THE EFFECT OF MYCOBACTERIAL ADJUVANTS ON IMMUNOGLOBULIN PRODUCTION IN THE GUINEA-PIG.

William Arnot Fleming, B.Sc.

SUMMARY

Ph.D. Thesis

April, 1968

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The effect of mycobacterial adjuvants on antibody production against soluble protein antigens has frequently been investigated. Such investigations have, however, been limited to the effect of such adjuvants on the production of $7S_{\gamma 1}$ and $7S_{\gamma 2}$ immunoglobulin antibodies of the guinea-pig, and the development of delayed-type hypersensitivity to the injected antigen.

In this thesis, the effect of mycobacterial adjuvants on the production of antibody in all immunoglobulin classes, $7S_{\gamma 1}$, $7S_{\gamma 2}$ and 19S, of the guinea-pig has been explored. The antigen used throughout the study was bacteriophage ØX174, which is known to stimulate the production of both 19S and 7S immunoglobulin antibodies in the guinea-pig. Levels of bacteriophage neutralising antibody in the serum of animals immunised with and without the use of adjuvants were measured using the bacteriophage neutralisation test on the eighth and twenty-first days after immunisation. The different immunoglobulins were separated from immune sera by chromatography on Sephadex G-200 or DEAE-cellulose, identified by immunoelectrophoresis, and their antibody content measured.

It was found that whereas mycobacterial adjuvants cause a marked increase in the production of 7S antibody, particularly of the $7S_{\gamma 2}$ immunoglobulin type, no such increase occurred in the production of 19S immunoglobulin antibody.

In an attempt to explain the lack of effect of mycobacterial adjuvants on the production of 19S antibody, synthesis of bacteriophage neutralising antibody by different lymphoid tissues from immunised guinea-pigs was studied *in vitro* at times when the predominant circulating antibody was of the 19S or 7S immunoglobulin type. These investigations produced the surprising result that, on a unit weight basis, red bone marrow was much more active in the synthesis of 19S antibody than any other lymphoid tissue studied. In contrast, lymph node tissue, particularly from lymph nodes close to the site of antigen injection, was more active in the synthesis of 7S antibody than either spleen or bone marrow.

The significance of these findings is discussed with reference to previously published work, the mode of action of mycobacterial adjuvants and the relationship between the production of 19S and 7S immunoglobulin antibodies.

UNIVERSITY OF GLASGOW

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'I should see the garden far better,' said Alice to herself, 'if I could get to the top of that hill: and here's a path that leads straight to it - at least, no, it doesn't do that -' ..... 'but I suppose it will at last. But how curiously it twists!'

Lewis Carroll.

'Through the Looking-Glass.'

PREFACE

The work detailed in this thesis was carried out during the tenure of a Research Fellowship of the National Institutes of Health, U.S.A. (1964 - 1966) and a Medical Research Council Grant (1966 - 1968). I would like to acknowledge the support of both these organisations.

I am grateful to my supervisor, Professor R. G. White, for his advice and encouragement during this study, and for much helpful discussion and criticism of the manuscript.

I would like to thank Dr. P. C. Wilkinson for his collaboration in the early part of this work.

The experiments described in this thesis were designed and carried out by the author. Part of this work has been published as follows:

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Immunology 13 603.

Fleming, W.A., Wilkinson, P.C., White, R.G. 1967
'Sites of biosynthesis of Immunoglobulins in Guinea-Pigs Immunised with Bacteriophage ØX174.'
Immunology 13 603.

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In this thesis, the effect of mycobacterial adjuvants on the production of antibody in all immunoglobulin classes, $7S_{\gamma 1}$, $7S_{\gamma 2}$ and 19S, of the guinea-pig has been explored. The antigen used throughout the study was bacteriophage ØX174, which is known to stimulate the production of both 19S and 7S immunoglobulin antibodies in the guinea-pig. Levels of bacteriophage neutralising antibody in the serum of animals immunised with and without the use of adjuvants were measured using the bacteriophage neutralisation test on the eighth and twenty-first days after immunisation. The different immunoglobulins were separated from immune sera by chromatography on Sephadex G-200 or DEAE-cellulose, identified by immunoelectrophoresis, and their antibody content measured.

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The significance of these findings is discussed with reference to previously published work, the mode of action of mycobacterial adjuvants and the relationship between the production of 19S and 7S immunoglobulin antibodies.

REVIEW OF THE LITERATURE

THE IMMUNOGLOBULIN RESPONSE TO ANTIGEN

a) The Immunoglobulins.

It has been known for some considerable time that the serum proteins of animals can be divided into two broad groups on the basis of their sensitivity to precipitation by ammonium sulphate. These two groups are globulins, precipitated by half saturation with ammonium sulphate, and albumins, precipitated by full saturation with ammonium sulphate. Early workers in the field of immunology soon found that antibodies precipitated in the globulin fraction of immune sera. Analysis of immune precipitates showed that the amount of globulin present in the precipitate was proportional to the antibody activity removed from the serum. Such observations led to the view that antibodies are globulins specifically adapted to combine with antigen (Mudd, 1932).

Antibody globulins have been shown to fall into two distinct classes on the basis of their molecular weights, as determined by ultracentrifugal analysis. Most animal species have been shown to possess an immunoglobulin with a molecular weight of approximately 160,000, and an ultracentrifugal sedimentation constant of 7 Svedberg (S) units, and an immunoglobulin with a molecular weight of 900,000, and a sedimentation constant of 19S. Both of these immunoglobulin classes have been

shown to contain antibody in immune sera from man, horse, sheep, cattle, guinea-pig, rabbits, mice, chickens and a large variety of other animals (Cohen & Porter, 1964).

Most of the work on immunoglobulins has been concentrated on those found in human sera. Human sera contain three major classes of immunoglobulin, distinguishable on the basis of their molecular weight, electrophoretic mobility and antigenic characteristics. A 19S immunoglobulin is present, known as IgM, and two distinct 7S components, IgG and IgA. The immunoglobulins IgG and IgM have readily recognisable equivalents in sera from most animals, but IgA appears to be peculiar to human sera. These three classes of immunoglobulin show distinct differences in antigenic structure, chemical composition and biological function which are summarised in Fig. 1.

Although IgA is considered to be peculiar to human sera, there are reports of similar immunoglobulins in immune sera from other species. Schultz (1959) and Heremans (1959) have suggested that the T-globulin of horses may be the equine equivalent of IgA. In the guinea-pig, a similar protein was reported to be present in sera after immunisation (White, Jenkins & Wilkinson, 1963). Such proteins differ in chemical composition, antigenic structure and electrophoretic mobility from the IgG of the same species, and may well be the species equivalent of IgA. Caution must be exercised in the nomenclature of such immuno-

FIGURE 1

Chemical, Physical and Biological Properties of the Immunoglobulins.

	IgG	IgA	IgM
Sedimentation rate	7S	7S 12S(colostrum)	19S
Molecular weight	150,000	150,000	900,000
% in serum	80	15	5
Light chains	κ λ	κ λ	κ λ
Heavy chains	γ	a	u
Carbohydrate content	2%	8%	10%
Agglutination	+	+	+++
Precipitation	+++	+	+-
Complement fixation	+	-	+
Lysis	+-	-	+++
Neutralisation			
Viruses	+	+	+
Toxins	+	?	-
Enzymes	+	?	-
Arthus reaction	+	-	+
Avidity	variable	variable	strong
Placental passage	+	-	-

Compiled from Cohen & Porter (1964) and Pike (1967).

globulins. White et al (1963) and Bloch, Kourilsky, Ovary and Benacerraf (1963) showed that the electrophoretically fast immunoglobulin of the guinea-pig, thought to be the equivalent of IgA had biological properties which were not possessed by IgA. In consequence, the electrophoretically fast and slow immunoglobulins of the guinea-pig were named $7S_{\gamma 1}$ and $7S_{\gamma 2}$ respectively.

Investigations of IgG from humans, rabbits and guinea-pigs, using techniques such as enzymatic digestion and reduction of the globulin molecule have led to the formation of a basic molecular structure, which is shown in Fig. 2 (Porter, 1962). The molecule is composed of two heavy (H) and two light (L) chains, joined as shown by disulphide bonds. The H chain has a molecular weight of 50,000, and carries the antigenic determinant specific for the particular immunoglobulin class. In the case of the human immunoglobulins these determinants are γ, IgG, a, IgA and u, IgM. The carbohydrate content of the immunoglobulin molecule is usually associated with the H chain. The L chain has a molecular weight of approximately 20,000, and carries antigenic determinants common to all classes of immunoglobulin in the one animal. There are two such determinants named κ and λ, which occur together in any animal, but only one of these determinants is present on the L chains of any particular immunoglobulin molecule. The antibody combining site

FIGURE 2.

The basic structure of the immunoglobulin molecule.

(Porter, 1962)

is thought to be situated in the Fab fragment isolated by papain digestion, and on the part of the H chain isolated by this method (Cohen & Porter, 1964a). The 19S immunoglobulin molecule breaks down into similar H and L chains on reduction and is thought to be a polymer of the basic unit, containing five units joined to each other by disulphide bonds (Kurkel, 1960).

It is not within the scope of this review to deal in detail with the various structural, antigenic and chemical differences among the different immunoglobulin classes. For this purpose, the review articles of Porter and Press (1962) and Cohen and Porter (1964) provide excellent coverage. A summary is given below of the properties of the three major classes of immunoglobulin in humans, IgG, IgA and IgM. The details for IgG and IgM are generally applicable to similar immunoglobulin classes in other animals, but the properties of IgA apply solely to the human immunoglobulin.

The Characteristics of the Human Immunoglobulins.

IgG

IgG forms about 80% of the total serum immunoglobulins in the human. It has a molecular weight of 150,000 and a carbohydrate content of 2%. The electrophoretic mobility of this class of immunoglobulin ranges from slow γ into the

fast β globulins. Antibody activity in this class is unaffected by treatment with 2-mercaptoethanol.

Many antibodies are found in this class of immunoglobulin, including antitoxins, antibody against Salmonella H antigens, mumps and poliovirus, and many complement fixing antibodies. IgG crosses the placenta and enters the circulation of the foetus.

IgA

IgA forms approximately 15% of the total circulating immunoglobulins. It has a molecular weight of 150,000, but polymeric forms occur. The carbohydrate content is about 8%. The molecule has an electrophoretic mobility in the fast γ and β region. Antibody activity in this class is sensitive to reduction with 2-mercaptoethanol (Pike, 1967).

Antibodies found in this class are a small proportion of ABO isohaemagglutinins and agglutinins against Brucella abortus. This immunoglobulin was long associated with reagins in allergic individuals, but recently a new class of immunoglobulin, IgE, has been suggested to be responsible for this activity (Ishizaka, Ishinaha & Hoinbrook, 1966). IgA does not cross the placenta, but is present in many body secretions such as saliva and colostrum (Cohen & Porter, 1964).

IgM

IgM forms about 5% of the total serum immunoglobulins. It has a molecular weight of approximately 1,000,000, and a carbohydrate content of 10%. It has an electrophoretic mobility which lies entirely within the fast β region. Antibody activity in this class is destroyed by treatment with 2-mercaptoethanol, which breaks the molecule down into smaller fragments with a molecular weight of 150,000. IgM is thought to be a polymer of the basic 4 chain immunoglobulin structure shown in Fig. 2, involving 5 or 6 such units.

Many antibodies, particularly agglutinating antibodies for particulate antigens are found in this class e.g. ABO isohaemagglutinins, anti-Salmonella O, and Rheumatoid factor. IgM may cross the placenta, but only in very small amounts.

b) The Heterogeneity of the Immune Response to Antigen.

The development of new immunochemical techniques and the increase in knowledge of the structure of the immunoglobulins has directed attention towards the heterogeneity of the immunoglobulins present in serum. The presence of three main classes in human serum is well established, and there is evidence to support the view that at least two further classes exist. The circulating immunoglobulins in other animals are not

as well understood, but there is considerable evidence for the occurrence of at least three immunoglobulin classes in guinea-pig, horse, rabbit and mouse, although the relationship of these classes to the analagous classes in man is not known.

Early workers noted that the injection of antigens into animals resulted after a short lag period in the appearance of agglutinating, precipitating or neutralising antibodies in the circulation, depending on the nature of the antigen injected. In view of the work briefly reviewed above, the major part of more recent work has been devoted to determining the part played by antibodies of the 19S and 7S immunoglobulin types in the immune response.

After a single injection of different antigens in the rabbit, Bauer and Stavitsky (1961) and Bauer, Mathies and Stavitsky (1963) noted that the early antibody response occurring 6-8 days after antigen injection was of the 19S immunoglobulin type. Using the passive haemagglutination test, 19S antibodies against diphtheria toxoid were detected up to 10 days after antigen injection, at 13 days, 19S and 7S antibodies were present together, and at 17 days the predominant antibody was of the 7S type with declining levels of 19S antibody. Schoenberg and his colleagues (1965) obtained similar results. These observations suggested that there was a sequence of antibody production, beginning with the appearance of antibody of the

19S type, followed by antibody of the 7S type.

Uhr and Finkelstein and Baumann (1963) and Uhr and Finkelstein (1963) have studied extensively the progression of antibody formation against bacteriophage in the guinea-pig. They found that 19S antibody appeared early in the circulation, reaching a peak level 7-10 days after antigen injection, while 18 days after antigen injection, circulating antibody was predominantly of the 7S type. After a second injection of antigen, high levels of 7S antibody appeared, but no 19S antibody. Similar observations have been reported in chickens using bovine serum albumin (Benedict et al, 1963) and bacteriophage (Rosenquist & Campbell, 1966; Fleming, Munro & Cuperlovic, 1967). In this case the second injection of antigen resulted in the appearance of both types of antibody, but the importance of the 19S component was much less. A similar situation has been reported in humans suffering from primary and recurrent infections of typhus (Murray et al, 1965). In primary typhus, only 19S antibody could be detected against the organism, whereas in recrudescent disease, the predominant antibody was of the 7S type. Sequential production of antibody of the 19S and 7S types has been reported in humans using Salmonella H antigens (LoSpalluto, Miller, Dorward & Fink, 1962) and bacteriophage (Uhr et al, 1962). Mice, rats, cattle, sheep, turtles, frogs and goldfish have also been reported to form 19S

and 7S antibodies sequentially after a single injection of antigen. It seems therefore that a variety of species the antibody first formed in response to antigen is of the 19S immunoglobulin type, followed by later synthesis of 7S immunoglobulin antibody.

The response of experimental animals to a second injection of antigen is usually more rapid that the first, with greater antibody production. However, 19S antibody appears to play little part in this accelerated and heightened response. Uhr and Finkelstein (1963) suggested that in the guinea-pig, no immunological memory exists for 19S antibody production. A second injection of bacteriophage given 9 days after the first resulted in a slight rise in the circulating 19S antibody levels. However, a second injection given when 19S antibody could no longer be detected in the circulation resulted in no rise in 19S antibody. These results suggested that the rise in 19S levels after re-injection at 9 days was the result of re-activation or continued activation of the same antibody forming cells as had initiated the original 19S response. The lack of response to later re-injection must be due to death or inactivation of such cells and the necessity to prime a new cell population. Uhr and Finkelstein (1963) and Bauer et al (1963) postulated that 19S antibody producing cells did not undergo replication after antigenic stimulus, and,

further, that persistence of antigen is necessary for the continued synthesis of antibody of the 19S type.

The 19S antibody levels in sera reach a peak rapidly after immunisation, usually 8-10 days, and decline sharply thereafter. This transitory appearance of 19S antibody can be explained on three counts:

1. As stated above, persistence of antigen may be necessary for the synthesis of 19S antibody. As antigen is eliminated from the body, the stimulus for antibody synthesis will then disappear.
2. Antibody of the 7S type is known to inhibit the appearance of 19S antibody (Sahiar & Schwartz, 1964).
3. Estimation of the half-life of various guinea-pig serum proteins show that 19S immunoglobulin has a $t_{\frac{1}{2}}$ of 25.8 hours, whereas 7S immunoglobulin has a $t_{\frac{1}{2}}$ of 5.5 days (Uhr & Finkelstein, 1963). This means that after synthesis of 19S antibody has ceased, the circulating levels would fall rapidly due to normal catabolic processes.

The explanation for the short life of the 19S response is most likely to be a combination of these factors. This does not explain why 19S antibody formation precedes 7S antibody formation.

It has been suggested that the cell populations

responsible for the synthesis of 19S and 7S antibodies are separate and independent. Schoenberg et al (1965) studied antibody production in fragments of rabbit spleen and found that antibody of the 19S type was produced by non-phagocytic mononuclear cells situated in the walls of the sinusoids of the red pulp, while plasma cells in the white pulp were the major source of 7S antibody. In contrast, Nossal, Ssenberg, Ada and Austin (1964) showed that single plasma cells from rats immunised with flagellin first synthesised 19S antibody, then a mixture of 19S and 7S antibody, and finally 7S antibody only. Evidence from foetal and neonatal animals shows that 19S antibody synthesis occurs in the absence of 7S synthesis, in the absence of an organised lymphoid system and in the absence of detectable plasma cells (Good & Papermaster, 1964). The question of the cellular origin of these different immunoglobulins has yet to be satisfactorily answered, but the answer will certainly throw light on the sequence of antibody synthesis after antigenic stimulus.

Although many species respond to antigen injection by sequential production of 19S then 7S antibody, this is not always the case. Bauer and Stavitsky (1961) showed that only 19S antibody was produced in rabbits after a single injection of sheep erythrocytes, and that two or more injections were required before 7S antibody appeared. Bauer et al (1963) found

that repeated injections of Salmonella O antigen resulted in the formation of 19S antibody only, with no subsequent 7S formation. Guinea-pigs do not appear to produce 19S antibody against ovalbumin after a single injection, whether in adjuvant mixtures or in saline (Wilkinson, 1967).

It appears that the physical nature of the antigen may influence the type of antibody produced. Soluble protein antigens generally stimulate the formation of 7S antibody with transitory 19S production early in the immune response (Bauer et al, 1963; Gray, 1964). This is also found to be the case for viruses (Graves, Cowan & Trautman, 1964; Cowan & Trautman, 1965), bacteriophage (Uhr, Finkelstein & Baumann, 1963), and simple haptens (Bauer, 1963). In contrast, particulate antigens e.g. Gram-negative bacteria, predominantly stimulate antibody of the 19S type in rabbits and humans (Pike & Schulze, 1964; LoSpalluto et al, 1962). The response of the guinea-pig to such antigens is, however of the 7S type (Bloch, Kourilsky, Ovary & Benacerraf, 1963). Torrigieni and Roitt (1965) demonstrated that the injection of thyroglobulin coated on acrylic resin particles into rabbits resulted in a more vigorous and sustained 19S antibody response than did injection of the same antigen in solution. Ada et al (1965) observed similar results in rats after injection of polymeric and monomeric flagellin. Heat-aggregated bovine serum

albumin causes greater production of 19S antibody in rabbits than does injection of non-aggregated albumin (Lindqvist & Bauer, 1966). It seems from these results that the larger the antigenic particle, the more likely it is to induce the formation of 19S antibody.

Another factor which has been shown to affect the sequential production of 19S and 7S antibody is the antigen dose injected. Uhr and Finkelstein (1963) noted that with small doses of bacteriophage only 19S antibody was formed in guinea-pigs, and that larger doses were required to induce the synthesis of 7S antibody. Similar results have been observed using bacterial somatic antigens (Robbins, Kenny & Suter, 1965) and poliovirus (Svehag & Mandel, 1962). Treatment of animals with endotoxin or X-irradiation has been shown to prolong the 19S response, as has treatment of animals with thorotrast (Uhr & Finkelstein, 1963b; Jenkins, Auzins & Reade, 1965).

The sequential appearance of antibodies of the 19S and 7S type as described above may be more apparent than real. Freeman and Stavitsky (1965) have shown, using the sensitive technique of radio-immunoelectrophoresis, the simultaneous appearance of 19S and 7S antibodies for human serum albumin and bovine γ-globulin in the serum of rabbits. Osler and his colleagues (1966) have obtained similar results,

using a radio-active tracer method for antibody determination, and have shown the presence of greater levels of 7S than 19S antibody in the circulation during the first week after antigen injection.

On a weight for weight basis, 19S antibody has been shown to be many times more efficient than 7S antibody in the agglutination of red blood cells (Greenbury, Moore & Nunn, 1963), bacteria (Pike, Schulze & Chandler, 1966) and in the neutralisation of bacteriophage (Finkelstein & Uhr, 1967). On the other hand, 7S antibody is only slightly more efficient than 19S in the precipitation of soluble protein antigens (Heremans, Vaerman & Vaerman, 1963). It may be that the detection of 19S antibody before 7S antibody after antigen injection in the situations described above, is an artefact produced by the techniques used in the estimation of antibody. Tests such as virus neutralisation, agglutination and haemagglutination will detect much smaller quantities of 19S antibody than 7S antibody due to the much greater efficiency of 19S antibody in carrying out these reactions.

c) <u>The Effect of Age on the Immunoglobulin Response.</u>

A description of the immunoglobulin response would be incomplete without mention of the age of the stimulated

animal. The work described above deals entirely with results from adult animals. The immunoglobulin response in young animals, particularly neonatal animals is somewhat different. Smith (1960) found that the neonatal antibody response of humans and rabbits to Salmonella H antigen was of the 19S type only with no production of 7S antibody. Further, this response occurred in the absence of any detectable plasma cells in the lymphoid tissues of such animals, supporting the view of Schoenberg et al (1965) that the two classes of antibody are synthesised by different cell types. Uhr, Dancis, Franklin, Finkelstein and Lewis (1962) showed that premature infants were able to make only a 19S response to bacteriophage at birth. If the injection was delayed until 2-4 weeks after birth, then formation of both 19S and 7S antibody occurred. Eichenwald (1963) has studied virus infections of the foetus in utero and has concluded that the foetus is able to respond to such infections with the production of antibody of the 19S immunoglobulin type, but is unable to produce 7S antibody. Foetal lambs also respond to the injection of antigens such as bacteriophage, B.C.G. and Salmonella sp. by the production of 19S antibody, but such animals do not produce antibody against soluble protein antigens (Silverstein et al, 1963). It appears therefore that a 19S antibody response can be mounted by the foetal and

immediately neonatal animal after antigenic stimulation, but synthesis of antibody of the 7S type does not occur. Further, under these circumstances, many animals have been shown to lack plasma cells in their lymphoid tissues. Such results argue that the ability to produce antibody of the 19S type arises earlier than that to produce 7S antibody. This provides indirect support for the views that the synthesis of these immunoglobulins is subserved by different cell populations, and that sequential production of 19S and 7S antibody occurs in adult life.

d) Other factors affecting the Antibody Response to Antigen.

The ability of animals to form antibodies of any immunoglobulin type can be affected by a variety of non-specific factors such as age (see above), sex, hormone balance, genetic constitution and nutritional status. Many drugs such as cortisone and adrenocorticotrophic hormone will cause severe suppression of antibody production, as will irradiation and the use of cytotoxic drugs e.g. nitrogen mustards, 'Imuran'. Knowledge of how to suppress the immune response is of extreme importance in this era of tissue transplantation.

One factor which has been shown to affect the immune response in the other direction i.e. to potentiate the immune

response, is the use of adjuvants in the injection of antigen. The effect of adjuvants is fully discussed in the following section.

ADJUVANT ACTIVITY

The term 'adjuvant' is derived from the Latin verb adiuvare, meaning 'to help'. An adjuvant was defined by Munoz (1964) as 'a substance which enhances the antibody response to antigens injected simultaneously with it or within a period of time closely spaced to the injection of antigen'. This definition must be extended to include hypersensitivity states induced by antigen, which may or may not be dependent upon circulating antibody, and hypersensitivity states suspected to be associated with the immune response.

For the purposes of this review, adjuvants will be dealt with under three main headings.

1. Substances, usually non-antigenic, which adsorb antigen.
2. Bacteria and bacterial products, other than Mycobacteria.
3. Cells of various mycobacterial species, and chemical fractions of such cells.

1. ADSORBENTS.

The original observations of Ramon (1925) and Glenny, Pope, Waddington and Wallace (1926), showing that antigens adsorbed on particulate material gave rise to better antibody production, stimulated the investigation of many other

substances for adjuvant activity. Glenny et al (1926) found that addition of a solution of potassium alum to formol toxoid caused enhanced antibody production. Glenny and Barr (1931) further showed that alum concentrated and to a certain extent purified the antigen, in addition to enhancing antigenicity. Such mixtures of antigen and alum have been found effective in the immunisation of animals (Glenny & Barr, 1931) and humans (Bergey, 1934). Similar results were obtained by Hektoen and Welker (1933) using a mixture of formol toxoid and aluminium hydroxide in guinea-pigs. Similar results were obtained using diphtheria and tetanus toxoids and typhoid bacilli in experimental animals (Mann & Spinka, 1946).

Many other substances have been credited with adjuvant activity, including tapioca (Schmidt & Steenberg, 1936; Ramon, 1937), polyvinylpyrrolidone (Amies, 1962), phosphorylated hesperidin (Moss et al, 1956), and beryllium (Salvaggio, Flax & Leskowitz, 1965). Both sodium and calcium alginate have been reported to have adjuvant activity (Slavin, 1930; Amies, 1959; Scherr, Markowitz & Skelton, 1965). Pernis and Parinetto (1962) and Wilkinson and White (1966) have demonstrated enhanced immune responses with soluble antigens adsorbed on silica. Fisher (1966) has shown that intravenous administration of colloidal carbon, thorotrast, saccharated iron oxide and polystyrene latex prior to injection of antigen causes an enhanced response to

sheep erythrocytes.

A variety of oils and related sybstances have been quoted as adjuvants. Lemoignic and Pinoy (1916) reported that incorporation of typhoid and paratyphoid vaccines in petroleum jelly and lanolin gave to higher agglutinin titres than the vaccines alone. The observation that lanolin gives rise to enhanced antibody production was confirmed by Freund and Bonanto (1944). Dresser (1961) showed that Oleic acid and sorbitol mono-oleate were effective in inducing antibody production to bovine gamma globulin in the mouse when given prior to the antigen. Similar results were obtained by Barrie and Cooper (1964) using triolein or ethyl stearate. Hexadecylamine, sphingosines, substituted hexadecane, and phrenosine have been shown to be effective in enhancing the response of experimental animals to polio and influenza vaccines (Younger & Axelrod, 1963). Gall (1966) has demonstrated adjuvant activity in a variety of surface active agents, including amines, quaternary ammonium compounds, guanidines, benzamidines and thiouroniums.

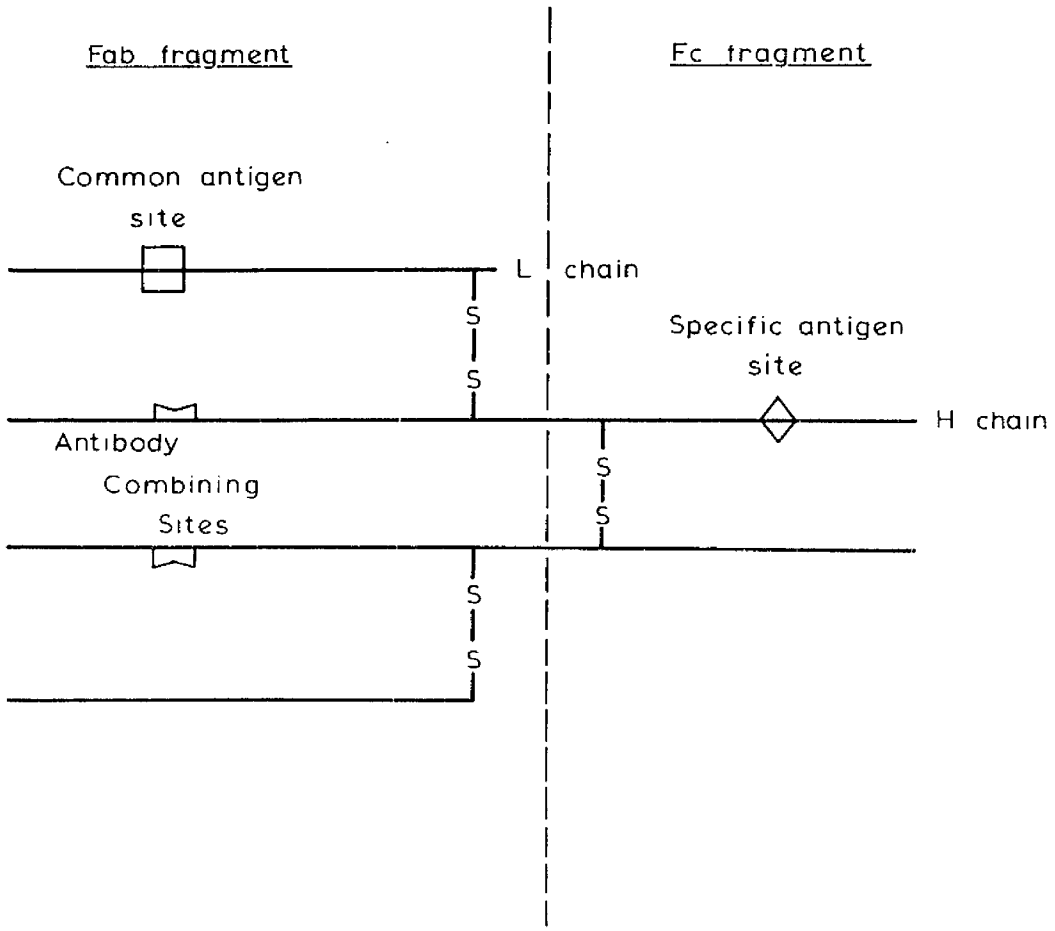
2. BACTERIAL ADJUVANTS.

A variety of both Gram positive and Gram negative bacteria have been shown to enhance antibody production to soluble and particulate antigens. Clark, Zellmer and Stone (1922) showed

that killed Gram positive cocci were adjuvant active, and Schultz and Swift (1934) obtained similar results using streptococci. They obtained enhanced sensitivity and antibody production to horse serum in rabbits. Swift and Schultz (1936a, b) and Burky (1934) demonstrated enhancement of antibody production by staphylococcal toxins. More recent, Holton and Schwab (1966) have shown that the mucopeptide fraction of the cell walls of β-haemolytic streptococci causes an increase in antibody production and skin sensitivity to bovine serum albumin in rabbits. The mucopeptide is more effective than comparable doses of cell walls or soluble cell extract.

Khanolkar (1924) reported that Pseudomonas aeruginosa was adjuvant active, an observation confirmed by Lipton and Steigman (1963). Ramon and Zoeller (1927) and Ramon (1931) worked extensively with typhoid vaccine, showing that this vaccine increased antitoxin titres against diphtheria and tetanus toxoids in animals and man.

Early work on Bordetella pertussis (Eldering, 1942; Parfentjeu et al, 1947) suggested that pertussis vaccine increased the sensitivity of mice to a number of stimuli, including bacterial and viral infection, histamine endotoxin and passive anaphylaxis. Further investigation by Greenberg and Fleming (1947, 1948) showed that injection of diphtheria toxoid with pertussis vaccine gave rise to enhanced antitoxin titres. Increased antibody responses were also reported for sheep


Fab fragment
Fc fragment
Common antigen site
L chain
S
S
Specific antigen site
H chain
Antibody Combining Sites
S
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S

erythrocytes, ovalbumin and human serum albumin (vid. Munoz, 1964). Farthing and Holt (1962) showed that Bord. pertussis cells enhanced the antitoxin response by causing earlier production of antibody, and a maintained differential over control animals.

The active principle of the many Gram negative organisms which show adjuvant activity appears to be endotoxin. Johnson et al (1956) purified endotoxin from Salmonella typhi, Pseudomonas aeruginosa, Serratia marcescens, and Brucella melitensis and demonstrated that such preparations when injected with antigen gave markedly increased antibody responses to soluble protein antigens. Farthing and Holt (1962) showed that endotoxin isolated from E. coli and Bord. pertussis was an effective adjuvant in guinea-pig, chicken and mouse. It would seem that endotoxin is not the only active principle from Bord. pertussis. Extracts of this organism which are not endotoxin will enhance antibody production, and the use of Bord. pertussis as an adjuvant increases the sensitivity of animals to anaphylaxis, an effect not usually associated with endotoxin (Munoz, 1963). Endotoxin has been shown to increase the resistance of mice to experimental virus infections (Wagner et al, 1959), to increase antibody production to bovine serum albumin in chickens (Lubecke & Sibal, 1962) and to accelerate the rejection of skin grafts in rabbits, an effect which is abolished if the animals are first made tolerant to endotoxin (Al-Askari et al, 1964).

3. MYCOBACTERIAL ADJUVANTS.

A review of the vast literature on this subject must be dealt with under four subheadings.

1. Effect of mycobacterial adjuvants on
   a) the circulating antibody response to antigen.
   b) the induction of the state of delayed-type hypersensitivity to antigen.
2. Qualitative alteration of the type of immunoglobulin produced in response to antigen.
3. The tissue reaction induced by injection of mycobacterial adjuvants.
4. The use of mycobacterial adjuvants in the production of experimental autoimmune disorders.

These four fields are, in fact, closely related, and much cross-reference is necessary.

1a) The effect of mycobacterial adjuvants on circulating antibody levels.

The observations of Lewis and Loomis (1924, 1926) showed that intraperitoneal injection of virulent tubercle bacilli a few days before injection of sheep erythrocytes or typhoid bacilli by the same route caused a considerable increase in the serum antibody levels against these antigens. Dienes (1928a, b, 1929) showed that injection of horse serum or

ovalbumin injected into foci of tuberculous infection in the guinea-pig gave rise to higher antibody titres than were obtained in uninfected animals.

The investigations of LeMoignic and Pinoy (1916) showed that incorporation of antigen, in this case typhoid bacilli, in water-in-oil emulsion gave rise to higher agglutinin titres in experimental animals and man than did antigen alone. Rabinovitsch (1897) showed that injection of tubercle bacilli incorporated in butter gave rise to intense tuberculin sensitivity. These results were confirmed by Grassberger (1899) using paraffin oil of high melting point, as did Coulaud (1935).

These two lines of investigation were merged by Freund, Casals and Hosmer (1937), who showed that incorporation of tubercle bacilli in paraffin oil not only increased the tuberculin sensitivity attained but also increased the antibody production against the tubercle bacillus, as measured by complement fixation. Over the following fifteen years, Freund and his co-workers dominated reasearch work in this field. It was found that injection of an aqueous solution of horse serum made up in water-in-oil emulsion containing killed tubercle bacilli induced greater antibody production in guinea-pigs than did injection of antibody alone (Freund & Bonanto, 1942; Freund & McDermott, 1942). Freund and Bonanto (1944) obtained similar results using diphtheria toxoid, but results obtained using

S. typhi as antigen were equivocal. In this case, water-in-oil emulsions raised the agglutinin titre obtained, but addition of mycobacteria to the injection mixture appeared to have little effect. The same workers also showed that such emulsions failed to induce antibody formation to pneumococcal polysaccharide in rabbits. Freund and Bonanto (1946) showed that the use of mycobacterial adjuvants in rabbits prolonged antibody synthesis against Salmonella sp. for periods in excess of three years. Similar results were obtained by Freund et al (1948).

Freund and his co-workers (1948) found that the composition of the injection mixture was of importance in obtaining an adjuvant effect. The adjuvant mixture used was prepared by mixing the aqueous solution of antigen in paraffin oil using an emulsifying agent e.g. Aquaphore (a wool-fat derivative) or Falba (a lanolin derivative) in the following proportions: aqueous phase 2-3.5 parts: Falba 1 part: paraffin oil 2 parts. It was found that increase in the paraffin oil tended to reduce the antibody titre obtained. Freund and Bonanto (1946) showed that peanut oil could not be substituted for paraffin oil, an observation confirmed by Freund et al (1948). These workers also showed that Arlacel A (Mannide monooleate) could be used as emulsifying agent.

A variety of hydrocarbon oils can be used as the oil phase in mycobacterial adjuvants. Shaw, Alvord and Kies (1946b)

showed that oils of carbon chain length C_{15}-C_{20}, all liquid at body temperature were effective. Oils of high C-chain length, which are solid at body temperature were ineffective, although this contradicts the observations of Coulaud (1935) the test systems used were different. Oils of short carbon chain length produced acute inflammatory reactions, and were apparently too toxic to the tissues to be effective. Barrie and Cooper (1964), have shown that certain oils are active as adjuvants *per se*, but this is a dose and time-dependent effect.

Many mycobacterial and related species have been shown to act as adjuvants when incorporated in water-in-oil emulsions, as have a few unrelated Gram positive and negative bacteria (see Fig. 3). *M. tuberculosis* var. *hominis*, *M. phlei* and *M. butyricum* have all been shown active (Freund *et al*, 1948) with the saprophytic mycobacteria showing, in some cases, a higher activity than tubercle bacilli. *Nocardia asteroides*, *C. diphtheriae* (Shaw et al, 1964a) and *C. parvum* (Neveu, Branellec & Biozzi, 1964) have also been shown to possess adjuvant activity. *M. bovis*, *M. avis* and *B.C.G.* have also been shown to be active (White, Bernstock, Johns & Lederer, 1958). White (1959) confirmed the activity of *N. asteroides*, and added to this *N. brasiliensis* and *N. rhodochrous*. Shaw *et al* (1964a) showed *C. rubrum* to be active, but this organism has since been shown to be a strain of *M. rhodochrous* (Gordon, 1965).

FIGURE 3

50% Effective Dose of Various Organisms Incorporated in Freund's Adjuvant in the Production of Experimental Allergic Encephalomyelitis.

