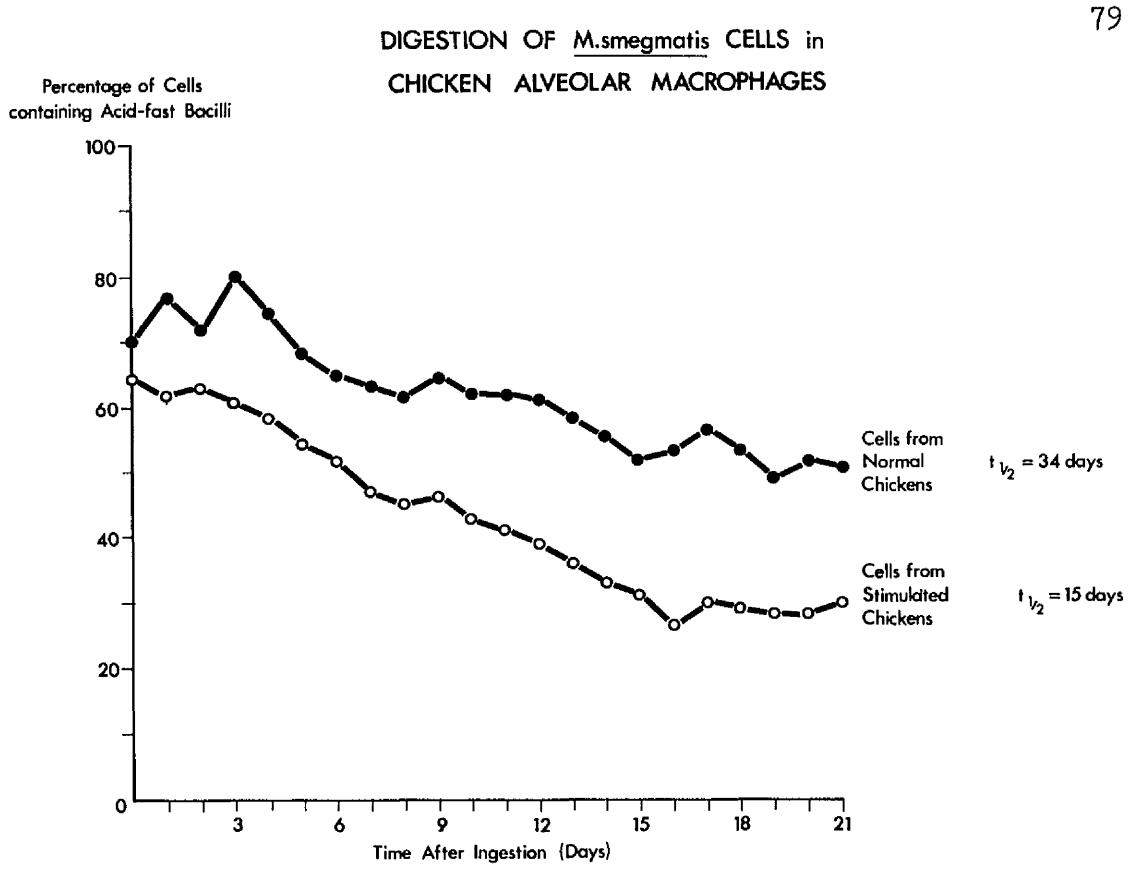


Fig 10: Comparison between alveolar macrophages of normal and M. smegmatis stimulated chickens in respect of the rate at which they digest heat-killed bacilli in vitro. (It should be noted that the normal cells phagocytosed 4-10% more organisms at day 0). Each point represents the mean of 3 tests.

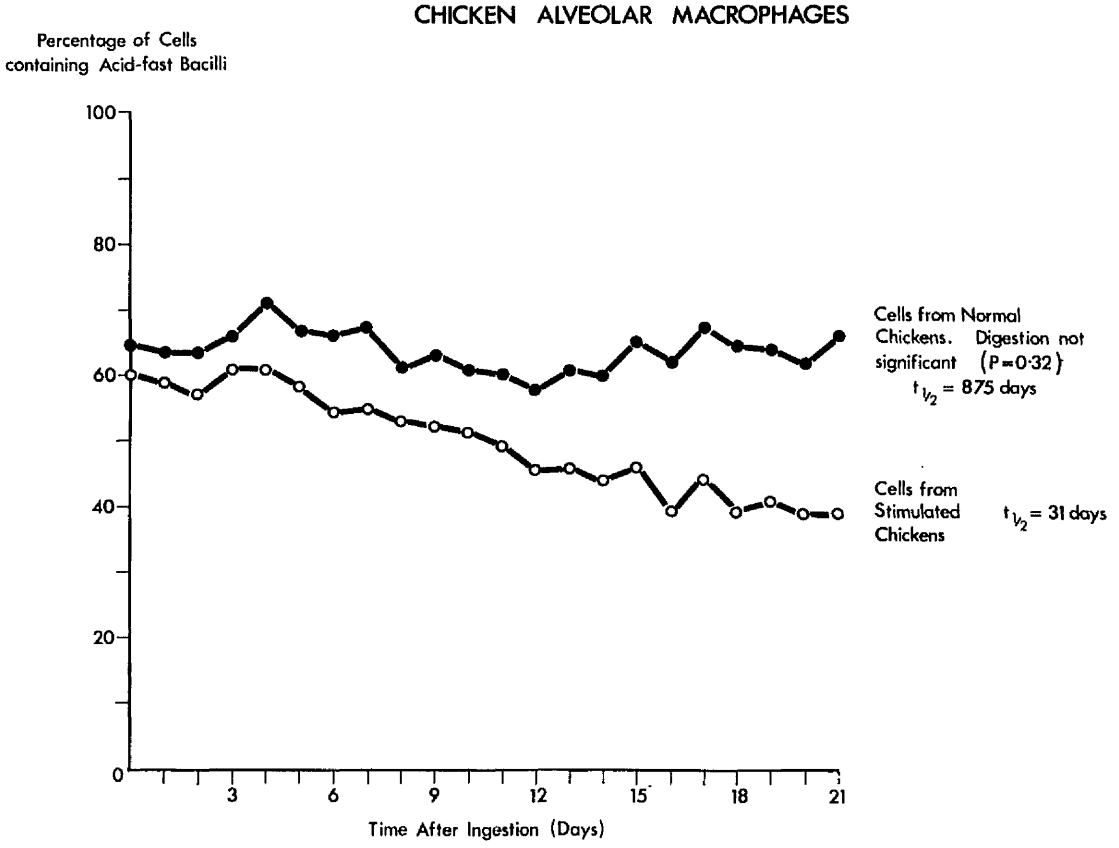


Fig 11: Comparison between alveolar macrophages of normal and M. avium stimulated chickens in respect of the rate at which they digest heat-killed bacilli in vitro. (It should be noted that the normal cells phagocytosed 4-10% more organisms at day 0). Each point represents the mean of 3 tests.

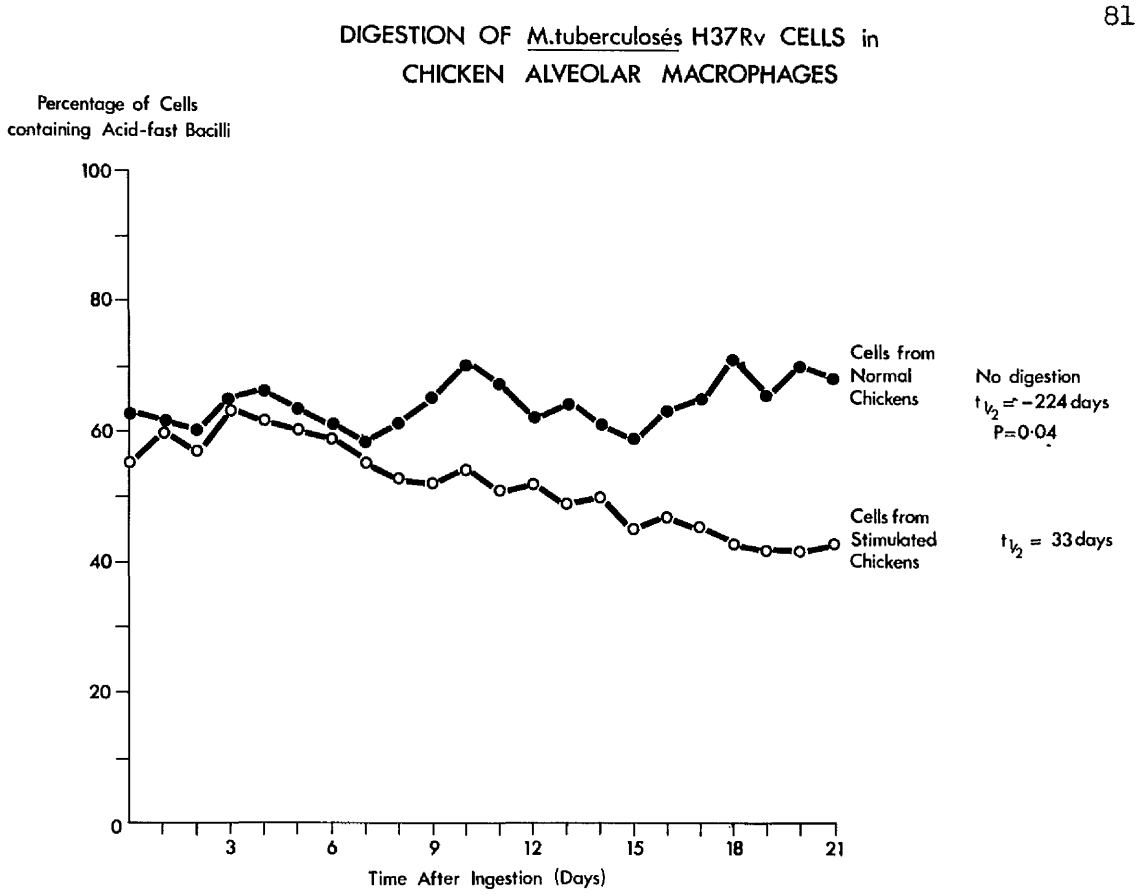


Fig 12: Comparison between alveolar macrophages of normal and M. tuberculosis H37Rv stimulated chickens in respect of the rate at which they digest heat-killed bacilli in vitro. (It should be noted that the normal cells phagocytosed 4-10% more organisms at day 0). Each point represents the mean of 3 tests.

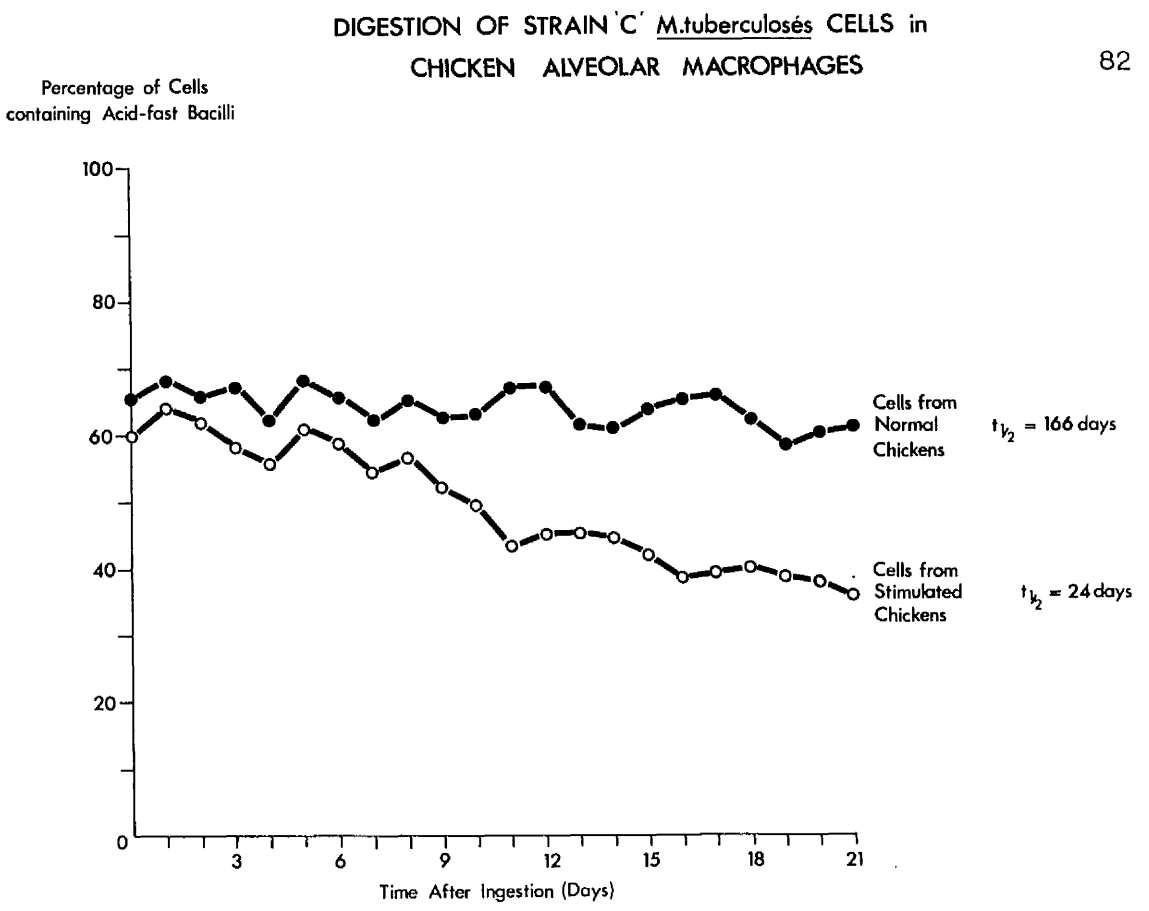


Fig 13: Comparison between alveolar macrophages of normal and Strain C M. tuberculosis stimulated chickens in respect of the rate at which they digest heat-killed bacilli in vitro. Each point represents the mean of 3 tests.

The results show that the three seprophytic organisms were digested more rapidly (Fig 8 - 10) than the pathogenic organisms (Fig 11 - 13). In studying Fig 8 - 13 it is obvious there is a descending curve over a period of 21 days but the slope is very gradual over this period suggesting that mycobacteria are degraded very slowly by macrophages. This is in marked contrast to Fig 6 and 7 in which a similar descending curve is seen for digestion of E. coli but over a time period of only 2 to 3 hours. It can also be seen by comparing the descending slopes for stimulated macrophages with those for normal alveolar macrophages that the stimulated macrophages consistently degraded mycobacteria more rapidly in vitro.

RESULTS Part (ii)

There was only a slight difference between the alveolar macrophages of the mouse and those of the chicken when they were compared for their ability to digest mycobacteria (Tables 4 and 5). Mouse macrophages were slightly more efficient than those of the chicken. Fewer mouse alveolar macrophages than chicken macrophages contained 6 to 10 or more stainable organisms after 15 days incubation in vitro. The saprophytic organisms were degraded more rapidly than the pathogenic.

CONCLUSIONS

1. Macrophages digest mycobacteria but the digestion is much slower than that of E. coli described in earlier sections.

2. Saprophytic mycobacteria are degraded more rapidly in either normal or stimulated cell preparations of chicken alveolar macrophages than pathogenic mycobacteria.

3. Mouse macrophages degrade both saprophytic and pathogenic acid-fast organisms slightly more rapidly than chicken macrophages.

Table 4

Intracellular degradation of different strains of Tubercle Bacilli within normal chicken alveolar macrophages *in vitro*

	M. tuberculosis (H37Rv)				*M. avium*			
Day	A	B	C	D	A	B	C	D
0	50	27	23	0	62	18	0	0
3	44	34	22	0	51	35	18	6
6	65	24	11	0	57	23	15	5
9	70	14	12	4	50	38	9	3
12	82	10	6	2	52	40	7	1
15	73	15	10	2	56	35	5	4

	M. phlei				*M. fortuitum*			
Day	A	B	C	D	A	B	C	D
0	55	35	10	0	61	27	12	0
3	51	25	16	8	43	32	10	15
6	42	29	18	11	40	34	21	5
9	45	33	15	7	48	31	18	3
12	43	30	22	5	40	35	21	4
15	48	35	12	4	43	30	25	2

100 macrophages containing tubercle bacilli were classified according to the number of stainable bacilli found within them. The figures represent the percentage of phagocytes containing the following number of tubercle bacilli: 1 to 2 (A), 3 to 5 (B), 6 to 10 (C), and more than 10 (D).

Table 5

Degradation of various acid-fast organisms *in vitro* by normal mouse alveolar macrophages

DAY (following incubation of mycobacterium with macrophages)	*M. phlei*				*M. fortuitum*			
	A	B	C	D*	A	B	C	D
0	51	40	8	1	46	36	12	6
3	63	34	3	0	57	30	10	3
6	65	32	3	0	62	23	15	0
9	48	43	9	0	56	41	3	0
12	40	33	27	0	50	28	22	0
15	42	30	28	0	64	31	5	0
	M. tuberculosis (H37Rv ATXC)				*M. avium*			
0	62	23	15	0	44	46	10	0
3	63	25	12	0	50	35	15	0
6	55	40	2	0	40	40	20	0
9	50	36	7	0	50	26	24	0
12	44	36	20	0	28	50	22	0
15	40	38	22	0	42	55	3	0

* The symbols A, B, C and D refer to the number of acid-fast organisms per cell as below:

A = % cells containing 1-3 acid-fast organisms
B = " 4-6 "
C = " 7-10 "
D = " > 10 "

C. INTRACELLULAR DEGRADATION OF FLUORESCEIN LABELLED CELL WALLS BY NORMAL MOUSE AND CHICKEN ALVEOLAR MACROPHAGES *in vitro*

PURPOSE:

To determine how effective "normal" chicken and mouse alveolar macrophages can degrade mycobacteria cell walls *in vitro*. Having established that chicken macrophages can digest whole mycobacteria, experiments were designed to study the digestion of subcellular fractions of mycobacteria.

In these experiments cell walls of *M. avium*, *M. phlei*, *M. fortuitum* and *M. tuberculosis* (H37Rv) (not defatted) were obtained by ultrasonic disruption of these organisms. The cell wall preparations were treated with proteinase to remove contaminating cellular sap. The cell walls were labelled with fluorescein isothiocyanate according to the procedure outlined in methods section.

In these experiments, normal mouse and chicken macrophages were incubated *in vitro* with such cell walls and studied using slide chambers as described and illustrated in methods. Cell wall degradation was assessed in terms of decrease of fluorescence, using a Leitz Ortholux microscope. Decrease of fluorescence was measured as detailed in the methods section. Readings were made daily over a period of 14 days, the day 0 sample being taken after 1 hour incubation at 37^{o} to allow phagocytosis of cell walls.

The results are shown in Figures 14 and 15.

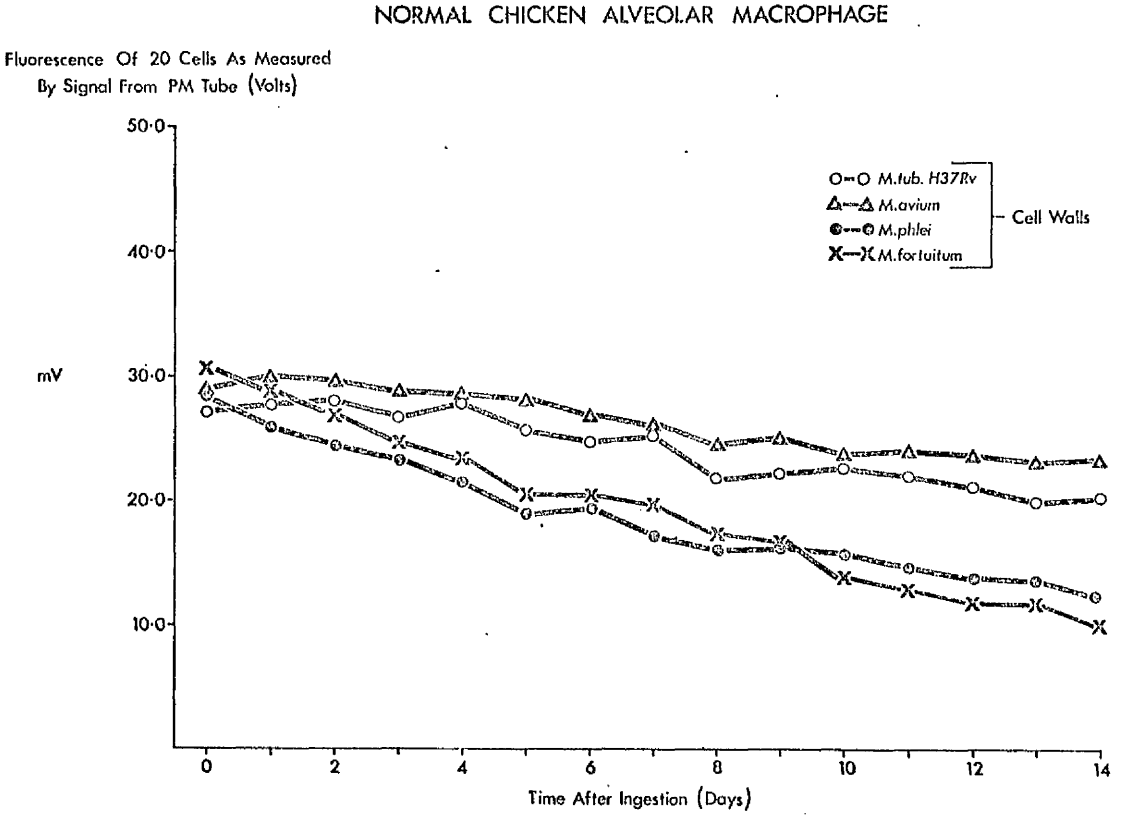


Fig 14: Comparison of degradation of fluorescein labelled cells of various strains of Mycobacteria by normal chicken alveolar macrophages in vitro.

It was shown that the pathogens, M. tuberculosis (strain C), M. tuberculosis (H37Rv) and M. avium were degraded by mouse and chicken macrophages but at a much slower rate than the saprophytic organisms. The results show that normal chicken macrophages digest the cell walls more slowly than the normal mouse alveolar cells. Thus these results confirm the preceding observations in which normal chicken and mouse alveolar cells were compared in their ability to degrade cells.

Figure 15 in comparison to Fig. 14 shows that mouse alveolar macrophages degrade the cell walls of the various acid-fast saprophytic and pathogenic organisms in vitro more rapidly than chicken macrophages. Plates 1 and 2 are examples of ingested FITC cell walls as seen using a combination of phase and ultra violet microscopy for these studies.

CONCLUSIONS

1. The cell walls from various acid-fast organisms were degraded in vitro by alveolar macrophages from normal mice and normal chickens.

2. The cell walls from saprophytic organisms were more rapidly degraded than the cell walls from pathogenic organisms by both mouse and chicken macrophages.

3. As observed previously, mouse macrophages degraded the cell wall preparations more rapidly than chicken macrophages.

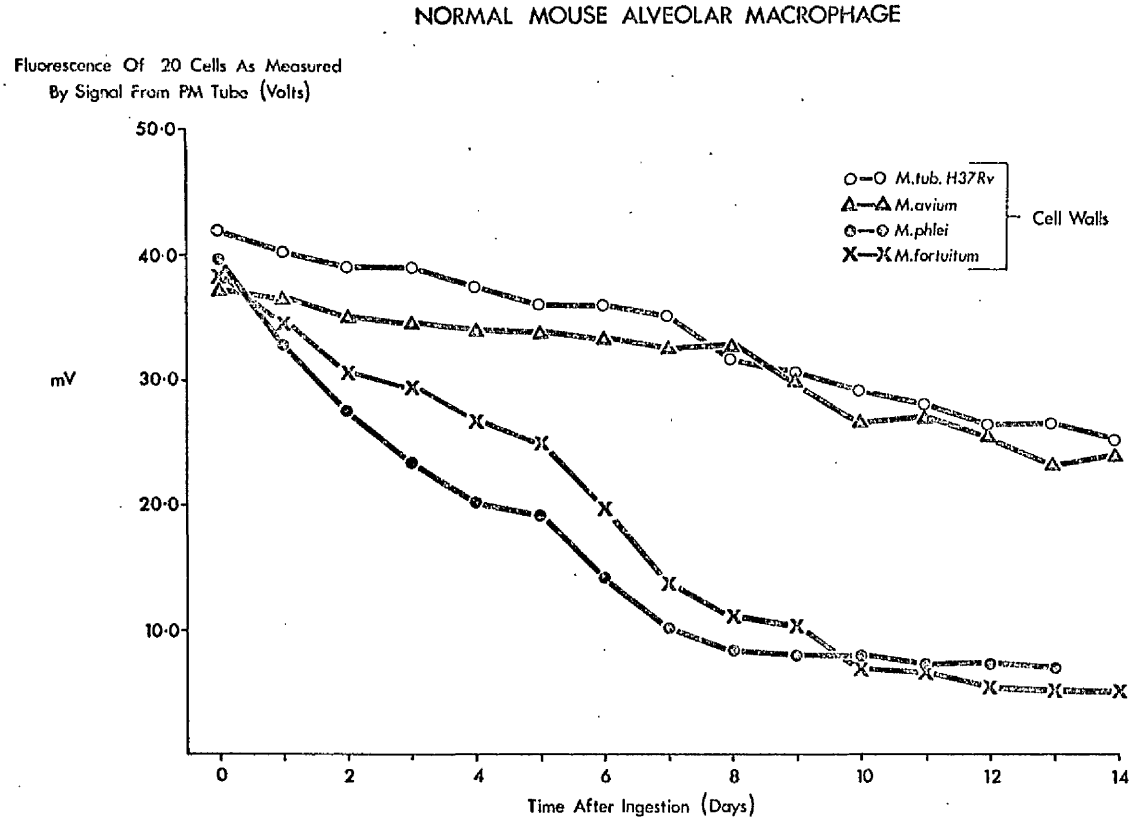


Fig 15: Comparison of degradation of fluorescein labelled cell walls of various strains of Mycobacteria by normal mouse alveolar macrophages in vitro.

