A STUDY OF IMMUNOLOGICAL MECHANISMS IN MICE

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Figure 1.
Diagram to illustrate the primary and secondary response. Taken from Glenny and Sudmersen (1921). Antitoxin production in a horse after two equal injections of toxin-antitoxin mixture.
A unique feature of an antibody response is its two phases; the primary and secondary responses. This phenomenon was first described by von Dungern (1903) and later in detail by Glenny and Sudmersen (1921) who showed that after a single injection of diphtheria toxoid, there was only a slight increase in the amount of circulating antibody in the rabbit. There was a long lag period between the injection of antigen and the first signs of a rise in the amount of circulating antibody. This response was called the primary response. When the same antigen was injected into a rabbit which had shown a primary response, there was a short lag period, followed by a rapid increase in the amount of circulating antibody. This was called the secondary response. These results of Glenny and Sudmersen are illustrated in Figure 1 by a diagram taken from their paper. A further interesting point that they made, was that a secondary response could be obtained even if the first injection of antigen had been made several years before.

The classical definition of a primary and a secondary response was on the empirical basis of the rise in circulating antibody after injections of antigen. In the light of work carried out since that of Glenny and Sudmersen, it is probably best to redefine the two phases of the antibody response. These phases could be called the phase of induction and the phase of production. The long lag period in a primary
response is the phase of induction which slowly becomes less evident as the antibody response moves towards the phase of production. The first signs of the phase of production is the slow rise in antibody seen in many primary responses. In a secondary response the phase of production is evident very soon after the injection of antigen and would therefore mask any inductive process which might still be taking place.

The major interest in the mechanism of antibody production lies in the highly specific process of induction. The phase of production is not uninteresting, but appears to be basically similar to other examples of protein synthesis, and is probably best considered as such. Most of the multitude of theories of antibody production are chiefly concerned with the inductive process. At the moment it is convenient to divide these theories into two broad categories: those which require that the induced state is heritable at the cellular level and those which do not require this. The hypotheses which postulate that the induced state is heritable either postulate that the antigen modifies the genotype or that it selects out clones of cells which are genotypically differentiated from antibody producing cells which produce other antibodies. The hypotheses which postulate that the induced state is not heritable all postulate that antigen or determinant groups persist within the induced cell to maintain the induced state. Some of the latter hypothetical mechanisms require the antigen as a template around which the complementariness of the antibody is
produced, while other hypotheses postulate that the persisting antigen elects the required aspect of the cell's genotype which results in the synthesis of the specific antibody.

Acquired immunological tolerance (tolerance) has often been considered as an immunological phenomenon. This is at first sight, natural enough when it is considered that the result of a state of tolerance is the specific inhibition of antibody specific to the tolerance inducing antigen. However, tolerance which may be a non-immunological phenomenon may be a situation which prevents antigen reaching the antibody producing mechanism. In theory, such a mechanism could operate at the cell surface, at which "self" and "not self" was recognised. Possibly "not antigen" (= self) is prevented from stimulating antibody production by the presence of highly specific enzymes in the cell which rapidly break down "self" components, such enzymes being adaptively produced in acquired tolerance (Humphrey 1956). A tolerance mechanism which prevents antigens from being antigenic would be non-immunological as the antibody producing capacity of the cell could still exist although incapable of being stimulated (assuming an "elective" mechanism of antibody production, see Part III).

An immunological tolerance mechanism would be one in which contact with antigen resulted in the destruction or specific inhibition of that part of the antibody synthesising mechanism which would normally result in the production of antibody to that antigen. This could consist of the killing of clones of
cells genotypically differentiated to produce the appropriate antibody or to a specific inhibition of the inductive phase of antibody production normally elected by that antigen.

It follows from this, that if the mechanism of tolerance is non-immunological, then the actual mechanism of tolerance need have no direct relationship with the actual mechanism of antibody production. If, however, tolerance was an immunological mechanism, then it is necessary to consider the mechanism of tolerance when considering the mechanism of antibody production.

As far as most theories are concerned, tolerance differs from antibody production in the relative paucity of hypothetical mechanisms. However, it is possible to evolve of theoretical grounds other mechanisms of tolerance induction which fall into the same categories as for antibody production. In this context, contact with antigen could result in a heritable change in the antibody producing cell or in a non-heritable change which would probably require the persistence of antigen for the maintenance of the induced state.

Both antibody production and tolerance are specific phenomena induced in response to an injection of antigen. That one of these mutually exclusive states should be induced at the expense of the other must be due to either of two factors: firstly, the basic difference may be due to the physiological state of the animal at the time of injection; or secondly, the difference may be due to the quantity of antigen
injected. If the former mechanism was operative, it would have to be postulated that the antibody producing cells of a young animal were all immature and that contact between an immature cell and antigen resulted in the induction of tolerance. Expansion of this hypothesis as the "stem line hypothesis" (Lederberg 1958) can explain the mechanism of immunological paralysis induced in adult animals.

Antibody production and tolerance could be considered as aspects of cellular differentiation. For instance, antibody production could be considered as a state of differentiation induced by the determinant groups of the antigen. A precise knowledge of the mechanism involved in either antibody production or tolerance would allow comparisons to be made. Already for instance, the experimental facts of both phenomena are sufficiently parallel with those obtained from experiments on the adaptive synthesis of enzymes in micro-organisms for useful analogies to be made. If experimental evidence could be obtained which favoured one or other of the many hypotheses of antibody production and tolerance, then it might be possible to devise a general theory of cellular differentiation. Such a theory would be useful if it included morphogenesis, embryogenesis, adaptive enzyme formation, antibody production and tolerance and was meaningful on a practical basis.

In the experiments described in Parts II and III of this thesis an attempt has been made to find out if the induced state in tolerance and antibody production is heritable at the
cellular level. Before these experiments could be started, certain requirements had to be met:

(1) It was necessary to study antibody production by induced cells. It was therefore essential that a system should be chosen in which a primary response and a secondary response were clearly separable. A further requirement, which was desirable rather than essential, was that an antigen which also induced tolerance should be used. It was essential to be able to measure the size of a secondary response, or the rate of antibody production during at least the first part of such a response.

A heterologous serum protein antigen seemed to be suitable to use, as the primary and secondary responses are clearly different (Talmage, Dixon, Bukantz and Damm 1951). Tolerance could be induced by this type of antigen (Hanan and Oyama 1954; Dixon and Maurer 1955) and the antigen-elimination technique (Talmage et al. 1951) seemed to offer a suitable quantitative test.

(2) The object of the experiments was to make induced cells divide in the absence of antigen and then to see what effect this had on the subsequent secondary response, or the subsequent state of tolerance. The most hopeful system where such an experiment could be carried out, seemed to be the transplantation of cells from immunised or tolerant donors into lethally irradiated recipients. Ford, Ilbery and Loutit (1957); Urso and Congdon (1957); and Mitchison (1957) have shown that isologous cells transplanted into an irradiated mouse multiply
and recolonise the host. It was necessary to use inbred strains of mice (isologous transfers of cells), so that the complications of a homograft reaction could be avoided.

(3) Unfortunately mice are well known to be poor and irregular producers of antibody to protein antigens (see Part I). Furthermore, the antigen-elimination technique had not previously been used in mice. It was therefore necessary to investigate reliable methods of immunising mice to protein antigens and to find out if the antigen-elimination technique was suitable for use in mice, before the main experiments could be commenced.

This thesis is therefore divided into three parts. Part I is concerned with methodology; Part II with tolerance; and Part III with the secondary response.
INTRODUCTION

This preliminary work was carried out in an effort to find a simple, reliable and quantitative method of estimating an antibody response to a serum protein antigen in mice. As was stated in the General Introduction, it was necessary to use a protein antigen in order to obtain a clear cut difference between a primary and a secondary response, thus enabling a study to be made of the cellular genetics of antibody production. Use has been made of the antigen-elimination technique in the mouse, although conventional techniques for detecting an immune response have also been employed. Emphasis has been placed on the antigen-elimination technique since it could be made quantitative more readily than the conventional techniques.

Unfortunately mice are notoriously poor producers of precipitins, unless a non-specific method of stimulating the antibody forming mechanism is used. Freund's adjuvant has been found to stimulate antibody production to bovine gamma globulin (BGG) in all mice tested. An attempt has been made to investigate the mode of action of adjuvants and of some of the criteria necessary for a heterologous serum protein to express its antigenicity.
Ransom (1900), in the course of a study of the duration of passive immunity, showed that goat antitoxin remained in the circulation for a considerable time when injected into goats. On the other hand, when he injected horse antitoxin into goats he found that the level of antitoxin decreased in the same way as the goat antitoxin for a few days but that the rate of decrease of the horse antitoxin was then greatly accelerated. Similar results were reported by von Behring and Kitashima (1901). The possibility that heterologous antitoxin (gamma globulin) was eliminated rapidly because of an immune response to the introduced serum, was discussed by von Dungern (1903) and Hamburger and Moro (1903).

The first use of such an elimination of heterologous antitoxin from the circulation as a measure of an immune response was made by Glenny and Hopkins (1923), who used equine diphtheria antitoxin as the antigen. After intravenous injection into rabbits, they found that the elimination of the antitoxin from the circulation of the rabbit was in three phases: a fairly rapid first phase, a second phase of slower, exponential elimination, and a final phase of very rapid elimination which immediately preceded a rise in anti-horse serum precipitins in the circulation of the rabbit. They interpreted the first phase of rapid elimination as being at least partly due to the equilibration of the concentration of antitoxin in the circulation with the extra-circulatory body fluids. The second phase was shown to be an exponential
elimination of antitoxin and this was interpreted as the turnover of antitoxin in a similar way to the turnover of the experimental animal's own serum proteins. The last phase of very rapid elimination they interpreted as being due to an immune response to the injected horse serum proteins in which antibodies precipitated the antigenic material out of the circulation. Opie (1923) studied the relation of the persistence of horse serum and egg albumin in the circulation to the appearance of specific precipitins. He concluded that antigen was rapidly removed from the circulation at the time when there was a rapid increase in the titre of precipitating antibody to the horse serum or egg albumin.

Talmage, Dixon, Bukantz and Dammin (1951) were the first to use the rate of elimination of iodine labelled heterologous serum protein antigens as a measure of an immune response. They used I^{131} labelled bovine serum protein antigens in rabbits. Their results substantially confirmed the findings and interpretations of Glenny and Hopkins (1923). The antigen-elimination technique has since been studied in greater detail (Dixon, Bukantz, Dammin and Talmage 1953). Laws and Payling-Wright (1952) and Laws (1952) have shown that rapid elimination of I^{131} label from the circulation is related to a rapid excretion of iodine in the urine, as iodide and as 3,5 diiodotyrosine. Weigle and others have used the "Farr technique" (Farr 1956, 1958a, 1958b) to study the formation and elimination of soluble complexes of antigen and antibody at the time of
rapid elimination of antigen bound radio-activity (Weigle and Maurer 1957a, 1957b; Weigle and Dixon 1958). Weigle and Dixon (1958) also investigated the effect of serum complement on the elimination of antigen-antibody complexes. Talmage and Dixon (1953) have studied the effect of Freund's adjuvants on the rate of antigen elimination during the phase of exponential elimination and found that the adjuvants had little effect in themselves in rabbits, although they may have helped to increase the amount of antibody produced in the final phase of elimination, thereby causing the rate of elimination in this final phase to be slightly greater than in controls without adjuvant.

The antigen-elimination technique has recently been used as a routine technique by several different groups of workers. Janeway (1951) and Gitlin, Latta, Batchelor and Janeway (1951) have studied hypersensitivity to protein antigens and the relation of this phenomenon to the rate of antigen elimination. Dixon, Maurer and Deichmiller (1954) have used the technique to study the specificity of the secondary response. Roberts and Dixon (1955) and Hobson, Porter and Whitby (1959) have studied adoptive immunity to serum protein antigens by means of this technique. Acquired immunological tolerance to bovine and human serum protein antigens has been demonstrated in rabbits by Cinader and Dubert (1955, 1956) and to bovine proteins by Smith and Bridges (1958) also in rabbits, by using the antigen-elimination technique.

All the work carried out with $^{131}$I labelled proteins and
cited so far, has been carried out in rabbits. Weigle and Dixon (1957) have extended the technique to guinea pigs, and in a very limited and unsatisfactory way to rats. Unfortunately no published work has been carried out using the technique in mice. An extension of the antigen-elimination technique to inbred mice, for the reasons previously stated, is therefore described here. Mice have been found to respond somewhat differently from rabbits.

MATERIALS

Animals

Mice of the CBA and A inbred strains, as well as an outbred strain were used. The inbred stocks were maintained in this laboratory by sib matings. The CBA strain used was obtained from University College, London. The A strain came from Bar Harbor, Maine, USA. The outbred mice were kindly supplied by Dr. Nigel Bateman, Animal Breeding Research Organisation, Roslin, Midlothian.

Mice used in experiments were of both sexes and were first immunised when 3-5 months old. After injections of iodinated protein, mice were given free access to food and to water containing 2 gms of potassium iodide per litre.

Control antisera were sometimes prepared in rabbits which were of both sexes and were supplied by the Small Animal Breeding Station, The Bush Estate, Midlothian.
Antigens

Several antigens were tested to see if they were suitable for use in mice with the antigen-elimination technique. Human serum albumin (HSA) was prepared from outdated human plasma supplied by the S.E. Scotland, Blood Transfusion Service. The albumin fraction was prepared by the cold ethanol fractionation technique of Cohn, Strong, Hughes, Mulford, Ashworth, Melin, and Taylor (1946). The other antigens were bovine serum albumin (BSA) (prepared by Armour and Co. by the technique of Cohn et al. (1946) and supplied as a freeze dried powder) and bovine gamma globulin (BGG) (also prepared by Armour and Co. but using the modification of the Cohn technique by Oncley, Melin, Richert, Cameron, and Gross (1949), method 9, and supplied as a freeze dried powder). The freeze dried proteins (BSA and BGG) were kept dry after the bottle had been opened by storing them at 2°C in a desiccator.

