The Antigenic Stimulus

by

J.M. Stark, M.B.,Ch.B.

A thesis submitted for the degree of

Doctor of Medicine

in the

University of Glasgow.

March, 1969.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

CONTENTS

Acknowledgments

My sincere thanks are due to Professor R.G. White for his constant support and encouragement throughout this work.

I am also extremely grateful to have had the enjoyable opportunity of collaborating with Professor White and Dr. Valentine French in the study of the immune response in the chicken.

I should like to record my indebtedness to Mr. J. S. Orr and Miss Jean Laurie, officers of the Regional Physics Laboratory, for many instructive discussions, as well as their advice on technical problems associated with equipment.

I wish to acknowledge the help of Dr. A. J. Harley of the National Lending Library for Science and Technology for his helpfulness in arranging the MEDLARS searches of the literature.

I have also to thank: Mr. R. Callander and Mr. J. Ramsden of the Medical Illustration and Photography Unit for their preparation of the diagrams for this thesis; Mr. T. Duffy and the staff of the animal house unit for their careful attention and help during the experiments; Mrs. Sheila McLaren for her skilled assistance in carrying out many of the antibody estimations.

I am also greatly indebted to Mrs. R. Dean for her typing of this thesis.

J.M.S.

"It is certain that all bodies whatsoever though they have no sense yet they have perception for when one body is applied to another there is a kind of election to embrace that which is agreeable and to exclude or expel that which is ingrate: and whether the body be alterant or altered, evermore a perception precedeth operation for else all bodies would be like one another."

Francis Bacon. 1620.

Quoted by Monod, Wyman, and Changeux (1965) in the context of allosteric transition of enzymic proteins (J.mol.Biol., 12,88).

Foreword

The immune response has been increasingly studied in the last decade. This interest has largely stemmed from the understanding provided by the work of Medawar, Burnet and their colleagues concerning the boundaries of the immune response. The momentum of research has been maintained for several reasons. Firstly the surgical practice of organ transplantation has emphasised the importance of immunological tolerance and suggests that a greater physiological and immunochemical knowledge might bring therapeutic rewards. Secondly work has been stimulated by the concept that malignant growth may not be "an inevitable genetic vice" but rather may somehow be related to failure of immunological surveillance (Burnet 1967). If the failure of the immune mechanism were fully understood and could be made good by appropriate stimulation there is again a chance of clinical benefit. Lastly the immunological model has been thought of as being potentially useful in the study of cell differentiation brought about by a specific stimulus although this model has proved perhaps an unexpectedly complex one (Watson 1966).

The work presented here is submitted as a contribution to the understanding of the physiology of the immune response. It is primarily concerned with the initiation of the reaction to the stimulus of antigen. The first study provides a reexamination in the chicken of the immune response

using labelled techniques whereby the point of recognition of the antigen is related to cellular and humoral changes. The second study hinges also on the point of recognition of an antigen, this time in an experimental model in mice. In this model a potential antigen not able by itself to stimulate an immune response is recognized and acted against as an antigen by virtue of the action of an immunological stimulant or "adjuvant" (L. adiuvare: to help). By further examination of the action of substance mimicking the putative actions of classical adjuvants, the mechanism of adjuvant action and in turn the switching-on of differentiation to antibody production are to some extent elucidated.

Chapter One

Introduction

An antigen is a macromolecular substance, most commonly protein in nature, which is able to stimulate an immune response when introduced into an animal host. This response takes the form of the production of specifically directed molecules or cells which can be demonstrated to react specifically in some immunological mode either *in vivo* or *in vitro* with the antigen concerned. This specificity is known from the work of Landsteiner to be directly related to the shape in space of part of the surface of the antigen molecule, such a specific stereochemical zone equivalent in size to a pentapeptide (Kabat, 1965) being termed a "determinant". The antibody molecule or the reacting cellular surface is complementary to these determinants to a greater or less degree of spatial exactitude. The mystery of the immune response lies in the ability of the animal host to produce such complementary protein surfaces against a gamut of foreign molecular shapes not before experienced. There is the further mystery that it does not do so against its own constituent macromolecules. The antibody molecules or immunoglobulins are known to be produced by plasma cells of the lymphoid tissues (Fagraeus, 1948) but the potentialities of any one such cell and the exact mechanism deciding its differentiation remain to be defined.

A logical course in attempts to elucidate this differentiation has been to follow the fate of antigen molecules. This has been extensively reviewed by Campbell and Garvey (1963). Humphrey (1956, 1960) however has emphasised that only a tiny minority of the total number of antigen molecules injected are likely to end in potentially responding immunological cells. Most would be katabolized non-specifically and so it would be difficult to devise an experiment to determine the crucial difference in handling which ends in antibody production. This he exemplified by observing in the rabbit the fate of γ-globulin complexed with goat anti-rabbit-globulin antibody, the globulins being labelled with different iodine radio-isotopes. No distinction could be made in the katabolism in the rabbit of the foreign (goat) or of self (rabbit) γ-globulin.

Richter, Larose and Rose (1964) were unable to detect antigen fragments by various immunological techniques in the blood or spleen of rabbits injected with human gamma-globulin, human serum albumin, and bovine gamma-globulin. The in-vivo and in-vitro studies of Levine and Benacerraf (1963) did not detect any difference in the katabolism of haptan-polylysine conjugates between the tissues of guinea pigs genetically able and those guinea pigs genetically unable (Levine, Benecerraf, and Ojeda, 1963a and 1963b) to respond immunologically to these antigens.

These observations point the lesson that gross observations of handling of antigen may not detect and consequently need not relate to differencesin the decisive handling of those antigen molecules directly concerned in antibody production. Deductions may have to be made tentatively from indirect effects rather than from direct observations of the fate of particular molecules which might lie beyond the finesse of present techniques. Nevertheless direct morphological evidence is available on the fate of particular antigen molecules. The problem has been to decide how significant their presence is in certain sites for antibody production. Sabin (1939) for example, noticing that dye-labelled antigen collected in reticulo-endothelial cells of various organs after intravenous injection in rabbits, attributed to these cells a direct antibody-forming function which would not be accepted today. More recently antigen (human serum albumin) has been seen by the fluorescent antibody technique to associate with dendritic macrophages of germinal centres in the chicken spleen (White, 1963). Nossal and his colleagues (1964) observed similar phenomena in rat lymph nodes using autoradiography with an antigen, flagellin, derived from Salmonella adelaide, heavily labelled with iodine-125. These findings were of interest in view of the close juxtaposition in these centres of macrophages, antigen, and lymphoid cells.

Macrophage Function and the Immune Response.

Other evidence had been gathering of direct participation by macrophages in steps before antibody production. In experiments with macrophages exposed to bacteriophage Fishman and Adler (1963) showed that ribonucleic acid (RNA) extracted from these macrophages when transferred to tissue culture or transplantation chambers could initiate the production of phage neutralizing antibody. Askonas and Rhodes (1965) working with haemocyanin in rats had brought evidence that a processing step took place in the macrophage. A product of this step, RNA-associated antigen, was passed on to a second (lymphoid) cell, this product being twenty times more efficient weight for weight than the original antigen in stimulating a secondary response.

Further evidence has come from Gallily and Feldman (1967) who observed the responses to antigen processed by irradiated and non-irradiated macrophages in an experimental model in mice using Shigella paradysenteriae as antigen. The antigen associated with the irradiated macrophages was less effective and the authors postulated an important role for these cells. Frei (1964) and Frei, Benacerraf, and Thorbecke (1965) had earlier shown that heat-aggregated bovine serum albumin (BSA) in the rabbit loses its immunising ability after the aggregates have been

phagocytosed by passage through a screening animal. This biologically filtered material was able to paralyse a second host against subsequent stimulation by the same antigen even when associated with a powerful adjuvant. This had led to the idea that processed antigen, i.e. antigen readily engulfed by macrophages will give rise to an immune response whereas unprocessed antigen unassociated with macrophages leads to paralysis of the antibody response. Dresser (1961) had been able to remove immunizing ability from bovine gamma-globulin solutions by ultracentrifugation thereby removing any aggregates present before injection into mice. Also Gamble (1966) has shown that aggregated human gamma-globulin is highly antigenic in mice and that aggregate-free preparations are poorly so.

The active participation of mature macrophages has been demonstrated by Martin (1966) in another immunological experiment. He showed that newborn rabbits need mature adult macrophages to be able to respond to bovine serum albumin as an antigenic stimulus. Unanue and Askonas (1968) have also shown in experiments in irradiated mice with Maia squinado haemocyanin as antigen that macrophage-associated antigen requires normal lymphoid immunocompetent cells before it can prime mice for a secondary response. Pinchuck, Fishman, Adler, and Maurer (1968) have taken this further by showing

that an immune response can be brought about in animals genetically not able to respond to a specific artificial polypeptidyl antigen by presenting the antigen to them in company with macrophages of one of several other responding species. There must therefore have been a latent antibody-forming potential in the lymphoid cells which only found expression with the help of donated macrophages. An additional feature of the responding population of lymphoid cells has been suggested by Cohen (1967) from his experiments with macrophage-RNA extracts from mice injected with sheep red blood cells. He found that the effect of extracts active in initiating antibody formation in spleen cells could be blocked by partially degraded RNA from immunized mice. From this he postulated that there are present specific receptors or recognition sites on the few cells competent to respond specifically.

The immunizing property of the macrophages is sensitive to ribonuclease (Askonas and Rhodes, 1965; Cohen,1967): any residual antigen, if present at all, in the transferable RNA must be very small indeed. Nevertheless it is not yet generally accepted that antigen is completely absent from these preparations. The macrophage system taking part may then be regarded as a catchment-and-transfer system for immunologically active molecules of the stimulating antigen, these molecules being transferred to lymphoid cells along with RNA.

The Digestibility of Potential Antigens.

The idea of antigen processing demands consideration of the katabolism of antigen molecules. In 1928 Wells provided evidence of the direct connection of antigenicity with proteolysis when by racemization of certain proteins he destroyed digestibility and antigenicity although the macromolecules concerned retained other protein characteristics. A different emphasis was placed by Humphrey (1956) when he discussed the possibly crucial relationship between digestion of proteins and the outcome of antigen administration. Protein digestion, he suggested, may be a possible means of recognition of foreign determinants on a protein surface whereby foreign groupings are less efficiently digested and by their relative persistence are antigenic. Similar ideas were put forward by Campbell (1957) in a speculative paper where he drew attention to the probability that antigenicity is often and perhaps always associated with incomplete intracellular fragmentation of material. These speculations relate antigenicity of a molecule to the particular host stimulated. The more closely related phylogenetically the donor and host species, the less likely it is that a protein derived from one will be a good antigen in the other because the appropriate digestive enzymes will be already available which would accurately fit the closely similar foreign substrate. This idea has been

extended (Medawar, 1960) so that the ability to be tolerant or non-responding might be thought of as being the ability to allow induction of appropriate katabolic enzymes. These would leave no undigested fragments to stimulate antibody formation.

Certain macromolecules are indigestible in mammalian tissues in which they consequently tend to persist. Examples are pneumococcal polysaccharide (Felton, 1949; Felton, Prescott, Kauffman, and Ottinger, 1955; Stark, 1955) and artificial polypeptide antigens composed of D-aminoacids (Gill, Carpenter, and Mann, 1967; Janeway and Sela, 1967). Such antigens readily produce paralysis of the antibody-forming mechanism when given in higher doses or in repeated injections (Janeway and Humphrey, 1968; Felton and Ottinger, 1942; Felton, Kauffman, Prescott, and Ottinger, 1955).

Immune Paralysis.

Billingham, Brent and Medawar (1953, 1956) and Hasek (1956) demonstrated how the immature immune apparatus of the mouse or chicken embryo might be specifically paralysed so that it might tolerate without reaction foreign tissue antigens. The introduction of foreign cells in embryonic life by injection(in the mouse) or by a parabiotic circulation (in the chicken) allowed sufficient exposure to bring about this specific paralysis (which had indeed been predicted by Burnet and Fenner (1949) in their theory of

antibody formation current at that time). The susceptibility of adult animals to a similar process emerged more slowly from studies with soluble protein antigens not associated with tissues or cells.

It was known that repeated large doses of protein antigen could depress the response. Glenny and Hopkins (1924) with horse gamma globulin (as diphtheria anti-toxoid) in the rabbit and Dixon and Maurer (1955) had had similar findings with repeated large infusions of heterologous plasma proteins, also in the rabbit. Dresser (1962a) using ethanol-fractionated bovine gamma globulin (BGG) demonstrated a protein overloading paralysis using doses in the 10-100 mg range. He was also able (Dresser 1962b) to show a depression of the response with tiny amounts (50-200 ug), the paralysed state resisting challenge by antigen with complete Freund's adjuvant. Similarly Battisto and Miller (1962) were able to depress the response in the guinea pig to BGG and a picryl chloride hapten by two intravenous injections into mesenteric veins of amounts varying from 10-1000 ug. The paralysis affected both cell-mediated delayed hypersensitivity and humoral antibody production when the animal was challenged with antigen in complete Freund's adjuvant.

Against this background Mitchison (1964) set out to determine the response of mature CBA mice to a purified antigen, bovine serum albumin (BSA) known to be poorly

antigenic in this species. Doses of BSA were given thrice weekly for periods of up to 16 weeks. From his findings Mitchison postulated two separate zones of induction of tolerance or immune paralysis, a "high" and a "low" zone of induction. A low zone of induction of specific tolerance was subsequently detected with flagellin in the rat (Shellam, 1966; Shellam and Nossal, 1968). It is not however detectable with all antigens in the normal animal. For example it is not present with diphtheria toxin or ribonuclease (Mitchison, 1968a) in the mouse.

Mitchison's BSA data gave rise to a concept which with Dresser (Dresser and Mitchison, 1968) he has subsequently developed. The two separate yet superimposed phenomena of immunization and (high-zone) tolerance induction are shown to have dissociated thresholds. The high-zone tolerance threshold they believe to be remarkably constant in terms of numbers of determinant units from observations with various antigens ($2x10^{17}$ to $5x10^{18}$ units). The immunizing threshold varies widely depending on whether a good (low immunizing threshold) or poor (high immunizing threshold) antigen is being considered.

Although paralysis might be explained by slow accumulation of antigen, Dresser and Mitchison find it difficult to accept that simple physical occupation or blocking of a

site is sufficient to inactivate the immunocompetent cell because of the relatively low forces of adhesion of such a blocking agent and the likelihood of dissociation. Death of the cell had been suggested as an explanation by Burnet in 1959. The cell may be killed by a process in which an antibody-like receptor on the surface of the immunocompetent cell reacts with antigen in the presence of complement (Nossal, 1966). There is some indirect evidence of this in that depression of complement in mice, either genetically determined or brought about by cobra-venom inhibitor (Azar, Yunis, Pickering, and Good, 1968), inhibits the development of immunological tolerance to human gamma-globulin.

Theories of Antibody Production.

Provided that antigen is not administered in a tolerizing dose an immune response is finally acted out by the differentiation and proliferation of a fraction of the total population of lymphoid cells. This response has been thought of in several ways since the beginning of the century.

Ehrlich (1900) in his side-chain hypothesis drew in the idea of specific complementary units or receptors which would combine with the surface of the antigenic (toxin) molecule. He suggested that by overcompensation, in keeping with ideas of the time in connection with healing, an excess of such side-chains would be produced. These would be nipped off and passed to the blood as antitoxin molecules. More

recent theories have retained the idea of interaction with receptors, either separately from cells as natural antibody (Jerne, 1955) or as part of the surface of a cell (Burnet 1959).

According to Jerne the natural antibody concentration of a particular specificity is reduced by the presence of the specific antigen and this leads to proliferation of the cells producing that antibody. Burnet modified this, believing that there was stimulation of specific clones directly by antigen finding its specific receptor on the surface of a lymphoid cell. This, like Jerne's theory implies a baffling number of preadapted clones. Later Burnet (1964) considered that the potentialities of the lymphoid population might be widened if Lederberg's assumptions (1959) on the hypermutability of the globulin gene were accepted.

The single potential of each cell, inherent in the original theory, has been questioned. Results such as those of Schechter (1965, 1968) have contributed to this. Members of a species may normally be able to respond to two similar antigens provided the antigens are given separately. If they are given together the potential response to one may be suppressed almost completely, indicating successful competition by one of the antigens. This favours the idea that antibodies to various determinants could be formed by the one cell, and that one determinant may achieve a preference in directing the response of that cell.

Eisen and Karush (1964) have also related immunological events to antigen interaction with natural bivalent antibody. They suggest that the repressive role of excess antigen in producing tolerance would be explained if Ag_2Ab complexes (with saturated bivalent antibody) were non-immunogenic but AgAb complexes (with unsaturated bivalent antibody) were immunogenic. The excess of antigen having thus saturated the free combining sites of the antibody molecules would prevent their interaction with and "switching on" of the immunocompetent cells.

A quite different theory of antibody formation, usually referred to as the direct template theory had been put forward by Breinl and Haurowitz (1930), Alexander (1931), and Mudd (1932). They held that the entry of the antigen molecule into the cell modified directly the structure of the antibody molecule as it was formed. This is not compatible with modern ideas of protein synthesis and structure (Watson, 1966). Burnet and Fenner (1949) also had put forward an "indirect template" theory. This explained the lack of reactivity to self antigens by assuming that self markers were carried by all the potential antigens of the host. It predicted that experience of an antigen embryonic life would prevent an antibody response later. It also noted that antibody production did not end with the elimination of detectable

antigen and regarded this as evidence that information had been stored genetically as an "indirect template". This Lamarckian view Burnet abandoned in favour of his clonal selection hypothesis with its Darwinian ideas of chance selection and proliferation.

Some stereospecific interaction between antigen or antigen derivative and some cellular structure is necessary at some point to stimulate the immune process. This may take place on the cell surface if selection of an unipotential cell is envisaged. If the cell is pluripotent, selection and derepression of a repressed process may take place or there may be direct physical modification of the translation phase of antibody molecule synthesis (Haurowitz, 1967). Smithies (1966) in a more complex hypothesis based on the interaction of nucleic acids with functionally incomplete genes again needs to assume such stereospecific interaction within the cell.

Antigenicity and Immunogenicity.

The increased knowledge of tolerance and its induction as paralysis in the adult animal where protein "antigens" manifestly do not generate an antagonistic response has led to some devaluation of the term "antigen". This has come about by its proleptic use when the writer has logically meant "potential antigen" not knowing the response to be expected in individuals of the species stimulated by the means used or in

the dosage used. The terms "immunogen" and "immunogenicity" have emerged in this situation (Dresser and Mitchison, 1968), these being related to substances inducing a positive immune response without the help of adjuvant. Substances needing adjuvant to produce such a response possess "antigenicity" but lack "immunogenicity". However even good "immunogens" may sometimes prove unsuccessful in producing a positive immune response, designedly so for example in the injection regimes used by Nossal (1966) to produce immune tolerance in rats towards the powerfully antigenic flagellin. Is this substance in this situation then to be regarded as an immunogen or antigen? It is in fact a good antigen presented in a non-immunogenic manner and it seems better to reserve "immunogenic" and the cognate terms for description of the mode of stimulation.

The Outcome of Adjuvant Action.

Immunological adjuvants are substances or mixtures of substances which when administered with an antigen enhance the response. Their mode of action will be discussed in detail later but at this point we need only recognize that the outcome of their activity may not only be quantitatively but also qualitatively different from the response without their presence. The simplest adjuvants which form depots of antigen may alter the response quantitatively, greatly increasing an already anticipated response. It is understandable

that if antigen continues to diffuse from a depot over a protracted period this could allow a greater and more prolonged but not necessarily qualitatively different result. An additional and theoretically more fascinating feature of the action of some adjuvants, probably not related to depot effects, is an alteration in the mode of expression of the immune response. More fundamentally still they may bring about an immune response against substances such as "self antigens" (the host's own antigens) which do not normally provoke any positive response. The mode of expression might be altered by production of a different antibody (Benacerraf, Ovary, Bloch, and Franklin, 1963; White, Jenkins, and Wilkinson, 1963) or the production of cell-mediated delayed hypersensitivity (Freund and McDermott, 1942; Freund, Thomson, Hough, and Pisani, 1948; Raffel, Arnaud, Dukes, and Huang, 1949). Illustrations of the immune response to self-antigens are to be seen in the production of experimental auto-immune disease in animals, e.g. thyroiditis, (Rose and Witebsky, 1956); aspermatogenesis (Freund, Lipton, and Thompson, 1953); and adjuvant arthritis (Pearson, 1956).

The qualitative differences in these adjuvant-mediated responses pose many questions against the several theoretical interpretations of the immune response set out above. Primarily how do adjuvants alter either a selective or an instructive process so that the mode of the response

is changed? How are they able to stimulate a response in individuals thought to be dispossessed of responding clones?

However qualitative the appropriate differentiation of a lymphoid cell, its origin will lie quantitatively in some alteration of crucial reagents in time, place, and concentration.

This outline is set out briefly as a background to the subsequent experiments and discussions. For extended discussion of these topics reference may be made to the reviews of Medawar (1960) and Leskowitz (1967) on tolerance; Campbell and Garvey, (1963) on fate of antigen; Mitchison (1966) on antigen recognition; Nossal (1967) on antibody production; and Dresser and Mitchison, (1968) on immune paralysis.

Chapter Two

The response in the chicken to a Soluble Protein Antigen

Antigens had been found to localize in association with dendritic macrophages of the germinal centres of lymphoid tissues by White (1963: human serum albumin in the chicken spleen) and Mossal, Ada, and Austin (1964: flagellin in rat lymph nodes). The relationship of these cells to the development of germinal centres and to the production of antibody had to be more closely defined. Accordingly a study was undertaken (White, French, and Stark, 1967) in the chicken with human serum albumin (HSA) given intravenously as antigen. The kinetics of antigen disappearance from the circulation and antigen localization in germinal centres were examined together with the appearance of antigen-producing cells in the spleen. The material submitted here extends the joint study previously reported with autoradiographic evidence of antigen localization, and gives also the detail of that part of the study where radio-isotope labelled HSA was used.

(Appendix B, "Germinal center formation and antigen localization in Malpighian bodies of the chicken spleen", White, French, and Stark, 1967, contains the associated information derived by the fluorescent antibody technique by my colleagues and has been appended in order that some of the deductions from the jointly obtained data may be discussed).

Materials and Methods

Chickens: White Leghorn cockerels, aged 6-14 weeks were used.

Antigen: Crystalline human serum albumin, "reinst" Fabwerke-Hoechst, was injected in 0.15 M saline without adjuvants.

Isotope labelling: HSA was trace labelled with radioiodine by the direct oxidation technique of Hunter and Greenwood (1962) using Chloramine T and thiosulphate-free isotope (IBS.3 iodine-131 or IMS.3 iodine-125 from the Radiochemical Centre, Amersham, England).

Different levels of activity were required for the Farr test (about 0.5 uC/ug) and autoradiography (up to 25 uC/ug). Previous experience had shown that 35-40% of the available activity could be attached to the protein. When high activities were required for autoradiography, the technique was modified in the manner of Ada, Nossal, and Pye (1964). For lower activities the protein was dissolved in a volume between 0.5 ml and 2 ml 0.1M sodium phosphate buffer at pH 7.5 and added simultaneously with 250 ug chloramine T in 0.25 ml buffer to the isotope. After addition of metabisulphite and iodide, as described by Hunter and Greenwood, the free iodide was separated from the labelled protein by passage through a 30 cm. G.25 Sephadex column, 1 cm. in diameter, equilibrated with 0.15M saline.

Antigen binding capacity - Estimation of serum antibody levels by means of the Farr Test

Quantitative estimation of antibody as antigen binding capacity was carried out by a modification of Farr's method (1958). The trace-labelled HSA was used at a concentration of 0.4 ug/ml. Dilutions of a serum under test were made in normal chicken serum. Dilution amounts of these in 0.1 ml. volumes, neat, 1/5, 1/25, 1/125, were placed in each of two tubes. Subsequently, 0.5 ml. of the trace-labelled antigen solution was added to each tube. After 18 hours at 4°, 0.4 ml. ammonium sulphate solution (saturated at 4°) was added to each tube. After allowing precipitation to take place for 2 hours at 4° the deposits were spun (15 min. at 2,500 r.p.m. in a model PR-2 International Centrifuge) and washed in 40 percent saturated ammonium sulphate. This washing procedure was repeated once more, the final precipitate being dissolved in 1 ml. normal saline. Four tubes containing 0.1 ml. normal chicken serum were included in the test. Two were treated as were the other sera under test to determine the minimal precipitation in the absence of antibody. To the other tubes 0.6 ml 10 percent trichloracetic acid (TCA) was added. The resulting precipitate was centrifuged and washed in 1 ml of TCA and resuspended for counting in 1 ml saline. The washed precipitates from all tubes and the supernates from the normal chicken serum and TCA tubes were counted in a well-type sodium iodide crystal scintillation counter.

The radioactive counts of the precipitate were plotted against the serum dilution on semilog paper and the dilution of 0.1 ml serum equivalent to 30 per cent precipitation determined (where 100 per cent total (TCA) precipitable protein counts minus the normal serum precipitate counts). From this was calculated the antigen binding capacity (ABC_{30}) in ug/ml.

Estimation of antigen (^{131}I-HSA) in the serum present as antigen-antibody complexes

Antigen (^{131}I-HSA) in combination with homologous antibody can be precipitated from chicken serum by 40 per cent saturation with ammonium sulphate. This differentiates such complexes from ^{131}I-HSA which is not precipitated by the same concentration of ammonium sulphate. Birds were injected intravenously with 10 mg HSA which had been previously labelled with 0.3 mC iodine-131. To 0.1 ml duplicate samples of serum, 0.5 ml saline and 0.4 ml saturated ammonium sulphate were added at 4°. The precipitate formed after preservation of the mixture for 18 hours at 4° was spun down in a refrigerated centrifuge (15 min. at 2500 r.p.m. in a Model PR-2 International centrifuge), washed twice in 40 per cent saturated ammonium sulphate and made up to 1 ml. To untreated 0.1 ml serum samples, 0.9 ml saline was added. The samples were counted in a scintillation counter and the precipitable HSA counts per 0.1 ml serum were expressed as a percentage of the total HSA counts per 0.1 ml serum in each daily sample.

Determination of half-life of chicken 7.7S immunoglobulin

The globulin used for these determinations was prepared from a pool of human chicken sera which was precipitated at 22.5 per cent saturation with ammonium sulphate. The serum was dialysed against 0.15M NaCl, 0.01M PO_4 pH 7.2 and trace-labelled with iodine-131 by the method of Hunter & Greenwood (1962). A 10-week old bird was injected with 1mg of this radiolabelled protein. Serum samples were collected at the 2nd, 4th, 7th, 9th, and 12th days after injection. The radioactivity of aliquots was estimated in a well-type (sodium iodide crystal) scintillation counter. The bird was provided during the period of observation with 0.01 per cent potassium iodide in half-strength physiological saline in place of drinking water.

Separation of antibody activity into macroglobulin and 7.7S components by sucrose-gradient ultracentrifugation.

Sera were collected from a bird every day from the fourth to the eighth day after injection with 1 mg of HSA. The sera were examined by ultracentrifugation in a manner similar to that described by Stanworth, James, and Squires (1961). After the sera had first been clarified by being spun for 30 min. at 10,000 r.p.m., samples of 0.25 ml were diluted in an equal volume of 0.15 ml saline and were placed on a sucrose density gradient (10 - 40 per cent sucrose in 0.1 M phosphate buffer at pH 6.8). Separations were performed on a M.S.E. Super Speed 50 Centrifuge using a 3 x 10 ml swing-out head. The temperature, initially 0°,

rose to 10-11° during the 16 hour runs at 30,000 r.p.m., with an average centrifugal force of 98,000g.

Fractions of 0.4 ml were obtained by piercing the bottom of the plastic centrifuge tube and their content of specific anti-HSA antibody was estimated by means of a modified Farr test. To aliquots of 0.1 ml of the resultant fractions, 0.5 ml of ^{131}I-HSA (0.2 ug protein) was added and allowed to stand at 4° overnight. Saturated ammonium sulphate at 4° (0.4 ml) was added. The mixture was left at 4° for 2 hours. After centrifugation at 4° the precipitate was washed once with 1.0 ml of 40 per cent saturated ammonium sulphate. The washed precipitates were counted in a well-type scintillation counter and the resultant counts of radioactivity plotted as shown in Fig 2.3. The optical density of each fraction was estimated in a spectrophotometer, readings being taken at a wavelength of 280 mu.

Preparation of Autoradiographs.

Five white Leghorn chickens 10 weeks old were injected intravenously with 40 ug of ^{125}I-HSA, which had been heavily labelled to a level of 25 uC per ug. At intervals (16 hr, 40 hr, 88 hr, 6, and 7 days) after the injection of antigen , spleens were obtained from the birds killed by an intravenous dose of pentobarbitone sodium. Sections were cut in a cryostat at -20° at a thickness of 4 u. After air-drying and fixation for 15 min. in absolute methanol at room temperature

they were dipped into Ilford Nuclear Research Emulsion K5 and exposed for 60 to 120 days. The slides were then developed by Microdol developer and stained with Leishman's stain or by the Unna-Pappenheim method of methyl-green pyronine.

Results

The primary response in the serum of chickens to an intravenous injection of human serum albumin

Six to eight week old birds. The antibody levels in the serum which follow an intravenous injection of 10 mg of HSA are expressed in Fig.2.1 as antigen binding capacities. Detectable antibody first made its appearance in the serum at four days after injection and increased to a maximum at 8 to 12 days. The peak was followed by a sharp drop to low levels of serum antibody at 16 days. No evidence was found of any later rise in the levels of antibody in sera which were estimated up to 42 days after injection.

In two birds given 1mg HSA, the maximum titre was detected on the ninth day with a highest value of 0.35ug/ml. In two further birds trace amounts, of about 0.03 ug/ml, were detectable between the eighth and tenth day.

10 to 14 week old birds. With a dose of 1 mg, antigen binding capacities of 39.3, 25.2, 12.0, 9.9, and 8.7 ug/ml were obtained on the 4th, 5th, 6th, 7th, and 8th days respectively. This series was submitted to ultracentrifugation.

Antibody Production to Human Serum Albumin in the chicken

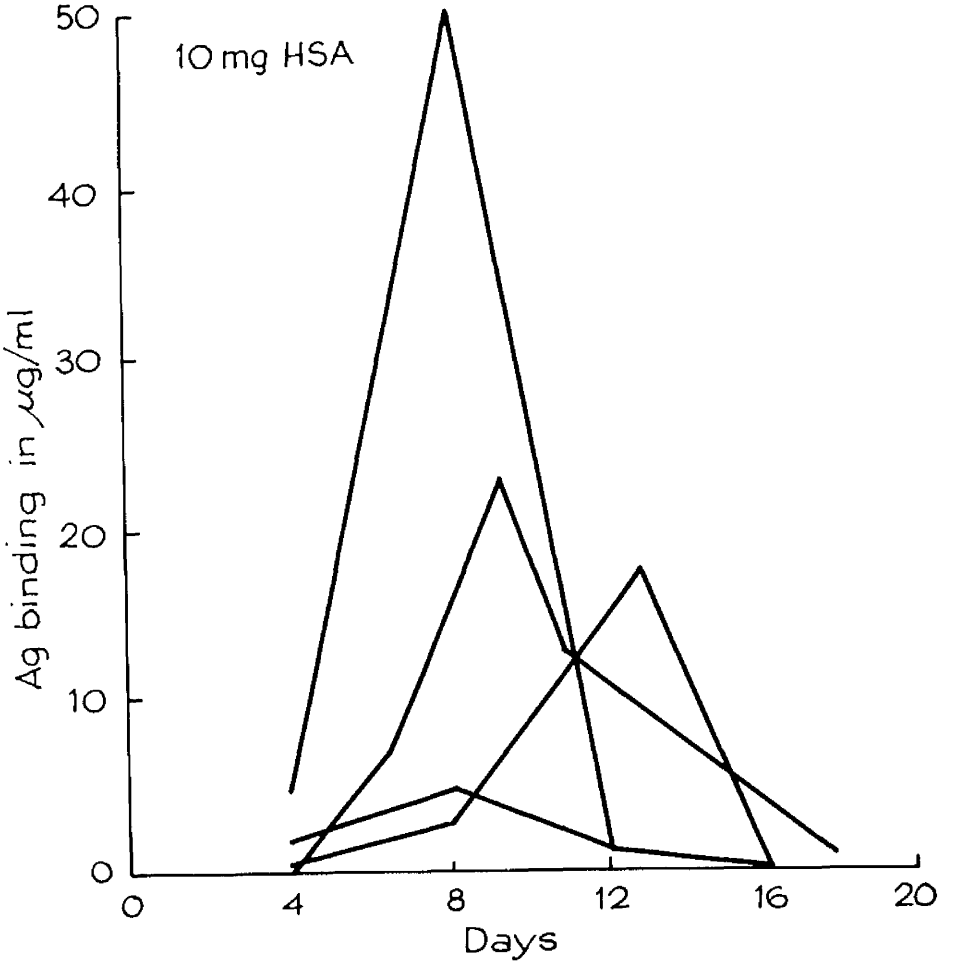


Figure 2.1 Serum antibody concentrations of individual chickens at varying time intervals after a single intravenous injection of 10 mg of human serum albumin (HSA). Antibody measured as antigen binding capacity in ug / ml.

In four birds, after injection of 40 ug HSA, ABC30 of 3.06 ± 0.51 ug/ml and 0.82 ± 0.14 ug/ml were obtained on the 10th and 18th days respectively.

Elimination of an intravenous injection of 10 mg of ^{131}I.HSA

The counts of radioactivity in blood samples obtained during the period of elimination of a 10 mg dose of ^{131}I.HSA are shown in Fig.2.2. After an initial period of two days when the antigen was eliminated with a uniform half-life of 1.25 days, the rate of elimination rapidly increased, the half-life shortening to 0.3 day. Almost all of the injected antigen was eliminated from the circulation by the 6th day after injection.

Antigen-antibody complexes in the circulation of chickens injected with ^{131}I.HSA

Antigen (^{131}I.HSA) which was present in the blood of chickens in the form of soluble complexes with homologous antibody was estimated as a percentage of the total antigen present. Complexes first appeared during the fourth day (Fig.2.2). The percentage of antigen complexed rose rapidly thereafter to 67 per cent on the sixth day. No more antigen complexed or free could be detected in the next serum sample taken. Free antibody could be detected towards the end of the fifth day when a small amount of antigen was still present.