Plate 1

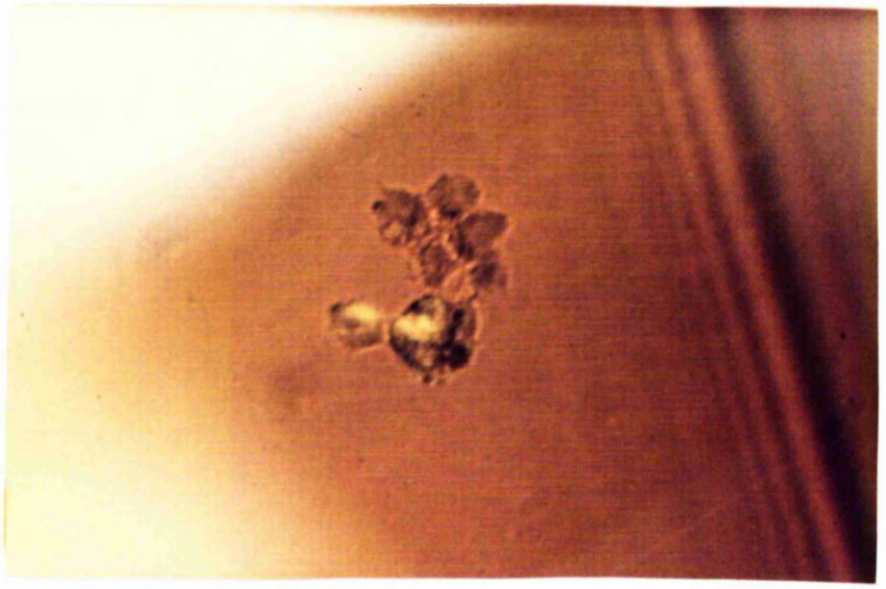
Fluorescent and phase micrograph of cultured chicken alveolar macrophages containing ingested labelled cell walls of M. avium (arrows) three days after phagocytosis in vitro. These culture preparations were studied using a slide chamber as outlined in Methods of this thesis.

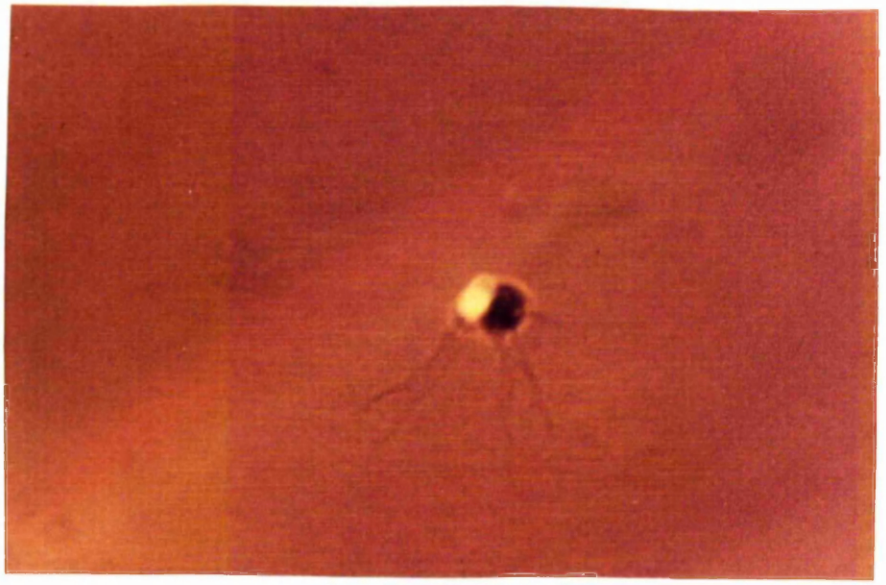
X-400

Plate 2

Fluorescent and phase micrograph of cultured mouse alveolar macrophage containing FITC labelled cell walls of M. tuberculosis (strain C) (arrow). This cell also shows peripheral 'spikes' (? very fine pseudopodia)

X-400





D. DEGRATATION RATE OF RADIOLABELLED *M. avium* AND *M. tuberculosis* (STRAIN C) CELL WALLS AND OF RADIOLABELLED CELLULOSE MEASURED *in vivo* BY TOTAL BODY COUNTS OF THE CHICKEN

PURPOSE:

The results of studies of degradation of ingested mycobacteria by macrophages *in vitro* stimulated an interest in the determination of the rate of degradation of mycobacteria cell walls *in vivo*. Therefore, in these experiments the degradation rate of radiolabelled mycobacterial cell walls was determined in living chickens.

Cell walls were labelled with ^{131}I. The method used for labelling was developed by Dr. I. McKay of this department. Groups of six nine-week old chickens (Babcock Supertan) were injected intravenously with radio-labelled cell walls as described below:

Group 1, each bird received 6 mg radiolabelled *M. avium* cell walls containing approximately 17 uCi ^{131}I.

Group 2, each bird received 6 mg radiolabelled *M. tuberculosis* (strain C) cell walls containing approximately 17 uCi ^{131}I.

Group 3, each bird received 6 mg radiolabelled ^{131}I cellulose powder (this substance is known to be inert and macrophages are unable to degrade it).

All preparations were suspended in saline, and injections were made into the basilic vein, a contributory of the branchial vein of the wing.

Whole-body radioemissions were counted in a γ-counter over a period from day 3 to day 20 after the injection. A standard (or baseline) estimation of whole-body radioactivity for each bird was estimated from the whole-body count immediately after injection, and this count has been termed the day 0 count.

The results are shown graphically in Figure 16.

It was observed from these results that there was rapid disappearance of the ^{131}I activity for 3-7 days after injection in those birds that received cell wall preparations, which contrasts with the moderate decline in the group which received cellulose. Thereafter, radioactivity declined at a comparable rate in both groups.

This second phase of isotope elimination could possibly be attributed to spontaneous release of isotope from the labelled preparations, an observation not uncommon in situations in which radioiodine is attached to the nucleus of a phenol. The first phase of isotope elimination might be due to enzymic degradation, but if so, it seems that only a part of the cell wall is being degraded, since the rapid decline in isotope levels does not continue beyond the first three to seven days. Possibly the first isotope to disappear is that attached to protein, which is dissolved or digested within the first week; the remainder of the isotope may be attached to a polysaccharide which resists digestion for a long time.

Indeed, the fact that the three isotope elimination curves are parallel from day 7 onwards implies that there is some cell wall

component which is not digested any faster than cellulose - i.e. is not digested at all.

CONCLUSION:

The results of these experiments show no evidence for digestion of mycobacteria (compared with the cellulose control) except during the first seven days after injection. This failure to show digestion may be technical, due to the indigestibility of the chemical site to which the radioisotope was linked, and may not reflect the capacity of the macrophage to digest the cell wall as a whole.

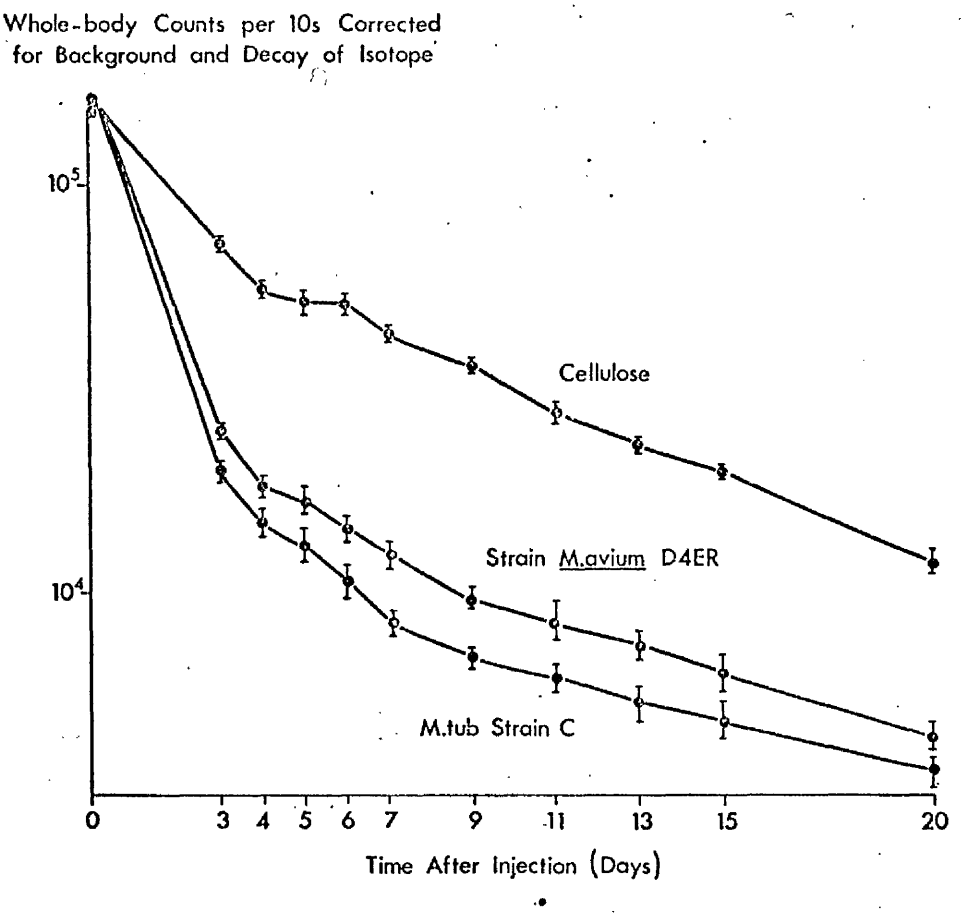


Fig 16 : Comparison of digestion rates of isotope labelled mycobacterial cell walls with an indigestible control (cellulose) in chickens.

E. EFFECT OF *M. avium* ON ACID PHOSPHATASE LEVELS IN LUNG AND LUNG EXUDATES OF THE CHICKEN *in vivo*

PURPOSE

In these experiments an attempt was made to determine what effect *M. avium* stimulation had on the levels of acid phosphatase in the lungs of the chicken.

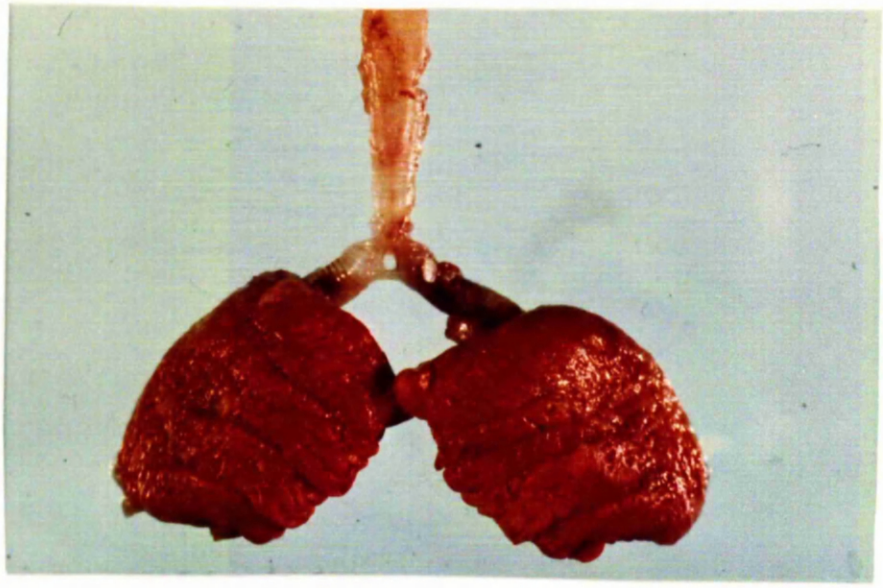
Acid phosphatase (phosphomonoesterase II) is widely distributed in nature, particularly in fungi and in the higher plants. In mammals, it is present in large amounts in the prostate glands, erythrocytes and polymorphonuclear leucocytes. Rabbit macrophages have been shown to contain active proteases, esterases, lipase, lysozyme and acid phosphatase (Dannenberg and Bennet, 1964; Cohn and Wiener, 1963; Leake and Myrvik, 1964). In rabbits stimulated with BCG inoculation macrophages have also been shown to contain acid phosphatase (Allison, *et al.*, 1961).

Consideration of the defensive role of these enzyme systems is complicated by the fact that there is evidence that some phagocytic cells are capable of adaptive changes following repeated exposure to particulate stimuli (bacterial organisms and other particulate material), which is manifested by increased phagocytic activity, increased enzyme synthesis and changes in cellular morphology (see Plate 3, showing hyperplasia of lungs of *M. avium* stimulated chickens).

HYPERPLASIA OF CHICKEN LUNGS (actual size)

Plate 3 : Dissected lung of normal chicken control (received i.v. 1 ml saline) 7 days following saline injection.

Plate 4A: Dissected lung of chicken stimulated with M. avium 7 days previously showing hyperplasia in the lungs of chicken.



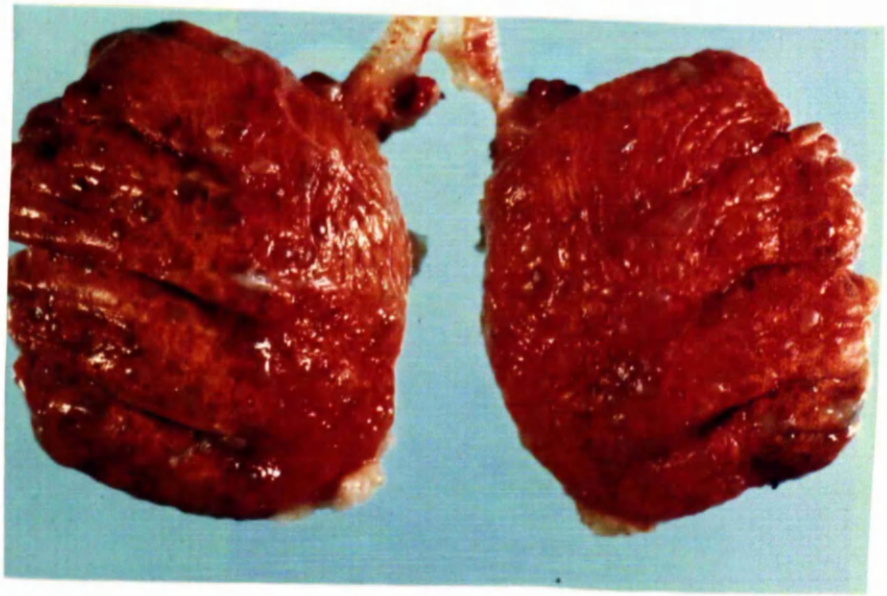
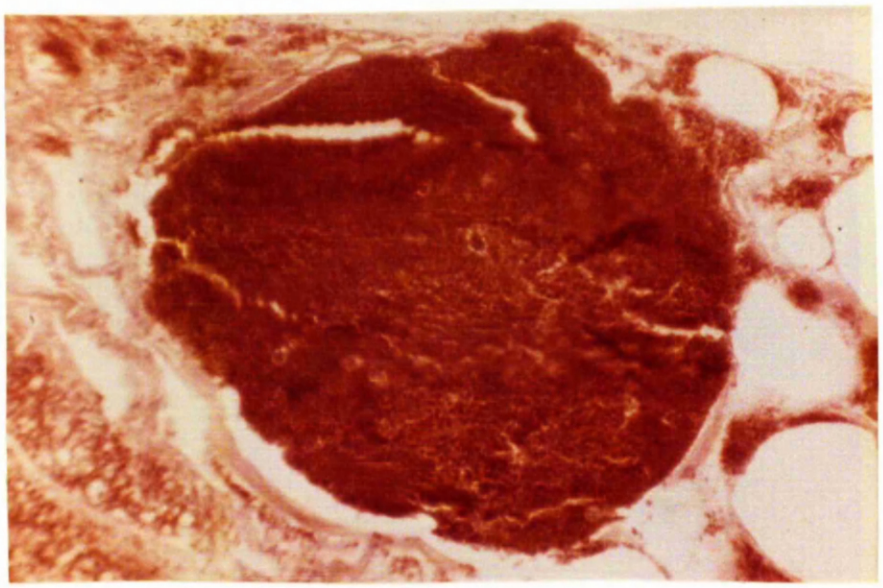
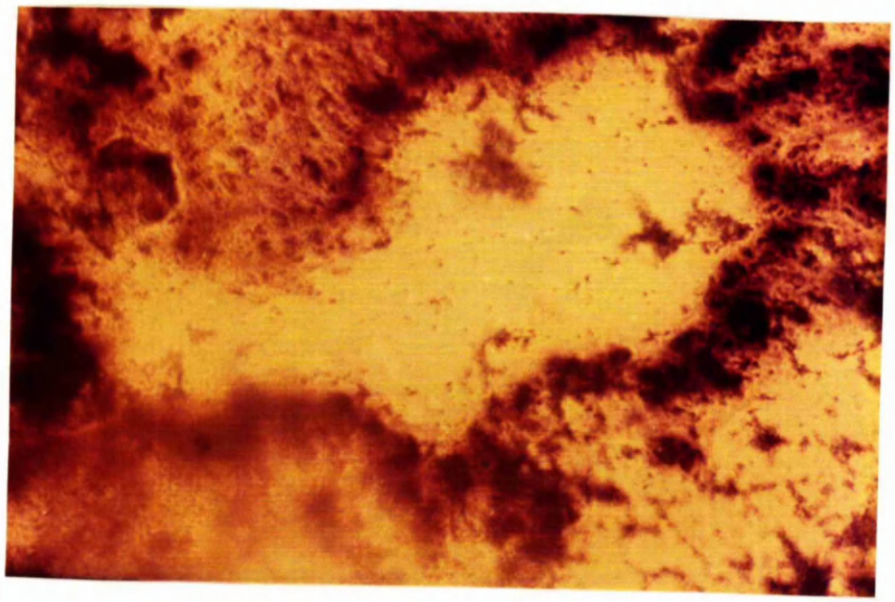


Plate 4B: Showing acute immune response in the chicken as a result of M. avium stimulation. (a) Granuloma: section cut through granuloma and stained for acid phosphatase. (b) Alveoli: showing concentration of acid phosphatase in macrophages around the periphery of air space.
7 days after injection.
The reddish brown areas show the presence of acid phosphatase within macrophages and the yellowish background indicates the air space.
Histochemical method of Takeuchi and Tanoue. X 400.



(a)



(b)

This experiment was designed to establish whether stimulation with mycobacteria increased the acid-phosphatase content of macrophages. Alveolar macrophages from the lungs of chickens treated in several ways were studied for enzyme activity.

(A) The first study was made using M. avium as the stimulating agent. Group 1, each bird was injected intravenously with 6 mg heat-killed M. avium suspended in saline. Group 2, an equal number of control chickens received saline intravenously.

(B) Two other groups of animals were used in these investigations. Group 3, each bird received 6 mg M. avium in saline injected intravenously. Group 4, each bird was injected with 6 mg M. butyricum intravenously.

Three test and three control animals were killed on days 3, 6, 9, 12 and 15 and lung exudates and lung tissues collected. The lungs and carcasses were weighed. The lung exudate cell count was determined using a Newbauer haemocytometer, according to methods discussed in previous sections.

RESULTS

A. The comparison of acid phosphatase content of whole lung and lung exudates from stimulated and unstimulated chickens is shown in Figure 17. The level of acid phosphatase in whole-lung homogenates

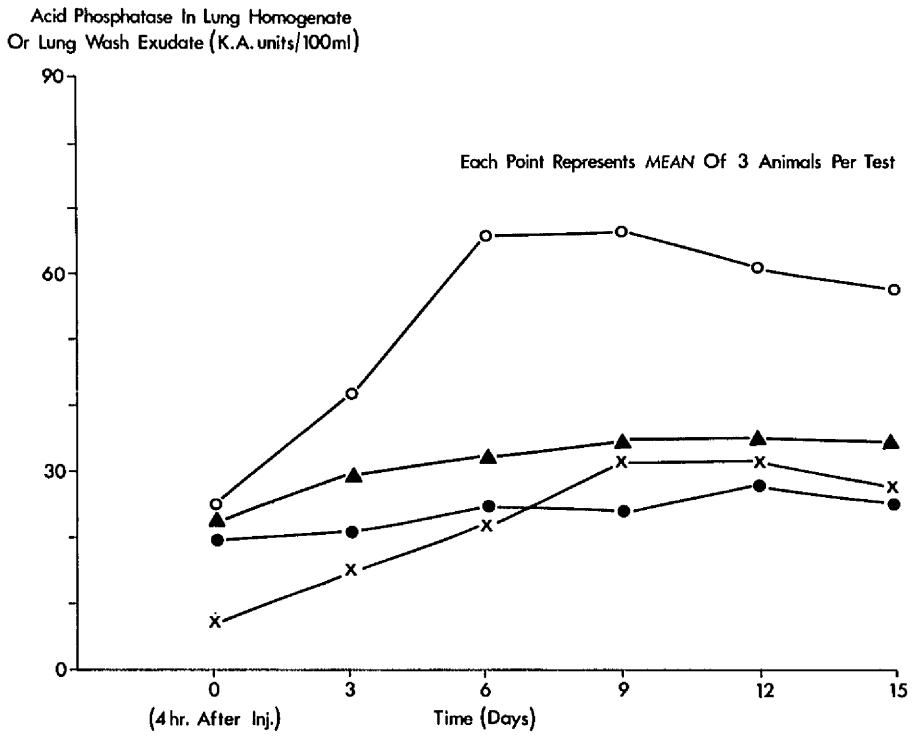


Fig 17: EFFECT OF *M.avium* ON ACID PHOSPHATASE LEVELS IN WHOLE LUNG AND LUNG EXUDATES OF THE CHICKEN IN VIVO.

O - whole lung homogenate
X - lung wash
} M. avium

▲ - whole lung
● - lung wash
} saline

Comparison of rises in acid phosphatase levels by M. avium stimulated (O = whole lung homogenate, X = lung wash exudate) and saline control (▲= whole lung, ● = lung wash).

from unstimulated birds rose slightly during the course of the investigation but did not exceed 50 K.A. units at any time. In contrast, lungs from chickens stimulated with M. avium showed marked increases in enzyme levels to a peak of 65 K.A. units. Enzyme levels in lung exudates showed no consistent difference between stimulated and unstimulated lung washings.

B. The previous experiment demonstrated that M. avium injected into chickens induced an increase in total acid phosphatase content in the lungs. However, this organism is a natural pathogen of birds and it was considered relevant to repeat the experiment using a non-pathogenic strain. A saprophytic strain M. butyricum was therefore chosen. The experimental outline is shown below.

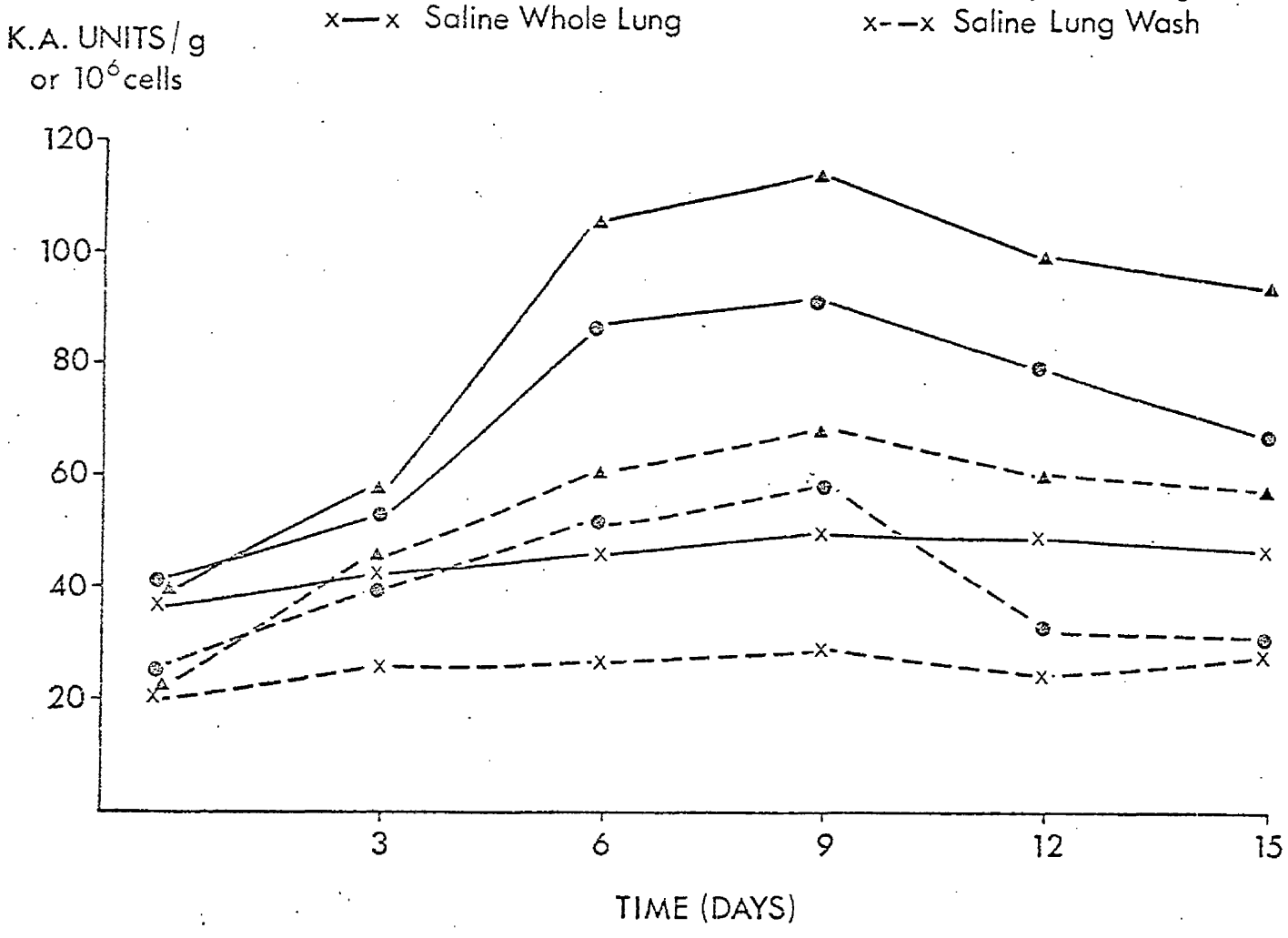
Group 3. Chickens in this group were inoculated with M. avium as before (see group 1 previous experiment).