METHODS

Immunisation

Immunising injections were made in either of three ways:

1. By intravenous, intraperitoneal or occasionally subcutaneous injection of protein solution, without the use of any adjuvants.

2. By the intravenous or intraperitoneal injection of protein solution, accompanied, often at the same time but sometimes at different times, by adjuvants or substances being
tested for adjuvant activity (adjuvanticity) which were injected subcutaneously. In some experiments the substance being tested for adjuvanticity was injected intravenously, in which case the protein was also injected intravenously but at a different time.

3. By the subcutaneous injection of water-in-oil emulsions which included the protein antigen in the water moiety. These were complete and incomplete Freund type adjuvant mixtures (Freund 1953).

Complete adjuvant was prepared by mixing 2 ml liquid paraffin containing 4 mgm per ml of heat killed, dried and powdered Mycobacterium tuberculosis with 1 ml Crill K 16 and 2 ml of 10% protein solution w/v. The mixture was converted to a water-in-oil emulsion by squirting it in and out through the nozzle of a 1 ml tuberculin syringe. The emulsion was tested by placing a small drop on the surface of cold water; it was judged to be water-in-oil if the drop did not spread.

Incomplete adjuvants were prepared by emulsifying protein solutions with Crill; with Crill plus liquid paraffin without mycobacteria; with liquid paraffin without mycobacteria; or with liquid paraffin containing mycobacteria.

A complete adjuvant without protein was also used in some

Kindly supplied by Messrs. Croda Ltd., Goole, Yorkshire.
Crill K. 16 is an oleic acid ester (mostly mono) of sorbitol.
experiments (saline Freund's). The protein solution was substituted by a saline solution (0.9% NaCl w/v) and the adjuvant emulsion prepared as already described.

A total of 0.2 ml adjuvant mixture was injected subcutaneously into a mouse, about 0.05 ml being injected into each of 4 sites in each mouse. When the adjuvant was made up with 10% protein solution, each mouse received 8 mgm of protein in the adjuvant mixture.

Substances being tested for adjuvanticity were injected by the route stated in the results section. The quantities of these substances* which were injected are also given in the results section.

Trace labelling the protein with iodine\[^{131}\]

Most techniques for labelling proteins unfortunately require that iodine be in solution as molecular iodine in order

* "Tweens" and "Spans" were kindly supplied by Honeywill and Stein Ltd., for Honeywill Atlas Ltd., Devonshire House, Mayfair Place, Piccadilly, W.L. "Lubrol" and "Dispersol", manufactured by ICI Ltd., were given to me by Dr. David Bell of the Agricultural Research Council, Poultry Research Centre, King's Buildings, Edinburgh 9. The polysaccharide of \( \text{Pasturella pestis} \) (Davies 1956) and the endotoxin of \( \text{Shigella dysenteriae} \) (Davies, Morgan and Moismann 1954) were kindly supplied by Dr. D.A.L. Davies, War Office, Microbiological Research Station, Porton, Wilts. The B.C.G. (Bacille de Calmette et Guerin) was kindly prepared by Dr. A. Wallace, The Laboratory, City Hospital, Greenbank Drive, Edinburgh 10. Some of this material was obtained direct and some was given to me by Dr. James Howard, Department of Surgical Science, University of Edinburgh, Teviot Place, Edinburgh 1, who had obtained it from the same source.
that the iodine can combine with the protein. A technique which did not require iodine to be free in solution was tried (Method 1) but was found to be unsatisfactory. It was therefore necessary to carry out the labelling process in a fume cupboard and behind lead shielding. In these circumstances there was insufficient space available in the laboratory for the relatively complex techniques of Hughes and Straessel (1950) or McFarlane (1956) to be used. It was therefore necessary to use a cheap, simple and reliable method which could be carried out in a confined space. Two methods were tried:

**Method 1** - This method is used by the Radio-Chemical Centre, Amersham, Bucks., for the labelling of HSA with I\(^{131}\) (Dr. J. S. Glover, personal communication). This method was tried for labelling BGG with I\(^{131}\). Three mCi of carrier free I\(^{131}\) as iodide in dilute sodium thiosulphate (catalogue No. IBS 1) were obtained from the Radio-Chemical Centre. A 2% solution of BGG in saline was prepared. 3.5 ml aliquots of the protein solution were mixed with 100, 10 and 2½ equivalents of Chloramine-T. The Chloramine-T (sodium derivative of N-chloro-p-tolulene sulphonamide, mol. wt. 281) was made up in 0.15 M. borate - NaOH buffer at pH 9.0-9.5 (Clark 1928; Hale 1958). 0.5 mgm of carrier iodine dissolved in 0.01 M. potassium iodide solution, was added to the carrier free iodine solution. Equal volumes of the radio-active mixture were added to the three protein/Chloramine-T solutions. The
mixtures were incubated at room temperature for half an hour. After incubation, uncombined iodide and Chloramine-T were removed from the protein solution by dialysis against 0.15 M. phosphate buffered saline (Sørensen's buffer; see Hale 1958) at pH 7.2, followed by many changes of saline.

The labelling of the protein is thought to take place in a complicated exchange reaction. Most of the label becomes attached to tyrosine.

**Method 2** - The protein was trace labelled with iodine$^{131}$ by a modification of the method of Wormall (Francis, Mulligan and Wormall 1951; Hemmings 1956). Carrier free $I^{131}$ as iodide in sterilised dilute phosphate buffer at pH 7 (catalogue No. IBS 2) was obtained from the Radio-Chemical Centre. Three millicuries was found to be sufficient to label 200 mgm protein using this technique.

For successful labelling, free iodine must be in solution before the addition of the protein. Unfortunately "IBS 2" contains a variable trace amount of sodium thiosulphate, sufficient in 4 out of 31 samples to completely reduce all the carrier free iodine added to the radioactive sample. It is possible to obtain "thiosulphate-free" iodide at a processing charge of £5. If this was done, it is possible that on the average the labelling efficiency would be twice that obtained by the following method. If this assumption were true, then the use of "thiosulphate-free" iodide would not be economically
justified unless 12 mC or more were being used each time. The following procedure has been found to be satisfactory: 20 mgs of iodine ($^{127}$I) was dissolved in 50 ml of 0.01 M. KI; 1.3 ml of this solution was added to 6.7 ml of saline in a colorimeter tube to form a standard. Another 1.3 ml was added to the carrier free radioactive solution. The radioactive mixture was then pipetted into sufficient saline in a colorimeter tube to make the volume 7.0 ml. The free iodine in the radioactive sample was adjusted to the same value as the standard in an "EEL" colorimeter with a blue filter, by the addition of extra iodine ($^{127}$I) from a saturated solution in 0.01 M. KI. The final volume was adjusted to 8.0 ml with saline. 200 mgm of protein was dissolved in 11 ml of saline; to this was added 1 ml of 25% solution of .880 ammonia (v/v), which raised the pH to 10.5-11.0. The 8 ml of radioactive iodine solution was then added dropwise to the 12 ml of protein solution, with continuous stirring. The mixture was allowed to stand for 40 mins. at room temperature ($16-20^\circ C$) and was then dialysed at $0^\circ C$ for 48 hours, against several changes of saline.

As an alternative to the dialysis the mixture was passed through an ion-exchange column (Permutit Deacidite FF, chloride form, 200 mesh); the pH of the protein solution was adjusted to 7 with N. HCl after it had left the column.

The amount of iodine used was sufficient to label the protein with 1 atom of iodine to every 100 molecules of protein at the theoretical maximum labelling efficiency of 50%.
However, the actual labelling efficiency achieved was from 8 - 15% so that the level of labelling was about 1 atom of iodine for 2 to 3 molecules of protein. This level of iodination has been found to cause no detectable antigenic change in the protein molecule by McFarlane (1956).

Oxidation of albumins

Albumins were oxidised prior to labelling, since pre-oxidation of -SH groups with unlabelled iodine at a slightly acid pH leaves tyrosine unchanged and free to react with labelled iodine at an alkaline pH (Dr. J.H. Humphrey, personal communication). The albumin solution was brought to pH 4.6 with glycine buffer (19 parts M. glycine to 1 part M. HCl). To a solution of M. KIO₃ was added sufficient HCl to release free iodine. This solution was added to the albumin dropwise until a trace of free iodine was detected with starch iodide paper (slightly acidified in the breath!). The solution was immediately passed through an ion-exchange column (Deacidite FF, chloride form, 200 mesh, 1 gm for 5 ml of solution). The solution was then made alkaline with ammonia and labelled with I¹³¹ using Method 2.

Estimation of antigen elimination

Mice were injected intravenously or intraperitoneally with 2 mgm of labelled protein. They were bled at two day intervals from a cut in the ventral tail artery. Using an automatic pipette, 0.025 ml of serum was then placed on a planchette
together with two drops of polyvinyl alcohol solution (a saturated solution of polyvinyl alcohol in 15% ethanol and 85% "teepol"). The radioactivity was measured with a Geiger-Muller tube with a thin end-window. Corrections were made for isotope decay. Preparation of serum was suitable when antigen elimination tests were being combined with antibody titrations, as the one bleeding would suffice for both tests. However, in some experiments in this part of the thesis and all experiments in Parts II and III of this thesis, the following modification was used: whole blood was taken up from a small cut in the ventral tail artery into a 0.05 ml automatic pipette and squirted directly into two drops of polyvinyl alcohol solution on a planchette. The pipette was washed out with dilute detergent solution, water and 3.4% sodium citrate solution before each bleeding.

Corrections for the dilution effect due to bleeding have been made in determinations of the biological half-life of labelled proteins; this was done by estimating the blood volume of the mouse, the quantity of blood removed at each bleeding and then calculating the percentage dilution. Confirmatory half-lives were obtained with injected but unbled mice. In these mice the radioactivity of the whole body was measured in a scintillation counter.

Tests were carried out with serum from mice injected with labelled BGG, to show that the radioactivity was a true measure of the concentration of antigen in the circulation. Protein-
bound radioactivity was determined by precipitation of all proteins in a serum sample with 10% trichloracetic acid. The radioactivity of the precipitate plus supernatant was then measured in a scintillation counter. The precipitate was centrifuged out of suspension and was washed twice with 5% trichloracetic acid. The remaining activity was expressed as a percentage of the total activity before washing. This is called the "non-specific precipitability". Radioactive serum samples were also treated with an excess of rabbit anti-BGG serum for 2 hours at 37°C. The precipitate was centrifuged out of suspension and washed once with saline. As before, the radioactivity was measured before and after washing and the amount of radioactivity precipitated expressed as a percentage of the total. This is called the "specific precipitability" of the label.

Calibration of the antigen-elimination curve

The rate at which labelled antigen was eliminated from a mouse in an immune response was calibrated. This was done by injecting non-immunised CBA mice with labelled antigen (2 mgm BGG-I\(^{131}\), labelled by Method 2). As will be subsequently shown, a primary response to this antigen is very unlikely. The mice injected with labelled BGG were then injected with an outbred mouse anti-BGG serum. Injecting different amounts of antibody into different groups of mice made it possible to relate the rate of elimination with the amount injected. The family of elimination curves so obtained could be then compared
with similar curves obtained in some experiments, especially those described in Parts II and III of this thesis.

In vitro tests were carried out on the serum used in this calibration of the elimination curves. These tests are described in detail in the next section; the mouse serum used in the precipitation of the labelled antigen (in vitro) was the same pool which was used in this test.

Six groups of 5 CBA males in each group, were injected intravenously with 2 mgm BGG-I\textsuperscript{131}. They were bled 2 days later and immediately afterwards were given an intraperitoneal injection of the outbred mouse anti-BGG serum. Different relative amounts of the antibody were injected into each of the experimental groups. The relative quantities are shown in Figure 8, in which 1 means 0.2 ml of the whole antiserum every 2 days. The bleeding and injection was repeated on the 4th, 6th, 8th and 10th days after the antigen injection. On the 12th day the mice were bled but not injected with antiserum. The bleedings were made at two day intervals so that the results would be comparable with the results obtained in other experiments. The injections of antiserum were also made at two day intervals so that the antibody and antigen had two days in which to reach equilibrium. The passive immunisation simulated a constant rate of antibody release into the circulation, although the elimination of antigen, if it had been followed at intervals shorter than two days, would have followed a series of steps. Injection of antibody at two day
intervals is also convenient as Dixon, Talmage, Maurer, and Deichmiller (1952) have shown that the half-life of mouse antibody globulin is about two days. This would mean that in the absence of antigen the total amount of antibody in the circulation of a mouse, at any one time, would never be more than twice the amount in a single injection.