Antibody Production to Human Serum Albumin
in the chicken

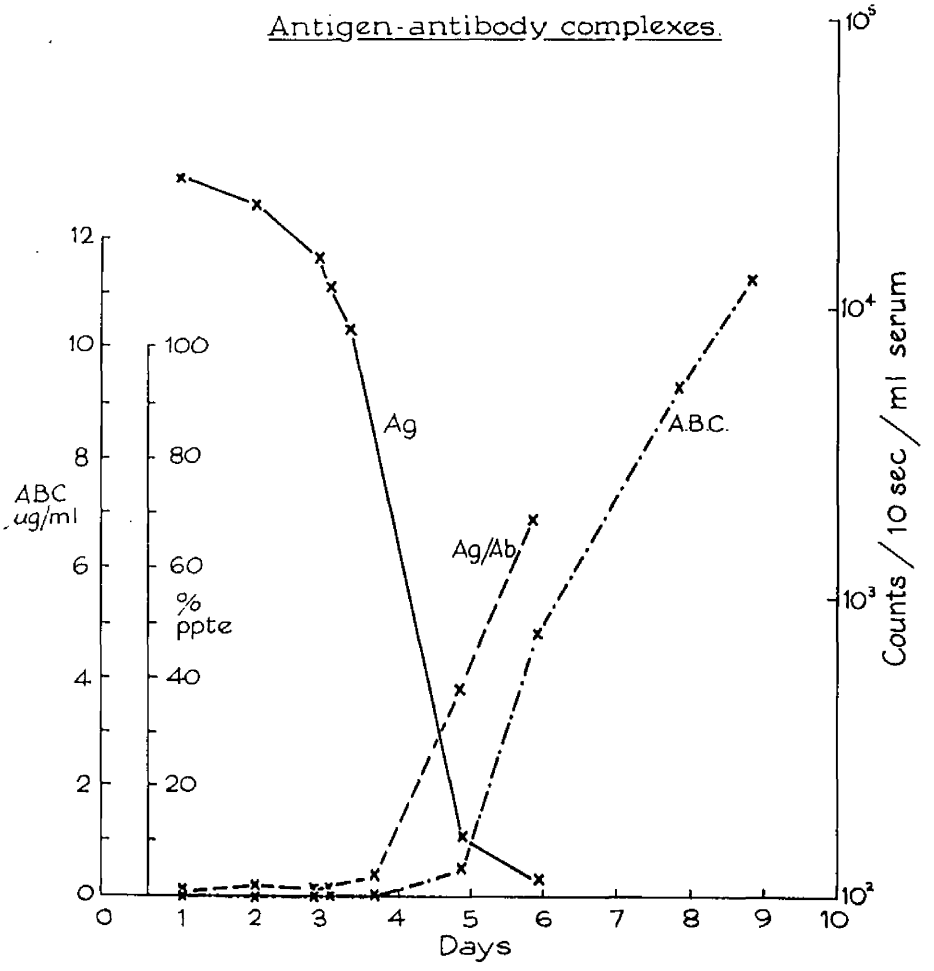


Figure 2.2 Curve of disappearance (Ag) of ^{131}I.HSA from the blood of a chicken. Curve of antibody to HSA (ABC) in the serum. The antigen which is complexed with antibody is plotted by the broken line Ag/Ab as a percentage of the total detectable circulating antigen.

Ultracentrifugation of sera from a ten week old chicken injected with 1mg HSA

The antibody activity of the 4th, 5th, and 6th day sera could be divided into rapidly sedimenting and slowly sedimenting moieties (Fig.2.3). The rapidly sedimenting antibody lay in the macroglobulin peak: the slowly sedimenting antibody was regarded as being comparable with the 7.7S antibody described by Tenenhouse and Deutsch, (1966). The 7th and 8th day sera contained only slowly sedimenting antibody.

The percentages of the two types of antibody in all sera examined were:

Day	Percentage rapidly sedimenting	Percentage slowly sedimenting	Total ABC_{30} ug/ml
4	10.1	89.9	39.3
5	5.3	94.7	25.2
6	2.4	97.6	12.0
7	nil	100	9.9
8	nil	100	8.7

Half-life of γ-globulin in 10 week old chicken

The half-life of the gamma globulin was found to be 2.25 days.

Autoradiography of Chicken Spleen Sections after injection of 40 ug ^{125}I.HSA

Antigen localization was denoted by exposed photographic grains derived from the disintegration of the iodine-125 attached to antigen molecules or to large fragments of these molecules (Humphrey, Askonas, Auzins, Schechter, and Sela, 1967). Large clusters of such grains were seen in

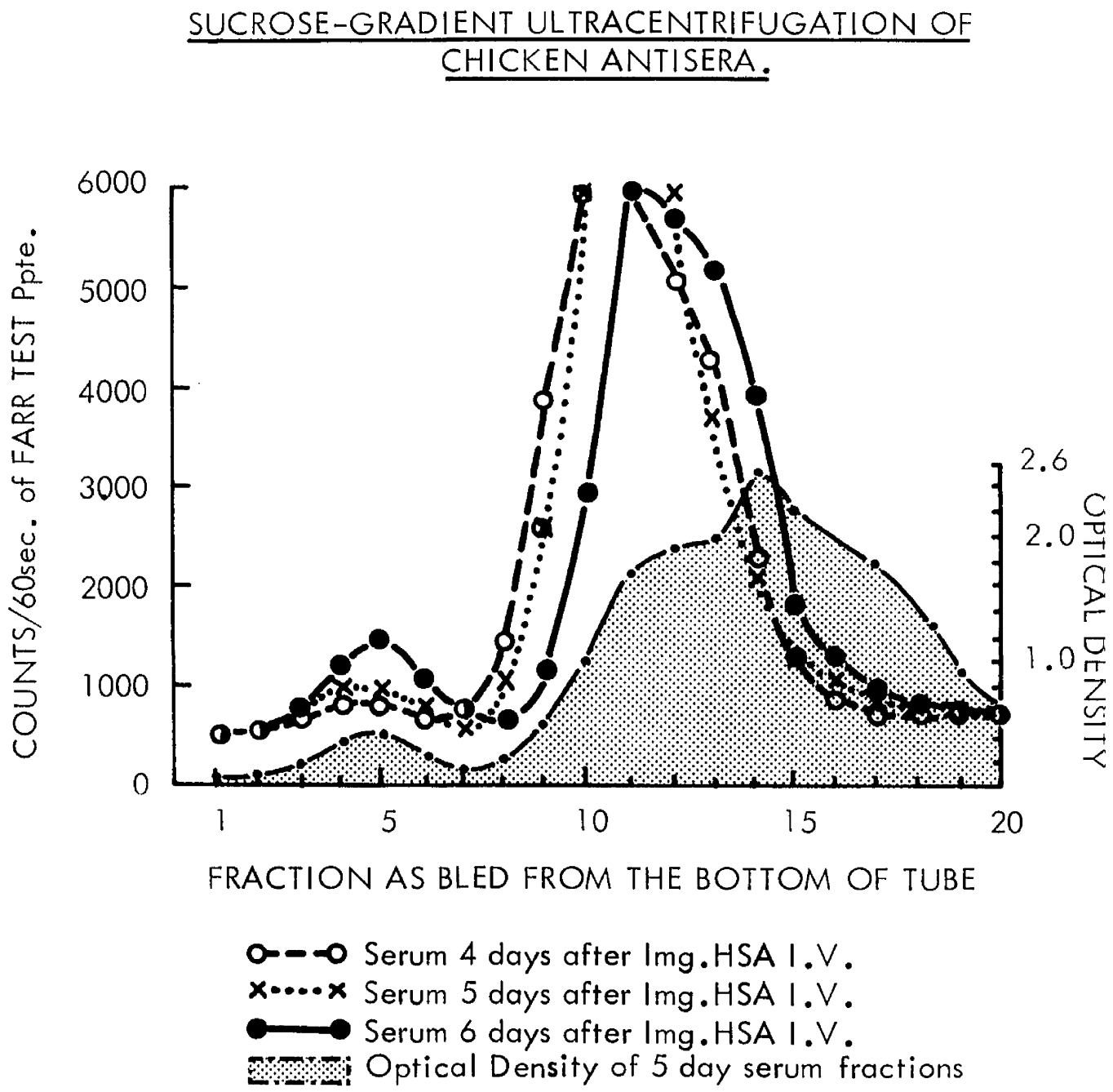


Figure 2.3 Antibody content of ultracentrifuged serum fractions

The counts in the specific precipitate produced by eacg fraction in a modified Farr test (antigen concentration = 0.4 ug / ml) are graphed for three days of the response. The optical density of the fractions of one serum (5-day serum) at 280mu are also shown.

sections taken at 40 hours after the intravenous injection of antigen but not at 16 hours afterwards when only a uniformly heavy background could be seen.

At 40 hours antigen was associated in the white pulp with sparse cells (Fig.2.4) which were interpreted as being macrophages. It was unusual to find any great number of these cells forming a pattern at this time although in places (Fig.2.5) more numerous macrophages could be found around the smaller arterioles.

At 88 hours the numbers of antigen-laden cells had greatly increased, these being distributed along the branches of the central arteriole of the white pulp (penicillar or penicillary arterioles) and extending from the periphery of the ellipsoid to the point of bifurcation of the central arteriole (Figs. 2.6, 2.7). Annular deposition of antigen in the white pulp around the ellipsoids was a feature of the localization at 88 hours after antigen injection (Figs. 2.8a and b). A zone of diffusely increased radioactivity extended round the periphery of the ellipsoid within which were several denser foci. This ring of increased antigen deposition was separated from the ellipsoid itself by at least one cellular layer.

Collections of antigen-laden macrophages, 14 to 16 in number, were to be found at this time at the bifurcation of the central arterioles (Figs. 2.9a and b). There was no displacement of the surrounding cells. This contrasted with

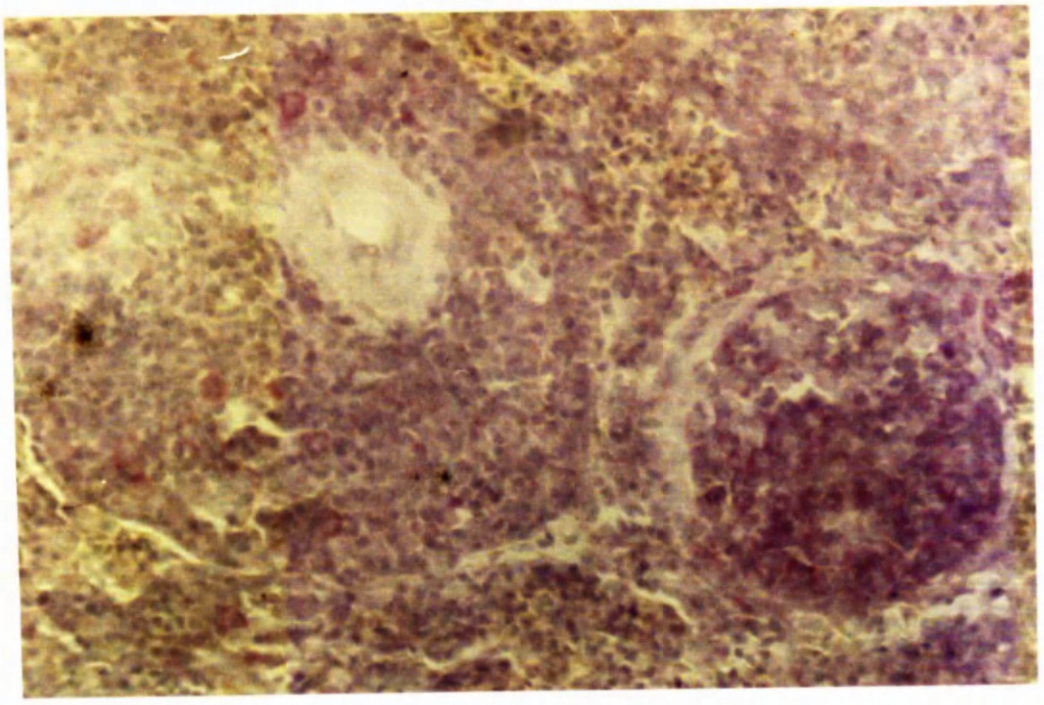


Figure 2.4 Spleen 40 hours after I.V. injection of 40 ug ^{125}I.HSA
A very few scattered grain foci are present in the white pulp on the left. None is present in the germinal centre (Bottom right).

Unna Pappenheim X 600

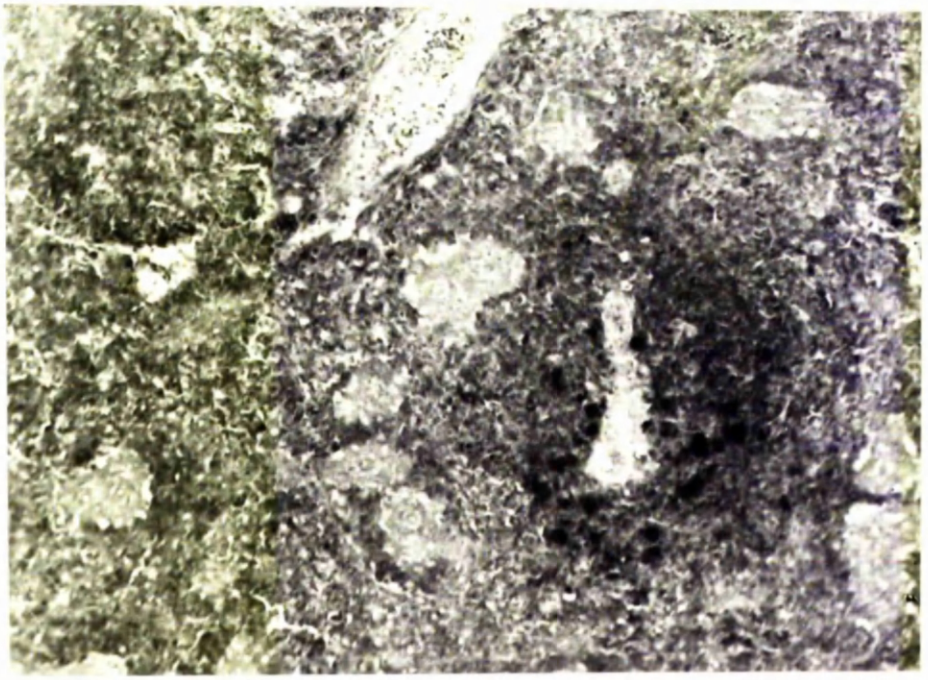


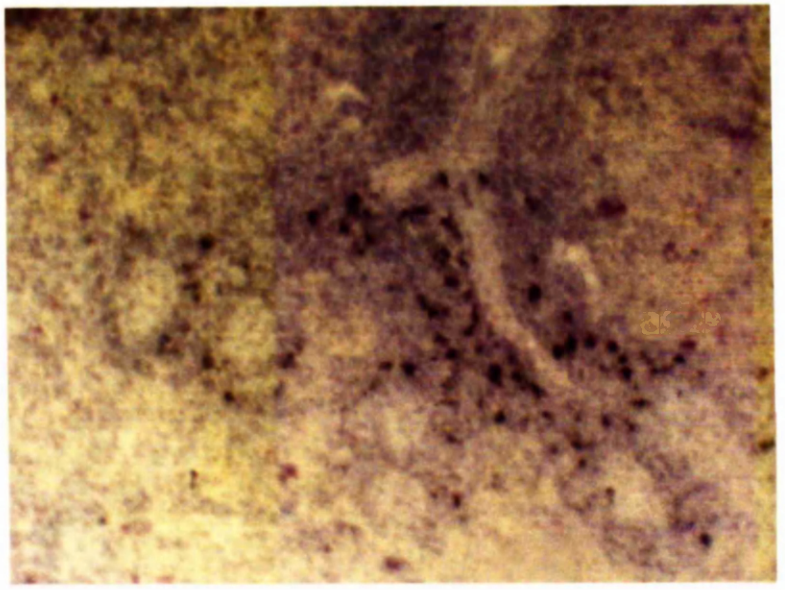
Figure 2.5 Spleen 40 hours after I.V. injection of 40 ug ^{125}I.HSA
Several grain clusters are present in this low-power field but only in the white pulp surrounding one arteriole.

Unna Pappenheim X 150

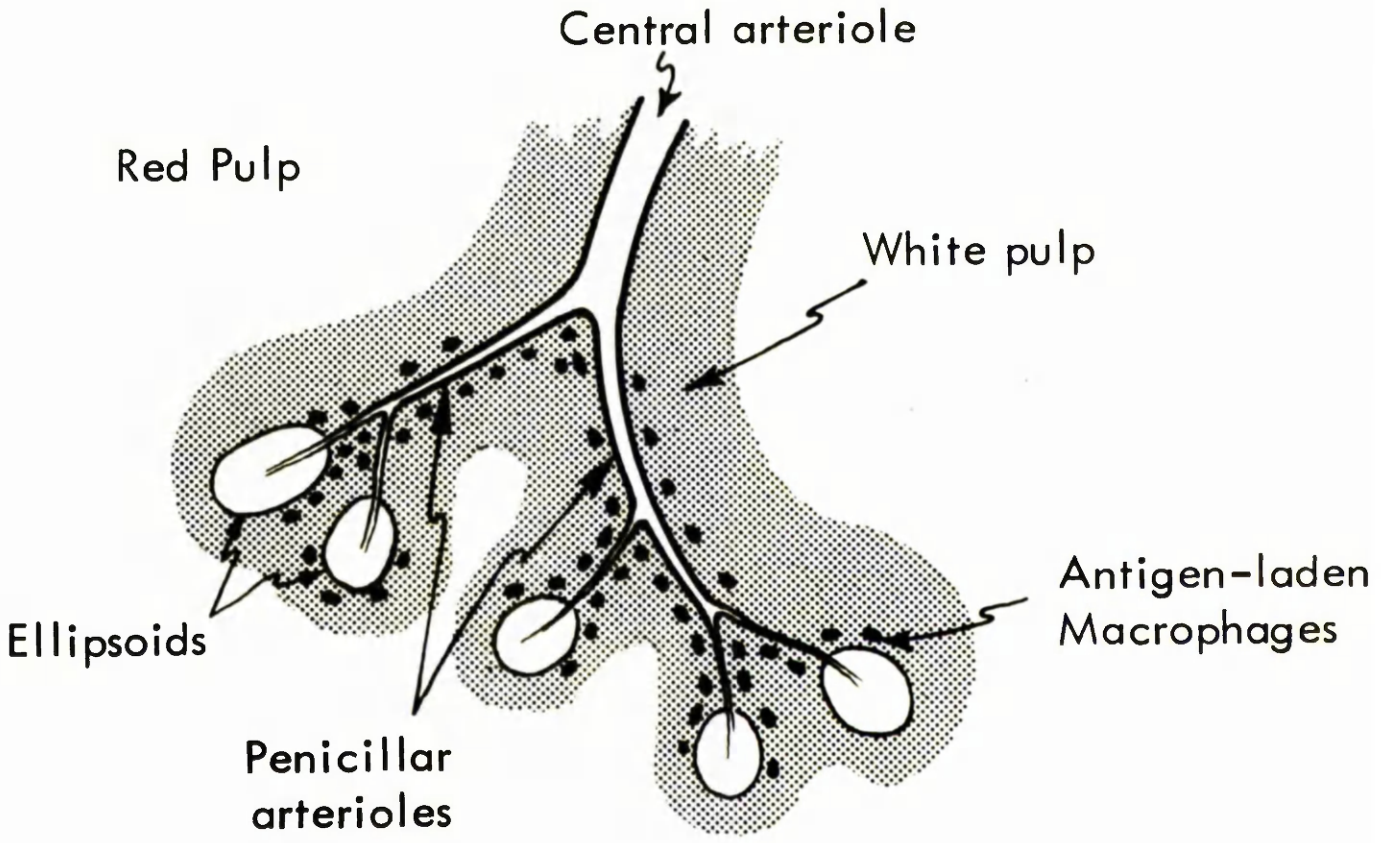
Autoradiograph of the Chicken Spleen

Figure 2.6 (with diagram) Spleen 88 hours after the I.V. injection of 40 ug ^{125}I.HSA

Dense grain foci attributed to antigen-bearing macrophages are distributed along the length of the penicillar arteriole from its point of origin at the bifurcation of the central arteriole. No HSA-bearing cells extend along the central arteriole itself. Several such cells are present at the periphery of some ellipsoids.



Leishman X 170



Autoradiograph of the Chicken Spleen

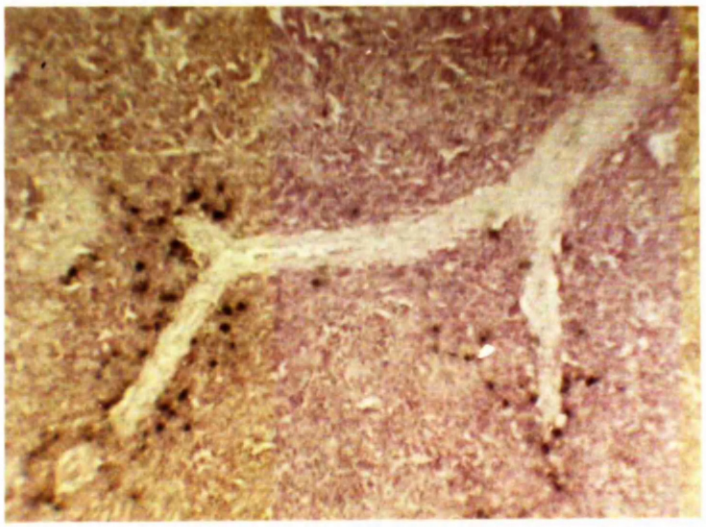


Figure 2.7 Spleen 88 hours after I.V. injection of 40 ug ^{125}I.HSA Antigen-bearing cells are again present along the distal penicillary arterioles. These cells do not surround the more proximal central arteriole.

Unna Pappenheim X 150

Autoradiographs of the Chicken Spleen

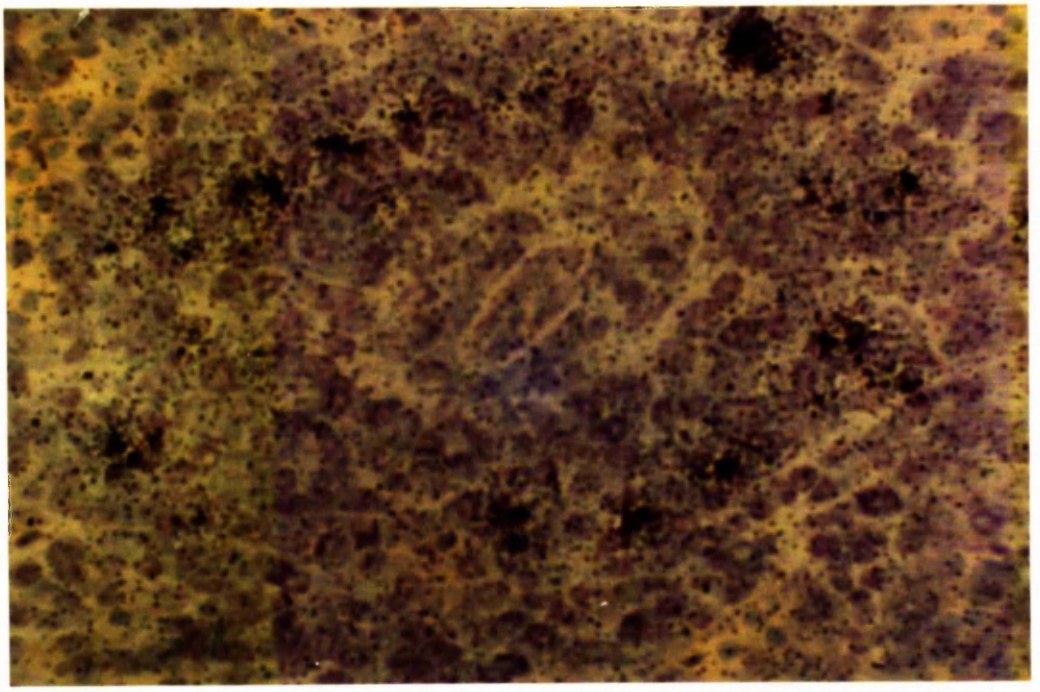


Figure 2.8a Spleen 88 hours after injection of 40 ug ^{125}I.HSA.
The periphery of an ellipsoid in the white pulp is outlined by a zone of increased grain density which includes a few denser foci.

Leishman X 800

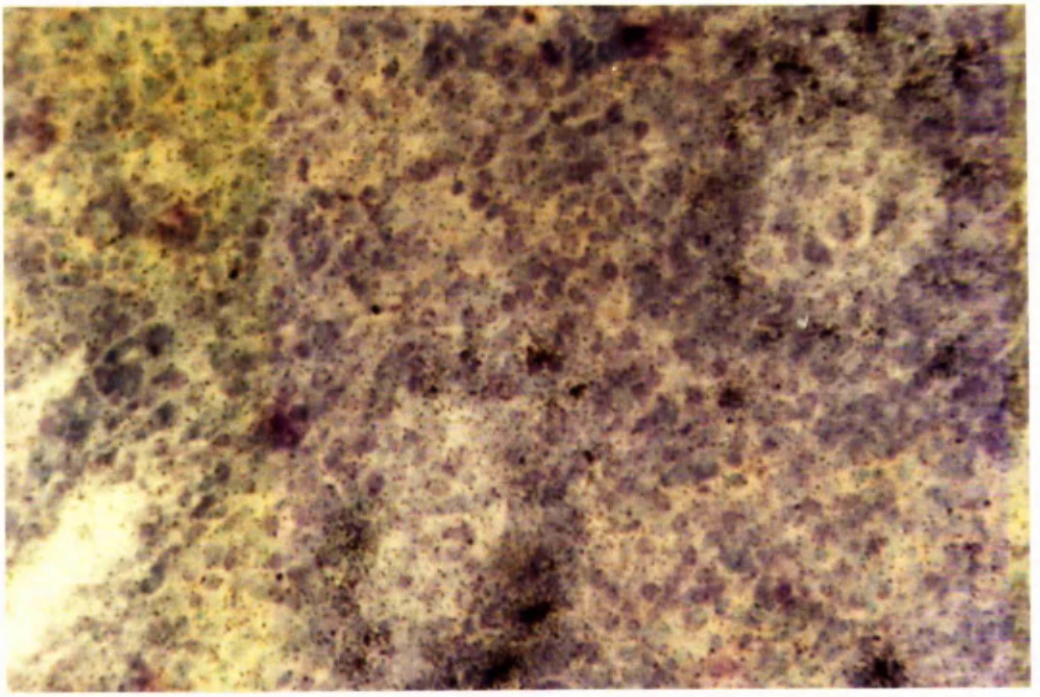


Figure 2.8b Spleen 88 hours after I.V. injection of 40 ug ^{125}I.HSA
Two other ellipsoids showing the same features as Fig.2.8a

Leishman X 600

Autoradiographs of the Chicken Spleen

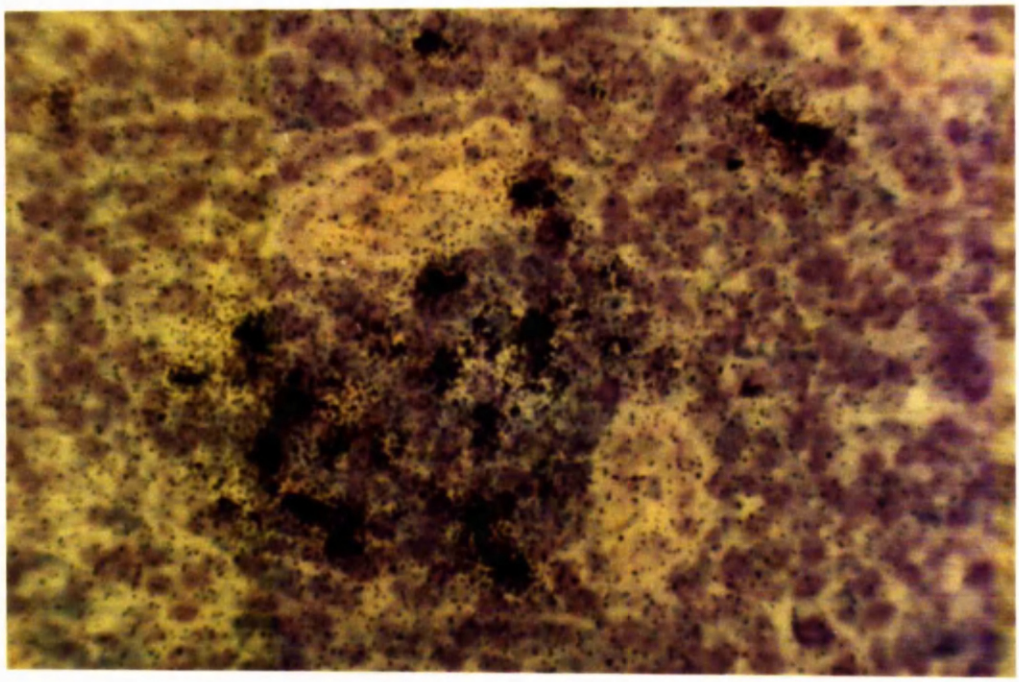


Figure 2.9a Leishman X 800

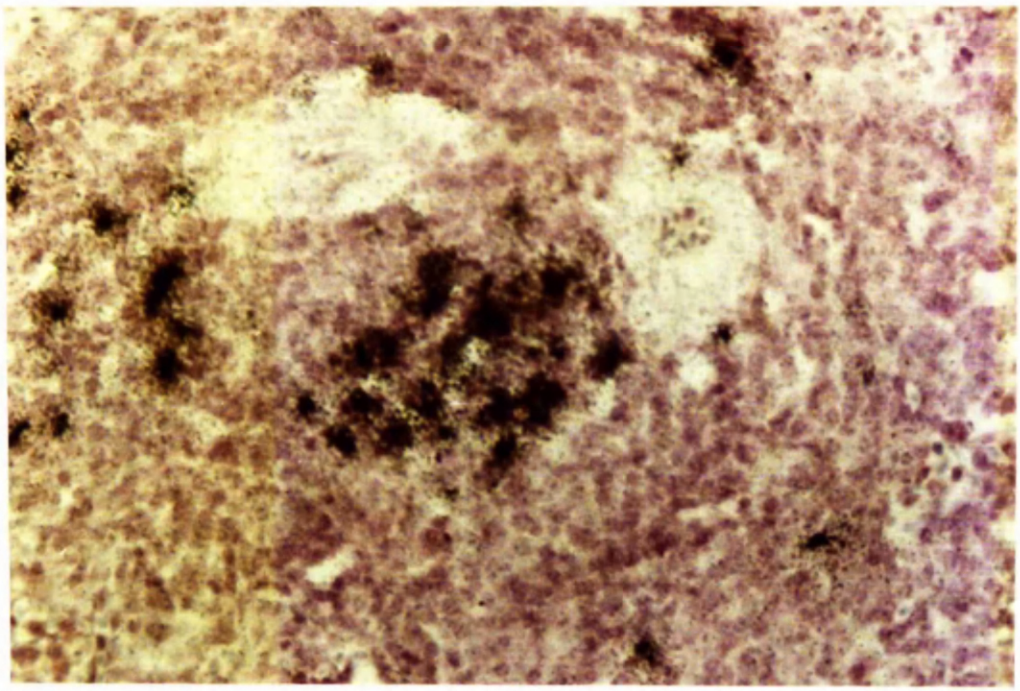


Figure 2.9b Spleen 88 hours after I.V. injection of 40 ug ^{125}I.HSA

Unna Pappenheim X 600

Figures 2.9a and 2.9b. Two examples of collections of grain foci (14-16 in number) denoting antigen-bearing macrophages clustering in the angle between two penicillary vessels.

the more fully developed germinal centres: around these the pattern of cells of the pulp could be interpreted as showing a displacement which might be attributed to an expansion of the macrophage-lymphoid mass. The centres present at this time were all closely associated with an arteriole (Figs. 2.10a and b). The larger centres often showed antigen-bearing cells limited to their more central areas (Fig.2.10b): the lymphoid cells on their outer fringes often did not have grains lying over them. Many of the cells of these centres had a more basophilic cytoplasm than the average cell of the white pulp when stained by Leishman's stain. Intensely basophilic cells were also seen at the periphery of centres (e.g. in Fig.2.11b around the centre not showing antigen localization).

Every centre did not show antigen localization at 88 hours. Table 1 below records the number of antigen-bearing cells and the number of lymphoid cells present in the centres of a typical section of spleen at this time and also at 6 days after HSA injection. Many centres of all sizes showed no antigen localization. These were less common in the section from the 6-day spleen.

Table 1

88 hour spleen.		6 day spleen	
Cell grain clusters.	Total cells in centre.	Cell grain clusters.	Total cells in centre
0	30	0	73
0	60	4	64
0	80	7	72
0	130	7	72
0	152	7	95
0	174	8	64
8	125	15	227
9	60	18	185
15	220	24	210
17	72	32	210
18	160		
20	120		

Autoradiographs of the Chicken Spleen

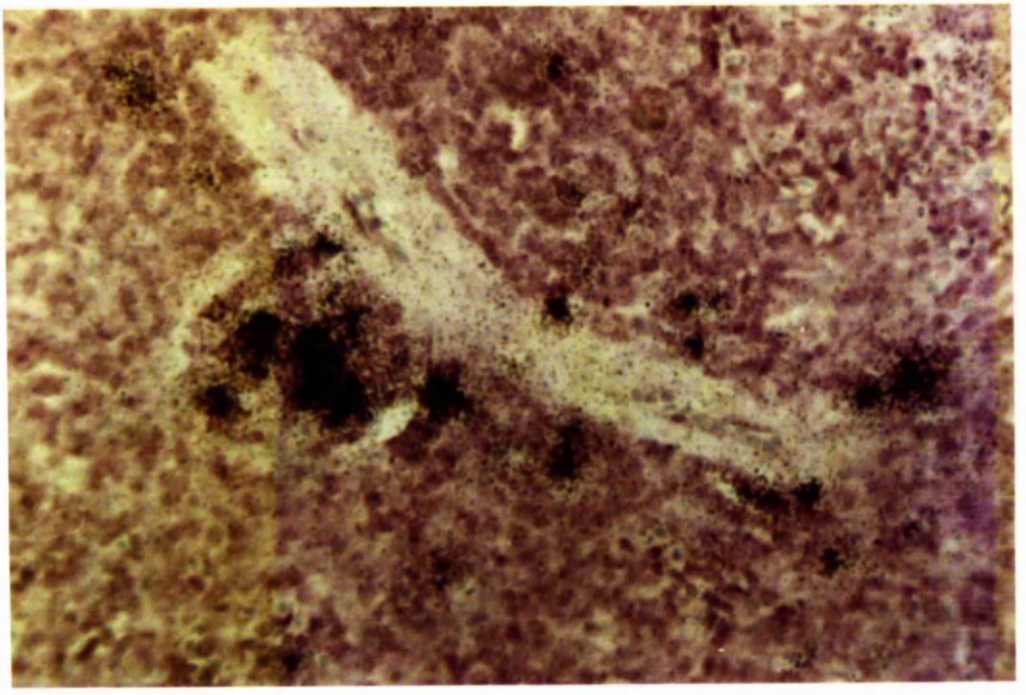


Figure 2.10a Spleen 88 hours after I.V. injection of 40 ug ^{125}I.HSA
A small germinal centre (diam.40u) containing 9 grain clusters developing close to the arteriolar adventitia.