Group 4. Each chicken in this group was injected intravenously with 6 mg M. butyricum in saline.

Group 5. Birds injected with saline as before to serve as controls.

The results of this investigation are shown in Fig 18 which shows that M. avium elevated the level of acid phospatase higher than that seen in M. butyricum stimulated chickens. It is probably that the differences between these mycobacteria in their capacity to stimulate production of acid phosphatase by macrophages reflect differences in cell-wall composition between the two organisms, M. avium presumably bearing macrophage-stimulating factors which are poorly represented in M. butyricum.

Fig 18 : Comparison of effect in rises of acid phosphatase in whole lung tissues of the chicken when stimulated by various strains of Mycobacteria.



The levels of acid phosphatase in M. avium stimulated chickens were about 20 per cent higher than those attained using the saprophytic organism M. butyricum.

CONCLUSIONS

1. Acid phosphatase levels are elevated in the chicken lung as a result of M. avium and M. butyricum stimulation.

2. Acid phosphatase levels are elevated as early as 3 hours after a single intravenous injection (6 mg) of the stimulating organisms.

3. The levels of acid phosphatase achieved in pulmonary macrophages after maximal stimulation were about 20% higher when M. avium was used as the stimulating as when M. butyricum was used.

4. As a result of stimulation with M. avium and M. butyricum, the levels of acid phosphatase were elevated in lung wash exudates (containing macrophages) as well as in the lung tissue itself.

F. THE EFFECT OF VARIOUS STRAINS OF MYCOBACTERIA ON THE SYNTHESIS OF ACID PHOSPHATASE IN THE LUNGS OF CHICKENS *in vivo*

PURPOSE

In this experiment the study described in the previous section was extended to a wider range of mycobacteria. Six strains of mycobacteria were studied to compare their ability to stimulate the lysosomal enzyme, acid phosphatase, in the lungs of chickens.

Seven groups of twenty-four 6-8 week old chickens were included in this experiment. One group of birds was injected intravenously with each of *M. avium*, *M. tuberculosis* (strain C), *M. tuberculosis* (H37Rv), *M. phlei*, *M. fortuitum* and *M. smegmatis*, and one group was kept as a control group (injected i.v. with saline). At three day intervals after injection, three birds from each group were killed and lung tissue was collected from each bird for histochemical studies.

Four grams of lung tissue from each bird were homogenised in 25 ml of saline at 4°C. After homogenisation, each homogenate was frozen and thawed five times and Triton X-100 added to final concentration of 1% v/v to insure total lysis of lysosomes. Aliquots of these preparations were then tested for acid phosphatase activity.

The results of this experiment are presented in Figure 19.

From Figure 19 it is clear that representative organisms of both pathogenic and saprophytic mycobacteria substantially elevated the acid phosphatase activity in the lungs of chicken. However, *M. avium* stimulated a greater increase in acid phosphatase activity than any of the other Mycobacteria studied.

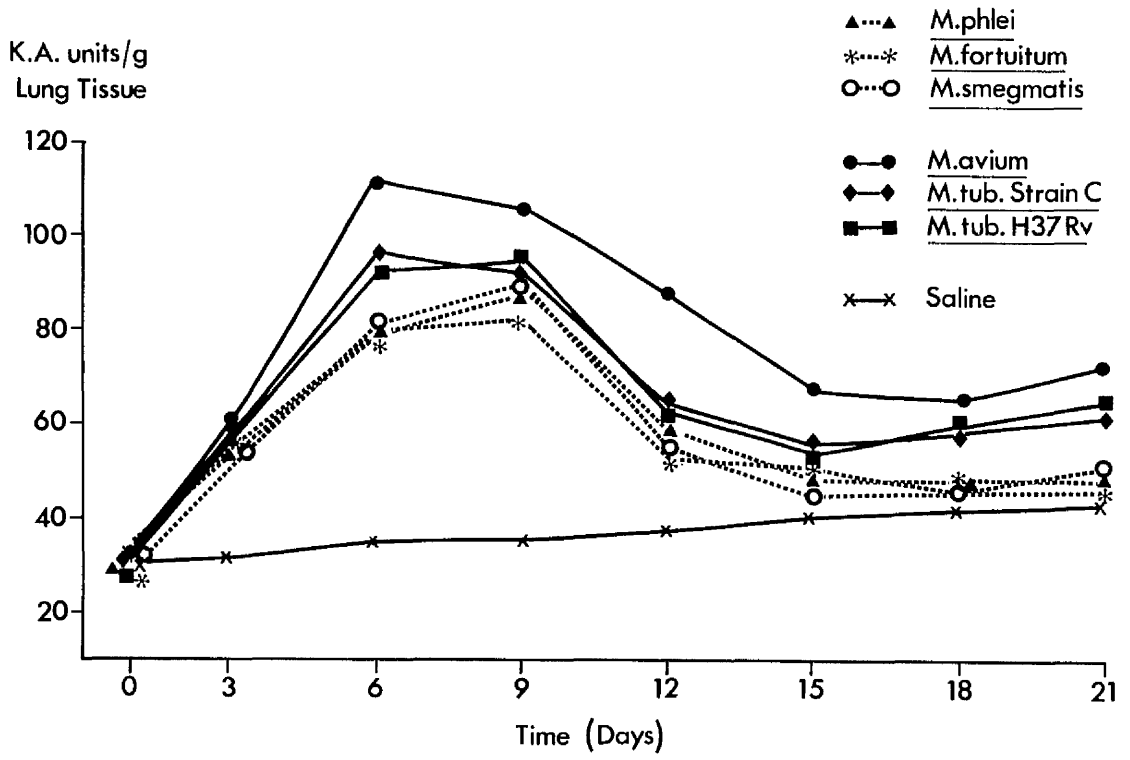


Fig. 19

The effect of various strains of Mycobacteria on acid phosphatase levels in vivo stimulation over extended period of time (21 days)

Further observations of enzyme function were made using lung tissue obtained from the birds used in the above experiment. When the animals were killed, tissue was obtained and numerous "imprint" preparations made on microscope slides. The slides were stained as recorded in the methods section for demonstration of lysosomal enzymes. Some preparations were stained with acridine orange.

RESULTS

Increased lysosomal enzyme content of lung tissue was observed in all birds stimulated with mycobacteria, and typical examples together with the appearances for control (unstimulated birds) are shown in Plate 6.

Slides with acridine orange (Plate 5 & 6) revealed the presence of "angry macrophages" in these preparations. This dye detects nucleic acid material and heavy staining is therefore taken to indicate increased cell metabolism and lysosomal activity. These slides therefore confirm the results obtained using enzyme assays.

PLATE 5

Stimulated ("angry") macrophages

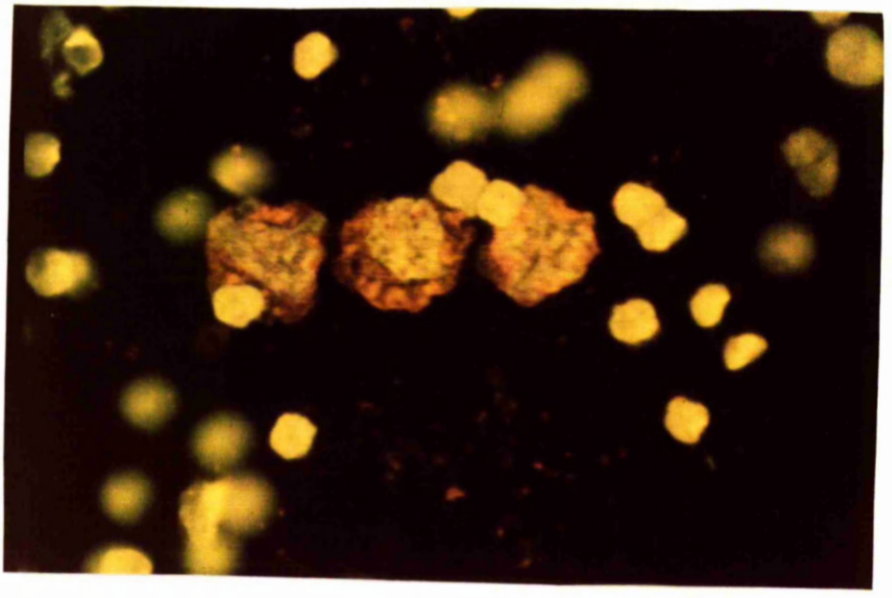
(*M. avium*)

PLATE 6

Normal macrophages

(Saline)

Chicken alveolar macrophages cultured on glass for two hours. The cells tend to be elongated, with well developed pseudopodia. (Ultraviolet light acridine-orange preparation): X 200.



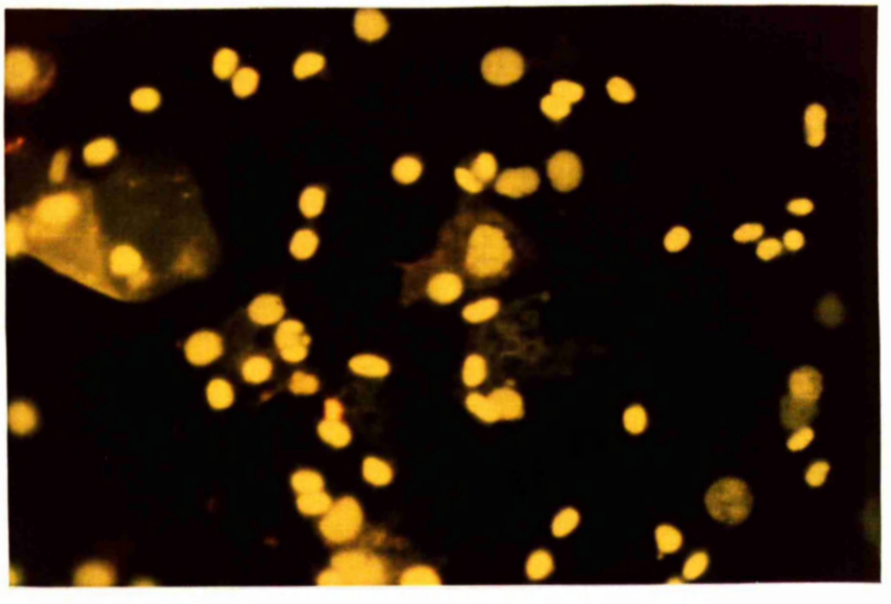


PLATE 7

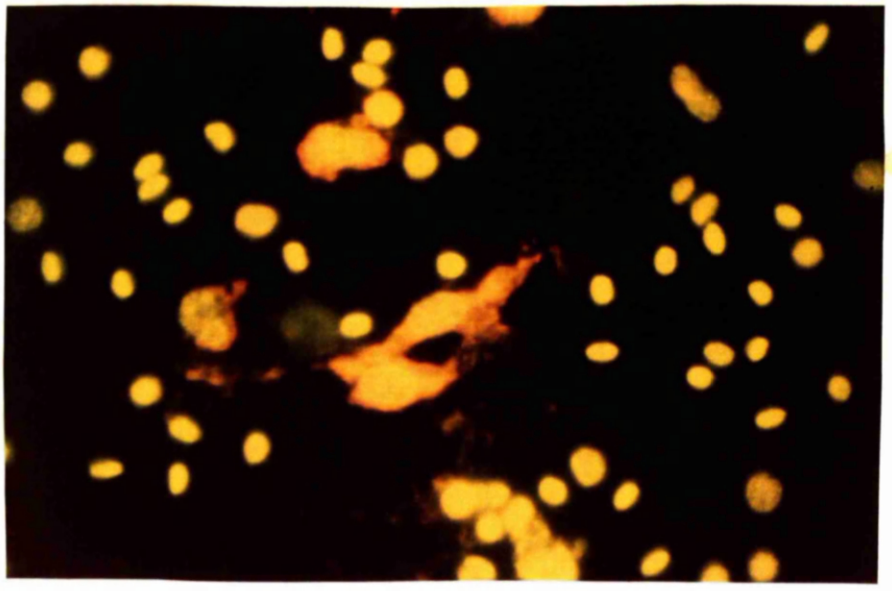
ATCC H37Rv Stimulated

Activated (M. avium) macrophages from lung wash of chicken. Acridine orange; X 400

PLATE 8

M. tuberculosis strain C stimulated

Macrophage from lung wash of chicken shows activated (M. avium) macrophage that has been allowed to spread on glass for two hours. Acridine orange. X 400.



When the results for enzyme levels are arranged in order of elevating acid phosphatase level the descending order is M. avium, M. tuberculosis (strain C), M. tuberculosis (H37Rv), M. smegmatis, M. phlei and M. fortuitum. It is therefore apparent that the pathogens show outright superiority in causing an increase of acid phosphatase levels. This could perhaps be thought to be animal species differences or differences in cell wall composition.

CONCLUSION

The biological property of stimulating the lysosomal enzyme system is possessed by a variety of mycobacteria. M. avium was more efficient than other strains in producing this effect in chickens. Many of the strains that stimulated a rise in acid phosphatase levels also caused hyperplasia of the lungs associated with granulomatous change.

(i) COMPARISON OF THE LYSOSOMAL ENZYME ACID PHOSPHATASE RESPONSE (in chickens) AFTER STIMULATION WITH *M. avium* SUSPENDED IN SALINE AND WATER-IN-OIL

PURPOSE

To determine the effect of *M. avium* suspended in either saline or water-in-oil emulsion, on macrophage lysosomal enzyme content with particular reference to acid phosphatase.

In previous experiments, it was found that *M. avium* suspended in saline caused a rapid rise of macrophage acid hydrolase content within one week. This experiment was designed to determine whether the same effect could be demonstrated when *M. avium* was incorporated in a water-in-oil emulsion.

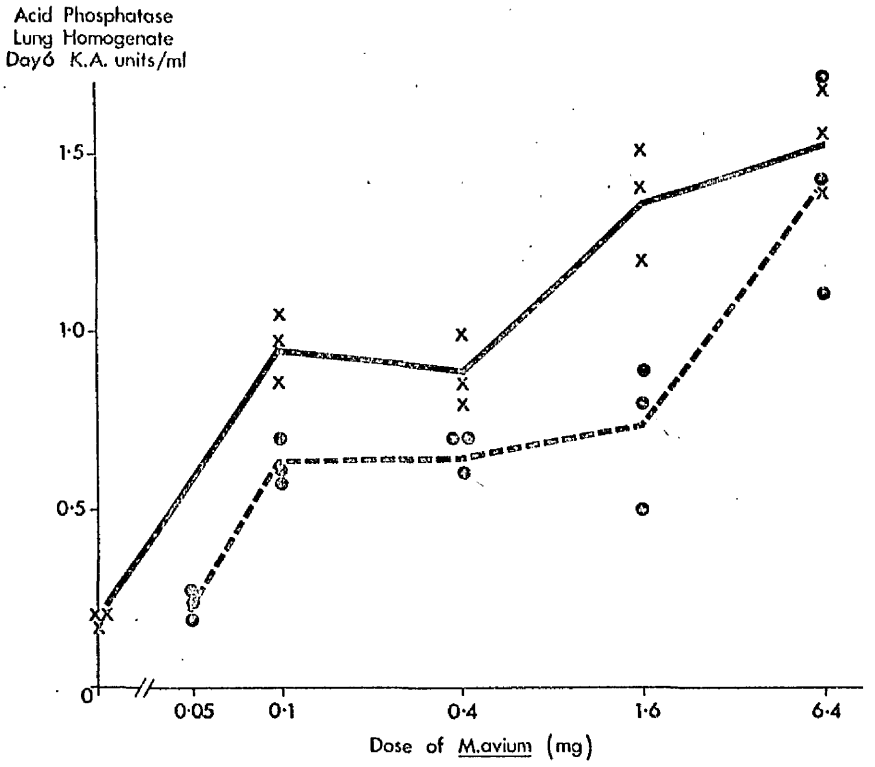
Ten groups each of 6 chickens at 6 - 8 weeks of age, received a single intravenous injection. The dose of bacteria was varied throughout the groups for both saline and oil emulsion suspended organisms to give amounts of 0.05, 0.1, 0.4, 1.6 and 6.4 mg. The animals were killed at the end of one week and lungs collected and homogenized as outlined in methods of this thesis. Aliquots were removed for acid phosphatase assay.

The results of these experiments are shown graphically in Figure 20.

When the assay was done soon after injection (6 days) it was seen that at the lower dose levels (0.1 mg to 1.6 mg) M. avium given in saline produced a sharper rise in acid phosphatase levels than did the same organism in water-in-oil emulsion. As the dose of M. avium was increased to 6.4 mg, the difference between the two preparations was no longer observed.

In a further experiment consisting of 12 test and 12 control birds, three birds were killed and enzyme assays performed on days 7, 14, 21 and 28 to determine the longer-term effects of the two methods of stimulation. After fourteen days the acid phosphatase levels were about 30 per cent higher in the animals inoculated with water-in-oil preparation than those that received the saline suspension (Fig 21). At day 21 the water-in-oil suspension provided a level of acid phosphatase which was 82% higher than the saline suspension.

It is clear from these experiments that the stimulation of pulmonary acid phosphatase is slower and more prolonged when the stimulating agent (M. avium) is suspended in a water-in-oil emulsion than when it is suspended in saline. It is possible that this is because the water-in-oil emulsion prevents rapid release of



DOSE-RESPONSE CURVES FOR STIMULATION OF PULMONARY ACID PHOSPHATASE LEVELS BY I.V. INJECTION OF HEAT KILLED *M.avium* IN SALINE (x—x) OR IN OIL PHASE OF WATER-IN-OIL EMULSION (o--o)

Fig. 20

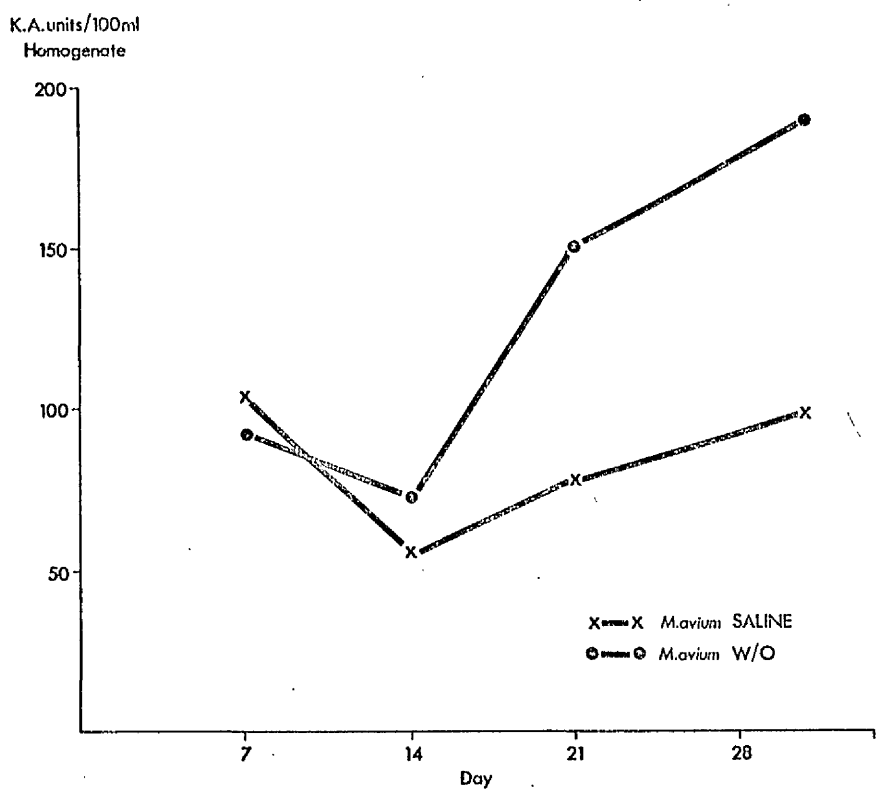


Fig. 21: Comparison of the effect on the activity of M. avium bacilli suspended either in water-in-oil emulsion or saline (6 mg per bird), and injected in a single intravenous injection. The effects on levels of acid phosphatase over varying periods of time in 28 days.

the mycobacteria from the site of deposition. Slow and prolonged release of the organism could thereby influence the macrophage acid phosphatase content by causing a continued stimulation of the macrophages.

Histological studies were made from tissues collected from these experiments and results are discussed below.

HISTOLOGY

The changes caused by the injections of M. avium in saline or water-in-oil emulsion became apparent as early as 6 - 7 days (Plates 9 and 10).

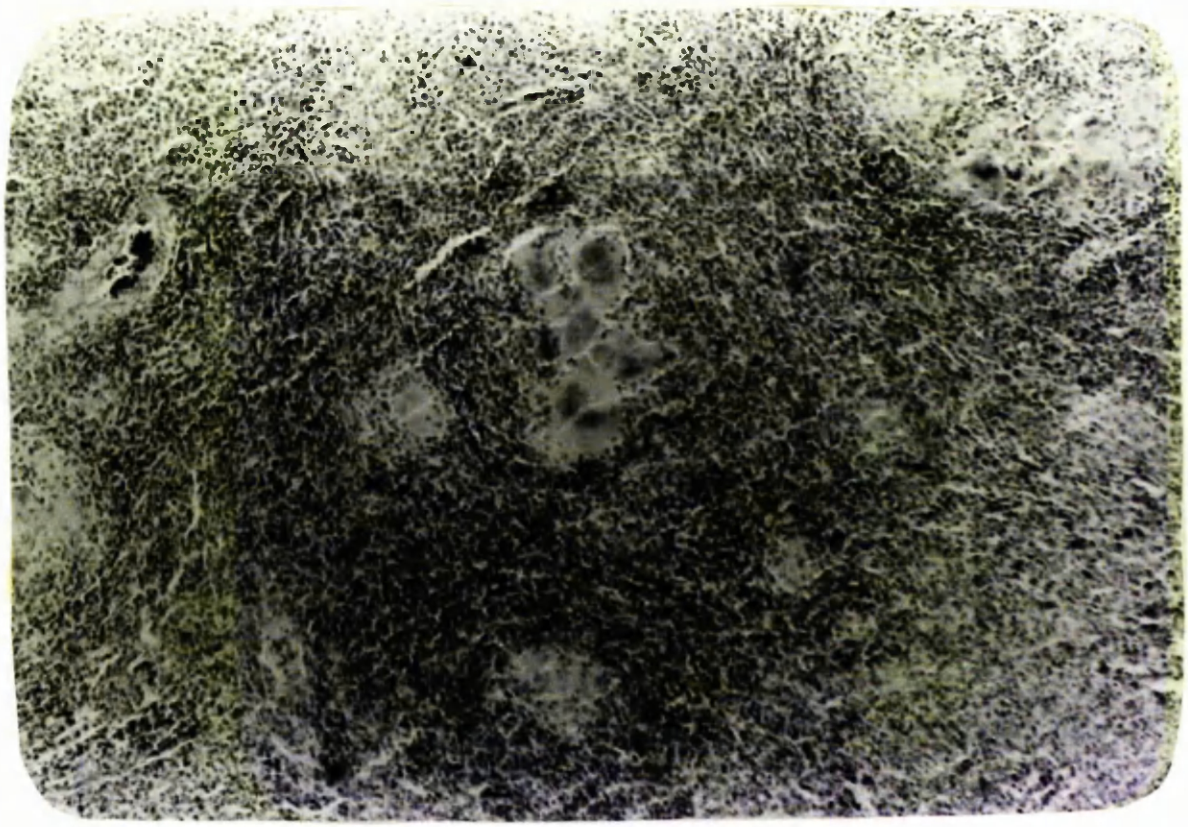
Lung - The pulmonary lesions took the form of focal granulomata in 6 out of 6 of the animals receiving either saline or water-in-oil emulsion preparations, being evidenced by numerous miliary nodules 1-2 mm in diameter scattered through all lobes. Microscopically the granuloma showed a predilection for subpleural locations but a random distribution was not uncommon. Focal aggregations of lymphoid tissue in peribronchial areas were not considered as evidence of localized lesions, since these were found commonly in the normal controls.

Plate 9 : Chicken lung collected 7 days after a single intravenous injection of physiological saline (1 ml). The section shows normal appearance of alveoli.

H & E X 1,200

Plate 10 : Chicken lung collected 7 days after a single intravenous injection of M. avium (6 mg/bird). The section shows granulomata and some cellular infiltration of the surrounding tissue.

H & E X 1,200



The liver and spleen showed slight hyperplasia and no sign of granulomata of epithelioid cells.

Liver - There were no miliary nodules located in the liver upon examination.

Spleen - There was evidence of reticuloendothelial hyperplasia in the red pulp, and considerable proliferation of plasmoblasts and plasma cells both in the mantle zone of the malpighian follicles and in isolated islands in the red pulp.

Kidney - Granulomata were not found in the kidneys of any of the animals.

The capacity of dead Mycobacteria to produce a tissue reaction quite similar to that typical of tuberculous infections has been known for a long time. Heat-killed saprophytic acid-fast bacilli in oil caused pulmonary lesions in chicken no matter what the dose, but only seldom when injected in a low dose in aqueous medium (saline). This was earlier observed in aqueous medium by Hagan and Lavine (1932). These findings were confirmed in rabbits (Rist, 1938), though dispersion of the Mycobacteria was not noted in the absence of mineral oil.

Therefore, the macrophage stimulating effects of mycobacteria in a water-in-oil emulsion were greater than those of saline suspensions of mycobacteria not only as judged by the elevation of acid phosphatase, but also judged from the overall size of the pulmonary lesions. These experiments also show that disseminated granulomata and elevation of levels of acid phosphatase can be produced by adjuvant-acitve mucobacteria without any added antigen, and are not dependent on the presence of the antigen with which the adjuvant is combined.

G. THE INFLUENCE OF M. avium ON PHOSPHOLIPASE A LEVELS IN LUNG TISSUES OF THE CHICKEN in vivo

PURPOSE

The purpose of this experiment is to determine whether M. avium affects the amount of phospholipase A within lung macrophages.