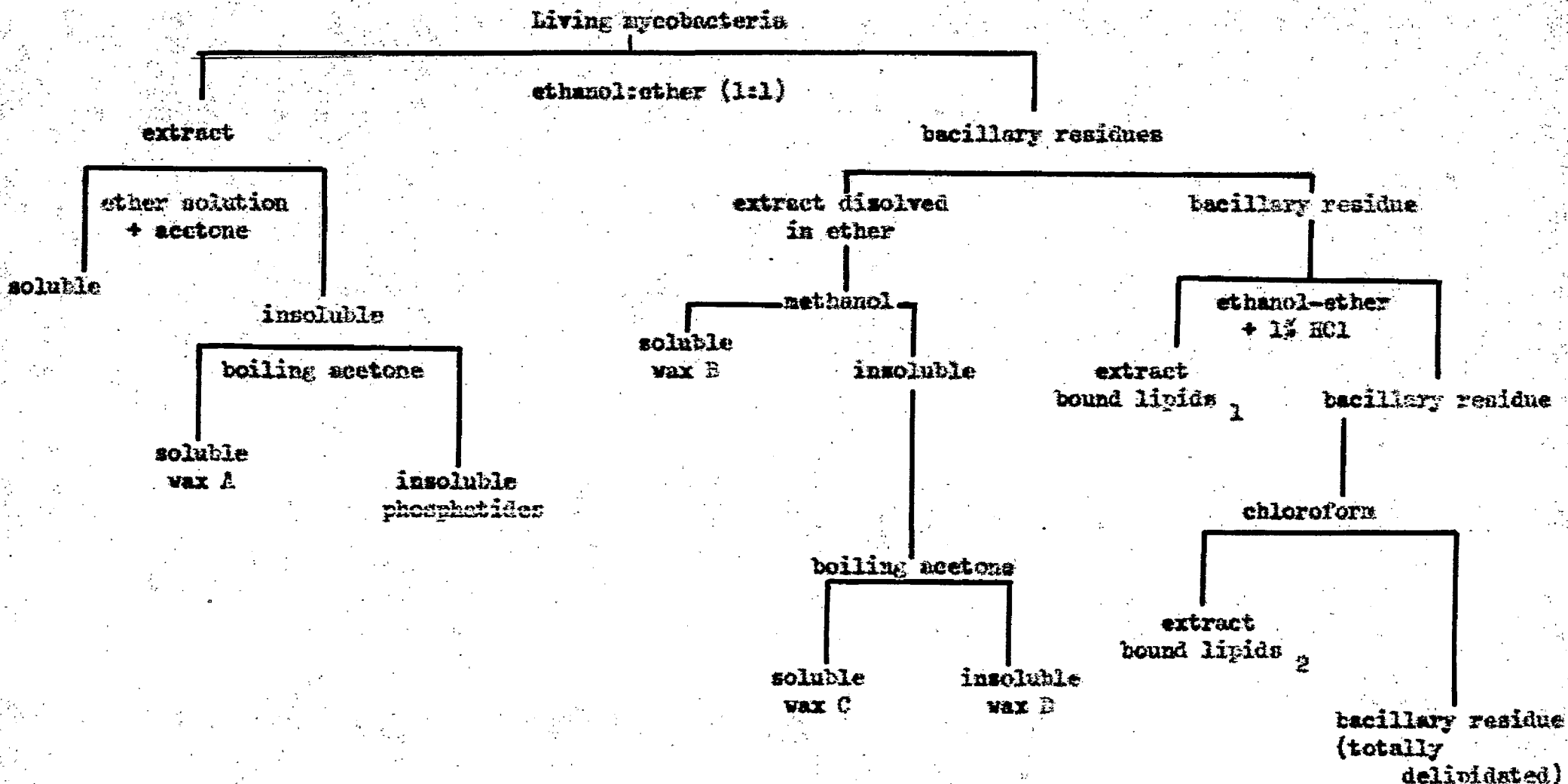
Organism	Dose (mg.)
M. tuberculosis var. *hominis* strains C, DT, PN	0.01
M. tuberculosis $H_{37}Ra$	0.01
Saprophytic mycobacteria	0.01
Nocardia asteroides	0.01
S. typhi	0.1
E. coli	0.1
Ps. aeruginosa	0.1
Bord. pertussis	0.02
C. rubrum	0.01
Staph. aureus	0.2
C. diphtheriae	2.0
B. cereus	5.0
Streptomyces sp.	0

(Shaw *et al*, 1964a)

The activity of such a large number of related species led to a search for the active principle common to all. Choucroun (1947) and Raffel (1948) established that a waxy substance isolated from tubercle bacilli by chloroform extraction, when injected along with tuberculoprotein, induced lasting tuberculin hypersensitivity. Raffel *et al* (1949) obtained similar results after injection of wax and ovalbumin in saline, with no detectable increase in serum antibody levels. The wax fraction used was 'purified' wax (Anderson, 1941). Using a similar wax fraction incorporated in water-in-oil emulsion with antigen, Raffel *et al* (1949, 1955) and White, Coons and Connolly (1955) demonstrated an enhancement of antibody production against ovalbumin.

White, Bernstock, Johns and Lederer (1958) using the extraction scheme shown in Fig. 4, showed that 40 micrograms wax D of *M. tuberculosis* str. Canetti incorporated in water-in-oil emulsion caused increased antibody production to ovalbumin. These workers also showed adjuvant activity in wax D fractions of *M. tuberculosis var Test* and *M. tuberculosis* $H_{37}R_v$. Wax D fractions of *B.C.G.*, *M. phlei*, *M. smegmatis*, bovine strains and *M. avis* were found to be inactive, as were various other chemical fractions from human strains including cord factor, wax C, mycolic acid, methyl mycolate and polysaccharide from wax D of strain Canetti. Although wax D from avian,

FIGURE 4 Scheme of Extraction of Mycobacterial Lipids.



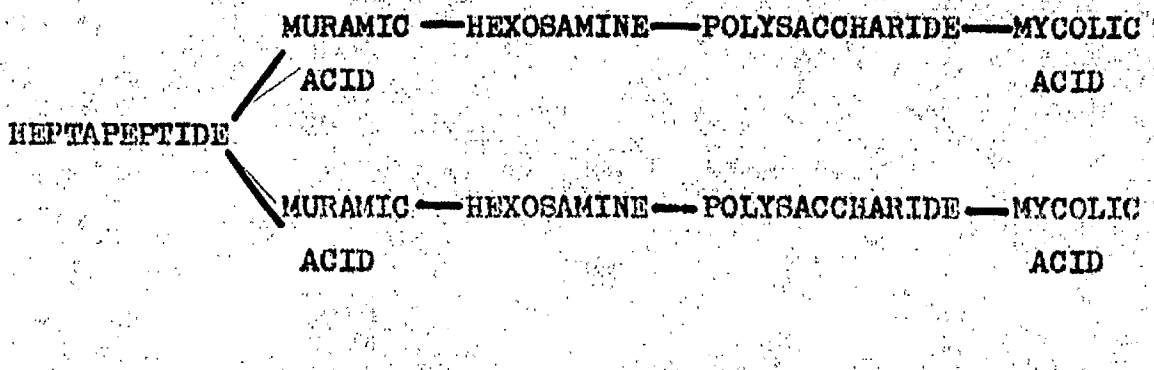
White et al (1958)

bovine and saprophytic strains were inactive, the delipidated bacterial residues showed adjuvant activity. These workers concluded that such activity was due to the presence of the amino acids alanine, glutamic acid and diaminopimelic acid, forming a peptide linked to a polysaccharide, in turn esterified to mycolic acid. The activity of delipidated bacterial residues was explained on the grounds that such cells probably contained a similar compound.

Asselineau, Buc, Jolles and Lederer (1958) showed that wax D fractions from human tubercle bacilli were peptidoglycolipids. Ultracentrifugal studies (Jolles, Samour & Lederer, 1962, 1963) showed that wax D was not a homogeneous substance, and that the peptidoglycolipid content varied from 10 - 90%. White, Jolles, Samour and Lederer (1964) showed that only peptide containing fractions of wax D from human strains could act as adjuvants. Further, peptide containing fractions with adjuvant activity were isolated in small quantity from M. phlei, M. avis and an atyoical mycobacterium. These fractions showed a similar amino acid content to human type wax D. Stewart-Tull and White (1964) demonstrated the presence of muramic acid in adjuvant active wax D fractions, further associating wax D with the cell wall. They proposed a structure for wax D (see Fig. 5) based on the mucopeptide structure of cell walls.

FIGURE 5

Hypothetical Structure of Wax D (Stewart-Tull & White, 1964).



The lack of activity of wax D fractions of saprophytic mycobacteria has been noted above. Recent evidence produced by Stewart-Tull and White (1966) has shown that wax D fractions of such organisms taken at an early stage in growth have a high content of peptidoglycolipid, and are adjuvant active. Similar fractions from old cultures of the same organisms are found to lack peptidoglycolipid, and to be inactive as adjuvants.

The quantitative effect of mycobacterial adjuvants on the response of guinea-pigs to ovalbumin was estimated by Fischel, Kabat, Stoerk and Bezer (1952). They showed that a quantitative increase in antibody formation occurred, correlated with local granuloma formation, and hyperplasia in

regional lymph nodes and spleens of the animals. More recently, Moore and Schoenberg (1966) compared the primary and secondary responses to M. butyricum in water-in-oil emulsion, but found little difference, except that antibody appeared slightly earlier after the second injection.

1b) The effect of mycobacterial adjuvants on the development of delayed-type hypersensitivity to soluble protein antigens.

The hypersensitive reaction is one in which an individual reacts to a substance which produces no reaction in normal individuals, or reacts to small doses of the substance, or in an exaggerated manner. Such reactions can be broadly divided into two types, based on the length of time after exposure to antigen required for the reaction to develop.

Immediate Hypersensitivity.

Briefly, immediate hypersensitivity is associated with circulating antibody, and can be passively transferred to a non-sensitive individual by serum from a sensitive subject. Such reactions take place within a short period of exposure to antigen, and dies down fairly quickly thereafter. The three main classes of immediate reaction are:

a) Arthus reaction

b) Anaphylaxis

c) Serum sickness.

Of these three, the Arthus reaction presents difficulty, having certain similarities to the delayed type reaction. Humphrey and White (1964) describe this reaction as follows. "When a small volume of solution of an otherwise harmless antigen is introduced into the skin of an immunised rabbit, the initial bled disappears, but within one or two hours a more diffuse swelling beginns to show and the local skin becomes hyperaemic. Petechial haemorrhages may appear, and the area of subcutaneous oedema and erythema continues to increase for several hours, after which the reaction gradually subsides. The more antibody is present in the rabbit, the more intense the reaction and the longer it takes to disappear."

Histology of the reaction shows involvement of platelets and polymorphs. Gross oedema appears. After 24 hours, the polymorphs degenerate, and many lymphocytes, macrophages and eosinophils appear. These cells persist for several days.

Delayed Hypersensitivity.

The presence of entire infectious agents, prolonged skin contact or the use of an adjuvant is usually required for the development of delayed hypersensitivity reactions. Such reactions cannot be passively transferred with serum from sensitised individuals, but can be using suspensions of nucleated blood cells, or cell suspensions of spleen, lymph

nodes or bone marrow from sensitive individuals. The cells active in this respect are believed to be lymphocytes. The reaction can be elicited in non-vascular tissues. The histology of such reactions has been extensively studied (Martins et al, 1964; Waksman, 1960). After injection of antigen into a sensitised animal, polymorphonuclear cells invade the site of injection. These are followed by large mononuclear cells, small lymphocytes and macrophages. The reaction takes 12-24 hours to develop, and may not reach a peak until 48 hours after exposure to antigen.

The most frequently described delayed hypersensitive reaction is the tuberculin reaction, first noted by Koch in 1891, and defined by Zinsser (1921) as

> "one in which there is no immediate effect, but within 4, 5 or more hours, a swelling becomes apparent which in the course of 12-24 hours results in a swollen oedematous area of varying intensity, often with a central necrotic spot, and, occasionally haemorrhage. This reaction may not reach its highest development until about 48 hours after injection, and is accompanied by distinct signs of inflammation, and some cell death."

Such reactions have been described as an early feature of the immune response to soluble protein antigens (Dienes, 1930) (Sell & Weigle, 1959) but these reactions are rapidly superceded by

reactions of the Arthus type.

Coulaud (1935) demonstrated that injection of killed tubercle bacilli in paraffin gave rise to intense and lasting tuberculin sensitivity. Raffel (1948) showed that delipidated tubercle bacilli failed to induce tuberculin sensitivity, but if the bacilli were first mixed with a chloroform extract of tubercle bacilli, tuberculin sensitivity was induced. Alcohol-ether extracts were ineffective in this respect. Raffel (1947) established that injection of tubercle wax with tuberculoprotein in saline produced tuberculin sensitivity, but that injection of tuberculoprotein alone failed to induce sensitivity. Choucroun (1948) stated that a chloroform soluble glycolipid, P_mK_o, would also cause tuberculin sensitivity when injected with tuberculoprotein. Raffel, Asselineau and Lederer (1955) identified this activity with the wax D fraction of tubercle bacilli.

Dienes and Schonheit (1927a, b, c, 1930) and Dienes (1928a, b, 1929) confirmed the observations of Lewis and Loomis (1924) showing that sensitivity to egg albumin could be induced in guinea-pigs by direct injection of antigen into a focus of tubercular infection. These workers observed that intratesticular injection of live tubercle bacilli in rabbit followed 24 hours later by an injection of ovalbumin in the same site resulted in the development of delayed sensitivity to

the antigen (1930). Similar results were obtained using intraperitoneal injections, and killed tubercle bacilli. These workers also noted that intratesticular injection of smallpox vaccine followed by egg albumin resulted in delayed hypersensitivity. Dienes (1930) demonstrated the specificity of the hypersensitive reaction using egg globulin, egg albumin and ovomucoid.

Casals and Freund (1938) sensitised Rhesus monkeys to tuberculin using killed tubercle bacilli in paraffin oil. Freund and McDermott (1942) showed that the use of killed tubercle bacilli in water-in-oil emulsions of horse serum albumin resulted in hypersensitivity of the tuberculin type to the antigen. White et al (1955) obtained delayed hypersensitivity to ovalbumin in guinea-pigs using purified wax of Anderson, and White et al (1958) obtained similar sensitivity to ovalbumin using wax D from a variety of strains of mycobacteria.

White et al (1955) studied the histology of the cornea of sensitive animals injected intracorneally with antigen. They found gross thickening of the cornea with intense cellular exudation throughout the whole thickness. Individual corneal fibres were grossly swollen. Corneas from non sensitive animals showed negligible changes. Voisin and Toullet (1963) studied the skin reactions produced by the use of mycobacterial adjuvants. They showed that vascular

permeability after an intradermal injection of tuberculin increased at the site of injection. This increase was intense, but transitory, lasting for 30-45 minutes. This was followed by a quiescent period of 30 minutes to 5 hours, depending on the dose of tuberculin and the degree of sensitivity of the animal. A second wave of vascular permeability then occurs, becoming maximal after 24 hours and disappearing after 72 hours. This increase is of moderate intensity, slowly progressive and long lasting. Voisin, Toullet and Voisin (1964) extended these observations to cover other hypersensitive reactions of the delayed type e.g. to soluble proteins, auto-antigens, contact hypersensitivity, homograft reaction, and showed that the increase in vascular permeability was a general phenomenon. They concluded that this constitutes a useful diagnostic feature for delayed hypersensitive reactions, especially where there is a concurrent Arthus reaction.

Qualitative effects of mycobacterial adjuvants on the immunoglobulin response of the guinea-pig.

Guinea-pigs given a single injection of a soluble protein antigen e.g. ovalbumin were generally thought to respond by production of a homologous population of antibody molecules, with similar electrophoretic mobility. White, Jenkins and Wilkinson (1963) found that injection of diphtheria toxoid or

ovalbumin in water-in-oil emulsion with added mycobacteria stimulated the production of two distinct precipitin arcs on immunoelectrophoresis. Injection of antigen in water-in-oil alone resulted in the appearance of only one precipitin arc. This single arc was designated the 'fast' arc due to its electrophorectic mobility. On similar grounds, the second arc obtained by inclusion of mycobacteria in the injection mixture was designated the 'slow' arc. Density gradient ultracentrifugation showed that both arcs appeared together, with a sedimentation constant of 7S. Consequently, these antibodies were designated $7S_{\gamma 1}$ (fast) and $7S_{\gamma 2}$ (slow).

Similar results were obtained by Benacerraf et al (1963) using protein antigens and hapten conjugates. These workers found that the slow arc appeared first in the course of immunisation, followed slightly later by the fast arc. Animals immunised intraperitoneally with hapten produced mainly fast migrating antibody. Common and distinct antigenic determinants were observed on the 'slow' and 'fast' antibodies.

The qualitative alteration in the type of immunoglobulin antibody produced may only hold true with respect to guinea-pigs. Coe (1966) has recently shown in the mouse that other factors may determine whether or not fast and slow antibody appear in response to antigen. The production of slow migrating antibody in the mouse was influenced by the use of

mycobacterial adjuvants, the antigen used, the strain of mouse and previous exposure of the mouse to antigen.

The properties of guinea-pig $7S\gamma_1$ and $7S\gamma_2$ immunoglobulins.

Separation of the two 7S immunoglobulins was first achieved by White et al (1963) using a stepwise elution technique on DEAE cellulose. These workers found that the $7S_{\gamma_1}$ fraction transferred passive cutaneous anaphylaxis much more efficiently than the $7S_{\gamma_2}$ fraction. This was confirmed by Ovary, Benacerraf and Bloch (1963). Bloch et al (1963) showed that only the $7S_{\gamma_2}$ immunoglobulin fraction would fix complement, and sensitise antigen-tanned red cells for lysis. Both fractions, however, would mediate passive cutaneous anaphylaxis in the rat.

The fractions differ in their adsorption and elution properties from DEAE cellulose. 80% of the $7S_{\gamma_2}$ fraction is eluted from DEAE by 0.02M phosphate buffer, pH 7.5 whereas $7S_{\gamma_1}$ appears at a concentration greater than 0.05M. They also differ antigenically, and in the electrophoretic mobility of their isolated heavy chains.

It has been suggested that the $7S_{\gamma_2}$ fraction of guinea-pig sera might be equivalent to the IgA fraction of human sera. However, this fraction of guinea-pig serum is transferred from maternal to foetal circulation (Bloch et al, 1963), a

property which human IgA does not have. However, due to their antigenically heavy chains, these two guinea-pig immunoglobulins must be considered as different classes.

The tissue response to mycobacterial adjuvants.

The effect of mycobacterial cells and chemical fractions of such cells in the production of chronic inflammation at the site of injection has long been known. Mycolic acids induce persistent necrotic lesions at the site of injection (Gerstl, Tennant & Pelzman, 1945). Delaunay, Asselineau and Lederer (1954) found that peptidoglycolipids from human tubercle bacilli, and glycolipids from bovine strains produced similar effects, and noted the presence of giant cells.

Suter and White (1954) demonstrated that injection of mycobacterial adjuvants into the footpad of guinea-pigs induced swelling of the injected foot, followed by desquamation and ulceration. Hyperplasia occurred in the regional lymph nodes. Histological studies showed a proliferation of macrophages, polymorphonuclear leukocytes and eosinophils in the injected foot. The lymph nodes also showed proliferation of macrophages. The liver was found to contain small foci of macrophages, with plasma cells, lymphocytes and polymorphs. Many granulomata of similar cell content were found in the lungs. Many macrophages

were detected in the sinusoids of the spleen.

White, Coons and Connolly (1955) detected epitheloid macrophages in the footpad granuloma. The cytoplasm of these cells was bulky, with an opaque or eosinophilic appearance. The nuclei were oval with clear-cut undulating nuclear membranes and an "empty" interior. Multinucleate giant cells were numerous. Antibody forming cells were uncommon, but when detected were of the plasma cell series. In contrast, in animals injected with water-in-oil emulsion, only slight macrophage proliferation occurred, with prominent fibroblast proliferation and young collagen fibres. These workers also studied the histology of the regional lymph nodes. Homolateral lymph nodes were from 4-15 times normal mass in animals receiving mycobacterial adjuvant. Sections of such nodes showed complete destruction of the normal architecture, with intensive macrophage proliferation. Numerous giant cells were present, but very few antibody containing cells could be seen. Other tissues, including liver, lungs and spleen showed the presence of granulomata.

Laufer, Tal and Behar (1959) studied the tissues reaction of mice, guinea-pigs and hamsters to mycobacterial adjuvants. Granulomatous lesions were detected in lungs, liver and spleen, but not heart or kidneys in all three species. Rupp, Moore and Schoenberg (1960) demonstrated the occurrence

of granulomata in the heart, kidneys and lungs of rabbits after intravenous injection of mycobacterial adjuvant. In a study of the effects of subcutaneous, intramuscular and intravenous injection of mycobacterial adjuvants, Steiner, Langer and Schultz (1960) showed the formation of disseminated lesions in lung, kidney, liver and adrenals after subcutaneous or intravenous injection. These workers detected no granulomata in spleen.

Wilkinson and White (1966) have related local tissue effects of mycobacterial adjuvants to the ability of such adjuvants to induce production of $7S_{\gamma_2}$ antibody and delayed hypersensitivity to soluble protein antigens (see Fig. 7).

The production of autoimmune disease.

It is a general rule that the body does not normally react immunologically against its own antigens. Ehrlich first noted that experimental animals did not make an immune response against their own proteins, and propounded the theory of 'horror autotoxicus'. This states simply that an animal will never produce antibodies against substances normally found in its own circulatory system. Burnet (1962) has suggested that the inability of the body to react to its own antigens is the result of a homeostatic mechanism operating in embryonic life and persisting into adult life. It is necessary that lymphoid cells should be able to recognise and to react against any foreign

materials e.g. bacteria, viruses, which may be found in the tissues. To mediate this recognition a dictionary of patterns, carried in the genetic material of the lymphoid cells, is necessary. Any such patterns corresponding to 'self' components are suppressed during embryonic life, and should such patterns arise by some means in later life, they in turn are suppressed by the same mechanism. Thus, the body is prevented from reacting against its own antigens.

Weiner (1952) explains the inability of the body to react against self components on the basis of immunological paralysis. He proposed that the antigenic constituents of the body reach antibody forming cells in such large amounts that the cells are unable to respond. A similar theory was proposed by Billingham, Brent and Medawar (1953) who suggested that animals never react immunologically against foreign homologous tissue cells to which they have been exposed sufficiently early in foetal life. This process was termed 'actively acquired tolerance'.

Whatever the mechanism which prevents the body from reacting against its own antigens may be, under certain circumstances it may break down, with a resultant immune response and damage to the tissues of the body. Such a result can be produced experimentally by the following methods:

1. Immunisation with foreign antigens similar to an animal's own tissues.

2. Immunisation with altered self-components.

3. Immunisation with self-components in mycobacterial adjuvants.

4. The transfer of immunologically competent cells from an allogeneic donor to a recipient made unable to reject them e.g. by the use of X-irradiation.

These experimental methods result in the development of an autoimmune disease with damage to the tissue injected as antigen. It is thought that the tissue damage occurring in these processes is mediated by a delayed hypersensitivity reaction (Burnet, 1963; Asherson, 1965).

Mycobacterial adjuvants have been found effective in the production of a number of experimental autoimmune diseases in animals, including allergic encephalomyelitis (Kies & Alvord, 1958), allergic thyroiditis (Witebsky & Rose, 1956) and disease of the adrenals (Colover & Glynn, 1958), heart (Kaplan & Craig, 1963) and kidney (Hunter, Hackel & Heymann, 1960). As an example of the production of such disease, it is proposed to discuss experimental allergic encephalomyelitis.

Experimental allergic encephalomyelitis.

Rivers, Sprunt and Berry (1933) and Rivers and Schwentker (1933) demonstrated that a demyelinating encephalomyelitis could be produced in Rhesus monkeys by repeated injections (up to 30) of nervous tissue over a long period of time. Subsequently,

Morgan (1947) and Kabat, Wolf and Bezer (1947) showed that a similar disease could be produced using 1-3 injections of nervous tissue in mycobacterial adjuvant. Animals developed neurological symptoms, and lesions of the brain and spinal cord, including vascular thrombosis, perivascular cuffing with inflammatory cells and demyelination. These lesions were accepted as the result of immunisation to central nervous tissue. Freund, Stern and Pisani (1947) and Kopeloff and Lopeloff (1947) produced a similar disease in guinea-pigs by the injection of guinea-pig nervous tissue in mycobacterial adjuvant. The symptoms were less severe than those observed in monkeys. The involvement of mycobacterial adjuvants in the production of this disease led to an investigation of the active fraction of the bacillus responsible for the effects of the adjuvant.

Waksman and Adams (1953) observed that lipopolysaccharide and wax D fractions of tubercle bacilli were active in the production of allergic encephalomyelitis in rabbits, when injected together with rabbit spinal cord. Colover (1954) found that totally delipidated tubercle bacilli were active when injected along with heated brain suspensions, but that none of the lipid fractions isolated showed any activity. Colover and Consden (1956) isolated an active fraction from tubercle bacilli which they concluded to be a lipoprotein.

White and Marshall (1958) demonstrated that wax D from a large number of strains of tubercle bacilli were active in the

production of allergic encephalomyelitis in the guinea-pig. These workers used 0.2 ml. of a water-in-oil emulsion containing 16 mg. homologous brain tissue and 1 mg. wax D fractions of M. phlei, M. avis, B.C.G. and other bovine strains were found to be inactive. They suggested that the activity of mycobateria in the production of allergic encephalomyelitis was due to the presence of a peptidoglycolipid in the cell and wax D fractions of the cell.

Freund and Stone, on the basis of the observations of Asselineau and Lederer (1953) that wax D constitutes approximately 6-8% of the dry weight of the tubercle bacillus, calculated that the amount of wax D required to act as a substitute for tubercle bacilli in the production of allergic encephalomyelitis should be 12-15 times less than the minimum amount of tubercle bacilli required. This was not found to be the case. The minimum dose of tubercle bacilli required to produce disease in the guinea-pig was found to be 0.02-0.04 mg. whereas the dose of wax D required to produce the same effect was 0.2 mg. These workers cast some doubt on the function of wax D in the production of allergic disease, concluding that, if wax D were the active fraction, then it is at least 10 times more active in situ than after isolation and purification.

It seems likely that wax D is the active fraction of the tubercle bacillus in the production of allergic encephalomyelitis. Stewart-Tull (1966) has suggested that the

lipid fractions obtained by Colover (1954) failed to act because they did not contain sufficient wax D, and were probably contaminated with other inactive waxes of the tubercle bacillus. Further, although purified wax D is relatively inactive when compared to the whole bacilli, wax D is a surface structure of the tubercle bacillus (White, 1967), and relatively more wax D will be exposed *in situ* than in a pure preparation.

The tissues reaction induced by wax fractions of the tubercle bacillus active in the enhancement of antibody formation against ovalbumin or the production of autoimmune disease are the same. Inactive fractions which are not effective in potentiating the antigenicity of ovalbumin or homologous brain tissue produce a very much reduced tissue reaction which lacks the epitheloid macrophages found in association with active fractions (White & Marshall, 1958).

Although mycobacterial adjuvants will regularly produce encephalitis in guinea-pigs, rabbits and monkeys, in some animals, it is not necessary to add mycobacteria to the injection mixture. Levine and Wenk (1961) showed that in the rat encephalitis ensued within a fortnight of injection of nervous tissue in simple water-in-oil emulsion. Similar results were obtained by Paterson and Bell (1962). In this case, the use of an oily emulsion is necessary, but the addition of mycobacteria to the emulsion does not increase the incidence of the disease.

Diseases of this type appear to be mediated through a delayed hypersensitivity reaction. Paterson (1960) showed that allergic encephalomyelitis in rats could be transferred by means of lymph node cell suspensions, but not by serum, from diseased animals. Stone (1961) obtained similar results in guinea-pigs. Batchelor and Lessof (1964) confirmed that attempts to reproduce the lesions of experimental allergic encephalomyelitis by passive transfer of sera from animals suffering from the disease were unsuccessful. It is characteristic of the delayed hypersensitivity reaction that it can only be transferred from one animal to another by the use of lymphoid cell suspensions. Since mycobacterial adjuvants have been shown on many occasions to increase the occurrence of delayed hypersensitivity to antigens, it seems likely that the experimental allergic diseases induced by such adjuvants are mediated by this type of reaction.

The results obtained in rats cast some doubt on this hypothesis. White (1967) has put forward two explanations to cover these results:

1. The disease of experimental allergic encephalomyelitis in this species is not due to delayed hypersensitivity to nervous tissue antigens, but is mediated by some other means.
2. The adjuvant principle of mycobacteria which increases delayed hypersensitivity successfully in many

experimental animals may fail to increase the response in the rat.

Paterson and Bell (1962) showed that, in contrast to their results in rats, the use of mycobacteria was essential for the production of encephalomyelitis. It may be, therefore, that the reaction to mycobacterial adjuvants is not a general phenomenon throughout the animal kingdom, and that species differences may affect the end results obtained.

As has been stated above, mycobacterial adjuvants have been used in the production of a variety of other experimental autoimmune diseases. The autoimmune process has been implicated in a number of human diseases which occur spontaneously. These include diseases such as thyroiditis, rheumatoid arthritis, ulcerative colitis, myasthenia gravis, systemic lupus erythematosus and many others. In such diseases circulating antibody against tissue components can be detected, but whether such antibodies are the cause of the disease, or the result of tissue damage arising from a disease process has yet to be shown. Asherson (1965) has suggested that tissue damage in autoimmune disease could occur by one or a combination of the following methods:

1. the killing of cells by cytotoxic antibody in the presence of complement.
2. antibody facilitating phagocytosis.
3. damage by soluble antigen-antibody complexes.

4. delayed hypersensitivity reactions.
5. synergistic action by circulating antibody and sensitised cells.

Whatever the mechanism of tissue damage may be, mycobacterial adjuvants supply a useful experimental model for autoimmune disease which may allow us to determine the cause and possible treatment of analagous disease in humans.

The Mode of Action of Mycobacterial Adjuvants.

Although mycobacterial adjuvants are well known to potentiate the immune response against antigens, little is known of how this effect occurs. Freund (1951) suggested that the absorption, destruction and elimination of antigen are retarded by the use of water-in-oil emulsions, but this does not explain the greater effect of the addition of mycobacteria to the injection mixture. Raffet et al (1949) has reported that injection of the wax fraction of the tubercle bacillus in saline solution of antigen results in the induction of delayed hypersensitivity to the injected antigen, without any increase in the production of circulating antibody. Inclusion of wax in a water-in-oil emulsion of antigen, however, results in increased antibody formation and the induction of a delayed hypersensitivity reaction to the antigen. It is possible, therefore, that the enhancement of antibody formation and the induction of delayed hypersensitivity by mycobacterial adjuvants are the results of two unrelated mechanisms.

Mycobacterial adjuvants produce a characteristic tissue reaction at the site of injection, in the spleen, bone marrow and in regional and more distant lymph nodes. Suter and White (1954) White et al (1955) and White and Marshall (1958) showed the local tissue reaction to involve large mononuclear cells, epithelioid macrophages, giant cells and lymphocytes. A similar cell population was found in the regional lymph nodes,

and plasma cell proliferation was shown to occur in more distant lymph nodes. It was suggested that the epithelioid cell was of considerable importance in the adjuvant activity resulting from the use of mycobacteria or their wax fractions. Further, Wilkinson and White (1966) have shown a close statistical correlation between the tissue reaction induced by mycobacterial adjuvants and enhanced antibody production and delayed hypersensitivity to the injected antigen (see Fig. 7).

Steiner et al (1960) have studied the effect of mycobacterial wax fraction injected separately and in combination with oil. They found that mycobacteria caused the formation of epithelioid cells in the reticulo-endothelial system, and proliferation of plasma cells. These effects were more evident when oil and mycobacteria were combined. In contrast, Bendixen (1963) investigated the importance of the tubercle fraction in the induction of delayed hypersenstivity, concluding that the oil acted solely as a solvent for the tubercle wax, but possessed no direct function itself.

The suggestion that a water-in-oil emulsion may retard the release of antigen (Halbert, Mudd & Smolens, 1946; Freund, 1951; White et al, 1955) has received considerable attention. Recently, Herbert (1966) has shown that the antibody levels reached after a single injection of antigen in water-in-oil emulsion can also be reached by giving a large number of

regularly spaced, minute injections of antigen alone. He suggests that such adjuvants may act by the slow release of minute quantities of antigen into the circulation over a long period of time. Freund (1951, 1956) has shown that excision of the site of injection of antigen in water-in-oil emulsion 30 minutes after injection does not prevent the formation of antibody, and further, such animals produce higher levels of antibody than animals injected with antigen in saline, without excision of the injection site. The titres obtained were somewhat less than those obtained when the water-in-oil emulsion was left in situ. Although these results are apparently contradictory, it may be that small droplets of antigen in water-in-oil emulsion are rapidly disseminated after injection to other parts of the body, and sufficient spread occurs to allow a heightened immune response through the mechanism postualted by Herbert. The work of Kosunen and Kaariainen (1966), showing that a significant proportion of the antigen present in water-in-oil emulsion is released into the circulation rapidly after injection, would support this viewpoint. Freund (1956) has postualted that oil facilitates the passage of antigen throughout the body.

The suggestion that the adjuvant activity of mycobacterial adjuvants may in part be due to the retention of antigen within the emulsion is contradicted by the observations of Dresser (1960) who showed that, in the mouse, antigen and mycobacterial adjuvant could be injected in different sites without loss of adjuvant

activity. Similar results were obtained, but by pre-treatment with adjuvant, by Houdayer, Metzger and Paraf (1965). These results suggest that mycobacterial adjuvants exert a non-specific effect on the antibody producting system. Nicol, Quantock and Vernon-Roberts (1966) have shown that water-in-oil emulsions stimulate the activity of the reticulo-endothelial organs of the mouse to a significant level. Addition of mycobacteria to such an emulsion results in even greater stimulation. In view of the recognised importance of phagocytosis as a crucial step in the induction of the immune response (Perkins & Leonard, 1963), this may prove to be at least part of the explanation for the activity of mycobacterial adjuvants.

Humphrey and Turk (1963) have shown that an unrelated delayed hypersensitivity reaction in animals results in increased antibody production against soluble protein antigens. In tuberculin positive animals, injection of diphtheria toxoid and tuberculin intracutaneously results in greater levels of circulating antibody than does injection of toxoid alone. These workers suggest that this is a result of an increase in the number of immunologically competent cells to which the toxoid has access due to the delayed hypersensitivity reaction. However, prior sensitisation to tuberculin has no effect on the response of guinea-pigs to antigen injected in mycobacterial adjuvant, although it does increase the response of guinea-pigs

injected with antigen in simple water-in-oil emulsion (Aron & Janicki, 1967). Wilkinson and White (1966) reversed this situation, and injected antigen in water-in-oil emulsion prior to the injection of antigen in mycobacterial adjuvants into guinea-pigs. They found that prior injection of antigen reduced both the occurrence of delayed hypersensitivity and the production of $7S\gamma_2$ antibody in these animals. Further, the tissue reaction to the adjuvant was less severe. No information was given on the total circulating antibody levels. These results suggest that the production of a delayed hypersensitivity state is important in the action of mycobacterial adjuvants.

There are three main hypotheses for the activity of mycobacterial adjuvants:

1. Retention and slow release of antigen at the site of injection.
2. Local and general stimulation of immunologically competent cells.
3. Induction of a state of delayed hypersensitivity with a resultant increase in antibody production.

The mode of action of such adjuvants is complex, and would be more fairly represented as a combination of the above three hypotheses. More recently, Stewart-Tull, Wilkinson and White (1965) have proposed a direct role for mycobacterial wax in antibody synthesis. Because this peptidoglycolipid has an affinity for, and binds to guinea-pig $7S\gamma_2$ immunoglobulin, these

workers have suggested that it may act as a derepressor of γ-globulin synthesis.

In the case of the experimental autoimmune diseases, the part played by mycobacterial adjuvants is not clear. Although many such disorders are mediated by delayed hypersensitive reactions, which mycobacterial adjuvants are known to induce, the normal homeostatic mechanisms of the body which prevent it reacting against self components must also be overcome. Freund (1956) has suggested that killed tubercle bacilli produce a cellular reaction resulting in allergic injury to certain organs if appropriate tissue antigens are injected in combination with mycobacterial adjuvant. He further suggested that the tissue antigen might combine with the mycobacterial fraction, forming a complex with tissue specificity, but not recognisable as 'self' to immunologically competent cells. In view of the results of Stewart-Tull et al (1965) described above, this is not unlikely. White and Marshall (1958) suggested that mycobacteria increase the immunological response of the body to iso-antigens, but this would argue that there is an immune response to 'self' antigens without the use of adjuvants. Burnet (1965) suggests that the use of mycobacterial adjuvants disrupts the normal homeostatic control of the body over the immune response, thus allowing immunological recognistion of 'self' as antigenic material.

In recent years, much attention has been focussed on the

part played by the lysosome in the immune response. The lysosome is an intracellular vesicle, bounded by a lipoportein membrane, which contains many lytic enzymes. Such organelles are abundant in lymphocytes and macrophages (Novikoff, 1963). It is thought that lysosomes join with vacuoles containing phagocytosed material in such cells, and the lysosomal enzymes then digest the foreign material. Weissmann (1964) has proposed three mechanisms whereby denaturation of normal tissue might result in the production of antibodies, and resultant autoimmune disease.

1. Foreign macromolecules encountered and engulfed by the cell are broken down by lytic enzymes normally held within the lysosome. After degradation, fragments of enhanced immunogenicity appear to become associated with the cytoplasmic RNA. Subsequently these antigen-RNA complexes are transferred to antibody forming cells. It is possible that degraded foreign macromolecules and degraded self macromolecules are processed similarly as 'antigen' by the macrophage, as described above.
2. Leakage of lysosomal enzymes from weakened lysosomes may lead to the breakdown of native macromolecules, resulting in the production of auto-antigenic fragments, which would be transferred to antibody forming cells.
3. Intracellular residence of micro-organisms and viruses

may lead to lysosomal fragility with the consequences outlined above.

In view of the gross inflammatory changes induced by mycobacterial adjuvants, involving both macrophages and lymphocytes, it is possible that lysosomal enzymes might be released. Degradation of the injected 'self' tissue into smaller antigenic fragments not recognisable as self might then occur, with resultant antibody production and the appearance of autoimmune disease.

Such a mechanism may also be responsible in part for the effect of mycobacterial adjuvants on antibody production against foreign antigens. The cellular reaction induced by simple water-in-oil emulsion contains few macrophages in comparison with that induced by mycobacterial adjuvant. It is possible that the greater number of lysosome containing cells around the site of antigen injection may be responsible for the greater enhancement of antibody production by mycobacterial adjuvants.

Sites of Antibody Synthesis.

Since the observation of Pfeiffer and Marx (1898) that the spleen of rabbits immunised with cholera vibrio contained antibody at a higher concentration than the blood, the synthesis of antibody by organs of the reticulo-endothelial system has been investigated by a variety of methods. In the late 19th century, both extraction of different organs, and estimation of the antibody content of the extract, and surgical ablation of selected organs were used as methods of investigation of the sites of antibody synthesis. In 1912, Carrel and Ingebrigsten applied the method of tissue culture to the investigation of tissues involved in the production of antibody.

Investigation of the sites of antibody synthesis has generally been carried out using one or more of the following methods:

a) Simple extraction of tissues from immunised animals by grinding, and comparison of the antibody content of the extract with that of serum.

b) Ablation of different organs by surgery, hormonal treatment or irradiation, and estimation of the effect of such treatment on antibody production. More recently, after 'blanket' ablation either by irradiation or treatment with immunosuppressive drugs, aglated tissues have been replaced by cell suspensions from homologous animals, and the ability of such cells to restore the

immune response of the treated animal observed.

c) In vitro culture of tissues from immunised animals, and estimation of the antibody formed during the period of incubation. Such formation of antibody is usually measured either by direct antigen-antibody reaction, or by the incorporation of carbon-14 labelled amino acids into newly synthesised antibody.

The above methods were all in use in one form or another in the early 20th century. A fourth method was developed much later. Coons, Leduc and Connolly (1955) developed the 'sandwich' technique of immunofluorescent staining. In this technique, frozen sections of the tissue under infestigation are exposed to a solution of antigen. The section is then washed, and treated with a fluorescent labelled antiserum against the antigen. Antigen is adsorbed to antibody containing cells, and the labelled antiserum is in turn adsorbed to antigen. On subsequent examination under the ultra-violet microscope, cells which adsorbed antigen fluoresce.

Most of the recent investigations on sites of antibody synthesis have utilised ablation techniques, in vitro culture techniques, the 'sandwich' technique or a combination of these three.

The literature on the subject of sites of antibody synthesis is vast, and the references far too numerous for the scope of this review. The review deals with the major sites of

antibody synthesis, mentioning only key references.

SPLEEN.

Early extraction studies established that the spleen played a part in the production of antibody. Pfeiffer and Marx (1898) showed that spleen contained agglutinins in higher concentration than did the serum of an immunised animal. Similar results were obtained by Freund (1927) and Topley (1930).

Splenectomy was observed to reduce antibody formation by Tizzoni and Cetani (1892). Similar results were obtained by Deutsch (1899) who observed that agglutinin production against typhoid bacilli was markedly reduced if the spleen were removed after, but not before, the injection of antigen. These results were confirmed by Hektoen (1909) and Luckhardt and Becht (1911). Wolfe *et al* (1950) demonstrated reduced precipitin production in chickens after splenectomy, and Taliaferro and Taliaferro (1950) obtained similar results in rabbits. Humans respond badly to foreign erythrocytes after splenectomy, as do rats (Rowley, 1950a, b).