Unna Pappenheim X 600

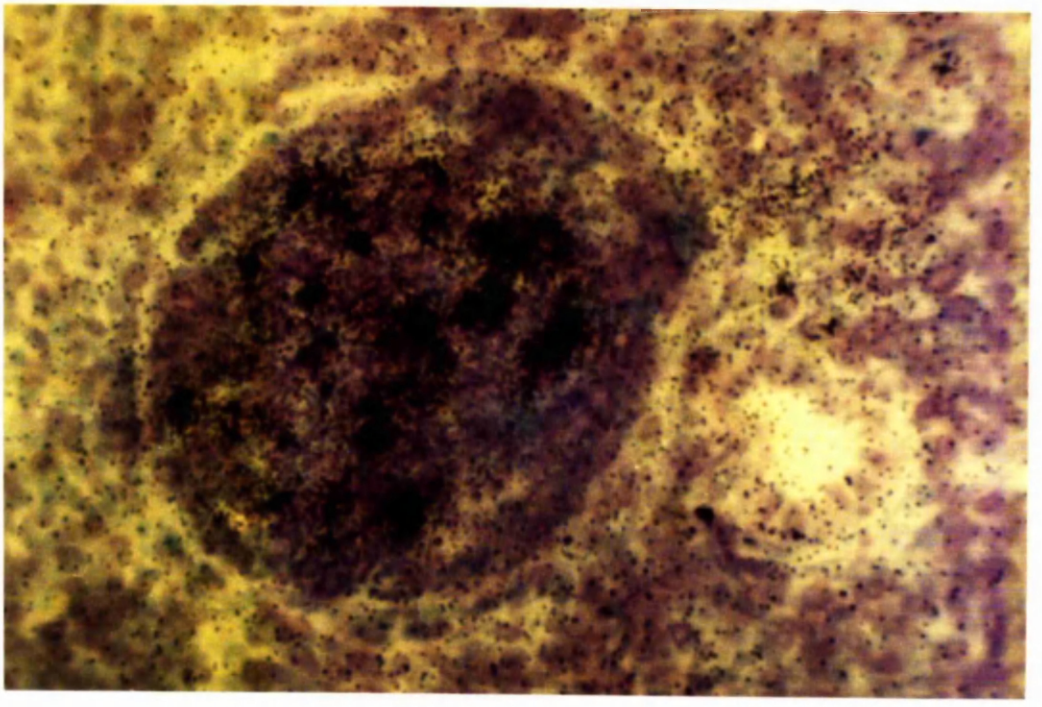


Figure 2.10b Spleen 88 hours after I.V. injection of 40 ug ^{125}I.HSA
A larger centre (diam.75u) containing about 16 grain clusters again closely related to an arteriole. Note that the outer layers of part of the centre are devoid of grain foci.

Leishman X 800

Autoradiographs of the Chicken Spleen

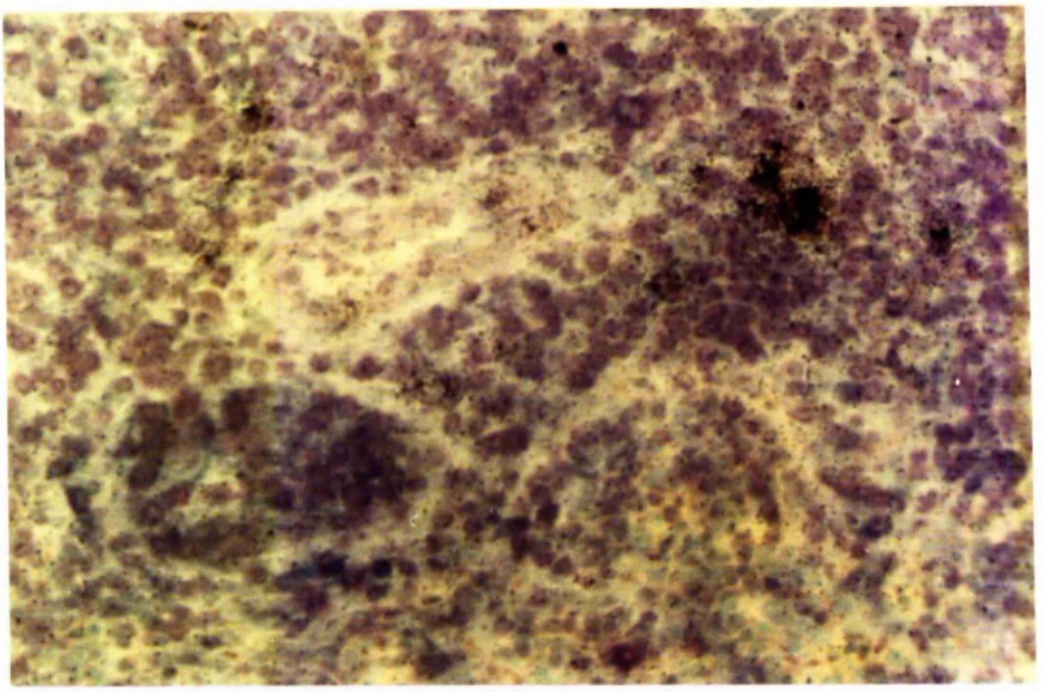


Figure 2.11a Spleen 88 hours after I.V.injection of 40 ug ^{125}I.HSA
A small centre not showing grain foci at this time. Antigen-laden cells are present in the white pulp.
Leishman X 600

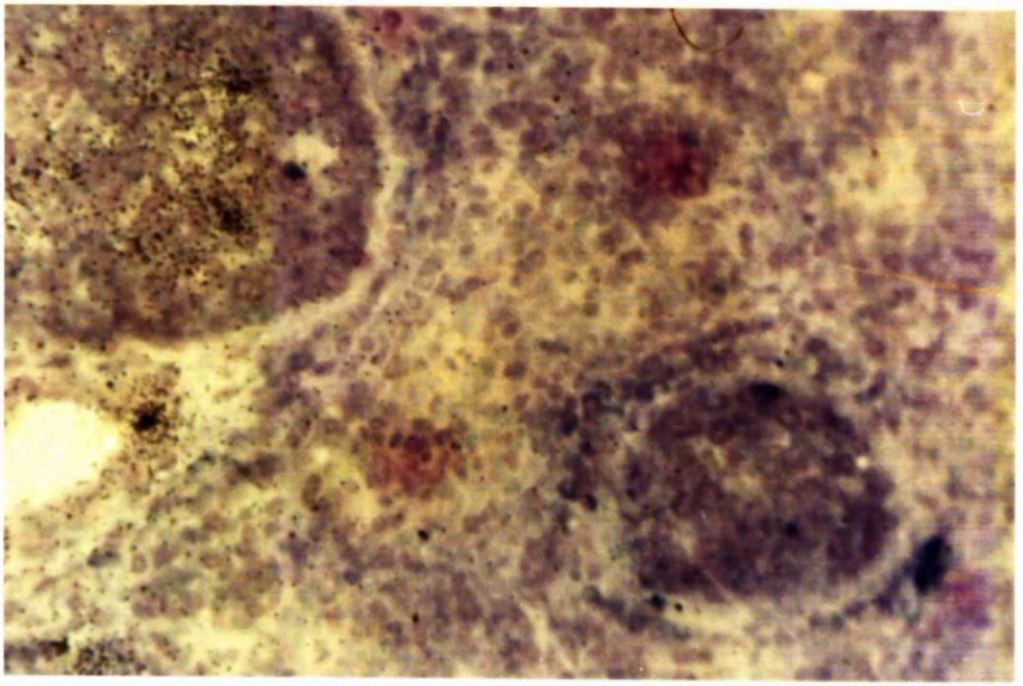


Figure 2.11b Spleen at 6 days after I.V. injection of 40 ug ^{125}I.HSA
Two centres, one without antigen localization, are present. Note also antigen-label in the vessel wall.
Leishman X 600

At the later times, six to seven days after antigen injection, the picture of antigen localization had altered. The periarteriolar pattern of antigen-laden macrophages had largely disappeared although some such cells could still be found in the white pulp (Fig.14b shows some around a small venule). Most of the remaining antigen was to be found within germinal centres although the centres varied very much in appearance. Thus some (Fig.2.12) showed a deposition limited to the more central areas of the centre (Fig.2.12) similar in siting to, but less intense than that found in some centres at 88 hours. Others showed heavy deposition with only slight expansion of the centre beyond the antigen-bearing cells (Fig.2.13, small centre). Some of the cells of these centres showed evidence of a high RNA content in their increased pyroninophilia with Unna Pappenheim staining. Other centres showed very slight deposition of antigen but also the increased pyroninophilia (Fig.2.13, large centre).

Antigen was also seen at this time commonly within the endothelium of arterioles and venules, something not seen at earlier stages (Figs. 2.14a and b, 2.12).

Discussion

The Antibody Response.

The findings here are similar to those of Hektoen (1918) using human serum as antigen in chickens with the early rise in antibody titre and the peak usually between the 8th and 12th day after injection of the antigen. The

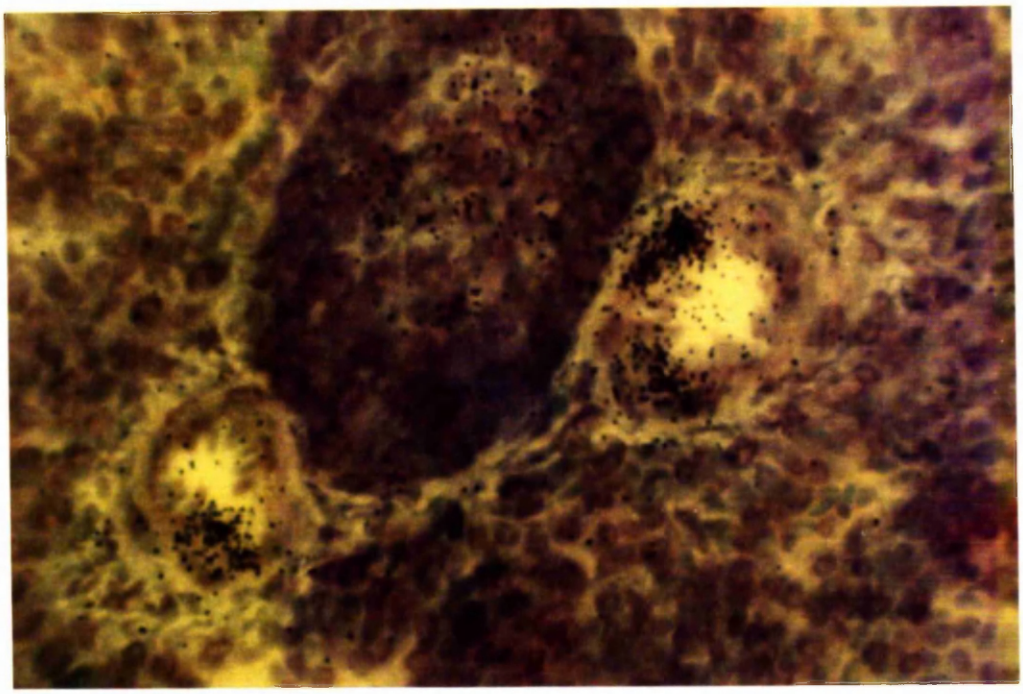


Figure 2.12 Spleen 6 days after I.V. injection of 40 ug ^{125}I.HSA
Germinal centre related to arterioles after their bifurcation. Note antigen localization mainly in central area of the centre and also in the walls of two arterioles.

Leishman X 800

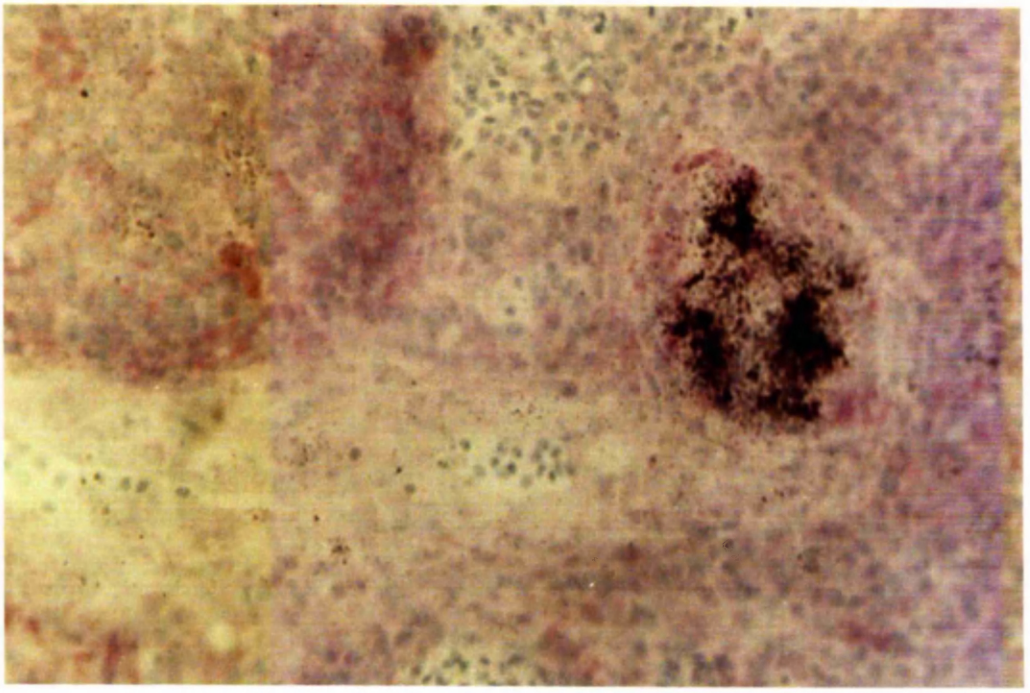


Figure 2.13 Spleen 6 days after I.V. injection of 40 ug ^{125}I.HSA
Small centre with cells heavily laden with antigen: part of larger centre with two faint foci of localization. Intense grain clusters are not present along the arteriole.

Unna Pappenheim X 600

Autoradiographs of the Chicken Spleen

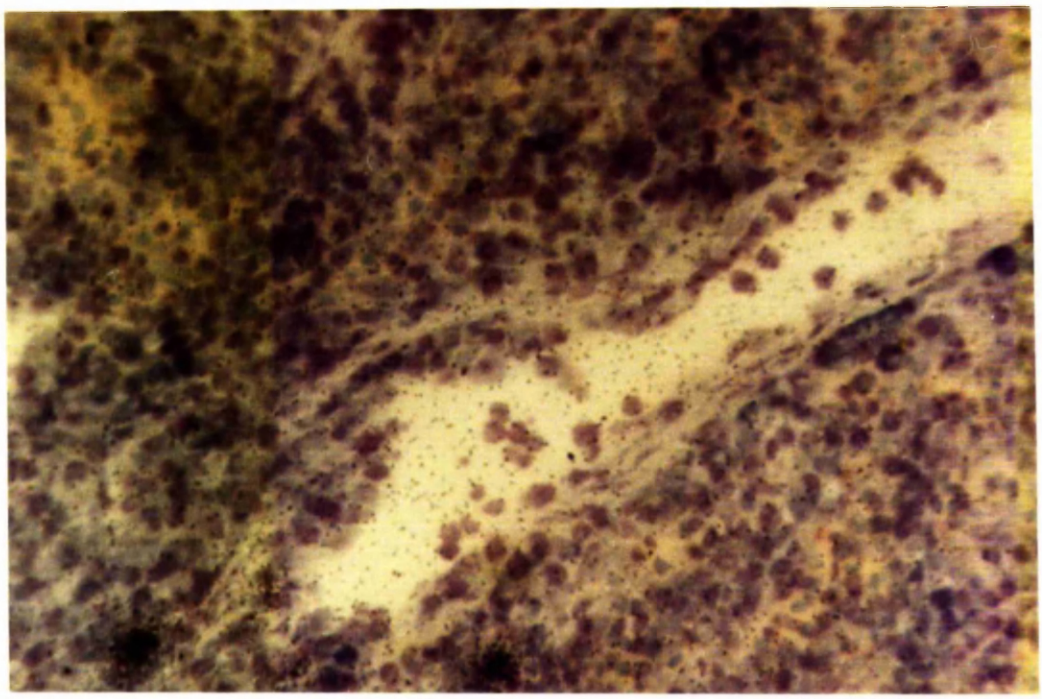


Figure 2.14a Spleen at 88 hours after I.V. injection of 40 ug ^{125}I.HSA Length of venule in white pulp. Localization of antigen in a few cells of the pulp but not in endothelial cells of the vessel.

Leishman X 600

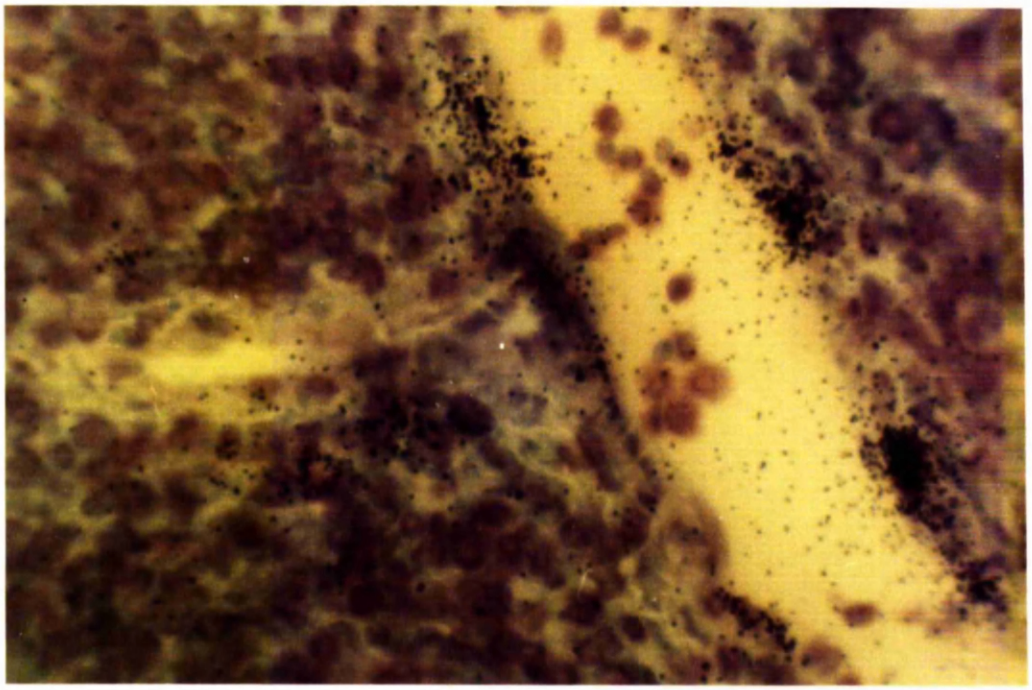


Figure 2.14b Spleen at 6 days after I.V. injection of 40 ug ^{125}I.HSA Length of venule in white pulp showing localization of antigen in endothelial cells of the vessel.

Leishman X 800

rapidity of the response of the chicken to this antigen is more akin in its time relationships to the secondary responses of other species, e.g. antibody production in the guinea pig, rabbit, and horse to diphtheria toxoid (Glenny and Sudmersen, 1921) or in the rabbit to staphylococcal toxin (Burnet, 1941). The rapid fall in the titre of antibody after the peak is in keeping with the half-life of 2.25 days found with the globulin preparation although some birds show evidence of shorter half-life of their antibody globulin.

The bird which received the 1 mg dose of HSA showed a peak titre at an earlier time, the levels of antibody falling from the fourth day onwards. This may represent the lesser delay reported by Cerny, Ivanyi, Madar, and Hraba (1965) in the response of the chicken to smaller doses of a protein antigen. The age of the chicken also influences the response, the younger birds responding less well to the smaller doses. In older, 10 to 12 week old birds, 40 ug HSA brought about an immune response whereas 100 ug was unable to do so in younger birds.

From his experiments on the primary and secondary responses of rabbits to Maja plasma as antigen, von Dungern (1903)deduced a direct relationship between the rate of disappearance of antigen from the bloodstream, and antibody production. Weigle (1960) related the disappearance of antigen (bovine serum albumin) from the bloodstream of rabbits to the production of antigen-antibody complexes. Such complexing did

not necessarily result immediately in the removal of complexed antigen from the bloodstream but only took place when the complex reached a certain size. The results obtained here in the chicken showed a faster sequence which did not allow the detection of such an intermediate stage. Acceleration of antigen elimination was already taking place when the complexes were first detected.

The small amounts of macroglobulin antibody present on the 4th, 5th and 6th days after the injection of 1 mg of antigen may have an important function in the localization of antigen at these times. The findings of Levenson and Braude (1967) in the guinea pig are of interest for they showed that antigen (Salmonella typhi Vi antigen attached to sheep red cells) captured by macrophages in the presence of early, macroglobulin antibody was not ingested into internal vacuoles of the cell but remained on the surface whereas antigen captured im the presence of late 7S antibody was ingested into internal vacuoles of the cell. This could have a decisive significance in the reactions of cell surfaces at this time. Rosenquist and Gilden (1963) did not find macroglobulin (19S) antibody by the Farr technique on ultracentrifugation of chicken antisera to bovine serum albumin given in large (40 mg) doses. Ivanyi, Valentova, and Cerny (1966) on the other hand found macroglobulin antibody in chicken responses to 1 to 5 mg HSA per kilogram body weight: the peak titre fell away after the fifth day of the response.

The Farr ammonium sulphate precipitation test was useful in the testing for chicken antibodies against this albuminous antigen: it detected macroglobulin antibody in the concentrations of reagents used (Pinckard, McBride, and Weir, 1967) and it obviated co-precipitation phenomena which vary the apparent performance of chickens with time and salt concentration (Hektoen, 1918; Goodman, Wolfe, and Norton, 1951).

Integration of Events.

The antibody results may now be integrated with the autoradiographic findings and also with the associated findings previously made by the fluorescent antibody technique (White, French, and Stark, 1967; Appendix B). The fluorescent antibody technique (FABT) had shown that (a) antibody-producing cells appeared in increasing numbers in the red pulp from 24 hours after antigen injection; (b) antibody globulin was associated with the antigen-bearing macrophages in germinal centres; (c) the production of immune tolerance by injections of HSA from hatching prevented antibody production and antigen localization.

The sequence starts with the appearance of antibody-producing cells in the red pulp comparable with those found by the FABT in the red pulp of the rabbit spleen (Coons, Leduc, and Connolly, 1955) after primary inoculation. At 40 hours a few scattered macrophages in the white pulp can be seen by autoradiography to have taken up antigen (Figs. 2.4 and 2.5) while, by the third day, antigen-antibody complexes

begin to make their appearance in the bloodstream. The macrophages localising antigen early are not scattered evenly throughout the pulp (Fig. 2.5). This is somewhat inconsistent with the view that localized antigen is entirely antigen that has been complexed previously in the bloodstream for this would be expected to produce a uniform distribution. Local influences may operate to increase the chances of localisation at a particular site. (In this respect Balfour and Humphrey (1967) have found preformed antibody to an artificial antigen in the cortices of mouse aortic lymph nodes which aided antigen localisation there). At 88 hours however a much greater degree of localisation has taken place at a time when the complexing of large amounts of antigen is causing a precipitous fall in the antigen content of the blood. The absolute amount of antigen localising in the chicken spleen is not large: Ivanyi, and Corny (1965) found that at the time of complex formation, the splenic uptake of labelled HSA was only 0.5 ug/g of tissue when doses of 0.5 mg HSA per kilogram body weight had been given.

There is some selectivity in the localisation, for the macrophages have a precedence in time over the endothelial cells within blood vessels (Figs. 14a and b). This precedence may be related to the specific recognition system for immunoglobulin which Patterson and Suszko (1966) have demonstrated in the macrophages of several species including avian species.

The localization of antigen around the ellipsoid suggests that antigen has emerged from the sheath and has been picked up by a net of macrophages which with their processes surround the ellipsoid. Solnitzky from dye studies in the dog (1937) however interpreted his findings as showing that cells of the ellipsoidal sheath phagocytose the dye and themselves subsequently migrate outside the ellipsoidal sheath. Nossal, Austin, Pye, and Mitchell (1966) noted that after intravenous injection of isotope-labelled flagellin into the rat, the antigen entered the white pulp of the Malpighian corpuscle of the spleen from the red pulp and showed a pallisading effect at the junction of the two zones. This was not observed at the junction of the red and white pulp in the chicken spleen at the times examined. The difference may be due in part to the fact that Nossal's observations were observations of the early arrival of uncomplexed antigen, not as here of the late arrival of antigen thought to be complexed to antibody. It is interesting that Nossal and his colleagues found localization of carbon particles in the analogous sites to those of HSA in the chicken spleen, namely in the periarteriolar macrophages of the rat spleen.

The appearances in the autoradiographs at 88 hours could be interpreted as suggesting that the antigen-bearing macrophages migrate proximally along the arteriole. The apparent forming-up of clusters of macrophages at the

arteriolar junctions (Figs. 2.9a and b) might then be taken as the first stages of the mixed agglutination reaction mooted as responsible for the formation of the germinal centres by White, French, and Stark (1967). The elements taking part in this reaction would be macrophages, antigen-antibody complexes, and also lymphocytes with some special affinity for the antigen. Antibody globulin is also present on the surface of these dendritic macrophages (Figure 10 of Appendix B) and it is known from other studies that the selectivity of these cells in localizing antigens depends on the presence of antibody. Humphrey and Frank, for example, examined the performance of the analogous dendritic cells of mammalian lymph nodes. Rabbits were used which had been rendered tolerant to the antigen to be injected and which consequently did not produce antibody. In these animals the dendritic macrophages of the cortex did not localize antigen whereas macrophages of the lymph node medulla did show a non-specific localization without subsequent germinal centre formation around them.

This requirement for antibody in order to localize antigen to dendritic cells is at variance with the findings of Nossal and Ada (1964) who found that dendritic localization took place in rats thought to have been made tolerant to flagellin by injection from birth. This conflicting result would only be explained if the tolerance had not been complete against this phylogenetically far removed substance, a suggestion subsequently put forward by Ada, Nossal, and Pye (1965).

Dendritic cells can phagocytose carbon particles (Miller and Nossal, 1964). However the function required of the web, if there is to be mixed agglutination, is attachment and maintenance of the antigen at the cell surface. The evidence of electron microscopy supports to some extent this external siting of antigen. Arguing from the negative evidence of the lack of ultrastructural features of active pinocytosis and ingestion into the elongated processes of the macrophages, Milanesi (1965) suggested that there is an architecture which allows the macrophage to present a large external surface with adherent unprocessed antigen to lymph pathways and consequently to lymphocytes within the centre. Nossal, Abbot, Mitchell, and Lummus (1968) with high resolution autoradiography also found evidence that the association of antigen with the cell membrane is mainly extracellular although there was some evidence of true entry of antigen into the nuclei of lymphocytes.

The lymphocytes taking part in the mixed agglutination reaction would be those with some surface complementary to the trapped antigen. There would be in this way a selection of cells: those with the greater closeness of fit, being more firmly adherent, would be more likely to be arrested in their passage through the area. If the sequence were repeated in new crops of germinal centres on restimulation with the same antigen, cells being trapped would become increasingly selected and accurate in their complementary fit.

White has suggested (1968) that the lymphoid cells accumulating in this way on the macrophage net might be stimulated to blast cell transformation by a mechanism similar to that by which antiserum against allotypic determinants can transform lymphocytes (Gell and Sell, 1965). In the forming germinal centre the fitting of the complementary surface of antigen against a receptor site on the lymphoid cell surface would in the analogy cause the transformation. Subsequent division of such cells would result in an expansion of the germinal centre. This agrees with the findings in figures 2.10b and 2.12 where the actively proliferating mass of lymphoid cells has expanded beyond the zone of antigen-bearing macrophages which themselves do not divide. Each centre would then include collections of lymphoid cells of similar if not the same specificity, being directed against the determinants of the one species of antigen molecule attached to the dendritic web.

The intimate association between macrophage and lymphoid cell envisaged here, whereby the one presents antigen to the other, is consistent with some in-vitro observations of their behaviour towards one another. McFarland, Heilman, and Moorhead (1966) for example have described special cytoplasmic projections by which lymphocytes can become attached to macrophages among other cells (and through which, they postulate, stimulating material may be acquired).

Sharp and Burwell (1960) have also described an interreaction, 'peripolesis', by which macrophages might supply material to lymphocytes.

The data in Table 1 show that there are few centres at six days which do not show some antigen localization. It is not possible to be certain that this always represents localization with a view to specific selection of lymphocytes as described above. The fact however that many centres do not show localization at 88 hours when there is much circulating complexed antigen must mean that the dendritic surface of their contained macrophages must either be already saturated or that the surfaces have not been accessible to such aggregates.

The studies reported here record the formation of a germinal centre as an event secondary in time to the production of antibody in the primary response. This is consistent with the findings of Leduc, Coons, and Connolly (1955) and White (1960) who only on secondary stimulation of rabbits found antibody-producing cells (as distinct from antibody-bearing macrophages) in the germinal centres of lymph nodes. The function of the centre is therefore likely to be that of an "immunizing organ" (Osterlind, 1938) in the sense of recruitment of cells for a subsequent response rather than as a furnisher of antibody for the immediate response (as Osterlind thought from his studies in the guinea pig with diphtheria toxin).

The dynamic formation of germinal centres (Conway, 1937) has been demonstrated in this study. The suggested interpretation of the findings would combine ideas of lymphoid proliferation (Flemming, 1885) and reaction to the presence of foreign material (Hellman and White, 1930). The reactants have now been demonstrated by modern techniques to include antibody and antigen.

What has not been established is any obvious localization of antigen in the red pulp in which the antibody-producing plasma cells are lying (Figure 3 of Appendix B). The initiation of their antibody production clearly does not require the presence of gross amounts of antigen and consequently from this study it is not possible to answer whether any macrophage cell has played any decisive part in the initiation of the response by these cells.

Chapter Three

Immunological Adjuvants and their Modes of Action

Adjuvants complicate antigenic stimulation in a manner which subsequently enhances the response. We have seen in the last chapter the framework of an immune response and it is timely to consider how immunological adjuvants might be able to alter this process.

Past work has shown that their contribution may be purely physical altering the behaviour of the antigen in the timing of its distribution; alternatively the adjuvant may have pharmacological effects which alter the behaviour of the responding cells; commonly these activities are combined in the one adjuvant preparation. They will be considered here in turn.

Physical Effects

Physical association of antigen and adjuvant so that antigen escapes slowly to reach immunocompetent cells has been found to be a useful means of stimulating a response. Thus diphtheria toxoid was co-precipitated with alum (Glenny and Barr, 1931) and this resulted in an "increased antigenic efficiency" as shown by the heightened and extended response. The slow release and absorption from the site of injection (Glenny, Buttle, and Stevens, 1931) was regarded as providing its own secondary stimulus.

Slow release is also the mechanism of action of water-in-oil emulsions. Freund (1947) records that 100 MLD of tetanus toxin in such a presentation was non-toxic for the mouse, clear evidence that only a tiny amount was escaping in unit time. Herbert (1966) has mimicked the slow escape associated with such adjuvants by giving repeated tiny doses of antigen (ovalbumin). He found that an improved response is set up and maintained just as with the adjuvant and inferred therefrom that the presence of formed antibody did not prevent further stimulation of antibody-producing cells. (It is worth commenting also that neither this regime nor the antigen in water-in-oil adjuvant produced a "low-zone" tolerance (Mitchison, 1964), at least a theoretical possibility with the prolonged antigenic stimulation produced by this type of adjuvant). The same author (1968) has also shown that the depot formed can gradually become functionally walled off although the immured material is still active if removed to another site.

Dale (1961) has demonstrated that there is a direct effect on the lymphatics of the ears of mice injected with water-in-oil emulsions, an appearance which she interprets as a "proliferation" of lymphoid vessels. Certain water-in-oil emulsions found by Freund to be less efficient adjuvants were also less able to bring about this proliferation.

Farr and Dixon (1960) made a telling observation regarding water-in-oil emulsions containing bovine serum albumin as antigen. The full effect in rabbits they found to be

dependent not on the total dose but on the concentration of antigen in the injection volume, the response increasing with concentration. This offers a clue to the local conditions which must prevail for the immune process to be initiated.

The physical combination of some component of the adjuvant material with the antigen may make it more resistant to digestion and excretion. Torrigiani and Roitt (1965) with the antigen, human thyroglobulin, attached to acrylic resin particles have demonstrated a qualitative change in the response in rabbits in that the 19S antibody response was selectively greatly increased. The authors suggest that there has been either prolonged persistence of antigen in macrophages or an increased initial uptake. Amies (1962) has also enhanced the response to particulate viral bodies by placing them in solutions of polyvinylpyrrolidone. This layers the particles with polymer which encourages the formation of aggregates. The enhanced response is then thought to be due to delayed absorption from the injection site (but increased phagocytosis might equally well be responsible).

The experiments of Adler and Fishman (1962) with diffusion chambers (filters of 0.1 u pore size) in the peritoneal cavities of rats and mice showed that their physical presence produced some adjuvant effect in the response against haemocyanin and ferritin as antigens. Since there was still an effect when the antigen was injected elsewhere in the

peritoneal cavity, the authors could not be certain whether the effect was due to the chamber acting as a physical depot or was the result of the cellular response on and around it.

Effects causing Altered Cellular Behaviour

Mycobacterial Infections An early and important observation was the increased ability of tuberculous guinea pigs compared with normal animals to produce antibody against sheep red cells (Lewis and Loomis, 1924). Mycobacterial infections were also shown to increase greatly the performance of the reticuloendothelial system (RES). In a carefully standardized carbon clearance test in mice (Halpern, Benacerraf, and Biozzi, 1953; Biozzi, Benacerraf, and Halpern, 1953) injections of live attenuated tubercle bacilli in the form of BCG brought about the more rapid removal of carbon particles (diam. 25mu) by a stimulated RES (Biozzi, Benacerraf, Grumbach, Halpern, Levaditi, and Rist, 1954). This stimulation was at its height 17-18 days after the injection of BCG and was associated with an increase in the weight of the liver and spleen thought to be due to hyperplasia of phagocytosing cells. Animals with BCG infections were also shown to produce antibody greatly in excess of normal against the 'H' and 'O' antigens of Salmonella typhi (Halpern, Biozzi, Stiffel, and Mouton, 1958).

Endotoxin

The influence of endotoxin on phagocytosis and antibody production was investigated in a parallel fashion. Johnson, Gaines and Landy (1956) reported the enhanced response to intravenous ovalbumin in rabbits when endotoxin (the lipopolysaccharide of Salmonella typhi) had also been given intravenously at the same time. They believed that host susceptibility to the toxic property of the substance was a prerequisite for adjuvant activity and showed that a dose sufficient only to produce a pyrexia in rabbits may be insufficient to produce an adjuvant effect. Biozzi, Benacerraf, and Halpern had previously (1955) demonstrated increased phagocytosis in mice injected with killed S. typhi or its endotoxin. The adjuvant effect of endotoxin (mainly from Bordetella pertussis) on antibody production against diphtheria toxoid has since been shown by Farthing and Holt (1962) in mice, rabbits and guinea pigs. They found that the inoculation of antigen and adjuvant together in time was important (together or within 24 hours of antigen) although the substances did not need to be given into the same site. They believed that the action was not a physical association of the two substances but a direct one on the host cells. This was also the view of Condie, Zak, and Good (1955) after work with meningococcal endotoxin. Some support for this lay in the findings of Ward, Johnson, and Abell (1959) who found a great increase in modified reticular cells in lymphoid tissue.

The stimulus to proliferation of cells in the haemopoietic tissues by endotoxin, well known in the context of radiation protection has been related mainly to the granular series and to a lesser degree, if at all, to the lymphoid elements (Smith, 1964; Smith, Alderman, and Gillespie, 1957, 1958; Smith, Marston, and Cornfield, 1959).