The lungs from 6-8 week old chickens that had been injected intravenously 7 days previously with 6 mg of M. avium, were obtained immediately after death. The lungs were homogenised as outlined in the methods section, and Triton X-100 (0.1%) was added to ensure complete lysosome lysis. Aliquots of these preparations were assayed for phospholipase A as described in the methods section.

The result of these experiments is shown in Table 6.

Table 6 shows the results of a short term experiment (7 days). The level of phospholipase A is changed following stimulation with M. avium and can be observed even after 7 days. These findings of elevation of phospholipase A in the macrophages of M. avium stimulated chicken are in general agreement with those of Munder et al., (1965) in their experiments using FCA to stimulate macrophages in vitro. However, Munder et al., (1969) reported that it was rather difficult to test the influence of FCA on the metabolism of macrophages in vitro, because the mixture of paraffin oil, sorbitol-laurate and mycobacteria is non-miscible with water. Even after extensive emulsification of the adjuvant with the cell suspension fluid, a two phase system will

reform within 10-30 minutes. Therefore, the experimental conditions are not easy to reproduce. Herbert (personal communication) has postulated and demonstrated this phenomenon in water-in-oil emulsion and in sha sha mice. Nevertheless, in all his experiments, Munder reported that contact with and/or phagocytosis of the emulsified adjuvant led to the formation of lysolecithin.

As early as 1 hour after injection a significant breakdown of lecithin and cephalin occurred and lysolecithin accumulated. However, in the experiments with alveolar macrophages of rabbits stimulated with FCA, no difference was found between the effect of FCA and Freund's incomplete adjuvant. Therefore, Munder et al., (1965) did further experiments to determine whether paraffin oil (Bayol F) or the emulsifier (Arlacel A) was the stimulation agent. Only the Arlacel A was reported to give significant formation of lysophosphatides in macrophages and Bayol F had no effect.

In the present experiment the phospholipase A content of lung homogenised tissue from animals injected with M. avium increased above that for control animals even though the M. avium was suspended in saline (Table 6). Therefore, it would appear that mycobacteria can initiate this activity even in the absence of the oils, which had been used by Munder et al., (1965) as carriers for the organisms.

Lysolecithin has been shown to stimulate phagocytosis (Burdzy et al., 1964; Kaboth, 1965), cause the spontaneous haemolysis of erythrocytes (Munder et al., 1965) and to enhance bacterial growth (Munder et al., 1965). It is thought that by labilizing cellular

membranes without disrupting them, lysophosphatides may stimulate cell division, a similar mechanism to that proposed for the surface-active vitamin A (Dresser, 1968). It is also thought that lysophosphatides may induce a better cellular uptake of antigenic material, or as for vitamin A, a better non-specific localization of antigenic material on the membrane by changing the membrane structure (Lucy, 1964). Activated phospholipase A could therefore have some adjuvant effect and further studies were carried out with pure phospholipase A in studies of the antibody response in the chicken.

TABLE 6

PHOSPHOLIPASE A ACTIVITY IN CHICEKN LUNG HOMOGENATES, MEASURED BY RATE OF CHANGE OF pH DUE TO RELEASE OF FATTY ACIDS FROM LECITHIN

(7 days after injection of *M. avium* or saline)

Test	Rate of change of pH (Δ pH/h)	Mean (for all chickens)	Standard error
M. avium group	0.103	0.173	± 0.035
	0.150		
	0.235		
	0.273		
	0.104		
Saline group	0.019	0.087	± 0.021
	0.080		
	0.146		
	0.107		
	0.086		

Time course of hydrolysis of lecithin to lysolecithin by phospholipase A of whole chicken lung homogenate.

(i) THE EFFECT OF PHOSPHOLIPASE A ON SERUM ANTI-HSA LEVELS IN THE CHICKEN

PURPOSE

In these experiments an attempt was made to investigate the adjuvant activity of pure phospholipase A (bees' venom) in the chicken.

On the basis of the finding in the preceding experiment (the effect of M. avium in stimulating the levels of phospholipase A in the lungs of chickens) it became of interest to investigate the adjuvanticity of pure phospholipase A in the chicken in vivo. Two experiments were designed to shed light on this question.

Experiment I

In these experiments three groups of 12 week old chickens (Thornber 404) were injected intravenously as follows:

Group 1 received 100 µg HSA in W.O.W. (8 animals)
Group 2 received 20 µg phospholipase A plus 100/µg HSA in W.O.W. (8 animals)
Group 3 received 200 µg phospholipase A plus 100/µg HSA in W.O.W. (8 animals)

One bird from each group was killed at day 6, 12, and 49 for histological studies. Blood samples for antibody assay were collected on days 6, 7, 8, 12, 15, 21, 28, 35 and 42.

The results of these experiments are shown graphically in Figure 22.

It can been seen from Fig 22 that during the first week after injection of phospholipase A plus HSA there was a sharp increase in antibody level which declined rapidly during the second week. This is the type of response one gets if HSA suspended in W.O.W. is injected alone as can be seen in the control experiment group.

After 21 days there is a further rise in antibody levels to day 35 using phospholipase A and thereafter a more moderate decline. After day 35 there is a slight rise in antibody levels in the control birds injected with HSA in W.O.W. without i.e. phospholipase A, and this level continues to rise to day 42. The differences in antibody levels between the two groups were small and this experiment therefore showed little or no enhancing effect of phospholipase A on antibody levels to HSA.

Experiment II

In another experiment four groups of adult hens, 12 weeks old, were injected intramuscularly as follows:

Group 1 received 40 µg HSA in W/O as a control group (3 animals)
Group 2 received 40 µg HSA plus 400 µg phospholipase A in W/O (3 animals)
Group 3 received 40 µg HSA plus 5 mg M. avium in W/O (3 animals)
Group 4 received 40 µg HSA plus 40 µg phospholipase A in W/O (3 animals).

Blood samples were taken on days 7, 9, 12, 14, 21, 28, 35, 42 and 49.

The results of these experiments are shown graphically in Figure 23.

It can be seen from the results that much more antibody was produced with FCA than with the other HSA and PL-A combinations studied. The differences between the antibody levels with and without phospholipase A were minimal.

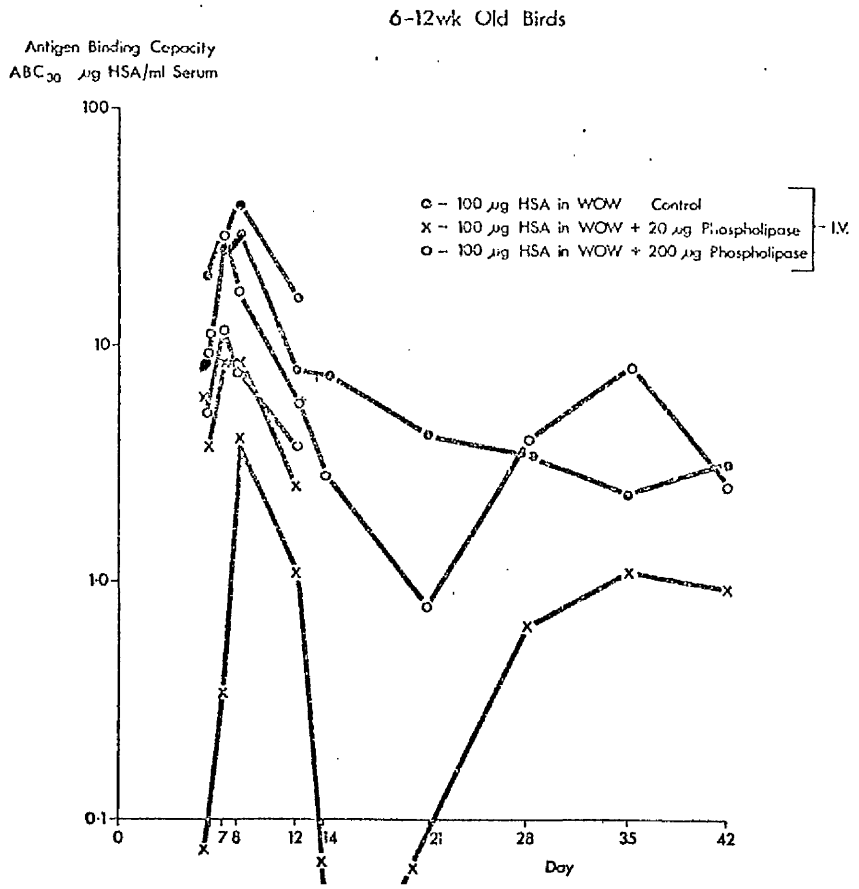
CONCLUSION

1. The differences between antibody levels in groups injected with HSA either with or without phospholipase A were negligible, in contrast to the group injected with HSA in FCA in which a clear adjuvant activity on antibody levels was observed.

2. It can be concluded from these experiments that phospholipase A has little adjuvant activity when injected into the chicken as judged by the levels of serum antibody attained.

DISCUSSION

Phospholipase A would be likely to show effects on phagocytic cells *in vitro* where the enzyme comes into direct contact with the cell surface and forms lysophosphatides thus altering the permeability of the membrane. However, phospholipase A injected *in vivo* is likely to become dispersed and degraded and the chances of its contacting the surfaces of lymphocytes or macrophages are small. It is therefore not surprising that adjuvant effects were not observed in the present experiments.



EXPERIMENT NO. I

Figure 22: Comparison of serum antibody responses in the chicken (values for individual birds) at various times after an intravenous injection of HSA plus phospholipase A, 200 µg and 20 µg in water-oil-water emulsion; with those from birds similarly injected with HSA in water-oil-water emulsion (control).

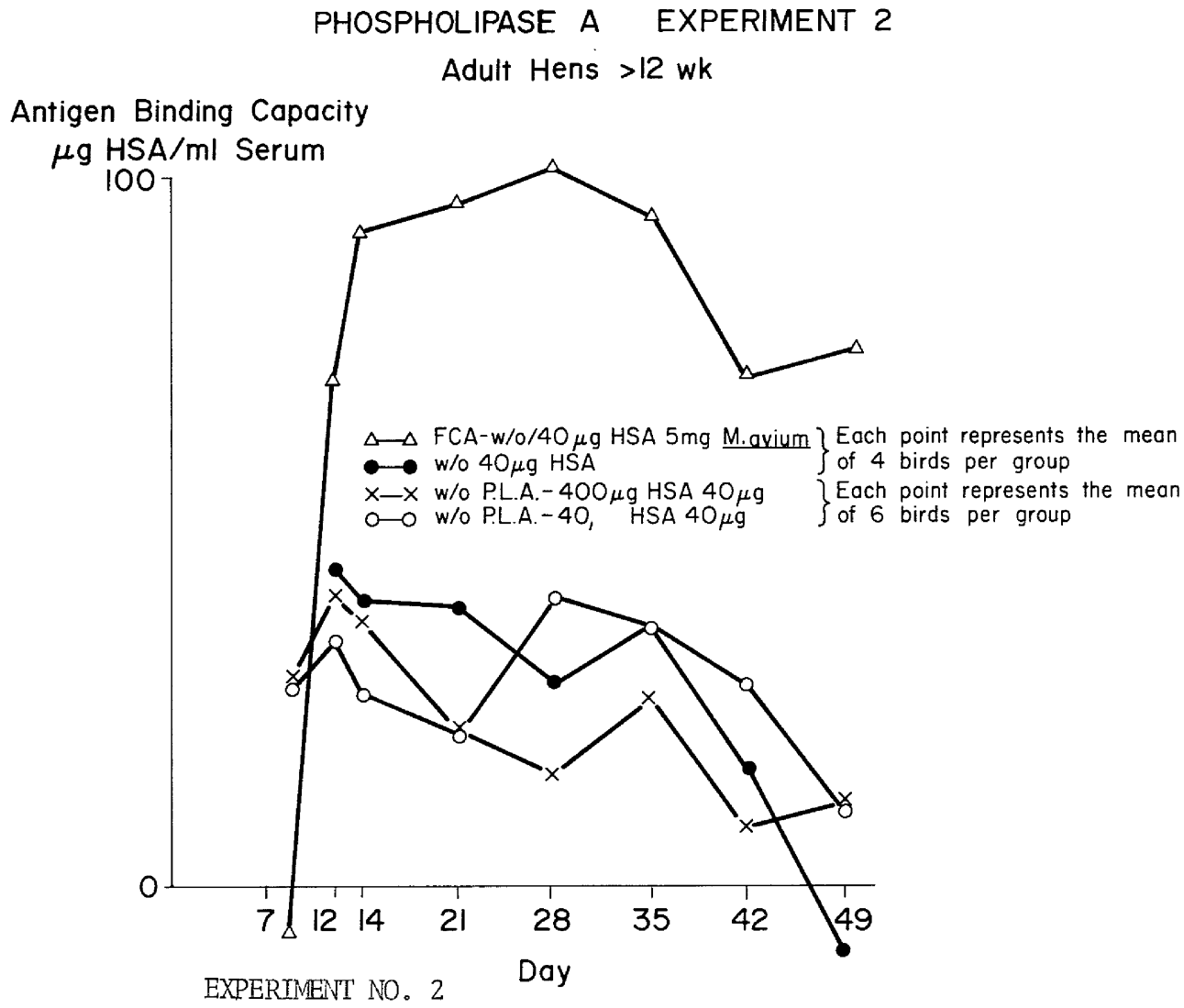


Figure 23: Comparison of serum antibody responses in the chicken (average values for groups of 4-6 birds) at various times after an intramuscular injection of HSA plus phospholipase A, 400 μg and 40 μg in water-in-oil emulsion; with those from birds similarly injected with HSA in water-in-oil emulsion (control), & with HSA plus M. avium (5 mg), to show the adjuvant effect.

H. EFFECT OF M. avium STIMULATION in vivo ON PULMONARY LEVELS OF GLYCOSIDASES IN THE CHICKEN. β-glucuronidase, β-galactosidase, α-mannosidase and N-acetyl-β-glucosaminidase

PURPOSE

The observation that phospholipase A and acid phosphatase levels were elevated in chicken lungs after M. avium stimulation, prompted investigation of other glycosidases associated with intracellular digestion: the levels of β-glucuronidase, β-galactosidase, α-mannosidase and N-acetyl-β-glucosaminidase from lung wash exudates and whole lung tissue homogenates were determined. In addition after M. avium stimulation, histochemical and enzyme assay studies of lung tissue were performed in order to demonstrate the accumulation of these enzymes in lung tissue.

In these experiments eight 6 week old chickens weighing around 800-1000 g each were injected with 6 mg intravenously of heat killed M. avium suspended in saline. A control group of (8) birds received saline alone.

All animals were killed at the end of one week and immediately after death the lungs were washed via the trachea (as outlined in methods section of this thesis) removed, weighed and homogenised. Aliquots of the homogenate were assayed for the various enzymes studied, by techniques outlined in methods.

Histochemical methods for lung tissue have also been described in methods section.

The results of enzyme assay are shown in Table 7. It can be seen that the levels of all enzymes studied were raised in lung exudate and homogenates in the animals stimulated with M. avium, when compared to control birds (i.e. unstimulated).

Histochemical studies of lung tissue are shown in Plates 11, 12 and 13. It can be seen that where galactosidases are increased in amount, there is concentration of these enzymes in the region of macrophage proliferation along the alveolar membrane. This effect was observed only in birds stimulated with M. avium and not in unstimulated controls.

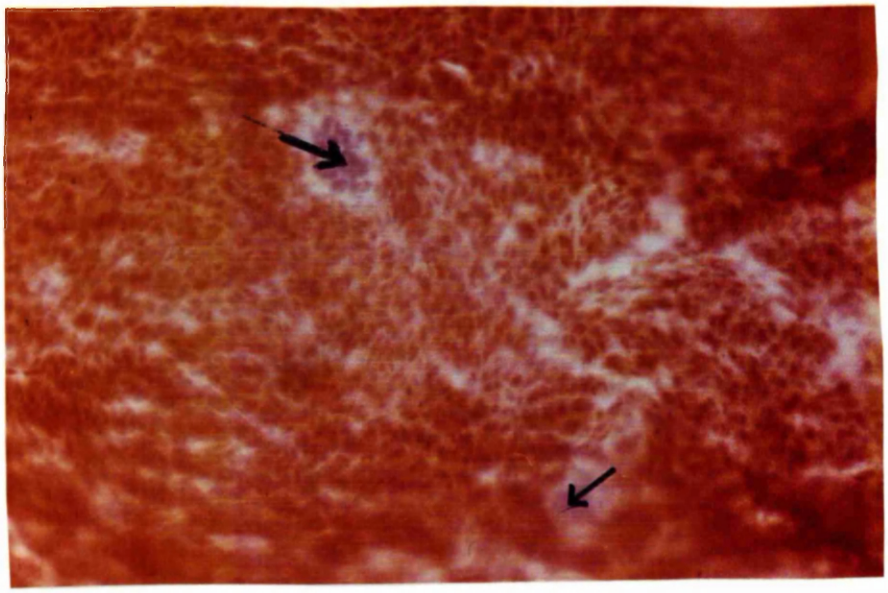
CONCLUSIONS

1. That levels of β-galactosidase, β-glucuronidase, α-mannosidase and N-acetyl-β-glucosaminidase are elevated in the chicken lung as a result of M. avium stimulation in vivo.

2. That the levels of these enzymes can be raised in a few days after M. avium stimulation.

Plate 11 : β-galactosidase in lung tissues of chickens after M. avium stimulation. The purple (arrow) shows the area of concentration of this enzyme in the lung tissue stained by the method of Rutenburg.
X 400.

Plate 12 : M-acetyl-β-D-glucosaminidase in chicken lung tissues after M. avium stimulation. The purple (arrows) show the distribution of this enzyme in the lung tissue as demonstrated in the method of Rutenburg.
X 400.



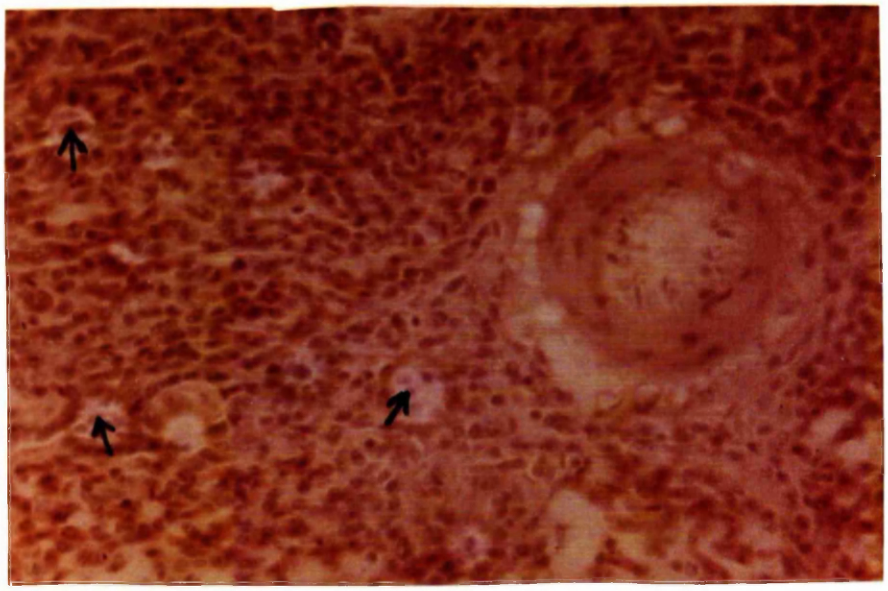


Plate 13 : β-galactosidase in chicken lung tissues after *M. avium* stimulation. The precipitated granules of this enzyme (arrows) are stained a strawberry red by the method of Rutenburg.
X 400.

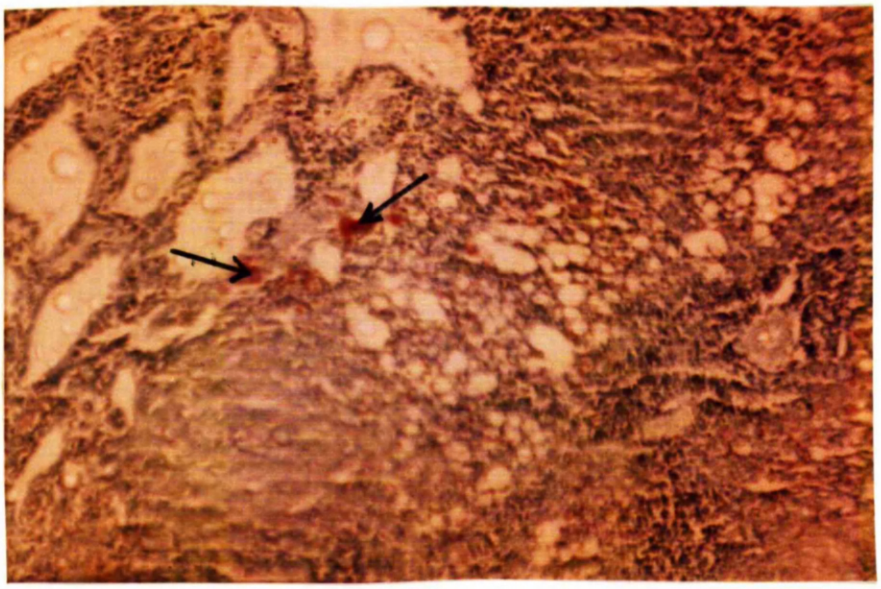


Table 7:

AVIAN GLYCOSIDASES

THE LEVELS OF β-GALACTOSIDASE, α-MANNOSIDASE, β-N-ACETYLGLUCOSAMINIDASE AND β-GLUCURONIDASE IN CHICKEN LUNG TISSUES 7 DAYS AFTER INTRAVENOUS INOCULATION OF *M. avium*

WHOLE LUNG HOMOGENATE			
β-galactosidase	β-glucuronidase	N-acetyl-β-glucosaminidase	α-mannosidase
M. avium injected chickens			
89.61 µg/g	193.82 µg/g	8.1 µg/g	7.5 µg/g
SALINE CONTROL			
11.55 µg/g	35.91 µg/g	0.21 µg/g	0.046
LUNG WASH EXUDATES			
β-galactosidase	β-glucuronidase	N-acetyl-β-glucosaminidase	α-mannosidase
M. avium injected chickens			
0.84 µg/10^6 cells	2.52 µg/10^6 cells	0.35 µg/10^6 cells	0.15 µg/10^6 cells
SALINE CONTROL			
0.02 µg/10^6 cells	0.005 µg/10^6 cells	Trace	Trace

Enzyme levels in homogenates or lung wash exudates were measured after incubation for 1-2 hour periods in the presence of Triton X-100. Details of other assay conditions are given in the Methods.

(a) THE EFFECT OF CELL WALLS OF *M. avium* AND *M. tuberculosis* (strain C) ON THE LEVELS OF ACID PHOSPHATASE, β-glucuronidase, β-galactosidase and α-mannosidase IN WHOLE CHICKEN LUNGS *in vivo*

In the experiments described previously whole organisms were used and the purpose of the experiments now described was to establish the effects of administration of proteinase-treated and non-treated cell walls (i.e. protein free) in stimulating the lysosomal acid hydrolases.

M. avium and *M. tuberculosis* (strain C) were chosen for this experiment because they had already been shown to cause greater stimulation of enzymes than other strains of mycobacteria.

Cell wall preparations were prepared from heat-killed *M. avium* and *M. tuberculosis* (strain C) by ultrasound disintegration. Aliquots of the cell wall preparations were treated with proteinase as outlined in the methods section. Other preparations were prepared by treating organisms in the same way but substituting a neutral solvent (saline) for the digestant (proteinase).

Three groups of chickens were used as follows: Group 1 received 6 mg (dry weight) *M. avium*, Group 2, 6 mg *M. tuberculosis* (strain C) per bird and Group 3, saline only.

The results are shown in Table 8.

The results show that there was a moderate but consistent difference (16 times out of 16) between the proteinase treated and

Table 8 : LYSOSOMAL ENZYME LEVELS IN CHICKEN LUNGS 7 DAYS AFTER INJECTION OF MYCOBACTERIAL CELL WALLS

	CELL WALL PREPARATIONS							
	Untreated Cell Walls				Treated (Pronase) Cell Walls			
	Acid Phosphatase	β-Glucuronidase	β-Galactosidase	α-mannosidase	Acid Phosphatase	β-Glucuronidase	β-Galactosidase	α-mannosidase
	K.A. units/100 ml*	µg/ml‡	µg/ml	µg/ml	K.A. units/100 ml	µg/ml	µg/ml	µg/ml
M. avium	165	32.42	27.33	16.49	146	28.63	25.51	12.73
	K.A. units/g	µg/g	µg/g	µg/g	µg/g	µg/g	µg/g	µg/g
	1,176.251 lung tissue	234.900	187.925	118.900	1,058.50	207.350	184.875	092.075
	K.A. units/100 ml	µg/ml	µg/ml	µg/ml	K.A. units/100 ml	µg/ml	µg/ml	µg/ml
M. tuberculosis Strain C	110	29.1	20.4	9.3	97.64	22.52	18.2	7.56
	K.A. units/g	µg/g	µg/g	µg/g	µg/g	µg/g	µg/g	µg/g
	797.50	210.975	147.900	67.425	707.600	163.125	131.950	54.375

* K.A. units/100 ml lung homogenate
‡ µg/ml lung homogenate

$$\text{Enzyme index} \quad \frac{\text{enzyme/ml}}{\text{total ml of homogenate}} = \text{enzyme/g}$$

MEAN OF 3 ANIMALS PER TEST

non-treated cell walls in elevating the levels of acid phosphatase, β-glucuronidase, β-galactosidase and α-mannosidase. However, the levels of these enzymes were higher with stimulation by M. avium cell walls than by M. tuberculosis (strain C) cell walls, and this confirms similar findings in previous experiments where whole organisms had been used. This could be because M. avium is naturally pathogenic for the chicken whereas M. tuberculosis (strain C) is not.

CONCLUSION

1. Cell walls can stimulate production of acid phosphatase and other hydrolases.

2. Proteinase-treated cell walls were still active in stimulating hydrolases and therefore the integrity of the peptide moiety of the cell is not necessary for stimulation of production of acid phosphatase or other acid hydrolases.