Antibody titrations

Antibodies to protein antigens have been titrated by haemagglutination techniques by several workers. These techniques involve the coating of surface stabilised erythrocytes with the antigenic protein. Boyden (1951) and Stavitsky (1954a; 1954b) coated sheep erythrocytes which had previously been treated with tannic acid. This method has its disadvantage in that the cell preparation must be prepared afresh every few days. McKenna (1957) attempted to overcome this disadvantage by first treating the sheep erythrocytes with formalin, so that the coated cells could be stored in the refrigerator or deep freeze. Formalin prevents the erythrocytes from lysing when frozen, or when stored at 0°C for a long time, and also stabilises the cell surface. Unfortunately sheep erythrocytes are spontaneously agglutinated in the presence of formalin, so it is necessary to treat the cell clumps by prolonged homogenisation in a Waring blender to get a suspension of single cells. Ingraham (1958) was able to overcome this disadvantage by using human group O cells, which do not spontaneously agglutinate if treated with formalin at 0°C. The human cells can also be coated with
protein in the presence of formalin. Ingraham describes several different methods. The one used here was as follows: Human group 0 erythrocytes were washed in phosphate-buffered-dextrose-saline and treated with formalin at 0°C. The phosphate-buffered-dextrose-saline was prepared by making a 0.9% solution of NaCl with 0.005 M. Sørensen's buffer pH 7.2-7.3 (\( \text{Na}_2\text{HPO}_4 \) plus \( \text{KH}_2\text{PO}_4 \)) (Clark 1928; Hale 1958) and 0.5% dextrose. This is called PBDS. After washing, a 50% suspension of cells in PBDS was poured into 4 volumes of 10% reagent grade formalin in PBDS, at 0°C. A 10% suspension of erythrocytes in 8% formalin was thus obtained. The suspension was stored for 10 days at 2°C and was resuspended each day. The erythrocytes were coated with the protein antigen (BGG), in the presence of formalin, by mixing 0.5 ml of 10% solution of protein in PBDS with 10 ml of the suspension of erythrocytes in formalin. To this mixture was added 2 ml of 0.5 M. McIlvaine's phosphate-citrate buffer at pH 6.0 (Hale 1958). The mixture was kept in suspension for 3 days at room temperature. The cells were then washed free of uncombined protein by washing several times in PBDS. The cells were stored at 2°C in PBDS-8% formalin. When required for use, the cells were washed with 8 or more changes of PBDS, left overnight at 2°C in a large volume of PBDS and washed twice more with PBDS before being made up as a 0.5% suspension in 1% normal rabbit serum in PBDS. Reliable negative patterns with non-immunised mouse serum, have always been obtained. Ingraham, however, states that some rabbit sera will spontaneously agglutinate coated cells and that others
will agglutinate cells if the serum is stored in the diluted and unfrozen state. Sera for titration were diluted two fold or five fold. Titrations were carried out in the usual manner, equal volumes of serum solution and coated erythrocyte suspension being mixed. This was usually 0.1 ml of each solution in a $\frac{3}{8}$ x 2" test tube. The agglutination patterns were read macroscopically.

In a limited number of experiments the results obtained with the haemagglutination technique were confirmed using a complement fixation technique (Kabat and Meyer 1948a; Sinclair 1957). The same procedure and the same sheep erythrocyte haemolysin used by Sinclair, were used in these experiments.

The presence of precipitating antibody was detected in sera using a simple ring-test (Kabat and Meyer 1948b). Quantitative estimations of the amount of precipitating antibody were made by a combination of the conventional antibody nitrogen estimations plus an estimation of the amount of antigen precipitated, using $^{131}$I labelled antigen. A serial dilution of BGG-$^{131}$I was made and to each tube was added a constant volume of antiserum. The mixture was incubated at 37°C for 2 hours, and the precipitate was then centrifuged out of suspension. The precipitate was washed once with a relatively large volume of saline. The amount of labelled antigen present in the precipitate was estimated. Similar experiments were carried out with unlabelled antigen, in which the total protein in the precipitate was estimated by a micro Kjeldahl
Figure 2.
The elimination of BGG-\textsuperscript{131}I labelled by Method 1, from the circulation of non-immunised CBA males. The numbers in the figure represent the equivalents of Chloramine-T used to label the three batches of BGG.
(Nessler) estimation (Umbreit, Burris and Stauffer 1957). The known amount of antigen in the precipitate could then be subtracted from the known total amount of protein in the precipitate, giving an estimate of the amount of antibody present.

In some experiments the presence of an immune response was tested for by a test for anaphylactic sensitivity. Five weeks after the first injection of BGG, mice were given an intravenous injection of 2 mgm BGG or BGG-\textsuperscript{131}I. The results were classified as a typical anaphylactic death, typical anaphylactic shock without death and no marked reaction. The symptoms observed were substantially the same as those described by Weisner, Golub and Hamre (1941). In a limited number of experiments the results obtained in the anaphylaxis experiments were compared with the results obtained in antigen-elimination experiments. If the protein antigen was injected by the intraperitoneal route, then deaths from anaphylaxis were reduced to negligible proportions.

RESULTS

Elimination of protein antigens

Figure 2 shows the result obtained when BGG was labelled by Method 1. It can be seen that BGG labelled by 100 equivalents of Chloramine-T was very unstable in vivo, since it was eliminated very fast from the circulation of non-immunised mice. When labelled with 10, and 2\textsuperscript{1/2} equivalents of Chloramine-T the protein was less unstable. It is possible that the elimination
Figure 3.
The elimination of BGG-I$^{131}$ labelled by Method 2, from the circulation of non-immunised CBA males. There is no indication of any response in these mice.
of BGG labelled with $2\frac{1}{2}$ equivalents of Chloramine-T may have been due to a primary response to the antigen, rather than being due to denaturation of the protein. However, it will be seen later in this section, that BGG labelled by Method 2 did not elicit a primary response when injected alone. It therefore appears that Method 1, even using only $2\frac{1}{2}$ equivalents of Chloramine-T, has modified the protein. Using this amount of Chloramine-T the efficiency of labelling was the same or less than the efficiency obtained by Method 2. In all subsequent experiments described in this thesis, Method 2 has been used to label proteins with $^{131}\text{I}$. 

The biological half-life of 25 different batches of BGG-$^{131}\text{I}$ labelled by Method 2, have been found to be the same. For example, the biological half-life of BGG-$^{131}\text{I}$ in 45 non-immunised adult CBA males and non-pregnant females was in the range 3.7-4.2 days (see Figure 3). In five A strain mice it was 3.2-3.8 days and in five outbred males it was 2.9-3.2 days. These figures exceed those of Weigle (1957), who used the method of Hughes and Straessel (1951) to label the protein; the half-life reported by Weigle was 1.5 days. The difference can be accounted for by differences in the strains of mice used, in the different techniques used to label the BGG or possibly to differences in different batches of BGG prepared by the Cohn fractionation technique. All experiments reported in this thesis were carried out with the same batch of BGG (BF. 0270).

Adult CBA mice were injected with HSA-$^{131}\text{I}$ and BSA-$^{131}\text{I}$. 
Figure 4.

The elimination of BSA-I$^{131}$ labelled by Method 2, from non-immunised CBA mice and from CBA mice immunised one month previously with BSA contained in Freund's adjuvant.
The biological half-life of both these proteins was about 20 hours: this is too short to detect a difference between immune clearance and normal turnover of the labelled protein. This was clearly seen in an experiment where some CBA mice had previously been immunised with Freund's adjuvant containing BSA (see Figure 4). The increase in the rate of removal of labelled BSA was so slight in these mice compared with that in non-immunised mice, that it became clear that of the protein antigens tried only BGG was practicable for use with the antigen-elimination technique in mice. The half-life of BGG-\( ^{131}I \) is sufficiently long for a primary response to be detected, as will be shown later.

Precipitation of the protein in serum, after an injection of BGG-\( ^{131}I \) and therefore containing radioactivity, showed that the non-specific precipitability of the label was always greater than 99%. Similar tests showed that the specific precipitability with rabbit anti-BGG serum, was greater than 95%. There was probably a certain amount of co-precipitation between BGG and mouse gamma globulin by the rabbit anti-BGG serum. These results suggest that reincorporation of \( ^{131}I \) onto serum proteins of the experimental mouse was insignificant.

A drop in specific precipitability, from more than 95% to between 80% and 90%, was observed at the time of rapid immune elimination of the labelled antigen (see next section). It seems likely that this was due to the presence of soluble complexes of antigen (BGG) and antibody (mouse gamma globulin),
which were not as easily precipitated by the rabbit anti-BGG serum as BGG alone. Weigle (1958) has demonstrated the presence of such complexes in rabbits at the time of immune elimination, using BSA-\(^{131}\) as the antigen.

No difference in any subsequent immune response has been detected when antigen solution was injected by the intravenous route or the intraperitoneal route. In the first two days after the intravenous injection of labelled BGG there is a 50-60\% drop in the amount of circulating antigen. This is more than can be accounted for solely by the exponential decrease in antigen concentration in the blood, observed in the second phase of elimination. This first apparent decline does not appear if the radioactivity of the whole body is measured in a scintillation counter. The decrease in circulating antigen is probably due to two factors: firstly a slow rate of equilibration of the labelled protein in the circulation with the extra-circulatory body fluids (Glenny and Hopkins 1923; Talmage et al. 1951); and secondly, to the rapid elimination of any protein denatured in the labelling process (McFarlane 1956). This drop is not so marked when the labelled antigen was injected intraperitoneally.

**Immunisation with BGG**

CBA mice rarely showed a primary response when injected with BGG-\(^{131}\) (labelled by Method 2) (see Figure 3). Furthermore, it has been shown that intravenous, intraperitoneal or
A primary response to BGG-I\textsuperscript{131} in CBA mice injected with mycobacteria powder as a suspension in saline, at the same time as the injection of antigen.
subcutaneous injection of BGG solution alone is an inadequate method of immunising mice so that a secondary response can subsequently be obtained. Subcutaneous injection of Freund's adjuvant containing BGG has been shown to immunise mice when they were later challenged by an intravenous or intraperitoneal injection of BGG-I₁³¹.

The phase of very rapid elimination was taken as an indication of an immune response. In certain experimental situations to be described, a primary response could be obtained. A primary response usually started between the 8th and the 12th days after the injection of antigen (Figure 5). In CBA mice a secondary response after immunisation with Freund's adjuvant a month previously, started between the 2nd and the 4th days after the injection of BGG solution (Figure 6).

Unless non-immunised mice are given a non-specific stimulus at the time of the intravenous injection of BGG, most do not show a primary response. The few mice which do show a primary response probably do so because of the presence of particulate matter in the BGG solution. No primary responses whatsoever were obtained in 26 mice which were injected with BGG-I₁³¹ which had been centrifuged at an R.C.F. of 30,000 g. for 15 minutes. A significant increase in the number of primary responses to centrifuged BGG-I₁³¹ was obtained by the following injections given at the same time as the antigen injection: firstly there was the subcutaneous injection of saline Freund's adjuvant; and secondly the intravenous injection of a suspension of
Figure 6.

A secondary response to BGG-\(^{131}\) in CBA mice injected with Freund's adjuvant containing BGG one month previously.
mycobacteria (2 mgm) in saline, shortly after the intravenous injection of labelled antigen (see Table 1).

Immunisation of mice was initially tried by intravenous injection of BGG solution. After the failure to obtain reliable immunisation by this means, several methods of immunisation were tried. A secondary response was always obtained after immunisation with Freund's adjuvant containing BGG injected subcutaneously; this has been used subsequently as the standard method of immunising mice to BGG.

Less clear cut results were obtained when components of the complete adjuvant were omitted. For instance, an oil-in-water emulsion of liquid paraffin (with or without mycobacteria) with BGG solution, injected subcutaneously was successful in immunising both CBA and A mice. However, incomplete Freund's adjuvant where the mycobacteria alone were omitted, successfully immunised 5 A mice but only 3 out of 5 CBA mice (see Table 1).

After immunisation with Freund's adjuvant containing BGG and challenge with BGG-\textsubscript{131} a month later, A strain mice gave a secondary response which started sooner than a comparable response in CBA mice. In A mice the secondary response started between days 1 and 2 after the injection of antigen, whereas it started between days 2 and 4 after antigen injection, in CBA mice.

The effect of partial denaturation or precipitation of the BGG before injection was tested. For instance, 5 mgm of alum
<table>
<thead>
<tr>
<th>Method of immunisation</th>
<th>Number of mice responding</th>
<th>Number of mice challenged</th>
<th>Type of response obtained when the mice were injected with 2 mgm of BGG-\textsuperscript{131}</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Not previously treated</td>
<td>3/45</td>
<td>-</td>
<td>Primary</td>
</tr>
<tr>
<td>2. Single iv. injection of 5 mgm BGG, 1 month previously</td>
<td>0/18</td>
<td>2/18</td>
<td>Secondary</td>
</tr>
<tr>
<td>3. Seven iv. injections of 5 mgm BGG at 3 day intervals. Last injection 1 month previously. Two mice died of anaphylaxis after the last injection. Of the remaining ten:</td>
<td>0/10</td>
<td>6/10</td>
<td></td>
</tr>
<tr>
<td>4. Saline Freund's adjuvant. sc. (i.e. without antigen in emulsion), 4 days previously</td>
<td>9/10</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>5. Same mice as in 4. rechallenged one month later</td>
<td>-</td>
<td>5/5</td>
<td></td>
</tr>
<tr>
<td>6. Injection of 2 mgm mycobacteria in saline at same time as injection of BGG-\textsuperscript{131}, iv.</td>
<td>5/5</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>7. Freund's adjuvant containing 8 mgm BGG for each mouse, sc.</td>
<td>-</td>
<td>25/25</td>
<td></td>
</tr>
<tr>
<td>8. Incomplete Freund's adjuvant (i.e. minus mycobacteria)</td>
<td>-</td>
<td>3/5</td>
<td></td>
</tr>
<tr>
<td>9. Emulsion of liquid paraffin (without mycobacteria) with BGG, 8 mgm/mouse, sc.</td>
<td>-</td>
<td>5/5</td>
<td></td>
</tr>
<tr>
<td>10. Emulsion of liquid paraffin containing 2 mgm mycobacteria per mouse, with BGG (8 mgm/mouse), sc.</td>
<td>-</td>
<td>5/5</td>
<td></td>
</tr>
</tbody>
</table>

iv = intravenous.
sc = subcutaneous.
precipitated BGG (Proom 1943; Dunsford and Bowley 1955) successfully immunised 5 CBA mice (Table 3). Two of the five mice injected with 2 mgm BGG-I$^{131}$ labelled by Method 1 using 2$^1_2$ equivalents of Chloramine-T, were rechallenged with 2 mgm BGG-I$^{131}$ labelled by Method 2, two months later. These two mice showed small but definite secondary responses.