Another observation on the action of an adjuvant has been made by Munoz (1961) with pertussis endotoxin. He has shown that it can produce an increased tissue permeability which may allow a slightly different tissue distribution of antigen, possibly of importance in adjuvant action. Johnson (1967) has also proposed that nuclear debris can act as an adjuvant being released as DNA or RNA from dead cells after the toxic action of endotoxin. He has brought circumstantial evidence that these substances have such an effect.

The interrelationships with infection by other organisms are interesting and complex: the toxic action of endotoxin cannot be neglected. Thus infection of mice with BCG, so that the RES is stimulated after 12-14 days, sensitizes them to endotoxin (Halpern, Biozzi, Howard, Stiffel, and Mouton, 1959; Suter and Kirsanow, 1961; Berry, Smyth, and Kolbye 1962). Stimulation of the RES by simple lipids also sensitizes mice to endotoxin (Cooper and Stuart, 1961). On the other hand endotoxin has protected mice against infection with, for example Mycobacterium fortuitum (Boehme and Dubos, 1958),

Salmonella enteritidis (Howard et al.1959), and Ectromelia (Gledhill, 1959), just as BCG (live or killed) has protected against acute staphylococcal death (Dubos and Schaedler, 1957). The protective action may lie in the increased phagocytosis possibly aided by increased production of non-specific serum opsonins (Jenkin and Palmer, 1960; Michael, Whitby, and Landy, 1961).

Exotoxins

Exotoxin was noted at an early stage to promote the antibody response (Glenny, Pope, Waddington, and Wallace, 1925). With diphtheria toxoid it was found that the more toxin (as opposed to toxoid) activity there was in an alum preparation the greater was the immune response obtained. Toxins of Clostridium welchii and Staphylococcus aureus could be substituted. This suggests that the effect is not due to the provision of important antigenic groups which might have been lost in the toxoiding process but to an unrelated phenomenon. The intimate nature of the cellular damage relevant to the adjuvant action of toxins has not been determined.

Simple Lipids

Lipids as well as making a physical contribution to adjuvant emulsions may alter cellular performance. Some lipids can greatly change the rates of phagocytosis (Cooper and West, 1962; Cooper and Houston, 1964; Conning and Hoppleston. 1966). Triolein for example can bring about a

striking change in the surface appearance of peritoneal macrophages as shown by the electron microscope (Carr, 1967). There is greatly increased formation and length of pseudopodial processes and more phagosomal vacuoles due to either greater absolute numbers or greater persistence of formed vacuoles.

Surface-active Agents.

Gall (1966) has drawn attention to the surface-active nature of many of the components of adjuvant mixtures and has attributed this quality to the colloidal metallic hydroxides (Glenny et al.,1925), lanolin (Freund and McDermott, 1942), saponin (Richou, Jensen, and Belin, 1963) and to proteins themselves. He has questioned whether more antigen can enter the immunocompetent cell through alterations in cell membrane permeability and using diphtheria toxoid as antigen in guinea pigs has pursued this in an investigation with a series of aliphatic nitrogenous bases. These are strongly polar molecules which cause large local granulomata. He found that the length of carbon chain and also the basicity of the molecules was related to their success as adjuvants.

Agents causing Inflammation.

Inflammation and abscess formation at the site of antigen inoculation increased antibody production to diphtheria toxoid in horses.(Ramon, 1925).

Inflammation implies release of chemotactic agents, attraction of varying cell types, cellular damage of

greater or less degree, increased phagocytosis, altered metabolism and rate of enzyme action due to change in local temperature, opening up of lymphatic drainage systems, alteration in tissue permeability, and formation of an acute abscess or chronic granuloma. Inflammation thus not only affords an opportunity for increased numbers of cells to take part in disposal of injected material but also necessarily means that many of these cells may be in a stimulated state (even if only in thermodynamic terms on account of the increased temperature).

In their adjuvant mixture Freund and McDermott (1942) brought together an inflammatory agent (killed tubercle bacilli) and an antigen depot (a water-in-oil emulsion, liquid paraffin emulsified with lanolin), the antigen used being horse serum. They thus hoped to obtain something of the effect of tubercle bacilli on antibody production (Lewis and Loomis, 1924) without the complications of infection. With this mixture, subsequently referred to as 'complete Freund's adjuvant' (CFA) they produced a profound stimulation of the immune response in guinea pigs. Not only were increased antibody levels achieved but in addition a state of sensitization akin to tuberculin sensitivity, so-called delayed hypersensitivity,(Zinsser, 1921), resulted towards the horse protein.

The activity of this type of adjuvant has been analysed in great detail. Much can be attributed to certain

wax fractions of the tubercle bacilli (Freund, Thomson, Hough, Somner, and Pisani, 1948; Raffel, Arnaud, Dukes, and Huang, 1949; White, Coons, and Connolly, 1955; White, Bernstock, Johns, and Lederer, 1958; White, Jolles, Samour, and Lederer, 1964) both in producing increased amounts of antibody and in inducing delayed hypersensitivity to pure protein antigens. Wax fractions can induce a widespread response in the plasma cells of spleen and lymph nodes as well as greatly stimulating the macrophages in the local granulomatous mass. Antibody-producing cells are however present in the granuloma (Askonas and Humphrey, 1955).

The framework produced by the mycobacterial adjuvant granuloma may be necessary to the setting up of delayed hypersensitivity which may require large numbers of participating cells. What must be judged is how essential is the granuloma for the induction of delayed hypersensitivity or of increased adjuvant activity. It is interesting that a similar histological picture has been produced when beryllium has replaced tubercle bacilli in CFA (Salvaggio, Flax, and Leskowitz, 1965). The reaction is less florid with fewer epithelioid cells: increased antibody to BSA was produced but not delayed hypersensitivity.

The apparent additional quality of the delayed-type hypersensitivity response may only represent an alteration in the proportion of cells committed to a particular course. Such cells may ordinarily respond in a minor way. From the

first Dienes (1930)regarded this hypersensitivity as a part, albeit minor, of the normal response preceding the humoral antibody response. This sector of the response has been encouraged by the adjuvant. An associated phenomenon has been the finding of specific slow or γ_2-antibody by immunoelectrophoresis of the sera of guinea pigs showing delayed hypersensitivity. (White, Jenkins, and Wilkinson, 1963; Benacerraf, Ovary, Bloch and Franklin, 1963).

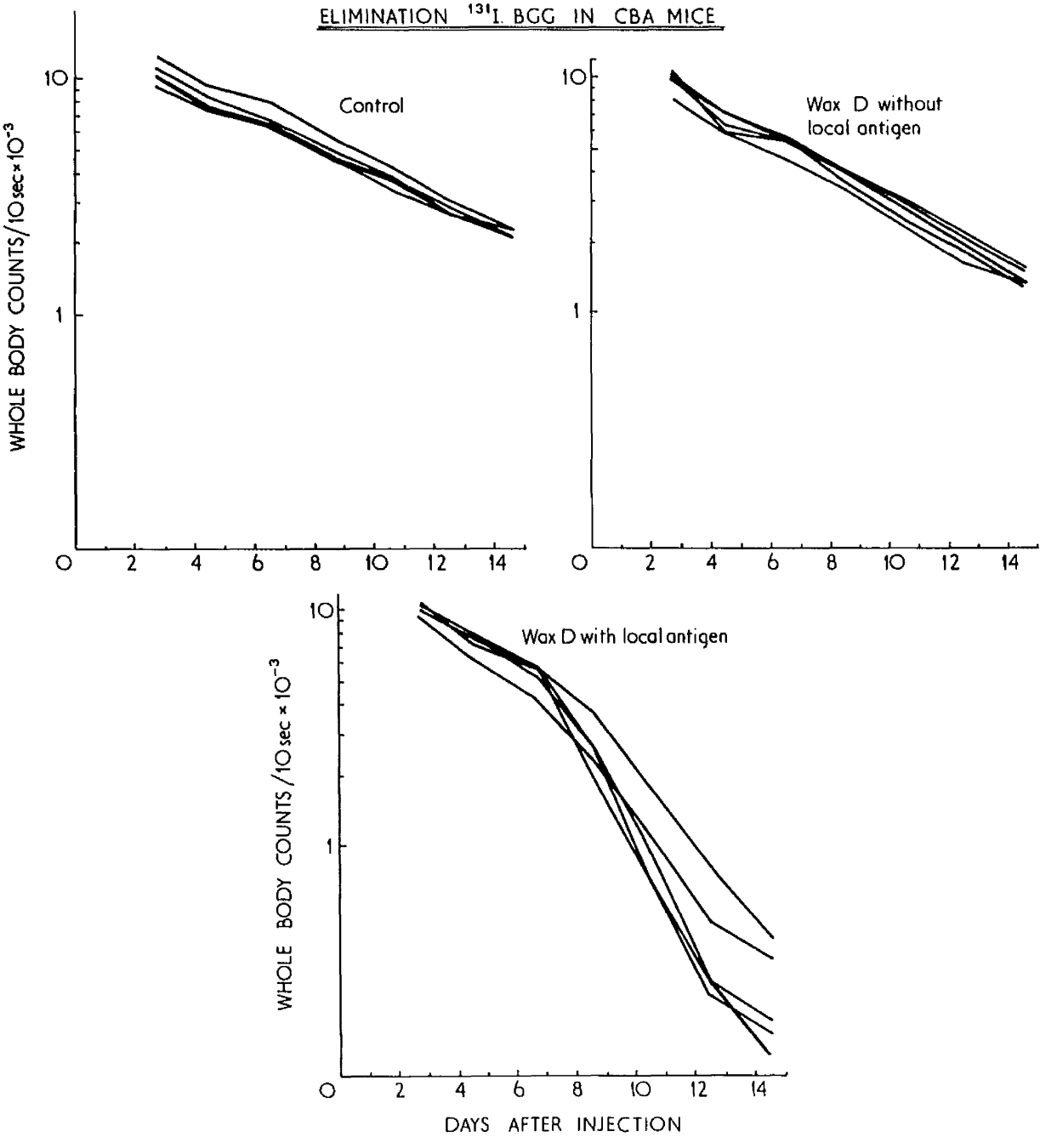
In practical terms adjuvants have their usefulness in improving the response to poor antigens. There are instances however where adjuvants allow a response to be set up when otherwise there would not only be no response but where a state of immune paralysis would be established. This will be discussed along with the experiments of the next chapter.

The subjects of bacterial adjuvants and adjuvant activity in the production of delayed hypersensitivity have been fully reviewed by Munoz (1964) and White (1967b) respectively.

Observation of Adjuvant Activity

The early experiments were first undertaken to examine the usefulness of an antigen elimination model (Dresser, 1960) in the examination for adjuvant activity of wax fractions derived from strains of Mycobacterium tuberculosis.

In this model a trace-labelled purified protein antigen, bovine gamma-globulin (BGG) is given intravenously to mice who initially katabolize the foreign protein as they do their own native protein (Dixon, Bukantz, Dammin, and Talmage, 1953; Freeman, Gordon, and Humphrey, 1958). Detectable amounts of this antigen are still in the bloodstream when antibody is first released from the lymphoid tissues. When this happens there is an acceleration of elimination usually on the seventh day or later after the administration of antigen (Fig.4.1c). If precautions have been taken to remove aggregates from the BGG (Dresser, 1961b), antibody formation is not initiated unless another stimulus in the form of an immunological adjuvant is given. The various substances to be examined for adjuvant activity can be tested for their ability to give this immunogenic stimulus after injection into subcutaneous sites. Those mice not showing the immune-type elimination on the primary exposure to antigen may have become latently immunized and will show an obviously immune-type elimination if the test is repeated



e 4.1 Influence of a subcutaneous adjuvant, with and without local antigen, on the elimination of a 1 mg I.V. dose of BGG.

Top left (a) Control group.
Top right. (b) Each mouse received 300 ug Wax D in 0.2 ml Bayol 55 subdivided for injection into four sites.
Bottom. (c) Each mouse received 300 ug Wax D and 100 ug BGG in 0.2 ml emulsion (saline, Bayol 55, and Arlacel A) similarly subdivided.

after an interval. Mice not responding on the first exposure may however have been immunologically paralysed specifically to the antigen and will not give an immune response on a second exposure even if attempts to encourage antibody formation are made by presenting the antigen in a highly immunogenic manner in complete Freund-type adjuvant (Dresser, 1962a and b).

After the initial observations, the interest came to lie not so much in the comparative activities of potential adjuvants as in how they achieved their effects and how host factors altered their performance.

Materials and Methods

Mice For most experiments CBA-strain male mice were used, 3-5 months old, 22-25 g in weight. In Experiment 8, 4-5 month old CBA female mice were used, one group being in the first week of lactation at the time of injection of antigen.

Antigen Bovine gamma globulin, ethanol fractionated (Cohn fraction II), batches nos. HL2970 and LB 1071.

Radiolabelling of Antigen The direct oxidation method of Hunter and Greenwood (1962) was used with iodine-131 as described in Chapter Two (p.21). Amounts of 100 mg BGG were labelled so that about 7uC of radioactivity were attached to each 1mg dose of antigen.

Preparation of Antigen to be injected. After radioiodination the antigen was spun for 30 min. at 30,000g in a 3 x 10 swing-out head of a MSE Super Speed 50 ultracentrifuge in order to remove aggregates. The contents of the upper two-thirds

of the tubes were removed, the O.D. estimated in a U.V. spectrophotometer at 280mu and the protein content derived from a standard graph. A final solution was then made up to contain 2mg antigen per 1ml of 0.15 ml saline.

Immunological Adjuvants

Adjuvant mixtures were prepared from the following reagents:

(a) Mineral Oil, (Bayol 55)
(b) Arlacel A, (as emulsifying agent)
(c) Antigen solution, (2.5 mg BCG per ml)
(d) Mycobacterium tuberculosis var. hominis, Weybridge strain C, heat-killed.
(e) Wax D, WL 52, from M.tuberculosis, strain Canetti.

Two types of adjuvant mixture were used:

1. a. Suspension in oil of M.tuberculosis, 1.5mg per ml. Bayol 55

   b. Solution in oil of Wax D, 1.5 mg per ml. Bayol 55.

2. Water-in-oil emulsions with antigen: Complete Freund-type adjuvants (CFA).

   These were prepared by suspending the mycobacteria in the oil (2.5mg per ml) or dissolving the Wax D in oil (2.5mg per ml). The oil was mixed with the Arlacel A and antigen solution in the proportions of 3:1:1 respectively: the mixture was emulsified by frequently repeated withdrawal and expression of the mixture through a fine needle until a uniform white emulsion of thick consistency was produced.

In Experimant 4, heat-killed Corynebacterium rubrum or Mycobacterium phlei substituted for M.tuberculosis in the two types of adjuvant.

Conditions of experiments.

The animals were kept in boxes of five or six, each experimental group being kept in the one box. Holes were punched in the ears according to a code of recognition.

The animals were put on a balanced diet (Diet 41, supplied by Wm. Pearson Ltd., Glasgow). There was no alteration of supply of feed during the period covered by the experiments. For drinking water 0.075 M saline was given to which 0.01 per cent potassium iodide had been added to promote iodine-131 excretion.

Injections of antigen were given intravenously through the tail vein in mice previously warmed. The adjuvants were given subcutaneously into the foot-pads of the hind limbs and into the anterior surfaces of the fore limbs, the total dose of 0.2ml being subdivided for this purpose. This meant that in the case of the CFA each animal received 300 ug of bacteria or Wax D and 100 ug of antigen. The same quantities of bacteria or wax were in 0.2ml volumes of the oil suspensions or solutions.

Experiments 1, 2, and 3 were performed during the early summer months of 1966. The mouse room had a southern exposure and a large, completely sealed window area: at times despite air conditioning the mouse room became very warm. The later experiments were carried out at ambient temperatures of 18° to 20°.

Counting of Radioactivity

The radioactivity in each animal was determined every second day from the second to the fourteenth day by placing the mouse in a centred container within a large Nuclear Enterprises plastic well scintillation counter. The time of

counting varied from 10 seconds to one minute. Counts of a standard source (about 1uC) were also taken at each counting session. All counts were corrected for radioactive decay and variation in performance of the apparatus, by multiplying each by the ratio of (the count of the standard on the first day) to (the count of the standard on day of count). The counts were then plotted on semilog paper and the rates of elimination (as half lives in days) obtained graphically from the plots.

Thyroid Diets

Thyroxine (Eltroxin, Glaxo) was incorporated in the following amounts:

Thyroid diet A:	0.2mg	Eltroxin	in 600 g diet
Thyroid diet B:	0.6mg	Eltroxin	in 300 g diet
Thyroid diet C:	3.0mg	Eltroxin	in 300 g diet

For convenience and thoroughness of mixing the Eltroxin tablets were first ground down with 20 g of sucrose and this mixed with pulverized diet. Amounts of the mixture were made up into a wet mash which was placed in a dish in each cage. The dishes were refilled daily. Mice were placed on these diets (Experiments 6 and 7) for one week before and two weeks after the primary injection of antigen.

Raising of Ambient Temperature

In Experiment 9 the mice were kept in cages under infra-red lamps so that at the top of the cages the temperature was 30°. Mice were kept under these conditions for three days before injection of antigen.

High Carbohydrate diet.

In Experiment 10 the mice were given brown bread (approximately 85 per cent carbohydrate) in place of Diet 41 in the fortnights before and after the administration of the antigen.

Experimental Section

Dresser (1960) had shown that the antigen (EGG) need not be presented with the killed mycobacteria at the local site of injection for the organisms to exert their adjuvant effect. The following experiments were set up firstly in Porton white mice, then in CBA mice to see if this could be reproduced as a base-line for further investigations. (In these experiments it is important to be clear about the two possible causes of increased elimination. There may be a generalized increase due to an increased rate of protein katabolism. Whether this takes place or not there may be acceleration of elimination usually after the seventh or eighth day due to the formation of immune complexes and their rapid phagocytosis and breakdown).

Experiment 1

Porton White Mice in three groups of five were injected subcutaneously with 0.2ml mycobacteria-in-oil, ten days before, four days before, and on day of I.V. injection of EGG respectively. These mice as well as a control group of five mice were injected with 1 mg ^{131}I.EGG intravenously

and elimination observed by whole body counting every second day.

The elimination rates (in terms of the biological half-life in days) of the antigen are shown on Table 4.1 for the first eight days of elimination. There was a significant difference between each mycobacterial group ($p < 0.01$ by Student's "t" test) and the control group in their rates of elimination. Because of the extremely rapid disappearance of the antigen it was not possible to detect any superimposed acceleration of its removal by antibody.

The animals were therefore after a further two months reinjected intravenously with antigen to reveal any secondary responses, known to be more accentuated in their speed of antigen elimination (von Dungern, 1903; Dresser,1960). The results are recorded in the table: several of the animals in the control group (3 out of 5) as well as many test mice showed a secondary response.

This strain of mouse was regarded as unsatisfactory for several reasons. Firstly with the rapid elimination there was little antigen left to show a response beginning only on the seventh day. Also slight changes of rate of elimination were not easily detectable at this speed. In addition too many of the control mice were producing a primary response without the aid of adjuvant. Subsequent experiments were carried out in CBA mice shown by Dresser to katabolize this antigen more slowly.

Table 4.1

Rates of Elimination of ^{131}I.BGG and Immune Responses in two strains of mice after various treatments.

Treatment	Mean Half-life of Elimination up to Day 8 (in days ± S.D.)	Primary Immune Responses	Secondary Immune Responses
Porton White Mice			
Nil	1.98 ± 0.163	-	3/5
M.Tuberculosis in oil 10 days before, S.C.	1.54 ± 0.05	-	2/5
M.Tuberculosis in oil 4 days before, S.C.	1.34 ± 0.314	-	3/5
M.Tuberculosis in oil On day of I.V.BGG,S.C.	1.6 ± 0.1	-	4/5
CBA Mice			
Nil	4.38 ± 0.66	0/5	0/5*
M.Tuberculosis in oil 10 days before, S.C.	4.14 ± 0.456	0/5	0/5*
M.Tuberculosis in oil 4 days before, S.C.	4.03 ± 0.17	0/5	0/5*
M.Tuberculosis in oil On day of I.V. BGG, S.C.	2.83 ± 0.14	0/4	0/5*
M.Tuberculosis in CFA with BGG, S.C. on day of I.V. BGG	2.96 ± 0.252	5/5	NT
CBA Mice			
Nil	5.0 ± 0.134	0/5	NT
Wax D in oil on day of I.V. BGG.	4.12 ± 0.277	0/5	NT
Wax D in oil with Bayol 55, Arlacel A and BGG. On day of I.V. BGG.	4.18 ± 0.392	5/5	NT

NT: Not tested.

*: Stimulated with complete Freund's adjuvant containing BGG.

Experiment 2

The above experiment was repeated in CBA mice: in addition a further group of five mice were given CFA subcutaneously. After two months all except the last group were again tested by reinjection of 1 mg ^{131}I.EGG and also given CFA subcutaneously.

The results are shown in Table 4.1. The mice in the two groups given the mycobacteria on the day of injection showed a significantly faster elimination (mean half-lives, 2.83 and 2.96 days; $p < 0.01$ by Student's "t" test) than the control group (mean half-life, 4.38 days). Only those mice receiving CFA showed a primary immune elimination. The others showed an immune paralysis when subsequently tested with CFA on a further elimination experiment two months later.

Experiment 3

Groups of five mice were given subcutaneous injections of Wax D in oil and in another group, CFA with Wax D in the oil phase. The elimination rates of antigen given on the same day to these animals and also to control animals are shown in Table 4.1 and Fig.4.1. The elimination rates in both groups receiving Wax D are significantly faster (4.12 and 4.18 days, mean half lives) than the control group (5.0 days mean half life, $p < 0.01$ by Student's "t" test). Only that group receiving the CFA showed a primary immune elimination. These results in the CBA mice

differed from those of Dresser in that neither the mycobacteria nor indeed the wax had been able to stimulate an immune response unless placed in the tissues with the antigen in a water-in-oil emulsion.

There was an interval of six months before the next experiments of this type were carried out.

Experiment 4

Two other bacterial species in addition to M.tuberculosis, C.pubrum and M.phlei were used separately in adjuvant mixtures. Groups of five mice were given either a suspension in oil of one of the species or were given the CFA type of preparation. Along with a control group, these mice were injected intravenously with 1mg ^{131}I.BGG.

Table 4.2 records the results. All groups showed an obvious primary immune elimination. (Fig.4.2 for the example of the M.tuberculosis in oil group). In addition all elimination rates in the preimmune phase were accelerated significantly over the control group ($p<0.01$ in all cases except the M.tuberculosis group when $p<0.05$ by Student's "t" test).

Experiment 5

Further groups were set up with M.tuberculosis and Wax D in the two types of adjuvant preparation. Primary immune responses to I.V. BGG were obtained in all cases (Table 4.2 second part). Control groups showed an elimination rate in the first eight days of 2.96 days as against 2.72 days for the Wax D in oil group, not significantly different on this occasion ($p< 0.1$ by Student's "t" test).

Elimination of ^{131}I.BGG in CBA mice

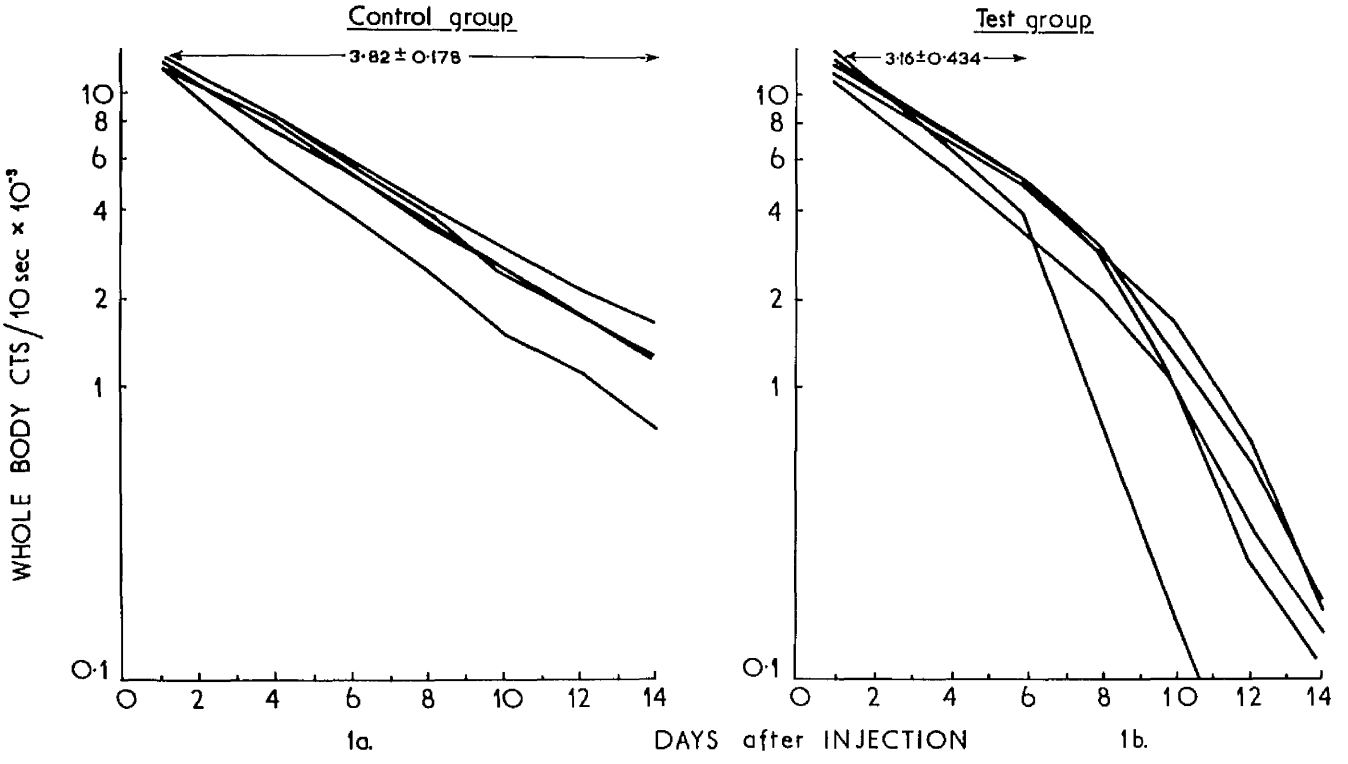


Figure 4.2 Influence of a subcutaneous adjuvant (without local antigen) on the elimination of a 1 mg I.V. dose of BGG.

The mice in the test group received 300 ug of killed M.tuberculosis var. hominis in 0.2 ml Bayol 55 subdivided for subcutaneous injection into four sites.

Table 4.2

Rates of Elimination of ^{131}I.BGG and Immune Responses in CBA mice after various treatments.

Treatment (by subcutaneous injection).	Elimination Rate (Half-life in days) up to Day 8.	Primary Immune Responses.
Nil	3.82 ± 0.178	0/5
CFA with *M. tuberculosis*	2.58 ± 0.16	5/5
M. tuberculosis in Bayol 55	3.16 ± 0.434	5/5
CFA with *C. rubrum*	2.3 ± 0.155	5/5
C. rubrum in Bayol 55	2.5 ± 0.18	5/5
CFA with *M. phlei*	2.4 ± 0.29	3/3
M. phlei in Bayol 55	2.58 ± 0.37	4/4
Nil	2.96 ± 0.09	0/5
CFA with *M. tuberculosis*	2.54 ± 0.167	5/5
M. tuberculosis in Bayol 55	2.26 ± 0.27	5/5
CFA with Wax D	2.38 ± 0.13	5/5
Wax D in Bayol 55	2.72 ± 0.23	5/5

Assessment of Experiments 1 to 5

It was difficult to explain the apparent contradiction between the early (Experiments 2 and 3) and the late results (Experiments 4 and 5) in the CBA mice which in the one case had not produced antibody unless CFA was used and in the other produced antibody when mycobacteria (or one of the substitutes) in oil had been used as adjuvant. The animals were of the same strain, age, and sex. The antigen was undoubtedly older on the second occasion: it had been kept under dry conditions but ageing could have caused some denaturation. Any denatured molecules would have been expected to be eliminated quickly however and would have been unlikely to affect the elimination rate much after the first 48 hours (Freeman, 1965,1966). The only difference in conditions which could be thought of (and this retrospectively) was the warmth of the early summer which might have affected the metabolic rates of the mice. Interest was focussed on the rate of katabolism for other reasons. Firstly there was some suggestion from the experiments with the Porton White mice that very rapid katabolism of antigen predisposed to an immune response. (This of course could have a quite different genetic basis). Secondly in Experiments 4 and 5 the elimination times were faster in the control groups than in Experiments 2 and 3. Lastly the adjuvants themselves accelerated katabolism of antigen in the pre-immune phase. The question posed was whether faster elimination contributed

to the adjuvant action. Attempts were therefore made to increase katabolism with thyroid hormone and observe if this alone could instigate immune-type elimination.

Experiment 6

Two groups of five mice were maintained on thyroid diets A and B as described above in the Materials and Methods section, during the seven days before and fourteen days after primary injection. Their elimination rates of ^{131}I.BGG were observed on two occasions at an interval of two months.

Table 4.3 records the results of the two exposures to antigen. In the primary exposure the rates of elimination of the thyroid groups had not been significantly increased over that of the control group ($p > 0.10$ in the group with the lower mean half-life). There was no evidence of any superimposed immune-type acceleration after the seventh day.

The secondary exposure to antigen resulted in acceleration of elimination by the sixth day in all mice in the thyroid groups over the rate prevailing in the period day 2 to day 4, the final mean half-lives being not more than 2.42 days. In the control group only one mouse showed acceleration (to a half-life of 0.8 day on day 4).

This was suggestive evidence that the mice had been immunized during the primary exposure to antigen while on the thyroid diet. As the amount of thyroid hormone in the diet had not produced a significantly increased katabolic

Table 4.3

Rates of Elimination of ^{131}I.EGG in mice receiving oral thyroxine during Primary exposure to antigen.

Group	Elimination Rate (half-life in days)	No. of mice showing Alteration of Elimination Rate* on		
	Days 2-4	Day 4	Day 6	Day 8
Control (5 mice)				
Primary exposure	3.58 ± 0.15	-	-	-
Secondary exposure	3.7 ± 0.36	1 (0.8d)	-	-
Thyroid diet A (5 mice)				
Primary exposure.	3.5 ± 0.25	-	-	-
Secondary exposure	3.24 ± 0.41	1 (1.0d)	4 (2.25 ± 0.34d)	-
Thyroid diet B (5 mice)				
Primary exposure	3.74 ± 0.15	-	-	-
Secondary exposure	3.2 ± 0.23	1 (1.5d)	4 (2.42 ± 0.19d)	-

(* The altered half-lives are shown in brackets below the no. of mice).

rate detectable in the preimmune period, it was decided to give a higher dosage of thyroid in the diet and observe the response to antigen.

Experiment 7

A group of mice was maintained on thyroid diet C during the seven days before and fourteen days after the primary injection of antigen. Antigen elimination tests were performed also ten weeks later, and again six months after the secondary exposure.

The results are shown in Figure 4.3.

Figure 4.3. During the primary exposure to antigen the mean half-life of the trace-labelled protein in the control group was 3.7 days: in the thyroxine-treated group the mean half-life was 2.76 days with no evidence of immune elimination on or after the sixth day. Ten weeks later however on the sixth day after a second dose of antigen there was significant acceleration in the thyroxine-treated group, the mean half-life decreasing from 2.72 days to 2.08 days at that time. A third exposure six months after the second showed a further accentuated immune reaction in this group (Figure 4.3). Control mice gave no response on the secondary exposure to antigen (one mouse died in this group before secondary exposure). On the tertiary exposure, one control mouse gave a mild response (half-life accelerating from 3.8 to 3.3 days after the sixth day).

Figure 4.3 Legend

Elimination of ^{131}I.BGG in CBA mice

The mice in the test group were fed on a diet containing 3 mg 'Eltroxin' (Glaxo) per 300 g for seven days before and 14 days after the primary exposure to antigen.

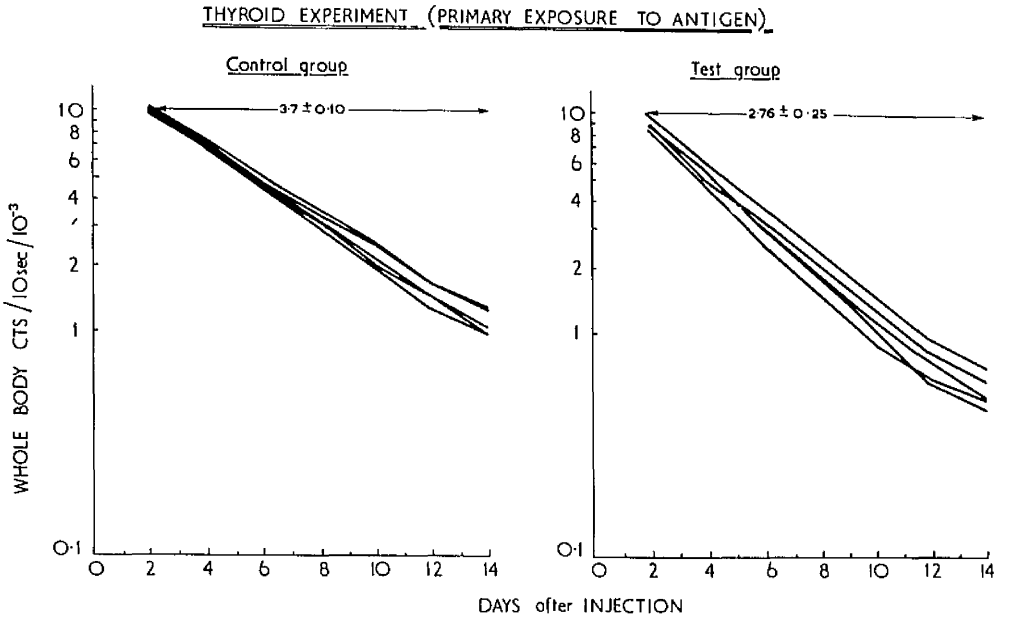
Top (a) Primary exposure to antigen in control and test groups

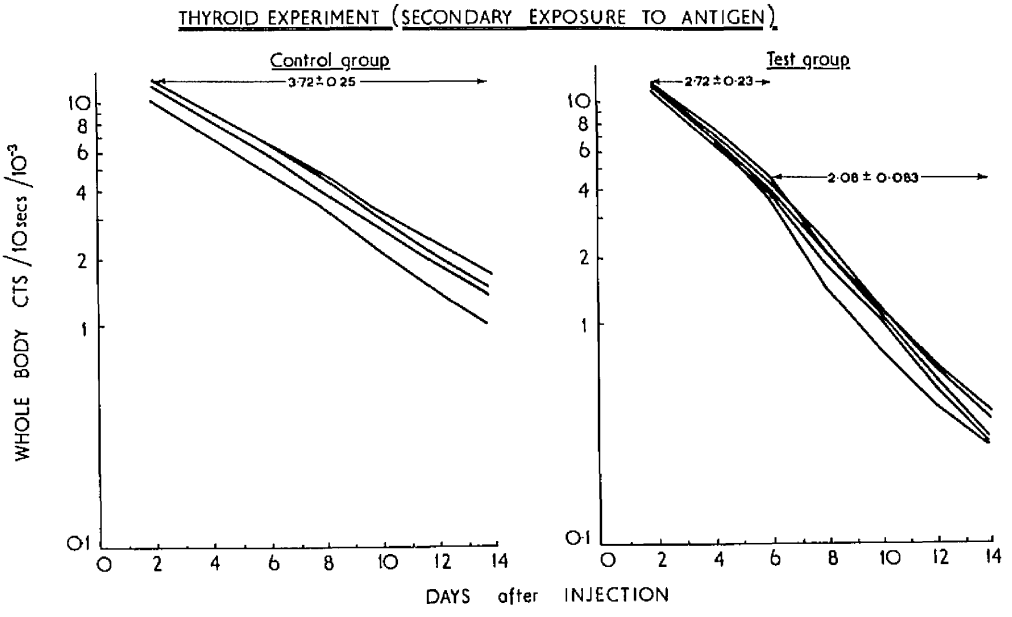
Middle (b) Secondary exposure to antigen ten weeks later.