In vitro cultures of spleen tissue from immunised animals have provided further evidence for antibody production by spleen. Carrel and Ingebrigsten (1912) suggested that spleen tissue from non-immunised animals would produce haemagglutinins when cultured *in vitro* in the presence of foreign erythrocytes. These results were not generally confirmed, but Parker (1937)

did demonstrate antibody formation *in vitro* in spleen cultures from rabbits immunised with guinea-pig erythrocytes or bacteria. Fagraeus (1948b) and Thorbecke and Keuning (1953) demonstrated antibody production in spleens from rabbits immunised with typhoid and paratyphoid bacilli.

Askonas and White (1956) and Askonas and Humphrey (1958) demonstrated the incorporation of C-14 labelled amino acids into anti-ovalbumin by spleen tissue removed from guinea-pigs and rabbits previously immunised with ovalbumin in mycobacterial adjuvant. King and Johnson (1963) detected antibody production after culture of spleen tissue in both primary and secondary responses, and further showed that the capacity of the spleen to produce antibody could be transferred using cell suspensions to x-irradiated recipients. Mitchison (1957) demonstrated transfer of antibody formation by spleen cell suspensions in young chickens.

van Furth (1966) has shown the incorporation of C-14 labelled amino acids into immunoglobulins of the IgG, IgA and IgM classes by human spleen tissue.

LYMPH NODES.

Hektoen (1915) and Murphy (1914) noted that x-irradiation of animals resulted in a depletion of lymphoid tissue, and a reduced ability to respond to the injection of antigen. Murphy and Sturm (1919) and Nakahara (1919) showed that dry heat

stimulated lymphoid proliferation, and there was a concomitant increased antibody response to the injection of antigen.

McMaster and Hudack (1935) demonstrated quite clearly the production of antibody by lymph nodes. After injection of antigen into the ear of a mouse, antibody was detected in the regional nodes earlier and frequently in higher concentration than in the serum, and earlier than antibody was detected in more distant nodes. Injection of each ear with a different antigen showed that the regional nodes first produced antibody against the antigen with which that ear was injected. In vitro studies of antibody production by lymph nodes from immunised rabbits showed little activity (De Cara & Angevine, 1943; Fagraeus, 1948b).

Thorbecke and Keuning (1953) demonstrated antibody formation in vitro by lymph nodes from rabbits hyperimmunised with paratyphoid bacilli. Harris and Harris (1954a, b) showed that antibody formation could be transferred from immunised donors to non-immune recipients using lymph node cell suspensions. This transfer was found to be more efficient if the recipients were first irradiated. Askonas and White (1956) and Askonas, White and Wilkinson (1965) demonstrated the incorporation of C-14 labelled amino acids into anti-ovalbumin by various lymph nodes from immunised guinea-pigs.

Studies on isolated single cells from lymph nodes have proved effective in the detection of antibody production by lymph node cells. Nossal (1959a, b) measured the immobilisation

of a motile Salmonella species by single lymph node cells from immunised rabbits. 2.3% of the cells studied from animals given a single injection of antigen showed antibody production. The proportion increased to 14% after a second injection of antigen. Similar results were obtained by Makela (1964) and Attardi et al (1959, 1964).

Harris et al (1963) using rabbits homozygous for each of an allelic pair of allotypes of γ-globulin as donors and recipients, demonstrated clearly that the antibody formed in recipient animals was antigenically of the donor type, and therefore formed by the donor cells.

Synthesis of immunoglobulins within lymph nodes has been detected both by immunofluorescent techniques (Burtin, 1960) and tissue culture - radioimmunoelectrophoresis (Levene et al, 1961; van Furth, 1966).

BONE MARROW.

Carrel and Ingebrigsten (1912) demonstrated agglutinin formation in bone marrow from guinea-pigs, but until the early 1940's the investigation of bone marrow as an antibody producing organ was much neglected. De Gara and Angevine (1943) studied bone marrow from rabbits after repeated injection of both soluble and particulate antigens; they consistently detected precipitating and agglutinating antibody. Fagraeus (1948b) cultured bone marrow from rabbits immunised with bacteria, and

demonstrated the production of agglutinins. The activity detected in bone marrow was much less than that of spleen from the same animals.

Thorbecke and Keuning (1953) studied bone marrow from rabbits given multiple injections of paratyphoid vaccine. After a 24 hour period of culture, bone marrow from such animals was consistently found to have produced agglutinins, but to a lesser extent than spleen from the same animal. These workers could detect no antibody formation in bone marrow from animals given only one injection of antigen. Askonas and White (1956) demonstrated the high contribution of the bone marrow to overall synthesis of antibody after a single injection of ovalbumin in the guinea-pig. Further, these workers showed that bone marrow played an important part in the synthesis of non-specific γ-globulin. Similar results were observed in rabbits given a single injection of alum-precipitated ovalbumin (Askonas & Humphrey, 1958).

Transfer of bone marrow tissue from immune to non-immune, x-irradiated recipients, has further demonstrated the involvement of bone marrow in antibody synthesis. Hobson, Porter and Whitby (1959) demonstrated antibody production against bovine γ-globulin using this method, and Garver, Santos and Cole (1959) obtained similar results using erythrocytes as antigen.

Burtin (1960) investigating the cells responsible for the production of IgM in humans, failed to detect any antibody

producing cells in bone marrow. Similarly, Langevoort et al (1963) demonstrated production of antibody against bovine γ-globulin in cultures of rabbit spleen and lymph nodes after a single injection of antigen, but no activity was detected in bone marrow cultures. Gengozian et al (1961) and Friedman (1964) showed that the bone marrow does not synthesise antibody in a primary response, but does so in a secondary response.

These negative observations are contradicted by the observations of Askonas and White (1956) and Askonas, White and Wilkinson (1965) which show active synthesis of antibody against ovalbumin in the bone marrow of guinea-pigs after a single injection of antigen. These workers, however, injected the antigen in mycobacterial adjuvant. van Furth (1966) has demonstrated the incorporation of carbon-14 labelled amino acids into immunoglobulins of IgG, IgA and IgM specificity by normal and pathological bone marrow, cultured in vitro from humans.

ANTIBODY FORMATION BY OTHER ORGANS.

The liver, although a prominent organ in the reticulo-endothelial system, seems to play very little part in the synthesis of antibody. Miller and Bale (1954) showed that isolated, perfused rat liver would incorporate carbon-14 labelled amino acids into albumin, β-globulin and fibrinogen, but that little labelled γ-globulin was produced. In contrast, if

the eviscerated carcase of the rat were perfused, only labelled γ-globulin was produced. Askonas and White (1956) and Askonas and Humphrey (1958) studied the incorporation of labelled amino acids into antibody by liver cultured *in vitro*. These workers showed that, on a unit weight basis, liver was only slightly active in the synthesis of antibody, and that the contribution of liver to overall antibody synthesis was, therefore, extremely small.

Lung and kidney have been shown to synthesise antibody. Bjorneboe and Gormsen (1947) demonstrated the presence of large numbers of plasma cells in the fatty tissue of the kidney of rabbits after repeated injection of antigen. Askonas and White (1956) and Askonas and Humphrey (1958) demonstrated the incorporation of labelled amino acids into antibody against ovalbumin and into non-specific γ-globulin, by kidney and lung tissue from immunised guinea-pigs and rabbits. van Furth (1966) detected synthesis of IgG and IgA, but not IgM, in tissue cultures of human lung.

It appears that the granuloma caused by the injection of mycobacterial adjuvants may synthesise antibody. Askonas and Humphrey (1958) studied the granuloma in rabbits immunised with ovalbumin, and showed that the granuloma made the major contribution to antibody production compared with the spleen, lymph nodes and bone marrow. In contrast, Askonas and White (1956) who investigated a similar system in guinea-pigs showed

that the contribution of the granuloma to overall antibody synthesis was very low, although small amounts of specific antibody were synthesised. Similar investigations by Askonas *et al* (1965) in the guinea-pig produced the same result. It has been known for some time, however, that the local site of antigen injection will produce antibody (Oakley, Warrack & Batty, 1954).

It is generally accepted that the thymus does not normally produce antibody. The organ does not show proliferation of plasma cells after administration of antigens by normal routes, and no antibody is produced (Fagraeus, 1948a; Thorbecke & Keuning, 1953; Askonas & White, 1956). Culture experiments have shown that thymic tissue will produce very small quantities of γ-globulin (Askonas & White, 1956; Asofsky & Thorbecke, 1961) and van Furth (1966) has shown the synthesis of IgG by human thymus tissue *in vitro*. If, however, antigen is injected directly into the thymus, then proliferation of plasma cells and antibody formation occurs (Marshall & White, 1961). Thymus cells have been shown by cell transfer experiments to produce antibody (Stoner & Hale, 1955; Dixon, Weigle & Roberts, 1957). It appears then that the thymus does not under normal circumstances synthesise antibody, but that the cells of this organ are capable of antibody synthesis if antigenically stimulated. Marshall and White (1961) postulated that the normal thymus possessed a barrier which prevented the passage of antigens from the bloodstream, thus preventing any antigenic

stimulus from reaching the thymic cells.

As can be seen from this brief review, antibody synthesis can occur in many different sites in the animal body. For a more comprehensive review of the sites of antibody synthesis see Wilson and Miles (1961) and McMaster (1957).

Cellular sites of synthesis of 19S and 7S immunoglobulins.

The great majority of the work described above has been concerned with the total antibody synthesis by any given lymphoid organ, and few investigations have been made on the relative importance of different lymphoid organs in the synthesis of different classes of immunoglobulin antibody. Qualitative results from investigations using the radio-immunoelectrophoresis/tissue culture technique of Hochwald, Thorbecke and Asofsky (1961) have shown that the major lymphoid organs of the body are able to synthesise immunoglobulins of all classes (Asofsky & Thorbecke, 1961; Thorbecke, Hochwald, Asofsky & Jacobson, 1964; van Furth, 1966). These results provide no information on the quantitative production of antibodies by such tissues.

Askonas et al (1965) investigated the relative importance of guinea-pig lymphoid tissues in the production of $7S\gamma_1$ and $7S\gamma_2$ immunoglobulin antibodies after injection of ovalbumin in mycobacterial adjuvant. It was found that no particular tissue synthesised $7S\gamma_2$ or $7S\gamma_1$ antibody exclusively.

These workers did show that any lymphoid tissue studied *in vitro* synthesised more $7S\gamma_2$ than $7S\gamma_1$ immunoglobulin, which, since $7S\gamma_2$ is the predominant circulating type, is not unexpected.

As can be seen, although it is accepted that lymphoid organs are responsible for the synthesis of antibody, we have very little knowledge of the effect of antigenic stimulation on the production of antibody of different immunoglobulin types by such organs. Another approach to the problem has been the investigation of the cell types responsible for antibody synthesis.

The work of Bjorneboe and Gormsen (1947) and Fagraeus (1948a, b) early implicated the plasma cell series as the cells responsible for the synthesis of antibody. Fagraeus (1948b) studied the *in vitro* production of antibodies by rabbit lymphoid tissues after multiple injections of antigen, and was able to show a close correlation between the synthetic activity of such tissues and their content of plasma cells. Keuning and van der Slikke (1950) immunised rabbits with whole paratyphoid bacilli, and cultured white and red pulp of spleen *in vitro*. Antibody formation was detected in the red pulp, and correlated with the presence of large numbers of immature and mature plasma cells.

The development of the fluorescent antibody 'sandwich' technique (Coons, Leduc & Connolly, 1955) made the identification of antibody producing cells an easy matter. Sections of tissue

from immunised animals are first treated with a solution of the injected antigen, washed, then treated with a fluorescein-conjugated antiserum against the antigen. If antibody containing cells are present in the section, antigen will combine with this at the cell surface, and conjugated antiserum will, in turn, combine with antigen. On subsequent examination of the section under ultra-violet microscopy, antibody-containing cells will fluoresce. Using this technique, Coons et al (1955) were able to detect antibody forming cells in the lymph nodes and spleen of hyperimmunised rabbits. White (1958) showed that two separate populations of plasma cells exist in the lymph nodes of rabbits given a simultaneous injection of ovalbumin and diphtheria toxoid, one producing antibody against ovalbumin, the other against diphtheria toxoid.

Many other workers have demonstrated the presence of antibody in mature and immature plasma cells (Nossal, 1959a, b; White, 1961; Hulliger & Sorkin, 1963; Schoenberg et al, 1965). However, these observations have been made when the predominant circulating antibody is of the 7S immunoglobulin type, and presumably also the antibody synthesised by the cells detected. This technique, however, detects only the presence of antibody, and gives no information on the immunoglobulin type of such antibody.

With the development of understanding of the antigenic structure of the immunoglobulin molecule, it became possible to

produce specific antisera which would react with only one class of immunoglobulin. Using such antisera, conjugated with fluorescein, much information should become available on the cells responsible for the synthesis of the different immunoglobulin classes. Pernis and Chiappino (1964) have shown that only one antigenic type of heavy and light chain is synthesised in any one cell, and presumably therefore only one antigenic class of immunoglobulin. Benier and Cebra (1965) studied human splenic tissue, demonstrating the presence of IgG and IgA in different plasma cells, but gave no information on IgM.

Although the plasma cell has been shown to be the source of 7S immunoglobulin antibodies, considerable argument surrounds the cell responsible for the synthesis of 19S immunoglobulin antibodies. Curtain (1961) reported 19S-specific fluorescence in large monocytoid cells in the bone marrow of patients suffering from macroglobulinaemia. Similar results have been reported in macroglobulinaemic patients (Kritoman et al, 1961) and in patients suffering from rheumatoid arthritis (Mellors et al, 1959). Synthesis of 19S immunoglobulins has been shown to occur in large mononuclear cells in humans unable to synthesise 7S immunoglobulins, and having no detectable plasma cells (Cruchaud et al, 1962). These observations have been made on patients with disease known to involve the immunoglobulins in one way or another, and may bear no relation to the normal processes of the body.

Schoenberg et al (1965) have studied extensively the cellular source of 19S and 7S immunoglobulins produced by the rabbit in response to antigen injection. These workers consistently detected 7S immunoglobulins in the plasma cells of the non-follicular white pulp of splenic tissues. In contrast, the only cells found to show fluoresence using a specific anti-rabbit 19S immunoglobulin were present in the walls of the sinusoids of the red pulp of the spleen. These cells were non-phagocytic mononuclear cells. In rabbits which produced only 19S antibody, this type of cell was the only one to show fluorescence using the sandwich technique, whereas in animals which produced both 19S and 7S antibody, both types of cell were shown to fluoresce. Antibody of the 19S type appeared first in the circulation and mononuclear cells were first to show fluorescence. The appearance of fluorescent plasma cells lagged behind that of fluorescent mononuclear cells, as did the appearance of circulating antibody of the 7S type.

It has been suggested that these mononuclear cells are precursors of plasma cells, which proceed as they mature from the synthesis of 19S immunoglobulin to the synthesis of 7S immunoglobulin (Nossal et al, 1964). However, Nossal (1964) and Cruchaud (1962) have shown continued synthesis of 19S immunoglobulin in such cells in single cell studies on tissues from humans and immunised animals. It is possible that a cell may change over from the synthesis of a polymeric form of the

immunoglobulin molecule to a monomeric form, the change being brought about by the loss or gain of a single enzyme. However, the change of synthesis from 19S to 7S also requires a change in the antigenic structure of the H chain of the immunoglobulin molecule to give 7S specificity. This is less easy to envisage. It seems reasonable to suggest that the production of antibodies of the 19S and 7S immunoglobulin types is the result of the development of two independent cell lines. Much more evidence is required before this statement can be made with certainty.

The situation is further confused by numerous reports of specific 19S fluorescence in plasma cells. Kunkel (1960) and Seligmann (1960) both report the presence of IgM in plasma cells, and in no other cells in humans. Burtin (1960) was unable to obtain any identifiable fluorescent cells when looking for IgM in human lymph nodes, spleen and bone marrow. van Furth (1966) reports that small and medium lymphocytes and plasma cells contain IgM in normal human lymphoid tissues.

It should be possible to correlate the activity of any organ in the synthesis of 19S immunoglobulin with the number of 19S containing cells which that organ contains. Such a study has not yet been fully attempted. Further, if the hypothesis of Uhr and Finkelstein (1963b) and Schoenberg et al (1965) holds good, that the 19S producing cell does not divide after antigenic stimulation, such an approach to the problem would not be particularly helpful.

The production of antibody necessitates the presence, within a cell, of a protein factory. Such a cellular factory can be recognised by the presence of an organised endoplasmic reticulum with many associated ribosomes, a prominent Golgi apparatus and a prominent nucleolus within the nucleus. The investigations of Fagraeus (1948b) using normal histological stains showed that immature and mature plasma cells have a large amount of cytoplasmic ribonucleic acid. Electron microscopic studies on plasma cells have shown the presence of the necessary apparatus for protein synthesis in such cells, (Harris, Hummeler & Harris, 1966). The lack of these intracellular organelles is one of the main arguments against the involvement of lymphocytes in the active synthesis of antibody. No parallel studies have yet been reported on the mononuclear cells which many workers have shown to contain 19S immunoglobulin.

Although both the cellular and organ sites of synthesis of antibodies of the 7S type are, at present, fairly well understood, our knowledge of the sites of 19S antibody synthesis is scant and confusing. Much work remains to be done in this field of immunology.

MATERIALS AND METHODS

Preparation of meat extract (nutrient) broth and nutrient agar.

These media were prepared from a basic ox-heart infusion broth as described below:

Ox-heart infusion broth

Minced ox-heart (fat-free)	1 lb
Peptone (B.D.H.)	10 g
Sodium chloride	5 g
Tap water	1 litre

These constituents were mixed together, and steamed at 100°C for 5 hours. The resultant broth was then filtered through coarse filter paper, and the pH adjusted to 8.4. The broth was then steamed for a further hour, and left standing overnight to allow precipitated phosphates to settle. After settling, the broth was again filtered through coarse filter paper.

Meat extract broth

Ox-heart infusion broth	1 litre
1% para-amino benzoic acid	5 ml

The pH of 1 litre of ox-heart infusion broth was adjusted to 7.6 and 5 ml 1% p.a.b.a. added. The broth was then bottled in 250 ml amounts and sterilised by autoclaving at 5 p.s.i. for 30 minutes.

For use as a culture medium, the contents of one bottle were transferred aseptically to a sterile 500 ml flask. For use as a diluent in the bacteriophage neutralisation test, 10 ml aliquots were transferred aseptically to sterile 6 x $\frac{3}{8}$" test tubes,

and stored at 4°C.

Nutrient agar

This medium was prepared using Oxoid agar no. 3.

Ox-heart infusion	1 litre
Oxoid agar no. 3	24 g
Distilled water	1 litre

1 litre ox-heart infusion broth, as described above, was made up to 2 litres with distilled water. Oxoid agar no. 3 was added to a final concentration of 1.2% (12 g/litre), and the mixture autoclaved at 15 p.s.i. for 15 minutes. The broth-agar mixture was then filtered through $\frac{1}{8}$" cellulose wadding, and the pH adjusted to 7.6. 10 ml 1% p.a.b.a. were then added, the agar distributed into 250 ml bottles and sterilised by autoclaving at 5 p.s.i. for 30 minutes.

0.6% Nutrient agar for use in the Bacteriophage Neutralisation Test. This was prepared as above, with the substitution of 6 g Oxoid no. 3 agar per litre for 12 g.

Preparation of peptone water for use in the preparation of coliphage X174.

Peptone (B.D.H.)	20 g
Sodium chloride	5 g
Distilled water	1 litre

The constituents were mixed and dissolved by steaming for 20 minutes. The pH was then adjusted to 8.2, and steaming continued for a further 20 minutes. The solution was then filtered, and the pH adjusted to 7.6. This medium was sterilised by autoclaving at 5 p.s.i. for 30 minutes.

10% horse blood agar

10% blood agar plates were prepared by the aseptic addition of 1 ml sterile citrated horse blood to 9 ml molten, nutrient agar, the whole mixture being poured into a Petri dish.

Medium for tissue culture - Eagle's medium.

Eagle's medium was prepared from 3 stock solutions prepared as described below:

Solution 1

NaCl	6.4 gm	$Fe(NO_3)_3$	0.001 gm
KCl	0.4 gm	L-glutamine	0.2 gm
$CaCl_2$	0.2 gm	Cristamycin	
$MgSO_4.7H_2O$	0.2 gm	+Antimycotic	0.5 ml
$Na_2HPO_4.2H_2O$	0.14 gm	Phenol red	1.5 ml
Dextrose	4.5 gm	(1% solution)	

+ - antimycotic - 0.01% n-butyl-p-hydroxy benzoate

Dissolve each ingredient in water, and make up to one litre with

distilled water. Then add 2.75 gm $NaHCO_3$.

Solution 2

L-arginine mono hydrochloride	0.42 gm
+Cystine	0.24 gm
Histidine monohydrochloride	0.192 gm
Isoleucine	0.524 gm
Leucine	0.524 gm
Lysine monohydrochloride	0.73 gm
Phenylalanine	0.33 gm
Threonine	0.476 gm
Tryptophan	0.08 gm
Tyrosine	0.362 gm
Valine	0.47 gm
Methionine	0.15 gm
Inositol	0.035 gm

+ - dissolve in the smallest volume N NaOH (0.1-0.3 ml)

Heat sterile distilled water to 56°C, add the amino-acids, dissolve and store at -20°C.

Solution 3

Choline chloride	0.05 gm
Folic acid	0.05 gm
Nicotinamide	0.05 gm
Pantothenic acid	0.05 gm

Pyridoxal hydrochloride	0.05 gm
Riboflavin	0.005 gm
Thiamine hydrochloride	0.05 gm

Dissolve in distilled water, and make to a final volume of 100 ml. Store at -20°C.

Preparation of Eagle's medium from stock solutions.

The three solutions 1, 2 and 3 were made up finally in the following proportions:

1	100 ml
2	5 ml
3	0.4 ml

To this mixture, sterile, dialysed, heated sheep serum was added to a final concentration of 10%, and the medium flushed with CO_2 until it turned orange-red. The medium was sterilised by millipore filtration. Sterility was checked by plating out 5 ml aliquots of medium on 10% blood agar and nutrient agar plates, which were then incubated at 37°C overnight and examined for bacterial growth.

Medium was made up from the stock solutions only as required, and used within 36 hours of preparation. It was stored at 4°C during sterility tests before use.

Tris - EDTA buffer

This buffer was prepared as required from two stock solutions:

Solution A 0.1 M Tris (Trishydroxymethyl aminomethane)

Solution B 0.01 M EDTA (Ethylene-diaminetetra-acetic acid, disodium salt)

To obtain a buffered solution of pH 8.2, 90 ml solution A was mixed with 10 ml solution B. This buffer was used in the preparation of coliphage ØX174, and in its storage.

Tetrazolium solution for the development of bacteriophage plaques

2:3:5 triphenyl tetrazolium chloride (B.D.H.)	0.1 g
Meat extract broth	100 ml

The tetrazolium was dissolved in the broth by shaking, and the solution stored at $4^{o}C$.

Mycobacterium tuberculosis

Heat-killed, lyophilsed cells of M. tuberculosis var. hominis strain C were supplied by the Central Veterinary Laboratories, Ministry of Agriculture, Fisheries and Food, Weybridge, Surrey.

Bacteriophage ØX174

The original suspension of coliphage ØX174 and its host organism E. coli strain C were kindly supplied by Dr. J. D. Pitts, Institute of Virology, University of Glasgow.

Maintenance of E. coli strain C

The organism was maintained throughout the series of experiments by fortnightly subculture on nutrient agar (Oxoid) slopes in Universal containers. Subcultures were kept at room temperature. Cultures used for experimental purposes were checked for purity before use by plating on nutrient agar plates, and single colonies used to inoculate cultures for test purposes.

Titration of coliphage ØX174

Coliphage ØX174 was titrated using the agar layer technique of Hershey (vide Adams, 1959). Tenfold dilutions of the bacteriophage stock ranging from 10^{-1} to 10^{-12} were made in 0.15 M saline. 0.5 ml of each bacteriophage dilution was added to 2 ml of a 4 hour culture of E. coli str C in nutrient broth, and mixed thoroughly. 0.5 ml of this bacteriophage-host cell mixture were then added to each of three tubes containing approximately 4 ml melted 0.6% nutrient agar

maintained at a temperature of 50°C, mixed thoroughly, and the entire mixture poured over the surface of a hardened layer of nutrient agar in a Petri dish. After the top layer of agar had solidified, plates were incubated at 37°C overnight, and any plaques then visible were counted after treatment as described below. The titre of the phage stock suspension was then calculated from the equation given below:

$$\text{Titre} = N \times 10^{n+1}$$

where N = average number of plaques for three plates at any one dilution

n = $\log_{10}$ dilution of phage stock.

Use of Tetrazolium to resolve bacteriophage plaques

Tetrazolium was used to facilitate the counting of bacteriophage plaques, as described by Pattee (1966). After bacteriophage plates had been incubated overnight to allow the development of plaques, the plates were flooded with 10 ml of a 0.1% solution of 2:3:5 triphenyl tetrazolium chloride (B.D.H.) in meat extract broth. The plates were then incubated for a further 20 minutes at 37°C, then the broth was carefully poured off. Each plaque was then visible as a sharply defined clear area against an intensely red background. This technique greatly facilitates the accurate counting of bacteriophage

plaques. The red background produced is due to precipitation of tetrazolium as a red, insoluble compound by viable E. coli cells. This is a reduction reaction.

Preparation of stock suspensions of coliphage ØX174

Coliphage ØX174 was prepared by the agar layer technique (Adams, 1959). Bacteriophage, in sufficient concentration to give barely confluent lysis, is added to a 4 hour culture of E. coli str C in nutrient broth. Aliquots of this mixture are added to molten sloppy agar at 50°C and mixed thoroughly. This mixture is then poured over the surface of a prepared agar plate, allowed to set and the plate incubated for 14-18 hours. To determine the concentration of bacteriophage necessary to give confluent lysis, the suspension of bacteriophage is first titrated by the method given above.

Sufficient coliphage ØX174 to cause confluent lysis was added to 30 ml of a 4 hour culture of E. coli str C in nutrient broth, grown at 37°C. 1 ml aliquots of this bacteriophage-bacterium mixture were added to approximately 4 ml 0.6% nutrient agar, and the whole mixture poured over the surface of prepared nutrient agar plates. After the sloppy agar layer had set, the plates were incubated for 14-18 hours.

After incubation, the plates were inspected, and any showing more than faint traces of bacterial growth were discarded. The remaining plates were quickly frozen in a -70°C deep freeze,

then allowed to thaw at 37°C. Any fluid extruded from the thawed plates was collected, centrifuged at 6000 r.p.m. for 20 minutes to remove debris, and stored at 4°C. The upper layer of sloppy agar was scraped off the surface of the thawed plates, and gently shaken in its own volume of peptone water for 30 minutes. This agar suspension was then centrifuged at 6000 r.p.m. to remove agar and bacterial debris, and the supernatant carefully withdrawn. The supernatant and the fluid collected from the plates after thawing were pooled.

This crude suspension of bacteriophage was brought to 40% saturation with ice-cold saturated ammonium sulphate solution, and allowed to stand at 4°C overnight. On standing, a precipitate formed which was harvested by centrifugation at 9000 r.p.m. for 45 minutes. The supernatant was discarded, and the precipitate resuspended in a small volume (5-10 ml) of Tris - EDTA buffer, pH 8.2. This suspension was allowed to stand at 4°C for 12 hours and was then centrifuged at 6000 r.p.m. to remove any remaining debris. The supernatant was collected, and further centrifuged 40,000 r.p.m. for 5 hours. Centrifugation at this speed sediments the bacteriophage as a dark brown pellet in the base of the centrifuge tube. The supernatant was discarded, and the pellets of bacteriophage suspended in 4-5 ml of Tris - EDTA buffer, pH 8.2 and stored at 4°C.

This method consistently yielded bacteriophage suspensions with a titre in the region of 10^{12} to 10^{13}

plaque forming units per ml.

Preparation of adjuvant mixtures and immunisation procedures.

Coliphage ØX174, prepared as described above, was suspended in saline at a concentration of 2×10^{90} plaque forming units (PFU) per ml. This suspension was incorporated in a water-in-oil emulsion made up in the following proportions: saline suspension of phage, 1 part: Arlacel A (mannide monooleata, Atlas Powder Co., Delaware, U.S.A.) 1 part: Bayol 55 (a paraffin oil, Esso Petroleum Co., Essex) 3 parts. This mixture was emulsified into a white opaque viscous fluid by vigorous shaking followed by further mixing using a syringe and a no. 1 hypodermic needle.

The second type of adjuvant mixture used was prepared exactly as above, with the addition of 0.2 mg heat-killed M. tuberculosis str C per animal to be injected. All animals injected with adjuvant mixtures received a total injection of volume of 0.5 ml.

Three groups of guinea-pigs were immunised as follows:

Group 1: Injected into the left hind footpad with 2×10^{9} PFU of coliphage ØX174 in 0.5 ml water-in-oil emulsion with added mycobacteria.

Group 2: Injected as above with 2×10^{9} PFU of coliphage ØX174 in water-in-oil emulsion (lacking Mycobacteria).

Group 3: Injected with 2×10^9 PFU of coliphage ØX174, in saline. Half of the animals studied in this group were injected subcutaneously in the left hind foot pad. The other half were injected intravenously by an ear vein.

Collection of guinea-pig sera.

Guinea-pig sera were obtained by one of two methods.

a) Animals were killed on the 8th or 21st day after injection of antigen, using a chloroform/air mixture. When a heart beat was no longer discernible, the skin was cut away from the rib cage, and the rib cage carefully removed. The heart was thus exposed, and excised over a Petri dish and the blood collected. Up to 20 ml of blood could be collected in this way.

b) In experiments involving two bleedings of the one animal, the first bleeding was made by cardiac puncture. The animal was anaesthetised with Nembutal (Pentobarbitone sodium), 40 mg per kg body weight, injected intraperitoneally. The anaesthetic took 20-30 minutes to take effect. The animal was then laid on its back, and blood drawn from the heart using a sterile 2 ml syringe and a no. 1 needle. The needle was inserted at a narrow angle through the rib cage until the beating of the heart could be felt. The plunger of the syringe was then gently withdrawn until 2 ml of blood had been

collected.

To obtain sera, blood samples ere left for 2 hours at 37°C then at 4°C overnight. Serum was then drawn off carefully using a Pasteur pipette and stored at -20°C.

Tissue culture of guinea-pig lymphoid tissues.

Guinea-pigs were killed on the 8th or 21st day after a single injection of antigen. Immediately after death, samples of spleen, bone marrow and lymph nodes were removed and placed in ice-cold Eagle's medium. The tissues were gently teased out to give small fragments: these were then washed thrice in ice-cold Eagle's medium by centrifugation in order to remove possible contamination by serum proteins.

Known weights of tissue were incubated at 37°C in a constant temperature water bath for 6 hours in 2 ml Eagle's medium with 10% added sheep serum in an atmosphere of 5% CO_2, 95% O_2. Gentle agitation was maintained throughout the period of incubation. At the end of this period of incubation, the tissue samples were disrupted by freezing and thawing thrice in a CO_2/ethanol mixture, and grinding with a sterile glass rod. The cell debris was removed by centrifugation at 12,000 r.p.m. for 30 minutes at a controlled temperature of 4°C. The supernatant was then carefully drawn off and assayed for activity against bacteriophage.

The bacteriophage neutralisation test.

Antibody activity against coliphage ØX174 was assayed by a phage neutralisation test based on that described by Uhr and Finkelstein (1963). A mixture of bacteriophage and guinea-pig anti-phage serum was allowed to react at 37°C and samples of the mixture, taken at different times, titrated for infective phage. Due to the large size of the plaques formed by coliphage ØX174, the maximum number of plaques that can be counted on a normal Petri dish is about 200 and the coliphage was therefore appropriately diluted before use.

The appropriate dilution of coliphage ØX174 was prepared and incubated at 37°C for 30 minutes. Then 0.1 ml of this phage dilution was added to 0.9 ml of the test serum or serum dilution which had also been pre-incubated at 37°C. 0.1 ml samples of this phage-antiserum mixture were withdrawn at given time intervals up to 30 minutes, and the sample immediately diluted in 9.9 ml ice-cold nutrient broth to stop the antigen antibody reaction. 0.5 ml aliquots of this dilution were then added to 2 ml of a 4 hour culture of E. coli str C and assayed for bacteriophage as described on page

It is not possible to obtain a sample for time 0 in this system. The concentration of bacteriophage present at time 0 was estimated by substituting 0.9 ml normal guinea-pig serum for the test serum. This also acted as a control for the effect of normal guinea-pig serum on the bacteriophage.

Calculation of guinea-pig serum antiphage antibody levels.

The neutralisation of bacteriophage by specific antibody follows first order kinetic as expressed by the equation shown in Fig. 6. Use of phage neutralisation test, therefore, gives rise to results expressed as inactivation constants (k). This k value, while an indirect measure of the antibody present, is characteristic for any given serum, and all serum antibody levels are given as k values.

Treatment of guinea-pig sera with 2-mercaptoethanol.

Treatment of guinea-pig sera with 2-mercaptoethanol was carried out as follows. One part of 2M 2-mercaptoethanol was added to 19 parts of serum to give a final concentration of 2-mercaptoethanol of 0.1M. The mixture of serum and 2-merceptoethanol was then incubated at 37°C overnight, and then dialysed against 0.02M iodoacetanide in phosphate buffered saline, pH 7.2, overnight. After dialysis, bacteriophage neutralisation tests were carried out on the treated serum, with similarly treated sera from non-immune animals as controls.

Fractionation of guinea-pig sera.

1. Diethyl amino ethyl cellulose chromatography

Fast (γ_1) and slow (γ_2) globulin fractions of guinea-pig sera were separated on DEAE cellulose (Whatman DE50) as described by White et al (1963). Separation was attained using a

FIGURE 6

Calculation of k values of guinea-pig anti-bacteriophage sera.

k values are calculated from the following equation:

$$k = \frac{2.3D}{t} \log_{10} \frac{N_o}{N}$$

where D = dilution of serum

t = time in minutes

N_o = phage assay at time 0

N = phage assay at time t

k is a velocity constant with the dimensions of minutes^{-1}.

(from Adams, 1959)

All k values in the series of experiments described have the dimensions minutes^{-1}.

stepwise elution technique.

4.5 gm DEAE were added to 250 ml 0.01M phosphate buffer, pH 7.5, and the DEAE allowed to settle. The supernatant was carefully poured off to remove the finest particles. This process was repeated twice. The slurry was then poured to form a column and the column equilibrated by passing through it 1.5 litres of 0.01M phosphate buffer overnight.

After equilibration of the column, 3 ml of guinea-pig serum, dialysed against the same buffer for 16 hours, was added to the column. The slow 7S fraction was eluted using 0.01M phosphate buffer, pH 7.5, the fast 7S fraction using 0.1M buffer and a crude 19S fraction using 0.15M buffer.

This method yielded a very pure $7S\gamma_2$ fraction, but the $7S\gamma_1$ fraction was always contaminated by $7S\gamma_2$. The crude 19S fraction obtained showed many other serum proteins.

Protein concentrations of the column eluate were estimated at a wavelength of 280 mu, using a Unicam spectrophotometer. Protein peaks were concentrated by dialysis against polyethylene glycol (Carbowax 20M, G.T. Gurr) to the original serum volume. Immunoelectrophoretic analysis of the fractions was carried out against rabbit anti-guinea-pig globulin, to check identity and purity.

2. Sephadex G-200 chromatography

Sephadex G-200 was used to separate the 19S and 7S immunoglobulin fractions of guinea-pig sera.

A slurry of 4 gm Sephadex G-200 in 0.15M NaCl was prepared and allowed to stand for 36-48 hours. The slurry was then poured to form a column of approximate dimensions 100 x 1 cm. 1 ml. of guinea-pig serum was then added to the column, and eluted using 0.15M NaCl. Fractions were collected using a Beaumaris fraction collector, and protein content estimated as above. Protein peaks were pooled and concentrated. Fractions thus obtained were checked by immunoelectrophoresis.

Elution from Sephadex usually took from 6-8 hours, and was generally carried out overnight.

The corneal test for delayed-type hypersensitivity.

The corneal test was used as a measure of delayed-type hypersensitivity and was carried out on the 19th day after immunisation.

2 drops of 2% cocaine hydrochloride were instilled into each eye of the guinea-pig to deaden the nerves, and the animal left for 2-4 minutes. A suspension of coliphage ØX174 was injected containing 10^{10} PFU was injected into the cornea of the left eye, and 10^{11} PFU per ml into the right eye, using a size 30 needle (Imperial standard wire gauge) on a tuberculin syringe. The tests were examined 24 and 48 hours later. Corneal reactions were scored as follows:

0 : no change other than a small area of opacity about the site of injection.

1 : slightly increased opacity extending beyond the site of injection.

2 : greater opacity covering a large area of the cornea.

3 : considerable thickening and grey opacity of the whole extent of the cornea, usually accompanied by chemosis.

Immunoelectrophoresis of guinea-pig sera.

a) Preparation of the electrophoresis plate

A rectangular dish was placed on the bench in a suitable site which was marked. Into this dish was poured rough 5% agar which was allowed to set in situ. The surface of this hard agar was subsequently used as a flat surface for the pouring of agar plates, provided that the same area of bench was always used. A clean sheet of glass (20 x 10 cm) was lightly siliconed on the upper surface, and excess silicone washed off with tap water. The sheet was dried and placed on the surface of the 5% agar in the dish. Two filter paper wicks (20 x 5 cm) were cut from Whatman no. 3 paper, placed parallel to the long edges of the glass plate so that they overlap it by 1 cm, and clipped in position.

Electrophoresis medium:

1. Barbitone buffer, 0.05M, pH 8.4

sodium barbitone 10.3 gm

barbitone 1.84 gm

distilled water to 1 litre

2. Ionagar

Ionagar no. 2 (Oxoid) 8.0 gm

merthiolate 0.1 ml

barbitone buffer to 1 litre

This agar was stored in approximately 100 ml amounts. One bottle of Ionagar prepared as above was melted and poured over the glass plate to give an agar layer of thickness 1-2 mm. The agar was allowed to set in situ and transferred to the refrigerator for use the following day.

b) Electrophoresis

The prepared plate was cut, with its wicks, from the agar. Troughs and wells were cut using a no. 1 cork borer, a ruler and a scalpel. The well has a diameter of 0.4 cm. It was separated from the troughs on either side by 0.4 cm of agar. Each trough was 0.4 cm wide. The plate was cut using a template drawn on graph paper.

The agar plate was then placed over the central compartment of a Shandon electrophoresis tank, and the filter paper wicks immersed in 0.05M barbitone buffer in the electrode compartments on either side. Guinea-pig sera were then placed in the wells, and a small amount of bromophenol blue added to 2 of the sera. This attaches itself to the serum albumin and forms a marker for the progress of protein separation. A

constant voltage of 150-200 volts was then applied to the plate. When the blue spot reached the edge of the plate, the current was switched off, and the troughs filled with the appropriate antisera. At this stage, the filter paper wicks were cut away. The plate was left in a damp chamber for 48 hours and examined for precipitin lines. The plate was then immersed in 0.15M saline for 24 hours, and washed in running tap water for 36-48 hours.

c) Staining of electrophoresis plates

The stain used was Chlorazol black, made up as follows:

Chlorazol black E (Azo black) G.T. Gurr	200 mg
Ethylene glycol	25 ml
Distilled water	75 ml

Dissolve the stain in ethylene glycol, add water and filter. The plate must be stained while still wet.

The plate is immersed in stain, and left for 1-2½ hours until the agar is black. The plate is then removed from the stain and decolourised by washing in running tap water for 24 hours. The plate was then preserved by drying at 37°C.

Preparation of rabbit anti-guinea-pig globulin sera for immunoelectrophoresis.

The globulin fraction of guinea-pig serum was precipitated by 40% saturation with ammonium sulphate. The precipitate was collected by centrifugation, redissolved in saline and dialysed

against 0.15M saline for 24 hours to remove any residual ammonium sulphate. The protein content of the solution thus obtained was then measured using a Unicam Spectrophotometer at a wavelength of 280 mu and adjusted to approximately 30 mg/ml by appropriate dilution.