I. EFFECTS OF VARIOUS CORYNEFORMS AND 'CLASSICAL' PROPIONIBACTERIA ON THE ACID HYDROLASES IN CHICKEN LUNGS *in vivo*

Immunologists in recent years, notably Halpern and his colleagues (1963) in France, have used several strains of anaerobic coryneforms (*C. parvum*) as adjuvants and as inhibitors of tumour growth. O'Neill *et al.*, (1973) observed that coryneforms were as effective as mycobacteria in stimulating particle clearance (K) by the reticuloendothelial system as described by Howard (1959).

Corynebacteria are specific stimulators of macrophages and since *Mycobacteria* were shown in preceding experiments of this thesis to be active stimulators of macrophages, and of lysosomal enzymes, it seemed important to study the effect of corynebacteria specifically on the lysosomal enzymes. However, the anaerobic coryneforms cell wall structure and biochemistry are similar to mycobacteria. Biological effects, like chemotaxis *in vitro* and granulomatous lesions they produce *in vivo*, however, are rather different from those of the mycobacteria. This is discussed further below, but it might be possible that the two types of organism have different effects on macrophage physiology.

Experiment A

In these experiments, 6-8 week old chickens were injected intravenously with one of the following organisms (6 mg/bird):
There are four groups of corynebacteria *serologically* grouped according

to Johnson and Cummins (1972) thus 4 groups of bacteria are all represented in the above design. The experimental groups of animals used were as follows. They are designated by capital letters to contrast with the organisms with which they were injected (whose serological groups are numbered 1-4). Group A, M. avium, Group B, P. avidum (4982, Serological Group 4), Group C, P. arabinosum (8981 'classical' propionibacteria), Group D, P. granulasum (0507 Serological Grou 3), Group E, C. parvum C (NCTC Serological Group 3), and Group H saline alone.

The animals were killed after one week and the lung tissues collected. The method used in preparing these tissues and that of the lung wash are outlined in the methods section of this thesis.

Aliquots from these preparations were assayed for β-glucuronidase and β-galactosidase as before and the results of these determinations are shown in Table 9.

The coryneform bacteria are not as efficient in stimulating lysosomal enzymes as mycobacteria. Indeed, M. avium produced twice as much stimulation as the most active of the coryneforms (Seroligical Group 1). Nevertheless, some of the coryneforms did stimulate increase in lysosomal enzyme levels when compared with controls, this difference being present both in tissue homogenates and lung washings. The increase in enzyme content was one or more times above control values.

All organisms of this corynebacteria group, with one notable exception of C. parvum C showed a high degree of activity in

comparison to the 'classical' propionibacterium P. arabinosum (8901) and saline controls.

Organisms from Serological Group 4 were better than the organisms from serological group 3 or than the 'classical' propionibacteria at increasing the level of activity of various lysosomal enzymes (Table 9).

Table 9: THE EFFECT OF INJECTION OF *M. avium* AND VARIOUS CORYNEFORMS ON N-ACETYL-β-GLUCOSAMINIDASE AND α-MANNOSIDASE LEVELS IN THE LUNG OF CHICKENS 7 DAYS AFTER A SINGLE INJECTION

	Enzyme level in Whole Lung Homogenate				Enzyme level in Lung Wash			
	N-Acetyl-β-Glucosaminidase		α-Mannosidase		N-Acetyl-β-Glucosaminidase		α-Mannosidase	
Organism Injected	per ml of tissue homogenate	per gem of lung tissue	per ml	per gm	per ml	per 10^6 cells	per ml	per 10^6 cells
M. avium	12.30	891.750	2.38	172.550	1.09	30.520	0.86	2.4080
P. avidum 4982	1.91	138.475	0.75	54.375	0.63	18.640	0.37	0.9360
P. granulosum 0507	1.31	94.975	0.37	25.825	0.41	11.480	0.19	0.5320
P. arabinosum 8901	0.90	63.225	0.54	39.170	0.19	o.5320	0.28	0.7840
P. parvum "C"	0.64	44.960	0.26	18.850	0.22	0.6160	0.45	1.2600
Saline Control	0.37	25.825	0.15	10.875	0.11	0.3080	0.28	0.7840

(Mean of 3 birds per test)

Experiment B

EFFECTS ON THE LEVELS OF LYSOSOMAL ENZYMES N-acetyl-β-glucosaminidase AND α-mannosidase LEVELS IN THE LUNGS OF CHICKENS STIMULATED WITH VARIOUS CORYNEFORM BACTERIA *in vivo*

It has been shown in part A of this experiment that coryneform bacteria will stimulate lysosomal enzymes to varying degrees (those enzymes which are responsible for bacterial digestion). It is therefore the purpose of this experiment to assay two other lysosomal enzymes, N-acetyl-β-glucosaminidase and α-mannosidase. These enzymes are also thought to be important in bacterial digestion.

Six groups of 4 birds per group, of 6-8 week old chickens were used. The groups received the following organisms intravenously:

Group A received 6 mg *M. avium*; Group B, 6 mg *P. avidum* (4982 Serological Group 4); Group C, 6 mg *P. granulosum* (0507, Serological Group 3); Group D, 'classical' propionibacteria (5958); Group E, carbon particles (16 mg/ml) 1 ml/100 g body weight; and Group F, saline as a negative control.

The animals were killed after one week and the tissues and lung wash exudates prepared as in part A of this experiment.

The results are set out in Table 10.

It can be seen that *M. avium* greatly increased the levels of both N-acetyl-β-glucosaminidase and α-mannosidase. *P. avidum* (4982

Table 10: ENZYME LEVELS IN CHICKENS WHOLE LUNG HOMOGENATE
(7 days after injection of various bacteria or carbon)

Organisms	Serological Group	N-acetyl-β-glucosaminidase (per gram lung tissue) µg/g	α-mannosidase (per gram lung tissue) µg/g
M. avium		8.1	7.5
P. avidum No. 4982	IV	2.24	0.091
P. granulosum No. 0507	III	1.53	0.031
"Classical" Propionibacteria (No. 5958)		0.856	0.016
Carbon		0.65	0.026
Saline		0.47	0.032

ENZYME LEVELS IN CHICKEN LUNG EXUDATES
(7 days after injection of various bacteria or carbon)

Organisms	Serological Group	N-acetyl-β-glucosaminidase (per gram lung tissue) $\mu g/10^6$ cells	α-mannosidase (per gram lung tissue) $\mu g/10^6$ cells
M. avium		0.921	0.365
P. avidum No. 4982	IV	0.503	0.126
P. granulosum No. 0507	III	0.241	0.094
"Classical" Propionibacteria (No. 5958)		0.079	0.058
Carbon		0.052	0.062
Saline		0.058	0.042

Serological Group 4), and P. granulosum also provided an increase although this was less dramatic than that for M. avium. The 'classical' propionibacterium (NCTC 5958) and carbon were much less active, although they did have some stimulatory effect. The levels of enzymes assayed were all higher in the test birds than in the saline injected controls.

CONCLUSIONS Experiment A

1. M. avium and P. avidum (4982 Serological Group 4) increased the levels of N-acetyl-β-glucosaminidase and α-mannosidase levels in the lung tissues and lung wash exudates of stimulated chickens. P. granulosum (0507 Serological Group 3) caused an increase in the N-acetyl-β-glucosaminidase levels only.

2. Carbon did not increase the enzyme levels significantly when compared to saline control.

3. 'Classical' propionibacteria did not increase significantly the level of enzymes when compared to both the saline and the carbon treated groups.

DISCUSSION

The results clearly show that the three strains, M. avium, P. avidum (4982 Serological Group 4) and P. granulosum (0507 Serological Group 3) were all excellent stimulants of the lysosomal enzyme system of macrophages in chickens. Their effects were demonstrated after a stimulation of one week in vivo.

The results agree with the work of Bohme and Bouvier (1960) on the effects of fractions of C. parvum in stimulating the reticulo-endothelial system of the mouse. Bohme and Bouvier claimed that this stimulation lasted up to 8 to 10 weeks after injection of the organisms. In the present experiments, these bacterial stimulants have not been studied for longer than one week and it is quite possible that they could elicit their effect over a much longer period.

From these results using different coryneforms, and 'classical' propionibacteria in the chicken, one can conclude that most known bacterial adjuvants in the chicken are good stimulants of the lysosomal enzyme system.

It must be pointed out that the organisms designated C. parvum (like other anaerobic coryneforms) are different from the 'classical' aerobic Corynebacteria, in that there are differences in their cell wall composition, serology and character of the cell lipids.

Many strains of coryneforms also produce a highly specific macrophage chemotactic factor, and can stimulate acid phosphatase production by macrophages in vitro.

I have tested 12 strains of such coryneform organisms for their ability to cause an increase in lysosomal enzyme levels, and observed that the effect reaches its highest level of activity at about 6-7 days. The activity was quantitated, at this time by measuring the level of acid phosphatase, β-glucuronidase, β-galactosidase, α-mannosidase and N-β-acetyl-glucosaminidase in the saline washout, and in the whole lung tissues per gram tissue (wet weight.) The

experiments here and later in the thesis show that they all possess the ability to stimulate lysosomal enzymes in macrophages, but that quantitative differences exist between the three groups. Mycobacteria are the most effective, coryneforms are moderately effective and classical propionibacteria have poor activity. In this respect it is interesting that classical propionibacteria do not have the macrophage chemotactic activity found in coryneform bacteria (Wilkinson et al., 1973a).

CONCLUSIONS Experiment B

1. That C. parvum and related anaerobic coryneforms cause two effects on macrophages in the chicken lungs.
    1. an increase in hydrolase activity per cell
    2. an increase in total number of cells (macrophage proliferation).

2. That 'classical' propionibacteria, although they stimulated lysosomal enzymes in the chicken lung more than saline controls, had a very feeble effect compared to the anaerobic coryneforms. The latter organisms were 8-10 times as effective as 'classical' propionibacteria in stimulating macrophage hydrolases.

3. The stimulative effect of anaerobic coryneforms is not as great as that of Mycobacteria.

J. THE LEVELS OF THE PROTEOLYTIC ENZYME CATHEPSIN-D IN THE LUNGS OF *M. avium* STIMULATED CHICKENS

PURPOSE

In these experiments an attempt was made to assay the proteolytic enzyme Cathepsin-D from lung tissue homogenate of *M. avium* stimulated chickens.

The experiment was done in two parts. Part (A) was a short term experiment (one week) and part (B) was a more extended experiment (15 days).

Experiment A (short term):

In these experiments 6 mg heat-killed *M. avium* was injected intravenously into a group of 8 chickens. The control group of 4 birds received saline alone. After 7 days the animals were killed and the lung exudates collected by washing the lungs via the trachea. Lung tissue was also collected. The preparations of lung wash exudates and lung tissue homogenates were prepared as outlined earlier in methods. Aliquots of each were studied for Cathepsin-D activity, quantitatively.

Experiment B (extended term): (Group of 12 test and 12 control birds.)

Same as Experiment A but for extended period of time (15 days).

The results are shown in Figure 24.

The results indicate that Cathepsin-D levels in the whole lung tissues (lysosomes) in the groups receiving *M. avium* were elevated

as compared to the controls. The levels began to increase after Day 1 (from the time of injection) to a peak on Day 6 which was maintained throughout the duration of the experiment (15 days).

CONCLUSION

Cathepsin-D levels were found to be raised as a result of *M. avium* stimulation.

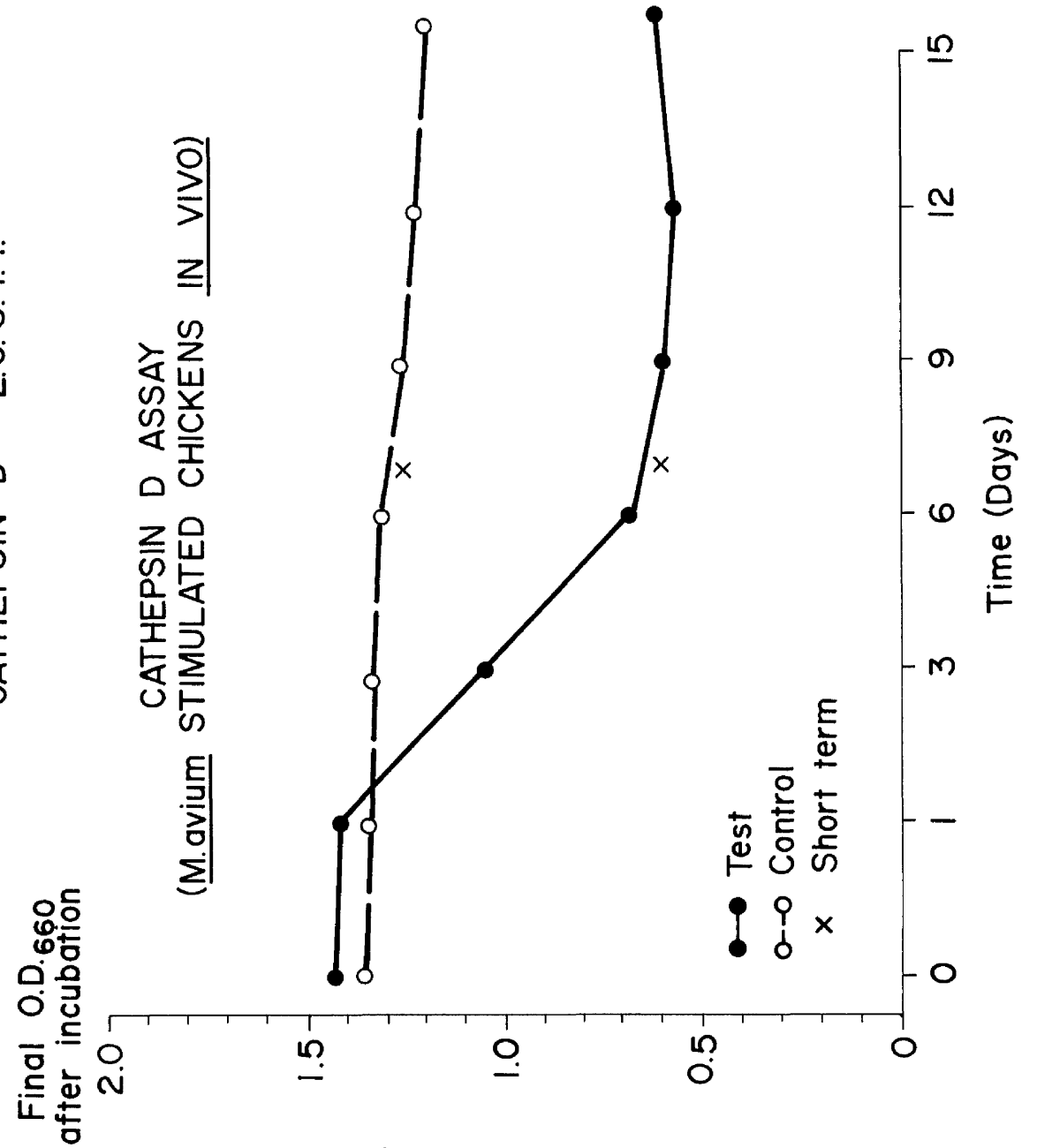


Fig 24: Levels of Cathepsin D in Whole Lung homogenates from chickens stimulated with a single intravenous dose (6 mg) of M. avium, over varying periods of time in 15 days. (Combined results of experiments A and B). Each point represents the mean of three tests on three animals and the changes in acid denatured haemoglobin by the tissue homogenates as measured in change (Δ) in optical density at 660 nM at 37°C.

(i) THE STIMULATION OF LYSOZYME LEVELS IN CHICKEN LUNG TISSUES FOLLOWING INJECTION OF *M. avium* AND VARIOUS CORYNEFORM BACTERIA *in vivo*

Purpose

The purpose of these experiments was to determine the effect of *M. avium* and coryneform bacteria on the level of lysozyme in the lungs of chicken.

In all experiments below, lysozyme in tissue homogenates from stimulated chickens was assayed using viable cultures of *Micrococcus lysodeikticus* an organism which is very sensitive to the lytic effect of this enzyme. To obtain standardisation of the assay, egg-white lysozyme was used as a positive control. Full details of the procedure are outlinedin Methods.

Experiment A

In these experiments a large group of chickens 6-8 weeks old were injected intravenously with 6 mg each of *M. avium* suspended in saline. A control group received saline alone. Three animals were killed each day from both test and control groups over a 14 day period and lung tissues collected immediately after death and homogenised for lysozyme assays.

Experiment B

In these experiments fourteen groups of animals received one of the various heat-killed coryneform bacteria (6 mg) intravenously as follows:

Organism	Serological group (Johnson and Cummins, 1972)
Mycobacterium avium	
P. avidum (0575)	4
C. parvum (3085)	1
P. avidum (4982)	4
C. anaerobium (578)	2
C. parvum (1383)	1
C. liquefaciens (814)	1
C. parvum (0208)	1
C. granulosum (0507)	3
P. freudenreichii	'Classical' propionibacterium
P. jensenii	
P. rubum	
P. arabinosum	
Saline control.	

Experiment A

The results of this experiment are shown graphically in Figure 25.

It can be seen from these results that M. avium elevated the levels of lysozyme in chicken lung following a single intravenous injection of 6 mg. The height of activity was between day 7-9 and thereafter declined. There was a slight increase in the control animals but as pointed out in earlier experiments, i.e. acid phosphatase assays, this could be the result of age of the bird.

Experiment B

The effects of various coryneform bacteria on lysozyme levels in the lungs of chicken are shown in Table 11.

M. avium elicited the greatest response, followed by P. avidum (serological group 4) and then C. parvum 1383 (serological group 1) in stimulating the levels of lysozyme in the lungs of chickens. Most of the coryneform bacteria (i.e. those which are classified in serological groups 1-4) were generally efficient in stimulating the lysozyme response, whereas the propionibacteria (unclassified in the serological scheme) were poor in this respect. However, as can be seen, the latter group did stimulate an increase of enzyme which was up to three times the level recorded in controls.

CONCLUSIONS

1. M. avium and various strains of coryneform bacteria elevated levels of lysozyme significantly in the lungs of chickens.

2. That classical propionibacteria had comparative little effect on lysozyme levels in the lungs of chickens in vivo.

TABLE 11

EFFECT OF VARIOUS HEAT-KILLED BACTERIA (6 mg IN SALINE, INJECTED i.v.) ON LYSOZYME CONTENT OF CHICKEN LUNG 7 DAYS LATER

Stimulating organism injected into chickens	Strain No.	Serological group of organism (Johnson and Cummins, 1972)	Concentration of lysozyme in lung (µg lysozyme/g wet tissue) Mean of 3 birds
M. avium			115.1
P. avidum	0589	IV	19.3
C. parvum	10390	I	2.4
P. avidum	4982	IV	24.5
C. anaerobium	578	II	9.85
C. parvum	6134	I	12.3
C. liquefaciens	2764	I	8.2
C. parvum	3085	I	8.5
C. granulosum	6290	II	6.2
P. freudenreichii	10470		3.1
P. jensenii	5960		1.40
P. rubum	5958		1.10
P. arabinosum	8901		0.92
Saline			0.74

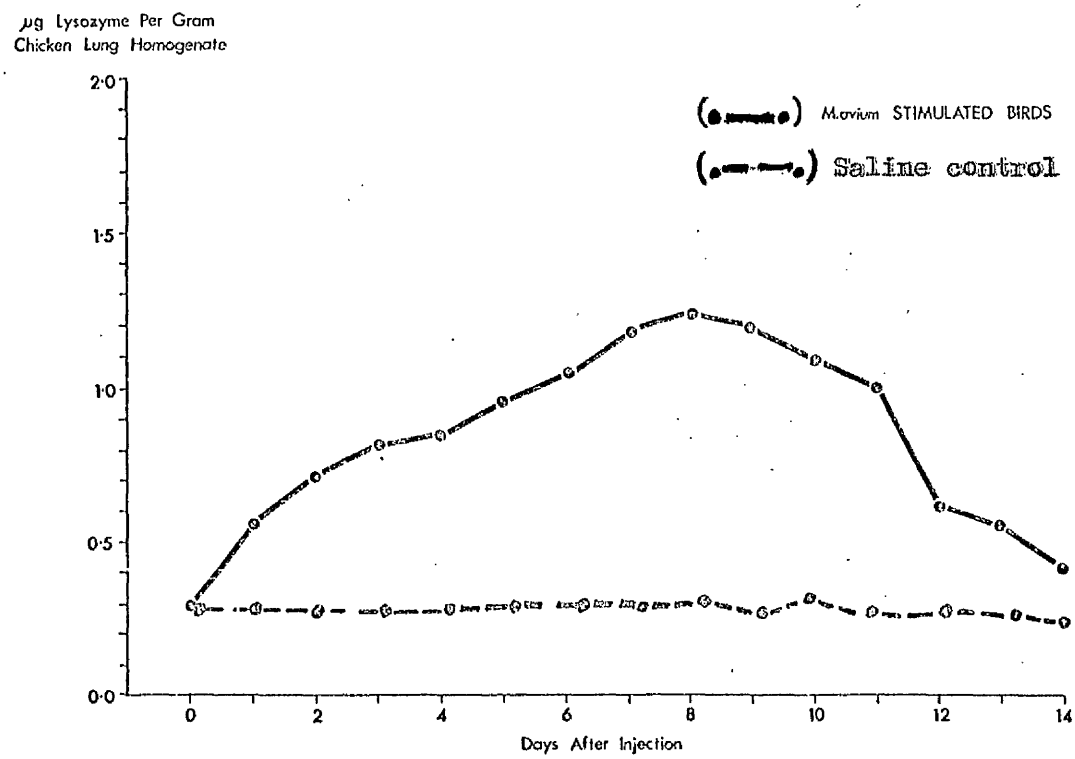


Fig 25: LYSOZYME LEVELS in chicken lung at various times after injection of M.avium into chickens. Each point represents the mean of the enzyme levels in the lungs of 3 birds.

K. THE EFFECT OF CORYNEBACTERIA AND MYCOBACTERIA ON SHORT-TERM SECRETION OF LYSOSOMAL ENZYMES BY PHAGOCYTIC CELLS *in vitro*

It has been shown already in the foregoing experiments that mycobacteria and some anaerobic coryneforms have a marked effect on enzyme levels in phagocytic cells, and that the ability to enhance these enzyme levels correlates very well with the ability to stimulate chemotaxis and phagocytosis. It has still not been shown, however, whether the bacteria cause the stimulation of enzyme levels by acting directly on the phagocytic cells, or whether the effect depends on other cells or humoral factors. Nor has it been shown whether or not the effect depends wholly on enzyme synthesis, or whether, especially in the short-term, the effect is one of activation or release of enzymes already synthesized.

It was the purpose of this experiment to answer these questions by allowing mouse peritoneal macrophages to remain in contact with the bacteria *in vitro*, and then to assay the whole suspension and also the extracellular fluid for enzymes released during the first two hours of incubation.

Most of the tests were carried out both in the presence and in the absence of plasma. This was done in order to see whether some bacteria stimulate the macrophages only in the presence of plasma, an effect that has been reported in studies of the chemotactic effects of mycobacteria (Symon *et al.*, 1972). In some of the experiments, Puromycin and Actinomycin D, inhibitors of protein synthesis, were added before incubation to ascertain whether the effects observed were

dependent on protein synthesis. The specificity of action of the bacteria on enzyme levels was investigated by assaying lactic dehydrogenase as well as the enzymes directly concerned with non-specific immunity. This LDH assay should indicate whether the lytic enzymes were being actively and selectively secreted, or whether they were merely being released as a result of increased membrane permeability. If the membrane became leaky, both LDH and the lysosomal enzymes would be released (Dresser, 1958). On the other hand, specific actions of bacteria on lysosomes should not influence the distribution of LDH.

To obtain pure unstimulated mouse macrophage cultures, 9-10 week old mice (BALB C) of both sexes were injected intraperitoneally with sterile Gey's medium immediately after death by cervical dislocation. Immediately the abdomen was kneaded as outlined under Methods and the macrophages were collected, pooled and processed as outlined under Methods. The macrophage culture numbers were adjusted to 10^6 cells per ml and groups of cultures were set up from this suspension. Each aliquot of cells contained one of the following substances: Gey's medium (negative control), casein (1 mg/ml), 10% human plasma, M. tuberculosis H37Rv, M. tuberculosis H37Rv + 10% human plasma, C. diphtheroides (814), C. diphtheroides (814) + 10% human plasma, P. avidum (4982), P. avidum (4982) + 10% human plasma, M. tuberculosis strain C and M. tuberculosis strain C + 10% human plasma, puromycin D (500 µg/ml) + P. avidum (4982), actinomycin (500 µg/ml) + P. avidum (4982).

The plasma was used to give a baseline value for comparison with the various preparations containing plasma plus bacteria. Gey's

medium was used as a negative control, and casein was used as a known positive control.

The cultures were incubated in a 37°C water bath equipped with a mechanical shaker for 2 hours and immediately frozen at the end of incubation period. The suspensions were frozen and thawed five times and 0.1% Triton X-100 added; aliquots were taken from these suspensions for enzyme assays.

The results of these experiments are shown in Table 12A and 12B.

It can be seen from these experiments that addition of M. tuberculosis strain C, M. tuberculosis H37Rv and the various Coryneforms to macrophage cultures caused changes in the levels of the lysosomal enzymes acid phosphatase, β-glucuronidase, β-galactosidase, and one of the cytoplasmic marker enzymes, lactic dehydrogenase.

These changes are detailed in Table 12A, B. In the absence of plasma, the test bacteria caused an elevation of enzyme levels in the macrophages. This elevation was most pronounced when acid phosphatase was measured. The change in the other glycosidase levels was more inconstant. Few experiments are reported here and statistical analysis (courtesy of Dr. I. McKay) showed that the elevation of acid phosphatase levels attained when macrophages were incubated with casein or with P. avidum 4982 assumed statistical significance, Table 12A, B, but that the remainder of the results did not. Further experiments would be required to confirm statistically that M. tuberculosis or C. diphtheroides 814 caused an elevation of macrophage lysosomal enzyme levels. No constant pattern of change was evident when the cytoplasmic enzyme lactic dehydrogenase, was measured.