The effect of antibody-antigen complexes was tested. CBA mice were injected intravenously with passive isologous anti-BGG serum (0.5 ml hyper-immune serum) followed 24 hours later by an intraperitoneal injection of 2 mgm BGG. That this procedure was successful in conferring immunity was shown 3 weeks later when the mice were rechallenged with 2 mgm BGG-I$^{131}$ and all showed definite secondary responses. However, injections of 1.0 ml of homologous (outbred mouse) and heterologous (rabbit) anti-BGG sera, followed as above by an injection of BGG, failed to immunise the mice so that a subsequent secondary response could be obtained (Table 2). These preliminary results will have to be repeated before this can be considered as a reliable observation.

The rise of circulating antibody in both primary and secondary responses was shown by both the haemagglutination technique and the complement fixation technique. Similar results were obtained with both techniques. The results summarised in Figure 7 are based on complement fixation titres. The rise in circulating antibody in a primary response was found to occur between the 7th and 10th days after the
TABLE 2

Experiments to show the effect of antigen–antibody complex on the immunisation of CBA mice to the antigen (BGG).

Both primary and secondary injections of antigen were 2 mgm BGG–I\(^{131}\).  

<table>
<thead>
<tr>
<th>Antibody injection</th>
<th>Second injection of BGG–I(^{131})</th>
<th>Category of secondary response, see Figure 9</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\frac{1}{2}) ml CBA anti-BGG serum (ca. 1 mgm Ab protein/ml). 1 day before the first injection of antigen</td>
<td>22</td>
<td>0 1 2 3 4</td>
</tr>
<tr>
<td>5 x 0.2 ml whole outbred mouse anti-BGG serum (ca. 1 mgm Ab protein/ml) at two day intervals starting 2 days after the first injection of antigen</td>
<td>35</td>
<td>3/5 1/5 1/5 - -</td>
</tr>
<tr>
<td>As above, but (\frac{1}{2}) the amount of outbred mouse serum</td>
<td>35</td>
<td>4/5 - - 1/5 -</td>
</tr>
<tr>
<td>As above, but 0.2 ml of rabbit anti-BGG serum (3 mgm Ab protein/ml)</td>
<td>28</td>
<td>4/5 1/5 - - -</td>
</tr>
</tbody>
</table>

\(\#\) days between first injection of antigen and the second injection of antigen.
Figure 7.

A diagram illustrating the elimination of BGG-I$^{131}$ (solid line) and the rise of specific antibody (dashed line). Each line represents the mean of 5 mice. The primary Ab response was obtained after immunisation with Freund's adjuvant containing BGG. The primary Ab elimination curve is the same experiment as figure 5. Both secondary responses were obtained in the same mice after intravenous injection of BGG-I$^{131}$ into mice previously immunised with adjuvant. The dotted line illustrates the elimination of BGG-I$^{131}$ in mice not given an adjuvant stimulus at the time of antigen injection.
Figure 8.
The elimination of BGG-\(^{131}\text{I}\) from non-immunised CBA mice due to passive immunisation with different amounts of outbred mouse anti-BGG serum (ca. 1 mgm Ab protein/ml). The number '1' represents 0.2 ml of the antiserum injected every two days. The other numbers represent multiples of this amount, also injected every two days.
subcutaneous injection of Freund's adjuvant containing BGG. The same mice were rechallenged a month later by an intravenous injection of BGG-1\textsuperscript{131} (the antibody titres were combined with an antigen-elimination experiment). The rise in circulating antibody in a secondary response started on the 4th day after antigen injection (Figure 7).

The results of the passive immunisation experiment are illustrated in Figure 8. This experiment was designed to calibrate the antigen-elimination curves. Each block on the diagram represents the elimination of labelled BGG from the circulation of five mice. It can be seen that the rate of elimination is graded according to the quantity of antibody injected into the mice every 2 days. The result of this experiment is compared in Part III with results obtained in adoptive immunisation experiments described in that section. It is possible by this means to estimate the relative rates of antibody production during the first part of a secondary response. The results of the estimation of the amount of antibody to BGG in the outbred mouse antiserum showed that there was about 1 mg/ml antibody protein per ml. This means that the group of mice which received 0.2 ml of whole antiserum (No. 1) every 2 days would receive about 0.2 mg/ml antibody protein. In vitro 1 ml of this antiserum would only precipitate 15% of 2 mg/ml BGG. This implies that soluble complexes as well as precipitate are removed from the circulation, as the in vivo efficiency of the antibody appears to be
Figure 9.
Diagram to illustrate the arbitrary categories into which the secondary responses obtained in "adjuvant" experiments, are classified. These categories are utilised in Tables 2-5.
much greater than the in vitro precipitating efficiency of the same antibody.

The adjuvanticity of different substances

Results have been presented (see above) which indicate that saline Freund's adjuvant injected subcutaneously can lead to a primary response to a solution of BGG injected intravenously at the same time or very shortly afterwards. A separate injection of mycobacteria had the same effect. It was thought that this result indicated a possible technique whereby one of the functions of an adjuvant could be tested. Several substances have been tested in this way for an effect that has been called "adjuvanticity". These substances were injected subcutaneously and centrifuged BGG solution was injected intravenously at the same time. One month later the mice were challenged with an intravenous injection of BGG-^131 and the rate of antigen elimination in a secondary response used as a measure of the effect of the adjuvant used. The different responses obtained have been divided into 5 categories which are illustrated in Figure 9.

Experiments have been carried out to see if stimulants of phagocytic activity also stimulated the immunisation of mice by means of BGG solution. Reticulo-endothelial system (RES) stimulants and depressants have been injected, together with the intravenous injection of 5 mgm of centrifuged BGG in solution. One month later the mice were challenged with
Experiments designed to show any relationship between reticulo-endothelial stimulants or depressants and adjuvanticity.

Mice challenged by ip. injection of 2 mgm BGG-\(^{131}\) one month after injection of 5 mgm BGG.

CBA males, 3-4 months old at the time of the original treatment.

<table>
<thead>
<tr>
<th>First antigen injection</th>
<th>Treatment</th>
<th>Category of secondary response</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>5 mgm alum precipitate BGG</td>
<td>ip. NIL</td>
<td>-</td>
</tr>
<tr>
<td>5 mgm centrifuged BGG in soln.</td>
<td>iv. NIL</td>
<td>10/10</td>
</tr>
<tr>
<td></td>
<td>sc. NIL</td>
<td>4/4</td>
</tr>
<tr>
<td></td>
<td>iv. 2 mgm indian ink(\text{#})</td>
<td>8/8</td>
</tr>
<tr>
<td></td>
<td>iv. infected with B.C.G. 8 days before BGG injection</td>
<td>8/8</td>
</tr>
<tr>
<td></td>
<td>iv. Infected with B.C.G., 35 days before BGG injection</td>
<td>8/8</td>
</tr>
<tr>
<td></td>
<td>iv. 25 (\mu) gm Pasturella lipopolysaccharide iv. one day before BGG injection</td>
<td>4/5 \text{</td>
</tr>
<tr>
<td></td>
<td>iv. 25 (\mu) gm Shigella endotoxin, iv. one day before BGG</td>
<td>5/5</td>
</tr>
</tbody>
</table>

\(\text{\#}\) Gunther Wagner, Special Pelikan Ink for injection.

+ Route of injection, (ip. = intraperitoneal).

++ Route of injection, (ip. = intraperitoneal).
BGG-\textsuperscript{131} and the elimination of the labelled antigen was followed. Some of the RES stimulants which have been used are diethylstilbestrol (Biozzi, Halpern, Bilbey, Stiffel, Benacerraf, and Mouton 1957), infection with B.C.G. (Howard, Biozzi, Halpern, Stiffel and Mouton 1959; Biozzi, Halpern, Benacerraf and Stiffel 1957) and the injection of bacterial endotoxins (Biozzi, Benacerraf and Halpern 1955; Thomas 1957) although the later do depress the phagocytic index for 24-12 hours before a stimulation effect becomes manifest. Injections of carbon particles have been shown to depress the phagocytic index (measured by the uptake of saccharated iron oxide) (Benacerraf, Biozzi, Halpern and Stiffel 1957).

The results of these experiments, designed to show the adjuvanticity of various substances are shown in Tables 3, 4 and 5. Results of the experiments using RES stimulants and depressants are shown in Table 3. Of the mice infected with B.C.G. some were injected with 5 mgm centrifuged BGG 6 days after infection and others 35 days afterwards. This is because Biozzi, Halpern, Benacerraf and Stiffel (1957) have shown that the stimulation of the RES reached a peak about 20 days after infection with B.C.G. and quickly declines so that a month after infection the stimulation is scarcely higher than in the controls.
TABLE 4

Similar to Tables 3 and 5. Combining possible adjuvants with the RES stimulating effect of diethylstilbestrol dipropionate.

First antigen injection, 5 mgm centrifuged BGG in saline solution, iv.
Second antigen injection, 2 mgm BGG-I\(^{131}\) one month later, ip.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Category of secondary response</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.4 ml Olive oil sc. at same time as first BGG injection</td>
<td>0  1  2  3  4</td>
</tr>
<tr>
<td>0.4 ml Olive oil sc., containing 2½ mgm diethylstilbestrol/ml, at same time as first injection of BGG</td>
<td>2/5 1/5 2/5 - -</td>
</tr>
<tr>
<td>0.4 ml Crill K 16(^{\text{\textregistered}}) sc., at same time as the first injection of BGG</td>
<td>- - - - 8/8</td>
</tr>
<tr>
<td>0.4 ml Crill K 16 sc., containing 2½ mgm diethylstilbestrol/ml, at the same time as the first injection of BGG</td>
<td>1/8 1/8 - - 6/8</td>
</tr>
<tr>
<td>NIL</td>
<td>5/5 - - - -</td>
</tr>
</tbody>
</table>

\(^{\text{\textregistered}}\) Crill K 16 is a commercial preparation of sorbitol monoleate, but contains other esters of sorbitol as impurities. Manufactured by Croda Ltd., Goole, Yorks.
TABLE 5

Experiments designed to show the adjuvanticity of certain substances. All the substances being tested for adjuvanticity were injected subcutaneously.

The first injection of antigen was 5 mgm of centrifuged BGG injected intravenously one day after the substance being tested for adjuvanticity.

The second injection of antigen was 2 mgm BGG-\textsuperscript{131} iv, one month later.

<table>
<thead>
<tr>
<th>Substance being tested for adjuvanticity</th>
<th>Category of secondary response</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>25 \textsuperscript{+} gm Pasturella lipopolysaccharide*</td>
<td>-</td>
</tr>
<tr>
<td>1-2 mgm killed mycobacteria as a suspension in saline</td>
<td>-</td>
</tr>
<tr>
<td>0.2 ml/Freund's adjuvant (no antigen)</td>
<td>-</td>
</tr>
<tr>
<td>0.4 ml Crill K,16 (mainly sorbitol monoleate)</td>
<td>-</td>
</tr>
<tr>
<td>0.2 ml &quot; &quot; &quot; &quot;</td>
<td>-</td>
</tr>
<tr>
<td>0.2 ml Span 80 (sorbitol monoleate)+</td>
<td>1/5</td>
</tr>
<tr>
<td>0.2 ml Tween 40 (polyoxyethylene sorbitol mono-palmitate)+</td>
<td>2/5</td>
</tr>
<tr>
<td>0.2 ml Tween 80 (polyoxyethylene sorbitol monoleate)+</td>
<td>3/5</td>
</tr>
<tr>
<td>0.2 ml Special Arlacel A (manitol monoleate)+</td>
<td>4/5</td>
</tr>
<tr>
<td>0.2 ml Liquid paraffin BP</td>
<td>4/5</td>
</tr>
<tr>
<td>Mil (control)</td>
<td>8/8</td>
</tr>
</tbody>
</table>

* N.B. In Table 5 Pasturella lipopolysaccharide shown to have little adjuvant effect when injected intravenously.


N.B. 0.4 ml of "Lubrol" and "Dispersol" (anionic detergents - I.C.I. Ltd.) killed CBA mice within 24 hours, when injected subcutaneously.
TABLE 6

Experiments in which the effect of Crill K 16 was measured by anaphylactic sensitivity.

First injection of antigen, 5 mgm centrifuged BGG iv, one day after the injection of adjuvant sc.

Second injection of antigen, 2 mgm BGG-I\textsubscript{131} iv, 35 days later.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Category of anaphylactic response</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.4 ml Crill K 16 sc, one day before first injection of antigen</td>
<td>NIL          7/17          10/17</td>
</tr>
<tr>
<td>0.4 ml Crill K 16 containing 2\frac{1}{2} mgm diethylstilbestrol/mouse, sc. one day before the first injection of antigen</td>
<td>1/8          2/8          5/8</td>
</tr>
<tr>
<td>Freund's adjuvant containing 8 mgm BGG per mouse</td>
<td>4/8          3/8          1/8</td>
</tr>
</tbody>
</table>
DISCUSSION

Methodology

(a) Antigen-elimination

The results show that the technique of antigen-elimination developed by Talmage et al. (1951) in the rabbit is also practicable in the mouse. There are, however, two differences between rabbits and mice in the application of this technique. First, only some of the antigens suitable for rabbits are also suitable for this technique in mice; for example, albumins are eliminated too rapidly to make it possible to distinguish readily between immune and non-immune elimination. Second, there is the failure of immunisation after intravenous injection of protein antigens in mice, although Talmage et al. (1951) have, among many other authors, shown that this method of immunising rabbits to heterologous serum protein antigens is effective.