This solution was then incorporated into a mycobacterial adjuvant mixture made up in the following proportions:

Antigen in saline solution with 1 mg added *M. tuberculosis*	1 part
Arlacel A	1 part
Bayol 55	3 parts

The antigen/adjuvant emulsion thus obtained was injected into a rabbit subcutaneously behind the ankle of both hind legs. 0.5 ml was injected into each leg. After 28 days, a similar dose of antigen dissolved in 1 ml saline was injected in divided doses as follows: 0.4 ml into one hind limb, 0.4 ml into the centralateral fore limb and 0.2 ml intravenously into an ear vein. Rabbits were bled from an ear vein 7-14 days after the second injection, in sufficient quantity to yield 15-20 ml serum after separation.

Preparation of pure rabbit antisera against guinea-pig 19S and 7S immunoglobulins.

Pure rabbit antisera were prepared by the method described by Goudie, Wilkinson and Horne (1967). The 19S immunoglobulin fraction of guinea-pig serum, obtained and purified by DEAE chromatography and gel filtration, was immunoelectrophoresed against impure rabbit anti-guinea-pig 19S. The precipitin arc thus obtained was carefully cut out, care being taken to avoid contamination with other unwanted precipitin arcs, and the excised piece of agar washed in saline for 48 hours. The agar was then broken up and suspended in 1 ml water-in-oil emulsion containing 1 mg heat-killed M. tuberculosis per animal to be injected.

Approximately 0.1 ml of this mixture was injected into the popliteal lymph nodes (both legs) of a rabbit. One month later a second injection was given, containing the same precipitin line emulsified in saline. This was injected as follows: 0.4 ml subcutaneously into one hind limb: 0.4 ml subcutaneously into the contralateral forelimb: 0.2 ml intravenously. Blood samples were obtained 10-12 days later, and examined immunoelectrophoretically. Cross reaction of this antiserum with 7S immunoglobulin, due to similar light chains, was removed by absorption with 25 ug guinea-pig $7S\gamma_2$ globulin.

Antiserum specific for guinea-pig 7S immunoglobulins were prepared similarly.

RESULTS

The kinetics of neutralisation of coliphage ØX174 by guinea-pig antisera.

The use of k values as a measure of serum levels of coliphage neutralising antibody is dependent on the logarithmic neutralisation of the bacteriophage by the antiserum. To show that this held true for guinea-pig sera the following experiments were carried out. Sera from three guinea-pigs immunised 21 days previously with a single intravenous dose of 2×10^9 PFU of coliphage ØX174 were tested in two ways. First, the sera were diluted 1:10 in saline, and a full bacteriophage neutralisation test carried out to demonstrate that neutralisation of coliphage was logarithmic with respect to time. Second, the sera were diluted 1:10, 1:100 and 1:1000 in saline, and coliphage neutralisation allowed to take place over a fixed period of time. The k values thus obtained for the different dilutions of one serum were then calculated and compared.

The results obtained are shown in Plate 1 and Table 1. As can be seen from Plate 1, neutralisation curves of the three sera tested are straight lines if infective phage remaining is plotted against time on a semi-log scale, showing that the neutralisation reaction progresses logarithmically with time. Further, the k values obtained for the three dilutions of individual sera are equivalent, within a 5% error, showing that the k value is independent of dilution.

These results show that the k value of a serum bears

a constant relationship to the level of coliphage neutralising antibody. It was concluded that such k values could reliably be used as a means of compering the levels of coliphage neutralising antibody in different sera.

The effect of adjuvants on serum antibody levels against coliphage ØX174 in guinea-pigs on the eighth day after a single injection.

Antibody levels in sera taken post mortem or by cardiac puncture were determined using the bacteriophage neutralisation test, and the results expressed as k values. The following groups of animals were studied:

1. guinea-pigs injected via the footpad with coliphage ØX174 in water-in-oil emulsion with added mycobacteria. The dose of mycobacteria used was 0.2 mg per animal.
2. guinea-pigs injected via the footpad with coliphage ØX174 in water-in-oil emulsion without added mycobacteria.
3. guinea-pigs injected via the footpad with coliphage ØX174 in saline.
4. guinea-pigs injected intravenously with coliphage ØX174 in saline.

The immunising dose of coliphage ØX174 was 2×10^9 PFU in all groups of animals. The k values for the sera from these animals are shown in Table 2.

Animals in all four groups consistently showed

TABLE 1

k values at different dilutions of guinea-pig sera taken 21 days after a single injection of bacteriophage ØX174 in saline.

Serum no.	*k value at dilution 1:10	1:100	1:1000
1.	0.45	0.439	0.457
2.	0.37	0.371	0.385
3.	0.39	0.385	0.40.

*k has the dimensions of minutes^{-1}

PLATE 1.

Neutralisation curves for sera from three guinea-pigs 21 days after intravenous immunisation with 2×10^9 PFU coliphage ØX174.

All sera were diluted 1:10 in 0.15M saline.

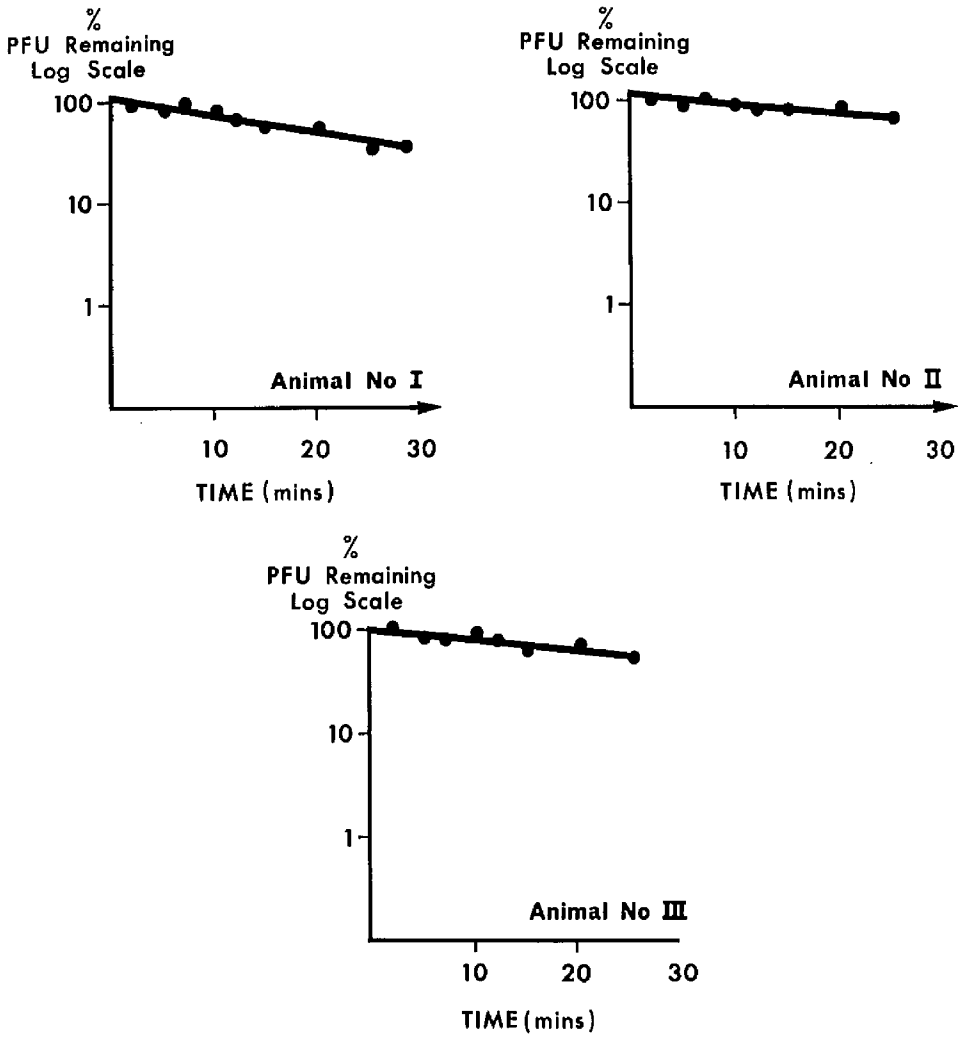

%
PFU Remaining
Log Scale
100
10
1
Animal No I
10
20
30
TIME (mins)
%
PFU Remaining
Log Scale
100
10
1
Animal No II
10
20
30
TIME (mins)
%
PFU Remaining
Log Scale
100
10
1
Animal No III
10
20
30
TIME (mins)

TABLE 2

*k values for guinea-pig sera taken 8 days after immunisation with coliphage ØX174 in adjuvant mixtures and in saline.

k			
W/O emulsion + mycobacteria	W/O emulsion	Saline (i/v)	Saline via footpad
0.04	0.03	0.01	
0.28	0.143	0.111	
0.33	0.035	0.09	
0.076	0.153		0.11
0.16	0.77		0.105
0.23	0.101		0.135
0.105	0.46	0.114	
0.135	0.081	0.291	
0.17	0.337	0.162	
0.29	0.095		0.079
0.65	0.16		0.393
0.337	0.021		0.131
0.205	0.447		
0.012	0.201		
Arithmetic mean values			
0.214	0.217	0.129	0.159

*k is the first order neutralisation constant for the reaction between coliphage and specific antiserum, and has the dimensions of minutes^{-1}.

detectable levels of serum antibody at 8 days. From 14 animals injected with coliphage in water-in-oil emulsion, the range of *k* values obtained was 0.021 to 0.77, with an arithmetic mean of 0.217. The range of *k* values from animals injected with coliphage in water-in-oil emulsion with added mycobacteria was 0.012 to 0.65 with an arithmetic mean of 0.214. The range of results from animals injected with coliphage in saline intravenously and *via* the footpad was 0.01 to 0.291, mean 0.129 and 0.079 to 0.393, mean 0.159 respectively. These results were obtained from groups of 6 animals.

It appears from these results that the incorporation of coliphage in water-in-oil emulsion before injection results in higher serum antibody levels than does injection of antigen in saline by the same route, or intravenously. However, the addition of mycobacteria to water-in-oil emulsions of coliphage does not have any effect on serum levels of coliphage neutralising antibody in comparison with antibody levels in sera from animals injected with coliphage in simple water-in-oil emulsion.

It was therefore concluded that water-in-oil emulsions exert a slight potentiating effect on antibody production as measured by serum levels of neutralising antibody on the eighth day after antigen injection.

The effect of 2-mercaptoethanol treatment on the neutralising antibody levels of sera taken 8 days after a single injection of coliphage.

Treatment of sera with 0.1M 2-mercaptoethanol, a reducing agent, is known to abolish almost entirely the activity of 19S antibody. In order to determine the immunoglobulin responsible for the coliphage neutralising activity in sera from the groups of animals described above, selected sera from each group were treated with 2-mercaptoethanol, the k value for each serum being determined before and after treatment.

The results obtained in this investigation are shown in Table 3. Coliphage neutralising activity in all sera dropped to negligible levels, as measured by k values, after treatment with mercaptoethanol. No difference in sensitivity of antibody to mercaptoethanol was observed in sera from animals injected with coliphage in water-in-oil emulsion with or without added mycobacteria, or in saline.

It can therefore be accepted that with each of the methods of antigen administration used, the predominant class of antibody present in the serum 8 days later is macroglobulin (19S) antibody.

TABLE 3

k values of guinea-pig 8 day antiphage sera before and after treatment with 0.1M 2-mercaptoethanol.

		*k		
		W/O emulsion + mycobacteria	W/O emulsion	Saline
1.	Before	0.29	0.3	0.11
	After	0.009	0.009	0.001
2.	Before	0.33	0.77	0.135
	After	0.005	0.021	0.003
3.	Before	0.23	0.101	1.11
	After	0.007	0	0.005
4.	Before	0.117	0.08	-
	After	0.0042	0.007	-

*k has the dimensions of minutes^{-1}.

Distribution of coliphage neutralising antibody in 19S and 7S immunoglobulin fractions obtained by Sephadex G-200 filtration of guinea-pig sera.

Molecules of appreciably different molecular weight, such as 7S and 19S immunoglobulins can be effectively separated from each other by filtration on Sephadex G-200. In order to confirm the results obtained from treatment of 8 day sera with 2-mercaptoethanol, sera from animals injected with coliphage in water-in-oil emulsion with and without added mycobacteria, and in saline, were fractionated on Sephadex G-200 using 0.15M saline as eluent. The elution pattern for such a fractionation is shown in Plate 2.

Three protein peaks were obtained, as judged by the absorbency of the eluate on a U-V spectrophotometer. The volume of eluate containing these peaks was concentrated to the original serum volume, and the identity of the protein present determined by immunoelectrophoresis. The first protein peak was thus shown to contain the 19S immunoglobulin fraction and the second peak to contain the 7S immunoglobulin fraction. These fractions were tested for neutralising activity against coliphage ØX174 by the bacteriophage neutralisation test. The results of immunoelectrophoresis of the protein fractions are shown in Plate 3, and of the bacteriophage neutralisation tests in Table 4. In all cases, the greater part of the antibody activity present was detected in the fraction shown to contain the 19S

PLATE 2.

The elution pattern obtained by chromatography of guinea-pig serum on Sephadex G-200.

The fractions designated 1-5 were collected, and each fraction concentrated to the original volume of serum using Carbowax 20M.

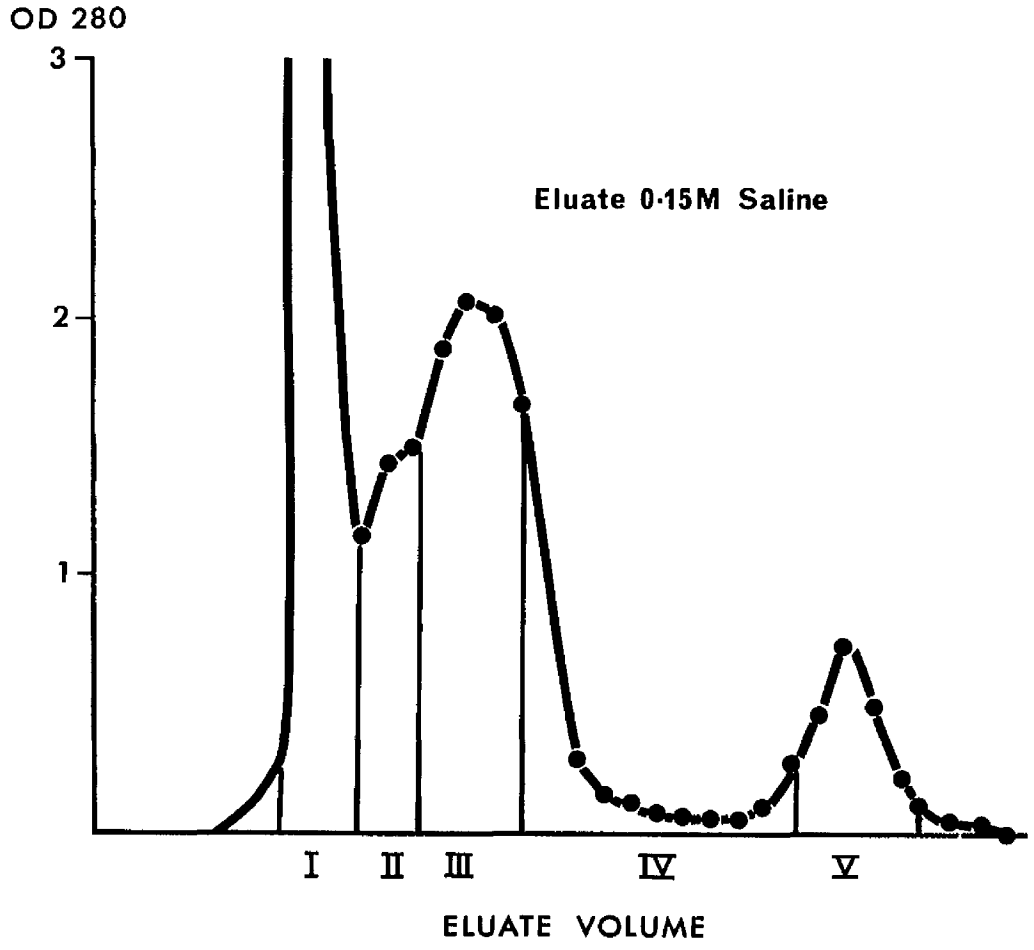

OD 280
3
2
1
Eluate 0·15M Saline
I
II
III
IV
V
ELUATE VOLUME

PLATE 3.

Immunoelectrophoresis of the fractions of guinea-pig serum obtained by chromatography on Sephadex G-200.

Wells :- a) Guinea-pig whole serum.

b) Sephadex fraction 1.

c) Sephadex fraction 2.

d) Sephadex fraction 4.

e) Sephadex fraction 3.

f) Sephadex fraction 5.

Troughs - all troughs contained rabbit anti-guinea-pig globulin.

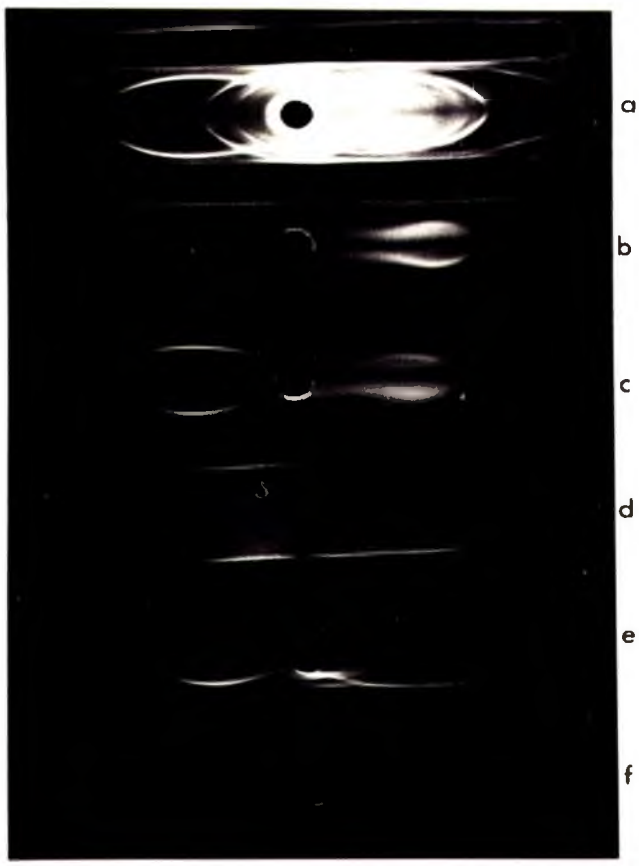

a
b
c
d
e
f

TABLE 4

Distribution of coliphage neutralising antibody in 19S and 7S immunoglobulin fractions of guinea-pig sera taken 8 days after injection of coliphage ØX174 in adjuvant mixtures or saline.

	Antibody (k x 10)	
	19S ex Sephadex G-200	7S ex Sephadex G-200
W/O emulsion with	0.105	0.001
added mycobacteria	0.093	0
W/O emulsion	0.051	0.005
W/O emulsion	0.16	0.011
Saline	0.04	0.003
Saline	0.075	0.006

k has the dimensions of minutes^{-1}.

immunoglobulins by immunoelectrophoresis. These results confirm those obtained from the treatment of sera with 2-mercaptoethanol, showing that eight days after injection of coliphage ØX174 in adjuvants and in saline, the antibody present in the serum is predominantly of the 19S immunoglobulin class.

The effect of adjuvants on serum antibody levels against coliphage ØX174 on the 21st day following a single injection.

Coliphage neutralising antibody levels in sera obtained post mortem from guinea-pigs killed on the 21st day after a single injection of antigen in adjuvant mixtures or in saline were determined using the bacteriophage neutralisation test. The results were expressed as k values, and are shown in Table 5. The groups of animals studied were as described above.

The injection of coliphage in water-in-oil emulsion produced antibody levels with k values in the range 0.53 to 1.82, arithmetic mean, 0.94. Addition of mycobacteria to the water-in-oil emulsion of antigen resulted in antibody levels with k values in the range 0.306 to 3.406, arithmetic mean 2.11. The mean results in these two groups are the mean for 12 animals in each case.

Injection of coliphage in saline resulted in antibody levels with k values in the range 0.16 to 0.356, mean 0.227 in six animals injected via the footpad, and 0.279 to 0.56, mean 0.398 in six animals injected intravenously.

TABLE 5

*k values for guinea-pig sera taken 21 days after immunisation with coliphage ØX174 in adjuvant mixtures and in saline.

k			
W/O emulsion + mycobacteria	W/O emulsion	Saline (i/v)	Saline via footpad
2.506	1.21		0.16
0.306	1.17		0.205
2.300	0.81	0.45	
2.910	0.89	0.37	
2.070	0.53	0.39	
1.810	0.805		0.23
2.931	1.82		0.356
3.406	0.95	0.34	
1.290	0.735	0.56	
1.537	0.615	0.28	
2.212	0.81		0.219
2.009	0.92		0.193
Arithmetic mean values			
2.11	0.94	0.277	0.227

*k has the dimensions of minutes^{-1}.

These results show that the use of water-in-oil emulsions of antigen results in much higher neutralising antibody levels in the serum on the 21st day after a single injection than does injection of antigen in saline either intravenously or *via* the footpad. Further, the addition of mycobacteria to water-in-oil emulsions of antigen induced the highest antibody levels obtained, with a mean *k* value over twice as great as that for the group of animals injected with antigen in simple water-in-oil emulsion, and five times greater than the mean *k* value obtained for animals injected with coliphage in saline.

It was concluded from these results that the inclusion of coliphage in a water-in-oil emulsion prior to injection results in a definite increase in antibody levels in the serum, 21 days after injection, compared with serum antibody levels in animals injected with coliphage in saline. Further, addition of mycobacteria to the injection mixture results in even greater levels of serum antibody at this time.

Distribution of antibody in $7S_{\gamma 1}$ and $7S_{\gamma 2}$ immunoglobulin fractions obtained by DEAE cellulose chromatography of guinea-pig sera taken 21 days after a single injection of coliphage.

Antibody activity in guinea-pig sera is confined to 3 immunoglobulin classes, $7S_{\gamma 1}$, $7S_{\gamma 2}$ and 19S. These classes can be separated by chromatography on DEAE-cellulose, an ion

exchange resin, due to their different molecular charge. In order to determine in which of these fractions the major part of coliphage neutralising antibody was to be found 21 days after a single injection of antigen in adjuvants or in saline, sera from such animals were fractionated on DEAE cellulose columns.

Fractionation of guinea-pig sera was carried out using a stepwise elution technique to separate the $7S\gamma_1$ and $7S\gamma_2$ immunoglobulin fractions. The elution pattern of guinea-pig serum from DEAE cellulose, using 3 elution steps of 0.01M and 0.15M phosphate buffer, pH 6.5, is shown in Plate 4. Immunoelectrophoretic analysis showed that the first protein peak obtained contained only $7S\gamma_2$ immunoglobulin. The second peak (eluted with 0.1M buffer) contained mainly $7S\gamma_1$ immunoglobulin along with other electrophoretically 'fast' proteins, but this fraction always showed the presence of a certain amount of $7S\gamma_2$ immunoglobulin. The third peak, eluted with 0.15M buffer, was found to contain the 19S immunoglobulin fraction. The results of immunoelectrophoresis of these fractions are shown in Plate 5.

The $7S\gamma_1$, $7S\gamma_2$ and 19S immunoglobulin fractions thus obtained were tested for coliphage neutralising activity using the bacteriophage neutralising test. The results, expressed as $\underline{k}$ values are shown in Table 6. Both 7S fractions showed coliphage neutralising activity in all sera studied, whether the sera were from animals injected with coliphage in adjuvant mixtures or in

PLATE 4.

The elution pattern obtained by stepwise elution of guinea-pig serum from DEAE-cellulose, using phosphate buffers of increasing molar strengthh, and at a constant pH of 6.5.

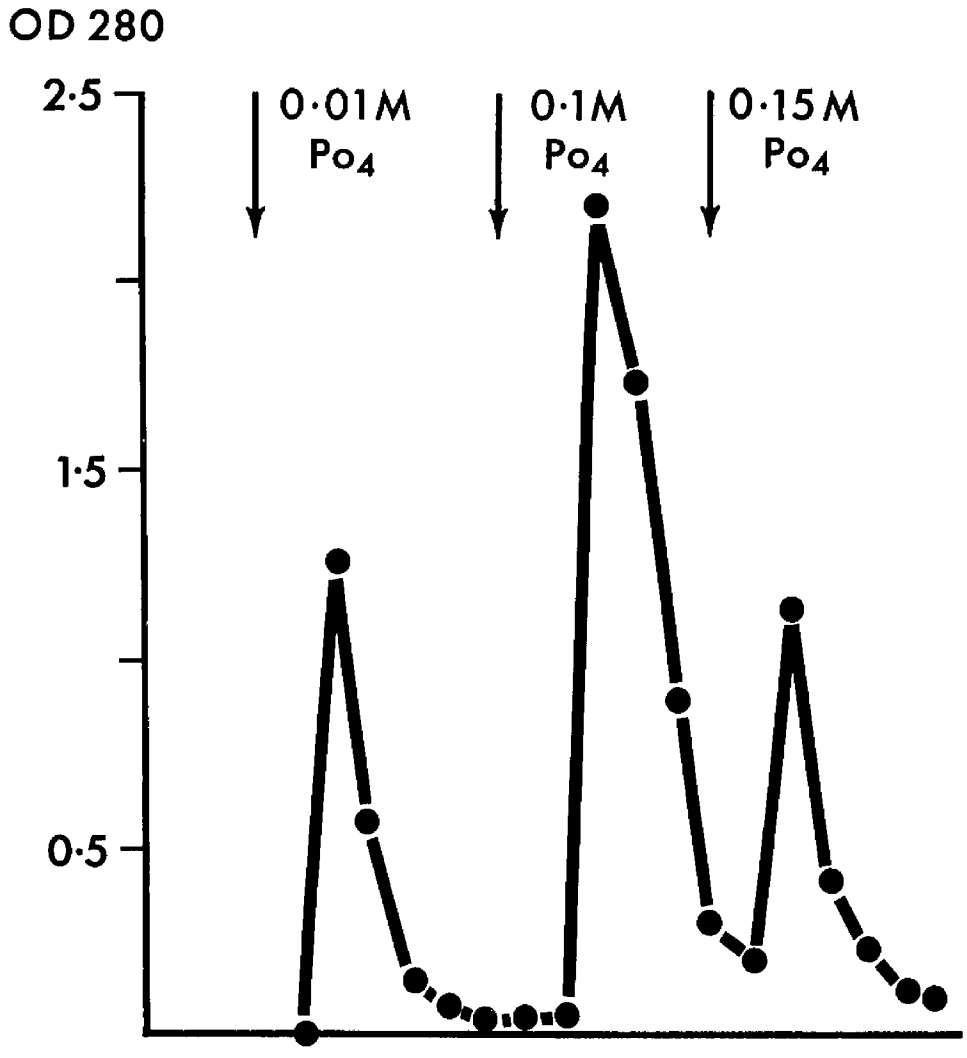

OD 280
2·5
1·5
0·5
0·01M
Po4
0·1M
Po4
0·15M
Po4

PLATE 5.

Immunoelectrophoresis of guinea-pig 7Sγ_1, 7Sγ_2 and 19S immunoglobulin fractions obtained by chromatography on DEAE-cellulose.

Wells :- 1. 7Sγ_2 fraction eluted with 0.01M phosphate buffer, pH 6.5

2. Guinea-pig whole serum.

3. 19S fraction eluted with 0.15M phosphate buffer, pH 6.5

4. 7Sγ_1 fraction eluted with 0.1M phosphate buffer, pH 6.5

Troughs :- All troughs contained rabbit anti-guinea-pig globulin.


1
2
3
4

TABLE 6

Antiphage activity of 19S and 7S fractions obtained by DEAE cellulose chromatography of 21 day guinea-pig sera.

Antigen injected in	k value of fraction		
	$^{7S}\gamma_2$	$^{7S}\gamma_1$	19S
W/O + mycobacteria	0.23	0.115	0.69
W/O + mycobacteria	0.32	0.215	0.19
W/O + mycobacteria	0.197	0.142	0.087
W/O	0.014	0.207	0.115
W/O	0.023	0.115	0.059
W/O	0.09	0.043	0.216
Saline	0.029	0.097	0.022
Saline	0.019	0.101	trace
Saline	0.036	0.14	0.003

k has the dimensions of minutes^{-1}.

saline. The activity of the $7S_{\gamma 1}$ immunoglobulin fraction was similar in sera from animals injected with antigen in water-in-oil emulsion with and without added mycobacteria. The activity of this fraction in animals injected with antigen in saline was less than that where water-in-oil emulsions were used.

The activity of the $7S_{\gamma 2}$ fractions of sera from animals injected with antigen in saline and antigen in water-in-oil emulsion, and no difference was evident between them. The activity of the $7S_{\gamma 2}$ fraction of sera from animals injected with coliphage in water-in-oil emulsion with added mycobacteria were high, and 5-10 times greater than the same fractions of sera from animals injected with antigen in water-in-oil emulsion or saline.

The crude 19S immunoglobulin fraction obtained by DEAE cellulose chromatography of these sera was also tested for the presence of coliphage neutralising antibody. Such fractions from animals injected with antigen in water-in-oil emulsion with and without added mycobacteria were found to have high phage neutralising activity in comparison with similar fractions from sera from animals injected with coliphage in saline.

It was concluded from these results that the use of water-in-oil emulsions of antigen with added mucobacteria selectively increases the production of antibody of the $7S_{\gamma 2}$ type. The incorporation of antigen in water-in-oil emulsion with or without added mycobacteria results in increased antibody of the

$7S_{\gamma 1}$ and 19S immunoglobulin classes in comparison with similar fractions from animals injected with coliphage in saline, in sera taken 21 days after a single injection of antigen.

Immunoelectrophoretic investigations on serum immunoglobulin fractions obtained by DEAE cellulose chromatography.

In view of the results obtained above, it was decided to check the immunoglobulin fractions tested by immunoelectrophoresis against a rabbit antiserum known to contain antibody specific for guinea-pig $7S_{\gamma 1}$, $7S_{\gamma 2}$ and 19S immunoglobulins. The results obtained are shown in Plate 6. As can be seen, immunoelectrophoresis of the $7S_{\gamma 2}$ immunoglobulin fraction *ex* DEAE resulted in a single precipitin line in the fast region, showing that such fractions were pure. The $7S_{\gamma 1}$ fraction, however, showed quite clearly the presence of two precipitin lines, one of which corresponded to that obtained for $7S_{\gamma 2}$ fractions. The $7S_{\gamma 1}$ fraction obtained by DEAE chromatography was therefore contaminated with $7S_{\gamma 2}$ immunoglobulin. Immunoelectrophoresis of the 19S immunoglobulin fraction *ex* DEAE showed the presence of only one precipitin line, in the position expected for 19S immunoglobulin, and no precipitin lines were apparent in the 7S region.

These results remove the possibility that the antibody activity observed in 19S immunoglobulin fractions *ex* DEAE reported above might be due to contamination with other immunoglobulin fractions. It was therefore concluded that the

PLATE 6

Immunoelectrophoresis of guinea-pig 7Sγ_1, 7Sγ_2 and 19S immunoglobulin fractions ex DEAE-cellulose against a rabbit antiserum containing precipitating antibody against these three components of guinea-pig serum only.

Wells ;-

a) 7Sγ_2 ex DEAE.

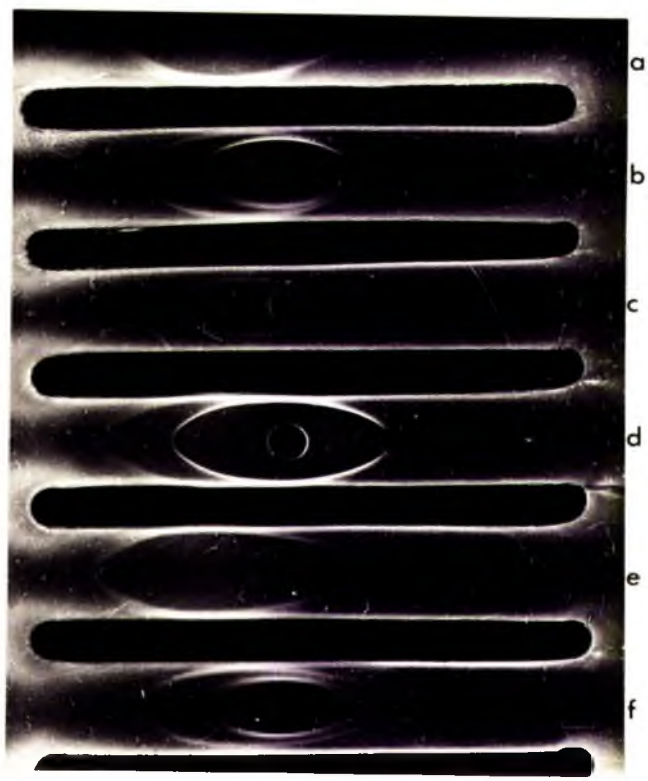
b) 7Sγ_1 ex DEAE.

c) 19S ex DEAE.

d) 7Sγ_1 ex DEAE.

e) 7Sγ_2 ex DEAE.

f) whole guinea-pig serum.


a
b
c
d
e
f

use of water-in-oil emulsions of antigen results in the persistence of 19S antibody production up to 21 days after a single injection of antigen.

The effect of 2-mercaptoethanol treatment on the neutralising antibody levels in sera taken 21 days after a single injection of coliphage in adjuvants and in saline.

In view of the above results showing that injection of coliphage in water-in-oil emulsion resulted in persistence of 19S antibody production, three sera from each of the groups injected with coliphage in water-in-oil emulsion with and without added mycobacteria and in saline were treated with mercaptoethanol. Levels of coliphage neutralising antibody present in the sera before and after treatment were measured using the phage neutralisation test, and the results expressed as k values (see Table 7).

As can be seen, sera from animals injected with coliphage in saline were least sensitive to treatment with mercaptoethanol, and in one case, the k value increased after treatment. The reason for this is not known. Sera from animals injected with coliphage in water-in-oil emulsions with or without added mycobacteria were somewhat more susceptible to treatment with mercaptoethanol, suggesting that such sera contained a higher proportion of mercaptoethanol sensitive antibody than did sera from animals injected with antigen in saline.

TABLE 7

The effect of 0.1M 2-mercaptoethanol on coliphage neutralising antibody in guinea-pig sera taken 21 days after immunisation with coliphage in adjuvants or in saline, as judged by k values determined before and after treatment.

		k		
		W/O emulsion + mycobacteria	W/O emulsion	Saline
1.	Before	2.506	0.81	0.45
	After	2.13	0.79	0.43
2.	Before	2.931	0.53	0.23
	After	1.712	0.32	0.39
3.	Before	1.29	1.82	0.279
	After	1.31	1.51	0.201

k has the dimensions of minutes^{-1}.

Taken along with the results obtained from DEAE fractionation of similar sera, these observations supply supporting evidence for the persistence of 19S antibody production in animals injected with antigen in water-in-oil emulsions.

Delayed hypersensitivity to coliphage ØX174 after a single injection of antigen in adjuvant mixtures and in saline.

Delayed hypersensitivity to coliphage ØX174 was detected using the corneal test. A bleb of a suspension of coliphage ØX174 containing 10^{11} PFU/ml was injected into the cornea of the test guinea-pigs, and the eyes examined 24 and 48 hours after injection. The results are shown in Table 8. Of 11 animals injected with antigen in water-in-oil emulsion with added mycobacteria, 8 showed a delayed hypersensitive reaction to the antigen. In animals injected with antigen in water-in-oil emulsion, 7 out of 11 showed a delayed reaction. In 11 animals injected with antigen in saline, 1 gave a definite delayed response, and 2 gave reactions which were doubtful positives. There was no distinct difference in the frequency of the intensity of the reactions induced by injection of antigen in water-in-oil emulsion with and without added mycobacteria.

It was concluded that delayed hypersensitivity to coliphage was more likely to arise after injection of antigen in water-in-oil emulsion, but that the addition of mycobacteria

TABLE 8

Results of corneal tests done 19 days after injection of guinea-pig with coliphage ØX174 in adjuvant mixtures and saline, and read at 24 and 48 hours after test.

W/O emulsion + mycobacteria		W/O emulsion		Saline	
24 hr	48 hr	24 hr	48 hr	24 hr	48 hr
1	2	0	0	0	1
3	3	½	1	½	0
½	1	1	2½	0	½
0	1½	0	0	0	0
0	2	1	3	½	0
1	0	½	1	0	0
0	3	0	0	0	0
½	0	0	1	0	0
0	1	1	1	½	0
1	1½	2	3	0	0
0	0	½	0	0	½

Total no. of animals in each group giving a positive reaction at 48 hours.

8/11	7/11	3/11

to such emulsions had no additional effect.

In vitro studies of the synthesis of coliphage neutralising antibody by lymphoid tissues of the guinea-pig after injection of coliphage ØX174 in adjuvants and saline.

Studies on the sera of guinea-pigs injected with coliphage ØX174 showed that although mycobacterial adjuvants induced a marked increase in 7S immunoglobulin antibody against coliphage, no such increase occurred early in the immune response, when 19S antibody was predominant in the circulation. The experiments described below were designed to determine whether the effect of mycobacterial adjuvants on serum antibody levels could be explained on the basis that the different lymphoid organs of the guinea-pig were not of the same importance in the synthesis of 19S and 7S immunoglobulins.

Kinetics of neutralisation of coliphage ØX174 by supernatants of lymphoid tissue cultures from immunised animals.

To determine whether neutralisation of coliphage by supernatants from tissue cultures of lymphoid tissues from immunised guinea-pigs followed first order kinetics, the following experiment was carried out. A guinea-pig was injected with 2×10^9 PFU of coliphage ØX174 intravenously and killed 21 days later. Spleen, bone marrow and lymph node tissue were removed and cultured *in vitro* for 6 hours. The tissues

PLATE 7.

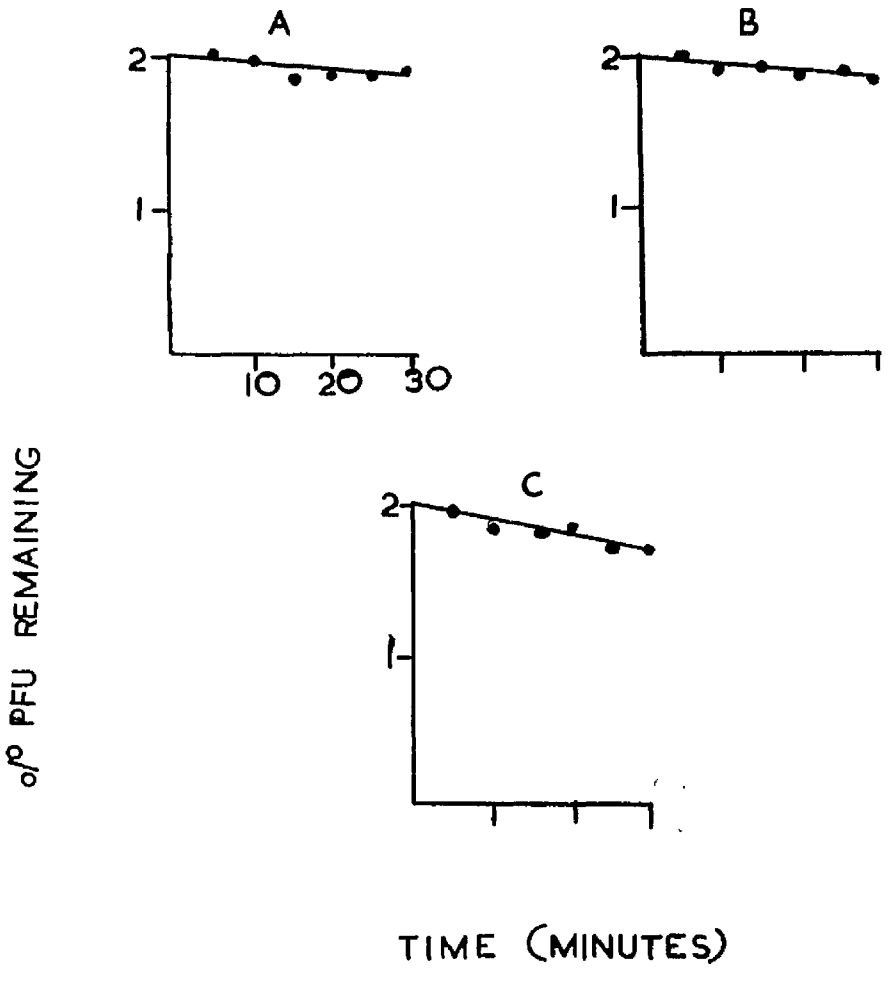
Neutralisation curves for the neutralisation of coliphage ØX174 by the supernatants from 6 hour cultures of lymphoid tissues removed from a guinea-pig 21 days after the injection of 2×10^9 PFU coliphage ØX174 in saline into the left hind footpad.