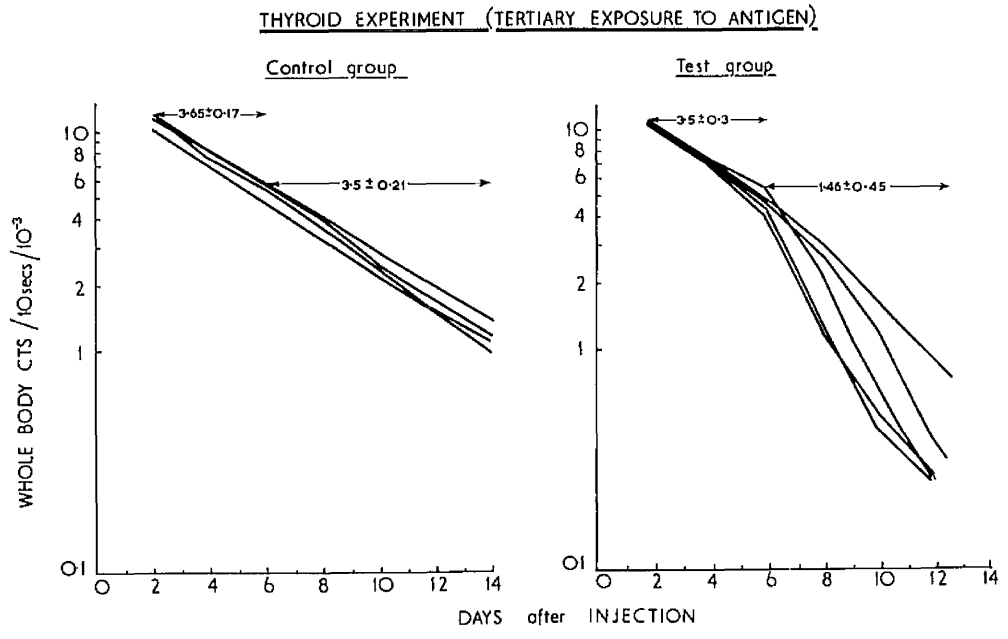
Bottom (c) Tertiary exposure to antigen a further six months late

The numbers quoted in the diagram represent the mean biological half-lives ± S.D. in days of the ^{131}I.BGG obtained graphically over the periods indicated.

(Legend on facing page)







The thyroxine-treated mice have thus formed antibody on the second exposure to BCG although none was detectable in the primary response which lacked any immune elimination after the sixth day. The accelerated katabolism may have been crucial in establishing this response but it cannot alone produce as great an adjuvant effect as the mycobacteria after which the primary immune response is well defined.

It was considered worthwhile examining the responses in female mice in the physiological hypermetabolic state of lactation.

Experiment 8

A group of six lactating female mice in the first week of lactation and control groups of five male and six female mice were submitted to the antigen elimination test with ^{131}I.BCG.

The results of primary exposure, and secondary exposure two months later, are shown in Table 4.4. The mean half-life of elimination of antigen in the non-lactating females did not differ significantly from that of the male group ($p > 0.10$ by Student's "t" test). The lactating females eliminated the antigen significantly faster ($p < 0.01$ in both cases).

On secondary exposure, the male controls showed immune elimination in four mice out of five, as also did five of the six non-lactating females. Four out of the six lactating females also showed such immune elimination, but two did not do so.

Table 4.4

Comparative Rates of Elimination of ^{131}I.BGG in Female CBA Mice.

Group	Elimination Rate (half-life in days)	No. of mice showing Alteration of Elimination Rate*		
	Days 2-4	Day 4	on Day 6	Day 8
Male (5 mice)				
Primary	3.08 ± 0.31	-	-	-
Secondary	3.06 ± 0.75	2 (0.6d)	2 (2.3d)	-
Female (6 mice)				
Primary	2.76 ± 0.24	-	-	-
Secondary	2.45 ± 0.30	3 (0.5d)	2 (1.4d)	-
Female (Lactating, 6 mice)				
Primary	1.96 ± 0.27	-	-	-
Secondary	2.23 ± 0.44	2 (1.3d)	1 (1.6d)	1 (1.8)

(* The altered half-lives are shown in brackets below the no. of mice).

Although the antigen in this experiment in some unexplained way was more likely to induce an immune response, the fact that two lactating mice did not become immunized suggests all the more strongly that rapidity of elimination of the antigen by itself is not sufficient to bring about the induction of the immune response.

Experiment 9

Mice were kept under warm conditions (at 30°) as described in the Material and Methods section. In this way it was hoped to slow down their metabolic rate (Pennicuik, 1967) and possibly alter the ability of M.tuberculosis in oil to induce an immune response. A group of mice at 18° and a group at 30° were injected with adjuvant; a second group at each temperature acted as controls.

Table 4.5 records the results. Control mice show a significantly faster rate of elimination of the antigen (mean half-life 3.48 days) than do the warm mice (mean half-life 3.84 days, $p<0.01$). Both groups receiving adjuvants show evidence of a primary immune response but the rate of immune elimination in the warm mice is significantly slower (mean half-life 1.7 days) than the control group (1.175 days: $p<0.05$).

The ratio of the protein katabolic rates (30°: 18°) is 3.84 : 3.48 in the control groups, i.e. 1.1:1.0. The ratio of the protein katabolic rates in the preimmune phase of the adjuvant groups is 3.1 : 2.67, i.e. 1.17 : 1.0.

Table 4.5

Effect of Raised Ambient Temperature on Elimination Rates of ^{131}I.BGG in CBA mice.

Other Treatment		Elimination Rates (half-life in days) at Ambient Temperatures of 19°	30°
NIL (5 mice)		3.48 ± 0.08	3.84 ± 0.173
M. tuberculosis in Bayol 55 (5 mice)	Days 2-8	2.67 ± 0.264	3.1 ± 0.56
	Days 8-14	1.175 ± 0.34	1.7 ± 0.31

Table 4.6

Effect of High Carbohydrate Diet on Elimination of ^{131}I.BGG in CBA mice.

Other Treatment	Elimination Rates of Mice On Normal Diet	On Carbohydrate Diet
NIL (5 mice)	3.22 ± 0.19	3.98 ± 0.054
Wax D - in oil S.C. (4 mice) Days 2-7	2.72 ± 0.47	3.27 ± 0.09
Days 7-14	1.58 ± 0.38	2.2 ± 0.52

The ratio of the immune rates of elimination in the adjuvant groups is 1.7 : 1.17, i.e. 1.45 : 1.0. That is to say that the immune elimination in the mice at 18° is faster, compared to the group at 30°, than might be expected from their metabolic rates alone.

This would be in keeping with a greater degree of antibody production even allowing that the quicker immune elimination in the 18° mice might be partially due to a generally faster metabolic rate.

Experiment 10

By eliminating the specific dynamic action of protein in the diet it was hoped that a significant slowing would be effected in the metabolic rate which again might affect the performance of an adjuvant. The test groups were given a high carbohydrate diet as described in the Materials and Methods section.

One group of mice on normal and one on high carbohydrate diet were given Wax D in oil as adjuvant. A second group on each diet acted as controls.

The results are recorded in Table 4.6. The katabolic rates were slowed in the mice receiving the carbohydrate diet. (Mean half-life of 3.98 to 3.22 days, $p < 0.01$): the rates of immune elimination were also significantly slower in these mice. (2.2 as compared with 1.58 days).

The ratio of the katabolic rates is 3.98 : 3.22, i.e. 1.25 : 1.0. The ratio of the preimmune rates of

elimination in the adjuvant group is 3.27 to 2.72 days, i.e. 1.2 : 1.0. The ratio of the immune rates of elimination in the adjuvant groups is 2.2 : 1.58, i.e. 1.41 : 1.0.

That is to say the rate of immune elimination is faster in the group receiving normal diet than would be expected from the different rates of metabolism alone.

Discussion

In these experiments in adult mice, the induction of tolerance to a purified protein antigen, BGG, is being observed: after the initial exposure the later presentation of the antigen in CFA does not result in an immune response. The antigen has been shown to include both a major and a minor component (Dresser, 1963). The latter comprises about 10 per cent of the total protein present and can induce antibody formation specific to itself without the aid of adjuvant. Although this may cause an apparent prolongation of the elimination rates on secondary and subsequent exposure, it does not alter the main implications of the immune-type elimination seen when antibody against the major component is formed.

The Presence of Antigen at the Site of Adjuvant Injection

The later experiments agreed with those of Dresser (1960) which showed that mycobacterial adjuvants without local antigen were able to produce a demonstrable immune elimination of the intravenously injected antigen.

Nevertheless if circumstances are unfavourable to the establishment of the response as they appear to have been in the earlier experiments (Expmts. 2 and 3), the increased local concentration of antigen clearly made it easier for antibody formation to be set up. The adjuvant mixtures lacking emulsified antigen must therefore be regarded as weaker adjuvants. This would be in accordance with the findings of Farr and Dixon (1960) who demonstrated the importance of local concentration of antigen as opposed to total amount of antigen in stimulating an antibody response.

Substitutes for M.tuberculosis.

Wax D,WL 52, a peptidoglycolipid derived from M.tuberculosis, strain Canetti (White, Jolles, Samour, and Lederer, 1964), C.rubrum, and M.phlei satisfactorily substituted for M.tuberculosis in these experiments. These agents have previously been shown to have adjuvant effects in other antigen systems such as the induction of acute encephalitis (White et al., 1964: Shaw, Alvord, Fahlberg, and Kies, 1964).

In the present experiments they produced an antibody response to EGG when they were themselves injected in an oily medium without local antigen. They also produced the accelerated katabolism of protein in the preimmune phase noted in the experiments with M.tuberculosis.

Acceleration of Antigen Katabolism

The increased rate of protein katabolism in the later experiments (mean half-lives in the control groups of

3.82, 2.96, 3.58, 3.48, and 3.22 days as compared with 4.38 and and 5.0 days) may have been important in creating the changing experience with the adjuvants lacking emulsified antigen. The observations were difficult to explain in other terms as has been discussed above unless some change in the room temperature was accepted as a valid reason for the difference. This had some support from the experiment in which the ambient temperature was raised to 30° for in this situation the katabolic rate fell. However the weaker type of adjuvant was still able to induce an immune response although less antibody was produced if the rate of immune elimination is taken as quantitative indication of antibody production (Dresser, 1965).

Thyroid hormone The thyroxine in the diet induced an immune response in Experiments 6 and 7 which was detectable at the time of the secondary response. In Experiment 6 the mice had not shown a katabolic rate during the primary exposure significantly increased over the control mice.

Thyroid hormone has been known for a long time to have an influence on the performance of the non-specific body defences (Marbé, 1909) and can indeed stimulate antibody production in several species including man (Long and Shewell, 1955; Long, 1957). It has also produced hyperplasia of lymphoid tissue in guinea pigs (Ernström and Gyllensten, 1959; Gyllensten, 1962) and in the fowl (Höhm, 1959). The nature of the action in the current experiments is a matter for speculation but two negative points can be made. Firstly the

ability of thyroxine to induce an immune response to BGG is not dependent on accelerating the katabolic rate during the first exposure as shown by the experience of Experiment 6. Secondly, acceleration of the katabolic rate by itself as shown in Experiment 7. (mean half-life of 2.76 days compared with 3.7 days in the control group) does not bring about the definite primary response achieved by the injected adjuvants.

Lactating mice The experience in these mice confirms the last point made with the thyroid mice, namely that hyperkatabolic rates need not of themselves bring about an immune response. This was the finding in two mice of this group.

Slowing of Antigen Katabolism

The two procedures which brought about a slowing of antigen katabolism, namely the warm environment and the carbohydrate diet, were interesting in that they also reduced the amount of antibody produced against the antigen on the evidence of the immune elimination rates, even when allowance has been made for altered rates of phagocytosis in the different metabolic conditions.

Although the degree of alteration of adjuvant action is minor, the experiments perhaps suggest how the process of keeping concentrations of protein antigens temporarily above tolerance-inducing thresholds might be aided. If the findings could be extrapolated to the human species, they would suggest that the following proposition might be examined, namely that a high protein diet as opposed

to a high carbohydrate diet would contribute to a different likelihood of diseases associated with immune sensitization, especially so in a cold climate.

Postulated Increases in Temperature.

No recordings of body temperature were made during the experiments. Nevertheless the hypermetabolic state produced for example by the thyroid hormone is likely to result in an increased production of heat which might not be compensated for by cooling mechanisms. There would then be as in clinical hyperthyroidism in man a raised body temperature. The footpad granulomata produced by the injection of the mycobacterial adjuvants may also produce local and general rises in temperature. It is interesting to speculate on the possible effect of such a rise in temperature on the katabolism of protein molecules within a digestive vesicle of a cell.

Figure 4.4. shows a hypothetical case where an array of identical molecules of a protein with a repeating structure is being attacked by two endopeptidases A and B, each assumed to have a preference for specific parts of the repeating structure (Dixon and Webb, 1964) and not to interfere with each other spatially. At T^{o} the number of peptide bonds being broken by each enzyme in unit time is the same and fragments of a particular type are produced. If the temperature is raised to $(T + n)^{o}$ the rate of action of each enzyme will not be affected equally if one has a

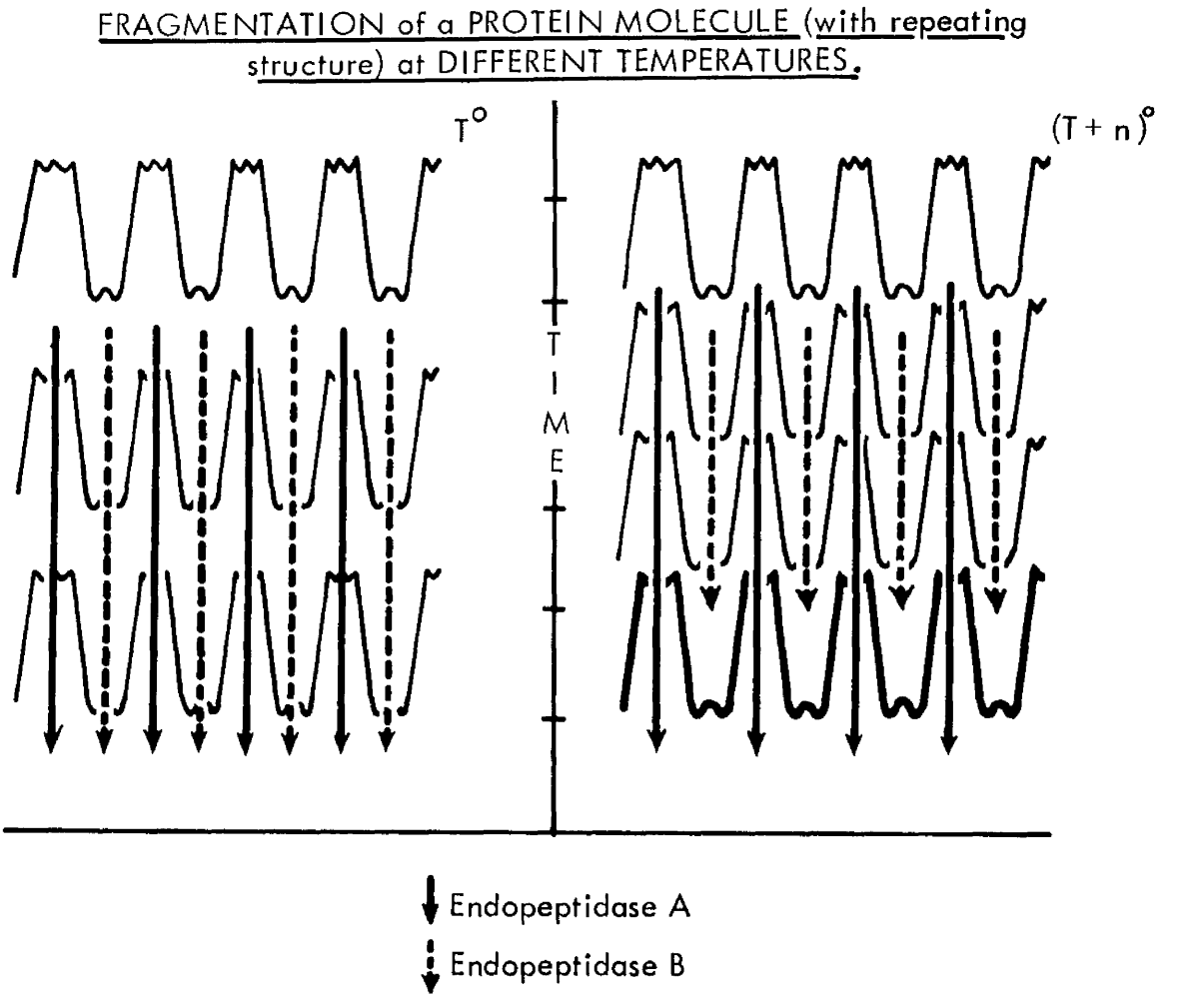


Figure 4.4

Proteolysis of a hypothetical protein with a repeating polypeptide structure by two endopeptidases at two temperatures, T° and $(T+n)^{\circ}$. The descending arrows show the number of peptide bonds split as time elapses. The diagram demonstrates a possible outcome if the rates of action of the two enzymes are not equally accelerated by the increase in temperature.

greater temperature coefficient than the other. The digestive process will then produce at certain points of time fragments of a type not produced at the lower temperature.

The real situation in the breakdown of protein molecules may be quite different: the enzymes for example will not break down all bonds of one molecule before attacking those of another. The principle will however hold that a population of different sub-species of molecules will be present in the digestive vesicles of cells carrying out the digestion at different temperatures. If the time allowed for digestion is long, both will achieve breakdown to the same degree. If there is some intermediate event, different populations of molecules will take part in the reactions.

From the work of Lapresle (1955) it seems that hidden determinants of an antigen uncovered by proteolysis in vivo may stimulate antibody production: this implies that fragmentation of antigen ordinarily takes place and helps to direct antibody specificity. The hypothesis presented here suggests that different antibodies may be stimulated at different temperatures. Such rises in temperature might result in antibodies being produced against fragments which did not exist in the course of digestion at lower temperatures. In this way antibody might be formed against an antigen incapable of stimulating antibody formation at lower temperatures.

Kovorkov (1964) does record changes in rabbit antibody in animals exposed for short times to hypo- and

hyper-thermia. In both, reduced titres of antibody of lower avidity were produced against a bacterial species.

In poikilotherms temperature has unusual effects on antibody production. (Bisset, 1948; Rees, Perkins, and Elek, 1963). Toads immunized in the cold do not produce antibody but are latently immunized and on being warmed to 27° after long intervals produce antibody spontaneously. The phenomenon is analogous to that of the action of tetanus toxin in frogs. The toxin is inactive until the frogs are warmed. The active material in both cases may have been stored in a crucial site to become active under different conditions. These effects with temperature in poikilotherms do not offer any insight into immunological effects of hyperpyrexia in mammals.

Adjuvants and the Tolerant State

Various influences will prevent the induction of the tolerant state to BSG. In addition to mycobacterial adjuvants, Dresser (1961) has shown that lipid and lipidophilic substances can prevent the induction of tolerance. Using the same model Claman (1963) has shown that endotoxin will also induce antibody formation. Claman and Bronsky (1965) also showed that antibody production could also be initiated by actinomycin D, an agent known to inhibit the synthesis of DNA-dependent RNA.

Adjuvants can also prevent the induction of the specific tolerant state by other protein antigens. Paraf,

Fougereau, and Metzger (1963) have shown this with bovine serum albumin (BSA) in adult mice using Freund's complete adjuvant (even when the latter was given 10 days after the antigen). Pinckard, Weir, and McBride (1967) have shown the same effect in rabbits, using BSA as antigen and Corynebacterium parvum as adjuvant. A viral infection has also been shown (Mergenhagen, Watkins, and Dougherty, 1967) to prevent the induction of tolerance to human gamma globulin in mice.

Many of these findings are in keeping with the idea that if a non-specific cellular stimulus is added to the specific antigenic stimulus, cells are stimulated to produce antibody where otherwise tolerance would ensue. (Dresser, 1960; Claman, 1963; Talmage and Pearlman, 1963).

Adjuvants can sometimes terminate established tolerance: for example in studies of humoral antibody formation and cellular localization of antigen (1968a and b), Lind was able to demonstrate in rats termination of tolerance to flagellin by complete Freund's adjuvant. Using the same adjuvant however Weigle (1962) was unable to terminate tolerance to a purified mammalian antigen in rabbits.

Humphrey (1964a and b) has shown with several purified mammalian proteins that tolerance in rabbits does not end abruptly nor does spontaneous antibody production invariably follow. Tolerance can persist despite removal of free antigen by antibody passively administered or by the lapse of time. Thus the antibody production in some

of the experiments detected at the time of secondary exposure to antigen may represent a true stimulation of antibody producing cells by the adjuvant procedure (at the time of first exposure) rather than the early release from tolerance by more rapid elimination of antigen.

In the present experiments the adjuvant procedures have interfered with the decisive process which results in tolerance. The handling of potential antigen molecules and the possible outcome of their arrival at certain sites in the cell at that time will be discussed more fully in the next chapter.

Chapter Five

The Handling of Injected Materials

Clearance, Katabolism, and Elimination

At this point what is known of the kinetics of the handling of injected materials might appropriately be considered. As has already been emphasized, digestibility is an important attribute of an antigen: something of value might therefore be deduced from the contrasting kinetic features of the disposal of digestible and indigestible entities.

Indigestible Colloidal Particles

The kinetics of carbon particle disposal or other similar inert colloidal suspensions are known particularly from the work of Halpern, Biozzi, and Benacerraf and their colleagues. In studies in various species (Halpern et al., 1954) where the removal from the bloodstream of these materials is observed, the graph of elimination obeys the following kinetic equation:

$$\frac{\text{Log } c_1 - \text{log } c_2}{t_1 - t_2} = k$$

where c_1 and c_2 = concentration at times t_1 and t_2 respectively.

This equation represents exponential elimination of such foreign particles as they are removed by the reticulo-endothelial system (RES). The value "k" varies inversely with the amount initially injected and therefore differs in this particular from a true first order reaction where "k"

would be independent of initial dose. Saturation of some surface is probably taking place as is suggested by repeated exposures. The clearance rate decreases with each successive dose although it is observed that it is probably impossible to saturate the system completely. Aschoff (1924) who had defined the performance of the RES by quite different morphological means also believed that it could not be saturated.

The data from Equation 1 are derived from observations made over a very short time (minutes in the instance of carbon) and relate only to removal from the bloodstream. They tell little of the fate of the material thereafter. The crucial "surfaces" are gradually replaced although materials such as carbon may remain in RES cells for quite long periods.

Janeway and Humphrey (1968) have also demonstrated the prolonged retention in the tissues of the relatively indigestible polypeptide substances composed of unnatural D-aminoacids.

Digestible Proteins

Digestible protein substances are not only removed from the circulation but are subsequently katabolized and the basis of the kinetics of their removal is consequently the more complicated.

A hypothesis has been elaborated by Brambell (1966) to explain the findings with immunoglobulins. He derived his hypothesis from first observing transmission of γ-globulin molecules across cellular barriers such as the placenta (in the rabbit) and the intestinal wall (in the rat). When observations made in these systems were taken with the findings of the sensitization of tissue cells with γ-globulin, the importance became evident of selective competition with other γ-globulin molecules or with certain fragments (Fc pieces) of other γ-globulin molecules. These molecules were thought to be competing for 'receptors' on the external surface of cells. Other γ-globulin fragments (Fab pieces) and other protein molecules did not take part in the competition. An area of the cell surface bearing such a receptor might subsequently become invaginated and form the wall of a pinocytotic vesicle. This vesicle would traverse the cell, be assimilated into the cell surface of the opposite side and there release the attached molecule of γ-globulin. Considering how this might affect protein breakdown Brambell pointed out that enzyme action was much more likely to be upon molecules unattached to membrane but free to meet enzymes within the digestive vesicle. It follows from this that the rate of katabolism varies with the concentration. A high concentration will mean a relatively high rate of katabolism, the amount attached to receptors being limited and a great excess being free. A low concentration will mean that a proportionately high amount will be attached to the

receptors and that very much less proportionately will be free to be digested.

The expression governing the half-life ($T_{\frac{1}{2}}$) is as follows:

$$T_{\frac{1}{2}} = \frac{c \ln 2}{a(c-b)} \qquad \text{(Equation 2)}$$

where c = the concentration of γ-globulin.
b = minimum concentration of γ-globulin which saturates the receptors.
a = the proportion of globulin pinocytosed per day.

This graphs as a rectangular hyperbola and has been shown to fit the data of Fahey and Sell (1963) in the mouse (Brambell, Hemmings, and Morris, 1964).

The contrast with the kinetics of carbon clearance is great. In the one case saturation of a receiving surface leads to blockage of removal: on the other increasing saturation of a receiving surface leads to a more rapid breakdown and removal of molecules.

In the guinea pig there is no evidence of such sequestration of γ-globulin molecules on receptors as the rate of katabolism is apparently independent of concentration (Bell, 1964). It is not known whether similar receptors are important in the breakdown of other native proteins. Nevertheless it is worth considering whether this concept of receptors, intravesicular digestion, and specific competition, evolved by Brambell in the context of immunoglobulin transmission, sensitization, and katabolism, might be transferred to the situation where foreign protein molecules have been introduced

into the system and to which there is a subsequent immune response.

Surface receptors are acceptable in the immune context. The competition for receptors might be either between similar self molecules of the host and the foreign protein molecules or between partially digested fragments and intact molecules of the foreign protein.

Katabolism of a Potential Antigen and Related Events

A Hypothesis

In view of the facts set out in Chapter One on the digestibility of potential antigens, it is not unreasonable to assume that a digested fragment of the antigen molecule is able to take part in an immunologically important process in a way in which the intact molecule cannot. If the further assumption is made that a membrane-associated phenomenon is important in the reaction, a possible deduction might be that smaller fragments penetrate the membrane whereas the larger do not do so. Penetration is assumed here to provide the immunogenic stimulus and is further assumed to follow directly reception of the smaller fragments on the specific receptor. Thus the end-result of the arrival of foreign molecules in the digestive vesicle will depend on successful competition by the smaller fragments for the receptor.

The probability of such an event will be

influenced by extracellular and intracellular features. Extracellular features of importance would be the number of appropriate receptors, the avidity with which they combine with the foreign protein, the external concentration of the foreign protein, the length of time of exposure before invagination as a pinocytotic vesicle, and competition with native molecules with some affinity for the same receptor. Intracellular features would be the rate of endocytosis, the rate of digestion, and the relative digestibility of different parts of the antigen molecule.

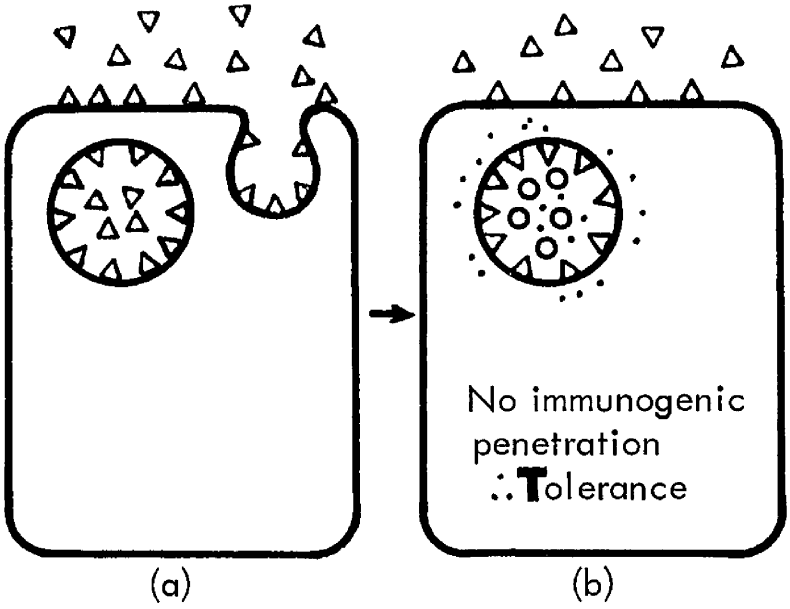
These events taking place in an immunologically competent cell are portrayed in the accompanying series of diagrams in several immunological situations. One cell here represents the total surface area of these cells carrying the entire complement of specific receptors: similarly the membrane of the pinocytotic vesicle represents the total vesicular surface which has invaginated from the external surface. Small amino-acid fragments which are non-immunogenic are assumed to penetrate the vesicular membrane without difficulty.

Reception of Antigen Molecules and their derived Fragments

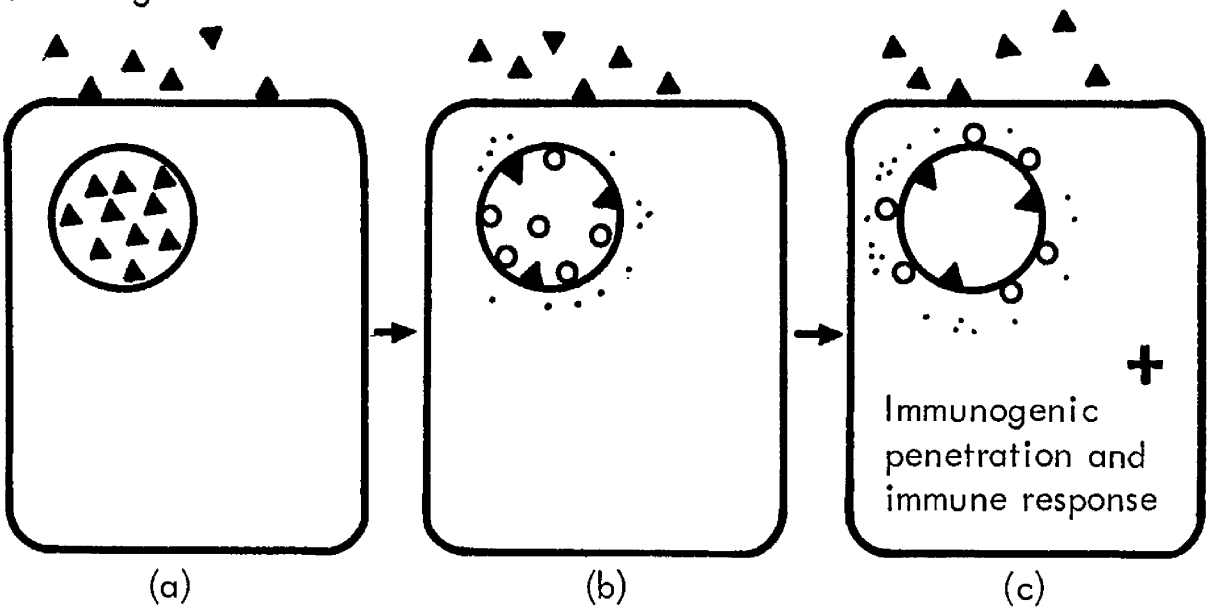
1. Self Protein Molecules. In this situation the number of self molecules is so high that the receptors of the different specifities are fully blocked before any invagination of the membrane takes place. There is thus a very low probability of an immunogenic fragment penetrating the membrane as the

RECEPTION of ANTIGEN MOLECULES and THEIR DERIVED FRAGMENTS

1. Self Protein



2. Foreign Protein



▲ Intact antigen molecule
△ Intact host-protein molecule
O Partially digested antigen, potentially immunogenic
⁘ Further digested antigen, non-immunogenic

receptors which will allow them to do so are preemptively occupied by intact molecules. There would therefore be no immune response against self antigens.

An alternative explanation is of course that the population of cells which might have responded has been eliminated at an early stage of development (Burnet, 1959) or has become exhausted by terminal differentiation (Sterzl, 1966).

(Figure 1)

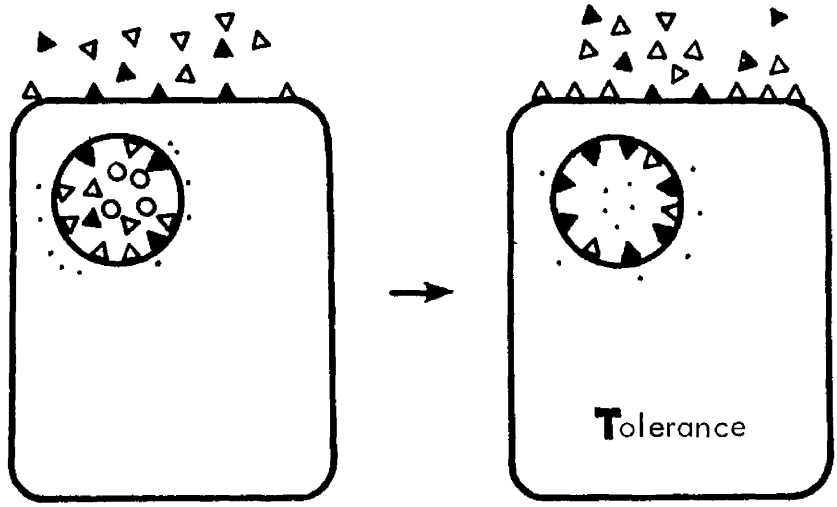
2. Foreign Protein Molecules. Here there are fewer antigen molecules in the environment and there is therefore a low chance of occupation of receptors with intact molecules before invagination. There is consequently a higher probability of a fragment reaching a specific receptor, penetrating the membrane and causing the necessary differentiation to bring about an immune response.

(Figure 2)

3. Poor antigen, similar to host protein. In this example the immune response is poor or there is immune paralysis because there are either very many self molecules competing for the same receptors or there are very few cells which could respond. If it is accepted that the potentially responding cells are not eliminated in the process that produces tolerance (Dowden and Sercarz, 1967) the tolerant state would persist as long as the molecules remain on the receptor.