(b) Adjuvants

Failure to achieve immunisation in rats and mice, and occasionally in rabbits, after intravenous injection of protein solutions, has been described by many workers. They have devised several methods of achieving effective immunisation. Boyd and Malkiel (1940) showed that simultaneous intravenous injection of horse haemoglobin and Monilia sp. into rabbits, elicited an immune response to the protein, but injection of the haemoglobin alone elicited a slight or no response. Lipton, Stone and Freund (1956) showed that a reliable response to proteins could be elicited in rats only after immunisation.
with adjuvants of the Freund type. Weisner, Golub and Hamre (1941) found that the response of mice to intraperitoneal or intravenous injection of egg albumin was variable, but Wheeler, Brandon and Petrenco (1950) and Havas and Andre (1955) succeeded in obtaining a reliable response to the protein by using Freund's adjuvant. Barr, Fulthorpe and Llewellyn-Jones (1957) have used diphtheria and tetanus toxoids in mice. They showed that mice were far more refractory to immunisation with toxoid solutions than were guinea-pigs. Adsorption of the toxoids onto alum or aluminium phosphate, greatly decreased the difference in response between the two species. Cameron (1956) immunised W-Swiss mice with BGG and other protein antigens. He gave a course of 3 injections of different amounts of protein solution and found that immunisation was poorest when the injection was made intravenously. The subcutaneous and intraperitoneal routes were more successful in conferring immunity, although the degree of success also depended on the antigen used. His figures support the view that W-Swiss mice probably show a greater proportion of responding individuals after intravenous injection of BGG than do CBA mice.

The results reported here confirm the previous work. Intravenous, intraperitoneal and subcutaneous injection of BGG alone failed to elicit an immune response. However, the intravenous route has been shown to be effective with BGG solution if a non-specific stimulus was given at the same time. One non-specific stimulus used in this way was saline Freund's
adjuvant. Subsequently, however, other substances have been found to have adjuvant activity (adjuvanticity) when injected subcutaneously at the same time as the intravenous injection of centrifuged BGG. It is clear from the results that it is not necessary for the antigen to be incorporated in a water-in-oil emulsion for the adjuvant to be effective. This is at first sight contrary to the findings of Freund, Thomson, Hough, Sommer and Pisani (1948), who used rabbits and found that some water-in-oil emulsions were not effective in sustaining antibody production, whereas water-in-oil emulsions were effective. In their case, however, it was not simply a question of the presence of an immune response to the protein antigen but to the augmentation and prolongation of a response which could always be obtained, even after intravenous injection of the protein. It appears from the results obtained in mice that one of the chief functions of the adjuvant was to activate the antibody forming mechanism. The increase in titre of antibody in rabbits immunised with a water-in-oil emulsion of antigen solution and the prolongation of the response, is probably due to the slow release of antigen from the emulsion in addition to the stimulating effect. It seems likely that the slow release of antigen may increase the titres of antibody in mice too, as very high relative titres of precipitating antibody were obtained in mice immunised with Freund's adjuvant containing BGG. The most profound adjuvant effects seem to be obtained with Freund's adjuvants which can stimulate an animal to produce
large amounts of antibody to protein antigens, where antigen alone would elicit the production of only small amounts, or no antibody. Furthermore, delayed hypersensitivity to protein antigens can be induced by means of Freund's adjuvant mixtures, and possibly by no other way. Auto-immune disorders such as allergic encephalomyelitis can also be induced by injections of brain tissue in Freund's adjuvant. The effect and use of adjuvants of this type have recently been reviewed by Freund (1953), Edsall (1953), Pappenheimer and Freund (1959) and Paterson (1959).

Other adjuvant techniques were known before the highly successful Freund's adjuvants were developed. For instance, adsorption of protein antigens onto relatively large particles or the formation of protein precipitates is well known to increase the effectiveness of the proteins as antigens. One of the most effective techniques has been the precipitation of the proteins by means of alum. Glenny, Pope, Waddington and Wallace (1929) precipitated toxoid by means of potassium alum and found that the antibody response which resulted from its injection was greater than if the non-precipitated toxoid had been injected. Schmidt (1932) obtained similar results with aluminium hydroxoid and Holt (1947) used aluminium phosphate. Freund and Bonanto (1941), Proom (1943), Miller, Humber and Dowrie (1944), Salk (1941; 1945) and Barr et al. (1957) have obtained similar results using various combinations of protein antigens and different alums. In this thesis, experiments
have been described (see Table 3) in which alum precipitated
BGG (Proom 1943) successfully immunised CBA mice, whereas
injection of the non-precipitated BGG failed to elicit any
response whatsoever.

Several workers have shown that the simultaneous injection
of two antigens often results in higher individual titres than
if one or other of the two antigens had been injected alone.
The first recorded experiment which showed this (or any)
adjuvant effect was by Pasteur and Joubert (1877), who showed
that an injection of anthrax bacteria plus the bacteria of
septicemia, into guinea-pigs gave a greater immunity to anthrax
than the injection of anthrax bacteria by themselves. Mostly
using bacterial toxoids in combination with other bacterial
toxoids or with vaccines, Ramon (1923, 1926, 1936, 1939);
Sacquepee, Pilod and Jude (1936a, 1936b); Bigler and Werner
(1941) and Lapin (1942) have all shown similar phenomena,
generally called the "Ramon effect" or "antigenic-synergy".
However, McLean and Holt (1940) failed to observe this effect
when they used tetanus toxoid together with T.A.B. vaccine.

There is a certain amount of controversy about the effect
of antibody-antigen complexes as adjuvants. Barr, Glenny and
Randall (1950) and Mason, Robinson and Agerholm-Christensen
(1955) have shown that passively transferred antibody, except
in very low amounts, inhibits subsequent antibody production in
humans or guinea-pigs injected with toxoid shortly after the
passive immunisation. Dixon, Talmage and Maurer (1952)
obtained similar results with serum protein antigens, although the preliminary results reported in this thesis appear to contradict this, at least as far as isologous antibody seemed to cause a subsequent injection of antigen solution to immunise CBA mice. Zingher and Park (1923), Uhr, Salvin and Pappenheimer (1957); Rosenberg, Chandler and Fischel (1959) and Hughes and Wollins (1959) have shown that an antibody-antigen complex can induce a state of delayed hypersensitivity to the antigen. Uhr et al. prepared the antibody-antigen complex in vitro but the other workers carried out their experiments in a similar fashion to those described in this thesis.

Adjuvants and adjuvanticity

Landsteiner (1945) considered that the following factors affected the immune response to an injected antigen: rate of antigen uptake by antibody producing cells and the stimulation of cellular activity; particle size; to which could be added the possible formation of antigen depots after adjuvant injections.

(a) Antigen depots - Glenny et al. (1926) suggested that antibody titres were increased due to a depot of antigen persisting at the injection site. Holt (1949) showed that the main mass of injected antigen had to be removed fairly soon after the injection if the subsequent response was to be inhibited. He therefore suggested (Holt 1950) that intracellular microdepots were formed. Similar experiments by
Freund and Lipton (1951, 1955) with Freund's adjuvant, showed that excision of the main bulk of the injected material a few hours after injection did not inhibit the subsequent response (allergic encephalomyelitis). The results of Freund et al. (1948) show that the slow release of antigen from a water-in-oil emulsion prolongs the time during which antibody is produced to the antigen. Except with non-metabolisable pneumococcal polysaccharide (Felton 1949) there is no evidence that intracellular microdepots of antigen can exist for long periods. The literature cited so far seems to indicate that macrodepots at the injection site may very well prolong and even increase the antibody titre.

(b) Particle size - Good antibody responses can be obtained when particulate antigens are injected intravenously. This has been shown to apply to proteins which can be made particulate by precipitation with alum or adsorption onto kaolin. It is possible that particle size merely increases the rate of antigen uptake by the antibody producing cells. As there appears to be no evidence that this hypothesis is incorrect, this will be considered below.

(c) Stimulation of antigen uptake and cellular activity - It was thought possible that adjuvanticity was similar to stimulation of phagocytic activity. Experiments were carried out to test this hypothesis in which known stimulants of the reticulo-endothelial system were used at the same time as the injection of antigen. One of the most marked stimulants of
the phagocytic index is infection with B.C.G. Mice have been infected with B.C.G. and injected with 5 mgm BGG (centrifuged) at the time the effect of the infection was at its height and afterwards as a control. This procedure was not successful in conferring immunity. Similarly, another RES stimulant, diethylstilbestrol, was found to depress immunity stimulated by another adjuvant substance. Bacterial endotoxin and lipopolysaccharide were injected intravenously where they have a maximum effect on the phagocytic activity of the RES, first depressing it for 24 hours but subsequently increasing it. No immunity to BGG was stimulated this way, yet when lipopolysaccharide was injected subcutaneously, and the BGG intravenously, all the mice tested showed at least some immunity to BGG. Intravenous injections of carbon particles had no effect, although such an injection would probably be classed as an RES depressant. It seems evident that phagocytosis and the induction of immunity are unrelated. This seems to be confirmed by the observation of Benacerraf, Biozzi, Halpern and Stiffel (1957) and Stiffel, Biozzi, Benacerraf and Halpern (1957) who showed that a 3 day old rat had a higher phagocytic index than an adult rat, whereas, as Freund (1930) has pointed out, a rat of this age cannot produce antibody. A possible conclusion is that RES stimulation or a general increase in phagocytosis is not the same thing as adjuvanticity.

Several workers have shown that Haemophilus pertussis vaccine has an adjuvant effect. Malkiel (1953), who reviews
the literature, shows that on the basis of anaphylaxis, increased sensitivity of the mouse to antigen-antibody complex starts immediately the pertussis was injected. Malkiel claims that pertussis increases the sensitivity of the mouse to histamine. However, previous workers have shown that antibody production was enhanced when antigen and pertussis were injected together. It is possible, therefore, that the so called "Ramon effect" or "antigenic synergy" was a similar effect in which one of the antigens acted in a similar way to pertussis. At least some of the antigens which exhibited this effect were toxoids. It is possible that the stimulation of antibody production was due to a non-specific factor which caused irritation and cellular or systemic damage, which stimulated antibody production. It is fairly easy to imagine a certain amount of residual toxicity in a toxoid, which could act in this way.

Ramon (1923) showed that sterile tapioca when injected caused inflammation at the site of injection and also showed that this injection had a marked adjuvant effect. Mann and Welker (1949) showed that dogs produced only small amounts of precipitating antibody to bovine serum albumin. Injections of filtered egg albumin caused toxaemia and at the same time stimulated the production of much larger amounts of antibody to bovine serum albumin. The water-in-oil Freund's adjuvant causes "irritation" which results in cells migrating to the site of the injection and forming a granuloma. The experiments
reported in this thesis show that olive oil and non-ionic detergents all have adjuvanticity (see Tables 4 and 5). The experiments of Raffel (1948), Raffel and Tourney (1948), White, Coons and Connelly (1955) and Yamamura, Yasaka, Nakamura, Yagaguchi, Ogawa, Endo and Takenchi (1955) show that much of the adjuvant effect of mycobacteria could be due to a wax fraction, possibly a phospho-lipid. All these results suggest that one effect of adjuvants is to stimulate the antibody producing cells to activity.

The mechanism of adjuvanticity

It is possible that a bacterium, bacterial toxin or virus is its own adjuvant. Presumably the antibody producing mechanism has evolved as a specific defence against such materials. It therefore seems very possible that in the highly artificial situation when serum protein antigens are being used, that the "particulate" and the "irritation" aspects of normal adjuvant action are both missing. It seems probable from an evolutionary (and teleological) point of view, that adjuvanticity is normally a part of antigenicity in the broadest sense. However, the fact that the adjuvanticity may be separate from antigenicity when BGG is used in mice, allows the mechanism of adjuvanticity to be investigated.

The lipid or lipidophilic nature of most adjuvant materials suggests that the irritation caused by adjuvants might be a result of dissolution or damage of the lipid component of cell
membranes; one effect of this results in the release of histamine from mast cells, leading to local or systemic inflammation. Damage to the cell membrane of antibody producing cells, either directly by the adjuvant or indirectly by substances released by cells damaged by the adjuvant, might set in motion either or both of two reactions: firstly, immature antibody producing cells might be stimulated to divide and mature, thus becoming immunisable; or secondly, damage to the membrane might stimulate the uptake of antigen in a form suitable for initiating antibody production.

The results of Coons, Leduc and Connolly (1955) suggest that cell division occurs during the induction period of an immune response. Nowell (1959) has shown that a factor in serum from infected rats stimulates mitosis in bone marrow cells grown in vitro. If the mechanism of adjuvanticity was to stimulate cell division only, then it would have to be postulated that only dividing or newly divided cells were capable of being immunised. It is for this reason, that the second hypothetical mechanism looks more plausible.

Damage to the membrane might lead to a process of its regeneration. This in turn might lead to parts of the old membrane being taken inside the cell, or to the initiation of pinocytosis or phagocytosis. The stimulation of pinocytosis by proteins and other substances has been reported by several workers (Edwards 1925; Mast and Doyle 1934; Chapman-Andresen and Prescott 1956; and Clark 1959). However, it is possible
that antigen is not taken up in this manner when the antibody producing cells are stimulated by adjuvant. For instance, the damaged cell membrane might become "sticky" to foreign (and other) substances, perhaps in much the same way as serum albumins can adsorb dyes and haptenic materials from the serum (Karush and Sonnenberg 1949; Karush 1954; and Markus and Karush 1958). In this way antigen may be taken up attached to membrane in a suitable form to initiate antibody production.