Table 12A : THE EFFECTS OF CORYNEBACTERIA AND MYCOBACTERIA ON SHORT-TERM STIMULATION OF LYSOSOMAL ENZYME ACTIVITY IN PHAGOCYTIC CELLS (MOUSE) IN VITRO

Test	No. of Test	Acid phosphatase (K.A.units/ml) mean	No. of Test	β-galactosidase 10^{-12} µg/ml/cell mean	No. of Test	β-glucuronidase 10^{-12} µg/ml/cell mean	No. of Test	Lactic dehydrogenase 10^{-12} µg/ml/cell mean
Gey's Medium	3	0.360	2	1.56	3	1.78	3	0.961
Casein (1 mg/ml)	5	1.28	3	0.922	4	2.510	2	0.63
10% Plasma	2	1.04	2	0.990	2	1.91	1	0.55
10% Plasma (No cells)	2	2.581	2	0.540	2	0.54	NR	-
M.tuberculosis H37Rv 100 µg/ml	2	0.402	NR	-	2	0.88	1	0.27
M.tuberculosis H37Rv 100 µg/ml + Plasma	2	0.860	NR	-	2	1.23	1	0.37
C.diphtheroides 814 500 µg/ml	5	0.833	3	1.672	4	2.56	2	1.41
C.diphtheroides 814 500 µg/ml + Plasma	4	1.42	4	1.180	4	3.06	2	2.77

All incubations at 37°C. Macrophages (10^6/ml) suspended in Gey's solution containing one of the above listed combinations (plasma casein). Cells were exposed to these preparations for 2 hours. Values were expressed as mean K.A.units/ml for acid phosphatase and µg/10^6 cells for β-glucuronidase, β-galactosidase and lactic acid dehydrogenase. The percent change in enzyme release is compared with enzyme release from untreated control cells.

10^{-12} = the amount of enzyme/cell

Table 12B : THE EFFECTS OF CORYNEBACTERIA AND MYCOBACTERIA ON SHORT-TERM STIMULATION OF LYSOSOMAL ENZYME ACTIVITY IN PHAGOCYTIC CELLS (MOUSE) IN VITRO

Test	No. of Test	Acid phosphatase (K.A.units/ml) mean	No. of Test	β-galactosidase 10^{-12} µg/ml/cell mean	No. of Test	β-glucuronidase 10^{-12} µg/ml/cell mean	No. of Test	Lactic dehydrogenase 10^{-12} µg/ml/cell mean
P.avidum 4982 500 µg/ml	4	1.14	3	1.48	4	2.01	3	1.01
P.avidum 4982 + Plasma	5	1.28	4	1.15	4	3.05	2	2.62
M.tuberculosis Strain C 100 µg/ml + Plasma	3	1.22		1.26		3.08	1	4.46
P.avidum 4982 500 µg/ml + Puromycin	5	1.27	5	1.05	5	2.65	5	0.35
P.avidum 4982 500 µg/ml + Actinomycin D	5	1.13	5	1.65	5	2.86	5	0.61

All incubations at 37°C. Macrophages (10^6/ml) were suspended in Gey's solution containing one of the above listed combinations (puramicin or actinomycin D). Cells were exposed to these preparations for 2 hours. Values were expressed as mean K.A. units/ml for acid phosphatase and µg/10^6 cells for β-glucuronidase, β-galactosidase and lactic acid dehydrogenase. The percent change in enzyme release is compared with enzyme release from untreated control cells.

10^{-12} = the amount of enzyme/cell

It can be seen that plasma (human) alone without the cells itself contains a high level of the various lysosomal enzymes. It can also be seen that the levels of enzymes in macrophages are, however, elevated even higher than the baseline plasma levels when the macrophages were incubated in plasma together with the test organisms.

Conclusions

1. That M. tuberculosis strain C, M. tuberculosis H37Rv and the various Coryneforms cause an elevation of the level of lysosomal enzymes, particularly acid phosphatase in mouse peritoneal macrophages after a short term incubation in vitro.

2. It cannot be concluded that these bacteria cause an increase in synthesis of these enzymes over such a short time period. It is more probably that release or activation of preformed enzyme is being measured. Experiments where inhibitors of protein synthesis (actinomycin, puromycin) were added to the medium indicated that part at least of the elevation in enzyme levels was not due to synthesis of new protein.

3. The experiments in which the bacteria were incubated with plasma to see if a complement or other factor was activated which could stimulate lysosomal hydrolases were difficult to interpret because of the content of such enzymes in the plasma itself.

(i) EFFECT OF BACTERIAL CYTOPLASMIC MATERIALS ON RELEASE OF LYSOSOMAL ENZYMES FROM MOUSE PERITONEAL MACROPHAGES *in vitro*

It was observed in one of the preceding experiments that whole organisms (Table 9) caused selective release of lysosomal enzymes in chicken macrophages *in vivo*. An attempt was made to determine what effect cytoplasmic (bacterial) materials (soluble material after removal of cell walls) had on cellular release of enzymes from normal mouse macrophages *in vivo*.

Large numbers of normal macrophages, from 7-8 week old female Balb C mice were collected by injecting intraperitoneally 3 ml sterile Gey's medium as was described in Methods of this thesis. Counts were made and cell numbers adjusted to 10^6 per ml.

Bacterial cytoplasmic materials were collected by lysing *P. avidum* (4982), *P. granulosum* (0507), *P. rubrum* (5958), *P. arabinosum* (8901) and *C. parvum* C by ultrasound suspended in Gey's solution.

The cytoplasmic materials were differentially separated by centrifugation and separated into 7 cultures of macrophages in a concentration of 1 in 10 cytoplasmic material to macrophage culture. Gey's solution was used as negative control. This mixture was allowed to incubate for 2 hours at $37^{o}C$. At the end of the incubation period, macrophages were removed by centrifugation, and aliquots taken for assay of acid phosphatase, β-glucuronidase and β-galactosidase. The results are shown in Table 13.

Table 13 The Effects of Cytoplasmic Materials from lysates of anaerobic coryneforms on Lysosomal Enzyme Release from Mouse Peritoneal Macrophages in vitro

Cellular Material	Serological Group	Acid Phosphatase K.A. units/ml	β-glucuronidase µg/ml	β-galactosidase µg/ml
P. avidum (4982)	4	0.239	0.960	1.060
P. granulosium (0507)	3	0.240	1.520	1.270
P. rubrum (5958)		0.200	1.730	1.250
P. arabinosum (8901)		0.215	1.450	1.792
C. parvum C		0.263	1.691	1.850
Gey's solution		0.215	1.824	1.200

All incubations carried out at 37°C with cytoplasmic material and macrophage suspended in Gey's solution. Effect of cytoplasmic material (cellular sap) is compared with Gey's normal control.

The results show that cytoplasmic materials of coryneform bacteria have no significant stimulative activity on enzyme release from macrophages *in vitro*.

L. TIME COURSE OF ACCUMULATION OF CELLS, PRODUCTION OF LYSOSOMAL ENZYMES AND CHANGES IN CHEMOTACTIC ACTIVITY IN THE PERITONEAL FLUID OF MICE INJECTED WITH *M. tuberculosis* (strain C) OR GLYCOGEN

The experiments in the previous section showed that mycobacteria and anaerobic coryneforms stimulate the hydrolases of mouse peritoneal macrophages on incubation for a short period *in vitro*. These bacteria have been shown earlier to stimulate chemotaxis of peritoneal macrophages *in vitro*, the anaerobic coryneforms by a direct effect (Wilkinson *et al.*, 1973a) and the mycobacteria by activating plasma (Symon *et al.*, 1972). It is important therefore to try to show how these effects are related in a more physiological situation, namely following injection of bacteria or other stimulants *in vivo*. Snyderman *et al.*, (1971) showed that after injecting glycogen into the peritoneum of mice chemotactic factors for neutrophils appeared in the peritoneal fluid. Later Wilkinson *et al.*, (1973b) showed that glycogen and *M. tuberculosis* injected into the guinea pig peritoneum caused production of chemotactic factors for macrophages in the peritoneal fluid. However these experiments were only carried over 4 days and little difference was seen between the two stimuli over this short period. Mycobacteria cause a chronic granuloma, glycogen does not. Therefore it is important to determine the macrophage-stimulating properties of mycobacteria compared to glycogen over a longer period (of weeks) and the experiments described below were designed to do this. The effects of intraperitoneal injection into mice of mycobacteria and of glycogen on chemotactic factor production in the peritoneal cavity, and on the number of cells accumulating in the peritoneum, and on the hydrolase content of these cells was compared over a period of 15 days after injection.

Mice were injected intraperitoneally with heat-killed M. tuberculosis strain C (2 mg in saline) or with glycogen (60 µg in saline). At various times (up to 15 days) after injection, six mice of each group were killed and the peritoneal cavities washed out with Gey's solution. Total and differential counts were obtained, and the three enzymes, acid phosphatase, β-glucuronidase and β-galactosidase, were assayed after lysis of the cells as described in Methods.

Samples of the peritoneal washings were centrifuged to remove cells and the supernate assayed for its ability to attract macrophages in vitro.

The results of these experiments are shown in Table 14 and Table 15.

It can be seen from these experiments that mycobacteria and glycogen both increased chemotactic activity in the peritoneal exudate. This increase is seen within twenty-four hours after injection of the stimulant, falling in the second day and flattening on the third day only to decline on the fourth day and thereafter. This activity pattern correlated with the increase and fall in macrophage counts during the first few days after injection.

After stimulation with glycogen, the enzyme levels per 10^6 cells rise to an elevated level at day two although at this time the cell counts are on the decline. The enzyme levels began to decline at day three, but thereafter there is a gradual elevation in enzyme levels over fifteen days at a time when cell counts in the peritoneum have

Table 14: Time Course of Accumulation of Cells, Production of Lysosomal Enzymes and Changes in Chemotactic Activity in the Peritoneal fluid of Mice with *Mycobacterium tuberculosis* Strain C *in vivo*

Time after injection (days)	Cell Counts in Peritoneal Washings					Enzyme Assays in Washed Cells from Peritoneal Exudates (Assayed After Lysis of Cells)			Chemotactic Activity of Cell free Supernate from Peritoneal Washings: Distance Migrated by Normal Macrophages toward Supernate in 30 min; Mean of Filters* (µm)
	Differential Cell Count of 100 Cells								
	Macrophages	Lymphocytes	Mast cells	Eosinophils	Neutrophils	Acid phosphatase (K_eA. units/10^6 cells) mean	β-glucoronidase (µg 4-nitrophenol produced per 10^6 cells)	β-galactosidase (µg 4-nitrophenol produced per 10^6 cells)	
1	45	17	16	15	7	4.6	11.7	15.6	50
2	56	15	12	11	12	9.0	17.6	25.5	54
3	59	20	9	8	4	0.77	3.3	4.1	52
4	62	17	7	9	6	1.75	6.6	11.6	46
8	71	15	3	1	10	9.2	9.7	13	35
12	80	10	3	2	5	48.1	14.1	17.4	25
15	85	10	2	3	5	57.5	25	35.8	32

Each figure represents pooled peritoneal washings from 6 mice.

*Negative control: pooled peritoneal washings of 6 normal mice caused normal macrophages to migrate 24 µm

Table 15

TIME COURSE OF ACCUMULATION OF CELLS AND PRODUCTION OF LYSOSOMAL ENZYMES IN THE PERITONEAL CAVITY OF MICE INJECTED WITH GLYCOGEN

Time Course of Accumulation of Cells, Production of Lysosomal Enzymes and Changes in Chemotactic Activity in the Peritoneal Fluid of Mice with Glycogen in vivo.

	CELL COUNTS					ENZYMES			
Time (days)	Macrophages	Lymphocytes	Mast cells	Eosinophils	Neutrophils	Acid Phosphatase (K.A. units/ 10^6 cells)	β-glucoronidase µg/ 10^6 cells	β-galactosidase µg/ 10^6 cells	Chemotactic activity of the peritoneal fluid. Migration of macrophages (µm in 130 mins)
	(per cent of total cell count in 5 ml)								
1	59	36	4	5	6	0.5	1.25	0	58
2	82	14	0	2	2	6.1	9.3	31	78
3	48	29	9	9	5	23.2	7.3	14.5	64
4	47	32	5	8	8	14.3	16.1	20.4	78
8	35	30	6	9	20	12.5	10.0	15.1	49
12	33	28	6	8	25	6.5	7.5	9.0	35
15	32	30	5	5	28	0.8	2.3	5.3	28

All three biological effects, i.e. the accumulation of macrophage, elevation of enzyme levels in the cells, and production of chemotactic factors in peritoneal fluid are quantitated. Comparisons are made of peritoneal washings fluids and cell lysed from the peritoneal. Each result represents pooled peritoneal washings from 6 mice.

returned to normal and the chemotactic activity of the peritoneal washing likewise.

M. tuberculosis stimulates activity of the various lysosomal enzymes to a sharp peak on the second day. The levels drop sharply on the third day although cell counts are high, but thereafter there is a gradual rise in levels up to twenty-one days, while at the same time the cell counts remained elevated for 15 days.

Although the three enzymes show a similar pattern in elevation or decline of levels, acid phosphatase always gave the highest levels followed by β-galactosidase and β-glucuronidase respectively. Thus both glycogen and M. tuberculosis caused an elevation in lysosomal enzyme levels both in the short term, over the first few days and in the long term, after three weeks. The enzyme activity seen three weeks after stimulation was higher than that seen earlier. At three weeks, stimulated mouse peritoneal macrophages contain endoplasmic reticulum and may be synthesizing substantial amounts of enzyme (R. McInroy, personal communication). In terms of cell counts, the early macrophage response to M. tuberculosis and to glycogen is similar. However, there are many more macrophages present in the peritoneum three weeks after injecting M. tuberculosis than after glycogen. Therefore, although hydrolase production per cell is not greatly different when the two stimuli are compared, the total enzyme produced by mycobacterium stimulated cells is much greater than that produced by glycogen-stimulated cells.

Conclusions

A. EFFECTS OF M. tuberculosis strain C

1. All three biological effects, i.e. the accumulation of cells, elevations of enzyme levels in those cells, and production of chemotactic factors in the peritoneal fluid, were demonstrable.

2. The chemotactic effect is of particular interest since it has not previously been reported that the stimulating agents used endow the peritoneal fluid with chemotactic activity for macrophages. This finding lends support to the belief that the attraction of macrophages into the peritoneal cavity may indeed depend on chemotaxis, rather than on changes in vascular permeability, or on immobilization of cells. The chemotactic activity observed showed a steady decline throughout the experiment, approaching the negative control levels by day 12. This contrasted markedly with the time course of the other biological effects studied.

3. All the enzymes assayed, expressed as enzymic activity per 10^6 cells, appeared to show a short-term increase during days 1 and 2, and a sharp fall on day 3. Thereafter, the three enzyme levels all showed a steady increase throughout the remainder of the experiment, the increase in acid phosphatase being particularly marked.

   This observation would be compatible with the view that the cells possess a short-term mechanism by which enzymes may be activated or in some way be made more easily available, as well as a longer term mechanism by which the synthesis of lysosomal enzymes can be stimulated.

4. The cells attracted into the peritoneal were predominately macrophages, but lymphocytes, mast cells, eosonophils were always present and sometimes neutrophile were found too. It is difficult to draw any firm conclusion about the changes in cell numbers with time.

   The total cell counts were markedly elevated from day 1 to day 15, but there was no clear tendency to increase or decrease, except that the figure obtained on day 3 was higher than usual, a result to which we cannot at the moment attach much significance.

B. EFFECT OF GLYCOGEN

1. In contrast to mycobacteria the cell counts in the peritoneal cavity after stimulation with glycogen remained elevated for only a short time and then returned to normal. On the other hand the few remaining cells after 15 days showed evidence of higher hydrolases.

2. The ability of glycogen to stimulate the formation of chemotaxic factors in the peritoneal cavity appeared to be more short lived than that of mycobacteria.

PART FOUR

DISCUSSION

THE DIGESTION OF KILLED BACTERIA BY MACROPHAGES

The main functions of phagocytes in higher and lower animals are the segregation and digestion of effete cells, particulate matter and damaged macromolecules.

These functions are regarded as belonging to the non-specific or natural immunity mechanisms; as such they might be regarded as part of the genetic endowment of the body. Nevertheless, the activity of a given population of macrophages is altered by experience. The presence of acid hydrolases in their cytoplasmic lysosomes is an adaptive energy requiring process (Cohn and Morse, 1960). For this reason alone, the function of individual cells and of different anatomical populations of macrophages might be expected to differ -- their activity being dependent on their past experience.

In the early experiments of this thesis a comparison was made between various micro-organisms in respect of their phagocytosis and rate of intracellular digestion within macrophages from two different anatomical sites: the peritoneal cavity and lung of the adult fowl. Attempts were also made to activate the macrophages and increase their ability to digest intracellular killed bacteria. Use has been made of two types of bacteria: Escherichia coli and mycobacteria.

The designation of a macrophage as "normal" or "stimulated" is questionable. Nevertheless, for the sake of clarity in this thesis "normal" is used when phagocytic cells are collected from animals which had not received any added stimulus; when "stimulated" cells are

referred to, the term covers cells which have been collected from animals after intravenous or other parenteral injection of an appropriate stimulus.

In the first experiment the rate of digestion in vitro of E. coli (heat-killed and viable organisms) was compared in the macrophages obtained from the lungs and peritoneal cavity of "normal" chickens.

It was found in culture preparations that "normal" peritoneal macrophages digested a given quantity of killed E. coli in approximately half the time taken by "normal" alveolar macrophages to digest the same quantity of the same organisms (Fig 6, page 73). It was further found that normal peritoneal macrophages degraded viable E. coli organisms faster than normal alveolar macrophages under similar conditions where the culture contained 25 per cent homologous chicken serum (which was necessary for adequate digestion). Alveolar macrophages therefore have less killing and degrading power for micro-organisms than peritoneal macrophages, although the former contain more lysozyme and acid phosphatase than the latter. This result would be surprising if it were certain that lysozyme and acid phosphatase were essential for degradation of E. coli by macrophages. However, the relative importance of different metabolic and enzymatic functions for the bactericidal activity of macrophages is still unclear. In neutrophils the peroxidase system is known to be of importance for killing E. coli (Klebanoff, 1967), but this system is poorly represented in macrophages. It is possible that some as yet unidentified bactericidal mechanism is better represented in peritoneal than in alveolar macrophages.

Most studies concerned with mechanisms of intracellular killing and digestion of micro-organisms have been done with stimulated peritoneal or alveolar macrophages, that is to say, with cells taken following injection of an evoking agent intraperitoneally or intravenously, in order to increase the numbers of macrophages in the lung or peritoneal cavity (Cohn and Morse, 1960). Mycobacteria were used by earlier authors as the organisms of choice to stimulate activity of both the alveolar and peritoneal macrophages and were also frequently employed in the work described in this thesis to elicit "stimulated" cells. Stimulated peritoneal macrophages were found to degrade E. coli slightly faster than normal peritoneal macrophages (Fig 6, page 73). Possible reasons for this difference are discussed below.

In 1960 Jenkin and Benacerraf demonstrated that peritoneal macrophages from mice infected with BCG, phagocytosed and killed virulent bacteria more effectively than peritoneal cells from normal mice. Cohn and Morse (1960) showed that phagocytosis is an energy requiring process and that during the ingestion of particles whether inert or of bacterial origin, oxygen consumption, glucose utilization and lactic acid production increase. Macrophage populations might therefore exhibit differences in bactericidal powers because of differences in their metabolic activity or in their capacity to respond to a metabolic stimulant. Pavillard and Rowley (1962) measured oxygen consumption of macrophages and demonstrated that guinea pig alveolar cells have higher respiration rates than mouse peritoneal cells. Undoubtedly species differences contribute to this finding but Karnovsky

et al., (1970), comparing guinea pig alveolar and peritoneal macrophages, also concluded that alveolar cells had a higher respiratory rate than peritoneal cells.

It is probable that the respiration rates measured for alveolar macrophages are elevated as a consequence of their in vivo phagocytic experience with dust particles, etc. Stimulated cells may therefore show enhanced metabolic activity compared to normal cells and their capacity to degrade bacteria more rapidly may partly result from this.

It is not possible to exclude that antibody to E. coli or complement was playing a part in this killing since the culture medium contained 25 per cent fresh normal homologous chicken serum. It is possible that some natural antibody was present in this serum, but no known source of antibody was added to the medium. However, natural antibody, if present, would be at very low levels and it is more likely that the rapid killing observed was due to the degradative capacity of the macrophages alone unaided by antibodies or complement.

Digestion of mycobacteria by chicken lung macrophages:

In contrast to the rapid degradation of E. coli by macrophages (within a few hours) mycobacteria are degraded very slowly by the same cells, the period required being one of weeks. This may reflect differences in cell-wall structure between the two types of bacterium. E. coli cell walls do not contain the peptidoglycolipids found in the cell walls of mycobacteria. The presence of the

peptidoglycolipid may make an important contribution to the indigestibility of mycobacteria since macrophages lack enzymes capable of attacking this unusual molecule.

Few studies have aimed at measuring the rate of degradation of heat-killed mycobacteria within macrophages. Suter (1952), studied the degradation of heat-killed mycobacteria (strain R1Rv) and BCG and found there was little or no degradation of these organisms in vitro in studies carried over seven days. In the present experiments, a similar study was continued over a much longer period of time, i.e. for three to four weeks, and it was only after around two weeks of incubation that appreciable numbers of organisms were found to be damaged through the degradation process by the lysosomal enzymes of macrophages. This is shown in the electromicrographs in plates 14, 15, and 16.

The fate of viable Mycobacterium tuberculosis (strain H37 Rv or H37Ra) within guinea pig peritoneal macrophages and maintained in tissue culture, was studied by Hsu (1971), who found that the intracellular virulent mycobacteria had a lag phase of two days followed by a logarithmic multiplication. In contrast, he found no growth of avirulent bacteria nor did they multiply within the host macrophages - here too the incubation time was very short, an interval of 5 days.

The virulence of Mycobacteria depends on their relative capacity to multiply within host macrophages and cause cell destruction and/or death of the animal. Suter (1952), Mackaness and associates (1954), and Berthrong and Hamilton (1958) independently designed tissue culture experiments to illustrate this point. Macrophages harvested

Plate 14

Ultrastructural features of the cytoplasm of a "normal" chicken alveolar macrophage. Normal appearance of mitochondria (M) and smooth endoplasmic-reticulom, Golgi complex (Go), and lysosomes of varying sizes are depicted. X 60,000.

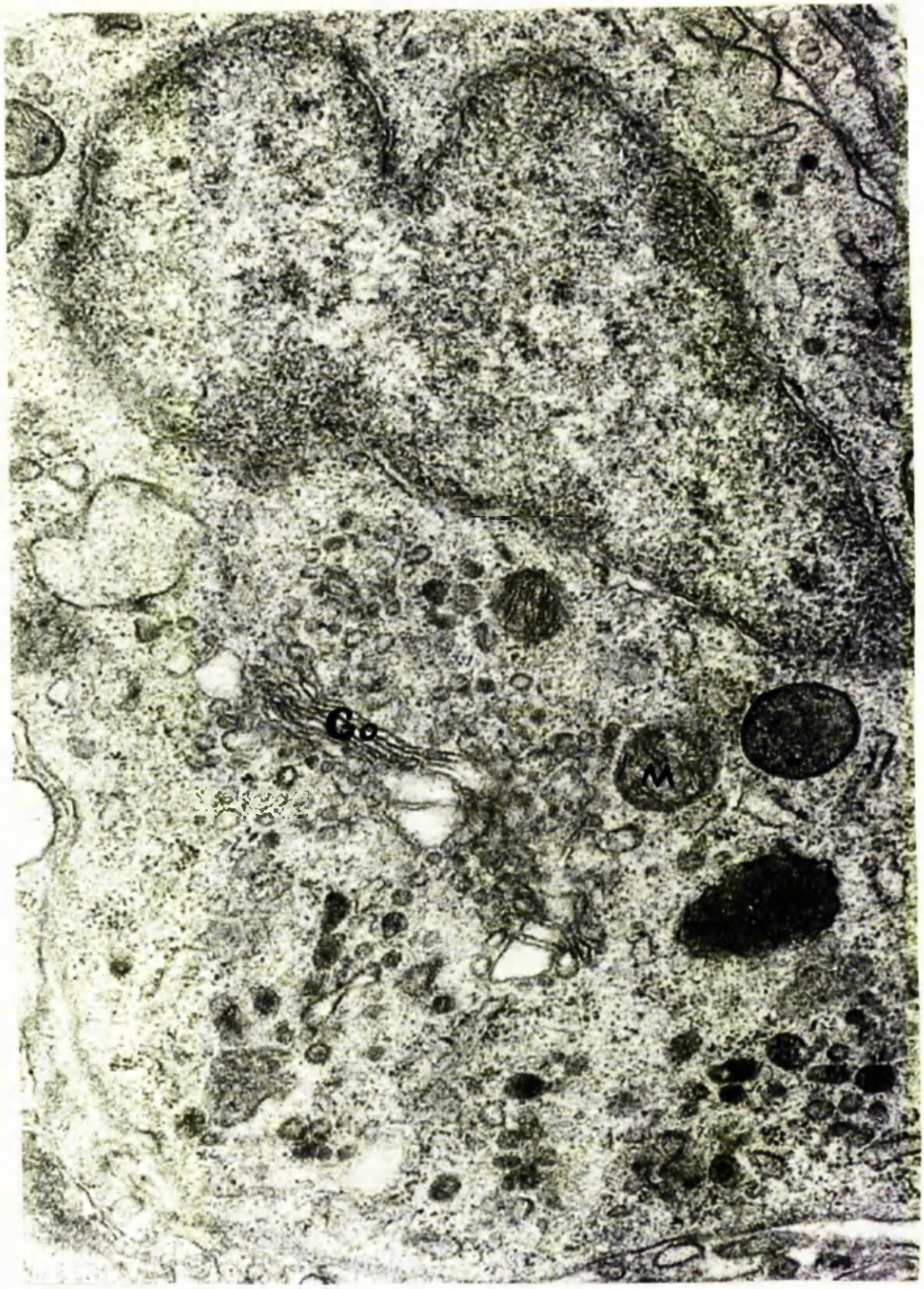

Go
M

Plate 15

Ultrastructural features of the cytoplasm of a sectioned chicken alveolar macrophage from an animal 7 days after receiving intravenous injection of M. avium. Increasing degrees of bacillary damage, with myelin figure development (*) and increased number of ribosomes are depicted. X 70,000.