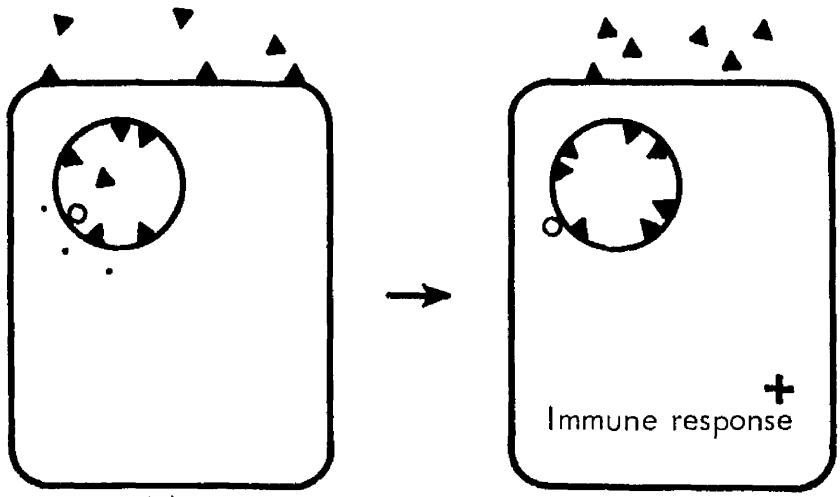
RECEPTION of ANTIGEN MOLECULES and THEIR DERIVED FRAGMENTS

3. Poor antigen, similar to host-protein.



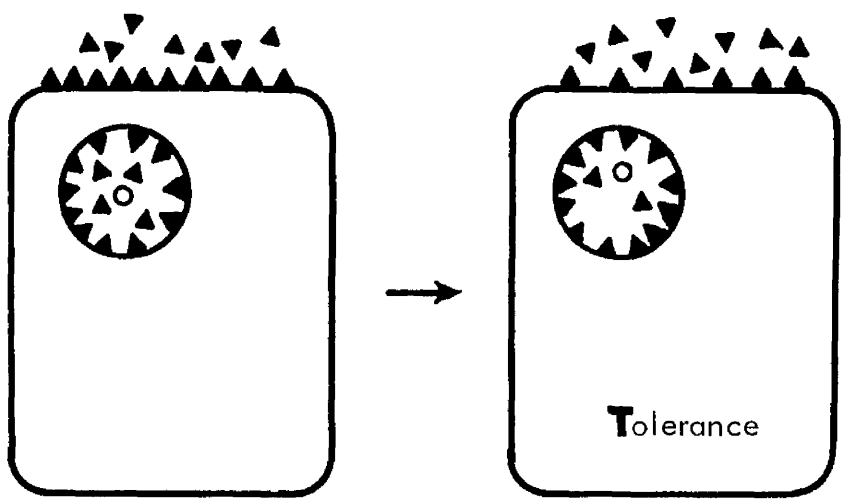
(a) (b)

4. Poor antigen, relatively indigestible, in low dosage.



(a) (b)

5. Poor antigen, relatively indigestible, in high dosage.



(a) (b)

The findings of Dresser and Gowland (1964) who used leporine γ-globulin in the rabbit and found a convincing degree of paralysis or those of Phillips (1966) in chickens with turkey γ-globulin are examples of this situation.

(Figure 3)

4. Poor antigen, relatively indigestible, in low dosage.
Although the antigen is not digested rapidly the few molecules introduced into the system do not preempt all the receptors as intact molecules.

(Figure 4)

5. Poor antigen, relatively indigestible, in high dosage.
The slow breakdown of the antigen means that it is very unlikely that an immunogenic fragment will reach a receptor before all are occupied by intact molecules. Tolerance is therefore a likely outcome.

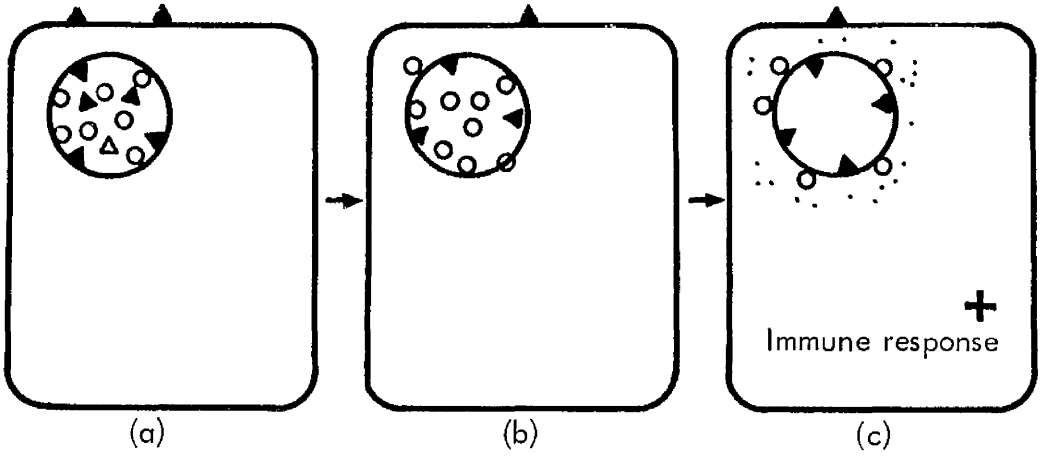
(Figure 5)

6. Adjuvant activity: accelerated ingestion and digestion.
The accelerated ingestion and more rapid breakdown would mean the early presence of fragments within the vesicles before the surface was saturated. Antibody production would therefore be encouraged.

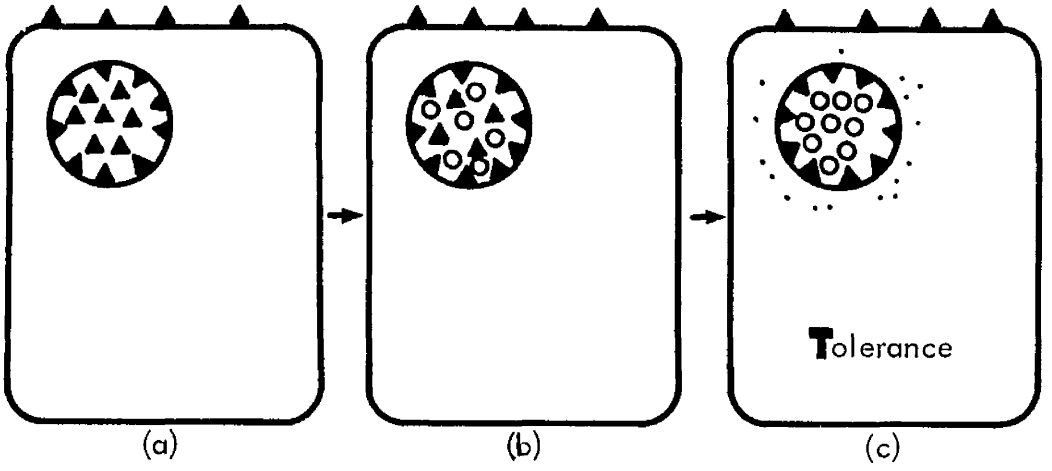
Some of the experiments with the classical adjuvants cited in Chapter Four provide evidence of this accelerated katabolism of the antigen. Although it may be said that such katabolism does not necessarily reflect the behaviour of the immunologically competent cell (ICC) it

RECEPTION of ANTIGEN MOLECULES and THEIR DERIVED FRAGMENTS

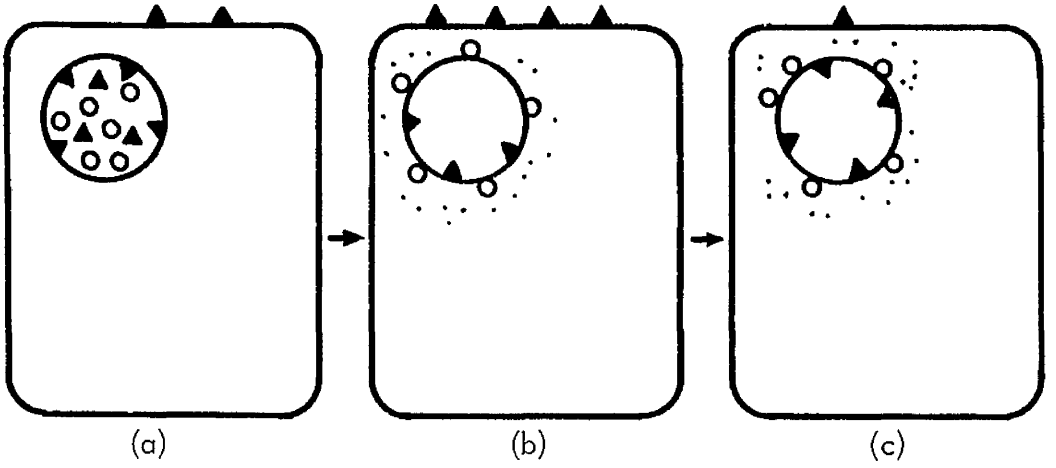
6. Adjuvant activity: accelerated ingestion and digestion.



7. Adjuvant activity: high dose of antigen beforehand.



8. Adjuvant activity: altered membrane permeability.



does reflect accurately the time relationships in the changing concentrations of externally present antigen. This of itself could have the postulated effect on the time relationships of the saturation of the surface receptors of the ICC. (Figure 6)

7. Adjuvant activity: high dose of antigen beforehand.

This would be exemplified by the paralysis observed by Dresser (1962a) in mice given the poorly antigenic EGG. Subsequent stimulation by antigen in CFA was unable to stimulate antibody production because of the postulated blockage by large intact molecules. (Figure 7)

8. Postulated Adjuvant Activity.

The hypothesis here outlined is one of specific permeability of a vesicular membrane by means of a specific receptor. If however the permeability of the membrane is altered by non-specific means enhanced penetration by immunogenic fragments would be expected and the immune response improved. This possibility is explored in the next chapter. (Figure 8)

Chapter Six

The Relationship of Membrane Permeability to Antigenic Stimulation

In the hypothesis elaborated in the last chapter, interest had been placed on the arrival of antigen-derived fragments on to receptors of the lysosomal vesicles. Such reception was suggested as effecting the passage of the fragment across the vesicular membrane, regarded here as a decisive immunogenic event. Any agent non-specifically altering the permeability of such membranes would be expected to alter the immune response. Such an agent was vitamin A (Dingle, Lucy, and Fell, 1961; Lucy, Dingle, and Fell, 1961; Dingle, 1961; Fell, Dingle, and Webb, 1962), known to release proteolytic enzymes from the vesicles within cells and to allow the enzyme to spread throughout the cell and also into tissue fluids.

It was consequently decided to use vitamin A in the antigen elimination model, the vitamin being given by the oral route to obviate effects due to tissue reaction to the suspending oil (Shaw, Alvord, and Kies, 1964). The vitamin had been previously used in another immunological model in the guinea pig (White, 1968) in which unsuccessful attempts had been made to stimulate delayed-type hypersensitivity with vitamin A present in a water-in-oil emulsion incorporating antigen. Thyroid was therefore again used to simulate the increased katabolism noted as being produced by the

classical adjuvants (Chapter 4, Expts 1, 2, and 3) because it was thought it might change the kinetics of arrival of antigen into pinocytotic vesicles in favour of an immune response. The formal experiments are set out below.

Materials and Methods

Antigen Elimination Model. This was as described in Chapter Four with ^{131}I.EGG as antigen in CBA male mice. Whole body counting determined the excretion of iodine-131 after intracellular katabolism of the labelled antigen in mice saturated with iodide from their drinking water.

Vitamin A "Avoleum" (British Drug Houses) was used, containing 30,000 i.u. Vitamin A per g. Mice were given 0.3 ml. by stomach tube on the three days up to and including the day of intravenous injection of antigen.

Thyroid Thyroid diet C was used as previously described (p.57). Mice were fed for the seven days before and 14 days after intravenous injection of antigen.

Experiment 1

Four groups of 5 mice were set up as follows:

Group 1	Normal diet.
Group 2	Thyroid diet C.
Group 3	Normal diet and vitamin A orally.
Group 4	Thyroid diet and vitamin A orally.

The mice were given 1 mg ^{131}I.EGG intravenously and their radioactivity counted every second day thereafter.

Results The results are shown in Figure 6.1. Immune-type elimination was observed in the groups receiving vitamin A with or without thyroid diet.

The elimination rates in the preimmune period were (in days):

Group 1	3.6 ± 0.22	
Group 2	2.76 ± 0.35	
Group 3	3.56 ± 0.38	
Group 4	2.86 ± 0.25	(One mouse died in this group)

There was no evidence that vitamin A had accelerated the protein katabolism during the preimmune phase (Group 3). Various supporting experiments were now set up to determine

1) the amount of vitamin A required to produce the immunizing effect. For this purpose, dilutions were made in olive oil (1/5 and 1/25). A control group was set up receiving olive oil alone.
2) when the vitamin had to be given in relation fo the intravenous dose of antigen to achieve the effect.
3) whether arachis oil, in which the vitamin is dissolved in "Avoleum", acts as an adjuvant when given by the oral route.
4) whether vitamin D influenced the performance of vitamin A. (This vitamin is naturally found with vitamin A in fats and has a steroid structure. It was thought there might be some contrary or complementary action in membranes which have evolved in the presence of the two agents).

Elimination of ^{131}I.BGG in CBA mice.

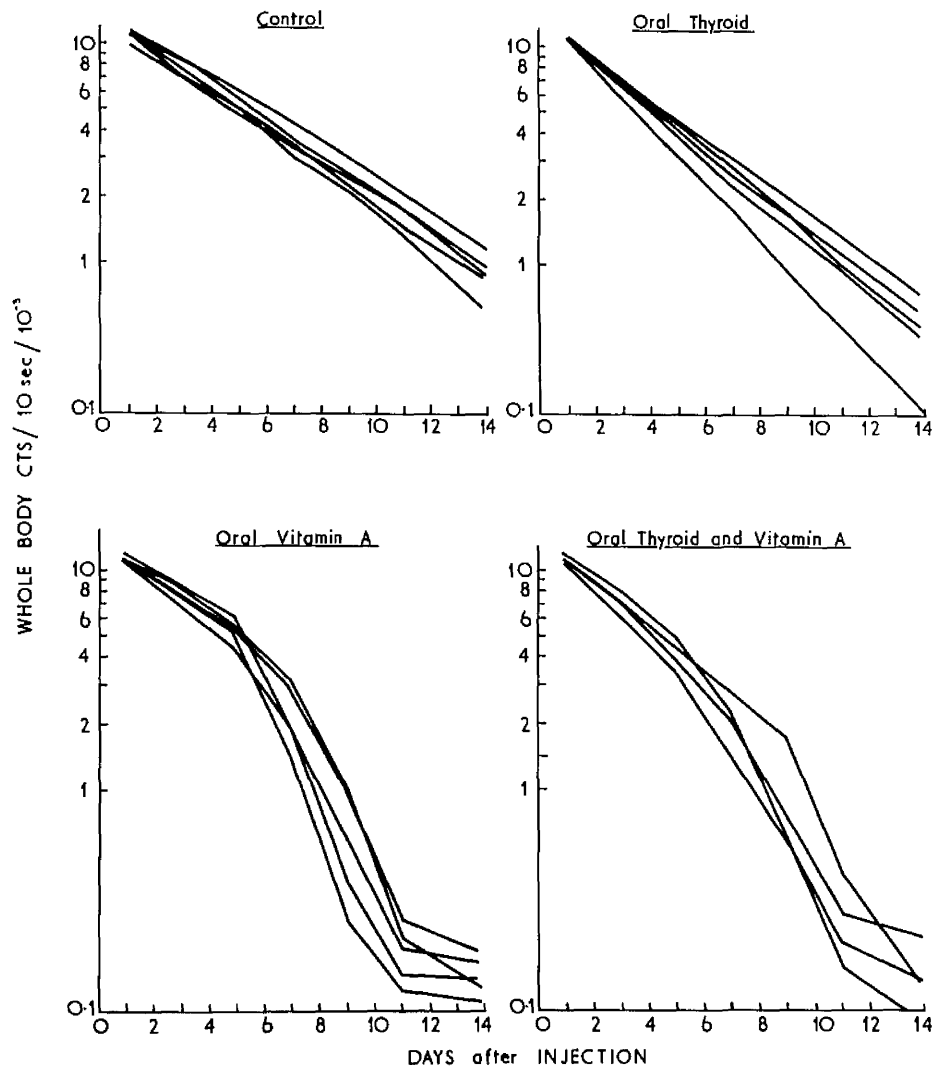


Figure 6.1 Influence of thyroid hormone and vitamin A on the elimination of a 1 mg I.V. dose of BGG.

The mice in the different groups shown above received:

(top left) a. Normal diet.
(top right) b. Diet incorporating 3 mg thyroxine per 300 g diet in the 7 days before and 14 days after injection of BGG.
(bottom left) c. 10,000 i.u. vitamin A orally on the three days before injection of BGG.
(bottom right) d. A combination of (b) and (c).

Results. The results are shown in Table 6.1. The activity of vitamin A was much reduced at a 1/5 dilution and non-existent at 1/25 as judged by its ability to influence this response. With the dosage used a minimum of two days' treatment was required to produce an immune response but whether this preceded or followed the intravenous injection was not important. The activity was reduced considerably when treatment ended four or seven days beforehand. Arachis oil, olive oil, and Radiostol (vitamin D2, B.D.H.) did not affect the response nor did Radiostol in the dose used affect the adjuvant performance of the Vitamin A.

It was also important to establish the effect of vitamin A on other antigen systems. Therefore experiments were set up as follows:

Experiment 2

Materials and Methods: Mice Adult male Porton White (PW) and CBA mice.

Antigen Human serum albumin (HSA)

Antigen Binding Capacity Primary Response Neat mouse serum and a 1/4 dilution (using as diluent normal rabbit serum itself diluted 1/4 in 0.15 M saline) were set up in 0.1ml amounts with 0.5 ml of 0.1 ug/ml ^{131}I.HSA solution in 0.15 M saline. The antigen had previously been labelled by the method of Hunter and Greenwood (1962) with the isotope to an activity of approx. 1uC/ug. The test was then carried out as described previously (p.22).

Secondary Response A similar procedure was used, but the antigen concentration was increased to 0.4 ug/ml and the 1/4 dilution extended serially to 1/256.

Table 6.1

Effect of various oral treatments on the induction of an immune response to ^{131}I.BGG in CBA mice

Treatment	No. of Primary responses
Group 1	
All agents, 0.3 ml for 3 days before antigen.	
Olive oil.	0/4
Avoleum	4/4
Avoleum diluted 1/5 in olive oil	1/4
Avoleum diluted 1/25 in olive oil	0/4
Group 2	
Avoleum on days recorded relative to day of antigen injection, 'D'	
-2, -1, D	4/4
-1, D	3/3
D	2/4
D, +1	4/4
D, +1, +2	4/4
No treatment.	0/4
Group 3	
Avoleum on days recorded relative to day of antigen injection.	
-10, -9, -8	3/6
-6, -5, -4	2/6
-2, -1, D	6/6
No treatment.	0/6
Group 4	
All agents, 0.3ml for 3 days before antigen injection.	
Arachis oil	0/5
Avoleum	5/5
Radiostol (3,000 i.u.per ml, BDH)	0/5
Avoleum (0.15ml) + Radiostol (0.15ml)	5/5

Treatment Twelve PW mice were given 1mg HSA intravenously after six of their number had received oral treatment with 0.3ml Avoleum daily for three days. The mice were bled on the 7th, 10th, and 14th days after the antigen injection. The mice were reinjected with 1mg HSA and bled again on the 7th, 10th, and 14th days after the secondary stimulation. After separation, sera were stored at -20° until estimation of ABC_{30}.

Ten CBA mice were also used, five having received the same treatment with Avoleum. They were given 10ug HSA and bled on the 14th day only.

Results

PW mice These are shown in Figures 6.2 and 6.3. The mice receiving vitamin A yielded tiny amounts of antibody on the primary response, the ABC_{30} values being obtained by extrapolation. The antibody content rose from the 7th to the 14th day in these mice whereas the control mice showed no response. On the secondary stimulation, the vitamin A treated mice showed high levels of antibody which were already declining between the 7th and 14th days. One control mouse also showed an antibody response gradually increasing over this period.

CBA mice The sera of those mice that had received vitamin A showed a mean ABC_{30} on the 14th day of the primary response of 0.28 ± 0.04 ug/ml. The ABC_{30} of the control mice were all less than 0.06 ug/ml.

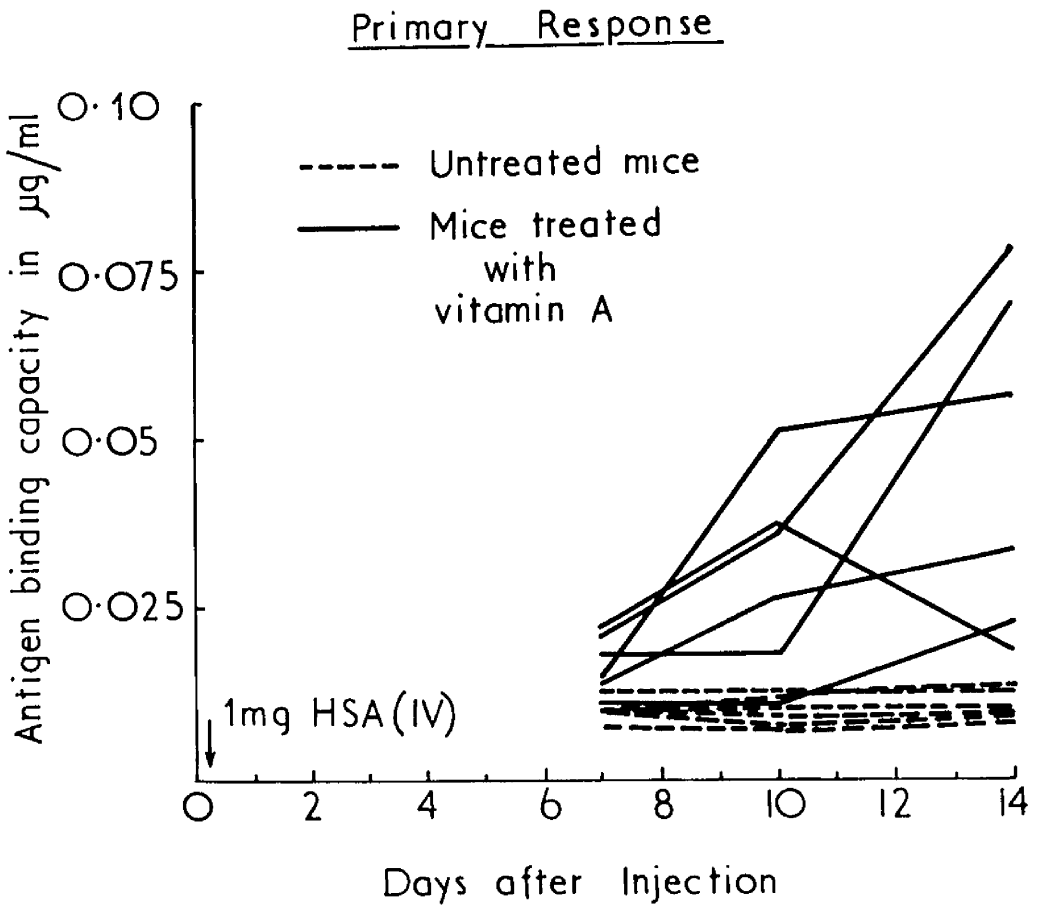


Figure 6.2 **Influence of oral vitamin A on the response to human serum albumin (HSA)**

The primary antibody responses to 1 mg HSA of control (-----) and test mice (——) are shown, measured as antigen binding capacity in ug / ml. Samples were taken on the 7th, 10th, and 14th days after injection. The test mice received 10,000 i.u. of vitamin A on the three days before injection of antigen.

Antibody response to Human Serum Albumin in Mice.

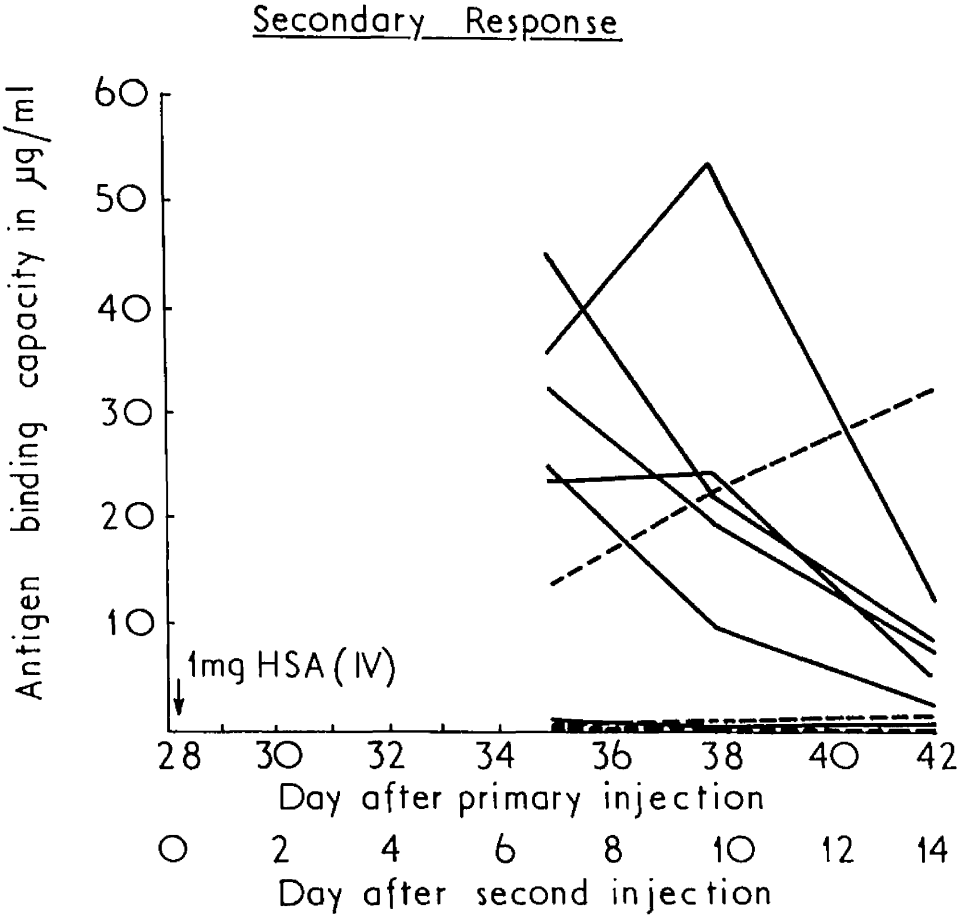


Figure 6.3 Influence of Oral Vitamin A on the response to human serum albumin (HSA)

The secondary antibody responses to 1 mg HSA, measured as antigen binding capacity of control (-----) and test mice (———) are shown. HSA had been given 28 days after the primary stimulation with the same dose. Vitamin A was given only at the time of the primary stimulation. Samples were taken on the 7th, 10th, and 14th days after secondary exposure to antigen.

As the oral administration of vitamen A had had an adjuvant effect in mice, it was decided to repeat the procedure in guinea pigs. These animals were to receive also incomplete Freund's adjuvant and observations would be made to see if the response was modified.

Materials and Methods.

Guinea pigs Fifteen Dunkin-Hartley guinea pigs were used, 6-7 months old, c. 1250 g in weight.

Footpad Injections Injections into the left footpad were made of water-in-oil emulsions (Incomplete Freund's adjuvant) made up from Bayol 55, Arlacel A, and 0.15 M Saline containing 50 mg HSA per ml., in the proportions of 3:1:1. as previously described (p.55).

One group of guinea pigs as a positive control for delayed-type hypersensitivity received also Wax D (White, Jolles, Samour, and Lederer, 1964), it being dissolved in the oil phase at a concentration of 1 mg per ml.

Each guinea pig received an injection of 0.2 ml emulsion, ten guinea pigs receiving emulsion without Wax D, and five receiving the injection with Wax D i.e. each animal received 2 mg HSA and those receiving Wax D received 200ug. wax.

Vitamin A Avoleum (2.5 ml) was introduced by stomach tube on the three days before adjuvant injection into five guinea pigs receiving incomplete Freund's adjuvant.

Antigen binding capacity This was estimated in the manner previously described for chicken antibody (p.22) except that the sera were diluted in normal guinea pig serum. The test sera were obtained when the animals were killed on the 21st day after footpad injection.

Corneal Test At the 18th day, injections of approximately 0.02 ml of a 20 mg/ml solution of HSA in 0.15 M saline were made tangentially into the cornea so as to produce a circular opalescent zone, the cornea having been first anaesthetized with 4% cocaine hydrochloride. The eyes were inspected on the 19th and 20th days.

Intradermal Test On the 18th day 0.1 ml of a 1 mg per ml solution of HSA was injected intradermally. The skin thickness was measured by skin-measuring forceps at 48 hours.

Results The findings are summarised in Table 6.2. Only those animals which had received complete Freund's adjuvant with Wax D showed evidence of delayed-type hypersensitivity in the strongly positive corneal reactions (White et al. 1964) and much increased dermal thickening. Antibody production was not increased in the group receiving vitamin A but was apparently lower, although not significantly so.

Table 6.2

Effect of oral vitamin A on the immune response in the guinea pig to Freund's incomplete adjuvant.

Group	Corneal No.	Corneal Test	Dermal Thickness mm	Footpad Swelling mm	ABC_{30} (ug/ml)
Freund's Incomplete Adjuvant	1	+	6.4	8.5/8.0	20.4
	2	+	4.3	8.4/7.7	32.4
	3	-	4.4	8.4/7.9	48.0
	4	+	4.3	7.6/7.6	90.0
	5	-	4.9	8.0/7.9	7.2
Freund's Incomplete Adjuvant + Oral Vitamin A.	1	-	5.3	8.2/7.6	18.0
	2	+	4.2	8.2/8.2	10.2
	3	Died			
	4	-	3.8	8.3/8.3	7.8
	5	-	4.0	8.5/7.2	9.0
Freund-type Adjuvant with Wax D.	1	++	6.7	>10/ 9.9	4.2
	2	+++	4.2	>10/ >10	12.0
	3	Died			
	4	++	6.6	>10/9.7	20.0
	5	++	8.0	> 10/ >10	84.0

Discussion

The experiments with vitamin A have been successful in demonstrating adjuvant activity with two antigens in mice and are consistent with the hypothesis outlined in Chapter Five. However in the guinea pig experiment no such activity has been demonstrated either on antibody production or in delayed-type hypersensitivity. It may have been that by presenting the HSA in incomplete Freund's adjuvant the response could not be improved upon, at least as regards antibody stimulation. Insufficient vitamin A may have been given: weight for weight the guinea pigs were given about five times less vitamin than the mice. However local vitamin A in high concentration at the site of injection had also been unable to promote delayed-type hypersensitivity (White, 1968).

The activity of vitamin A bears some resemblance to that of endotoxin in that delayed-type hypersensitivity is not produced and also in that no local granuloma is required for its adjuvant action. The latter is especially demonstrated by the oral administration of the vitamin. Some of the activities of endotoxin have also been related to action on lysosomal membranes (Weissman and Thomas, 1962). The fact that M. tuberculosis can sensitize mice to endotoxin (Suter and Kirsanow, 1961) suggests that products of the mycobacteria might also affect membrane performance directly or indirectly.

Vitamin A has been examined in several other immunological systems. Antibody titres to prophylactic

agents such as TAB and diphtheria toxoid have been reported as being improved by an increased intake of vitamin (Scaglione, 1938; Schmeckebier, 1945). Acute hypervitaminosis A has been shown by Uhr, Weissman, and Thomas (1963) to depress previously induced delayed-type hypersensitivity; this the authors related to the reduced inflammatory responses of animals whose lysosomes have been depleted of many active enzymes (Weissman, et al. 1963). Hypervitaminosis A also depressed the production by adjuvants of experimental thyroiditis and antibodies against thyroid in guinea pigs (Jancsz, Flad, Koffler, and Miescher, 1967). However in this experiment it is interesting to note that the thyroid glands of animals receiving only the vitamin showed some lymphoid infiltration.

Dresser (1968a and b) using BGG as antigen has also demonstrated in mice adjuvant activity of vitamin A given by subcutaneous injection, and has suggested an interpretation of the mode of action based on observations of lysosomal behaviour before cellular division (Allison and Malucci, 1964) or transformation (Hirschhorn, Kaplan, Goldberg, Hirschhorn, and Weissman, 1965). He believes that release of lysosomal enzymes such as ribonuclease by vitamin A provides a stimulus to division. This is regarded as crucial in antigenic stimulation, one cycle of division of a lymphoid cell in the presence of antigen being enough to set off the immune response. Unless, however, this allows the passage of information regarding the shape of antigen to some effector site, it is difficult to see how this division alone can

result in specific differentiation. If the lymphoid cell is regarded as multipotent, then there must be penetration of some moiety from the outside of the cell which selects the response. Although Franzl (1962) has found that antigen molecules sediment with the lysosomal fraction of lymphoid tissue, little more is at present known of the intimate intracellular fate of protein molecules (Ryser, 1968).

In explaining the action of his surface-active molecules Gall (1966) has thought in terms of more antigen entering immunocompetent cells by altered membrane permeability. The basicity of some of the agents used by Gall may have further enhanced the process by stimulating pinocytosis (Allison, 1968)

Membrane permeability and Vitamin A.

Molecules pass through membranes in various ways: by simple diffusion along a channel, by dissolving in the lipid phase of the membrane, and redissolving in the aqueous phase on the other side, and also by movement along a series of adsorption sites (Harris, 1960). A specific adsorption mechanism such as the permease mechanism of Escherichia coli (Rickenburg, Cohen, Buttin, and Monod, 1956) has been suggested (Sorkin, 1960) as important in collecting antigen in immunocompetent cells. Reversible spatial changes or "gates" have been thought of as allowing larger molecules across membranes (Vidaver, 1966) and this would fit the behaviour of sugar molecules when observed crossing membranes (Stein, 1968)

Zechmeister (1962) has commented on the spatial sensitivity of the vitamin A molecule which has a conjugated linear unsaturated structure: a cis-trans change at one of the central double bonds causes an unusually great change in the shape of the molecule (Figure 6.4). Dingle has related this phenomenon to possible permeability changes when this substance is dissolved in the lipid of a micellar membrane (Lucy, 1964; Dingle and Lucy, 1965).

In another aspect of membrane behaviour requiring spatial changes of a grosser nature, namely phagocytosis, Nicol and Bilbey (1963) also relate significance in a molecule to a double bond and the groups on either side of it. Loss of the ethyl groups of 4:4' diethyl-dihydroxy stilbene (Figure 6.5) reduces tenfold the ability to increase phagocytosis

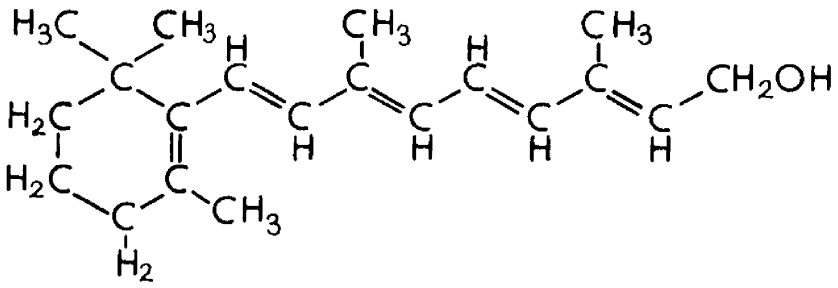
Activity of Bacterial Carotenoids

The activity of vitamin A might suggest that carotenoids present in bacteria (e.g.Prebble, 1968) may make some previously unsuspected contribution to adjuvant activity. Whether vitamin A or related carotenoids would assist or cut down the effect of mycobacterial adjuvants in inducing delayed-type hypersensitivity might be examined: they might or might not favour the setting up of a competing humoral antibody response.