A - tibial bone marrow

B - spleen

C - homolateral iliac lymph nodes.

% PFU remaining is plotted on a log. scale, where 2 represents 100%, and 1, 10%.


A
B
C
2
1
10
20
30
% PFU REMAINING
TIME (MINUTES)

were then disrupted by freezing and thawing, cell debris spun off and the supernatants collected.

A bacteriophage neutralisation test was carried out on the supernatants from each tissue and the results are shown in Plate 7. As can be seen, when infective coliphage remaining was plotted on a logarithmic scale against time, straight line curves were obtained in the case of each of the tissues studied. These results show that neutralisation of coliphage by culture supernatants from immune tissues follow first order kinetics as does neutralisation of coliphage by specific antisera.

Synthesis of coliphage neutralising antibody in vitro by various lymphoid organs of the guinea-pig 8 days after injection of coliphage in adjuvants and in saline.

Spleen tissue, lymph node tissue from the iliac nodes homolateral and contralateral to the site of footpad injection and homolateral tibial bone marrow were removed from guinea-pig killed on the eighth day after the injection of antigen, and cultured for six hours in vitro (see Materials and Methods). The following groups of animals were studied:

1. guinea-pigs injected via the footpad with antigen in water-in-oil emulsion with added mycobacteria (0.2 mg per animal).
2. guinea-pigs injected via the footpad with antigen in water-in-oil emulsion without added mycobacteria.

3. guinea-pigs injected *via* the footpad with antigen in saline.

4. guinea-pigs injected intravenously with antigen in saline.

After culture, the tissues were disrupted by freezing and thawing, cell debris spun off and the coliphage neutralising activity of the supernatant determined using the bacteriophage neutralisation test. In this system, the time for the phage neutralisation test was fixed at 30 minutes. To 0.9 ml tissue culture supernatant was added 0.1 ml of a suspension of coliphage ØX174 containing 10^6 PFU. The number of infective phage particles remaining in this mixture was determined after 30 minutes and the synthetic activity of the different tissues compared, using the number of phage particles neutralised by the culture supernatant per unit weight (0.1 gm) of tissue incubated as a basis for comparison. The controls for the phage neutralisation test in this system were 0.9 ml aliquots of sterile tissue culture medium.

The results obtained in the study of lymphoid tissues removed 8 days after a single injection of antigen are shown in Table 9. Coliphage neutralising antibody was consistently detected in the tissue culture supernatants from the tissues studied in all groups of animals. In comparing the synthetic activity of lymphoid tissues from any one animal, bone marrow cultures were found to give the levels of coliphage neutralisation

TABLE 9

Bacteriophage neutralising activity on a unit weight basis for lymphoid tissues cultured in vitro from guinea-pigs 8 days after injection of coliphage ØX174.

A. Animals injected with antigen in water-in-oil emulsion with added mycobacteria.

*in vitro activity / unit weight of tissue

Animal	Spleen	Homolateral iliac lymph node	Contralateral iliac lymph node	Bone Marrow
1.	3.4	2.7	4.7	16.0
2.	5.1	4.0	6.7	24.0
3.	2.5	7.3	-	13.8
4.	4.9	1.2	6.2	21.9
5.	2.8	-	1.7	18.1
6.	2.7	12.1	3.5	12.9

*activity = no. of PFU coliphage ØX174 neutralised x 10^{-3}/0.1 g tissue incubated.

TABLE 9 (cont.)

B. Animals injected with antigen in water-in-oil emulsion.

Animal	*in vitro* activity / unit weight of tissue			
	Spleen	Homolateral iliac lymph node	Contralateral iliac lymph node	Bone Marrow
1.	1.3	1.9	4.8	12.5
2.	2.7	1.6	5.8	18.7
3.	1.6	3.4	0.9	19.3
4.	3.8	8.3	6.7	16.5
5.	2.6	7.9	2.1	11.1
6.	6.9	4.3	-	21.1

TABLE 9 (cont.)

C. Animals injected with antigen in saline.

in vitro activity / unit weight of tissue

Animal	Spleen	Homolateral iliac lymph node	Contralateral iliac lymph node	Bone Marrow
1.	1.5	5.8	7.5	10.4
2.	0.9	4.0	3.1	9.2
3.	3.1	2.3	2.1	12.3
4.	1.2	4.5	0.8	15.6
5.	1.5	1.6	3.7	11.7
6.	2.8	2.4	6.3	8.3

Animals 1-3 received antigen subcutaneously in the footpad.

Animals 4-6 received antigen intravenously through an ear vein.

per unit weight of tissue incubated. This occurred in all groups of animals studied, irrespective of the injection mixture. The range of activity in bone marrow cultures was 8.3×10^3 to 24.0×10^3. In some cases, in animals injected with coliphage in water-in-oil emulsions with or without added mycobacteria, the iliac lymph nodes homolateral to the site of footpad injection showed a degree of activity approaching that of the bone marrow of the same animal, but in no case exceeding it. There was considerable variation between the activity of the iliac lymph nodes homolateral and contralateral to the site of footpad injection. In some animals the homolateral nodes showed the highest activity than the contralateral nodes: in other animals the opposite was the case. The activity of the spleen cultures was usually low, but in some cases exceeded that of the lymph nodes on a unit weight basis.

The mean values of activity obtained for each tissue relative to the activity of the homolateral lymph node in each animal are shown in Table 10. As can be seen, the distribution of activity in the groups of animals studied is essentially similar, although the bone marrow in animals injected with mycobacterial adjuvant could be considered to be more active in comparison with the bone marrow from other groups. Plate 8 shows the mean results obtained from the group of animals injected with coliphage in mycobacterial adjuvant.

PLATE 8.

Diagrammatic representation of the mean synthesis of coliphage neutralising antibody by lymphoid tissues *in vitro* from 6 guinea-pigs immunised 8 days previously with coliphage ØX174 in mycobacterial adjuvant.

The arrows indicate the range of activity observed.

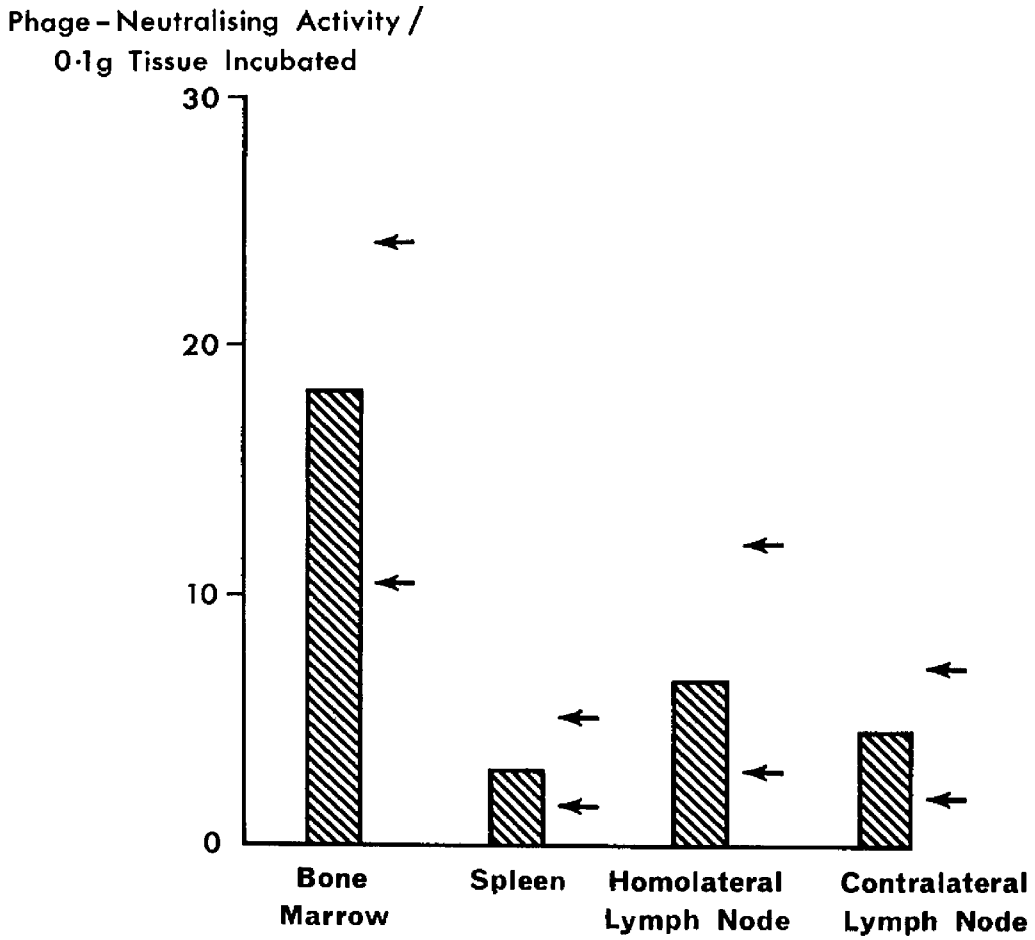

Phage - Neutralising Activity / 0·1g Tissue Incubated
30
20
10
0
Bone Marrow
Spleen
Homolateral Lymph Node
Contralateral Lymph Node

TABLE 10

Mean distribution of activity in the synthesis of coliphage neutralising antibody of various lymphoid tissues, relative to the homolateral iliac nodes (100%) from guinea-pigs immunised 8 days previously with coliphage in adjuvants or saline.

Group	Mean synthesis / 0.1 g tissue			
	Homolateral iliac node	Contralateral iliac node	Spleen	Bone Marrow
W/O emulsion + mycobacteria	100	198	146	727
W/O emulsion	100	148	87	537
saline (i/v)	100	169	77	475
saline (footpad)	100	99	62	315

Synthesis of coliphage neutralising antibody in vitro by various lymphoid organs of the guinea-pig 21 days after injection of coliphage in adjuvants and in saline.

Spleen, iliac lymph nodes homolateral and contralateral to the site of the footpad injection and homolateral tibial bone marrow from three groups of 6 animals each were studied. The groups of animals were injected with coliphage in water-in-oil emulsion with added mycobacteria, without added mycobacteria and with coliphage in saline respectively. In all animals the site of injection was the left hind footpad. Tissue culture and antibody estimations were carried out in the manner described above. The results are shown in Table 11.

In animals injected with coliphage in water-in-oil emulsion with added mycobacteria, the highest coliphage neutralising activity per unit weight of tissue was detected in the lymph nodes. The iliac lymph nodes contralateral to the site of antigen injection generally showed higher activity than the homolateral nodes in any one animal. The activity of spleen cultures was low on a unit weight basis. Bone marrow cultures from this group of animals showed variable coliphage neutralising activity. In two of the animals studied, bone marrow showed activity higher than that of the homolateral iliac nodes, but much less than that of the contralateral nodes. In two animals the activity of the bone marrow greatly exceeded that of the contralateral iliac lymph nodes and in the remaining two animals,

TABLE 11

Bacteriophage neutralising activity of lymphoid tissues cultured in vitro from guinea-pigs 21 days after injection of coliphage ØX174.

A. Animals injected with antigen in water-in-oil emulsion with added mycobacteria.

	*in vitro activity / unit weight of tissue					
Animal	Spleen	Homolateral iliac lymph node	Contralateral iliac lymph node	Bone marrow	Homolateral popliteal node	Cervical nodes
1.	3.3	4.8	11.6	5.3	-	-
2.	1.8	3.8	8.65	17.4	-	-
3.	0.7	2.1	9.2	18.1	-	-
4.	2.6	3.2	24.1	4.5	-	-
5.	4.3	7.2	15.1	6.4	4.9	1.4
6.	2.4	11.3	11.8	3.9	7.2	3.7

* Activity = no. of PFU coliphage ØX174 neutralised x 10^{-3}/0.1 g tissue incubated.

TABLE 11 (cont.)

B. Animals injected with antigen in water-in-oil emulsion.

Animal	*in vitro* activity / unit weight of tissue					
	Spleen	Homolateral iliac lymph node	Contralateral iliac lymph node	Bone marrow	Homolateral popliteal node	Cervical nodes
1.	2.9	6.6	10.9	4.5	-	-
2.	1.53	8.2	6.83	4.8	-	-
3.	1.7	15.2	7.9	13.6	-	-
4.	5.1	4.3	8.2	7.95	-	-
5.	0.8	2.7	12.1	3.4	10.5	1.8
6.	0.2	10.8	9.4	6.5	6.1	1.3

the activity of the bone marrow lay between that of the homolateral lymph nodes and the spleen. These observations were made on a unit weight basis.

Tissues from animals injected with antigen in water-in-oil emulsion without added mycobacteria showed a similar distribution of activity among the lymphoid tissues. In these animals, the activity of the contralateral lymph nodes was nearer to that of the homolateral iliac nodes than was the case in animals injected with mycobacterial adjuvant. The activity of spleen cultures was again low on a unit weight basis. Bone marrow activity was again found to be variable.

In animals injected with coliphage in saline *via* the footpad the results obtained show that the activity of the homolateral iliac lymph nodes was slightly greater than that found in the contralateral nodes, on a unit weight basis. Further, the activity of bone marrow cultures from these animals was consistently much lower than the activity of the lymph nodes studied, on a unit weight basis. Neutralising antibody production by spleen cultures was lower than that detected in cultures of lymph nodes and similar to that of bone marrow cultures on a unit weight basis.

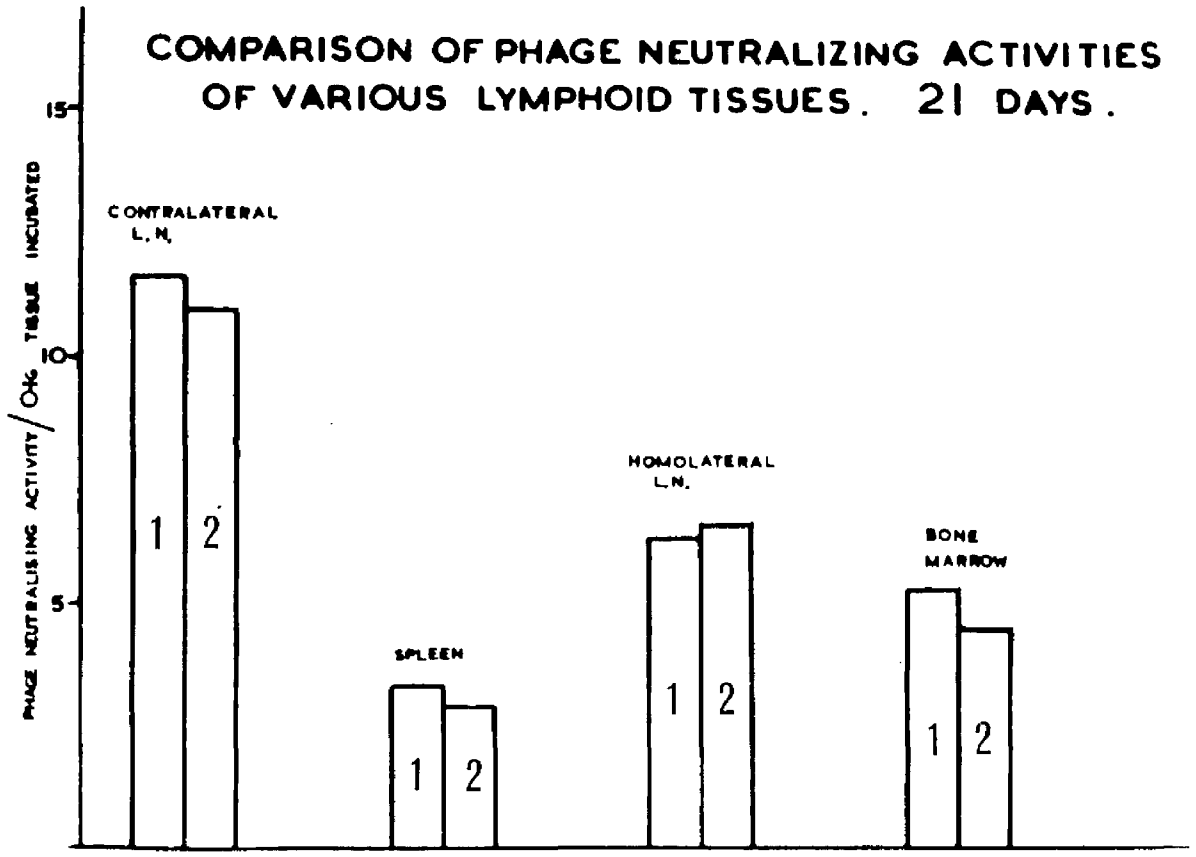
It was concluded from these results that, at a time when the predominant circulating antibody is of the 7S immunoglobulin type, the lymphoid organs most active in the synthesis of coliphage neutralising antibody on a unit weight basis, are

PLATE 9.

Diagrammatic representation of the synthesis of coliphage neutralising antibody by lymphoid tissues from two guinea-pigs immunised 21 days previously with

1 - coliphage ØX174 in mycobacterial adjuvant.

2 - coliphage ØX174 in water-in-oil emulsion.


COMPARISON OF PHAGE NEUTRALIZING ACTIVITIES OF VARIOUS LYMPHOID TISSUES. 21 DAYS.
PHAGE NEUTRALISING ACTIVITY / 0.1 c.c. TISSUE INCUBATED
15
10
5
CONTRALATERAL L.N.
1
2
SPLEEN
1
2
HOMOLATERAL L.N.
1
2
BONE MARROW
1
2

the lymph nodes close to the site of antigen injection. The synthetic activity of bone marrow cultures at this time is much less than at a time when the predominant circulating antibody is of the 19S immunoglobulin class. Synthetic activity in spleen cultures was consistently lower than in cultures of the other tissues studied. These results hold true whether antigen was injected in water-in-oil emulsion with or without added mycobacteria or in saline. Plate 9 shows the results obtained from two animals injected with coliphage in mycobacterial adjuvant.

Comparison of activity in the synthesis of coliphage neutralising antibody of in vitro cultures of bone marrow from different sites taken 8 days after injection of antigen.

In the studies on lymphoid tissues removed 8 days after injection of antigen, bone marrow from the tibia, homolateral to the site of antigen injection, consistently showed the highest synthesis of coliphage neutralising antibody. In order to determine whether this activity was due to the proximity of this bone to the site of antigen injection, the following experiments were carried out. Tibial bone marrow from the tibiae homolateral and contralateral to the site of footpad injection of coliphage in water-in-oil emulsion with added mycobacteria from two animals was cultured in vitro and the coliphage neutralising antibody present in the tissue culture supernatants determined. From

another animal, similarly immunised, bone marrow from the tibiae homolateral and contralateral to the sites of footpad injection, and from the homolateral humerus, was similarly studied. The results are shown in Table 12 and Plate 10.

As can be seen the activity of bone marrow from the tibiae homolateral and contralateral to the site of footpad injection is very similar. In one animal, the activity of the homolateral tibial marrow exceeded that of the contralateral, on a unit weight basis, by a factor of 7.1%, while in the other, the activity of the contralateral exceeded that of the homolateral by 5.3%. In the case of bone marrow from the humerus, the activity of such tissue on a unit weight basis, was 9% less than that observed in the homolateral tibial marrow of the same animal.

Attempts to examine sternal bone marrow from immunised guinea-pigs were unsuccessful due to the relatively small amount of marrow present in the sternum, and the technical difficulties in exposing it. Three cultures of sternal bone marrow attempted showed gross bacterial contamination, the only time in this study that such contamination was observed.

It was concluded from these results that the high activity of bone marrow cultures from the homolateral tibiae described above, 8 days after the injection of antigen, was not restricted to this bone alone. Further, such activity is probably representative of the total body bone marrow, and certainly of the bone marrow in the long bones.

PLATE 10.

In vitro synthesis of coliphage neutralising antibody by tibial bone marrow, homolateral and contralateral to the site of footpad injection, removed 8 days after injection of coliphage in mycobacterial adjuvant.

ANTIBODY ACTIVITY IN CULTURES OF HOMOLATERAL AND CONTRALATERAL TIBIAL MARROW. 8 DAYS.

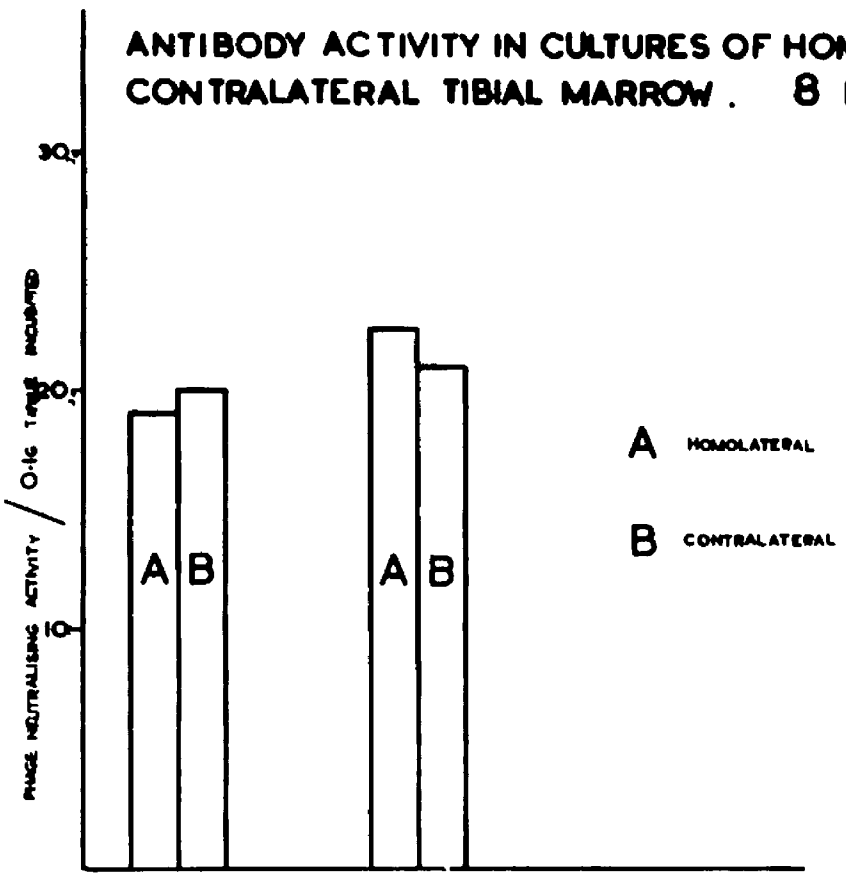


TABLE 12

Synthesis of coliphage neutralising antibody in vitro by bone marrow taken from different sites 8 days after antigen injection.

Animal	*Synthetic activity / 0.1 g bone marrow		
	Homolateral tibial	Contralateral tibial	Homolateral humerus
1.	19	20	-
2.	22.5	21	-
3.	17.3	-	15.8

*activity = no. of PFU coliphage ØX174 neutralised x 10^{-3}/0.1 g tissue incubated.

Synthesis of coliphage neutralising antibody in in vitro cultures of cervical and popliteal lymph nodes from animals immunised 21 days previously.

To determine the activity of lymph nodes closer to, and further from the site of antigen injection, in the synthesis of coliphage neutralising antibody, the following tissues were studied. In two out of each group of six animals, the lymphoid tissues of which were studied 21 days after immunisation, homolateral popliteal and cervical lymph nodes were studied. The results are given in Table 11. These nodes showed synthesis of antibody in all animals studied, and the popliteal node was found to be more active than the cervical nodes in all cases. In the case of animals injected with coliphage in saline, the activity of the popliteal node was higher than in any of the other nodes studied. In animals injected with antigen in water-in-oil emulsion with added mycobacteria, the activity of the popliteal node was less than that of either the homolateral or contralateral iliac nodes, and in animals injected with antigen in water-in-oil emulsion, the popliteal node from one animal showed lower activity than the homolateral iliac node, and in the other animal the position was reversed.

It was concluded from these results that the activity of lymph nodes in the synthesis of coliphage neutralising antibody was dependent on the distance of the node from the site of antigen injection.

Demonstration of the net synthesis of antibody for coliphage ØX174 by lymphoid tissues cultured in vitro.

The following experiments were carried out to determine whether antibody with antiphage activity could have been present in the lymphoid tissues before *in vitro* incubation, or was necessarily the product of active synthesis during the period of tissue culture. Samples of spleen, lymph node tissue and bone marrow were removed from two animals 8 days after a single injection of coliphage ØX174 in water-in-oil emulsion with added mycobacteria. Each tissue sample was divided into two approximately equal parts, and both washed in ice-cold tissue culture medium. One portion was then deep-frozen in a solid CO_2-ethanol bath and stored at -20°C. The other portion was cultured *in vitro* for six hours. After the period of incubation both portions of tissue were disrupted by freezing and thawing, and the supernatants examined for coliphage neutralising antibody. Similar experiments were carried out on tissues taken from two animals 21 days after immunisation with coliphage in water-in-oil emulsion with added mycobacteria.

In the case of tissues removed from animals 8 days after antigen injection (Table 13, Plate 11) the results show that approximately 25% of the coliphage neutralising antibody per unit weight of tissue detected after incubation *in vitro* was extractable from the tissues before incubation. In tissues removed 21 days after antigen injection (Table 14) similar results were obtained.

TABLE 13

Net synthesis of antiphage antibody in tissues removed from guinea-pigs 8 days after injection with coliphage ØX174.

Tissue	*Activity / 0.1 g tissue before incubation	after incubation	Net synthesis
Spleen	0.7	2.8	300%
	0.55	1.9	240%
Homolateral	1.7	6.1	259%
lymph node	2.4	8.1	248%
Contralateral	1.9	6.4	240%
lymph node	1.2	3.9	240%
Bone marrow	7	33	370%
	12	50	317%

*activity = no. of PFU coliphage ØX174 neutralised x 10^{-3}/0.1 g tissue incubated.

PLATE 11.

Coliphage neutralising antibody extractable per unit weight of tissue before and after *in vitro* incubation in lymphoid tissues removed from a guinea-pig 8 days after injection of coliphage ØX174 in mycobacterial adjuvant.

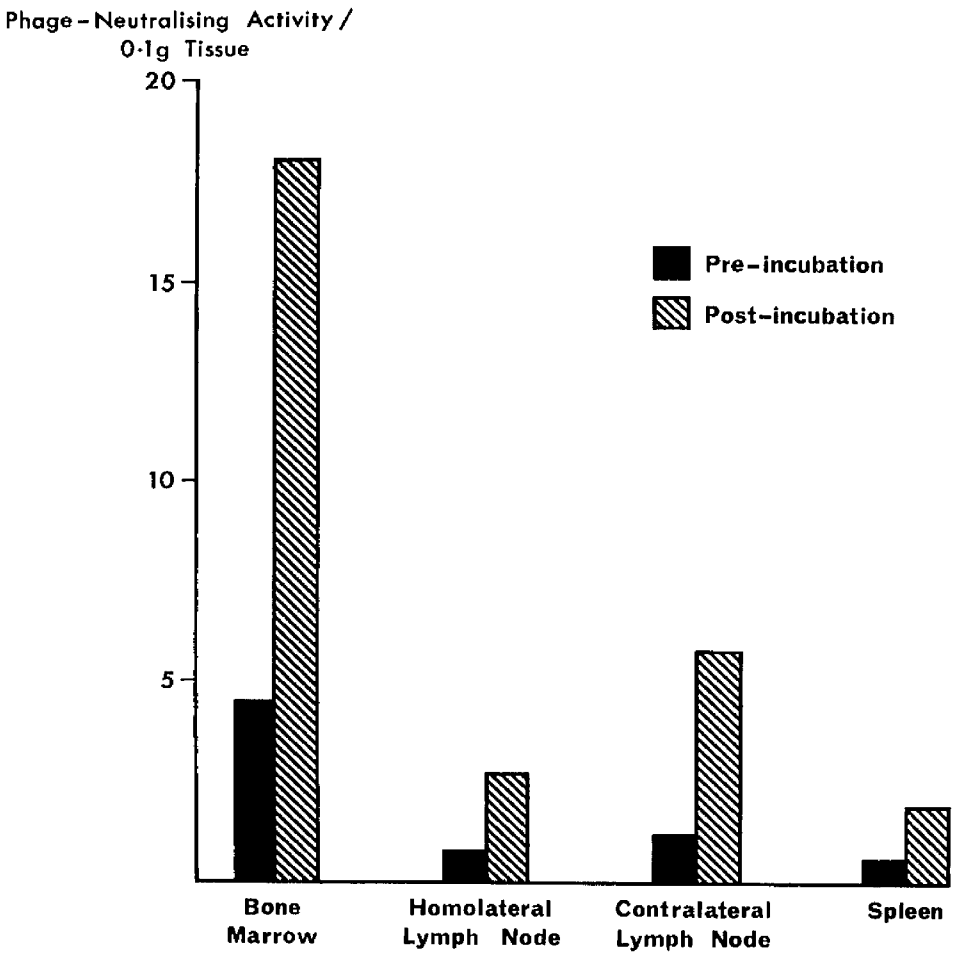

Phage-Neutralising Activity / 0·1g Tissue
20
15
10
5
Pre-incubation
Post-incubation
Bone Marrow
Homolateral Lymph Node
Contralateral Lymph Node
Spleen

TABLE 14

Net synthesis of antiphage antibody in lymphoid tissues removed from guinea-pigs 21 days after immunisation with coliphage ØX174.

Tissue	*Activity / 0.1 g tissue before incubation	after incubation	Net synthesis
Spleen	0.58	3.2	450%
	0	0	0
Homolateral iliac lymph node	1.80	9.6	433%
	0.37	1.8	387%
Contralateral iliac lymph node	0.91	3.1	240%
	1.7	8.7	411%
Bone marrow	1.4	6.3	350%
	2.1	8.9	327%

*activity = no. of PFU coliphage ØX174 neutralised x 10^{-3}/0.1 g tissue incubated.

The net synthesis which occurred in these tissues was slightly greater than that observed for tissues studied at 8 days. In one animal, coliphage neutralising antibody was not detected in spleen tissue before or after incubation. In view of the fact that other lymphoid tissues from this animal produced neutralising antibody, this result must have been due to technical failure.

As well as demonstrating that net synthesis of antibody had occurred during the period of incubation in vitro, the results of extraction studies supplied additional evidence to support the distribution of activity in the synthesis of coliphage neutralising antibody in lymphoid tissues 8 and 21 days after immunisation. From tissues studied 8 days after antigen injection, the greatest amount of antibody extractable from non-incubated tissues on a unit weight basis was in the bone marrow. Similarly, the greatest quantity of antibody extractable from lymphoid tissues 21 days after immunisation was in the lymph nodes.

The effect of Puromycin on the synthesis of coliphage neutralising by lymphoid tissues in vitro.

Puromycin is an antimetabolite known to inhibit the formation of protein by cells in vitro. In order to check the conclusion derived from the experiments described above, puromycin was incorporated in cultures of lymphoid tissues from immunised guinea-pigs to determine whether or not this drug would prevent

the synthesis of coliphage neutralising antibody.

Tibial bone marrow from a guinea-pig immunised 8 days previously with coliphage ØX174 in water-in-oil emulsion with added mycobacteria was divided into 4 portions. These portions were then incubated <u>in vitro</u> in the presence of puromycin at different molar concentrations as follows:

Sample 1 - tissue culture medium

Sample 2 - 3×10^{-4}M puromycin in tissue culture medium

Sample 3 - 3×10^{-5}M puromycin in tissue culture medium

Sample 4 - 3×10^{-6}M puromycin in tissue culture medium

After incubation, the neutralising antibody content of the supernatants was determined on a unit weight basis, and the reduction in activity caused by the presence of puromycin expressed as a percentage of the activity of the control culture (Sample 1). Similar experiments were carried out on contralateral iliac lymph nodes removed from three animals injected with coliphage as above 21 days previously.

The results are shown in Plate 12 (8 day) and Plate 13 (21 day). A concentration of puromycin of 3×10^{-4}M puromycin reduced the antibody detected after incubation by 80% compared to the antibody detected in cultures of the same bone marrow not exposed to puromycin. Little difference was observed in the effect of 3×10^{-5} and 3×10^{-6}M puromycin, the reduction observed approaching 50% in both cases.

Lymph node tissues taken 21 days after the injection of antigen showed a 70% reduction in antibody production when

PLATE 12.

The effect of different molar concentrations of puromycin on the synthesis of coliphage neutralising antibody in tissue culture by tibial bone marrow from guinea-pigs injected 8 days previously with coliphage ØX174 in mycobacterial adjuvant *via* the left hind footpad.

Synthesis per unit weight of tissue incubated is expressed as a percentage of the unit weight synthesis by bone marrow incubated in the absence of puromycin.

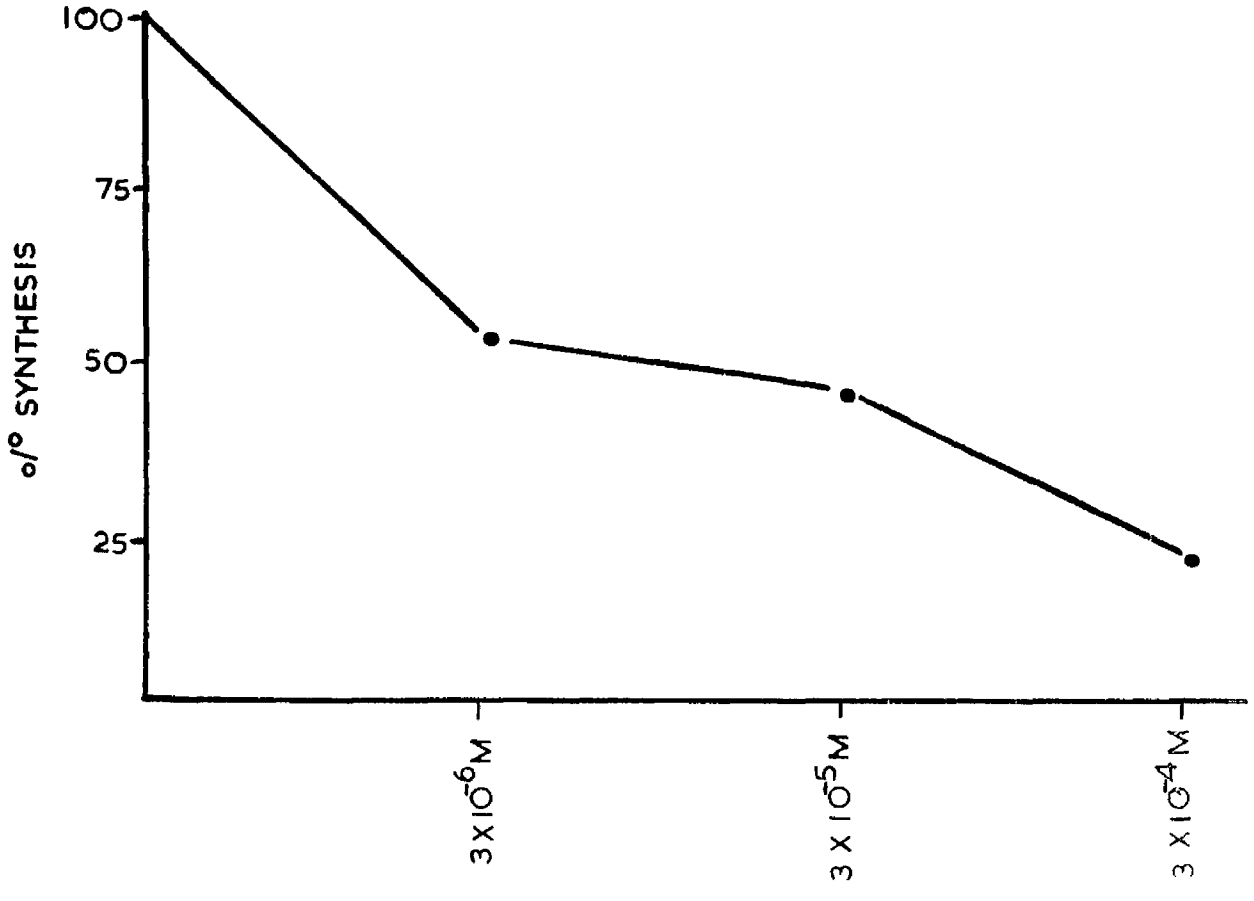
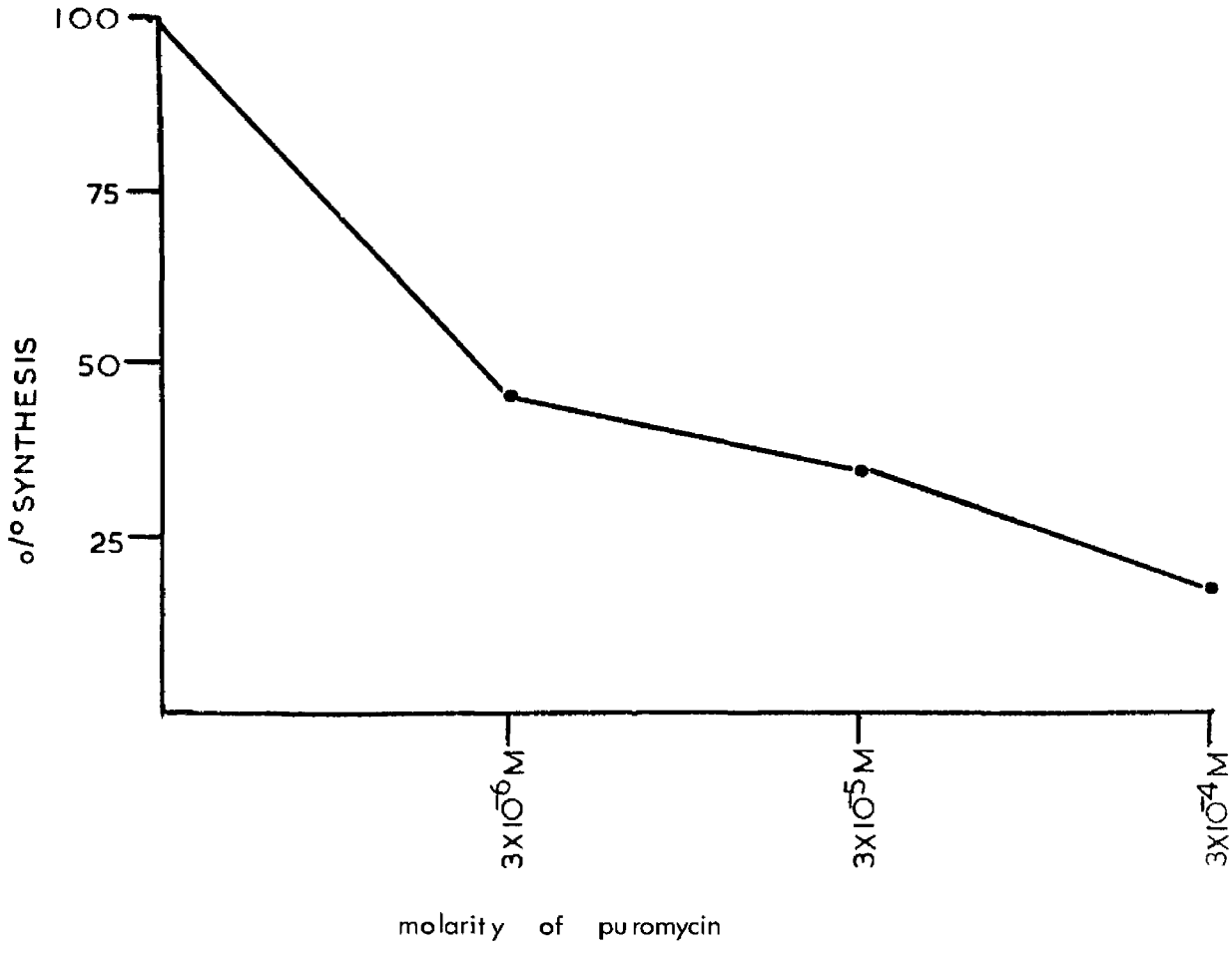

100
75
50
25
% SYNTHESIS
3 X 10⁻⁶ M
3 X 10⁻⁵ M
3 X 10⁻⁴ M
molarity of puromycin

PLATE 13.