Metchnikoff (1905) has drawn an analogy between the uptake of food materials by amoeboid cells and the defensive role of phagocytosis. He makes the point that defensive phagocytosis has probably evolved from a mechanism for recognising food materials foreign to the cell. The recognition of foreign (antigenic) material by the reticulo-endothelial cell and the antibody producing cell would on this basis be an evolutionary hangover. However, the stimulation of antibody producing cells to take up antigenic material appears to be non-specific. If this is so, antigenic and non-antigenic material would be sorted out by an intracellular tolerance mechanism. If this were true, tolerance could not be a recognition process acting at the cell surface (see discussion to Part II).

It is possible that eye protein, thyroid tissue and nerve tissue lack adjuvanticity in animals, in general, in much the same way as BGG does in mice. The stimulation of auto-immune disorders, when for instance nerve tissue is mixed with Freund's adjuvant, would be due to this tissue now gaining the
adjuvanticity that it normally lacked. It would follow from this that auto-immunity was not a phenomenon related to the failure of the tolerance mechanism. In Part II of this thesis, it is shown that tolerance can be induced to BGG and that Freund's adjuvant containing BGG cannot break that state of tolerance. Paterson (1959) claims that tolerance and paralysis can be induced to nerve tissue, and suggests that the normal lack of tolerance to such tissues was due to a lack of opportunity for inducing tolerance. This hardly seems to be a satisfactory explanation of how nerve tissue normally fails to elicit an immune response unless it is incorporated in Freund's adjuvant. There may in fact be two mechanisms whereby the formation of auto-immune antibodies is normally prevented: namely, tolerance and lack of adjuvanticity. It seems likely that the former mechanism operates for soluble and many tissue antigens, whereas the latter mechanism operates for a few tissues such as thyroid, nerve tissue and lens protein. The lack of tolerance to nerve tissue could be due to the lack of opportunity to induce tolerance or conversely to the lack of any necessity, during evolution, for the induction of tolerance due to a prior lack of adjuvanticity.
PART II: ACQUIRED IMMUNOLOGICAL TOLERANCE AND IMMUNOLOGICAL PARALYSIS TO BOVINE GAMMA GLOBULIN

INTRODUCTION

Acquired immunological tolerance has been defined as a state of immunological unresponsiveness to a particular antigen in an adult animal which is brought about by injections of the antigen in question into the animal when it was neonatal. Possibly the first descriptions of the phenomenon were by Traub (1936; 1938; 1939) who was studying the adaptation of a strain of choriomeningitis virus to a colony of mice. It is possible, however, that his observation showed the development of latency of the virus. Owen (1945; 1956) observed tolerance to erythrocyte antigens in cattle twins which had developed blood group chimaeras. Similar observations have been made in sheep (Stormont, Weir and Lane 1953) and in humans (Dunsford, Bowley, Hutchinson, Thompson, Sanger and Race 1953). Anderson,Billingham, Lampkin and Medawar (1951) have shown that monozygotic and many dizygotic cattle twins are tolerant to skin homografts taken from each other.

Tolerance to homografts has been artificially induced by injections of living cells from the donor of the graft or from donors of the same genetic constitution. Billingham, Brent and Medawar (1953; 1955; 1956) have used inbred strains of mice and Canon and Longmire (1952; 1954) have used chickens and rabbits. Woodruff and Simpson (1955) demonstrated tolerance
to homografts in rats injected with spleen cells from donors of the same strain and Medawar and Woodruff (1958) have shown that a skin graft at birth could induce tolerance to subsequent skin grafts from the same donors or donors of the same strain. Barnes, Ford, Ilbery, Koller and Loutit (1957), Barnes, Ford, Ilbery and Loutit (1958) and Trentin (1958) have discussed tolerance to skin grafts in radiation chimaeras.

There are no reports of tolerance being induced to skin grafts from distantly related or unrelated species. Tolerance to unrelated erythrocytes has been described by Hasek (1956) between ducks, geese, and guinea-fowls; by Simonsen (1955) between fowls and turkeys; by Hasek, Hrabá and Esslova (1956) and Hasek (1956) between fowls and turkeys; and by Nossal (1957) to sheep erythrocytes in rats. Buxton (1954) has described partial tolerance to Salmonella pullorum in chicks and Kerr and Robertson (1954) have described partial tolerance to Trichomonas foetus in calves. Burnet, Stone and Edney (1950) were unable to induce tolerance in chicks to human erythrocytes, flu virus or T2 bacteriophage. It seems very likely that some antigens are not capable of inducing tolerance in some animals; perhaps it is only antigens fairly closely related to an animal's own proteins or tissues which can induce tolerance.

Tolerance to protein antigens has been described by many workers. Wolfe, Tempelis, Mueller and Reibel (1957); Wolfe, Tempelis and Mueller (1958) induced tolerance to bovine serum
proteins and Stevens, Pietryk and Ciminera (1958) to human serum proteins, in chicks. Cinader, Pearce and Carter (1958) induced tolerance to bovine ribonuclease in rabbits. The induction of tolerance to heterologous serum protein antigens in neonatal rabbits has been demonstrated by Hanan and Oyama (1954), Dixon and Maurer (1955), Cinader and Dubert (1955; 1956) and Smith and Bridges (1958). Smith and Bridges showed that the duration for which tolerance lasted was directly related to the amount of antigen injected into the neonatal animal but that the state of tolerance could be made to persist by further injections of antigen. These results were interpreted as indicating that antigen must persist for the state of tolerance to be maintained. Similar results have recently been obtained by Terres and Hughes (1959) using bovine serum albumin in mice and by Mitchison (1959) using heterologous and homologous erythrocyte antigens in fowls.

For the moment, immunological paralysis can be defined as the induction of a state of immunological unresponsiveness in adult animals by means of massive injections of antigen, into the adult. Tolerance can therefore be distinguished from paralysis by two criteria: firstly, tolerance is induced in neonatal animals; and secondly, the doses of antigen required for tolerance are small in absolute terms when compared to the doses required to induce paralysis. The relationship between paralysis and tolerance defined in this way, will be discussed.

Felton (1940, 1949); Felton and Ottinger (1942); Felton, Kauffman, Prescott and Ottinger (1955) and Felton, Prescott,
Kauffman and Ottinger (1955) have described a state of immunological paralysis in mice injected with pneumococcal polysaccharides. These may not have been the first observations on the phenomenon, as Bordet (1897) showed that large injections of streptococci could paralyse phagocytosis of the bacterium and also showed that the phenomenon was highly specific. Both Felton and co-workers and Watson and Cromartie (1953) have shown that pneumococcal polysaccharide paralysis is highly specific. Coons (1954) has shown that detectable antigen persists at intracellular sites in paralysed mice. Stark (1955a; 1955b) has suggested that antibody is produced in these animals but that the persisting antigen prevents the antibody from being released. This was possibly confirmed by Dixon, Maurer and Weigle (1955) who claimed that $^{131}$I labelled passive antibody to the polysaccharide was broken down faster in paralysed mice than in non-paralysed mice. It is possible that the persisting antigen catalyses the rapid breakdown, intracellularly, of all the antibody produced in a cell. Sercarz and Coons (1959) were unable to demonstrate any antibody producing cells at all in mice paralysed to pneumococcal polysaccharide and to BSA (using gigantic quantities of the protein!). Johnson, Watson and Cromartie (1954) and Dixon and Maurer (1955) have been able to paralyse rabbits to serum protein antigens. The resulting state of immunological unresponsiveness was of short duration and differed from pneumococcal paralysis in mice, which is of very long duration.
This part of the thesis describes the induction of tolerance to bovine gamma globulin in CBA mice. Experiments have been carried out to determine the effect of altering three variables: firstly, the quantity of BGG injected within 12 hours of birth; secondly, mice were injected with BGG at different times after birth; and thirdly, the effect of the time between the tolerance injection and the challenge injection was also investigated. The mechanism of the loss of tolerance was also investigated by means of experiments designed to show the effect of cell division on the loss of tolerance, and the effect of extracellular antigen was determined by injections of anti-BGG antibody.

Experiments have been carried out to show the effect of dose size on the degree of immunological paralysis to BGG. This experiment has been carried out in sub-lethally irradiated and non-irradiated mice.

The presence of an immune response after prior injection of Freund's adjuvant containing BGG has been tested for by the antigen-elimination technique of Talmage et al. (1951) as described in Part I. In this part of the thesis (Part II) it is shown that the antigen-elimination technique can be used to detect tolerance to one component of BGG, despite an immune response to other components. It seems that if only antibody titrations had been used to detect tolerance in these experiments, that very few mice would have been found to be even partially tolerant. The possibility arises that cases of
partial tolerance mentioned in the literature, where antibody titrations alone were made (e.g. Hanan and Oyama 1954) may also have been due to a high degree of immunity to minor components masking the absence of antibody to major components. It follows that partial tolerance defined by the antigen-elimination technique in respect of one fraction of BGG is more precise than partial tolerance defined by antibody titres based on the overall amount of antibody to all the fractions of BGG. However, it seems probable that antibody titres are quantitatively more accurate than the antigen-elimination technique if the antigen is truly homogeneous.

MATERIALS

CBA mice were used. Mice injected shortly after birth were weaned when one month old and the sexes then kept separate. The antigens used were BSA (powdered fraction V) and BGG (batch No. BF 0270). The BGG was labelled with $^{131}$I by Method 2 described in Part I of this thesis.

METHODS

Induction of tolerance

During a course of tolerance injections the colony of breeding mice was examined twice a day. The baby mice were injected within 12 hours of birth or in some cases at longer intervals after birth. New-born mice received as much as 0.1 ml of solution, although 0.025 ml was found to be a more
satisfactory volume. The injections were made intraperitoneally by means of a tuberculin syringe. A 30 g. needle was inserted through the muscles of the shoulder, passed subcutaneously and then into the peritoneum. The hole made by the needle was sealed by a drop of "New Skin". If any of the injected mice showed signs of the antigen solution leaking, then the mouse was killed.

**Induction of paralysis**

Non-irradiated and irradiated CBA male mice aged 2½-3 months were given a single intraperitoneal injection of BGG. The quantities injected were 35 mgm, 70 mgm and 150 mgm, into both irradiated and non-irradiated mice. The mice were challenged by an injection of Freund's adjuvant containing BGG 2 months after the paralysis injection. The result of this challenge was tested a month later by the injection of 2 mgm of BGG-\(\text{I}^{131}\).

In another experiment, 150 mgm of BGG was injected into mice which had been immunised with Freund's adjuvant a month earlier. The mice were injected with BGG-\(\text{I}^{131}\) one month later.

**Antibody titrations**

The presence of precipitating antibody was detected by a ring test. In some sera antibody was titrated by the haemagglutination technique of Ingraham (1958) (see Part I of this thesis).

\* Manufactured by Harwood's Laboratories, St. Helens, Lancashire.
Ouchterlony gel diffusion tests

The double diffusion technique of Ouchterlony was used (Ouchterlony 1949, 1953). Agar was made into a \( \frac{1}{2}\% \) gel in saline containing 0.01% merthiolate and 0.06% Orange G (Wilson and Pringle 1955, 1956). The agar was clarified by the method of Feinberg (1956). Fifteen ml of the clarified agar was poured into petrie dishes, which had been made grease free by washing them in chromic acid, water and with a final rinse in ether. After the agar had set, wells were cut through to the glass with a cork borer. In spite of this, no leakage along the interface between the glass and the agar gel was ever observed. The wells were filled with sera and antisera and refilled after 24 hours. The lines of precipitation had usually become clearly visible after about 48 hours at room temperature. Gels were photographed and some were stained and preserved, for later reference, by the method of Uriel and Grabar (1956).

Starch gel electrophoresis

Some serum samples were analysed by means of starch gel electrophoresis (Smithies 1955; Poulik and Smithies 1958). The discontinuous buffer system of Poulik (1957) was modified by Lush (1959). The modified system was designed for use with fowl sera but has been found to be satisfactory with mouse sera. The starch gel was made up in 0.76 \( M \) tris (hydroxymethyl) aminomethane and 0.005 \( M \) citric acid (pH 9.0). The electrode vessel buffer was 0.03 \( M \) boric acid and 0.1 \( M \) NaOH (pH 8.9-9.1).
Sera were obtained from mice injected with 10 mgm BGG-I$^{131}$. The distribution of the labelled antigen amongst the serum fractions of the mouse serum was determined on the starch gel after electrophoresis. The bands of fractions from each serum sample were cut into strips 12 mm wide. Each strip was then cut into blocks 6 mm x 12 mm. The activity of each block was measured with a Geiger-Muller tube with a thin end-window. Histograms of the distribution of radioactivity along the strip were then drawn.

Stained and fixed starch gels were scanned photoelectrically. The apparatus was improvised but was found to give repeatable results when the same gel was rescanned. Comparisons were only made between serum samples run on the same block of starch gel.

**X-irradiation**

The mice were irradiated in a circular box 10.5 cm in diameter and 6.5 cm deep. The conditions of irradiation were 150 kvp, 5 mA and the filtration was 3 mm aluminium plus the plastic lid of the box. The X-ray source was above the box, which contained from 5 to 8 mice. The distance between the target and a point 1.5 cm above the floor of the box was 29.5 cm; the dose rate at that point was 65 r./minute. Under these conditions 950 r. was a lethal dose for 2-3 month old CBA mice (20 tested) but only about an LD$_{30}$ for CBA mice aged 5-7 months (25 tested).
In the experiments described in this part of the thesis (Part II), mice assumed to be tolerant to BOG were irradiated. Sub-lethal doses were used to determine the effect of X-irradiation on the maintenance of the state of tolerance. Lethal doses (700 r. for 5 week old mice) were used in experiments to determine the effect of cell division on the loss of tolerance. In these experiments different numbers of cells from tolerant mice were transplanted into the irradiated tolerant mice. Mice in the same age group which were also tolerant were similarly irradiated but were injected with spleen cells from non-tolerant donors. Finally non-tolerant mice were irradiated (950 r.) and injected with spleen cells from tolerant donors.