Size of Antigen Fragment and Delayed Hypersensitivity

The activity of vitamin A has been attributed here to changes in membrane permeability in the cell receiving

Figure 6.4 Structure of Vitamin A and Related Compounds



Vitamin A (alcohol)

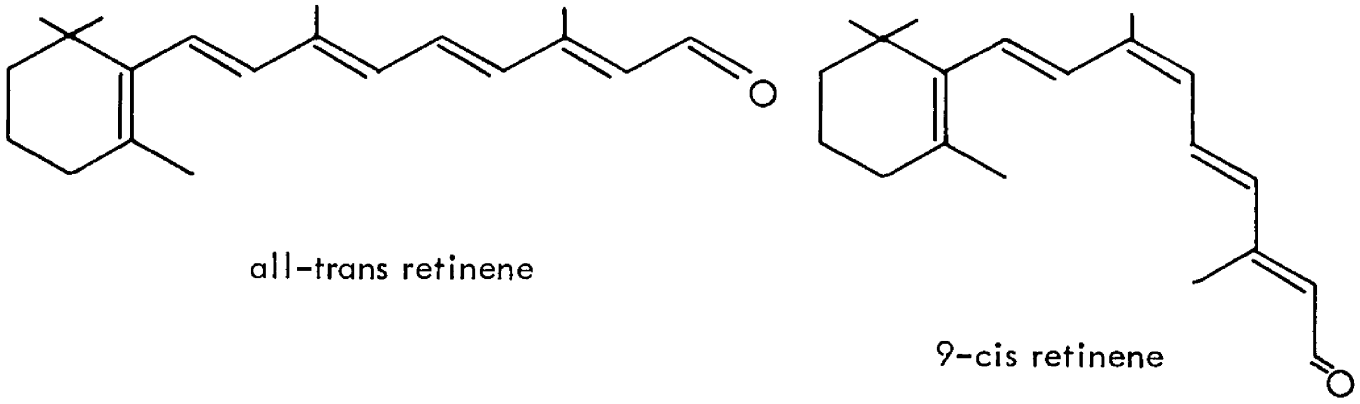
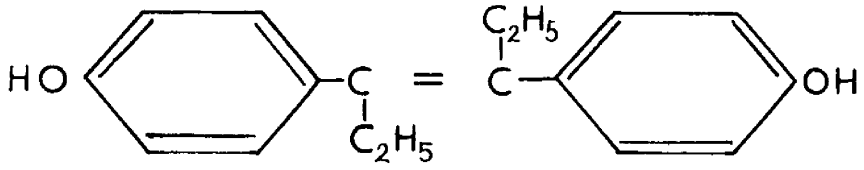
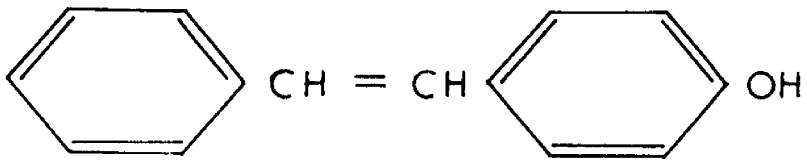


Figure 6.5 Structure of 4-4' Dihydroxy-Diethyl-Stilbene and Related Compound



4: 4' Dihydroxy-Diethyl-Stilbene



4: 4' Dihydroxy-Stilbene

antigen. Some speculations might be made on the assumption that the action of agents causing delayed hypersensitivity is also related to alterations in permeability to antigen. Larger parts of the antigen molecule take part in the delayed-type hypersensitivity reaction (Salvin and Smith, 1960 a and b; Levine, Ojeda, and Benacerraf, 1963; Benacerraf, Ojeda, and Maurer, 1963; Siskind, Paul and Benacerraf, 1966). If this is brought about by direct recognition on a surface of part of the molecule, the adjuvant which can cause delayed-type hypersensitivity may be altering membrane permeability so that larger pieces can penetrate to crucial sites to cause the different type of differentiation. Alternatively the reacting cells which such adjuvants attract may have a different innate permeability.

Adjuvant Induction of Clines.

If the differentiation to antibody formation or cell-mediated hypersensitivity were determined on the size of antigen fragment initially penetrating a membrane, a gradient of characters might be detected in those clones of immunologically competent cells responding at varying distances from a depot of diffusing membrane-active adjuvant substance. As the adjuvant concentration fell with distribution there would be concomitant reduction of the permeability of cellular membranes. A "geographically" graded "cline" (Huxley, 1938,1939) may consequently arise whose specificities are related to

progressively smaller determinant areas. This may have an importance in that clones specifically forming humoral antibody may inevitably be induced even with adjuvants developed to induce delayed-type cell-mediated hypersensitivity.

Chapter Seven

Conclusion

Some comments may now be made on the various interpretations of the work described in the thesis.

The study in the chicken demonstrates macrophages apparently presenting antigen to lymphoid cells. However the time sequence and relationship to antibody formation in other sites observed by the fluorescent antibody technique make it clear that this is a secondary event requiring previously formed antibody. The lymphoid cells are apparently caught in a macrophage net and are in close proximity with antigen with which they have some complementary surface affinity. Several questions are unresolved by the findings. The relationship of antigen to plasma cells is not demonstrated in the red pulp: it is still unanswered whether antigen arrives at these cells directly or after macrophage processing. In addition the function of the germinal centre can still only be surmised. The assumption has been made that the lymphoid cells gathering in the centre bear a receptor for antigen: the responding plasma cells must also have borne a similar receptor yet they differentiated in another way. The germinal centre may represent an earlier stage in the maturation of selected cells so that on a further occasion

they respond actively to the apposition of antigen on their surface by producing antibody.

The second study in the mouse has examined a situation where the host animal readily enters the state of tolerance to the antigen (DGG) used. Evidence from the literature has been cited for the importance of the digestion of antigens before antibody production. The observation of increased katabolism produced by mycobacterial adjuvants was thought possibly to change the kinetics of arrival of digested and undigested antigen at specific receptors. The use of thyroid in accelerating the antigen katabolism laid down some immunization which was detectable at the second stimulation. (The demonstration of the same phenomenon without significantly increased katabolism with the lower doses of thyroid implies that increased katabolism is not necessarily the mechanism of action of thyroid in this system). The studies of Hardy and Rowley (1968) with Sobey mice also bear on this for they showed that individuals tolerant to bovine serum albumin were ones that eliminated the antigen at a slow rate. With the DGG system the thyroid effect is weak compared with the primary response established by the mycobacterial adjuvants. The idea that has been put forward is that with a particular antigen alteration in the rate at which it is handled may affect the outcome in a particular species. (That is not to say that different comparative rates of katabolism of different antigens affect their

ability to induce tolerance).

Receptors within vesicles of immunologically competent cells are thought of as mediators of the next positive event, allowing the membrane to be penetrated at that point possibly by acting as the hinges of hinged "gates". In this way they would form part of a mechanism of specific permeability. When the membranes are made more permeable by vitamin A, the question arises whether it dispenses with the necessity of a receptor on the vesicular membrane or allows penetration after initial attachment to a receptor on a membrane relatively impermeable before treatment. Further examination of the action of vitamin A should be made to determine to what degree it alters avidity of attachment of proteins to particular membranes and in what manner the avidity of antibody itself is altered

The relationship of these findings to the action of classical mycobacterial adjuvants in inducing delayed-type hypersensitivity has not been proved. It is felt however that the concept of an influence on membrane permeability may prove useful in the further elucidation of their activities.

REFERENCES

Reference has been made to the following publications in the preparation for this thesis:

Ada, G.L., Nossal, G.J.V., and Austin, Caroline M. (1964)

Antigens in Immunity V. The ability of cells in lymphoid follicles to recognise foreignness.

Aust. J. exp. Biol. med. Sci., 42, 331

Ada, G.L., Nossal, G.J.V., and Pye, J. (1964)

Antigens in Immunity III. Distribution of iodinated antigens following injection into rats via the hind footpads.

Aust. J. exp. Biol. med. Sci., 42, 295

Ada, G.L., Nossal, G.J.V., and Pye, J. (1965)

Antigens in Immunity XI. The uptake of antigen in animals previously rendered immunologically tolerant.

Aust. J. exp. Biol. med. Sci., 43, 337

Adler, F.L., and Fishman, M. (1962)

Adjuvant effect of diffusion chambers on soluble antigens.

Proc. Soc. exp. Biol. Med., 111, 691

Alexander, J. (1931)

Some intracellular aspects of life and disease.

Protoplasma 14, 296

Allison, A.C. (1968)

Some effects of pharmacologically active compounds on membranes.

Br. med. Bull., 24, 135

Allison, A. C., and Mallucci, L. (1964)

Lysosomes in dividing cells, with special reference to lymphocytes.

Lancet, 2, 1371

Amies, C.R. (1962)

Immunity to vaccinia induced by small doses of active virus.

J. Hyg., Camb. 60, 483

Aschoff, L. (1924)

Das reticulo-endotheliale System.

Ergeb. inn. Med. u. Kinderheilk. 26, 1

Asherson, G.L. and Stone, S.H. (1965)

Selective and specific inhibition of 24-hour skin reactions in the guinea-pig.

Immunology, 9, 205

Askonas, Brigitte A., and Humphrey, J.H. (1955)

Antibody formation in slices of granulomata produced by adjuvant.

Biochem. J., 60, X.

Askonas, Brigitte A., and Rhodes, Joan M. (1965)

Immunogenicity of antigen containing ribonucleic acid preparations from macrophages.

Nature (Lond.) 205, 470

Azar, M.M. (1966)

Termination of acquired immunological tolerance in mice with antigen aggregates.

Proc. Soc. exp. Biol. Med., 123, 571

Azar, M.M., Yunis, E.J., Pickering, P.J., and Good, R.A. (1968)

On the nature of immunological tolerance.

Lancet, 1, 1279

Balfour, B.M., and Humphrey, J.H. (1967)

Localization of γ -globulin and labeled antigen in germinal centers in relation to the immune response.

In "Germinal Centers in Immune Responses" Ed. Cottier, H., Odartchenko, N., Schindler, R., and Cougden, C. C. Springer - Verlag, Berlin.

Battisto, J. R., and Miller, J. (1962)

Immunological unresponsiveness produced in adult guinea-pigs by parenteral introduction of minute quantities of hapten or protein antigen.

Proc. Soc. exp. Biol. Med., 111, 111

Benacerraf, B., Ojeda, A., and Maurer, P.H. (1963)

Studies on artificial antigens II. The antigenicity in guinea pigs of arsanilic acid conjugates of copolymers of D- or L- amino-acids.

J. exp. Med., 118, 945

Benacerraf, B., Ovary, Z., Bloch, K.J., and Franklin, E.C.(1963)

Properties of guinea pig 7S antibodies. 1. Electrophoretic separation of two types of guinea pig 7S antibodies.

J. exp. Med., 117, 937

Berry, L.J., Smythe, Dorothy S., and Kolbye, Susannah McC., (1962)

Effects of bacterial endotoxins on metabolism V. The hyperreactivity of mice infected with *Mycobacterium tuberculosis*, strain BCG

J. exp. Med., 116, 897

Bilbey, D.L.J., and Nicol, T. (1963)

The molecular basis of drug effect on R.E.S. activity. From "Role du système réticulo-endothélial dans l'immunité antibactérienne et antitumorale"

Colloques internationaux du Centre National de la Recherche Scientifique, no 115, Paris.

Billingham, R.E., Brent, L., and Medawar, P.B., (1953)

Actively acquired tolerance of foreign cells.

Nature, Lond., 172, 603

Billingham, R.E., Brent, L., and Medawar, P.B., (1956)

Quantitive studies on tissue transplantation immunity III Actively acquired tolerance.

Phil. Trans. R. Soc. Ser., B239, 357

Biozzi, G., Benacerraf, B., and Halpern, B.N., (1953)

Quantitative study of the granulopectic activity of the reticulo-endothelial system. II: A study of the kinetics of the granulopectic activity of the R.E.S. in relation to the dose of carbon injected. Relationship of the weight of the organs and their activity.

Br. J. exp. Path., 34, 441

Biozzi, G., Benacerraf, B., and Halpern, B.N., (1955)

The effect of *Salm. typhi* and its endotoxin on the phagocytic activity of the reticulo-endothelial system in mice.

Br. J. exp. Path., 36, 226

Biozzi, G., Benacerraf, B., Grumbach, F., Halpern, B.N., Levaditi, J. and Rist, N. (1954)

Etude de l'activité granulopexique du système réticulo-endothélial au cours de l'infection tuberculeuse expérimentale de la souris.

Ann. Inst. Pastour, 87, 291

Biozzi, G., Mene, G., Halpern, B.N., and Benacerraf, B. (1951)

Nouvelles recherches sur l'activité granulopexique du système réticulo-endothélial. Cinetique de l'épuration sanguine de carbone après des injections intraveineuses répétées et rapprochées d'encre de chine.

C. r. Séanc. Soc. Biol., 145, 499

Biro, C.E., and Garcia, Guadeloupe (1965)

The antigenicity of aggregated and aggregate-free human gamma-globulin in the rabbit.

Immunology, 8, 411

Bisset, K.A. (1948)

The effect of temperature upon antibody production in cold blooded vertebrates.

J. Path. Bact., 60, 87

Boehme, D., and Dubos, R.J. (1958)

The effect of bacterial constituents on the resistance of mice to heterologous infection and on the activity of their reticulo-endothelial system.

J. exp. Med., 107, 523

Boss, J. H., Bitterman, W., and Gabriel, M. (1966)

Histopathology of vitamin A induced autograft and homograft rejection.

Transplantation, 4, 293

Boyden, S.V., and Sorkin, E. (1961)

The absorption of antibody and antigens by spleen cells *in vitro*. Some further experiments.

Immunology, 4, 244

Brambell, F.W.R. (1966)

The transmission of immunity from mother to young and the catabolism of immunoglobulins.

Lancet, 2, 1087

Brambell, F.W.R., Hemmings, W.A., and Morris, I.G. (1964)

A theoretical model of γ-globulin catabolism.

Nature, Lond., 203, 1352

Breinl, F. and Haurowitz, F. (1930)

Chemische Untersuchung des Präzipitates aus Hämoglobin und Anti-Hämoglobin-Serum und Bemerkungen über die Natur der Antikörper.

Hoppe-Seyler's Z. physiol, Chem., 192, 45

Burnet, F.M. (1941) The production of antibodies.

Macmillan, Melbourne

Burnet, F.M. (1959)

The clonal selection theory of acquired immunity.

University Press, Cambridge

Burnet, F.M. (1964)

A Darwinian approach to immunity.

Nature, Lond., 203, 451

Burnet, F.M. (1967)

The impact of ideas on immunology.

Cold Spring Harb. Symp. quant. Biol., 32, 1

Burnet, F.M., and Fenner, F. (1949)

Production of antibodies. Second edition.

Macmillan, Melbourne

Campbell, D.H., (1957)

Some speculations on the significance of formation and persistence of antigen fragments in tissues of immunised animals

Blood, 12, 589

Campbell, D.H., and Garvey, Justine S. (1963)

Nature of retained antigen and its role in immune mechanisms.

Adv. Immun. 3, 261

Carr, I. (1967)

The cellular basis of reticulo-endothelial stimulation.

J. Path. Bact., 94, 323

Carpenter, C.B., Gill, T.J., and Mann, L.T. (1967)

Synthetic polypeptide Metabolism. III. Degradation and organ localization of isomeric synthetic polypeptide antigens.

J. Immun., 98, 236

Cerny, J., Ivanyi, J., Madar, J., and Hraba, T. (1965)

The nature of the delay in the immune response after administration of large doses of protein antigen in chicks.

Folio biol., Praha, 11, 402

Cinader, B., and Dubert, J.M. (1956)

Specific inhibition of response to purified protein antigens.

Proc. R. Soc., B146, 18

Claman, H.N. (1963)

Tolerance to a protein antigen in adult mice and the effect of non-specific factors.

J. Immun., 91, 833

Claman, H.N. and Bronsky, E.A. (1965)

Inhibition of antibody production and acquired immunologic tolerance by actinomycin.

J. Immun., 95, 718

Cohn, Z.A. (1962)

Influence of rabbit polymorphonuclear leucocytes and macrophages on the immunogenicity of Escherichia coli.

Nature, Lond., 196, 1066

Cohn, Z. A. (1964)

The fate of bacteria within phagocytic cells.

J. exp. Med., 120, 869

Cohen, E.P. (1967)

Conversion of non-immune cells into antibody-forming cells by RNA.

Nature, Lond., 213, 462

Cohn, Z. A. and Hirsch, J.G. (1960)

The influence of phagocytosis on the intracellular distribution of granule - associated components of polymorphonuclear leucocytes.

J. exp. Med., 112, 1015

Cohn, Z. A., Fedorko, Martha E., and Hirsch, J.G. (1966)

The in vitro differentiation of mononuclear phagocytes. V. The formation of macrophage lysosomes.

J. exp. Med., 123, 757

Condie, R.M., Zak, S.J., and Good, R.A. (1955)

Effect of meningococcal endotoxin on the immune response.

Proc. Soc. exp. Biol. Med. 90, 355

Conning, D.M., and Hoppleston, A.G. (1966)

Reticuloendothelial activity and local particle disposal. A comparison of the influence of modifying agents.

Br. J. exp. Path., 47, 388

Conway, Eleanor A, (1937)

Cyclic changes in lymphatic nodules.

Anat. Rec. 69, 487

Coons, A.M. Leduc, Elizabeth M, and Connolly, Jeanne M. (1955)

Studies on antibody production I. A method for the histochemical demonstration of specific antibody and its application to a study of the hyperimmune rabbit.

J. exp. Med. 102, 49

Cooper, G.N. and Houston, Barbara (1964)

Effects of simple lipids on the phagocytic properties of peritoneal macrophages II Studies on the phagocytic potential of cell populations.

Austr. J. exp. Biol. med. Sci. 42, 429

Cooper, G.N. and Stuart, A.E. (1961)

Sensitivity of mice to bacterial polysaccharide following alteration of activity of the reticulo-endothelial system.

Nature, Lond. 191, 294

Cooper, G.N., and West, Dawn (1962)

Effects of simple lipids on the phagocytic properties of peritoneal macrophages. 1. Stimulating effects of glyceryl trioleate.

Aust. J. exp. Biol. Med., 40, 485

Dale, M. Maureen, (1961)

The effect of components of adjuvant emulsions on lymphatics in the mouse's ear.

Br. J. exp. Path. 42, 297

Dannenberg Jr., A.M., Walter, P.C., and Kapral, F.A. (1963)

A histochemical study of phagocytic and enzymatic functions of rabbit mononuclear and polymorphonuclear exudate cells and alveolar macrophages.

II The effect of particle injection on enzyme activity: two phases of *in vitro* activation.

J. Immun., 90, 448

de Duve, C., Wattiaux, R., and Wibo, M. (1962)

Effects of fat-soluble compounds on lysosomes *in vitro*.

Biochem. Pharmac., 9, 97

Dienes, L. (1930)

The first manifestation of the developing hypersensitiveness.

Proc. Soc. exp. Biol. Med., 28, 75

Dingle, J.T. (1961)

Studies on the mode of action of excess of vitamin A. 3. Release of bound protease by the action of vitamin A.

Biochem. J., 79, 509

Dingle, J. T. (1968)

Vacuoles, vesicles, and lysosomes.

Br. med. Bull., 24, 141

Dingle, J.T. and Lucy, J.A. (1965)

Vitamin A, Carotenoids, and Cell Function.

Biol. Rev. 40, 422-61

Dingle, J.T., Lucy, J.A., and Fell, Honor, B. (1961)

Studies on the mode of action of excess of vitamin A 1. Effect of excess of vitamin A on the metabolism and composition of embryonic chick-limb cartilage grown in organ culture.

Biochem. J., 79, 497

Dixon, F.J., Bukantz, S.C. Dammin, G.J., and Talmage, D.W. (1953)

Fate of I-131-labeled bovine gamma globulin in rabbits. From 'The nature and significance of the antibody response'.

Columbia University Press, New York.

Dixon, F.J., and Maurer, P.H. (1955a)

Effects of large infusions of heterologous serum proteins on the serum protein metabolism of rabbits.

J. exp. Med., 101, 233

Dixon, F.J., and Maurer, P.H. (1955b)

Immunologic unresponsiveness induced by protein antigens.

J. exp. Med., 101, 245

Dowden, S.J., and Sercarz, E.E. (1967)

The X-Y-Z scheme of immunocyte maturation.

J. Immun., 98, 827

Dresser, D.W. (1960)

The elimination of ^{131}I-labelled protein antigens from the circulation of the mouse.

Immunology, 3, 289

Dresser, D.W. (1961a)

Acquired immunological tolerance to a fraction of bovine gamma globulin.

Immunology, 4, 13

Dresser, D.W. (1961b)

Effectiveness of lipid and lipidophilic substances as adjuvants.

Nature, Lond, 191, 1169

Dresser, D.W. (1962a)

Specific inhibition of antibody production. I. Protein overloading paralysis.

Immunology, 5, 161

Dresser, D.W. (1962b)

Specific inhibition of antibody production. II. Paralysis induced in adult mice by small quantities of protein antigen.

Immunology, 5, 378

Dresser, D.W. (1963)

Specific inhibition of antibody production. III. Apparent changes in the half-life of bovine gamma globulin in paralysed mice.

Immunology, 6, 345

Dresser, D.W. (1965)

Specific inhibition of antibody production. IV. Standardisation of the antigen-elimination test; immunological paralysis of mice previously immunized.

Immunology, 9, 261

Dresser, D.W. (1968a)

Adjuvanticity of vitamin A.

Nature, Lond., 217, 527

Dresser, D.W. (1968b)

An assay for adjuvanticity.

Clin. exp. Immun. 3, 877

Dresser, D.W., and Gowland, G. (1964)

Immunological paralysis induced in adult rabbits by small amounts of a protein antigen.

Nature, Lond. 203, 733

Dresser, D.W. and Mitchison, N.A. (1968)

The mechanism of immunological paralysis.

Adv. Immun., 8, 129

Dubos, R.J. and Schaedler, R.W. (1957)

Effects of cellular constituents of mycobacteria on the resistance of mice to heterologous infections.

J. exp. Med., 106, 703

Ehrlich, P. (1900)

On Immunity with special reference to cell life. Croonian lecture.

Proc. R. Soc., 66, 424

Eisen, H.N., and Karush, F. (1964)

Immune tolerance and an extracellular regulating role for bivalent antibody.

Nature, Lond., 202, 677

Ernström, V., and Gyllensten, L. (1959)

The histologic picture in thyroxin induced lymphatic hyperplasia. A quantitative examination on guinea pigs.

Acta path. microbiol. scand., 47, 243

Fagraeus, Astrid (1948)

The plasma cellular reaction and its relation to the formation of antibodies in vitro.

J. Immun., 58, 1

Fahey, J.L., and Sell, S., (1965)

Immunoglobulins of mice V. The metabolic (catabolic) properties of five immunoglobulin classes.

J. exp. Med., 122, 41

Farr, R.S. (1958)

A quantitative immunochemical measure of the primary interaction between I BSA and antibody.

J. inf. Dis., 103, 239

Farr, R.S., and Dixon Jr., F.J. (1960)

The effect of antigen concentration on the initiation of detectable antibody synthesis in rabbits.

J. Immun., 85, 250

Farthing, J.R., and Holt, L.B. (1962)

Experiments designed to determine the mechanism of the adjuvant activity of Gram-negative organisms upon antibody production.

J. Hyg., Camb., 60, 411

Fazekas de St. Groth, S., and Webster, R.G. (1966)

Disquisitions on original antigenic sin. 1. Evidence in man.

J. exp. Med., 124, 331

Fazekas de St. Groth, S., and Webster, R.G. (1966)

Disquisitions on original antigenic sin. 2.

J. exp. Med., 124, 347

Fell, Honor B., Dingle, J.T., and Webb,M. (1962)

Studies on the mode of action of excess of vitamin A 4. The specificity of the effect on embryonic chick-limb cartilage in culture and on isolated rat-liver lysosomes.

Biochem. J., 83, 63

Felton, L.D. (1949)

The significance of antigens in animal tissues.

J. Immun., 61, 107

Felton, L.D., Kauffmann, Gladys, Prescott, B., and Ottinger, Barbara, (1955)

Studies on the mechanism of the immunological paralysis induced in mice by pneumococcal polysaccharides.

J. Immun., 74, 17

Felton, L.D., and Ottinger, B. (1942)

Pneumococcus polysaccharides as a paralysing agent on the mechanism of immunity in white mice.

J. Bact., 43, 94

Felton, L.D., Prescott, B., Kauffmannm Gladys, and Ottinger, Barbara, (1955)

Pneumococcal antigenic polysaccharide substances from animal tissues.

J. Immun., 74, 205

Fishman, M. and Adler, G.L. (1963)

Antibody formation initiated in vitro. II Antibody synthesis in x-irradiated recipients of diffusion chambers containing nucleic acid derived from macrophages incubated with antigen.

J. exp. Med., 117, 595

Flemming, W. (1885)

Schlussbemerkungen über die Zellvermehrung in den lymphoiden Drüsen.

Arch. mikr. Anat., 24, 355

Florey, Lord, (1966)

The uptake of particulate matter by endothelial cells.

Proc. R. Soc. B 166, 375

Franzl, R.E. (1962)

Immunogenic subcellular particles obtained from spleens of antigen injected mice.

Nature, Lond, 195, 457

Freeman, T. (1965)

The assessment of homogeneity of iodine-labelled plasma proteins.

J. labelled Comp. 1, 312

Freeman, T. (1966)

Criteria for assessing the adequacy of iodinated proteins for metabolic studies

from 'Proceedings of the Conference on problems connected with the preparation and use of labelled proteins in tracer studies. Pisa, January 17-19, 1966'

Freeman, T. Gordon, J. and Humphrey, J.H. (1958)

Distinction between catabolism of native and denatured proteins by the isolated perfused liver after carbon loading.

Br. J. exp. Path., 39, 459

Frei, P.C. (1964)

Influence de la dimension des particles d'un antigène proteinique sur la réponse ou la tolèrance immunitaires.

C. r. Soc. Suisse Physiol., 64, 124

Frei, P.C., Benacerraf, B., and Thorbecke, G.J. (1965)

Phagocytosis of the antigen, a crucial step in the induction of the primary response.

Proc. natn. Acad. Sci. U.S.A., 53, 20

Freund, J. (1947)

Some aspects of active immunization.

Ann. Rev. Microbiol., 1, 291

Freund, J., Lipton, M.M., and Thompson, G.E. (1953)

Aspermatogenesis in the guinea-pig induced by testicular tissue and adjuvants.

J. exp. Med., 97, 711

Freund, J., and McDermott, Katherine (1942)

Sensitization to horse serum by means of adjuvants.

Proc. Soc. exp. Biol., 49, 548

Freund, J., Schryver, E.M., McGuiness, Martha B., and Geitner, Mary B. (1952)

Diphtheria antitoxin formation in the horse at the site of injection of toxoid and adjuvants.

Proc. Soc. exp. Biol. Med., 81, 657

Freund, J., Thomson, K.T., Hough, H.B., Somner, H.E. and Pisani, T.M. (1948)

Antibody formation and sensitization with the aid of adjuvants.

J. Immun., 60, 383

Gall, D. (1966)

The adjuvant activity of aliphatic nitrogenous bases.

Immunology, 11, 369

Gallily, Ruth and Feldman, M. (1967)

The role of the macrophage in the induction of antibody in X-irradiated animals.

Immunology, 12, 197

Gamble, C.N. (1966)

The role of soluble aggregates in the primary immune response of mice to human gamma globulin.

Int. Archs Allergy appl. Immun., 30, 446

Garvey, J. S., and Campbell, D.H. (1966)

Autoradiographic investigation of tissues after primary and multiple antigenic stimulation of rabbits.

Nature, Lond., 209, 1201

Glauert, A.M., Dingle, J.T., and Lucy, L.A. (1962)

Action of saponin on biological cell membranes.

Nature, Lond., 196, 953

Gledhill, A.W. (1959)

The effect of bacterial endotoxin on resistance of mice to Ectromelia.

Br. J. exp. Path., 40, 195

Glenny, A.T., and Barr, Mollie (1931)

Alum toxoid precipitates antigens.

J. Path. Bact., 34, 118

Glenny, A.T., Buttle, G.A.M. and Stevens, Muriel F. (1931)

Rate of disappearance of diphtheria toxoid injected into rabbits and guinea-pigs: toxoid precipitated with alum.

J. Path. Bact., 34, 267

Glenny, A.T., and Hopkins, Barbara, E. (1924)

Duration of Passive Immunity Part IV.

J. Hyg., Camb., 22, 208

Glenny, A.T., Pope, C.G., Waddington, Hilda, and Wallace, U, (1925)

Immunological notes IV. The effect of the addition of small amounts of toxin on the antigenic value of modified toxin.

J. Path. Bact., 28, 337

Glenny, A.T., and Südmersen, H.J. (1921)

Notes on the production of immunity to diphtheria toxin.

J. Hyg., Camb., 20, 176

Golub, E.S., and Weigle, W.O. (1967)

Studies on the induction of immunologic tolerance.

J. Immun., 99, 624

Goodman, M., Wolfe, H.R., and Norton, S (1951)

Precipitin production in chickens VI. The effect of varying concentrations of NaCl on precipitate formation.

J. Immun., 66, 225

Green, Ira, Vassalli, V. and Benacerraf, B. (1967)

Cellular localization of anti-DNP-PLL and anti-conveyor albumin antibodies in genetic nonresponder guinea pigs immunized with DNP-PLL albumin complexes.

J. exp. Med., 125, 527

Gunsalus, I.C., Conrad, M.E., and Trudgill, P.W. (1966)

Regulation of catabolic metabolism.

In "Impact of Basic Sciences in Medicine" Ed. by Shapiro, B. and Pryves, M. Academic Press, New York.

Gyllenston, L. (1962)

Influence of thyroxin on regeneration of the lymphatic tissue after corticosteroid-induced involution.

Acta path. microbiol. scand. 56, 29

Halpern, B.N., Benacerraf, B., and Biozzi, G. (1953)

Quantitative study of the granulopectic activity of the reticulo-endothelial System. I. The effect of the ingredients present in India ink and of substances affecting blood clotting *in vivo* on the fate of carbon particles administered intravenously in rats, mice, and rabbits.

Br. J. exp. Path. 34, 426

Halpern, B.N., Benacerraf, B., Biozzi, G., and Stiffel, C. (1954)
Facteures regissant la fonction phagocytaire du système réticulo-endothélial.

Rev. d'Hématol. 9, 621

Halpern, B.N., Biozzi, G., Howard, J., Stiffel., and Mouton, D. (1958)

Exaltation du pouvoir toxique d'*Eberthella typhosa* tuée chez la souris inoculée avec le B.C.G. vivant. Relation entre cette augmentation de la susceptibilité et l'etat fonctionnel du système réticulo-endothélial.

C. r. Séanc. Soc. Biol. 152. 899

Halpern, B.N., Biozzi, G, Stiffel, C., and Mouton, D. (1958)

Correlation entre l'activité phagocytaire du système réticulo-endothélial et la production d'anticorps antibactériennes.

C. r.Séanc. Soc. Biol. 152. 758

Halpern, B.N., Biozzi, G, Stiffel, C., Mouton, D. (1966)

Inhibition of tumour growth by administration of killed *Corynebacterium parvum*.

Nature, Lond., 212, 853

Halpern, B.N., and Dick, P. (1951)

Influence des substances hyperthermisantes sur l'activité granulopexique du système réticulo-endothélial.

C. r. Seanc. Soc. Biol. 145. 1035

Halpern, B.N., Dick, P., Biozzi, G., and Mene, G. (1951)

Influence du refroidissement sur l'activité granulopexique du système réticulo-endothélial.

C. r. Seanc. Soc. Biol. 145. 503

Hardy, D., and Rowley, D. (1968)

The production of antibody to bovine serum albumin in unresponsive (Sobey) mice.

Immunology, 14, 401

Harris, E.J. (1960)

Transport and accumulation in biological systems.

2nd edition Butterworth, London.

Hasek, M. (1956)

Tolerance phenomena in birds.

Proc. R. Soc, B 146, 67

Haurowitz, F. (1967)

The evolution of selective and instructive theories of antibody formation.

Cold Spring Harb. Symp. quant. Biol., 32, 559

Havas, H.F., and Andre, Janet. (1955)

A study of the enhancing effect of adjuvants on antibody formation in mice.

Br. J. exp. Path., 36, 171

Heise, E.R., Myrvik, P.N., and Leake, E.S. (1965)

Effect of Bacillo Calmette Guerin on the levels of acid phosphatase, lysozyme and cathepsin in rabbit alveolar macrophages.

J. Immun. 95, 125

Hektoen, L., (1918)

The production of precipitins in the fowl.

J. inf. Dis. 22. 561

Hellman, T., and White, G. (1930)

Das Verhalten des Lymphatischen Gewebes während eines Immunisierungs prozesses.

Virchows Arch. path. Anat. Physiol. 278, 221

Herbert, W.J. (1966)

Antigenicity of soluble protein in the presence of high levels of antibody: a possible mode of action of the antigen adjuvants.

Nature, Lond., 210, 747

Herbert, W.J. (1968)

The mode of action of mineral-oil emulsion adjuvants on antibody production in mice.

Immunology, 14, 301

Hirschhorn, R., Kaplan, J.M., Goldberg, A.F., Hirschhorn, K., and Weissman, G. (1965).

Acid phosphatase-rich granules in human lymphocytes produced by phytohemagglutinin.

Science, N.Y., 147, 55

Hohn, E.O. (1959)

Action of certain hormones on the thymus of the domestic hen.

J. Endocrinol., 19, 282

Howard, J.G. (1959)

Activation of the reticulo-endothelial cells of the mouse liver by bacterial lipopolysaccharide.

J. Path. Bact., 78. 465

Howard, J.G., Biozzi, G., Halpern, B.N., Stiffel, C., and Mouton, D., (1959)

The effect of BCG infection on the resistance of mice to bacterial endotoxin and Salmonella enteriditis infection.

Br. J. exp. Path. 40. 281

Humphrey, J.H. (1956)

The fate of antigen.

Proc. R. Soc. B 146, 34

Humphrey, J.H. (1960)

The fate of antigen and the importance of different routes of administration.

From "Mechanisms of Antibody Formation". Czechoslovak Academy of Sciences, Prague, p.39

Humphrey, J. H. (1964a)

Immunological unresponsiveness to protein antigens in rabbits. I The duration of unresponsiveness following a single injection at birth.

Immunology, 7. 449

Humphrey, J.H. (1964b)

Immunological unresponsiveness to protein antigens in rabbits. II The nature of the subsequent antibody response.

Immunology, 7. 462

Humphrey, J. H., Askonas, Brigitte, A, Auzins, Leva, Schechter, I., and Sela, M. (1967)

The localization of antigen in lymph nodes and its relation to specific antibody-producing cells. II Comparison of iodine-125 and tritrum labels.