* The myelin appearance is the description given by Armstrong et al., (1971) to the prominent concentric whorls which represent varying degrees of degradation of Mycobacteria within mouse peritoneal macrophages in vitro.

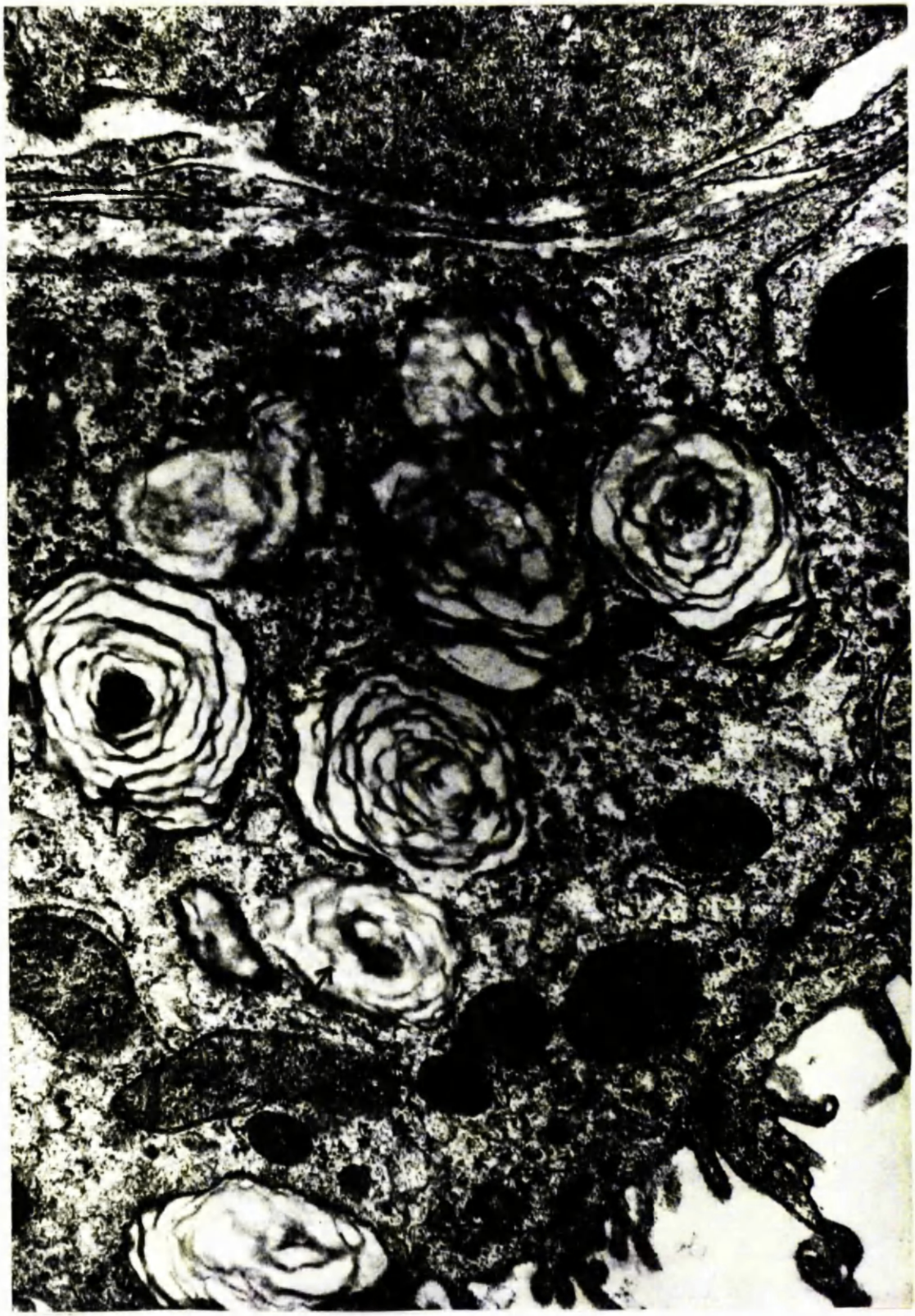


Plate 16

Ultrastructural features of the cytoplasm of a sectional chicken alveolar macrophage from an animal 7 days after intravenous injections of M. avium. Dense granules, or secondary lysosomes (gr), are abundant, varying both in size and density of their contents. Profiles of mitochondira (M) and of the Golgi complex (Go) and increase in ribosomes are also depicted. X 50,000.


(M)
(Gr)
(Ga)

from guinea pigs or rabbits, were infected with virulent or avirulent strains of mycobacteria, and maintained in tissue culture. Virulent organisms were reported to grow rapidly whereas avirulent organisms multiplied slowly or not at all within culture monocytes. These data were based upon direct microscopic enumeration of intracellular acid-fast bacteria in stained culture preparations.

The present investigation confirms the findings of these authors that the virulence of various mycobacterial organisms is a determinant of the extent to which they can be degraded within the host macrophages.

In further experiments macrophage cultures were exposed to various killed acid-fast organisms in vitro and studied over varying periods of time. These preparations were set up using flying cover slips and the method used for the comparison in digestion was patterned after the method Suther (1952) designed to study mycobacterial intra-cellular multiplication. In the present studies the number of stainable (acid-fast organisms) was used as an index of degradation. Mycobacteria will stain acid-fast whether alive or dead but not after digestion of the cell wall. Therefore, the loss of acid-fastness was used as a criterion that degradation of the cell was taking place and to determine how fast this was taking place.

Among the various strains of mycobacteria employed in studying the degradation of various acid-fast organisms, several strains that are classified as human pathogens, M. tuberculosis (H37Rv) and M. tuberculosis (strain C) and a host of organisms that are not recognised as pathogenic in man, M. fortuitum, M. smegmatis, M. phlei and M. avium

(avian pathogen) were used. It was found that the saprophytic organisms were degraded more rapidly than the pathogenic organisms (however, only M. avium is pathogenic in chickens). It was found that there existed some differences at day 0 in the number of organisms phagocytosed. The reason for this probably is the result of activation of phagocytic cells in the stimulated animals.

Earlier investigators have noted other differences in the biological activities of various strains of mycobacteria; for example, Freund (1947) found that M. butyricum was slightly better than M. phlei for stimulating antibody response, but found M. phlei slightly inferior to M. tuberculosis in this respect. From the cell wall studies, White, et al., (1964) found that certain fractions obtained by high-speed centrifugation of wax D preparations from M. phlei, M. tuberculosis, and several bovine strains of M. tuberculosis were adjuvant active. It is intriguing that White and his colleagues (1964) found that the bovine and saprophytic strains of mycobacteria studied had relatively little of the active wax D fraction, whereas the strains of human origin contained a large percentage of the active peptide containing glycolipid (peptidoglycolipid).

From all these observations it seems clear that the rate of degradation of mycobacteria is related closely to virulence and composition of the cell walls. The present investigation supports those of Suter (1952) and Hsu (1971) in suggesting that differences in cell wall composition, which may be responsible for differences in adjuvant activity or in virulence, may also determine the ease or difficulty with which individual strains of mycobacteria may be digested by macrophages.

INTRACELLULAR DEGRADATION OF FLUORESCEIN LABELLED CELL WALLS.
NORMAL MOUSE VERSUS NORMAL CHICKEN ALVEOLAR MACROPHAGES *in vitro*

In the experiments described above the digestion of whole bacteria by macrophages was described. In addition, a study of the digestion of isolated cell walls was undertaken. To elucidate the difference between different macrophage populations in their ability to degrade mycobacteria, comparisons of normal mouse and chicken alveolar macrophages were made using fluorescein labelled mycobacteria cell walls from *M. avium*, *M. phlei*, *M. fortuitum* and *M. tuberculosis* (strain H37Rv). These macrophage cultures were studied using a slide chamber technique described by Schrick, R., and Rabinowitz, Y. (1962) and modified in the present experiments for fluorescence studies as outlined in the Methods section of this thesis.

Degradation was assessed in terms of decrease of fluorescence. These readings were made daily over a period of 14 days. The reading for day 0 was taken after the macrophages had been allowed to phagocytise cell walls for one hour. The results are shown in Fig 14 and p 87 which represent the mean values of twenty individual cell readings in each cell wall preparation.

Thus the cell walls of acid-fast organisms were degraded by normal macrophages *in vitro*. Saprophytic organisms were degraded more quickly than pathogenic organisms by both mouse and chicken macrophages.

It can be seen that under identical conditions, mouse macrophages phagocytised more of the cell walls at day 0 than did chicken macrophages. The mouse macrophages degraded the cell walls of each species about twice as quickly as chicken macrophages under similar

conditions. Acid phosphatase levels were found to be highest in lung macrophages. It was shown (Rowley and Jenkin, 1962) that Salmonella typhimurium strain C5, which is highly virulent for mice but relatively avirulent for rats, can be effectively killed by mouse macrophages in vitro if the organisms used for challenge have been pretreated with normal rat serum. On the other hand, rat macrophages are able to phagocytise and kill this organism poorly in vitro, only if the bacterium have been pre-treated with mouse serum. This suggests that species immunity is determined by serum factors and that the cellular component is less specific.

The present investigation confirms that mouse macrophages are able to degrade acid-fast organisms more rapidly than chicken alveolar macrophages.

DEGRADATION RATE OF RADIO-LABELLED M. avium AND M. tuberculosis (STRAIN C) CELL WALLS AND OF RADIO-LABELLED CELLULOSE MEASURED in vivo

Experiments were designed to study the degradation rate of radiolabelled mycobacterial cell walls in chickens as a supplementary technique which might yield more detailed information than that obtained with fluorochrome-labelled cell walls described above. Contrast of radiolabel in vivo with fluorescein in vitro of mycobacteria cell walls was studied to determine the speed of degradation in vivo as compared to that for these organisms in vitro. Cellulose was used as an inert control material.

For these experiments cell walls were labelled with ^{131}I as outlined in the Methods section of this thesis. The birds all received either M. tuberculosis (strain C) or M. avium cell walls. A control group received ^{131}I labelled cellulose powder. The radioactivity of similar amounts of cell walls to those injected were measured on day 0 to give an estimate of the amount of whole body radioactivation.

The results of these experiments (Fig 16 p 93) showed rapid disappearance of the isotope during the initial 3 to 7 days after injection of cell walls. However, thereafter, the isotope disappeared more slowly with cell walls of both M. avium, M. tuberculosis (strain C) and with cellulose. It can be seen from Fig 16 that the disappearance rates for all three substances are similar.

The second phase of isotope disappearance could be attributed to spontaneous release of isotope from the labelled preparations, an observation which is not uncommon with preparations of radioiodine attached to the aromatic nucleus of a phenolic compound. The first phase of isotope elimination could be due to enzymic degradation. If this were the case it would appear that only a part of the cell wall is being degraded, because the rapid decline in isotope levels does not continue beyond the first three to seven days. Possibly the first isotope to disappear was that attached to protein molecules may be which dissolved or digested within the first week. The remainder of the isotope probably is attached to a polysaccharide which may resist digestion for longer periods of time.

The three isotope elimination curves are parallel from day 7

and until the end of the experiment (Fig 16 p 93) implying that there is some cell wall component which is not digested any faster than cellulose which is indigestible. Digestion could easily have been detected even if the half-life of the substance was as long as three weeks.

It was further noted that the digestion curves for M. avium cell walls and M. tuberculosis strain C cell walls show little difference. This is in contrast to the profound differences in adjuvant activity between these two organisms in the chicken (Aiyedun, White and McKay, unpublished, 1970), suggesting that slow digestibility alone is not an adequate explanation for the action of bacterial adjuvants.

In discussing the degradation rate of mycobacteria assessed by counting stainable organisms, or measuring degradation of various acid-fast organisms labelled with fluorescein, and ^{131}I, several factors must be taken into consideration in assessing the differences observed between different organisms.

1. There is a difference between the cell wall structure of saprophytic and pathogenic mycobacteria.
2. Some chemical entity from the pathogenic organisms may render them much more difficult to digest.
3. The pathogenic organisms may not be in the right position within the macrophage for digestion. That is, they may be in the cytoplasm or a vacuole and therefore not exposed to digestive enzymes (Armstrong et al., 1971).
4. Digestion may require a certain combination of enzymes to become

active on the cell walls, and these combinations may only operate if the composition of the cell wall permits. The organism might of course be resistant to the lysosomal hydrolases, as some investigators have proposed (Trouet, 1966), or perhaps even using them to their advantage (Brown et al., 1969).

EFFECT OF M. avium ON ACID PHOSPHATASE LEVELS IN WHOLE LUNG HOMOGENATE AND LUNG EXUDATES OF THE CHICKEN in vivo

Birds stimulated intravenously with M. avium were killed after one week and lung wash exudates were collected immediately after death. Whole lung tissue was also collected and both preparations prepared as outlined in the Methods section for assaying acid phosphatase levels.

The acid phosphatase levels in the lung wash exudate macrophages from stimulated chickens were approximately 30 per cent higher at peak than the levels in macrophages from the saline control group (Fig 17 p 96). In comparison, acid phosphatase levels were about twice as high in the stimulated birds as that of saline control group for the whole lung preparations.

In the second part of this experiment, M. avium and M. butyricum were compared for their effect on acid phosphatase levels in whole lung, lung homogenates and lung wash exudates.

The lung wash macrophages from M. butyricum-stimulated

chickens had acid phosphatase levels which were about twice as high as those of macrophages from saline injected controls. M. avium stimulated whole lung macrophages had acid phosphatase levels which were more than twice as high as the control levels. Lung-wash-exudate macrophages from the M. avium stimulated birds contained 9 per cent more acid phosphatase activity than M. butyricum stimulated birds (Fig 18 p 98).

The lung homogenates from stimulated animals also had elevated enzyme activities in comparison to the saline injected controls. Thus the birds injected with M. butyricum had 1.84 times the acid phosphatase level recorded for the control birds. Further, the M. avium stimulated chickens had a 130 per cent higher acid phosphatase level than the controls. Therefore, the enzyme level in the M. avium stimulated birds was 25 per cent higher than that of the chickens injected with M. butyricum.

It can be concluded that acid phosphatase levels are elevated in the chicken lung as a result of stimulation with M. avium and M. butyricum in vivo, and that this effect of stimulation was found to be significant within a few hours of injection (Fig 18 p 98).

Acid phosphatase has been regarded as a classical marker for lysosomal enzyme activity by earlier investigators (DeDuve et al., 1951; Cohn, Z. and Wiener, 1963), and it was therefore employed, in this series of experiments and, in conjunction with 2-glycerophosphate as substrate, as the original marker for lysosomal enzyme activity, but the activity of several other acid hydrolases was also studied and is discussed later in this thesis.

By light microscopy the tissues from the lung of stimulated birds show a concentration of acid phosphatase in the vicinity of the alveoli in tissues subjected to the conventional Rutenberg staining technique (Plate 4B). This evidence of the response to tuberculosis stimulation led us to anticipate similar findings using electron microscopy as a guide to the frequency of lysosome-phagosome fusion. Tissues infected 7 days previously, were heavily marked with the reaction product (Plate 15) after conventional glutaraldehyde fixation and Gomori staining. Differences in lysosomes and lysosome-phagosome fusion in macrophages containing intact or damaged organisms, are suggested by the distribution of the reaction product in many cells (Plate 16). Attention must be paid to the appearance of lysosomes of normal unstimulated tissues compared to that of tissues from birds stimulated with M. avium (Plates 15 and 16). Various degrees of fusion of lysosomes with phagosomes containing tubercle bacilli are shown in the stimulated lung macrophages. There is also an increase in ribosomes and rough endoplasm reticulum. The 'normal' macrophage preparations show the normal appearances of the lysosomes and the presence of smooth endoplasm reticulum denoting that the lysosomal enzymes have not been activated (Plate 14).

THE EFFECT OF SUSPENSION OF THE INJECTION MIXTURE OF *M. avium* IN EITHER SALINE OR IN WATER-IN-OIL EMULSION ON ITS CAPACITY TO ACTIVATE ACID PHOSPHATASE IN MACROPHAGES OF CHICKENS

The present experiments were designed to study the effect on acid phosphatase activity in macrophages of a bacterial stimulus, injected in a water-in-oil emulsion to allow the stimulating bacteria to be released slowly and over a long period. The results are presented in Fig 20 & 21 pp 106-7. Injection of *M. avium* in saline gave a significant increase in acid phosphatase activity over a short period of time, i.e. 7 to 8 days, while *M. avium* injected in a water-in-oil emulsion gave rise to a slightly (11 per cent) lower enzyme activity over the same time period. However, at a much later time, namely on the 28th day after inoculation, the geometric mean of the enzyme levels of the water-in-oil injected groups was nearly double that of the saline injected group.

The precise mechanism of the action of these water-in-oil injected preparations of *M. avium* in influencing enzymic activity, is not entirely clear. Material injected in such an emulsion enters the macrophages and is probably slowly released from the emulsion depot as described by Herbert (1966). The slow release of macrophage stimulatory material derived from the bacteria from the water-in-oil emulsion could account for the slow rise in acid phosphatase in the present experiments, because such material could reach macrophages over a much longer time period than the same material in aqueous medium.

Histological examination of the tissue changes caused by the mycobacterial preparations injected in either saline or in water-in-oil

emulsion showed gross granulomatous infiltration with gross hyperplasia in the lung at seven days (Plate 10). In the liver and spleen, there were no miliary nodules, but these organs showed slight hyperplasia and no sign of granulomata of epithelioid cells.

The capacity of dead mycobacteria to produce a tissue response quite similar to that typical of tuberculous infections has been described earlier (Freund, 1947) in a study involving the injection of mycobacteria in oil in guinea-pigs. Similarly, in this thesis, heat-killed saprophytic acid-fast bacilli injected in oil caused pulmonary lesions in the chicken regardless of the dose of bacteria injected, a finding rarely observed when an aqueous medium was used. A similar observation was made previously in rabbits by Hagan and Lavine (1932), and Rist (1938), who also found that dispersion of injected mycobacteria through the body of the animal was very limited in the absence of mineral oil from the suspending medium used for injection.

It has previously been reported that a commercial non-ionic surface active agent, Triton WR 1339, and other similar macromolecular products studied in the laboratory of Hart and colleagues, suppressed the development of experimental tuberculosis in the mouse (Cornforth, et al., 1951). The activity of Triton WR 1339 has been confirmed by Solotorovsky and Gregory (1952), who further reported a synergistic action with dihydrostreptomycin in murine tuberculosis. Hart and his colleagues defined more precisely the structural requirements of the molecule of these non-ionic surface-active agents and the mechanism of their action, and in their study they hypothesised that surface-active agents influence tuberculous infection by modifying the surface lipids of the tubercle bacillus within the monocytes.

Suramin (urea derivative of the amino-naphthalene-sulphonic acid series prepared from Triton X-100 by Hart et al., 1955) enhances a tuberculous infection in the mouse (Hart et al., 1955). Like Triton WR 1339, this substance is reported to remain intact within the circulation for long periods and is concentrated in macrophages (Jancso and Jancso-Gabor, 1952).

It is realised that even if the hypothesis that surface-active agents influence mycobacterial infection by modifying the cell walls of the bacteria is correct (Cornforth et al., 1951 and 1955), there are also other ways in which the host-parasite relationship could be altered by the presence of macromolecules within phagocytic cells - for example, by the suppression or enhancement of enzymic activity of the cell or of the parasite, or of both. Whatever may be the precise mechanism of action of these agents, it seems clear that they enter the monocytes and probably they provide a means of artifically influencing the operation of the cellular defenses in tuberculous infection.

The present results are consistent with the hypothesis of Hart et al., (1955) that the oil in an emulsion did show an affinity for the mycobacterial surface and could act to form a protective coat over the cell wall. It is probable that this protective coat requires to be penetrated by the action of various acid hydrolases in order that macrophage stimulating material may be released from the bacteria, which in turn will activate the formation of further lysosomal enzymes. The oily coat on the bacteria does not therefore inhibit the stimulating organisms from activating the acid hydrolases of macrophages but rather delays release of stimulating material thereby enabling the stimulus to

be maintained over a longer period and thus maintain a higher rise of enzyme activity over a prolonged period of time.

THE INFLUENCE OF *M. avium* ON THE METABOLISM OF PHOSPHOLIPIDS AND ON PHOSPHOLIPASE A LEVELS IN CHICKEN LUNG TISSUES *in vivo*

I have demonstrated in the earlier experiments that phagocytosis of whole mycobacterial organisms by macrophages will activate the acid hydrolases of these cells. I then became interested in another enzyme, phospholipase A (PL-A), known to have an indirect deleterious effect on macrophage membranes and which perhaps elicits an immune response (Munder *et al.*, 1965).

In the present experiments an attempt was made to determine what effect *M. avium* stimulation has on the levels of this enzyme in the lung tissues of the bird. An attempt to determine the cytotoxic effect of phospholipase A (PL-A) on the cellular membranes is described. Further, an attempt was made to determine whether these effects are due to normal intermediates of the phospholipid metabolism of cells.

The lungs from 6 to 8 week old chickens that had been stimulated one week earlier with a single intravenous injection of 6 mg of *M. avium* were obtained immediately after death. The lungs were homogenised as described in the Methods section of this thesis and aliquots of these preparations were assayed for phospholipase A. The results of these experiments are shown in Table 6 p 113. This table shows the cumulative mean Δ pH/h from 15 animals receiving *M. avium*. The method is based on the measurement of liberated fatty acid by

titration. A ΔpH/h change of 0.865 was observed for the group stimulated with M. avium. This value is approximately two times the Δ pH/h obtained from the group injected with saline. Thus the Δ pH/h for the control group was 0.438.

It is clear from these experiments that the activation of intracellular phospholipase A and the subsequent breakdown of lecithin to lysolecithin in vitro takes place to a higher degree in cells from chickens stimulated with M. avium than in cells from a control group.

Munder et al., (1969) have speculated that the 'adjuvanticity' of some of the known adjuvants may be associated with the same of similar cytotoxic mechanisms, i.e. adjuvants may act by activating or inhibiting one or more of the enzymes of cellular phospholipid metabolism. The action of phospholipase A on lecithin might thus lead to the accumulation of lysolecithin. The release of these substances could then explain the inflammatory reaction observed after the application of an adjuvant.

Lysolecithin until recent years had been considered solely as a strongly cytotoxic substance which only appears under rare pathological conditions. Studies of Lands et al., (1964), Oliveira and Vaughn (1964), Van Deene, L.L.M. and Damel, R.A. (1964) and Munder et al., (1965 and 1969) have, however, clearly shown that lysolecithin in small concentrations can be readily metabolised by cells and their membranes and is, in fact, an important intermediate of cellular phospholipase metabolism.

Dresser (1968), using vitamin A as an adjuvant, speculated whether the 'adjuvanticity' of an adjuvant might be caused by its ability

to damage cellular membranes, in particular lysosomal membranes, 'in this way stimulating cell division', which in turn would lead to immunity rather than tolerance in the presence of an antigen.

The results of the present investigation do not allow a decision as to whether the increased concentration of lysolecithin after stimulation with M. avium is due to an activation of an intracellular phospholipase A or to an inhibition of the acyltransferase system which would still allow phospholipase A to degrade lysolecithin. Both activation of the former or inhibition of the latter enzymes will lead to accumulation of lysolecithin. Munder et al., (1969) speculated that the increased formation of lysolecithin in macrophages upon exposure to chemicals known to be adjuvants may represent an endogenous mechanism by which the organism is able to produce its own real adjuvant at sites of inflammation.

The hypothesis that lysolecithin released by phospholipase A can act as adjuvant was tested and is discussed below.

THE EFFECT OF PHOSPHOLIPASE A ON SERUM ANTI-HSA ANTIBODY LEVELS IN THE CHICKEN

In these experiments an attempt was made to determine if phospholipase A is capable of producing an adjuvant action in chickens.

If it is true that adjuvants can activate an intracellular phospholipase A and that the surface-active lysolecithin formed is the common denominator for such an action of the rather diverse group of substances with adjuvant activity, then exogenous lysolecithin per se

should in itself have 'adjuvanticity' (Munder et al., 1969).

Results of experiments in which phospholipase A was injected into chickens show that its effect on antibody levels to HSA compared to the control group was small (Fig 22 p 117). The antibody response to HSA injected with PL-A was not significantly increased compared to that noted for HSA in oil. However, in a group injected with HSA in FCA the antibody levels were higher (Fig 23 p 118). It was concluded from these experiments that phospholipase A had little adjuvant activity as determined by the levels of serum antibody.

The speculation of Munder et al., (1967) that phospholipase A has adjuvant activity remains unsupported by the experiments reported in this thesis.

THE EFFECT OF STIMULATION WITH M. avium ON THE GLYCOSIDASES OF MACROPHAGES IN THE LUNG OF THE CHICKEN

As shown previously, acid phosphatase and phospholipase A levels in the chicken lung were raised when animals were stimulated with M. avium. The group of studies to be discussed presently were performed to assess the effect of M. avium on glycosidases, namely, β-glucuronidase, β-galactosidase, α-mannosidase and N-acetyl-β-glucosaminidase in the whole lung and lung wash exudates of the chicken.

Groups of 6 week old chickens were given a single intravenous injection of 6 mg of M. avium and killed one week later. Lung tissue and lung wash exudates were collected as outlined in earlier

experiments, and glycosidase enzyme levels in such preparations were estimated as outlined in the Materials section. It was found that β-glucoronidase, β-galactosidase, α-mannosidase, and N-acetyl-β-glucosaminidase levels were raised in M. avium stimulated birds. In comparison to saline controls, in enzyme assays of whole lung homogenates, of birds injected with M. avium showed that β-galactosidase levels were 8 times as high, β-glucoronidase by 5.4, N-acetyl-β-glucosaminidase by 39 and α-mannisoidase by 163 (see Table 7 p 121).