The effect of different molar concentrations of puromycin on the synthesis of coliphage neutralising antibody in tissue culture by the contralateral iliac lymph nodes from guinea-pigs injected 21 days previously with coliphage ØX174 in mycobacterial adjuvant *via* the left hind footpad.

Synthesis per unit weight of tissue incubated is expressed as a percentage of the unit weight synthesis by lymph node tissue incubated in the absence of puromycin.


100
75
50
25
% SYNTHESIS
3X10⁻⁶M
3X10⁻⁵M
3X10⁻⁴M
molarity of puromycin

cultured in the presence of 3×10^{-5}M puromycin. A concentration of 3×10^{-4}M puromycin caused an 83% reduction in antibody production.

These results confirm that net synthesis of antibody was occurring in the tissues studied under the experimental conditions used.

Investigation of lymphoid tissues from normal guinea-pigs for the production of coliphage neutralising antibody in vitro.

It is possible that the activity of lymphoid tissue culture supernatants in the neutralisation of coliphage might be due to some non-specific action. To remove this possibility tibial bone marrow, iliac lymph nodes and spleen from non-immunised guinea-pigs were cultured in vitro and the culture supernatants examined for the presence of coliphage neutralising antibody.

No neutralisation of coliphage was detected in the supernatants of cultures of lymphoid tissues from 3 normal non-immunised animals.

Neutralisation of coliphage ØX174 by lymphoid tissue culture supernatants from animals immunised with staphylococcal phage 80.

Two guinea-pigs were immunised with staphylococcal phage 80 in water-in-oil emulsion with added mycobacteria and one animal killed 8 days later, the other 21 days later. The sera of these animals contained antibody against the staphylococcal

phage as judged by the inhibition of plaque formation on a lawn plate of sensitive Staphylococcus sp. Investigation of bone marrow, spleen and lymph node tissue from these animals for the production of antibody against coliphage ØX174 produced uniformly negative results. In view of the small amount of supernatant available, only qualitative tests were carried out for activity of these culture supernatants against phage 80. These showed that the supernatants of the lymphoid tissue cultures contained antibody against the injected staphylococcal phage by inhibition of plaque formation on a lawn plate of Staphylococcus sp.

Since coliphage neutralising activity could not be detected in lymphoid tissue cultures from non-immunised guinea-pigs, or guinea-pigs immunised with staphylococcal phage, it was concluded that the neutralisation of coliphage observed in culture supernatants of lymphoid tissues from animals immunised with that bacteriophage was due to the presence of specific antibody in these supernatants.

Experiments to determine the immunoglobulin type of the antibody formed by lymphoid tissues of the guinea-pig in vitro 8 and 21 days after immunisation with coliphage ØX174.

The predominant coliphage neutralising antibodies present in the sera of immunised guinea-pigs 8 and 21 days after antigen injection are of the 19S and 7S immunoglobulin class respectively. To show that these were the immunoglobulin

antibodies synthesised by lymphoid tissues from immunised guinea-pigs at these times, the following experiments were carried out.

a) Treatment of 8 day lymphoid tissue culture supernatants with 2-mercaptoethanol.

The activity of tissue culture supernatants from lymphoid tissues removed from guinea-pigs 8 days after immunisation with coliphage ØX174 was measured before and after treatment with 2-mercaptoethanol. The results are shown in Table 15.

Treatment with 2-mercaptoethanol markedly reduced the ability of the culture supernatants studied to neutralise coliphage ØX174, the reduction in activity in all cases bar one being in excess of 95%. Controls of mercaptoethanol treated sterile culture medium were used.

These results suggest that the antibody produced by these tissues in vitro is of the 19S immunoglobulin class.

b) The effect of rabbit antisera specific for either guinea-pig 19S or 7S immunoglobulin on coliphage neutralisation by lymphoid tissue culture supernatants.

Rabbit antisera against guinea-pig 19S or 7S immunoglobulins were prepared as described in 'Materials and Methods'. Such antisera showed precipitation only with the immunoglobulin class against which they were directed on immunoelectrophoresis (Plate 14).

Culture supernatants of lymphoid tissues from two animals injected 8 and 21 days previously with coliphage ØX174 in

TABLE 15

The effect of 2-mercaptoethanol treatment on the phage neutralising activity of tissue culture supernatants from guinea-pigs taken 8 days after immunisation.

Animal	Tissue	Activity / unit weight Before 2ME	After 2ME
A	Spleen	2.8	0.15
	Homolateral lymph node	-	-
	Contralateral lymph node	1.7	0.1
	Bone marrow	18.1	1.9
B	Spleen	4.3	0.3
	Homolateral lymph node	6.9	1.0
	Contralateral lymph node	-	-
	Bone marrow	21.1	1.5
C	Spleen	2.1	0.05
	Bone marrow	15.9	4.0

Animals A, B and C all received antigen in water-in-oil emulsion with added mycobacteria.

water-in-oil emulsion with added mycobacteria were treated with rabbit anti-guinea-pig 7S and rabbit anti-guinea-pig 19S immunoglobulin, after determination of the antibody content of the supernatants. To 0.5 ml of culture supernatant was added an equal volume of a 1:10 dilution of specific antiserum. This mixture was incubated at 37°C for 1 hour, and the coliphage neutralising activity then estimated again. The results obtained after treatment of tissue culture supernatants with rabbit anti-guinea-pig 19S and 7S immunoglobulins are shown in Table 16.

In the case of tissues removed 8 days after antigen injection, the use of a specific anti-guinea-pig 19S serum caused considerable reduction of the coliphage neutralising ability of the culture supernatant. The use of an anti-7S serum, however had comparatively little effect. In contrast, in supernatants of lymphoid tissues removed 21 days after antigen injection, the use of anti-7S immunoglobulin serum markedly reduced the coliphage neutralising ability of the culture supernatants, while anti-19S serum had very little effect, except in the case of bone marrow.

It was concluded from these results that lymphoid tissues removed from immunised guinea-pigs 8 and 21 days after injection of antigen, when the predominant circulating antibodies are of the 19S and 7S immunoglobulin classes respectively, synthesise under the conditions of <u>in vitro</u> culture antibody of the same immunoglobulin class as is present in the serum.

PLATE 14.

Demonstration of the specificity of rabbit anti-guinea-pig 19S and rabbit anti-guinea-pig 7S immunoglobulin sera by immunoelectrophoresis.

Wells :- all wells contained guinea-pig whole serum.

Troughs :- 1. Rabbit anti-guinea-pig whole serum.

2. Rabbit anti-guinea-pig 19S immunoglobulin.

3. Rabbit anti-guinea-pig 7S immunoglobulin.

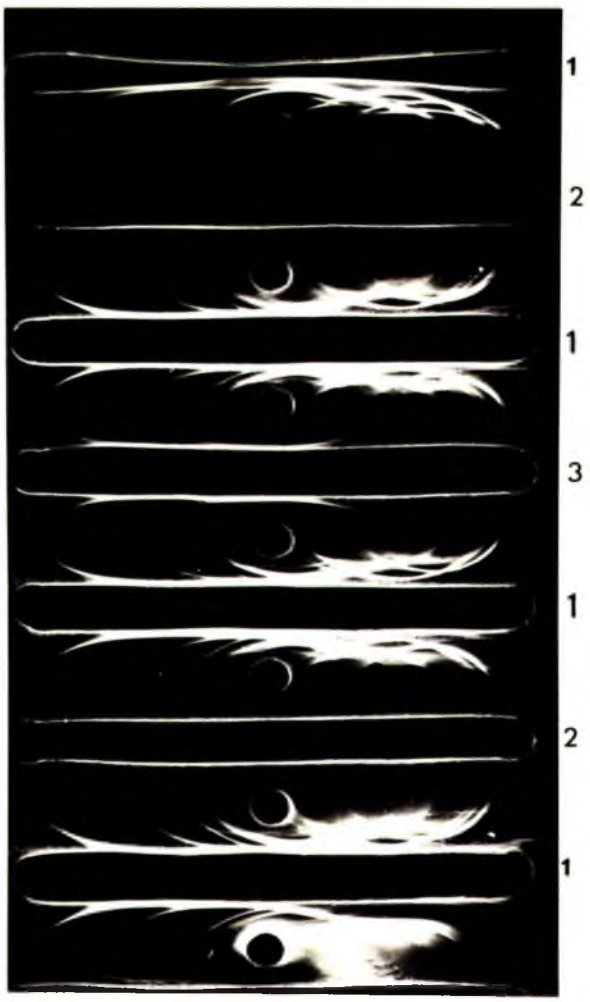

1
2
1
3
1
2
1

TABLE 16

The effect of specific anti-19S and anti-7S sera on the antibody present in culture supernatants from lymphoid tissues removed 8 days after immunisation with coliphage ØX174.

Tissue	Antibody activity/ unit weight of tissue		
	Pre-treatment	After anti-19S	After anti-7S
Spleen	3.4	0.01	3.25
Homolateral iliac lymph node	2.7	0	2.1
Contralateral iliac lymph node	4.7	0.6	4.6
Bone marrow	16.0	1.5	13.2

The effect of specific anti-19S and anti-7S sera on the antibody present in culture supernatants of tissues removed 21 days after immunisation with coliphage ØX174.

Tissue	Antibody activity / unit weight of tissue		
	Pre-treatment	After anti-19S	After anti-7S
Spleen	2.6	2.4	0.8
Homolateral iliac lymph node	3.2	3.3	0.05
Contralateral iliac lymph node	24.1	22.0	3.2
Bone marrow	4.5	2.4	2.7

DISCUSSION.

The effects of mycobacterial adjuvants on antibody production have been extensively investigated (see Review), but such investigations have been concentrated on the detection of the 7S immunoglobulin type, and no investigations have been published on the effects of such adjuvants on the production of antibody of the 19S immunoglobulin type.

Soluble protein antigens do not stimulate the production of 19S immunoglobulin antibody in the guinea-pig. The techniques of immunoelectrophoresis, quantitative precipitin testing and radio-immunoelectrophoresis have failed to detect any 19S antibody production against ovalbumin in the guinea-pig (Wilkinson, 1966). The choice of antigen for this study was dictated, therefore, by the requirement that it should stimulate the production of both 19S and 7S immunoglobulin antibodies in the guinea-pig. The antigen used, coliphage ØX174, has been shown to stimulate the production of both 19S and 7S antibodies in the guinea-pig (Uhr & Finkelstein, 1963). This antigen has several other advantages:

1. A highly sensitive technique, the bacteriophage neutralisation test, exists for the detection of bacteriophage neutralising antibody (Adams, 1959).
2. Relatively small amounts of bacteriophage will stimulate an antibody response in the guinea-pig.
3. Coliphage ØX174 is easily prepared, and a constant supply of fresh antigen for immunisation and antibody estimation can be maintained.

The immunising dose of coliphage ØX174 used throughout these experiments was 2 x 10^9 PFU per animal. This probably represents 20-25% of the total number of coliphage particles injected. This dose of antigen consistently stimulated the production of antibodies of the 19S and 7S immunoglobulin types in all animals injected.

Experimental Methods.

Before proceeding to a discussion of the results obtained in this study, it is first necessary to examine the validity of the bacteriophage neutralisation test as a measure of antibody.

The neutralisation of most viruses by specific antisera follows first order chemical kinetics. Such neutralisation follows a logarithmic course with time (Adams, 1959). This has been shown to hold good for a number of bacteriophages, including coliphage PC (Halmanson, Hersehey & Bronfenbrenner, 1942), actinophage (Bradley & Watson, 1963), T-even bacteriophages of E. coli (Fodor, 1957) and coliphage ØX174 (Uhr & Finkelstein, 1963; Brown & Patnode, 1964). These studies have shown that the rate of neutralisation of bacteriophage by specific antisera is independent of the absolute bacteriophage concentration, that the course of neutralisation proceeds logarithmically with time, and that deviation from logarithmic neutralisation occurs only when the reaction is allowed to go beyond 90-95% completion.

In view of reports by Uhr and Finkelstein (1963) and Bowman and Fatnode (1964) that not all preparations of coliphage ØX174 would show logarithmic neutralisation, each freshly prepared batch of coliphage ØX174 was tested against a standard rabbit antiserum, and any batch failing to show logarithmic neutralisation was discarded. Out of 21 batches of coliphage ØX174 prepared in this study, 4 were discarded for this reason.

The k values obtained by the bacteriophage neutralisation test are constant for any one antiserum (Adams, 1959; and see Table 1). The k values are not a direct measure of the antibody content of a serum, but a measure of the rate of the reaction between antibody and bacteriophage. This rate of reaction is, however, directly proportional to the antibody content of a serum, and the k value can therefore be used as a means for comparing the antibody content of different sera.

Such a use of k values is limited for the following reasons. The capacity of unit amounts of individual guinea-pig immunoglobulin antibodies to neutralise bacteriophage is not known. Finkelstein and Uhr (1967) have shown in dissociation studies that guinea-pig 19S antibody has a higher bacteriophage neutralising activity per unit weight than 7S antibody. No information is available on the realtive activities of guinea-pig $7S\gamma_1$ and $7S\gamma_2$ antibodies. The k values for one immunoglobulin type cannot therefore be directly compared with those for another. Further, circumstances are optimal for the detection of 19S

antibody because of the extreme sensitivity of the test and because of the greater avidity for bacteriophage of 19S than 7S antibody per unit weight of immunoglobulin. For these reasons it is only possible to compare directly k values of sera taken at the same time after immunisation when the predominant immunoglobulin types with antibody activity are similar.

The methods used for the separation of guinea-pig 19S, $7S\gamma_1$ and $7S\gamma_2$ immunoglobulins from whole serum in the studies described in this thesis are standard. Separation of 19S immunoglobulins from 7S was achieved by gel-filtration on Sephadex G-200, and the identity of the fractions thus obtained confirmed by immunoelectrophoresis. Gel-filtration has been shown to be a satisfactory method for the separation of high molecular weight immunoglobulins from those of low molecular weight (Killander & Flodin, 1962; Wilkinson & Carmichael, 1964).

Separation of guinea-pig $7S\gamma_1$ and $7S\gamma_2$ immunoglobulins was first achieved by ion-exchange chromatography on DEAE-cellulose using a gradient elution technique with increasing molarity of phosphate buffer (White et al, 1963). In this study, a stepwise elution technique, using phosphate buffer of three different molar strengths, was used, as described by Stewart-Tull et al (1965). Immunoelectrophoretic analysis of the $7S\gamma_1$ and $7S\gamma_2$ fractions thus obtained, showed that while a pure $7S\gamma_2$ fraction was

obtained by stepwise elution, the 7Sγ_1 fraction contained a small proportion of 7Sγ_2 immunoglobulin, and a large number of other serum proteins. A 19S immunoglobulin fraction was also obtained by this method. Antibody activity in these fractions was estimated using the bacteriophage neutralisation test.

Antibody activity due to 19S immunoglobulins was also detected indirectly by the treatment of whole serum with 2-mercaptoethanol. Reduction of whole serum using 2-mercaptoethanol breaks down the disulphide bonds of 19S antibodies, with the production of 7S subunits devoid of antibody activity (Deutsch & Morton, 1957; Grubb & Swahn, 1958; Kunkel, 1960). These units may re-aggregate non-specifically on the removal of the mercapto-ethanol, but this can be prevented by dialysis of the treated serum against iodoacetamide. Wilkinson and Carmichael (1964) have shown, however, that the loss of antibody activity is permanent, whether or not iodoacetamide is used.

EXPERIMENTAL RESULTS

The effect of mycobacterial adjuvants on antibody production to coliphage ØX174.

The results obtained from investigations of the effect of mycobacterial adjuvants on the production of antibody of different immunoglobulin classes against coliphage ØX174 show major differences from those obtained using soluble protein antigens. Against bacteriophage, all animals produced

neutralising antibody of the 19S type, detectable 8 days after immunisation, when antigen was injected in saline or in water-in-oil emulsions with and without added mycobacteria. Further, 21 days after a single injection of bacteriophage in saline, $7S_{\gamma 1}$ and $7S_{\gamma 2}$ antibodies were present in the serum of immunised guinea-pigs. The use of water-in-oil emulsions increased the antibody content of the $7S_{\gamma 1}$ fraction compared with similar fractions of sera from animals injected with bacteriophage in saline only. The addition of mycobacteria to such emulsions resulted in the production of the highest antibody levels, with increase in the antibody content of both $7S_{\gamma 1}$ and $7S_{\gamma 2}$ immunoglobulin fractions.

White and his colleagues (White, 1963; White et al, 1963, 1964; Wilkinson & White, 1966) have shown that the production of $7S_{\gamma 2}$ antibody against ovalbumin in the guinea-pig occurs after a single injection only if the antigen is injected in water-in-oil emulsion containing added mycobacteria. If mycobacteria are omitted from the injection mixture, serum antibody levels are lower, and the antibody produced is of the $7S_{\gamma 1}$ type exclusively. This is also the case when ovalbumin is injected in saline. Similar results were obtained by Benacerraf et al, (1963) At no time in these experiments was 19S antibody against ovalbumin detected whether quantitative precipitin testing, immunoelectrophoresis or radio-immunoelectrophoresis (Wilkinson, 1966).

After most methods of soluble protein antigen injection,

the guinea-pig responds principally by the production of 7Sγ_1 antibody. This has been shown to be the case for ovalbumin, human serum albumin and diphtheria toxoid (White et al, 1964; Wilkinson & White, 1966). Similar results have been obtained for anti-hapten antibody (Benacerraf et al, 1963). The results of investigations using the O-antigen of intact Escherichia coli cells showed that the predominant antibody produced was 7Sγ_2 rather than 7Sγ_1 (Bloch et al, 1963). As has been shown in the present study, synthesis of 7Sγ_2 antibody against a much smaller pargicle, coliphage ØX174, occurs without the addition of mycobacteria to the injection mixture, although the quantitative effects of mycobacterial adjuvants on 7S immunoglobulin antibody, particularly of the 7Sγ_2 type are still very striking.

The use of water-in-oil emulsions of coliphage enhanced the production of 19S antibody in comparison with saline as a vehicle for antigen injection, as judged by antibody levels in sera taken on the eighth day after a single injection of antigen. Adjuvants were not, however necessary to stimulate a 19S antibody response. Further, the addition of mycobacteria to water-in-oil emulsions of coliphage did not increase the level of 19S antibody attained: the mean levels of 19S antibody obtained using water-in-oil emulsions of antigen with and without added mycobacteria were not significantly different. This is the first report of an investigation of the effect of mycobacterial adjuvants on the production of 19S antibody in the guinea-pig.

There are two possible reasons for the apparent lack of a 19S antibody response against soluble protein antigens, which occurs in spite of the fact that 19S antiphage antibody is consistently detected after injection by similar routes and in similar adjuvant mixtures. First, as discussed above, the bacteriophage neutralisation test provides highly sensitive conditions for the detection of 19S antibody. Second, particulate antigens stimulate higher and more prolonged 19S responses than are attained using soluble proteins. Bauer, Mathies and Stavitsky (1963) found that while a single injection of soluble protein into rabbits resulted in an initial 19S response followed by a more prolonged 7S response, repeated injected of salmonella O-antigen resulted in a 19S response only. Turner and Rowe (1964) also showed a sustained 19S response to Salmonella typhi O-antigen in man. Torrigiani and Roitt (1964) showed that acrylic resin particles coated with thyroglobulin produced a more vigorous and sustained 19S response in rabbits than did thyroglobulin in solution.

Bacteriophage ØX174 is a relatively small particle, with a diameter of 225 Å (Tromans & Horne, 1961). The immunoglobulin response against this and other small virus particles may be expected to resemble more closely the response to soluble protein antigens than that which follows the injection of the larger bacteria and erythrocytes. Fahey and Horbett (1959) found most of the antibody against mumps virus in the 7S fraction of

human sera. Tokumara (1966) noted an initial rise in IgM antibody against herpes simplex virus in man, but later observed a predominance of IgG and IgA antibody. The production of early 19S antibody, followed by 7S antibody, has been observed after a single injection of coliphage ØX174 both in guinea-pigs (Uhr & Finkelstein, 1963) and chickens (Rosenquist & Campbell, 1966). In the chicken, the antibody response to coliphage ØX174 shows anintermediate position between the response to sheep erythrocytes and human serum albumin. The response to sheep erythrocytes was found to be entirely 19S antibody, that to human serum albumin entirely 7S antibody, and the response to coliphage showed an early 19S component, which was rapidly superceded by a 7S antibody response (Fleming, Munro & Cuperlovic, 1968).

The immunisation of guinea-pigs with coliphage ØX174 in water-in-oil emulsion resulted in the presence of neutralising antibody in the 19S immunoglobulin fraction of sera taken 21 days later. Antibody was not present in this fraction of sera from animals injected with antigen in saline. In this case, only 7S antibody was detected. This was shown by investigations on the 19S fractions obtained by DEAE fractionation of sera, and by the effect of 2-mercaptoethanol on sera taken from animals 21 days after immunisation.

It has been suggested that the continued synthesis of 19S antibody is dependent on a maintained antigenic stimulus (Uhr & Finkelstein, 1963). Such a stimulus would be provided by

the slow release of antigen from a water-in-oil emulsion 'depot' at the site of injection. A raised level of 7S antibody is known to inhibit the production of 19S antibody (Sahiar & Schwartz, 1964) but the mechanism of this action is not understood. Circulating antibody of the 7S type may rapidly clear the circulation of antigen, thus removing the stimulus for further production of 19S antibody. If this is so, then a further dose of antigen should result in the synthesis of 19S antibody. This has been shown to be the case for coliphage ØX174 in the guinea-pig (Uhr & Finkelstein, 1963). It is equally possible, therefore, that a constant, slow release of antigen from a water-in-oil emulsion may circumvent the inhibition of 19S antibody production by 7S. Further the inhibition of 19S antibody may not be due to the negative feedback mechanism postulated by Sahiar and Schwartz (1964), but may be due to a simple lack of circulating antigen.

In addition to studies on the effect of mycobacterial adjuvants on the serum levels of neutralising antibody against coliphage ØX174 a small number of observations were made on the development of dealyed-type hypersensitivity to coliphage after injection in adjuvant mixtures or saline. One of the criteria for assessing the adjuvant activity of mycobacteria and their chemical fractions is the production of delayed hypersensitivity (White, 1967). The delayed-type skin and corneal hypersensitivity reactions are a crucial feature of the adjuvant effect of

mycobacteria on the immune response of the guinea-pig to soluble protein antigens.

Delayed-type hypersensitivity to coliphage, as measured by the corneal test 21 days after antigen injection, developed in guinea-pigs injected with coliphage in water-in-oil emulsion with and without added mycobacteria. Animals injected with coliphage in saline failed to develop such a reaction. In the case of ovalbumin, the development of corneal hypersensitivity after a single injection of antigen has been shown to be dependent on the use of mycobacterial adjuvants (White, 1967) although the phenomenon can sometimes be reproduced using bentonite as an alternative adjuvant (Wilkinson & White, 1966). A close relationship has been demonstrated between the use of such adjuvants, the production of an adjuvant granuloma, the production of $7S_{\gamma 2}$ antibody against obalbumin and the development of corneal hypersensitivity to this antigen (see Fig. 7).

It appears that the use of simple water-in-oil emulsions of antigen is sufficient to induce the development of delayed-type hypersensitivity to coliphage ØX174. The number of animals tested, however, was small in comparison with the number tested by White in his investigations using soluble protein antigens. Much more experimental work must be done on this particular point before any conclusion may be drawn. The results do suggest that the close relationship between the use of mycobacterial adjuvants and the development of delayed

FIGURE 7

Correlation of footpad granuloma size with corneal response and the presence or absence of a 7S γ_2 globulin arc at 21 days after primary injection of ovalbumin in Freund's complete adjuvant.

No. of animals with	Marked footpad swelling ± ulceration	Moderate footpad swelling	Swelling negligible or absent	Totals
Strong corneal reaction	20	2	0	22
Moderate corneal reaction	6	6	4	16
Negative corneal reaction	2	4	9	15
γ_2 globulin arc present	18	8	1	27
absent	6	4	11	21

hypersensitivity to soluble protein antigens in the guinea-pig may not be applicable to other antigens.

Sites of synthesis of 19S and 7S immunoglobulin antibodies against coliphage ØX174 in the guinea-pig.

Lymphoid tissues from guinea-pigs immunised with a single injection of coliphage ØX174 in adjuvant mixtures and saline were studied *in vitro* 8 and 21 days after immunisation, when the predominant, circulating antibodies were of the 19S and 7S immunoglobulin classes respectively. Neutralising antibody against coliphage ØX174 consistently appeared in the supernatants of lymphoid tissue cultures at both these times. That the neutralising activity observed was due to the synthesis of specific antibody is supported by the following experimental evidence:

1. Neutralisation of coliphage by culture supernatants from lymphoid tissues of immunised guinea-pigs followed first order chemical kinetics, as did neutralisation of coliphage by specific antisera.
2. Culture of lymphoid tissues in the presence of the antimetabolite puromycin, which inhibits DNA-dependent RNA synthesis (Yarmolinsky & De La Haba, 1959), prevented the appearance of neutralising activity against coliphage.
3. Extraction studies on lymphoid tissues before and after *in vitro* culture showed that the coliphage neutralising

antibody extractable per unit weight of tissue incubated increased during the period of culture.

4. Culture supernatants from lymphoid tissues taken from non-immunised animals did not show any neutralising activity against coliphage ØX174.

5. The neutralising activity detected in culture supernatants was specific to the injected bacteriophage. Tissues from animals injected with a staphylococcal bacteriophage did not produce neutralising activity against coliphage ØX174 on *in vitro* culture.

The neutralisation of coliphage ØX174 by supernatants of lymphoid tissue cultures was therefore due to the synthesis of specific antibody during the period of *in vitro* incubation.

It is necessary to consider how far the synthesis of antibody by lymphoid tissues *in vitro* can be compared with their activity *in vivo*. Many workers have demonstrated the synthesis of antibody by lymphoid tissues *in vitro* up to 48 hours after removal from the animal body (Vaughan *et al*, 1960; La Via, 1962; Van Furth, 1966). However, studies on the rate of antibody synthesis *in vitro* have shown that maximal antibody production occurs during the first few hours of incubation (Dresser, 1965 Further, it has been shown that small pieces of tissue are mo efficient in the *in vitro* synthesis of antibody than are ce suspensions (Attardi *et al*, 1964; Dresser, 1965). With th possible exception of bone marrow, the tissues studied i

experiments described above were incubated as small fragments, and not as cell suspensions.

Although it is possible that the conditions of culture might alter the ability of cells to synthesise antibody, lymphoid tissues *in vitro* begin to synthesise antibody without a noticeable lag period, and maximal antibody production occurs within the first 6 hours of incubation (van Furth, 1966). Further, the activity of various lymphoid tissues in the *in vitro* synthesis of antibody can be correlated with the number of antibody producing cells present in such tissues (Fagraeus, 1948a). These results suggest that the tissues are carrying on with their normal function.

In the experiments on sites of synthesis of immunoglobulins antibodies against coliphage, it has been shown that net synthesis of antibody occurred during the period of incubation, both by extraction studies, and the use of puromycin. Further, the results of extraction studies before incubation of tissues showed a similar distribution of antibody content, on a unit weight basis, to that observed in culture supernatants after incubation. In view of these results, and the observations of other workers, it is reasonable to conclude that the activity of different lymphoid tissues in the synthesis of antibody *in vitro* is a fair reflection of the *in vivo* activity of the same tissues.

It is therefore possible to compare the relative importance of different lymphoid tissues in the synthesis of antibody by *in vitro* culture methods. This has been carried out

in this study by comparing the amount of antibody produced by different lymphoid tissues from the guinea-pig cultured *in vitro* under the same conditions. Antibody was measured indirectly by determining the number of coliphage particles neutralised in 30 minutes by tissue culture supernatants per unit weight (0.1 gm) of tissue incubated.

Eight days after immunisation of guinea-pigs with coliphage ØX174, bone marrow was found to be the lymphoid tissue most active, on a unit weight basis, in the synthesis of coliphage neutralising antibody. This was the case whether antigen was injected in water-in-oil emulsion with or without added mycobacteria, or in saline. Spleen was least active on a unit weight basis, and lymph nodes showed an activity intermediate between that of bone marrow and spleen. These results are summarised in Table 17. The antibody present in lymphoid tissues at this time was of the 19S immunoglobulin type, as was shown by its sensitivity to treatment with 2-mercaptoethanol and specific rabbit anti-guinea-pig 19S immunoglobulin antisera.

The lymphoid tissues found to be most active in the synthesis of coliphage neutralising antibody 21 days after antigen injection were the iliac lymph nodes homolateral and contralateral to the site of antigen injection. These results are summarised in Table 18. On a unit weight basis, spleen was the least active of the tissues studied; bone marrow showed extremely variable activity. In this series of experiments, the activity of the

TABLE 17

Mean distribution of activity in the synthesis of coliphage neutralising antibody of various lymphoid tissues, relative to the homolateral iliac nodes (100%), on a unit weight basis, from guinea-pigs immunised 8 days previously with coliphage in adjuvants or saline.

Group	Mean synthesis / 0.1 g tissue			
	Homolateral iliac node	Contralateral iliac node	Spleen	Bone Marrow
W/O emulsion + mycobacteria	100	198	146	727
W/O emulsion	100	148	87	537
Saline (i/v)	100	169	77	475
Saline (footpad)	100	99	62	315

TABLE 18

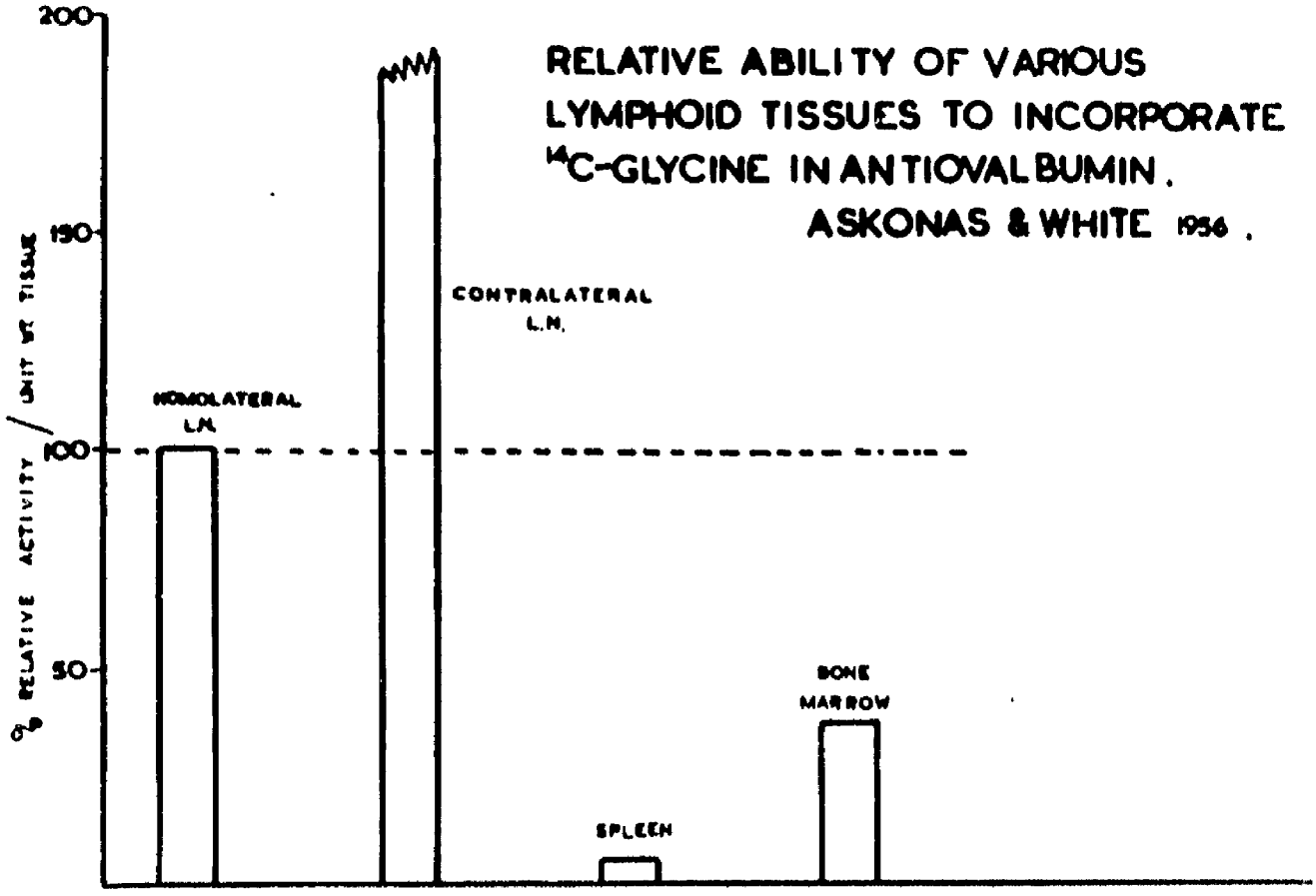
Mean distribution of antibody synthesis in lymphoid tissues, relative to the homolateral iliac nodes, (100%), on a unit weight basis, from guinea-pigs 21 days after immunisation with coliphage in adjuvants and in saline.

Group	Homolateral iliac node	Contralateral iliac node	Spleen	Bone Marrow	Popliteal node	Cervical node
W/O emulsion + mycobacteria	100	295	53	149	65	25
W/O emulsion	100	171	21	95	223	39
Saline (footpad)	100	91	51	41	150	15

lymph nodes homolateral and contralateral to the site of footpad injection was affected by the use of adjuvant mixtures. In animals injected with coliphage in mycobacterial adjuvant *via* the footpad, the contralateral iliac lymph nodes showed a much higher activity than the homolateral nodes, on a unit weight basis. This was true to a lesser extent of the same tissues from animals injected with coliphage in water-in-oil emulsion, but in animals injected with antigen in saline the position was reversed. These results are summarised in Table 18. Antibody activity in such culture supernatants was not susceptible to treatment with specific anti-guinea-pig 7S sera, showing the antibody present to be of the 7S immunoglobulin class.

The sites of synthesis of 7S antibody against coliphage ØX174 in the guinea-pig, 21 days after antigen injection in adjuvant mixtures or in saline are similar to those reported by Askonas and White (1956) (see Fig. 8) and Askonas *et al* (1965). These workers showed that 21 days after injection of guinea-pigs in the left hind footpad with ovalbumin in mycobacterial adjuvant, the lymphoid tissue most active in the synthesis of antibody against ovalbumin on a unit weight basis were the contralateral iliac lymph nodes. These workers also showed that bone marrow showed a higher synthetic activity under these circumstances than did spleen or homolateral iliac nodes. This distribution is essentially similar to that observed in response to the injection of coliphage in water-in-oil emulsion with and without added

FIGURE 8.


RELATIVE ABILITY OF VARIOUS
LYMPHOID TISSUES TO INCORPORATE
^{14}C-GLYCINE IN ANTIOVALBUMIN.
ASKONAS & WHITE 1956.
200
150
100
50
% RELATIVE ACTIVITY / UNIT WT TISSUE
HOMOLATERAL
L.N.
CONTRALATERAL
L.N.
SPLEEN
BONE
MARROW

mycobacteria.

In animals injected with coliphage in saline *via* the footpad, the distribution of synthetic activity was different. In this case, the homolateral iliac lymph nodes showed a slightly higher activity than the contralateral nodes, on a unit weight basis. McMaster and Hudack (1935) showed that lymph nodes regional to the site of injection of antigen produced antibody earlier and in greater quantities than did lymph nodes more distant from the site of antigen injection. The distribution of synthetic activity in these animals can therefore be considered the normal response to antigen injection. The altered distribution of synthetic activity seen in the homolateral and contralateral iliac lymph nodes of animals injected with coliphage in adjuvant mixtures can be explained on the basis of the tissue response to the injection of such adjuvants. Suter and White (1954) and White *et al* (1955) demonstrated pronounced hyperplasia of the regional lymph nodes in guinea-pigs injected with mycobacterial adjuvant *via* the footpad. This hyperplasia was found to be due to intense macrophage proliferation, with complete disruption of the normal architecture of the node. Few antibody containing cells are seen in sections of such nodes. Under such circumstances, the low synthetic activity of such tissues on a unit weight basis is easily understandable. However, the regional lymph nodes which undergo hyperplasia weight from 4-12 times as much as their contralateral equivalents. If the tissue culture activity

of a portion of such a node is representative of the whole node then the total contribution of such organs to overall antibody production will be significantly higher than that of any other lymph node. In this series of experiments the mean weights of the homolateral and contralateral iliac lymph nodes from animals injected with injected with mycobacterial adjuvant were 0.49 gm and 0.07 gm respectively. On the basis of the mean unit weight activity of these organs, the corresponding figures for total antibody production would be in the ratio of 2.5:1.

The high synthetic activity of bone marrow in the production of 19S antibody 8 days after antigen injection is in striking contrast to the distribution of synthesis of 7S antibody. Askonas and White (1956) calculated the total synthesis of antibody by lymphoid organs in the body as a whole by extrapolation from the synthetic activity of known weights of tissue from animals injected with soluble protein antigens. They concluded that bone marrow might contribute up to 50% of the overall calculated antibody synthesis, because of its large mass relative to the other lymphoid tissues, although on a unit weight basis the activity of the bone marrow was significantly lower than that of the lymph nodes. Similar results were obtained in rabbits by Askonas and Humphrey (1958). By the same token, if the activity of the tibial bone marrow in the present series of experiments is representative of the total bone marrow, then synthesis of 19S antibody against coliphage must be almost

entirely due to the synthetic activity of the bone marrow.

Synthesis of antibody by bone marrow cultures *in vitro* has been frequently reported. However, such synthesis has either been detected after hyperimmunisation of the animal, or investigation at such a time after a single injection of antigen that the antibody produced is of the 7S immunoglobulin class. No previous report on sites of synthesis of 19S antibody in response to a single injection of antigen could be found in the literature. It has been suggested that bone marrow is unable to take part in antibody production after a single injection of antigen (Langevoort *et al*, 1963; Gengozian *et al*, 1961; Friedman, 1964).