Immunology, 13, 71

Humphrey, J. H. and Frank, M.M. (1966)

The localisation of non-microbial antigens in the draining lymph nodes of tolerant, normal, and primed rabbits.

Immunology, 13, 87

Humphrey, J. H. and McFarlane, A.S. (1954)

Fate of homologous antibody in the rabbit.

Biochem. J., 57, 195

Humphrey., J.H. and Turk, J.L. (1963)

The effect of an unrelated delayed-type hypersensitivity reaction on the antibody response to Diphtheria Toxoid.

Immunology, 6, 119

Hunter, W.M. and Greenwood, F.C. (1962)

Preparation of iodine-131 labelled human growth hormone of high specific activity.

Nature, Lond., 194, 495

Huxley, J. (1938)

Clines: an auxiliary taxonomic principle.

Nature, Lond., 142, 219

Huxley, J.S. (1939)

Clines: an auxiliary method in taxonomy.

Bijdr. Dierk., 27, 491

Ivanyi, J., and Cerny, J. (1965)

The effect of protein antigen dosage on its elimination from the blood and organs.

Folio biol., Praha, 11, 335

Ivanyi, J., Hraba, T., and Cerny, J. (1964)

The elimination of heterologous and homologous serum proteins in chickens at various ages.

Folio biol., Praha, 10, 275

Ivanyi, J., and Valentova, Vera. (1966)

The immunological significance of taxonomic origin of protein antigen in chickens.

Folio biol., Praha, 12, 36

Ivanyi, J., Valentova, Vera, and Cerny, J. (1966)

The dose of antigen required for the suppression of the IgM and IgG antibody response in chickens. I. The kinetics and characterisation of serum antibodies.

Folio biol., Praha, 12, 157

Jancsz, A, Flad, H.-D., Koffler, D., and Miescher, P. (1967)

The effect of vitamin A on experimental immune thyroiditis.

Int. Archs Allergy appl. Immun., 31, 69

Janeway Jr. C.A., and Humphrey, J.H. (1968)

Synthetic antigens composed exclusively of L- or D- amino - acids. II Effect of optical configuration on the metabolism and fate of synthetic polypeptide antigens in mice.

Immunology, 14, 225

Janeway Jr. C.A., and Sela, M. (1967)

Synthetic antigens composed exclusively of L- or D-amimo-acids. 1. Effect of optical configuration on the immunogenicity of synthetic polypeptides in mice.

Immunology, 13, 29

Janoff, A., and Kaley, G. (1964)

Studies on lysosomes in tolerance shock and local injury induced by endotoxin.

In 'Bacterial Endotoxins'. Ed. M. Landy and W. Braun. Rutge's University Press.

Janoff, A., and McCluskey, R.T. (1962)

Effect of excess vitamin A on acid phosphatase content of guinea-pig peritoneal leucocytes.

Proc. Soc. exp. Biol. Med., 110, 586

Janoff, A., Weissman, G., Zweifach, B.W., and Thomas, L. (1962)

Pathogenesis of experimental shock. IV Studies on lysosomes in normal and tolerant animals subjected to lethal trauma and endotoxaemia.

J. exp. Med., 116, 451

Jenkin, C., and Palmer, D.L., (1960)

Changes in the titre of serum opsonins and phagocytic properties of mouse peritoneal macrophagesfollowing injection of endotoxin.

J. exp. Med., 112, 419

Jerne, N.K. (1955)

The naturalselection theory of antibody formation.

Proc. natn. Acad. Sci. U.S.A, 41, 849

Johnson, A.G. (1967)

Studies on the adjuvant action of bacterial endotoxins.

Symp. Series immunobiol. Standard., 6 221

Johnson, A.G., Gaines, S., and Landy, M. (1956)

Studies on the O antigen of Salmonella typhosa V. Enhancement of antibody response to protein antigens by the purified lipopolysaccharide.

J. exp. Med. 103, 225

Juhlen, L.(1958)

The effect of starvation on the phagocytic activity of the reticulo-endothelial system.

Acta physiol. scand. 43, 262

Kabat, E. A. (1966)

The nature of an antigenic determinant.

J. Immun., 97, 1

Kaplan, M.H., Coons, A.H., and Deane, H.W. (1950)

Localization of antigen in tissues. IV Cellular distribution of pneumococcal types II & III in the mouse.

J. exp. Med., 91, 15

Keilhack, H. (1935)

Das Verhalten der Serumeiweisskörper unter verschiedenen Stoffwechselbedingungen.

Naunyn-Schmiedebergs Arch. exp. Path. Pharmak., 180, 1

Kelly, Lola S., Dobson, E.L., Finney, Caroline R., and Hirsch, J. Dorothy. (1960)

Proliferation of the reticulo-endothelial system in the liver.

Am. J. Physiol., 198, 1134

Kevorkov, N.N. (1964)

Effect of body temperature change in experimental conditions on the blood protein spectrum and synthesis of antibodies.

(National Lending Library Translation from the Russian).
Zh. Mikr. biol, 41, 126

Landsteiner, K. (1945)

The specificity of serological reactions.

Republished in 1962 by Dover Publications, Inc., New York.

Langevoort, H.L., Asofsky, R., Jacobson, E.B., Vries, T.de., and Thorbecke, G.J. (1963)

Gamma globulin and antibody formation in vitro. II. Parallel observations on histologic changes and on antibody formation in the white and red pulp of the rabbit spleen during the primary response with special reference to the effect of endotoxin.

J. Immun., 90, 60

Lapresle, C. (1955)

Etude de la dégradation de la serumalbumine humaine par un extrait de rate de lapin.

Ann. Inst. Pasteur, 89, 654

Laufer, A., Tal, Chloe, and Behar, A.J. (1959)

Effect of adjuvant (Freund's type) and its components on the organs of various animal species. A comparative study.

Br. J. exp. Path., 40, 1

Lederberg, J. (1959)

Genes and antibodies.

Science, 129, 1649

Leduc, Elizabeth, M. Coons, A.M., and Connolly, Jeanne M. (1955)

Studies on antibody production II. The primary and secondary responses in the popliteal lymph node of the rabbit.

J. exp. Med., 102, 61

Leskowitz, S. (1967)

Tolerance.

Ann. Rev. Microbiol., 21, 157

Levenson, V.I., and Braude, N.I. (1967)

Different forms of interaction of macrophages with foreign antigens provided by early and late immune sera.

Folio biol., Praha, 13, 113

Levine, B.B. and Benacerraf, B. (1964)

Studies on Antigenicity. The relationship between in vivo and in vitro enzymatic degradability of hapten-conjugates and their antigenicities in guinea pigs.

J. exp. Med., 120, 955

Levine, B.B., Ojeda, A., and Benacerraf, B. (1963a)

Basis for the antigenicity of hapten-poly-L-lysine conjugates in random bred guinea pigs.

Nature, Lond., 200. 545

Levine, B.B., Ojeda, A., and Benacerraf, B. (1963b)

Studies on artificial antigens III. The genetic control of the immune response to hapten-poly-L-lysine conjugates in guinea pigs.

J. exp. Med., 118, 953

Lewis, P.A., and Loomis, D. (1924)

Allergic irritability. The formation of anti-sheep amboreceptor in the normal and tuberculous guinea pig.

J. exp. Med., 40, 503

Lind, Patricia E. (1968a)

The immune responses of normal and tolerant rats to Salmonella adelaide flagellin in Freund's complete adjuvant.

Aust. J. exp. Biol. med. Sci., 46, 179

Lind, Patricia E. (1968b)

Cellular localization of ^{125}I-labelled Salmonella adelaide flagellin following injection into the rat in the presence of Freund's complete adjuvant.

Aust. J. exp. Biol. med. Sci., 46, 189

Long, D.A. (1957)

The influence of the thyroid gland upon immune responses of different species to bacterial infection.

Ciba Fdn Colloq. Endocr., 10, 287

Long, D. A., and Shewell, Jennifer (1955)

The influence of the thyroid gland on the production of antitoxin in the guinea pig.

Br. J. exp. Path., 36, 351

Lucy, J.A. (1964)

Globular lipid micelles and cell membranes.

J. theoret. Biol. 7, 360

Lucy, J.A., Dingle, J.T., and Fell, Honor B. (1961)

Studies of the mode of action of excess of vitamin A 2. A possible role of intracellular proteases in the degradation of cartilage matrix.

Biochem. J., 79, 500

Lurie, M.B. and Ninos, G.S.(1956)

The effect of triiodothyronine and propylthiouracil on native resistance to tuberculosis.

Ann. Rev. Tuberc. 73, 434

Lurie, M. B., Zappasodi, P., Levy, R.S., and Blake, R.B. (1959a)

On the role of the thyroid in native resistance to tuberculosis I. The effect of hyperthyroidism.

Ann. Rev. Tuberc.,79, 152

Lurie, M. B., Zappasodi, P., Levy, R.S., and Blake, R.G. (1959b)

On the role of the thyroid in native resistance to tuberculosis. II The effect of hypothyroidism. The mode of action of thyroid hormones.

Ann. Rev. Tubercl., 79, 180

McFarland, W., Heilman, D.H., Moorhead, J.F. (1966)

Functional anatomy of the lymphocyte in immunological reactions in vitro.

J. exp. Med., 124, 851

Maclean Smith, J., and Dubos, R.J. (1956)

The effect of dinitrophenol and thyroxin on the susceptibility of mice to staphylococcal infections.

J. exp. Med., 103, 119

Marbé, S. (1910)

Les opsonines et la phagocytose dans les états thyroidiens. Résumé et conclusions.

C. r, hebd. Séanc. Acad. Sci., Paris. 69, 462

Martin, W. J. (1966)

The cellular basis of immunological tolerance in newborn animals.

Aust. J. exp. Biol. med. Sci., 44, 605

Medawar, P.B. (1960)

Theories of immunological tolerance.

In Ciba Foundation Symposium on "Cellular Aspects of Immunity".

Churchill, London.

Mego, J.L. and McQueen, J.D. (1965)

The uptake and degradation of injected labeled proteins by mouse liver particles.

Biochim. biophysic. Acta, 100, 136

Mergenhagen, S.E., Notkins, A.L., and Dougherty, Suanne F. (1967)

Adjuvanticity of lactic dehydrogenase virus: influence of virus infection on the establishment of immune tolerance to a protein antigen in adult mice.

J. Immun., 99. 576

Metchnikoff, E. (1905)

Immunity in Infective Diseases. (Translated by F.G. Binnie)

Cambridge University Press

Michael, J. G. Whitby, J.L., Landy, M. (1961)

Increase in specific bactericidal antibodies after administration of endotoxin.

Nature, Lond. 191, 296

Milanesi, S. (1965)

The ultrastructure of the dendritic cells of the lymph node follicles.

Extract from 'Atti del V Congresso Italiano di Microscopia Elettronica, Bologna, 5-7 Ottobre 1965'

Miller III, J.J., and Nossal, G.J.V. (1964)

Antigens in immunity VI The phagocytic reticulum of lymph node follicles.

J. exp. Med., 120, 1075

Miller W.S., and Smith, Jr, J.G. (1966)

Effect of acetylsalicylic acid on lysosomes.

Proc. Soc. exp. Biol. Med., 122, 634

Mitchell, Judith and Nossal, G.J.V. (1966)

Mechanism of induction of immunological tolerance. 1. Localising of tolerance inducing antigen.

Aust. J. exp. Biol. med. Sci., 44, 211

Mitchison, N.A. (1964)

Induction of immunological paralysis in two zones of dosage.

Proc. R. Soc., B 161, 275

Mitchison, N.A. (1966)

Recognition of antigen by cells.

Progress in Biophysics 16, 1

Mitchison, N.A., (1968a)

The dosage requirements for immunological paralysis by soluble proteins.

Immunology, 15, 509

Mitchison, N.A., (1968b)

Immunological paralysis induced by brief-exposure of cells to protein antigens.

Immunology, 15, 531

Mudd, S. (1932)

A hypothetical mechanism of antibody formation.

J. Immun. 23, 423

Munoz, J. (1961)

Permeability changes produced in mice by Bordetella pertussis.

J. Immun., 86, 618

Munoz, J. (1964)

Effect of bacteria and bacterial products on antibody response.

Adv. Immun. 4. 397

Noveu, T., Branellec, A., and Biozzi, G. (1964)

Propriétés adjuvantes de Corynebacterium parvum sur la production d'anticorps et sur l'induction de l'hypersensibilité retardée envers les protéines conjuguées.

Ann. Inst. Pasteur, 106, 771

Nossal, G.J.V. (1960)

Antibody production in single cells. IV. Further studies on multiply immunised animals.

Br. J. exp. Path., 41, 89

Nossal, G.J.V. (1966)

Immunological tolerance: a new model system for low zone induction.

Ann. N.Y. Acad. Sci., 129, 822

Nossal, G.J.V. (1967)

Mechanisms of antibody production.

Ann. Rev. Med., 18, 81

Nossal, G.J.V., and Ada, G.L. (1964)

Recognition of foreignness in immune and tolerant animals.

Nature, Lond., 1964, 201, 580

Nossal, G.J.V., Ada, G.L., and Austin, Caroline M. (1964)

Antigens in Immunity IV. Cellular localization of ^{125}I and ^{131}I-labelled flagellin in lymph nodes.

Aust. J. exp. Biol. med. Sci., 42, 311

Nossal, G.J.V., Ada, G.L., and Austin, Caroline M. (1965)

Antigens in Immunity X. Induction of immunologic tolerance to Salmonella adelaide flagellin.

J. Immun., 95, 665

Nossal, G.J.V., Austin, Caroline M., Pye, J., and Mitchell, Judith. (1966)

Antigens in Immunity XII. Antigen trapping in the spleen.

Int. Arch. Allergy appl. Immun., 29, 368

Nossal, G.J.V., Abbot, A., and Mitchell, Judith (1968)

Antigens in immunity XIV. Electron microscopic radioautographic studies of antigen capture in the lymph node medulla.

J. exp. Med., 127, 263

Nossal, G.J.V., Abbot, A., Mitchell, Judith, and Lummus, Z. (1968)

Antigens in immunity XV. Ultrastructural features of antigen capture in primary and secondary lymphoid follicles.

J. exp. Med., 127, 277

Osterlind, G. (1938)

Die Reaktion des lymphatischen Gewebes während der ausbildung der Immunitat gegen Diphtherietoxin.

Acta path. microbiol. scand., Suppl. 34

Ovary, Z. and Benacerraf, B. (1963)

Immunological specificity of the secondary response with dinitrophenylated proteins.

Proc. Soc. exp. Biol., 114, 72

Palmer, D.L., Rifkind, D., and Brown, D. (1966)

^{131}I-labeled colloidal human serum albumin in the study of reticulo-endothelial system function: the distribution of free and colloid-bound radioiodide.

J. lab. clin. Med., 68, 972

Paraf, A., Fougereau, M., and Metzger, J.J. (1963)

Etude sur la reactivation immunologique spontanée.

C. r. hebd. Séanc. Acad Sci., Paris 257, 312

Parish, C.R., Lang, P.G., and Ada, G.L. (1967)

Tolerance in adult rats to a purified protein, flagellin from Salmonella adelaide.

Nature, Lond., 215, 1202

Patterson, E., and Suszko, Irena, M. (1966)

Passive immune elimination and in vitro phagocytosis of antigen-antibody complexes in relation to species of origin of antibody.

J. Immun., 97, 138

Pearson, C.M. (1956)

Development of arthritis, periarthritis and periostitis in rats given adjuvants.

Proc. Soc. exp. Biol. Med., 91, 95

Pennycuik, Pamela R. (1967)

A comparison of the effects of a variety of factors on the metabolic rate of the mouse.

Aust. J. exp. Biol. med. Sci., 45, 331

Phillips, Julia M. (1966)

Tolerance of turkey γ-globulin in chickens.

Immunology, 11, 163

Pinchuck, P., Fishman, M., Adler, F.L., and Maurer, P.H. (1968)

Antibody formation. Initiation in non-responder mice by macrophage synthetic polypeptide RNA.

Science, N.Y., 160, 194

Pinckard, R. N., McBride, W.H., Weir, D.M. (1967)

Sensitivity and detection of 19S antibody by the Farr technique.

Nature, Lond., 215, 1084

Pinckard, R.N., Weir, D.M., and McBride, W.H. (1967)

Effects of Corynebacterium parvum in immunological unresponsiveness to bovine serum albumin in rabbits.

Nature, Lond., 215, 870

Ponfick, E. (1869)

Studien über die Schicksale körniger Farbstoffe im Organismus.

Virchows Arch. path. Anat. Physiol., 48, 1

Prebble, J. (1968)

The carotenoids of Corynebacterium fasciens strain 2Y.

J. gen. Microbiol., 52, 15

Prévot, A.-R., Halpern, B., Biozzi, G., Stiffel, Claude, Mouton, Denise, Morard, J.-C., Bouthillier, Yvonne, and Decreusefond, Cecile. (1963)

Stimulation du système réticulo-endothélial (S.R.E.) par les corps microbiens tués de Corynebacterium parvum.

C. r. hebd. Séanc. Acad. Sci., Paris. 257, 13

Raffel, S., Arnaud, L.E., Dukes, C.D., and Huang, J.S. (1949)

The role of the "wax" of the tubercle bacillus in establishing delayed hypersensitivity. II Hypersensitivity to a protein antigen egg albumin.

J. exp. Med., 90, 53

Ramon, G. (1925)

Sur la production des antitoxines.

C. r. Séanc. Acad. Sci., Paris 181, 157

Rees, T.A., Perkins, D.J., and Elok, S.D. (1963)

Studies on protein metabolism in toads in relation to immunity.

Comp. Biochem. Physiol., 8, 123

Ribbert, H. (1904)

Die Abscheidung intravenös injizierten gelosten Karmins in den Geweben.

Z. allg. Physiol., 4, 201

Richou, R., Jonson, R., and Bolin, C. (1963)

Sur l'accroissement de l'immunité antitoxique sous l'influence de l'addition de saponine à l'anatoxine staphylococcique.

C. r. hebd. Séanc. Acad. Sci., Paris, 252, 315

Richter, M., Larose, C., and Rose, B. (1964)

The metabolism of antigens in vivo.

Canad. J. Biochem., 42, 821

Rickenburg, H.V., Cohen, G.N., Buttin, G., and Monod, J. (1956)

La galactoside- perméase d'Escherichia coli.

Ann. Inst. Pasteur, 91, 829

Rose, N.R., and Witebsky, E. (1956)

Studies in Organ Specificity V Changes in the thyroid glands of rabbits following active immunization with rabbit thyroid extracts.

J. Immun., 76, 417

Rosenquist, Grace L., and Gilden, R.V. (1963).

Chicken antibodies to bovine serum albumen. Molecular size and sensitivity to 2-mercaptoethanol.

Biochim. biophys. Acta, 78, 543

Rowson, K.E.K. (1961).

The action of tetanus toxin in frogs.

J. gen. Microbiol., 25, 315

Ryser, H.J.-P. (1968)

Uptake of proteins by mammalian cells: an underdeveloped area.

Science, N.Y., 159, 390

Sabin, Florence R. (1939)

Cellular reactions to a dye-protein with a concept of the mechanism of antibody formation.

J. exp. Med., 70, 67

Salvaggio, J.E., Flax, M.H., and Leskowitz, S. (1965)

Studies on immunization. III. The use of beryllium as a granuloma-producing agent in Freund's adjuvant.

J. Immun., 95, 846

Salvin, S.B. and Smith, R.F. (1960a)

The specificity of allergic reactions. I. Delayed versus Arthus hypersensitivity.

J. exp. Med. 111, 465

Salvin, S.B. and Smith, R.F. (1960b)

Specificity of allergic reactions. II Azoproteins in the anamnestic response.

Proc. Soc. exp. Biol. Med. 104, 584

Sbarra, A. J., Shirley W., and Bardawil, W.A. (1962)

"Piggy-back" phagocytosis.

Nature, Lond., 194 255

Scaglione, G. (1938)

Influenza della vitamina A sulla formazione di agglutinino verso il tifo e paratifi negli organismi giovani.

Boll. Ist. Sieroter. Milano., 17, 408

Schechter, I. (1965)

Competition of antigenic determinants.

Biochim. Biophys. Acta, 104, 303

Schechter, I. (1968)

Antigenic competition between polypeptidyl determinants in normal and tolerant rabbits.

J. exp. Med., 127, 237

Schmeckebier, Mary (1945)

Variation in diphtheria antitoxin titers of human serum.

Ann. J. Dis. Child., 66, 25

Sela, M., and Fuchs, S. (1965)

On the role of charge and optical configuration in antigenicity.

In "Molecular and cellular basis of antibody formation" Czechoslovak Academy of Sciences, Prague.

Sell, S., and Gell, P.G.H. (1965)

Studies on rabbit lymphocytes in vitro. IV. Blast transformation of the lymphocytes from newborn rabbits induced by antiallotype serum to a paternal IgG allotype not present in the serum of the lymphocyte doners.

J. exp. Med., 122, 923

Sercarz, E., and Coons, A.H. (1959)

Specific inhibition of antibody formation during immunological paralysis and unresponsiveness.

Nature, Lond., 184, 1080

Sharp, J.A. and Burwell, R.G. (1960)

Interactive ('peripolesis') of macrophages and lymphocytes after skin homografting or challenge with soluble antigens.

Nature, Lond., 188, 474

Shaw, C.M., Alvord, E. C. Jr., Fahlberg, W.J., and Kies Marian W. (1964)

Substitutes for the myco-bacteria in Freund's adjuvants in the production of experimental "allergic" encephalo-myelitis in the guinea pig.

J. Immun., 92, 28

Shaw, C.M., Alvord, E.C. Jr. and Kies, Marian W. (1964)

Straight chain hydrocarbons as substitutes for the oil in Freund's adjuvants in the production of experimental "allergic" encephalomyelitis in the guinea pig.

J. Immun, 92, 24

Shellam, G.R. (1966)

A model system for the induction of immunological tolerance in adult rats.

Proc. Aust. Soc. med. Res., 2, 43

Shellam, G.R. and Nossal, G.J.V. (1968)

Mechanism of induction of immunological tolerance. IV The effects of ultra-low doses of flagellin.

Immunology, 14, 273

Siskind, G.W., Paul, W.E., and Benacerraf, B. (1966)

Studies on the effect of the carrier molecule on antihapten antibody synthesis. I. Effect of carrier on the nature of the antibody synthesized.

J. exp. Med., 123, 673

Smith, P.F. (1963)

The carotenoid pigments of mycoplasma.

J. gen. Microbiol., 32, 307

Smith, R.T. (1960)

Studies in the mechanism of immune tolerance . In "Mechanisms of antibody formation".

Czechoslovak Academy of Sciences, Prague.

Smith, R.T. (1961)

Immunological tolerance of non-living antigens.

Adv. Immun., 1, 67

Smith, R.T., and Bridges, R.A. (1958)
Smith, R.T., and Bridges, R.A. (1958)

Immunological unresponsiveness in rabbits produced by neonatal injection of defined antigens.

J. exp. Med., 108, 227

Smith, W. W. (1964)

Haemopoiesis promoting effect of endotoxin in irradiated animals.

In 'Bacterial Endotoxins' edited by M.Landy and W. Braun. Rutgers University Press.

Smith, W. W., Alderman, I.M., and Gillespie, R.E. (1957)

Increased survival in irradiated animals treated with bacterial endotoxins.

Am. J. Physiol., 191, 124

Smith, W. W., Alderman, I.M., and Gillespie, R.E.(1958)

Haemotopoietic recovery induced by bacterial endotoxin in irradiated mice.

Am. J. Physiol., 192, 549

Smith, W. W., Marston, R.Q., and Cornfield J. (1959)

Patterns of haemopoietic recovery in irradiated mice.

Blood, 14, 737

Smithies, O. (1966)

Somatic mutations and proteins.

Proc. R. Soc. B., 164, 320

Solnitzky, O. (1937)

The Schweigger-Seidel sheath (ellipsoid) of the spleen

Anat. Rec., 69, 55

Sorkin, E. (1960)

The cellular uptake of antigens and their intracellular fate from "Mechanism of Antibody Formation".

Czechoslovak Academy of Sciences, Prague, p61

Sorkin, E., and Boyden, S.V. (1962)

On the fate of rabbit antibody/antigen complexes in the presence of normal leucocytes in vitro.

Experientia 18, 127

Spiegelberg, H.L. and Weigle, W.O. (1965)

Studies on the catabolism of G subunits and chains.

J. Immun. 95, 1035

Stanworth, D. R., James, K., and Squire, J.R. (1961)

Application of zone centrifugation to the study of normal and pathological human sera.

Analyt. Biochem., 2, 324

Stark, O.K. (1955)

Studies on pneumococcal polysaccharide. II Mechanism involved in production of immunological paralysis by type I pneumococcal polysaccharide.

J. Immun., 74, 130

Stein, W.D. (1968)

The transport of sugars.

Br. med. Bull., 24, 146

Sterzl, J. (1966)

Immunological tolerance as a result of terminal differentiation of immunologically competent cells.

Nature, Lond. 209, 416

Stewart-Tull, D.E.S., Wilkinson, P.C., and White, R.G. (1965)

The affinity of a mycobacterial glycopeptide for guinea pig gamma-globulin.

Immunology, 9, 151

Stuart, A.E., Biozzi, G., Stiffel, C., Halpern, B.N. and Mouton, D. (1960).

The stimulation and depression of reticulo-endothelial phagocytic function by simple lipids.

Br. J. exp. Path., 41, 599

Suter, E. and Kirsanow, Eugenia M. (1961)

Hyperreactivity to endotoxin in mice infected with mycobacteria. Induction and elicitation of the reactions.

Immunology, 4, 354

Talmage, D.W., and Pearlman, D.S. (1963)

The antibody response: a model based on antagonistic actions of antigen.

J. theor. Biol., 5, 321

Tenenhouse, Harriet S., and Deutsch, H.F.(1966)

Some physical-chemical properties of chicken γ-globulins on their pepsin and papain digestion products.

Immunochemistry, 3, 11

Thorbecke, G.J., and Benacerraf, B. (1959)

Some histological and functional aspects of lymphoid tissue in germ-free animals.

Ann. N.Y. Acad. Sci., 78, 247

Thorbecke, G.J., Maurer, P.H., and Benacerraf, B. (1960)

The affinity of the reticulo-endothelial system for various modified serum proteins.

Br. J. exp. Path., 41, 190

Torrigiani, G., and Roitt, I.M. (1965)

The enhancement of 19S antibody production by particulate antigen.

J. exp. Med., 122, 181

Uhr, J.W., Weissman, G., and Thomas, L. (1963)

Acute hypervitaminosis A in guinea-pigs. II. Effects on delayed type hypersensitivity.

Proc. Soc. Biol. Med., 112, 287

Unanue, E.R. and Askonas, Brigitte A.

The immune response of mice to antigens in macrophages.

Immunology, 15, 287

Vidaver, G.A. (1966)

Inhibition of carrier flux and augmentation of counter flux shown by transport models not involving a mobile carrier.

J. theoret. Biol., 10, 301

von Dungern, E. (1903)

Die Antikörper. Resultate früherer Forschungen und neue Versuche.

G. Fischer, Jena

Waksman, B.H., and Adams R.D. (1953)

Tubercle bacillus lipopolysaccharide as adjuvant in the production of experimental allergic encephalomyelitis in rabbits.

J. inf. Dis., 93, 21

Ward, P.A., Johnson, A.G., and Abell, M.R. (1959)

Studies on the adjuvant action of bacterial endotoxins on antibody formation. III. Histologic response of the rabbit spleen to a single injection of a purified protein antigen.

J. exp. Med., 109, 463

Watson, J.D. (1965)

Molecular biology of the gene.

Published by W. A. Benjamin, Inc., New York.

Weigle, W.O. (1960)

The elimination of heterologous serum proteins from the blood of animals.

In "Mechanisms of Antibody Formation". Czechoslovak Academy of Sciences, Prague.

Weissman, G. (1965)

Studies of lysosomes VI. The effect of neutral steroids and bile acids on lysosomes *in vitro*.

Biochem. Pharmac., 14, 525

Weissman, G., and Dingle, J.T. (1961)

Release of lysosomal protease by ultraviolet irradiation and inhibition by hydrocortisone.

Exptl Cell Res., 25, 207

Weissman, G., and Thomas, L. (1962)

Studies on lysosomes I. The effects of endotoxin, endotoxin tolerance and cortisone on the release of enzymes from a granular fraction of rabbit liver.

J. exp. Med., 116, 433

Weissman, G., Uhr, J.W. and Thomas, L. (1963)

Acute hypervitaminosis A in guinea-pigs. I. Effects on acid hydrolysis.

Proc. Soc. exp. Biol. Med., 112, 284

Wells, H.G. (1928)

Antigens and their specificity. From "The Newer Knowledge of Bacteriology and Immunology". ed. by Jordan, E.O., and Falls, I.S.

University of Chicago Press, Chicago.

White, R.G. (1960)

The relation of the cellular responses in germinal or lymphopoietic centres of lymph nodes to the production of antibody.

From Mechanisms of antibody formation. Academy of Sciences, Prague.

White, R.G. (1963)

Functional recognition of immunologically competent cells by means of the fluorescent antibody technique.

From Ciba Foundation Study Group No.16 "The immunologically competent cell: its nature and origin."

Churchill, London.

White, R.G. (1967)

Antigen adjuvants.

From "Modern trends in Immunology" p.28 Ed. Cruikshank, R. and Weir, D.G.

Butterworth, London.

White, R.G. (1967)

Role of adjuvants in the production of delayed hypersensitivity.

Br. med. Bull., 23, 39

White, R.G. (1968)

Antigens and Adjuvants.

Proc. R. Soc. Med., 61, 1

White, R. G., Bernstock, L., Johns, R.G.S., and Lederer, E. (1958)

The influence of components of M. tuberculosis and other micobacteria upon antibody production to ovalbumin.

Immunology, 1, 54

White, R.G. Coons, A.M., and Connolly Jeanne, M. (1955)

Studies in antibody production III.
The Alum granuloma.

J. exp. Med. 102, 73

White, R.G., Coons, A.M., and Connolly Jeanne M. (1955)

Studies in antibody production IV. The role of a wax fraction of Mycobacterium tuberculosis in adjuvant emulsions on the production of antibody to egg albumin.

J. exp. Med. 102, 83

White, R.G., French, V., and Stark, J.M. (1967)

Germinal center formation and antigen localization in Malpighian bodies of the chicken spleen.

In "Germinal Centers in Immune Responses"
Ed. Cottier, H. et al.
Springer-Verlag, Berlin.

White, R.G., Jenkins, G.C., and Wilkinson, P.C., (1963)

The production of skin-sensitizing antibody in the guinea-pig.

Int. Archs Allergy appl. Immun., 22, 156

White, R.G. and Marshall A.H.E.(1958)

The role of various chemical fractions of M. tuberculosis and other mycobacteria in the production of allergic ancephalomyelitis.

Immunology, 2, 111

White, R.G., Jolles, P., Samour, D., and Lederer, E. (1964)

Correlation of adjuvant activity and chemical structure of Wax D fractions of mycobacteria.

Immunology, 7. 158

Zechmeister, L. (1962)

Cis-trans Isomeric Carotenoids, Vitamins A, and Aryl polyenes .

Springer Verlag, Wien.

Zinsser, H. (1921)

Studies on the tuberculin reaction and on specific hypersensitiveness in bacterial infection.

J. exp. Med., 34, 495

Appendix A

Use of Medlars Retrieval Service

The process of drawing the relevant references efficiently from the literature was aided by undertaking a computerized search on the subject of "Antigen Katabolism". The U.K. Medlars Information Retrieval Service searched for references over the period September 1963 to July 1967 and "up-dated" the search in July 1968. The form of this particular search is given overleaf.

The method has been outlined by Harley *(1966). Briefly a search is written in the vocabulary provided by the current Medical Subject Headings volume (MeSH) of the Index Medicus (part 2 of the January issue). A list of terms which the computer has to consider is chosen from this list. A search statement is also elaborated which instructs the computer on the particular combinations of these headings which it has to select. Three such combinations are allowed in each search: most conveniently these are usually at increasing levels of selectivity (See terms RA, RB, RC, in the example).

* U.K. Information Retrieval Service, A Handbook for Users by A.J. Harley. Published (1966) by the National Lending Library for Science and Technology, Boston Spa, Yorkshire, England.

The terms and search statement used were as follows:

ANTIGEN KATABOLISM

M1=ANTIGENS
M2=HAPTENS
M3=POLYSACCHARIDES, BACTERIAL
M4=TOXINS
M5=DIPHTHERIA TOXIN
M6=TETANUS TOXIN
M7=PROTEINS
M8=ALBUMINS
M9=BACTERIAL PROTEINS
M10=GAMMA GLOBULIN

M11=SUM M1-M10

M12=IMMUNOLOGY
M13=ANTIBODY FORMATION.
M14=IMMUNE TOLERANCE

M15=SUM M12-M14

M16=MACROPHAGES
M17=HISTIOCYTES
M18=RETICULOENDOTHELIAL SYSTEM
M19=PHAGOCYTOSIS

M20=SUM M16-M19

M21=ADJUVANTS, IMMUNOLOGIC
M22=FREUNDS ADJUVANT
M23=ENDOTOXINS
M24=TUBERCULOSIS
M25=THYROID GLAND
M26=HYPERTHYROIDISM
M27=HYPOTHYROIDISM
M28=MYXEDEMA
M29=CRETINISM

M30=SUM M21-M29

M31=METABOLISM
M32=PROTEIN METABOLISM
M33=BODY TEMPERATURE
M34=PEPTIDE HYDROLASES
M35=AMINOPEPTIDASES
M36=CARBOXYPEPTIDASES
M37=DIPEPTIDASES
M38=PEPTIDE PEPTIDOHYDROLASES
M39=CATHEPSIN
M40=CHYMOTRYPSIN
M41=PEPSIN
M42=TRYPSIN

M43=SUM M31-M42

S1=METABOLISM
S2=ENZYMOLOGY. M16-M18
S3=IMMUNOLOGY

RA=M11 and (M15 or S3) and (M20 or M43 or S1 or S2)

RB= M30 or S2

RC= S2

The searches yielded the following number of references:

Search Instruction	July 1967	"Up-dating" July 1968
RA	157	238
RB	8	14
RC	0	2

Of these, about 30 per cent were considered worth consulting: two thirds of that number were already known from general reading. An example of computer "print-out" is given to show that the headings printed with each reference tell much of its content and whether the reference is worth consulting.

Example of Computer Print-out

NOSSAL GJ ABBOT A MITCHELL J

ANTIGENS IN IMMUNITY. XIV. ELECTRON MICROSCOPIC RADIOAUTOGRAPHIC STUDIES OF ANTIGEN CAPTURE IN THE LYMPH NODE MEDULLA. ENG

J EXP MED 127 263-76 1FEB 68

ANIMAL EXPERIMENTS +*ANTIGENS FEMALE FLAGELLA IMMU IODINE ISOTOPES LYMPH NODES CYT +*MACROPHAGES PHYS MALE METHODS MICROSCOPY ELECTRON +PHAGOCYTOSIS PHYS PLASMA CELLS IMMU RADIOAUTOGRAPHY RATS SALMONELLA IMMU

+ : signifies that the following heading was included in the original search instruction.

* : signifies that reference is recorded under the following heading in the Index Medicus.