These enzyme levels were also elevated in the lung wash exudates of M. avium stimulated chickens in comparison to saline controls (Table 7 p 121).

The results suggest strongly that these enzymes are stimulated as was acid phosphatase and other acid hydrolases. They also lend support to the concept that these enzymes are mainly concerned with bacterial degradation.

The results described in this section confirm the intimate relationship between β-N-acetyl-glucosaminidase, β-galactosidase and β-glucuronidase established by Conchie et al., (1959). These authors observed very similar findings to those described in the present investigation. A further interesting observation was that tissues which contain the β-glucosidases also contain α-mannosidase.

Conchie et al., (1959), postulated that these various glycosidases may exert a concerted type of action in the catabolism of mucopolysaccharides and glycoproteins. This hypothesis is in agreement with the general lytic function attributed to the lysosomes. As discussed

by DeDuve (1959), the segregation of the hydrolases within impermeable particles probably represents a fundamental control mechanism of their lytic activity.

These results are in agreement with the hypothesis of Conchie et al., (1959), that an intimate relationship exists between acid phosphatase, β-glucuronidase and various other acid hydrolases, and where an elevation in the levels of one of these enzymes occurs, normally a similar increase of other lysosomal enzymes is observed.

FURTHER STUDIES OF THE EFFECT OF VARIOUS STRAINS OF MYCOBACTERIA ON ACID PHOSPHATASE LEVELS IN THE LUNGS OF CHICKENS

The experiments discussed below were designed to compare the activity of a number of different acid-fast organisms, which differ in cell wall structure, and some of which are pathogenic either in birds or in mammals, whereas others are non-pathogenic, on the levels of acid phosphatase activity in the chicken lung. Seven groups of 6 to 8 week old chickens received 6 mg of either M. avium, M. tuberculosis strain C, M. tuberculosis H37Rv, M. phlei, M. fortuitum or M. smegmatis in a single intravenous injection. The chickens were killed after one week and lung tissues were homogenised and aliquots assayed for acid phosphatase.

It was found from these experiments (Fig 19 p 101) that representative organisms both pathogenic and saprophytic produced substantial increases in acid phosphatase. The ability of the myco-

bacteria to stimulate acid phosphatase activity in descending order is as follows: M. avium, M. tuberculosis strain C, M. tuberculosis H37Rv, M. smegmatis, M. phlei and M. fortuitum.

The above findings were of interest because the levels of acid hydrolases achieved as a result of stimulation by the various mycobacteria have not previously been investigated. However, these organisms have been intensively studied for their adjuvant activity in stimulating an immune response in terms of antibody biosynthesis (White et al., 1970). It is possible that the stimulatory activity of these organisms on the acid hydrolases and their adjuvant activity in enhancing antibody production is closely related.

THE EFFECT OF CELL WALLS OF M. avium AND M. tuberculosis STRAIN C ON THE LEVELS OF ACID PHOSPHATASE, β-glucuronidase, β-galactosidase and α-mannosidase IN LUNGS OF CHICKENS

It was found in the experiments discussed above that the cell walls of mycobacteria were slowly degraded by normal and stimulated chicken and mouse macrophages. The following experiments were designed to determine the effect of cell walls of representative stimulatory mycobacteria on the acid hydrolases in vivo in chicken lung macrophages. In these experiments, cell wall preparations were prepared from heat-killed cultures of M. avium and M. tuberculosis strain C. Portions of the cell wall preparations were treated with proteinase as outlined in the Methods section, page 52, while untreated preparations were washed several times in saline.

All preparations were suspended in saline for injection. Each animal received 6 mg (wet weight) of one of the above preparations or saline (control group) intravenously.

There was a very consistant but small difference between the proteinase-treated and untreated cell walls in raising the levels of glycosidases acid phosphatase, β-glucuronidase, β-galactosidase and α-mannosidase was found (Table 8 p 123). The active constituent of M. avium and M. tuberculosis strain C responsible for stimulating an increase in lysosomal enzyme synthesis is in the cell wall itself and is not connected with components of intracellular materials.

Mycobacterial cell walls are thought to be chemically and antigenically more complex than other bacterial cell walls because of their content of peptidoglycolidpids (White, 1965). Characteristically, the mycobacteria have a high lipid content in their cell wall, as shown by the detailed works of Kotani et al., (1960) who demonstrated that significant amounts of the free lipids could be removed by treatment of bacteria with hot acetone before the mechanical disintegration of the cells.

Mycobacterial cell walls are not only good stimulators of the acid hydrolases of lysosomes but it is known that the BCG cell walls are capable of inducing tuberculin hypersensitivity and enhancing the resistance of animals to tuberculosis infection (Kotani et al., 1960; Larson et al., 1963). Therefore, the ability of the cell walls of mycobacteria to activate acid hydrolases in macrophages may be related to the capacity of cell walls from these organisms to evoke an adjuvant effect on antibody production, as was described by White et al., (1955a).

THE EFFECT OF ANAEROBIC CORYNEFORM BACTERIA ON ACID HYDROLASE LEVELS IN THE CHICKEN LUNG

In the investigation discussed in this section, an attempt was made to determine the effect of the anaerobic coryneform bacteria on levels of acid hydrolases in the lung of chickens. Various strains of Corynebacteria, serological groups 1 to 4 and some 'classical' propionibacteria strains, were included in these studies. It was found that whole lung tissue levels of acid phosphatase, β-glucuronidase, β-galactosidase, N-β-acetyl glucosaminidase and α-mannosidase were up to 5 times as high in the birds receiving anaerobic coryneforms as in the saline control group. In the chickens stimulated by injection of anaerobic coryneforms, levels in the lung wash were about 4 times higher than in the control group (Tables 9 and 10, pp 128 & 130).

In comparison, 'classical' propionibacteria did not produce a significant elevation in these enzyme controls (Tables 9 and 10).

The increases of these enzymes observed after injection of anaerobic coryneforms were only half those recorded after injection of M. avium (Tables 9 and 10).

The effect of cytoplasmic materials from various strains of coryneform bacteria on the levels of acid hydrolases on mouse peritoneal macrophages in vitro was also investigated (Table 13 p 149). These results show that no significant increases in the acid hydrolases of mouse peritoneal macrophages were produced by such materials.

Very little information is available on the effect of

coryneform bacteria in relationship to their ability to activate the acid hydrolases. However, various strains of Corynebacterium parvum have been studied by Halpern and colleagues (1963). They were found to be potent immunostimulants in experimental infections and in malignancies (Halpern et al., 1963). A remarkable feature of C. parvum is its inhibitory effect on tumour invasion or leukaemias in rodents. However, the results varied depending on the nature of the tumours, the genotypic relation between the donor and the recipient, and the route and timing of the administration of C. parvum. O'Neill et al., (1973) studied the role of anaerobic coryneforms on specific and non-specific immunological reactions. Of twenty-one strains of anaerobic coryneforms tested, fifteen strains were able to produce an increase of the phagocytic index in mice of at least 50 per cent (organisms tested were of each of the four main serological groups). Four organisms representing the 'classical' propionibacteria were also tested, but none of these caused an increase of phagocytic index in mice.

Tests in mice and chickens for an adjuvant action on serum levels of antibody failed to show any enhancement of primary responses, although a barely significant elevation of the secondary response in the mouse was observed. In the guinea-pig, a clear adjuvant effect on the levels of serum antibody in primary responses was observed. None of these organisms was able to enhance cell-mediated hypersensitivity, as shown by delayed-type skin tests or corneal reactions.

Anaerobic corynebacteria and their action in vitro in relation to immunopotentiation in vivo has also been studied by Wilkinson et al., (1973b) and the role of a macrophage-specific cytotaxin

from these organisms in chemotaxis of macrophages. Wilkinson et al., (1973a) found that many strains of these organisms produce a highly macrophage-specific chemotactic factor, and further found that the coryneforms also cause a burst of acid phosphatase production by macrophages in vitro and it is suggested that the macrophage-specificity of this chemotactic factor is related to its pattern of activation of lysosomal enzymes which differs from that of neutrophil cytotaxins. Production of this factor is closely related to the carbon clearance activity of many strains of these bacteria in mice. However, there is not a good correlation between chemotaxis and carbon clearance on the one hand and immunological adjuvant activity on the other Wilkinson et al., (1973a, 1973b). These results on short-term macrophage activation show some contrasts with the observations on long-term activation of macrophages by mycobacteria and anaerobic corynebacteria which are disucssed in a later section. It is probable that short-term activation depends on a 'metabolic burst' in the macrophage without new enzyme synthesis, whereas in the long term, enhanced biosynthesis of lysosomal enzymes is essential.

EFFECT OF M. avium ON THE LEVELS OF CATHEPSIN D AND LYSOZYME IN WHOLE LUNG TISSUES OF THE CHICKEN in vivo STUDIES

The results of these experiments (Table 11 p 140) show that M. avium stimulated a rise in lysozyme enzyme levels 5 or 6 times greater than P. avidum (serologocal group 4) and much greater than the number of serological group 1 organisms. In comparison, all of the

corynebacterial organisms evoked significant rises in this enzyme compared with those attained using the 'classical' propionibacteria. The 'classical' propionibacterial organisms did not significantly increase the level of lysozyme when compared to the levels of the control group (Table 11 p 139). If lysozyme had been produced solely by the breakdown of macrophages during homogenisation, this enzyme would have risen as high in the tissues of animals injected with coryneform bacteria. Further proliferation of macrophages in culture did not account for increased enzyme levels and it must therefore be concluded that the M. avium stimulated tissues were synthesizing more lysozyme than those of chickens injected with coryneform bacteria. This report shows that alveolar macrophages contain large amounts of lysozyme.

These increases in lysozyme levels in the tissues of stimulated birds were the results of injecting the organism in a saline suspension. Thus in contrast to the finding of Myrvik et al., (1962), who injected organisms in water-in-oil, this thesis has clearly demonstrated that similar increases in enzyme activity can be obtained by injecting the organism in a saline suspension.

In 1961 the enzyme lysozyme was initially reported in extracts obtained from peritoneal and alveolar macrophages reported (Brumfitt and Glynn, 1961; Glynn et al., 1966; Myrvik et al., 1962). It was found that the alveolar macrophages contain a much greater amount of lysozyme than the peritoneal macrophages (Pavillard and Rowley, 1962). The fact that the alveolar macrophages contain large amounts of lysozyme invites speculation therefore that the lysozyme content of such cells is related to the origin of these cells

(the lung) and to their presumed function as scavenger cells.

The reaction of lung macrophages to various stimuli had been the subject of several investigations including studies on the clearance of particulate matter and its disposition in the lung (Duthie, 1930; Wotten and Martin, 1951; Ross, 1939; Robertson, (1941). Fried (1934) reported that certain microorganisms which are fatal when introduced by the intraperitoneal, subcutaneous or hematogenous routes are harmless when the respiratory portion of the lungs is chosen as the route of infection. The apparent high resistance of the lung to infectious agents is mainly due to lysozyme and the fact that the lung contains an extensive surface area which in many instances is only one or two cells removed from the vascular compartment.

It would appear that the alveolar macrophage is faced with a great immunological task. This task would include phagocytosis and destruction of microorganisms which gain entrance to the lower respiratory tract. The lysozyme content of alveolar macrophages is related to this function.

Recently, Gordon et al., (1974) studied the in vitro synthesis and secretion of lysozyme by mononuclear phagocytes of mice and men. They found the production and secretion of lysozyme into the culture medium independent of the production of macrophage acid hydrolases, that increase and secretion of lysozyme occurs under conditions where acid hydrolases like N-acetyl-β-glucosaminidase, β-glucuronidase, β-galactosidase and Cathepsin D are neither accumulated nor secreted.

The name Cathepsin D was first applied to the acid protease

isolated from bovine spleen by Press et al., (1960). Interest in the fate of degrading organisms in macrophages has led me to investigate the proteolytic enzyme cathepsin D of the lungs of animals stimulated with M. avium.

By establishing valid assay conditions for eight experiments (3 animals per test) lung cathepsin D was thought to be associated with bacterial digestion. The results described in the present investigation show that M. avium stimulate cathepsin D activity.

Attention was drawn to the lung proteases by the striking granulomatous condition found after intravenous inoculation of mycobacteria. In granulomatous tissues as was reported in earlier experiments in this thesis, an increase in lysosomal enzymes exists. The protease cathepsin D has been reported by earlier investigators (Barrett, 1969) to be increased in inflammatory conditions, for example, rheumatoid arthritis, in various animal tissues. It was therefore of interest to determine the presence and levels of this enzyme in the lungs of mycobacterial stimulated birds.

Very little information is available in the literature concerning the stimulatory effect of microorganisms on this enzyme and as a result the present observation furnished some useful information on the effect of organism stimulation on this enzyme.

THE EFFECT OF CORYNEBACTERIA AND MYCOBACTERIA ON SHORT-TERM SECRETION OF LYSOSOMAL ENZYMES BY PHAGOCYTIC CELLS *in vitro*

It is possible that the rise in enzyme levels provoked by macrophage stimulating organisms correlates very well with the ability of these organisms to stimulate chemotaxis and phagocytosis. However, it was not shown whether the bacteria cause the stimulation of enzyme rises by acting directly on the phagocytic cells, or whether the effect depends on other cells or humoral factors. Nor was it known whether or not the effect depends wholly on enzyme synthesis, or whether, especially in the short-term, the effect is one of activation or release of enzymes already synthesized, or indeed, whether several different mechanisms which increase enzyme levels are activated.

In these experiments mouse peritoneal macrophages were allowed to remain in contact with the bacteria *in vitro*, and then the enzyme content of the whole suspension (cells + supernate) was assayed. Also the extracellular fluid was separated from the cells and was assayed separately for enzymes released during the first two hours of incubation.

Some of the tests were carried out in the presence of plasma. This was done in order to see if some bacteria stimulate the macrophages only in the presence of plasma, an effect that has been reported in studies of the chemotactic effects of mycobacteria (Symon *et al.*, 1972). In some experiments puromycin or actinomycin D, inhibitors of protein synthesis, were added before incubation to ascertain whether the effects observed were dependent on protein biosynthesis. The specificity of

action of the bacteria on enzyme levels was investigated by assaying the cytoplasmic marker enzyme, lactic dehydrogenase, as well as the lysosomal enzymes directly concerned with non-specific immunity. The lactic dehydrogenase assay should indicate whether the lytic enzymes were being actively and selectively secreted, or if they were merely being released as a result of increased membrane permeability. If the membrane becomes leaky as in the study of the effects of vitamin A on the cell membrane (Dresser, 1968), both LDH and the lysosomal enzymes would be released. On the other hand, specific action of bacteria on lysosomes should not influence the distribution of LDH.

It was found in the present investigation that addition of M. tuberculosis strain C, M. tuberculosis H37Rv, and the various coryneform bacteria to macrophage cultures caused changes in the levels of the lysosomal enzymes, acid phosphatase, β-glucuronidase, β-galactosidase and lactic dehydrogenase (Table 12A & 12B pp 145 & 146).

In the absence of plasma, the test bacteria caused an elevation of enzyme levels in the macrophages. The elevation was most pronounced when acid phosphatase was measured. The change in the other glycosidase levels was not constant. Few experiments are reported here but statistical analysis (courtest of Dr. I. McKay of this department) showed that the elevation of acid phosphatase levels attained when macrophages were incubated with casein or with P. avidum (serological group 4) assumed statistical significance (Table 12B p 145), but the remainder of the results did not. Further experiments would be required to confirm statistically if M. tuberculosis or C. diphtheroides etc., caused an elevation of macrophage lysosomal enzyme levels. No

constant pattern of change was evident when the cytoplasmic enzyme, lactic dehydrogenase, was measured.

It was found that plasma (human) alone without the cells, itself contains a high level of the various lysosomal enzymes. It can be seen that the levels of enzymes in macrophages are, however, elevated even higher than the baseline plasma levels when the macrophages were incubated in plasma together with the test organisms.

Some conclusions can be drawn from these findings, namely: M. tuberculosis strain C and M. tuberculosis H37Rv and the various coryneform bacteria did cause a rise in the release of lysosomal enzymes particularly acid phosphatase in mouse peritoneal macrophages after short term incubation in vitro. It could not be concluded that these organisms (mycobacteria and coryneform bacteria) caused an increase in synthesis of these enzymes over such a short time period, but it is more probable that release or activation of preformed enzyme is being measured.

Experiments using inhibitors of protein synthesis (actinomycin D and puromycin) indicated that part at least of the elevation in enzyme levels was not due to synthesis of new protein (Table 12B p 145).

It was difficult to assess the effect of plasma on activation of hydrolases in macrophages by bacteria or to demonstrate whether a complement or other serum factor was activated which could stimulate lysosomal hydrolases. The results of the experiments were difficult to interpret because of the content of such enzymes in the plasma itself.

TIME COURSE OF ACCUMULATION OF CELL AND OF PRODUCTION OF LYSOSOMAL ENZYMES AND CHANGES IN CHEMOTACTIC ACTIVITY IN THE PERITONEAL FLUID OF MICE INJECTED WITH *M. tuberculosis* STRAIN C OR GLYCOGEN

The anaerobic coryneform bacteria and mycobacteria have been shown earlier to stimulate chemotaxis of peritoneal macrophages *in vitro*, the anaerobic coryneform bacteria by a direct effect on the macrophages (Wilkinson *et al.*, 1973a), and the mycobacteria by activating plasma (Symon *et al.*, 1972). It was important therefore to attempt to show how these effects are related in a physiological situation, especially following injection of bacteria or other stimulants *in vivo*.

Snyderman *et al.*, (1971), showed that after injecting glycogen into the peritoneum of mice, chemotactic factors for neutrophils appeared in the peritoneal fluid. Later, Wilkinson *et al.*, (1973b) showed that glycogen and *M. tuberculosis*, when injected into the peritoneum of guinea-pigs, caused production of chemotactic factors for macrophages in the peritoneal fluid. However, these experiments were only carried over 4 days and little difference was seen between the two stimuli over this short time period. Mycobacteria were found to cause a chronic granuloma, whereas glycogen did not. Therefore the present investigation was designed to determine the macrophage stimulating properties of mycobacteria compared to glycogen over a longer period (of weeks).

Groups of 7-8 week old female Balb C strain mice received either 0.2 mg mycobacteria or 60 µg glycogen injections intraperitoneally.

Peritoneal macrophages were collected daily over a 15 day period and total cell counts were done in addition to differential counts (Table 14 p 153). Macrophages were removed from the peritoneal fluid and aliquots were assayed for acid phosphatase, ß-glucuronidase, and ß-galactosidase. At the same time the supernatant peritoneal fluid (after the macrophages had been removed by centrifugation) was examined for the presence of chemotactic factors. It was found that mycobacteria and glycogen both induced the formation of chemotactic factor in the peritoneal fluid from these stimulated animals (Tables 14 and 15, pages 153 and 154 respectively).

The results showed that chemotactic activity and rises in enzyme activity had occurred as early as twenty-four hours with glycogen and Mycobacteria. However, it was found that the glycogen stimulation did not remain elevated and both the chemotactic and enzyme levels dropped after three days while these activities from the mycobacteria stimulated animals remained elevated.

Mycobacteria were found to elicit a more significant rise in enzyme levels and chemotactic activity as early as the second day following injection, but for some inexplicable reason this activity dropped on the third day, although the total cell counts remained high. Thereafter, a gradual increase in levels of enzymes was seen from day four to day twenty-one with cell counts remaining elevated through to the 15th day (Table 14 p 153).

From the present investigation, several conclusions can be drawn concerning mycobacterial stimulation of peritoneal macrophages

in vivo, namely; (1) all three biological effects, i.e. the accumulation of cells in the peritoneum, elevation of enzyme levels in these cells and production of chemotactic factors in the peritoneal fluid, were demonstrable; (2) the chemotactic effect is of particular interest since it has not previously been reported that the stimulating agents used endowed the peritoneal fluid with chemotactic activity for macrophages. These findings lend support to the hypothesis that the attraction of macrophages into the peritoneal cavity may indeed depend on chemotaxis, rather than on changes in vascular permeability, or on immobilization of cells. The chemotactic activity observed showed a steady decline throughout the experiment, approaching the negative control levels by day 12 (Table 14 p 152). This contrasted markedly with the time course of the other biological effects studied. (3) All the enzymes assayed, expressed as enzymic activity per 10^6 cells, appeared to show a short-term increase during days 1 and 2, followed by a sharp fall on day 3 (Table 14 p 152), and thereafter, the three enzyme levels all showed a steady increase throughout the remainder of the experiment. The rise in acid phosphatase was found to be particularly marked.

This observation would be compatable with the view that the cells possess a short-term mechanism by which enzymes may be activated or in some way be made more easily available for use in degradation of bacteria (Myrvik et al., 1961), as well as a longer term mechanism by which the synthesis of lysosomal enzymes can be stimulated.

(4) The cells attracted into the peritoneum were predominantly macrophages, but lymphocytes, mast cells and eosonophils

were always present and some neutrophils were also found.

Glycogen did not act as significantly as mycobacteria in stimulating peritoneal macrophages in the long term. The conclusions from this experiment were: (1) in contrast to mycobacteria, the cell counts in the peritoneal cavity after glycogen stimulation remained elevated for only a short time and then returned to normal (Table 15 p 153). The few remaining cells 15 days after glycogen stimulation did, however, show evidence of rises in hydrolases. (2) The ability of glycogen to stimulate the formation of chemotactic factors in the peritoneal cavity appeared to be more short-lived than that of mycobacteria (Table 15 p 153). Thus, mycobacteria are capable of activating macrophages in the long term, as measured by chemotactic activity in vivo and by increases of the total numbers of cells arriving in the peritoneum over a period of three weeks. Glycogen only has short-term macrophage-activating properties. This adds support to the already considerable evidence for the role of mycobacteria as active chronic-granuloma inducing agents.

PART FIVE

SUMMARY AND ACKNOWLEDGEMENTS

This thesis describes studies of the events which occur when macrophages are exposed to mycobacteria or anaerobic coryneform bacteria *in vivo* or *in vitro*. It chiefly explores two aspects of these events. Firstly, the ability of macrophages to digest and degrade these organisms; and secondly, the effect of the organisms on the activity of the acid hydrolases of the macrophages. The studies on the degradation of bacteria by macrophages were performed using as a model system, the response of the pulmonary alveolar macrophages of the chicken obtained either from normal chickens or from chickens pre-injected with *M. avium* upon incubation with various test organisms including *M. avium*, a pathogenic organism in birds. *Mycobacterium tuberculosis* is not pathogenic in birds but has a unique cell-wall structure which may determine its immunopotentiating effects. Various non-pathogenic mycobacteria, as well as the anaerobic coryneform bacteria, are also known to have important immunopotentiating effects. In contrast to these chronic granuloma forming organisms, the handling of *Escherichia coli*, a bacterium not usually regarded as granuloma-forming, by macrophages, was studied.

The intracellular digestion studies showed that stimulated macrophages from the chicken lungs, i.e. macrophages induced by prior injection of *M. avium*, digested the various acid-fast organisms when incubated with them *in vitro* much more rapidly than those from normal non-stimulated chickens; it also showed that the saprophytic strains of mycobacteria were degraded more rapidly than the pathogenic strains by chicken and mouse macrophages. As indicators of bacterial degradation, the disappearance of mycobacteria and/or a reduction in the number of

stainable (acid-fast) organisms intracellularly in chicken lung macrophages was used.

The stimulation of the activity of a number of lysosomal hydrolases, namely acid phosphatase, β-glucuronidase, β-galactosidase, α-mannosidase, cathepsin D, phospholipase A, and N-acetyl-β-glucosaminodase in the macrophages of the chicken lung by contact with various mycobacteria and coryneform bacteria was studied. It was shown that M. avium stimulated rises in the activity of these enzymes in vivo to a much greater extent than other mycobacteria and coryneform bacteria after inoculation intravenously into the chicken. This rise in the levels of the lysosomal enzymes in macrophages was shown to be related to the enhancement of intracellular degradation of microorganisms by these macrophages.

It was shown that corynebacteria and mycobacteria caused selective release of mouse peritoneal macrophage lysosomal enzymes when incubated with these macrophages over a short time period in vitro. These organisms did not affect levels of the cytoplasmic enzymes, i.e. lactic acid dehydrogenase. In some experiments two inhibitors of protein synthesis were added before incubation to ascertain whether the effects observed were dependent on protein synthesis. The results showed that the enzymes released from mouse peritoneal macrophage in vitro on incubation with corynebacteria and mycobacteria were preformed enzymes and not newly synthesized.

This result is in contrast to the results obtained in the chicken lung where activation of hydrolases was studied over a long time period (three weeks) and where there is probably new synthesis of enzyme by macrophages.

The accumulation of cells in the peritoneal cavity of mice, production of lysosomal enzymes by those cells and the presence of chemotactic factors in the peritoneal fluid were studied over a time period of three weeks following intraperitoneal injection of mycobacteria into mice, using as a control stimulant, glycogen which was injected into a second group of mice. These experiments showed that the levels of acid phosphatase, β-glucuronidase and β-galactosidase in mouse peritoneal macrophages rose as a result of injection of both mycobacteria and glycogen. Mycobacteria elicited a greater and longer sustained level of these enzymes and also elicited emigration of macrophages into the peritoneal cavity over a longer time period than glycogen. It was further shown in these experiments that intraperitoneal injections of mycobacteria or glycogen led to the formation of chemotactic factors for macrophages which presumably attract the cells into the peritoneum. Injection of mycobacteria led to a persistence of such factors in the peritoneal cavity over a longer time period than glycogen.

The significance of these findings is discussed with reference to previously published work on the intracellular digestion of bacteria by macrophages, and on stimulation of lysosomal enzymes, and in the light of previous studies of the biological effects of the various strains of mycobacteria and coryneform bacteria.

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PART SIX

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