The presence of 19S immunoglobulin in cells in different tissues has, however, been shown. Burtin (1960) in a study of human tissues detected 19S immunoglobulin in cells of spleen and lymph nodes, but not in bone marrow. Curtain (1961) detected many 19S-containing cells in the bone marrow of a patient suffering from macroglobulinaemia. In a study of human tissues, both normal and pathological, van Furth (1966) detected 19S-containing cells in spleen, lymph nodes and bone marrow. This worker also showed, by a combination of tissue culture, immunoelectrophoresis and autoradiography, that human bone marrow was able to synthesise all three major classes of immunoglobulin (see Fig. 9). Bone marrow has also been shown to produce antibody by cell transfer experiments (Porter & Couch, 1959; Porter *et al*, 1959).

FIGURE 9

Synthesis of immunoglobulins by normal human tissues in vitro.

(from van Furth, 1967)

Organ	Autoradiography IgG	IgA	IgM
Spleen	++	++	+
Lymph node	++	+	+
Bone marrow	+++	++	+
Thymus	++	(+)	-

The intensity of the autoradiographic image is graded from

\- = negative

(+) = just visible

to

+++ = very dark

The experiments described in this thesis provide important evidence for the involvement of bone marrow in antibody production in response to a single injection of antigen. It is evident that guinea-pig bone marrow plays an important part in antibody synthesis against coliphage, most particularly in the synthesis of antibody of the 19S immunoglobulin type.

GENERAL DISCUSSION

The injection of soluble protein antigens in water-in-oil emulsion with added mycobacteria typically results in higher levels of serum antibody than are produced in mycobacteria are omitted from the injection mixture. The large volume of previous work on this topic has been concerned primarily with the effect of such adjuvants on 7S immunoglobulins. The work of White *et al* (1963) and Benacerraf *et al* (1963) has shown that the use of mycobacterial adjuvants preferentially stimulates the production of $7S_{\gamma 2}$ immunoglobulin antibody against soluble protein antigens in the guinea-pig. No investigations have been published on the effect of such adjuvants on the production of antibody of the 19S immunoglobulin type.

In the present thesis, the effect of mycobacterial adjuvants on the antibody production in all three immunoglobulin classes of the guinea-pig has been explored. The results show that this effect is specific for immunoglobulin class.

Mycobacteria have no enhancing effect on the production of 19S immunoglobulin antibody against coliphage ØX174. In contrast, serum levels of 7S antibody against coliphage are markedly raised by the use of mycobacterial adjuvants, particularly levels of $7S_{\gamma 2}$ antibody. The response of guinea-pigs to a single injection of coliphage in mycobacterial adjuvant differs from that described for soluble protein antigens (White et al, 1963) in two respects. First, the use of water-in-oil emulsions with or without added mycobacteria results in the persistence of a 19S antibody component, when sera from animals injected in saline contain antibody only of the 7S immunoglobulin type. Second, the use of mycobacterial adjuvants is not necessary for the stimulation of $7S_{\gamma 2}$ antibody production, although such adjuvants markedly increase the formation of this class of antibody.

In an effort to explain the lack of effect of mycobacterial adjuvants on the serum levels of antibody of the 19S immunoglobulin type, the major lymphoid tissues of the guinea-pig were studied in vitro to determine the sites of synthesis of these antibodies, 19S and 7S, at a time when the predominant circulating antibody was of that particular type. These investigations produced the surprising result that the bone marrow of the guinea-pig was almost entirely responsible for the synthesis of neutralising antibody of the 19S type against coliphage. On a unit weight basis, this tissue was much more active than any other lymphoid tissue studied.

In contrast, the distribution of activity in the synthesis of 7S neutralising antibody against coliphage was similar to that already reported by Askonas and White (1956) and Askonas *et al* (1965) for soluble protein antigens. In this case, lymph nodes close to the site of antigen injection were most active in the synthesis of antibody on a unit weight basis. Even in these animals, however, the bone marrow would make a significantly high contribution to the overall synthesis of antibody, on the basis of its unit weight activity.

A variety of mechanisms have been postulated for the mode of action of mycobacterial adjuvants. A commonly accepted explanation is that this adjuvant exerts its effect through the lymphatic system, probably by a combination of the properties of allowing slow release of antigen from the site of injection, and of producing a characteristic tissue reaction (see Review). The effect of mycobacterial adjuvants on serum levels of neutralising antibody for coliphage were greatest at a time when the lymph nodes regional to the site of antigen injection were most active in the synthesis of antibody, whether animals were injected with antigen in adjuvants or in saline.

The great importance of the bone marrow in the synthesis of 19S antibody against coliphage provides an explanation of the lack of effect of mycobacterial adjuvants on serum levels of this type of antibody. Synthesis of 19S antibody is a short-lived phenomenon when compared with the synthesis of 7S antibody

(Uhr & Finkelstein, 1963). It has been shown that the decay rate of circulating 19S antibody is very similar to the rates of catabolism of antigens. This has been shown for pneumococcal polysaccharide in the mouse (Siskind & Paterson, 1964) and Heterologous serum proteins in the rabbit (Richter et al, 1965). Further, the level of 19S antibody attained in the circulation is proportional to the antigen dose. This has been shown using the somatic antigen of Salmonellae (Landy et al, 1965) and coliphage ØX174 (Uhr & Finkelstein, 1963). These findings have led to the hypothesis that the production of 19S antibody is dependent on the persistence of circulating antigen which continuously primes short-lived antibody producing cells.

Further, Uhr and Finkelstein (1963) have supplied good evidence for the lack of a mechanism for 'immunological memory' in the 19S antibody system.

These results, and the results from the investigation here presented, argue that two distinct populations of cells are concerned in the manufacture of 19S and 7S antibodies. The results do not support the concept that the production of 19S and 7S are undertaken by the same cells working in a sequence of early 19S succeeded by later 7S production (Nossal et al, 1964). The activation of the two populations of cells will depend on the conditions governing access of antigen to the tissues containing these cells. The bone marrow appears to be the major location of 19S producing cells. Since this organ lacks a lymphatic supply, antigen must reach it exclusively

by haematogeneous dissemination. This concept is supported by the similarity of the activity of bone marrow from different sites in the synthesis of 19S antibody against coliphage. If local diffusion of antigen into the bone marrow took place, then the activity of such bone marrow should be higher than that of bone marrow from other sites. Kosunen and Kaariainen (1966) have shown that a considerable proportion of the coliphage injected in water-in-oil emulsion with added mycobacteria is released almost immediately into the blood stream.

The concept of haematogeneous spread of antigen as the mechanism of induction of 19S antibody synthesis is also supported by the ability of passively transferred antibody of either 7S or 19S type to inhibit the production of 19S antibody in experimental animals (Sahiar & Schwartz, 1964) (Wigzell, 1966). Such passively transferred antibody would rapidly remove any circulating antigen, thus removing the stimulus for 19S antibody production.

There is considerable evidence to support the view that 19S and 7S antibodies are produced by separate cell populations. Smith (1960) has shown that in the immediate neonatal period, human children can mount a 19S antibody response, but fail to produce a 7S response until at least two months of age. Such children do not appear to have any plasma cells or plasma cell precursors in their lymph nodes. Kalmutz (1962) and La Via,

Rowlands and Block (1963) have demonstrated antibody production, presumably of the 19S type, in the neonatal opossum, which does not develop plasma cells or germinal centres in lymphoid tissues until some time after birth. Eichenwald (1963) has shown that the human foetus *in utero* can produce 19S antibody, but not 7S. It has been suggested that cells producing 19S antibody are not of the plasma cell type. Schoenberg and his colleagues (1965) have described large mononuclear cells in the sinusoids of the spleen as the source of 19S antibody in the rabbit, and Curtain (1961) described similar cells in humans with macroglobulinaemia.

Antibody production in the draining lymph nodes 21 days after antigen injection is likely to have been stimulated by antigen which travelled *via* the lymphatic system. If retention of antigen at the site of injection, with slow release to the local lymph nodes, is an important factor in adjuvant action, a clear adjuvant effect might be expected on antibody produced predominantly by lymph nodes, but not to the same extent in bone marrow to which antigen is probably rapidly disseminated *via* the bloodstream. The different effect of mycobacterial adjuvants and water-in-oil emulsions of antigen on the production of coliphage neutralising antibody 21 days after antigen injection provides further support for the concept that the production of 19S and 7S antibodies is subserved by different populations of cells.

BIBLIOGRAPHY.

Ada, G.L., Nossal, G.J.V. Austin, C.M. 1965 in 'Molecular and Cellular Basis of Antibody Formation' ed. J. Sterzl p.31 Czech. Acad. Sci., Prague.

Adams, M.H. 1957 in 'Bacteriophages' p454, Interscience.

Al-Askari, S., Zweimann, B., Laurence, H.S., Thomas, L., 1964 J. Immunol. 93 742

Amies, C.R. 1959 J. Path. Bact. 77 435

Amies, C.R. 1962 J. Hyg. 60 483

Anderson, R.J. 1941 Chem. Rev. 29 225

Aron, S.A., Janicki, B.W. 1967 Proc. Soc. exp. Biol. Med. 126 518

Asherson, G.L. 1965 Abs. World Med. 38 145

Askonas, B.A., Humphrey, J.H. 1958 Bioch. J. 68 252

Askonas, B.A., White, R.G. 1956 Brit. J. exp. Path. 37 61

Askonas, B.A., White, R.G., Wilkinson, P.C. 1965 Immunochemistry 2 329

Asofsky, R., Thorbecke, G.J. 1961 J. exp. Med. 114 471

Asselineau, J., Lederer, E. 1953 Fortschr. Chem. organ. Naturstoffe 10 170

Asselineau, J., Buc, H., Jolles, P., Lederer, E., 1958 Bull. Soc. Chem. Biol. 40 1953

Attardi, G., Colm, M., Horibata, K., Lennox, E.S. 1964 Bact. Rev. 23 213

Barrie, J.U., Cooper, G.N. 1964 J. Immunol. 92 529

Batchelor, J.R., Lessof, M.H. 1964 Rec. Adv. Med. 32

Bauer, D.C. 1963 J. Immunol. 91 323

Bauer, D.C., Mathies, M.J., Stavitsky, A.B. 1963 J. exp. Med. 117 889

Bauer, D.C., Stavitsky, A.B. 1961 Proc. nat. Acad. Sci. 47 1667

Benacerraf, B., Ovary, Z., Bloch, K.J., Franklin, E.C., 1963 J. exp. Med. 117 937

Bendixen, G., 1963 Acta path. microbiol. Scand. 57 222

Benedict, A.A., Brown, R.J., Hersh, R.T. 1963 J. Immunol. 90 599

Benier, G.M., Cebra, J.J. 1963 J. Immunol. 95 246

Bergey, D.H. 1934 J. inf. Dis. 55 72

Billingham, R.E., Brent, L., Medawar, P.B. 1953 Nature 172 603

Bjørneboe, M., Gormsen, H., Lundqvist, F. 1947 J. Immunol. 55 121

Bloch, K.J., Kourilsky, F.M., Ovary, Z., Benacerraf, B. 1963 Proc. Soc. exp. Biol Med. 114 52

ibid. 1963a J. exp. Med. 117 965

Bowman, B.U., Patnode, R.A. 1964 J. Immunol. 92 507

Bradley, S.G., Watson, D.W. 1963 J. Immunol. 90 782

Burky, E.L. 1934 J. Allergy 5 466

Burnet, F.M. 1962 Med. J. Australia i 1

Burnet, F.M. 1965 Brit. Med. J. i 338

Burtin, P. 1960 in CIBA Foundation Sym. 'Cellular Aspects of Immunity' ed. Wolstenholme & O'Connor p.214.

Carrel, A., Ingebrigsten, R., 1912 J. exp. Med. 16 287

Casals, J., Freund, J., 1939 J. Immunol. 36 339

Choucroun, N. 1947 AMer. Rev. Tuberc. 56 203

Coe, J.E. 1966 J. Immunol. 96 744

Cohen, S., Porter, R.R.	1964	Adv. Immunol. 4 287
Cohen, S., Porter, R.R.	1964a	Bioch. J. 90 278
Colover, J.	1954	Brain 77 435
Colover, J., Consden, R.	1956	Nature 177 749
Colover, J., Glynn, L.E.	1958	
Coons, A.H., Leduc, E.H., Connolly, J.M.	1955	J exp. Med. 102 49
Coulaud, E.	1935	C. R. Soc. Biol. 119 360
Cowan, K.M., Trautman, R.	1965	J. Immunol. 94 858
Cruchaud, A., Rosen, F.S., Craig, J.M. Janeway, C.A., Getlin, D.	1962	J. exp. Med. 115 1141
Curtain, C.C.	1961	Austral. J. exp. Biol. Med. 39 391
Degara, P.F., Angevine, D.M.	1943	J. exp. Med. 78 27
Delaunay, A., Asselineau, J., Lederer, E.	1954	C.R. Soc. Biol. 145 650
Deutsch, L.	1899	Ann. Inst. Pasteur 13 639
Deutsch, H.F. Morton, J.L.	1957	Science, 125 600
Dienes, L.	1928a	J. Immunol. 15 141
Dienes, L.,	1928b	J. Immunol. 15 153
Dienes, L.,	1929	J. Immunol. 17 531
Dienes, L.,	1930	Z. Immunitats. 68 13
Dienes, L., Schonheit, E.W.	1927a	J. Immunol. 14 42
Dienes, L., Schonheit, E.W.	1927b	J. Immunol. 14 52
Dienes, L., Schonheit, E.W.	1927c	J. Immunol. 14 61
Dienes, L., Schonheit, E.W.	1930	J. Immunol. 19 41
Dixon, F.J., Weigle, W.O., Roberts, J.C.	1957	J. Immunol. 78 56
Dresser, A.M.	1965	Immunology 9 483
Dresser, D.W.	1961	Nature 191 1168
Eichenwald, H.E.	1963	cited by Good and Papermaster, 1964.
Fagraeus, A.	1948a	Acta Med. Scand. Supp. 204

Fagraeus, A.,	1948b	J. Immunol. 58 1
Fahey, J.L., Horbett, A.P.	1959	J. biol. Chem. 234 2645
Farthing, J.R., Holt, L.B.	1962	J. Hyg. 60 411
Fischel, E.E., Kabat, E.A., Stoerk, H.C., Bezer, A.E.	1952	J. Immunol. 69 611
Fisher, S.	1966	Immunology 11 127
Fleming, W.A., Munro, J.A., Cuperlovic, C.	1968	in preparation.
Fodor, A.R.	1957	J. Immunol. 79 227
Freeman, M.J., Stavitsky, A.B.	1965	J. Immunol. 95 981
Friedman, H.	1964	Proc. Soc. exp. Biol. 2 526
Freund, J.	1927	J. Immunol. 14 101
Freund, J.	1951	Amer. J. Clin. Path. 21 645
Freund, J.	1956	Adv. Tuberc. Res. 7 130
Freund, J., Bonanto, M.V.	1942	J. Immunol. 45 71
Freund, J., Bonanto, M.V.	1944	J. Immunol. 48 325
Freund, J., Bonanto, M.V.	1946	J. Immunol. 52 231
Freund, J., McDermott, K.	1942	Proc. Soc. exp. Biol. Med. 49 548
Freund, J., Stone, S.H.	1959	J. Immunol. 82 560
Freund, J., Casals, J., Hosmer, E.P.	1937	Proc. Soc. exp. Biol. Med. 37 509
Freund, J., Lipton, M.M., Thompson, G.E.	1953	J. exp. Med. 97 711
Freund, J., Stern, E.R., Pisani, T.M.	1947	J. Immunol. 57 179
Freund, J., Thompson, K.J., Hough, H.B., Sommer, H.E.	1948	J. Immunol. 60 383
van Furth, R.	1966	Immunology, 11 1
Gall, D.	1966	Immunology, 11 369

Garver, R.M., Santes, G.W., Cole, L.J.
1959 J. Immunol. 83 57
Gongozian, N., Makinodan, T., Shekerdii, I.C.
1961 J. Immunol. 86 113
Gorstl, B., Tennent, R., Pelzman, 6.
1945 Amer. J. Path. 21 1007
Glenny, A.T., Barr, M. 1931 J. Path. Bact. 34 131
Glenny, A.T., Pope, C.G., Waddington, H., Wallace, U.
1926 J. Path. Bact. 29 31
Good, R.A., Papermaster, B.W.
1964 Adv. Immunol. 4 1
Gordon, R., 1965 J. gen. Microbiol. 43 329
Goudie, R.B., Wilkinson, P.C., Horne, C.W.
1966 Lancet 11 1224
Grassberger, G. 1899 cited by Freund, 1956
Graves, J.H., Cowan, K.M., Trautman, R.
1964 J. Immunol. 92 507
Greenberg, L., Fleming, D.S.,
1947 Can. J. Pub. Health 38 279
Greenberg, L., Fleming, D.S.
1948 Can. J. Pub. Health 39 131
Greenbury, C.L., Moore, D.H., Nunn, L.A.C.
1963 Immunology 6 421
Grey, H.M. 1964 Immunology 7 82
Grubb, R., Svalm, D. 1958 Acta. path. microbiol. Scand. 43 305
Halbert, S.P., Mudd, S., Smolens, J.
1946 J. Immunol. 53 291
Harris, T.N., Harris, S. 1954a J. Immunol. 72 148
Harris, S., Harris, T.N. 1954b J. exp. Med. 100 269
Harris, T.N., Dray, S., Ellsworth, B., Harris, S.
1963 Immunology 6 169
Harris, T.N., Hummeler, K., Harris, S.
1966 J. exp. Med. 123 161

Hektoen, L. 1909 J. inf. Dis. 6 78

Hektoen, L. 1915 J. inf. Dis. 9 103

Hektoen, L., Welker, W.H. 1933 J. inf. Dis. 53 309

Herbert, W.J. 1966 Nature 210 747

Heremans, J.F. 1959 Clin. Chim. Acta 4 639

Heremans, J.F., Vaerman, J.P., Vaerman, C. 1963 J. Immunol. 91 11

Hobson, D., Porter, K.A., Whitby, J.L. 1959 Brit. J. exp. Path. 40 15

Hochwald, G.M., Asofsky, R., Thorbecke, G.J. 1961 J. exp. Med. 114 459

Holton, J.B., Schwab, J.H. 1966 J. Immunol. 96 134

Houdayer, M., Metzger, J., Feraf, A. 1965 Progr. immunobiol Stand. 2 63

Hulliger, L., Sorkin, E. 1963 Nature 198 299

Humphrey, J.H., Turk, J.L. 1963 Immunology 6 119

Humphrey, J.H., White, R.G. 1964 'Immunology for Students of Medicine' 2nd edn. Blackwell, Oxford.

Hunter, L.P., Hackel, D.B., Heymann, W. 1960 J. Immunol. 85 319

Ishizaka, K., Ishizaka, T., Hornbrook, M.M. 1966 J. Immunol. 97 75

Jenkin, C.R., Anzins, I., Reade, P.C. 1965 Austr. J. exp. Biol. Med. Sci. 43 607

Johnson, A. G., Gaines, S., Landy, M. 1956 J. exp. Med. 103 225

Jollès, P., Samour, D., Lederer, E. 1962 Arch. Bioch. Biophys. suppl. 1 203

Jollès, P., Samour, D., Lederer, E. 1963 Bioch. Biophys. Acta 78

Kabat, E.A., Wolf, A., Bezer, A.E.
1947 J. exp. Med. 88 417
Kalmanson, G.M., Hershey, A.D., Dronfenbronner, J.
1942 J. Immunol. 45 1
Kalmutz, S.E. 1962 Nature 193 851
Kaplan, M.H., Craig, J.M. 1963 J. Immunol. 90 725
Keuning, F.J., van der Slikke, L.B.
1950 J. Lab. Clin. Med. 36 167
Khanolkar, V.R. 1924 J. Path. Bact. 27 181
Kies, M.W., Alvord, E.C. 1958 Nature 182 1106
King, Y.M., Johnson, A.G. 1963 J. Immunol. 90 672
Kopelloff, L.M., Kopelloff, N.
1947 J. Immunol. 57 229
Kosunen, T.U., Kaariainen, L.J.
1966 Ann. Med. exp. et Biol. Fenn. 44 32
Kritzman, J., Kunkel, H.G., McCarthy, J., Mellors, R.C.
1961 J. Lab. Clin. Med. 47 905
Kunkel, H.G. 1960 in 'The Plasma Proteins' vol. 1 p279 ed. Putnam. Academic Press, London.
Landy, M., Sanderson, R.P., Jackson, A.L.
1965 J. exp. Med. 122 483
Langevoort, H.L., Asofsky, R.M., Jacobson, E.B., de Vries, T., Thorbecke, G.J.
1963 J. Immunol. 90 60
Laufer, A., Tal, C., Bohar, A.J.
1959 Brit. J. exp. Path. 40 1
La Via, M.F. 1962 Arch. Path. 74 116
La Via, M.F., Rowlands, D.T., Block, M.
1963 Science 140 1219

LeMoignic, Pinoy 1916 C.R. Soc. Biol.

Levene, H.I., Franklin, E.C., Thorbecke, G.J. 1961 J. Immunol. 86 440

Lewis, P.A., Loomis, D. 1924 J. exp. Med. 40 503

Lewis, P.A., Loomis, D. 1926 J. exp. Med. 43 263

Lindqbist, K., Bauer, D.C. 1966 Immunochemistry 3 373

Lipton, M.M., Steigman, A.J. 1963 J. Immunol. 90 512

LoSpalluto, J., Miller, W.J., Dorward, P., Fink, CW. 1962 J. Clin. Invest. 41 1415

Luckhardt, A.B., Becht, F.C. 1911 Amer. J. Physiol. 28 257

Luecke, D.H., Sibal, L.R. 1962 J. Immunol. 89 539

Makela, O. 1964 Immunology 7 9

Mann, L.S., Spinka, I.M. 1946 J. Immunol. 53 209

Marshall, A.H.E., White, R.G. 1961 Brit. J. exp. Path. 42 379

Martins, A.B., Raffel, S. 1964 J. Immunol. 93 937

McMaster, P.D. 1953 in 'The Nature and Significance of the antibody Response' p13, ed. A.M. Pappenheimer, Columbia Univ, Press.

McMaster, P.D., Hudack, S.S. 1935 J. exp. Med. 61 783

Mellros, R.C., Heimer, R., Corcos, J, Korngold, L. 1959 J. exp. Med. 110 875

Miller, L.L., Bale, W.F. 1954 J. exp. Med. 99 125

Mitchison, N.A. 1957 Folia biol.(Prague) 3 72

Moore, R.D., Schoenberg, M.D. 1966 Brit. J. exp. Path. 47 60

Morgan, I.M. 1947 J. exp. Med. 85 131

Moss, J.N., Boiler, J.M., Martin, G.T.
1956 J. Immunol. 76 363

Mudd, S. 1932 J. Immunol. 23 423

Munoz, J. 1963 J. Immunol. 90 133.

Munoz, J. 1964 Adv. Immunol. 4 397

Murphy 1914 J. exp. Med. 20 397

Murphy, , Sturm 1919 J. exp. Med. 29 1

Murray, E.S., O'Connor, J.M., Gaon, J.A.
1965 J. Immunol. 94 734

Nakahara, T. 1919 J. exp. Med. 29 17

Niveu, T., Bronellec, A., Biozzi, G.
1964 Ann. Inst. Past. 106 771

Nicol, T., Quantock, D.C., Vernon-roberts, B.
1966 Nature 209 1142

Nossal, G.J.V. 1959a Brit. J. exp. Path. 40 118

Nossal, G.J.V. 1959b Brit, J. exp. Path. 40 301

Nossal, G.J.V., Szenberg, A. Ada, G.L., Austin, C.M.
1964 J. exp. Med. 119 485

Novikoff, A.B. 1963 Coll. Int. Centre Natl. Rech. Sci. 115 67

Oakley, C.L., Warrack, H.G., Batty, I.
1951 J. Path. Bact. 63 33

Osler, A.g., Mulligan, J.J. Rodriquez, E.
1966 J. Immunol. 96 334

Ovary, Z., Benacerraf, B., Bloch, K.J.
1963 J. exp. Med. 117 951

Parker, R.C. 1937 Science 85 292

Paterson, P.Y. 1960 J. exp. Med. 111119

Paterson, P.Y., Bell, J. 1962 J. Immunol. 89 78.

Rivers, T.M., Sprunt, D.M., Berry, G.P.
1933 J. exp. Med. 58 39

Robbins, J.B., Kenny, K., Suter, E.
1965 J. exp. Med. 122 385

Rosenquist, G.L., Campbell, G.R.
1966 Immunology 10 169

Rowley, D.A. 1950a J. Immunol. 64289

Rowley, D.A. 1950b J. Immunol. 65 515

Rupp, J.C., Moore, R.D., Schoenberg, M.D.
1960 Arch. Path. 70 43

Sahiar, K., Schwartz, R.S. 1964 Science 145 395

Salvaggio, J.E., Flax, M.H., Leskowitz, S.
1965 J. Immunol. 95 846

Scherr, G.H., Markowitz, A.S., Skelton, L.Z.
1965 J. app. Bact. 28 174

Schmidt, S., Steenberg, E. 1936 Acta path. microbiol. Scand. 13 401

Schoenberg, M.D., Stavitsky, A.B., Moore, R.D., Freeman, M.J.
1965 J. exp. Med. 121 577

Schultze, H.E. 1959 in 'Bildung der Antikorper' 10 Coll. Ges. Physiol. Chemie in Mosbach p146 Springer, Berlin.

Schultz, M.P., Swift, H.F. 1934 J. exp. Med. 60 323

Seligmann, M. 1960 CIBA Foundation Sym. 'Cellular Aspects of Immunity' p224. ed. Wolstenholme & O'Connor Churchill, London.

Sell, S., Weigle, W.O. 1959 J, Immunol, 83 257

Shaw, C.M., Alvord, E.C., Fahlberg, W.J., Kies, M.W.
1964a J. Immunol. 92 28

Shaw, C.M., Alvord, E.C., Kies, M.W.
1964b J. Immunol. 92 24

Silverstein, A.M., Uhr, J.W., Kramer, K.L., Lukes, R.J.
1963 J. exp. Med. 117 799

Pattee, P.A. 1966 J. Bact. 92 787

Perkins, E.H., Leonard, M.R. 1963 J. Immunol. 90 228

Pernis, B., Chiappino, G. 1964 Immunology 7 500

Pernis, B., Parinetto, F. 1962 Proc. Soc. exp. Biol. 110 390

Pfeiffer, R., Marx, L., 1898 Z. Hyg. Infectkr. 27 272

Pike, R.M. 1967 Bact. Rev. 31 157

Pike, R.M., Schulze, M.L. 1964 Proc. Soc. exp. Biol. Med. 115 829

Pike, R.M., Schulze, M.L., Chandler, C.H. 1966 J. Bact. 92 880

Porter, K.A., Couch, N.P. 1959 Brit. J. exp. Path. 40 32

Porter, R.R. 1962 in 'Basic Problems in Neoplastic disease' ed. Gellhorn & Hirschberg. p 177 Columbia Univ. Press.

Porter, R.R., Press, E.M. 1962 Ann. Rev. Bioch. 31 625

Rabinovitsch, L. 1897 Z. f. Hyg. 26 90

Raffel, S. 1948 J. inf. Dis. 82 267

Raffel, S., Arnaud, L.E., Dukes, C.D., Huang, J.S. 1949 J. exp. Med. 90 53

Raffel, S., Asselineau, J., Lederer, E. 1955 CIBA Foundation Sym. 'Experimental Tuberculosis' p174 Churchill, Ltd.

Raffel, S., Torney, J.E. 1948 J. exp. Med. 88 485

Ramon, G. 1931 Ann. Inst. Past. 47 339

Ramon, G. 1937 Rev. Immunol. 3 193

Ramon, G., Zoeller, C. 1927 Ann. Inst. Past. 41 803

Richter, M., Zimmermann, S., Haurowitz, F. 1965 J. Immunol. 94 938

Rivers, T.M., Schwentker, F.F. 1935 J. exp. Med. 61 689

Siskind, G.W., Paterson, P.Y.
1964 J. Immunol. 93 489

Slavin, D., 1950 Nature 165 115

Smith, R.T. 1960 in 'CIBA Foundation Sym. Cellular Apects of Immunity' p348 ed. Wolstenholme & O'Connor. Churchill, London.

Steiner, J.W., Langer, B., Scahtz, D.L.,
1960 Arch. Path. 70 424

Stewart-Tull, D.E.S. 1966 personal communication

Stewart?Tull, D.E.S., White, R.G.
1964 J. gen. Microbiol, 34 43

Stewart?Tull, D.E.S., Whits, R.G.
1966 Immunology 12 349

Stewart-Tull, D.E.S., Wilkinson, P.C., White, R.G.
1965 Immunology 9 151

Stone, S.H. 1961 Science 134 619

Stoner, R.D., Hale, W.M. 1955 J. Immunol. 75 204

Suter, E., White, R.G. 1954 Am. Rev. Tuberc. 70 793

Svehag, S., Mandel, B. 1962 Virology 18 508

Swift, H.F., Schultz, M.P. 1936a J. exp. Med. 63 705

Swift, H.F., Schultz, M.P. 1936b J. exp. Med. 63 725

Taliaferro, W.H., Taliaferro, L.G.
1950 J. inf. Dis. 87 37

Thorbecke, G.J., Keining, F.J.
1953 J. Immunol. 70 129

Thorbecke, G.J., Hochwald, G.M., Asofsky, R., Jacobson, E.B.
1964 in 'Protides of the Biological Fluids' p125 ed. Peeters. Elsevier, Amsterdam.

Tokumara, T. 1966 J. Immunol. 97 248

Topley, W.C. 1930 J. Path. Bact. 33 339

Torrigiani, G., Roitt, I.M. 1965 J. exp. Med. 122 181
Tromans, W.J., Horne, R.W. 1961 Virology 15 1
Turner, M.W., Rowe, D.S. 1964 Immunology 7 659
Uhr, J.W., Finkelstein, M.S. 1963 J. exp. Med. 117 457
Uhr, J.W., Finkelstein, M.S., Baumann, J. 1962 J. exp. Med. 115 655
Uhr, J.W., Dancis, J., Franklin, E.C., Finkelstein, M.S., Lewis, E.W. 1962 J. clin. Invest. 41 1509
Vaughan, J.H., Dutton, A.H., Dutton, R.W., George, M., Marsten, R.Q. 1960 J. Immunol. 84 258
Voisin, G., Toullet, F. 1963 Ann. Inst. Past. 104 169
Voisin, G., Toullet, F., Voisin, J. 1964 Ann. Inst. Past. 106 353
Wagner, R.R., Snyder, R.M., Hook, E.W., Luttrell, C.N. 1959 J. Immunol. 83 87
Waksman, B.H. 1960 in 'CIBA Foundation Sym. Cellular Aspects of Immunity' p280 ed. Wolstenholme & O'CONNOR. Churchill, London.
Waksman, B.H., Adams, R.D. 1953 J. inf. Dis. 93 21
Weissmann, G. 1964 Lancet ii 1373
White, R.G. 1958 Nature 182 1383
White, R.G. 1959 in 'Mechanisms of Hypersensitivity'. Little-Brown, Boston.
White, R.G. 1961 Sci. Basis of Med. Ann. Rev. p31
White, R.G. 1963 B.M.B. 19 207
White, R.G. 1967 B.M.B. 23 39
White, R.G., Bernstock, L., Johns, R.G.S., Lederer, E. 1958 Immunology 1 54
White, R.G., Jenkins, G.C., Wilkinson, P.C. 1963 Int. Arch. Allergy appl. Immunol. 22 156

White, R.G., Jolles, P., Samour, D., Lederer, E.
1964 Immunology 7 158

Wiener, A.S. 1952 Ann. Allergy 10 535

Wigzell, H. 1966 Ann. Med. exp. Fenn. 44 209

Wilkinson, P.C., Carmichael, D.S.
1964 J. Lab. Clin. Med. 64 529

Wilkinson, P.C., White, R.G.
1966 Immunology 11 229

Wilkinson, P.C. 1967 personal communication

Wilson, G.S., Miles, A.A. 1961 Topley & Wilson's Principles of Bacteriology and Immunity, 4th ed. Arnold, London.

Witebsky, E., Rose, N.R. 1956 J. Immunol. 76 408

Wolfe, H.R., Norton, S., Springer, E., Goodman, M., Herrick, C.A.
1950 J. Immunol. 64 179

Yarmolinsky, M.B., De La Haba, G.L.
1959 Proc. nat. Acad. Sci. 45 1721

Youngner, J.S., Axelrod, V. 1963 J. Immunol. 90 1

Zinsser, H. 1921 J. exp. Med. 34 495

The Effect of Adjuvants on Biosynthesis of 19S and 7S Antibody against Bacteriophage ΦX174 in the Guinea-Pig

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any significant difference between the mean k values of any of the four groups ($P>0{\cdot}05$). Thus antibody levels obtained by use of the adjuvants tested, simple water-in-oil emulsions, water-in-oil emulsions with added mycobacteria and bentonite were not significantly higher than those obtained by injection of antigen in saline alone.

The antibody obtained from selected 8-day sera was treated with 2-mercaptoethanol in order to determine whether it was 19S or 7S immunoglobulin. Sera from animals in each of the above mentioned groups were tested. In all cases, the k value following reduction dropped to negligible levels. Although our previous unpublished observations have shown that 7S antibody levels in sera from guinea-pigs immunized with ovalbumin may drop following treatment with 2-mercaptoethanol, the fall in antibody activity is usually less than 50 per cent of the original level using the doses of mercaptoethanol described here. It was, therefore, concluded that 8-day antibody against ΦX174 was almost entirely of the γM immunoglobulin type as Uhr and Finkelstein (1963) had previously stated. Thus, addition of adjuvants to injection mixtures containing phage ΦX174 had an insignificant effect on γM antibody levels in sera taken 8 days later.

TABLE 1

PHAGE NEUTRALIZATION LEVELS (k VALUES) OBTAINED FROM SERA TAKEN FROM GUINEA-PIGS 8 DAYS AFTER A SINGLE INJECTION OF BACTERIOPHAGE ΦX174 IN VARIOUS ADJUVANTS

Guinea-pigs injected with antigen in:			
Complete Freund's adjuvant	Incomplete Freund's adjuvant	Bentonite	Saline
0·4	0·30	0·8	1·1 (footpad)
2·8	1·43	11·5	1·05 (footpad)
3·3	0·31	3·5	1·35 (footpad)
0·76	1·53	1·2	0·1 (i.v.)
1·6	7·7	1·15	0·9 (i.v.)
2·3	1·01	9·2	1·04 (i.v.)
1·05	4·6	1·4	1·11 (i.v.)
1·35	0·81	2·76	0 (i.p.)

Phage was injected in Freund's adjuvants *via* the footpad; in bentonite *via* the peritoneum. The route of injection of saline-injected animals is indicated in the table.

EFFECTS OF ADJUVANTS ON SERUM ANTIPHAGE LEVELS AT THE 21ST DAY OF THE PRIMARY RESPONSE TO ΦX174

Antibody levels in sera from guinea-pigs bled at 21 days after a single injection in various adjuvant mixtures were again determined by the phage neutralization test. The k values for phage neutralization for sera from the same four groups of animals are shown in Table 2. Clear differences were seen between the serum antibody levels of adjuvant injected and non-adjuvant injected groups. High levels of antibody (median value 22·7) were found in sera from guinea-pigs injected *via* the footpad with complete Freund's adjuvant. Sera from animals injected by the same route with the water-in-oil emulsion lacking in mycobacteria yielded intermediate levels of antibody (median value 8·1). Antibody levels in animals injected via the footpad or intravenously with ΦX174 in saline alone were considerably lower (median value 3·4). The groups of guinea-pigs

injected intraperitoneally with bentonite-adsorbed phage behaved inconsistently. Two of these animals showed the highest serum antibody levels in the whole experiment. Others showed no antibody at all.

TABLE 2

PHAGE NEUTRALIZATION LEVELS (k VALUES) OBTAINED FROM SERA TAKEN FROM GUINEA-PIGS 21 DAYS AFTER A SINGLE INJECTION OF BACTERIOPHAGE ΦX174 IN VARIOUS ADJUVANTS

Guinea-pigs injected with antigen in:			
Complete Freund's adjuvant	Incomplete Freund's adjuvant	Bentonite	Saline
25·06	12·1	36·6	1·6 (footpad)
3·06	11·7	34·0	2·1 (footpad)
23·0	8·1	0·8	4·5 (i.v.)
29·1	8·9	0	3·7 (i.v.)
20·7	5·3	0	3·9 (i.v.)
18·1	8·05	0	3·4 (i.v.)
22·7	8·1	—	2·3 (footpad)

7S γ_1- AND γ_2-ANTIBODY AGAINST PHAGE 21 DAYS AFTER PRIMARY IMMUNIZATION

Sera from selected guinea-pigs from the groups injected with phage in complete mycobacterial adjuvant, in incomplete adjuvant and in saline alone which had been taken 21 days after primary immunization were fractionated on DEAE-cellulose columns to separate the 7S γ_1- and 7S γ_2-globulin antibody fractions. The separated fractions were then tested for phage neutralizing activity. The results of these experiments showed

TABLE 3

RESULTS OF CORNEAL TESTS 19 DAYS FOLLOWING INJECTION OF GUINEA-PIGS WITH BACTERIOPHAGE ΦX174 IN VARIOUS ADJUVANT MIXTURES

Adjuvant used			
Complete Freund's adjuvant	Incomplete Freund's adjuvant	Bentonite	Saline
2	0	1	1
3	1	0	0
0	3	1	0
1½	0	0	0
2	3	0	0
0	1	1	0
0	0	0	0
1	1		0

The scoring of corneal tests is described in 'Materials and methods' (see text).

that phage neutralizing activity was present in both fractions in all of the sera tested. Injection in incomplete Freund's adjuvant gave rise to higher antibody levels in both the γ_2- and the γ_1-fraction than did injection in saline alone and injection in complete Freund's adjuvant gave rise to still higher levels of antibody of both types. Due to scarcity of available material k values were not calculated for these fractions. The neutralization

curve for both globulins was, however, similar in the sera tested. Thus, adjuvants were effective in raising the level of both γ_1-globulin antibody and γ_2-globulin antibody against bacteriophage ΦX174 in 21-day guinea-pig sera.

DELAYED HYPERSENSITIVITY

The results of corneal tests with phage performed 19 days after primary immunization in groups of guinea-pigs injected with ΦX174 in various adjuvant mixtures are shown in Table 3. Animals injected with phage in saline gave negative corneal tests. Bentonite injected animals also showed negligible corneal reactions. Some of the animals in both the group injected with complete Freund's adjuvant and that injected with incomplete Freund's adjuvant gave positive corneal responses.

DISCUSSION

The immunoglobulin response of the guinea-pig against bacteriophage ΦX174, as described above, shows certain differences from that previously described when soluble proteins were used as test antigens (White *et al.*, 1963; Wilkinson and White, 1966). Against bacteriophage, all animals produced γM antibody detectable at 8 days after primary injection. Against ovalbumin, γM antibody was not detected either at 8 days or at any other time following primary immunization, whether quantitative precipitin tests, immunoelectrophoresis or radioimmunoelectrophoresis against ^{131}I-labelled antigen were used to detect it (authors' unpublished results). Moreover, 21 days after immunization 7S γ_1- and γ_2-globulin antibodies were both present following the injection of phage in saline alone. The use of water-in-oil adjuvants increased the level of antibody of both types and the incorporation of mycobacteria in these emulsions led to still higher γ_1- and γ_2-globulin antibody levels. On the other hand, 21 days after primary immunization with ovalbumin, γ_2-immunoglobulin antibody production against protein antigens only occurred when guinea-pigs were injected with mycobacterial or silica adjuvants (Wilkinson and White, 1966).