The irradiation techniques described here have been used in experiments described in Part III of this thesis.

**Cell transfers**

Donor mice were killed, their spleens removed by dissection, placed in Hank's solution at pH 7.2 (Hanks and Wallace 1949) and minced with a stainless steel rotary mincer. The suspension of cells so formed was allowed to stand for 2 minutes so that the larger clumps of tissue could settle. The supernatant of more or less free cells was then transferred to another container. Known numbers of cells were injected intravenously into irradiated recipients or on one or two occasions into not irradiated recipients. Details of the procedure are given in Part III of this thesis.
Figure 10.
An example of the wide variation in secondary response in mice injected with BGG shortly after birth. CBA mice injected with 5 mgm BGG/gm body weight, 6 days after birth. Injected with Freund's adjuvant containing 8 mgm BGG per mouse at two months after birth. BGG-I$^{131}$ injected at 3 months after birth.
Figure 11.

Diagram to illustrate the arbitrary categories into which the wide range of secondary responses in tolerance experiments can be classified. These categories are utilised in the Tables summarising the results of tolerance and paralysis experiments.
Tolerance to BGG

After injections of different amounts of BGG at different intervals after birth and subsequent challenge with adjuvant, mice showed many different responses when the results of the challenge were tested by the antigen-elimination technique (see Figure 10). To enable the results to be presented in tabular form these responses have been arbitrarily classified into 5 categories, which are illustrated in Figure 11. Full tolerance (TT) is defined as the exponential elimination of BGG-¹³¹ at the same rate as in non-immunised controls; with a half-life of 3.7 days or longer. In partial tolerance (T) the BGG-¹³¹ is still eliminated exponentially but with a half-life reduced to 3.0-3.7 days. In both tolerant categories the antigen is eliminated at a rate proportional to the total amount of antigen present in the mouse (i.e. exponential elimination). The two immune categories (++) and (+) are lesser responses than that obtained in immunised controls (+++). In the immune categories the rate of elimination of antigen is not proportional to the total amount of antigen present but is increased by another factor, which is probably antibody. Beside these qualitative differences, there are the obvious quantitative differences between the five categories. In Figure 11 the line between (+++) and (++) represents the elimination of BGG-¹³¹ due to about 8 times more antibody being produced than that represented by the line between (+) and (T). This relative figure is based
TABLE 7

The effect of the size of the tolerance-inducing injection made within 12 hours of birth.

<table>
<thead>
<tr>
<th>Mgments of BGG injected into baby CBA mice 12 hrs. after birth</th>
<th>Challenged with Freund's adjuvant containing BGG 2 months after birth, and injected with BGG-\textsuperscript{131}I 1 month later</th>
</tr>
</thead>
<tbody>
<tr>
<td>(mgms/gm body wt.)</td>
<td>Category of response (see Fig. 11)</td>
</tr>
<tr>
<td></td>
<td>TT</td>
</tr>
<tr>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>1.3 (1 mgm/gm)</td>
<td>-</td>
</tr>
<tr>
<td>2.0 (1.5 mgm/gm)</td>
<td>-</td>
</tr>
<tr>
<td>3.3 (2.5 mgm/gm)</td>
<td>-</td>
</tr>
<tr>
<td>6.5 (5 mgm/gm)</td>
<td>-</td>
</tr>
<tr>
<td>10 (8 mgm/gm)</td>
<td>2 M</td>
</tr>
</tbody>
</table>

M = Male.  
F = Female.
The effect of injecting the same amount of antigen at different times after birth.

<table>
<thead>
<tr>
<th>Time after birth at which baby CBA mice received a single injection of 10 mgm BGG</th>
<th>Challenged with Freund's adjuvant containing BGG 2 months after tolerance inducing injection, and injected with BGG-I$^{131}$ 1 month later</th>
</tr>
</thead>
<tbody>
<tr>
<td>Category of response (see Fig. 11)</td>
<td>Total mice</td>
</tr>
<tr>
<td>TT</td>
<td>T</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>12 hrs.</td>
<td>2</td>
</tr>
<tr>
<td>2 days</td>
<td>-</td>
</tr>
<tr>
<td>4 days</td>
<td>-</td>
</tr>
<tr>
<td>9 days</td>
<td>-</td>
</tr>
<tr>
<td>28 days</td>
<td>-</td>
</tr>
</tbody>
</table>
TABLE 9

The effect of injecting the same amount of antigen, relative to the mean body weight at the time of injection, at different times after birth.

<table>
<thead>
<tr>
<th>Time after birth at which baby CBA mice received a single injection of BGG (8 mgm per gm)</th>
<th>Challenged with Freund's adjuvant containing BGG 2 months after the tolerance inducing injection, and injected with BGG-I(^{131}) one month later</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time of injection</td>
<td>Amount</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>12 hrs.</td>
<td>10 mgm</td>
</tr>
<tr>
<td>5 days</td>
<td>14 mgm</td>
</tr>
<tr>
<td>9 days</td>
<td>28 mgm</td>
</tr>
<tr>
<td>17 days</td>
<td>49 mgm</td>
</tr>
<tr>
<td>30 days</td>
<td>80 mgm</td>
</tr>
</tbody>
</table>
TABLE 10

The effect of the length of the interval between a course of tolerance inducing injections and challenge with adjuvant.

<table>
<thead>
<tr>
<th>Time after tolerance inducing injection of adjuvant</th>
<th>Injected with BGG-$^{131}$ 1 month after challenge with Freund’s adjuvant containing BGG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Category of response (see Fig. 11)</td>
</tr>
<tr>
<td></td>
<td>TT</td>
</tr>
<tr>
<td>2 months$^+$</td>
<td>2</td>
</tr>
<tr>
<td>4 months</td>
<td>1</td>
</tr>
<tr>
<td>6 months</td>
<td>-</td>
</tr>
<tr>
<td>6 months$^+$</td>
<td>2</td>
</tr>
</tbody>
</table>

$^x$ died of anaphylaxis when injected intravenously with BGG-$^{131}$.

$^+$ same mice, after challenge adjuvant at 2 months and injection at 3 months; these mice were injected with 2 mgm BGG at 5 months and rechallenged at 6 months (2 mice died before re-challenge).
TABLE 11

The effect of the length of the interval between a course of tolerance inducing injections and challenge with adjuvant.

<table>
<thead>
<tr>
<th>Time after tolerance of challenge with adjuvant</th>
<th>Injected with BGG-\textsubscript{131} \textsuperscript{1} 1 month after challenge with Freund's adjuvant containing BGG</th>
<th>Category of response (see Fig. 11)</th>
<th>Total mice</th>
<th>No. of litters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>TT</td>
<td>T</td>
<td>+</td>
</tr>
<tr>
<td>2 months\textsuperscript{+}</td>
<td></td>
<td>-</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>4 months</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5 months\textsuperscript{+}</td>
<td></td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

\textsuperscript{+} same mice, after challenge with adjuvant at 2 months and injection at 3 months; these mice were then rechallenged at 5 months (1 mouse died before rechallenge).
on the passive immunisation experiments in Part I, and which are illustrated in Figure 8.

The dose and time of administration of BGG to baby mice were varied as stated in Tables 7, 8 and 9. In all experiments in these Tables the mice were challenged with Freund's adjuvant 2 months after the tolerance injection. Table 7 shows that there was an increasing tendency for mice to be tolerant as the amount of antigen injected within 12 hours of birth was increased. Table 8 shows that the degree of tolerance decreased when the same amount of antigen (10 mgm) was injected at increasing intervals after birth. Table 9 shows a similar result to Table 8, except that the dose of antigen was proportional to the mean bodyweight of the litter, with the result that the decrease in the degree of tolerance was less marked.

Tables 10 and 11 show that the degree of tolerance decreased with longer intervals before challenge. Mice given a single injection of 10 mgm BGG at birth were still tolerant when challenged 4 months later. When other mice, similarly treated at birth, were challenged 6 months after birth they were found to be partially immune. Mice found to be tolerant at 2 months after birth were rechallenged at 6 months after birth and were all still tolerant. The BGG injected in challenging and testing the mice, plus an extra 2 mgm BGG which was injected 5 months after birth, was sufficient to maintain the state of tolerance. It is very possible that the Freund's adjuvant provided a long lasting source of antigen which was
released into the circulation very slowly. A similar result was obtained after mice had been injected with 10 mgm BGG in a course of injections ending on the 4th day after birth (Table II). This course of injections was $2^{1/2}$ mgm BGG within 12 hours of birth, $2^{1/2}$ mgm 2 days after birth and 5 mgm 4 days after birth.

The results show that a single intraperitoneal injection of 10 mgm BGG within 12 hours of birth was the most successful way of inducing tolerance to BGG. The course of three tolerance injections was found to be less satisfactory (Table II). This is unfortunate as the single injection of 10 mgm at birth caused a very high mortality amongst CBA mice (more than 75% dead). The mortality was very much less after a course of injections or with a single injection of a smaller quantity of BGG.

In a test for specificity of the induced state of unresponsiveness, one litter of CBA mice were injected with 10 mgm BSA within 12 hours of birth. No inhibition of the immune response to BGG was observed when these mice were subsequently injected with BGG-$I^{131}$. No attempt was made, however, to see if the mice were in any degree tolerant to BSA.

Sera for antibody titrations were prepared from tolerant and immune mice which had been placed in the (TT), (T) and (+++) categories after antigen-elimination tests. High titres of antibody were obtained in sera from all groups. For instance, 6 days after the injection of BGG-$I^{131}$ into mice previously
Figure 12.
Photograph of the gel diffusion experiment designed to illustrate the presence of antibody to one major component of BGG together with the persistence of the other major component of BGG, in tolerant mice.
Figure 13.
Diagram of figure 12, in which a possible interpretation of the result is presented.
Figure 14.
Photograph of a control experiment in which outbred mouse anti-BGG serum is diffused against normal CBA serum. There is no sign of any precipitation.
injected with Freund's adjuvant, the mean titre in 5 immune mice was 9.5 and in 5 tolerant mice the mean titre was 8.0 (Nos. = log₂ titre) in haemagglutination tests. Antibody to BGG was also detected by precipitation in sera from tolerant mice.

Reference to the antigen-elimination curves in tolerant mice showed that about half the antigen injected after challenge was eliminated very rapidly, as if in an immune response. For example, in mice never previously injected with BGG, there was on the 8th day after injection of 2 mgm BGG-I¹³¹ about 20% of the labelled antigen remaining in the circulation. In challenged tolerant mice, however, only about 10% of the antigen remained in the circulation at a similar time after the injection of 2 mgm BGG-I¹³¹, albeit the rate of exponential elimination of the labelled antigen remaining in the circulation was the same in both groups of mice.

Sera from challenged tolerant mice and from not challenged and non-tolerant mice, which had been injected with 10 mgm BGG a week previously, were tested against an outbred-mouse anti-BGG serum, by the double diffusion technique of Ouchterlony. The results are illustrated in Figure 12. Figure 13 is a diagram of this result in which a possible interpretation of the results is presented. In control experiments, the outbred-mouse anti-BGG serum gave no trace of precipitation against serum from CBA mice which had never been injected with BGG (Figure 14). The presence of two major components plus several minor components, in BGG, was demonstrated in a gel-diffusion experiment, using rabbit
Figure 15.
Photograph of a gel diffusion experiment in which hyperimmune rabbit anti-BGG serum is run against 2% BGG for 12 hours. Two bands visible.
Figure 16.

Same as figure 15, refilled once and run for a further 24 hours. Two major bands visible plus 2 or 3 minor bands.
Figure 17.
Same as figures 15 and 16, run for a further 24 hours. The right hand pair of wells a control, with BSA as the antigen.
Photograph of a stained starch gel. The left-hand band is the distribution of serum proteins from a tolerant mouse. Right is a normal (not tolerant) mouse serum sample, and centre is a sample of BGG solution.
Figure 18.

Diagram to illustrate the starch gel electrophoresis experiment. T is serum from mice found to be tolerant to BGG by the antigen-elimination technique. C is serum from control (non-immunised) mice. Subscript a represents the distribution of labelled antigen. Subscript s represents the distribution of all the proteins in the mouse serum. The sera for this experiment were obtained 6 days after the injection of 10 mgm BGG-\(^{131}\)I.
anti-BGG serum (see Figures 15, 16 and 17).

The persisting component of BGG-I$^{131}$ in tolerant mice, after injection of adjuvant and challenge with BGG-I$^{131}$, was analysed by means of starch gel electrophoresis. Two CBA males judged to be tolerant by the antigen-elimination technique and two CBA males which were not tolerant nor had they been challenged, were each injected with 10 mgm of BGG-I$^{131}$. They were bled 4 and 6 days later. Figure 18 is a composite diagram which illustrates the distribution of radioactivity on the electropherogram (Figure 19), from sera obtained 6 days after the injection of BGG-I$^{131}$. It is therefore possible to compare the serum from the tolerant mice with the serum from the controls. It can be seen that the fraction of BGG-I$^{131}$ which persisted was that which moved slightly towards the cathode (pH 9.0). No detectable difference can be seen in the overall distribution of the protein fractions in the two sera. In sera obtained only 4 days after the injection of labelled antigen the difference in distribution of radioactivity was much less. This probably indicates that at least some of the BGG-I$^{131}$ which moved towards the anode was being eliminated in an immune response to that fraction.