It is remarkable that when guinea-pigs are injected with protein antigens in incomplete Freund's adjuvant without mycobacteria, they consistently synthesize only 7S γ_1-globulin antibody. Earlier work in this laboratory reported this to be the case for three protein antigens; ovalbumin, human serum albumin and diphtheria toxoid. Benacerraf *et al.* (1963) reported similar findings for anti-hapten. However, when the same workers examined the response to a particulate antigen, the O-antigen of intact *E. coli* cells (Bloch, Kourilsky, Ovary and Benacerraf, 1963), they found that the antibody produced was 7S γ_2-globulin rather than 7S γ_1-globulin. As shown in the present study, 7S γ_2-globulin antibody against a much smaller particle, ΦX174, was synthesized without the addition of mycobacteria to the injected adjuvant mixture, although the quantitative effects of mycobacterial adjuvants on levels of both 7S γ_2- and 7S γ_1-globulin antiphage antibody were still very striking. A further difference between the immune response to soluble proteins and that to phage was seen when corneal tests were used to evaluate delayed hypersensitivity. Mycobacterial adjuvants are necessary to induce delayed hypersensitivity against protein antigens in the guinea-pig (White, Coons and Connolly, 1965). However, in the present experiments, positive corneal tests following a test

injection of bacteriophage were seen in a proportion of the animals immunized with antigen in water-in-oil emulsion which did not contain mycobacteria.

The incorporation of adjuvants with bacteriophage in injection mixtures had a negligible effect on levels of 19S anti-phage antibody subsequently attained as evaluated in sera taken on the 8th day following immunization. Adjuvants were not necessary to stimulate a 19S antibody response and the 19S antibody levels observed in guinea-pigs given phage in water-in-oil emulsions with and without mycobacteria or in mixtures with bentonite were not significantly higher than in those given phage in saline alone. This failure of adjuvants to exert an effect on 19S antibody levels may be related to the sites at which such antibody is synthesized and to the mode of access of antigen to these sites of antibody production discussed in the following paper (Fleming, Wilkinson and White, 1967). The effect of adjuvants on the duration of the γM response has not been reported here but is the subject of a further study now in progress.

It is necessary at this stage to discuss the validity of the use of k values derived from phage neutralization assays as a measure of immunoglobulin levels. As the capacity of unit amounts of individual guinea-pig immunoglobulins to neutralize bacteriophage is not known, k values obtained for one immunoglobulin type cannot be compared directly with those obtained for another. Finkelstein and Uhr (1966) have shown that guinea-pig 19S globulin has a higher phage neutralizing activity per unit weight than 7S globulin, although no information is available concerning the activity of 7S γ_1- and 7S γ_2-immunoglobulins. Circumstances for detection of γM anti-phage antibody are, therefore, optimal: (a) because of the extreme sensitivity of the phage neutralization test, and (b) because of the greater avidity of 19S than of 7S antiphage per unit weight. For these reasons it is not possible to compare antibody levels in sera taken 8 days after immunization with those in sera taken at 21 days.

Antibody of the γM-immunoglobulin type has not been detected in guinea-pigs following the injection of soluble protein antigens either by the authors of the present communication or in previously published reports. There are two possible reasons for the apparent absence of 19S antibody against soluble proteins when 19S antiphage is consistently present following injection of phage by similar routes using the same adjuvants. Firstly, as discussed above, the phage neutralization technique provides optimal conditions for detection of 19S antibody. Secondly, particulate antigens stimulate higher and more prolonged 19S antibody responses than are attained using soluble proteins. Bauer, Mathies and Stavitsky (1963) found that while primary injection of protein antigens stimulated an initial 19S response followed by a more prolonged 7S response, repeated injection of *Salmonella* O antigen resulted in 19S responses only, not 7S. Turner and Rowe (1964) also showed a sustained 19S antibody response against *S. typhi*-O in man. Torrigiani and Roitt (1965) showed that thyroglobulin coated onto acrylic resin particles stimulated a more vigorous and sustained 19S response in rabbits than the same antigen in solution. However, bacteriophage ΦX174 is an extremely small particle. Tromans and Horne (1961) report a maximum diameter of 225 Å; only slightly larger than an antibody molecule. The immunoglobulin response against this and other small virus particles may well resemble more closely the response after stimulation by soluble proteins than that which follows injection of much larger particles such as bacteria and red cells. Fahey and Horbett (1959) found most of the antibody against mumps virus in the 7S (IgG) fraction of human sera. Tokumaru (1966) noted an initial rise in IgM antibody against herpes simplex virus in man. At 21 days a predominance of IgA and IgG antibody was observed.

These responses against viral antigens are similar to those reported after injection of protein antigens in many species and indeed similar to those reported against bacteriophage ΦX174 in this paper.

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REFERENCES

ADAMS, M. H. (1959). *Bacteriophages*. Interscience, New York.

BAUER, D. C., MATHIES, M. J. and STAVITSKY, A. B. (1963). 'Sequences of synthesis of γ_1-macroglobulin and γ_2-globulin antibodies during primary and secondary responses to proteins, salmonella antigens and phage.' *J. exp. Med.*, **117**, 889.

BENACERRAF, B., OVARY, Z., BLOCH, K. J. and FRANKLIN, E. C. (1963). 'Properties of guinea-pig 7S antibodies. I. Electrophoretic separation of two types of guinea-pig 7S antibodies.' *J. exp. Med.*, **117**, 937.

BLOCH, K. J., KOURILSKY, F. M., OVARY, Z. and BENACERRAF, B. (1963). 'Properties of guinea-pig 7S antibodies. IV. Response to *E. coli* bacteria.' *Proc. Soc. exp. Biol. (N.Y.)*, **114**, 52.

FAHEY, J. L. and HORBETT, A. P. (1959). 'Human gamma globulin fractionation on anion exchange columns.' *J. biol. Chem.*, **234**, 2645.

FINKELSTEIN, M. S. and UHR, J. W. (1966). 'Antibody formation. V. The avidity of γM and γG guinea pig antibodies to bacteriophage ΦX174.' *J. Immunol.*, **97**, 565.

FLEMING, W. A., WILKINSON, P. C. and WHITE, R. G. (1967). 'Sites of biosynthesis of immunoglobulins in guinea-pigs immunized with bacteriophage ΦX174.' *Immunology*, **13**, 613.

HERSHEY, A. D., KALMANSON, G. and BRONFENBRENNER, J. (1943). 'Quantitative methods in the study of the phage–antiphage reaction.' *J. Immunol.*, **46**, 267.

STEWART-TULL, D. E. S., WILKINSON, P. C. and WHITE, R. G. (1965). 'The affinity of a mycobacterial glycopeptide for guinea-pig γ-globulin.' *Immunology*, **9**, 151.

TOKUMARU, T. (1966). 'A possible role of γA-immunoglobulin in herpes simplex virus infection.' *J. Immunol.*, **97**, 248.

TORRIGIANI, G. and ROITT, I. M. (1965). 'The enhancement of 19S antibody production by particulate antigen.' *J. exp. Med.*, **122**, 181.

TROMANS, W. J. and HORNE, R. W. (1961). 'The structure of bacteriophage ΦX174.' *Virology*, **15**, 1.

TURNER, M. W. and ROWE, D. S. (1964). 'Characterization of human antibodies to *Salmonella typhi* by gel-filtration and antigenic analysis.' *Immunology*, **7**, 639.

UHR, J. W. and FINKELSTEIN, M. S. (1963). 'Antibody formation. IV. Formation of rapidly and slowly sedimenting antibodies and immunological memory to bacteriophage ΦX174.' *J. exp. Med.*, **117**, 457.

WHITE, R. G., COONS, A. H. and CONNOLLY, J. M. (1955). 'Studies on antibody production. IV. The role of wax fraction of *Mycobacterium tuberculosis* in adjuvant emulsions on the production of antibody to egg albumin.' *J. exp. Med.*, **102**, 83.

WHITE, R. G., JENKINS, G. C. and WILKINSON, P. C. (1963). 'The production of skin-sensitizing antibody in the guinea-pig.' *Int. Arch. Allergy*, **22**, 156.

WILKINSON, P. C. and WHITE, R. G. (1966). 'The role of mycobacteria and silica in the immunological response of the guinea-pig.' *Immunology*, **11**, 229.

Sites of Biosynthesis of Immunoglobulins in Guinea-Pigs Immunized with Bacteriophage ΦX174

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Sites of Biosynthesis of Immunoglobulins in Guinea-Pigs Immunized with Bacteriophage ΦX174

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Summary. The sites of biosynthesis of 19S and 7S antibody in the guinea-pig were determined by assay of the phage neutralizing activity in *in vitro* cultures of lymph node, bone marrow and splenic tissues from guinea-pigs immunized with bacteriophage ΦX174. Per unit weight of tissue, much higher levels of 19S antibody were synthesized by bone marrow tissue cultures than by those of the spleen or the lymph nodes draining the site of antigen injection. On the other hand, the levels of synthesis of 7S antibody per unit weight of tissue were higher in cultures of the homolateral and contralateral lymph nodes draining the site of injection into the footpad than in cultures of spleen or bone marrow. Evidence was presented that the phage neutralizing activity observed was, in fact, due to synthesis of specific antibody.

INTRODUCTION

The results of *in vitro* investigations of the sites of synthesis of guinea-pig 7S anti-ovalbumin, 21 days after a single injection of ovalbumin in complete Freund's adjuvant into the left hind footpad, were reported by Askonas and White (1956) and Askonas, White and Wilkinson (1965). These investigations showed that the maximum *in vitro* synthesis of anti-ovalbumin per unit weight of tissue incubated occurred in the popliteal and flank (iliac) lymph nodes contralateral to the site of injection. Synthetic activity was also found in the homolateral popliteal and iliac lymph nodes, but relatively little in the spleen and bone marrow.

Certain major differences between the response to soluble protein antigens and that to bacteriophage ΦX174 were observed in experiments described in the preceding paper (Wilkinson, Fleming and White, 1967). Injection of ovalbumin, whether in saline or in various adjuvant mixtures, gave rise to no detectable 19S antibody response. In contrast, after injection of bacteriophage in saline or in adjuvant mixtures, a 19S antibody response was consistently detected 8 days after primary immunization. However, no significant difference in serum γM antibody levels was observed whether the bacteriophage was injected in saline or in adjuvant mixtures. On the other hand, 21 days after injection 7S antibody was consistently detected against both ovalbumin and bacteriophage, and injection of antigen in adjuvants caused marked increases in the level of serum antibody obtained.

In view of the failure of adjuvants to enhance the 19S response, experiments were undertaken in which the sites of synthesis of guinea-pig 19S antiphage antibody were

determined and compared by *in vitro* methods with those of synthesis of 7S antibody later in the primary response. The suggestion that the sites of biosynthesis of 7S and 19S antibody globulins against the same antigen might be dissimilar is explored in the investigations described below.

MATERIALS AND METHODS

Freund-type complete and incomplete adjuvant mixture

Bacteriophage ΦX174, prepared as described by Wilkinson *et al.* (1967), was suspended in saline at a concentration of 2×10^{10} particles/ml. This suspension was incorporated in a water-in-oil emulsion made up in the following proportions; saline suspension of phage, 1 part; Arlacel A, 1 part; Bayol 55, 3 parts. Each guinea-pig was injected with 0·5 ml of the above, as a well-emulsified mixture (incomplete Freund-type adjuvant mixture). Other animals were injected with the above mixture with the addition to each dose of 200 μg heat killed *Mycobacterium tuberculosis* strain C, kindly supplied by the Ministry of Agriculture, Fisheries and Food, Weybridge, Surrey (complete Freund-type adjuvant mixture). This complete Freund's adjuvant mixture was well emulsified before injection.

Four groups of guinea-pigs were immunized as follows:

Group 1. Injected into the left hind footpad with 2×10^9 plaque forming units (PFU) of ΦX174 in complete Freund-type adjuvant made up as above.

Group 2. Injected as above with 2×10^9 PFU of ΦX174 in incomplete Freund-type adjuvant (lacking in mycobacteria).

Group 3. Injected with a phage–bentonite mixture, prepared as described by Wilkinson and White (1966), intraperitoneally, 0·3 ml in each flank.

Group 4. Injected into the left hind footpad with 2×10^9 PFU of ΦX174 in 0·5 ml of saline. In addition a further group received an injection of staphylococcal phage 80 in complete Freund-type adjuvant mixture into the left hind footpad.

Preparation and in vitro *culture of various lymphoid tissues*

Guinea-pigs were killed on the 8th or 21st day after a single injection of antigen. Immediately after death, samples of spleen, bone marrow and the lymph nodes draining the site of injection (i.e. the flank nodes, homolateral and contralateral, in footpad injected animals, and mesenteric nodes in bentonite injected animals) were removed and placed in ice-cold Eagle's medium (Paul, 1959). The tissues were gently teased out to give small fragments; these were then washed thoroughly three times in ice-cold Eagle's medium by centrifugation in order to remove possible contamination by serum antibody. Known weights of tissue were incubated at 37° in a constant temperature waterbath for 6 hours in 2 ml Eagle's medium with 10 per cent calf serum in an atmosphere of 5 per cent CO_2, 95 per cent O_2. Gentle agitation was maintained throughout the period of incubation. At the end of this period of incubation, the tissue samples were disrupted by freezing and thawing three times and the cell debris removed by centrifugation at 12,000 rev/min for 30 minutes at a controlled temperature of 4°. The supernatant was then assayed for antiphage activity using the phage neutralization test.

Phage neutralization tests

Antibody activity against phage was assayed by a phage neutralization test based on the method of Uhr and Finkelstein (1963), and described by Wilkinson *et al.* (1967). A

mixture of bacteriophage and the tissue culture supernatant was allowed to react at 37° for 30 minutes. After this time, samples of the mixture were rapidly diluted in order to stop further antigen–antibody reaction, and the number of un-neutralized infective phage particles remaining was estimated using the agar layer technique. Blank values were obtained by mixing phage with sterile uninoculated tissue culture medium in place of the tissue culture supernatant.

Inactivation of phage by its antibody follows first order kinetics, expressed by the following equation:

$$k = \frac{2{\cdot}3D}{t} \log \frac{P^0}{P},$$

where P_0 is the initial bacteriophage titre, P is the bacteriophage titre at time t in minutes, D is the dilution of antiserum and k is the first order inactivation constant, expressed in minutes^{-1}. Using tissue culture supernatants in a preliminary experiment, a plot of log P_0/P against time gave a straight line showing that neutralization of phage by tissue culture supernatants followed the above equation.

In this series of experiments, where t is fixed at 30 minutes then log (P_0/P) is proportional to the concentration of antibody present. All results have, therefore, been expressed as the number of phage particles neutralized per unit weight (0·1 g) of tissue incubated, and not as k values.

RESULTS

RELATIVE *in vitro* ACTIVITIES OF VARIOUS LYMPHOID ORGANS OF THE GUINEA-PIG IN THE BIOSYNTHESIS OF ANTIBODY TO PHAGE ΦX174 AT 8 DAYS AFTER ANTIGEN INJECTION

Fig. 1 shows the antiphage activity of supernatants from tissue cultures prepared from the following groups of animals.

(1) Guinea-pigs injected with phage in complete Freund's adjuvant *via* the footpad.
(2) Guinea-pigs injected with phage in incomplete Freund's adjuvant *via* the footpad.
(3) Guinea-pigs injected with phage in saline *via* the footpad.
(4) Guinea-pigs injected with phage in bentonite intraperitoneally.

Antiphage activity was consistently present in all lymphoid tissue culture supernatants from the four groups of animals. From any one animal, bone marrow consistently gave the highest level of antiphage activity per unit weight of tissue incubated. In some cases the iliac lymph nodes homolateral to the site of injection also showed a high activity, but in no case did this activity exceed that of the bone marrow from the same animal. Further, considerable variation was found in the level of activity of the iliac lymph nodes homolateral and contralateral to the site of injection. In some cases, the activity of the homolateral nodes exceeded that of the contralateral nodes and vice-versa. The activity of the spleen was consistently low. In the case of animals injected with phage in bentonite, where the mesenteric lymph nodes only were studied, the activity found per unit weight of tissue incubated was intermediate between that found in bone marrow and spleen.

It was concluded that the highest level of synthesis of antibody against phage per unit weight of tissue, 8 days after a single injection of ΦX174, occurred in the bone marrow.

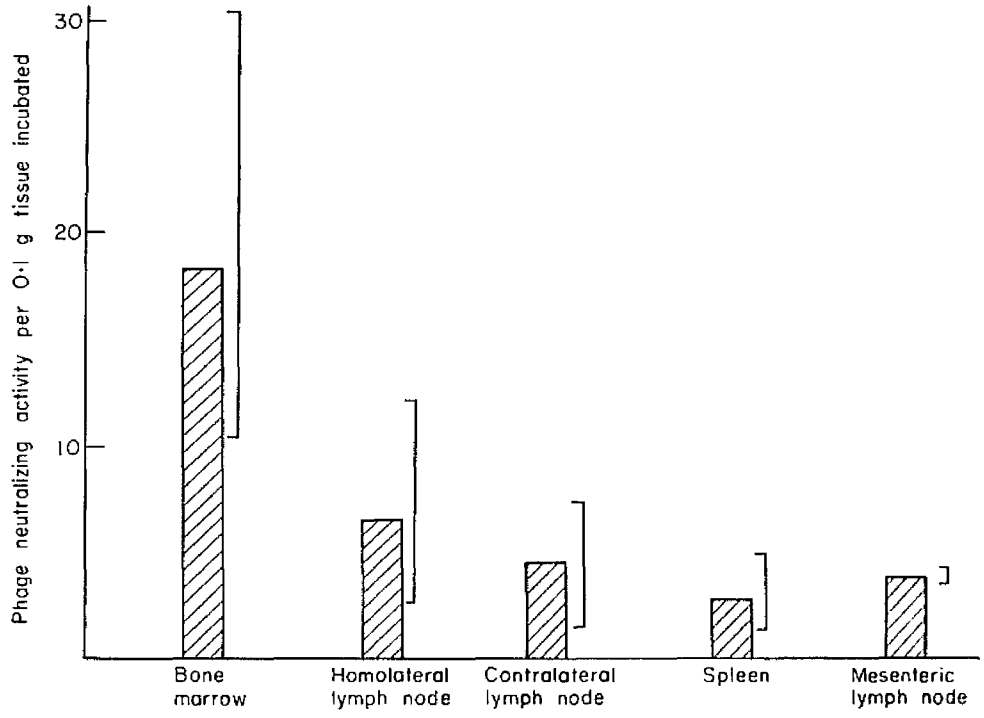

FIG. 1. Phage neutralizing activity of various lymphoid tissues taken 8 days after a single injection of ΦX174. Note that the columns represent levels of activity in tissues from a single typical guinea-pig. The upper limit of the brace indicates the highest level of phage neutralizing activity observed in a given tissue from the whole group of guinea-pigs. The lower limit of the brace indicates the lowest level of activity observed.

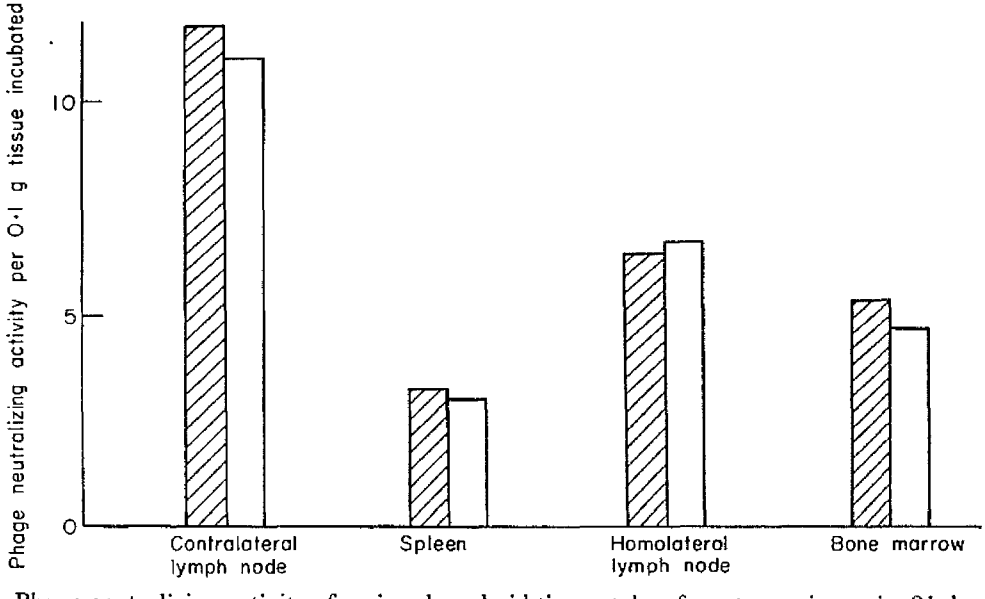

FIG. 2. Phage neutralizing activity of various lymphoid tissues taken from two guinea-pigs 21 days after a single injection of ΦX174. Cross-hatched columns, guinea-pig given complete Freund's adjuvant; open columns, guinea-pig given incomplete Freund's adjuvant.

RELATIVE *in vitro* ACTIVITIES OF VARIOUS LYMPHOID ORGANS OF THE GUINEA-PIG IN THE BIOSYNTHESIS OF ANTIBODY TO PHAGE ΦX174 AT 21 DAYS AFTER INJECTION OF ANTIGEN

As seen from Fig. 2, the major antiphage activity per unit weight of tissue in animals killed on the 21st day after a single injection with ΦX174 was found in the lymph nodes.

In animals injected with phage in incomplete and complete Freund's adjuvant *via* the footpad, the iliac lymph nodes contralateral to the site of injection showed maximal antiphage activity. In these animals, the spleen and bone marrow also showed antiphage activity, but consistently less than was found in the lymph nodes on a unit weight basis. The spleen again had the lowest activity of the four tissues studied. In animals injected with phage in saline *via* the footpad, the activity of the contralateral iliac lymph nodes was not greatly different from that of the homolateral lymph nodes whereas the activity of the spleen and bone marrow was significantly lower.

It was, therefore, concluded that 21 days after a single injection of ΦX174 using various adjuvants, the highest synthesis of antibody per unit weight of tissue occurred in the lymph nodes. Smaller amounts were synthesized by bone marrow and spleen.

NET SYNTHESIS OF ANTIPHAGE ANTIBODY *in vitro*

Experiments were carried out to determine whether the antiphage antibody detected after tissue culture was present in the tissues before incubation, or was actively synthesized during the period of incubation.

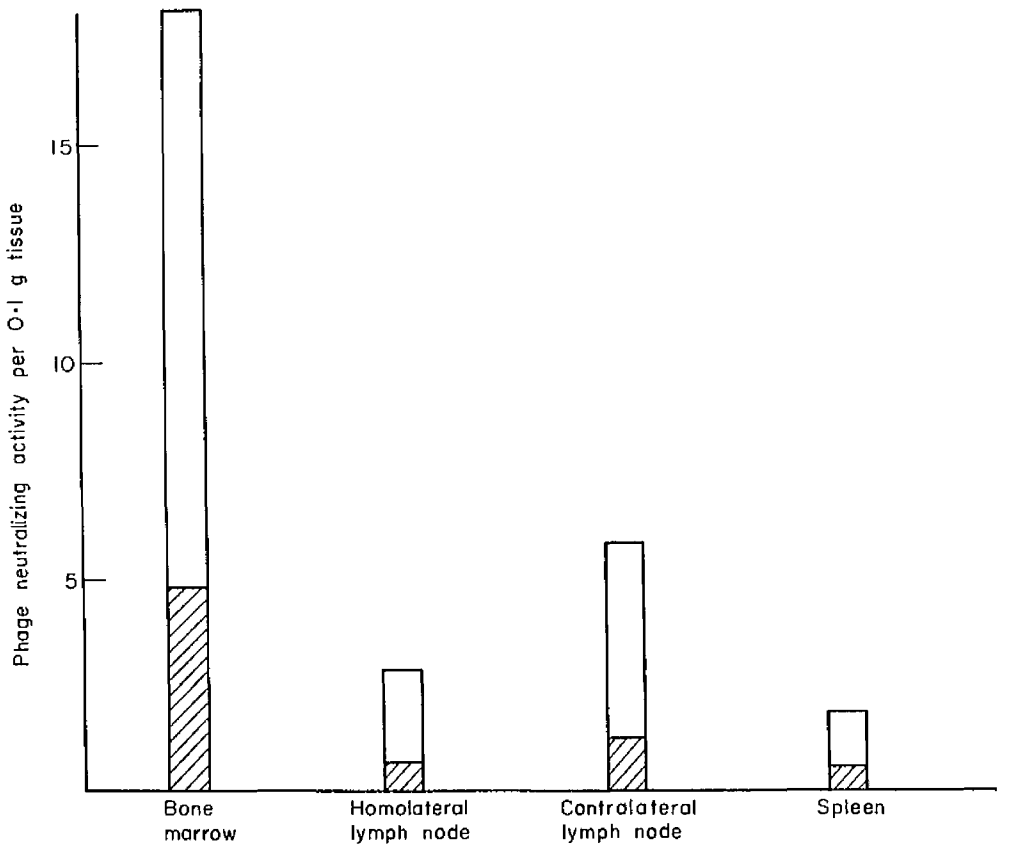

FIG. 3. Phage neutralizing activity of various lymphoid tissues taken 8 days after a single injection of ΦX174, before and after *in vitro* incubation. Open columns, post-incubated; cross-hatched columns, pre-incubated.

Samples of spleen, bone marrow and lymph nodes were removed from an animal 8 days after a single injection of antigen in complete Freund-type adjuvant mixture. Each sample was divided into two approximately equal parts, and both were placed in tissue

culture medium. One portion was immediately deep-frozen in a solid CO_2–ethanol mixture and the other was incubated as described above (see 'Materials and methods'). After the period of incubation, both portions of tissue were disrupted by freezing and thawing thrice and the supernatants examined for antiphage activity. The results (Fig. 3) showed that approximately 20 per cent of the activity per unit weight of tissue incubated was present in the tissues before incubation. The remaining 80 per cent was, therefore, presumed to have arisen by *de novo* synthesis of newly formed antibody during the 6-hour period of *in vitro* incubation.

EFFECT OF PUROMYCIN ON BIOSYNTHETIC ACTIVITY OF BONE MARROW CULTURES

A sample of bone marrow from an animal immunized 8 days previously with ΦX174 was divided into four portions. These samples were then tissue cultured in the presence of the anti-metabolite, puromycin, incorporated in the tissue culture medium at different molar strengths as follows:

Sample 1: tissue culture medium only.
Sample 2: 3×10^{-4} M puromycin in tissue culture medium.
Sample 3: 3×10^{-5} M puromycin in tissue culture medium.
Sample 4: 3×10^{-6} M puromycin in tissue culture medium.

After incubation the antiphage activity of the supernatants was determined. A concentration of 3×10^{-4} M puromycin was found to reduce the antiphage activity to a level comparable with that obtained from non-incubated tissue, i.e. approximately 20 per cent of the activity of the portion incubated in tissue culture medium only.

In view of these findings, and since puromycin is known to inhibit protein synthesis at the point of transfer of amino acids from RNA to protein, it was concluded that *de novo* synthesis of protein, and consequently of antibody, was occurring under the conditions of tissue culture used.

COMPARISON OF SYNTHETIC ACTIVITY FOR ANTIBODY TO ΦX174 IN TISSUE CULTURES OF BONE MARROW FROM THE TIBIAE HOMOLATERAL AND CONTRALATERAL TO THE SITE OF INJECTION

Red bone marrow from the tibiae homolateral and contralateral to the site of injection was removed from an animal 8 days after a single injection with ΦX174 in complete Freund's adjuvant *via* the footpad. Samples from each were cultured separately, and their ability to synthesize antibody against phage determined. As seen in Fig. 4, little variation in the activity of cultures of bone marrow from these sites was found. It was concluded, therefore, that the activity of the tibial bone marrow bore no relation to its proximity to the site of antigen injections, i.e. the left hind footpad.

INVESTIGATION OF TISSUES FROM NON-IMMUNIZED ANIMALS

Samples of spleen, bone marrow and iliac lymph node tissue from four healthy uninjected guinea-pigs were cultured *in vitro*, for a 6-hour period and examined for the production of antiphage activity as described above. In none of these cultures was detectable neutralization of ΦX174 found in the supernatant.

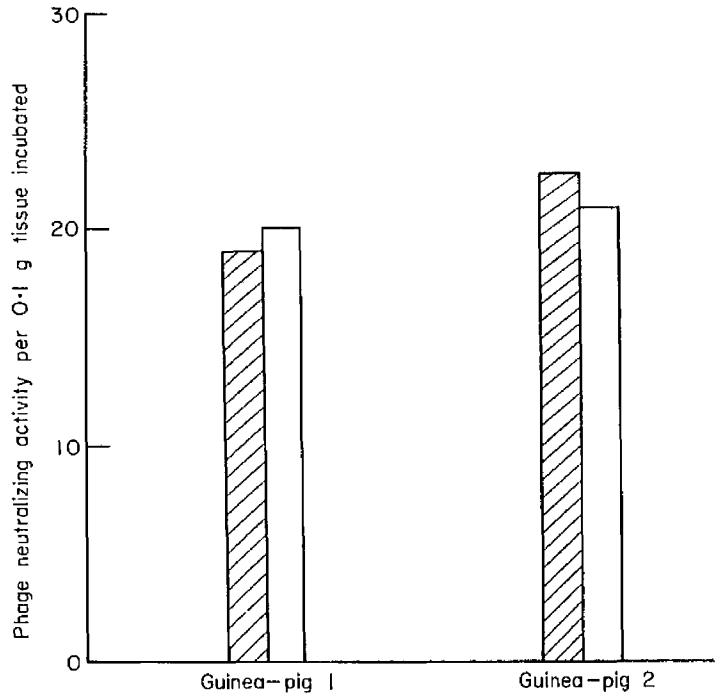

FIG. 4. Levels of phage neutralizing activity in tissue cultures of bone marrow taken from the tibiae homolateral (cross-hatched columns) and contralateral (open columns) to the site of injection of two guinea-pigs with ΦX174 8 days previously.

TABLE 1

THE EFFECT OF TREATMENT WITH 2-MERCAPTOETHANOL ON LEVELS OF PHAGE NEUTRALIZING ACTIVITY IN LYMPHOID TISSUE CULTURES TAKEN 8 DAYS AFTER IMMUNIZATION WITH BACTERIOPHAGE ΦX174

	Phage neutralizing activity per 0·1 g of tissue	
	Before treatment with 2-mercaptoethanol	After treatment with 2-mercaptoethanol
Guinea-pig No. 1: injected by the footpad		
Spleen	2·8	0·15
Homolateral lymph node	—	—
Contralateral lymph node	1·7	0·1
Bone-marrow	18·1	1·9
Guinea-pig No. 2: injected by the footpad		
Spleen	4·3	0·3
Homolateral lymph node	6·9	1·0
Contralateral lymph node	—	—
Bone marrow	21·1	1·5
Guinea-pig No. 3: injected intraperitoneally		
Spleen	2·1	0·1
Mesenteric lymph node	3·5	3·4
Bone marrow	15·9	4·0

INVESTIGATION OF TISSUES FROM ANIMALS INJECTED WITH A STAPHYLOCOCCAL PHAGE

Bone marrow, spleen and lymph nodes from guinea-pigs immunized against staphylococcal phage 80 were cultured *in vitro* over a 6-hour period and examined for the production of neutralizing activity in the supernatant against the homologous staphylococcal

phage and against phage ΦX174. Although the tissues studied showed neutralizing activity against the staphylococcal phage, they showed no activity against ΦX174.

From these results it was concluded that the observed activity of tissues from animals immunized with ΦX174 was specifically due to the injection of that phage.

EFFECT OF 2-MERCAPTOETHANOL ON THE PHAGE NEUTRALIZING ACTIVITY OF SUPERNATANTS FROM CULTURES OF TISSUES OF ANIMALS KILLED 8 DAYS AFTER IMMUNIZATION

Selected tissue culture supernatants were treated with 2-mercaptoethanol after determination of their antiphage activity. The antiphage activity was again determined after treatment. As shown in Table 1, treatment with 2-mercaptoethanol almost totally eliminated the antiphage activity of all but one of the supernatants indicating that the antibody formed *in vitro* by these lymphoid tissues 8 days after a single injection of phage was of the 19S immunoglobulin type.

DISCUSSION

The present series of experiments in which bacteriophage ΦX174 was injected in various adjuvant mixtures into the guinea-pig footpad, shows that the pattern of antibody biosynthesis among the various lymphoid organs 21 days after injection was similar to that described for ovalbumin by Askonas and White (1956) and Askonas *et al.* (1965). In all cases, the highest level of synthetic activity for antibody against phage, per unit weight of tissue incubated, was found in the iliac lymph nodes. When complete Freund's adjuvant (including 200 μg heat killed mycobacteria) or incomplete Freund's adjuvant (lacking mycobacteria) was used, higher levels of activity were found in the iliac nodes contralateral to the site of injection than in the homolateral. Smaller activities per unit weight of tissue were produced at sites more distant from the injection such as bone marrow and spleen.

By contrast, when animals were investigated at 8 days after injection of the guinea-pig with phage, the highest level of synthetic activity per unit weight of tissue was found in the bone marrow, and phage neutralizing activity was consistently higher in this tissue than in any of the lymph nodes or in the spleen. As was shown in experiments in which the activity of tibial bone marrow homolateral and contralateral to the site of injection into the footpad was tested, and also as demonstrated by the results from animals receiving phage intraperitoneally in bentonite, the high activity of the bone marrow was not related to proximity to the site of injection. It could not, therefore, be explained by local permeation of antigen from the injection site into adjacent bone marrow. This antibody synthesized 8 days after primary injection was of the γM-immunoglobulin type. Treatment of all but one of the tissue culture supernatant fluids taken at this time with 2-mercaptoethanol eliminated phage neutralizing activity. Further, treatment with 2-mercaptoethanol and DEAE-cellulose chromatography demonstrated γM antibody in serum taken from these animals at the same time (Wilkinson *et al.*, 1967).

The bone marrow was, therefore, the major site of synthesis of γM-antibody against bacteriophage in the guinea-pig. Taking the synthetic activity per unit weight of tissue of the homolateral iliac lymph nodes as an arbitrary 100 units, the relative activities per unit weight of the other tissues studied were as follows: bone marrow, 460 units; contralateral iliac nodes, 107 units ; spleen, 63 units. Askonas and Humphrey (1957) calculated

the total synthesis of antibody by lymphoid tissues in the body as a whole by extrapolation from synthetic activity of known weights of tissue and concluded that bone marrow made a major contribution to the overall calculated antibody synthesis against protein antigens because of its large mass relative to other lymphoid tissues. By the same token, if the activity of tibial bone marrow in the present series of experiments is representative of total bone marrow in the body, then synthesis of γM-antibody against phage must be almost entirely due to the synthetic activity of bone marrow.

The possibility has been considered that the phage neutralizing activity of the tissue culture supernatant fluids of incubated lymphoid tissues may have been due to an agent other than specific antibody. That the activity was in fact due to specific antibody is supported by the following evidence: (1) Neutralization of phage by tissue culture fluids followed first order chemical kinetics as does neutralization of phage by specific antiserum. (2) The activity of tissue culture fluids taken 8 days after immunization was abolished by treatment with 0·1 M 2-mercaptoethanol. The activity of serum taken at 8 days after immunization and presumably dependent on 19S antibody was similarly abolished by treatment with 2-mercaptoethanol. This suggests that, at the same time as a 19S antibody response is detectable in the serum, similar 19S antibody is being synthesized by lymphoid tissues. (3) The antibiotic puromycin which inhibits DNA dependent RNA synthesis (Yarmolinsky and De La Haba, 1959) prevented the appearance of phage neutralizing activity. (4) The activity was specific to the injected phage. Animals injected with a staphylococcal phage did not show phage neutralizing activity against bacteriophage ΦX174. (5) Tissues taken from unimmunized animals failed to show phage neutralizing activity after *in vitro* culture.

The injection of protein antigens in complete Freund-type adjuvant mixture typically results in higher levels of serum antibody than are produced if mycobacteria are omitted from the injection mixture. The large volume of previous work on this topic has been concerned in the main with effects on 7S γ_1- and 7S γ_2-immunoglobulins. It is, therefore, of interest that the levels of phage neutralizing activity achieved by various tissues at 8 days after primary immunization were unaffected by the presence or absence of any of the three adjuvant mixtures including complete Freund's adjuvant mixture. However, in the present work, the tissues taken from animals at 21 days after injection which produced the highest levels of phage neutralizing activity were those from animals injected with complete Freund-type adjuvant mixture including 200 μg heat-killed *M. tuberculosis* per animal.

The production of 19S antibody per unit weight of tissue at 8 days is predominantly undertaken by the bone marrow. In contrast, the production of 7S γ_1- and γ_2-antibody per unit weight of tissue at 21 days is predominantly undertaken by the lymph nodes. This argues that two distinct populations of cells are concerned in the manufacture of 19S and 7S antibody and does not support the concept (Nossal, Szenberg, Ada and Austin, 1964) that production of 19S and 7S antibody are undertaken by the same cells working in a sequence of early 19S succeeded by later 7S antibody production. The activation of the two populations of cells will depend on the conditions governing access of antigen to these tissues. Thus phage particles injected via the footpad will be disseminated predominantly by the blood stream (Kosunen and Kääriäinen, 1966). Such antigen will reach the bone marrow, which lacks a lymphatic supply, exclusively by haematogenous dissemination. On the other hand, antibody production in the draining lymph nodes at 21 days is likely to have been stimulated by antigen which travelled *via* the

lymphatics. If retention of antigen at the site of injection is an important factor in adjuvant action, a clear adjuvant effect might be expected on antibody production in lymph nodes but not to the same extent in bone marrow to which antigen is rapidly disseminated. The differential effect of mycobacterial and other adjuvants on production of phage neutralizing antibody at 21 days supports the concept that the production of 19S and 7S antibody is subserved by different populations of cells.

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REFERENCES

Askonas, B. A. and Humphrey, J. H. (1958). 'Formation of specific antibodies and γ-globulin *in vitro*. A study of the synthetic ability of various tissues from rabbits immunised by different methods.' *Biochem. J.*, **68**, 252.

Askonas, B. A. and White, R. G. (1956). 'Sites of antibody production in the gunea-pig. The relation between *in vitro* synthesis of anti-ovalbumin and γ-globulin and distribution of antibody-containing plasma cells.' *Brit. J. exp. Path.*, **37**, 61.

Askonas, B. A., White, R. G. and Wilkinson, P. C. (1965). 'Production of γ_1-, and γ_2-antiovalbumin by various lymphoid tissues of the guinea-pig.' *Immunochemistry*, **2**, 329.

Kosunen, T. U. and Kääriäinen, L. J. (1966). 'Studies on circulation and elimination of viruses in the guinea-pig.' *Ann. Med. exp. Fenn.*, **44**, 32.

Nossall, G. T., Szenberg, A., Ada, G. L. and Austin, C. M. (1964). 'Single cell studies on 19S antibody production.' *J. exp. Med.*, **119**, 485.

Paul, J. (1959). *Cell and Tissue Culture*. Livingstone, Edinburgh.

Uhr, J. W. and Finkelstein, M. S. (1963). 'Antibody formation. IV. Formation of rapidly and slowly sedimenting antibodies and immunological memory to bacteriophage ΦX174.' *J. exp. Med.*, **117**, 457.

Wilkinson, P. C. and White, R. G. (1966). 'The role of mycobacteria and silica in the immunological response of the guinea-pig.' *Immunology*, **11**, 229.

Wilkinson, P. C., Fleming, W. A. and White, R. G. (1967). 'The effect of adjuvants on biosynthesis of 19S and 7S antibody against bacteriophage ΦX174 in the guinea-pig.' *Immunology*, **13**, 603.

Yarmolinsky, M. B. and De La Haba, G. L. (1959). 'Inhibition by puromycin of amino-acid incorporation into protein.' *Proc. nat. Acad. Sci. (Wash.)*, **45**, 1721.