**Loss of tolerance**

Mice given a course of tolerance injections which totalled 10 mgm by the 4th day after birth, were divided into two groups. One group was retained as a control and the mice of the other
Figure 20.

CEA mice challenged three months after a course of tolerance-inducing injections (see text).

BGG-\textsuperscript{131}I injected 16 weeks after birth.
Figure 21.
Same as figure 20, but mice given 500 r. of X-irradiation 3 weeks before challenge (injection of Freund's adjuvant).
group were given 500 r. of X-irradiation when they were 1 month old. Both groups were challenged with Freund's adjuvant containing BGG 1 month later, which was followed a month after that by an injection of BGG-I\textsuperscript{131}. The results are illustrated in Figures 20 and 21. It can be seen that the degree of tolerance is considerably less in the mice which were irradiated.

An experiment was designed to show the effect of removal of extracellular antigen on the maintenance of the state of tolerance. Mice injected with a course of tolerance injections totalling 10 mg/m\textsuperscript{2} by the 4th day after birth, were injected with \(\frac{1}{2}, 1\) or \(2\) mls of anti-BGG serum. Heterologous (rabbit anti-BGG), homologous (outbred-mouse anti-BGG) and isologous CBA mouse anti-BGG) antisera were used. In all the experiments the mice were challenged when they were 2 months old. Large quantities of heterologous, homologous and isologous antiserum injected 3, 4 and 6 weeks after the tolerance injection failed to decrease the degree of tolerance observed after the mice had been challenged 2 months after the tolerance injection. In one experiment, mice were injected with heterologous antiserum 1 week after the tolerance injection. These mice showed a tendency towards a decrease in the degree of tolerance, when they were compared with mice which had not been injected with antiserum at that time, although the controls showed several fully immune mice, which makes the result inconclusive. These mice were challenged when 2 months old. Some of the results obtained after the injection of rabbit antiserum are shown in
Figure 22.
CBA males irradiated 700 r. \( \frac{5}{2} \) weeks after a course of tolerance-inducing injections. Immediately after irradiation injected with 90 million spleen cells from tolerant CBA males (same group as recipients, but not irradiated). Injected with Freund's/BGG (8 mgm BGG/mouse) at \( \frac{8}{2} \) weeks after birth. BGG-\( I^{131} \) injected at 3 months after birth.
Figure 23.
Same as figure 22 but only 11 million cells (same pool).
Figure 24.

Same as figures 22 and 23. Combined means of four groups of mice. The numbers on the diagram represent the number of tolerant spleen cells transferred.
Table 13, Expt. 31.

When adult cells were injected into baby mice at the time of the tolerance injection, there was no definite subsequent break from the state of tolerance (Table 13, Expt. 32b). There is the possibility of a very slight tendency towards a break from tolerance, but this is not significant.

It is thought that isologous lymphoid cells injected into irradiated mice undergo cell division (see discussion in Part III). It is also thought that varying the size of the original inoculum controls the amount of division that the transplanted cells must undergo to recolonise the irradiated host. CBA mice which had been given a course of tolerance injections were given 700 r. of X-irradiation when they were 5 weeks old. Groups of these mice were then injected with different numbers of spleen cells from another group of tolerant mice of the same sex which had not been irradiated. One month later these mice were challenged with Freund's adjuvant containing BGG and a month after that they were injected with BGG-I$^{131}$. The results of this experiment are illustrated in Figures 22, 23 and 24 (see also Table 14). If it is accepted that the smaller number of cells had to divide more than the larger number of cells transplanted, then it can be seen that the degree of tolerance decreases with an increasing amount of cell division. The evidence that transplanted cells divide in irradiated hosts is discussed in Part III of this thesis.
Further attempts were made to break the state of tolerance by injecting spleen cells from non-tolerant CBA mice into tolerant mice; 100 million cells were injected into tolerant mice 1 month after the tolerance injection. These mice were challenged when they were 2 months old. They were found to be still tolerant although there was a slight tendency towards a break from tolerance. Further experiments will have to be carried out to see if tolerance can be significantly broken by larger numbers of non-immunised cells or by different injection programmes.

When spleen cells are transplanted from tolerant mice to non-tolerant irradiated (950 r.) mice, and the recipients were subsequently challenged with Freund's adjuvant, the recipient mice showed immune (+++ ) responses. A similar result was obtained when non-tolerant (not immunised) cells were transplanted into irradiated tolerant mice. When spleen cells from tolerant mice were transplanted into not tolerant and non-immunised mice, and the recipients were not challenged with Freund's adjuvant but were injected with BGG-I$^{131}$ a month later, these recipients showed no signs of an immune response. Any antigen which might have been persisting within the tolerant cells, was unable to immunise the host.

**Paralysis to BGG**

The effect of different paralysing doses of BGG injected into non-irradiated adult mice is shown in Figures 25, 26, 27,
**Figure 25.**

Control for the paralysis experiments (figures 26-30). Adult CBA males, irradiated 500 r. (not injected with BGG) injected with adjuvant/BGG two months later and BGG-\(^{131}\text{I}\) another month after that. Also a group of mice not injected with adjuvant.
Figure 26.
Three month old CBA mice, irradiated with 500 r. and injected 70 mgm BGG immediately afterwards. Two months later injected with adjuvant/BGG and BGG-I\textsuperscript{131} another month after that.
Figure 27.

As for figure 26, but only 35 mgm.
Figure 28.

As for figure 26 but not irradiated (70 mgm BGG).
Figure 29.
As for figure 26 but not irradiated and only 35 mgm BGG.
Figure 30.

Paralysis experiment, combined means. Solid lines represent irradiated mice and dotted lines represent mice which have not been irradiated. Numbers represent the quantity of BGG (in mgm) injected 2 months before challenge.
28, 29 and 30. These mice were challenged with Freund's adjuvant 2 months after the paralysing dose, and a month after that they were injected with BGG-I\textsuperscript{131}. A similar experiment was carried out with mice given 500 r. of X-irradiation immediately before the paralysing injection. It can be seen that in the conditions of this experiment 150 mgm BGG was sufficient to partially paralyse a non-immunised CBA mouse, whereas 70 mgm had only a partial effect and 35 mgm hardly any detectable effect at all. In the irradiated mice, however, even 37 mgm BGG had a marked inhibitory effect on the subsequent immune response to BGG (Table 12). Irradiated controls not injected with BGG also showed a slight inhibition of response when compared with non-irradiated mice which also had not been injected with BGG previous to the challenge with Freund's adjuvant (see Figure 25). In this preliminary experiment it is therefore difficult to distinguish the possible increase in paralytic effect of the antigen in the irradiated mice from the direct inhibitory effect of the irradiation. Schwartz and Dameshek (1959) have recently shown a similar phenomenon in rabbits using 6 mercapto-purine instead of X-rays. The whole topic of the inhibition of the immune response by means of X-irradiation has been reviewed by Taliaferro and Taliaferro (1951).

In one experiment 150 mgm of BGG was injected into mice which had been immunised with Freund's adjuvant containing BGG a month previously. When BGG-I\textsuperscript{131} was injected at the same
TABLE 12

Experiments designed to show the effect of injecting large quantities of protein antigen (BGG) into irradiated and non-irradiated adult mice.

Mice were challenged with Freund's adjuvant containing 8 mgm BGG for each mouse, 2 months after the massive injection of antigen. All CBA males, 3 months old at time of massive injection.

<table>
<thead>
<tr>
<th>Irradiation</th>
<th>Dose of BGG (immediately after irradiation)</th>
<th>Category of secondary response</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>NIL</td>
<td>TT T + ++ +++</td>
</tr>
<tr>
<td>None</td>
<td>35 mgm</td>
<td>- - - - 5/5</td>
</tr>
<tr>
<td>None</td>
<td>70 mgm</td>
<td>- - - 3/5 2/5</td>
</tr>
<tr>
<td>None</td>
<td>150 mgm</td>
<td>- - 3/5 2/5 -</td>
</tr>
<tr>
<td>500 r.</td>
<td>NIL</td>
<td>- - 2/5 3/5</td>
</tr>
<tr>
<td>500 r.</td>
<td>35 mgm</td>
<td>- - 3/6 2/6 1/6</td>
</tr>
<tr>
<td>500 r.</td>
<td>70 mgm</td>
<td>- - 5/5 -</td>
</tr>
<tr>
<td>500 r.</td>
<td>150 mgm</td>
<td>- 2/5 3/5 -</td>
</tr>
</tbody>
</table>
TABLE 13

Experiments to test various procedures to see if they would reduce or abolish the state of tolerance to BGG. Tolerance was induced by a course of injections starting within 12 hours of birth, and totalling 10 mgm BGG by day 4 after birth. Mice challenged with Freund's BGG at 2 months after birth.

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Treatment</th>
<th>Category of secondary response</th>
</tr>
</thead>
<tbody>
<tr>
<td>34</td>
<td>NIL</td>
<td>-  2/5  3/5</td>
</tr>
<tr>
<td>34</td>
<td>500 r.</td>
<td>-  -  -  4/4</td>
</tr>
<tr>
<td>32b</td>
<td>NIL</td>
<td>2/3 1/3 -</td>
</tr>
<tr>
<td>32b</td>
<td>3.5 x 10^6 father's spleen cells ip, on day of birth</td>
<td>- 1/4 3/4 -</td>
</tr>
<tr>
<td>32b</td>
<td>10 x 10^6 father's spleen cells ip, on day of birth</td>
<td>- 1/4 3/4 -</td>
</tr>
<tr>
<td>32b</td>
<td>3.5 x 10^6 2 day old baby CBA mouse spleen cells ip, on day of birth</td>
<td>3/4 1/4 -</td>
</tr>
<tr>
<td>31</td>
<td>NIL</td>
<td>2/5 3/5 -</td>
</tr>
<tr>
<td>31</td>
<td>0.5 ml rabbit anti-BGG serum (3 mgm Ab protein/ml) 3 weeks before Freund's adjuvant injection</td>
<td>3/10 6/10 1/10</td>
</tr>
<tr>
<td>31</td>
<td>2 ml as above</td>
<td>2/4 2/4 -</td>
</tr>
</tbody>
</table>

* Freund's adjuvant (BGG) injected 16 weeks after birth (not 8 weeks).
TABLE 14

Experiment designed to show the effect of cell division on the subsequent degree of tolerance. It is assumed that cell division will be greatest in the recipients injected with the smallest inoculum of spleen cells from tolerant donors. Both recipients and donors were given a course of tolerance inducing injections, starting within 12 hours of birth, and totalling 10 mgm by 4 days after birth.

CBA males, donors 5½ weeks old, given 700 r. irradiation, followed by iv, injection of cells. At 2 months of age the mice were challenged with Freund's adjuvant containing BGG.

<table>
<thead>
<tr>
<th>Number of tolerant cells injected</th>
<th>Category of secondary response</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TT</td>
</tr>
<tr>
<td>90 million</td>
<td>-</td>
</tr>
<tr>
<td>45 million</td>
<td>-</td>
</tr>
<tr>
<td>22.5 million</td>
<td>-</td>
</tr>
<tr>
<td>11 million</td>
<td>-</td>
</tr>
</tbody>
</table>

Challenged with 2 mgm BGG–I$^{131}$ 1 month after adjuvant injection.
time as the injection of 150 mgm BGG, the secondary response observed was not detectable for about a fortnight. If the BGG-I$^{131}$ was injected a month after the injection of 150 mgm BGG into immune mice, then very little if any inhibition of the immune response was observed.

**DISCUSSION**

The evidence that antigen must persist for tolerance to be maintained

The degree of tolerance in mice has been found to be related to the dose of BGG injected, when the injection was made at 12 hours after birth (Table 7). It has also been shown that the degree of tolerance decreases with time but that further injections of antigen whilst the mouse is still tolerant can result in the state of tolerance being prolonged. The results are compatible with the hypothesis of Smith and Bridges that tolerance lasts only as long as antigen remains in the body.

The degree of tolerance in these experiments was measured by the antigen-elimination technique of Talmage et al. (1951). Tolerant mice did not show an immune elimination of labelled BGG, although the total amount of antigen present a few days after the antigen injection was less than that in non-immunised controls, even though the same quantity of antigen had been injected. This has been shown to be due to the presence of an immune response to at least one component of BGG in "tolerant"
(TT and T) mice.

Cinader and Dubert (1955; 1956) have shown that tolerance is highly specific, even when protein antigens are used. Billingham, Brent and Medawar (1956) showed that homograft tolerance was very specific. The gel diffusion tests attempted to show that in the experiments described in this part of the thesis, tolerance was specific for one fraction of BGG, despite an immune response to other fractions of BGG. Similarly, mice injected with BSA at birth showed no signs of tolerance to BGG. These results therefore confirm the earlier work.

Antibody to BGG was present in sera from tolerant mice in only very slightly lower amounts than in immunised controls. It was shown in the Ouchterlony tests that tolerant mice retained only one detectable fraction of BGG and quickly developed large amounts of precipitating antibody to the other fraction(s). The tolerated fraction of BGG was tentatively identified, by means of starch gel electrophoresis, as that fraction of BGG which moved towards the cathode.

A considerable variation was observed in the degree of tolerance in groups of mice receiving the same treatment. Cinader and Dubert (1956) suggested that a similar variation in the degree of tolerance that they observed in rabbits was possibly due to genetic variation in the experimental animals. The experiments described here have been carried out in a highly inbred strain of mice, so that genetic variability could not be
the explanation of the variation in the degree of tolerance that was observed. It seems more likely that much of the variation is due to variable factors in the period between the tolerance injection and the challenge injection or possibly to a highly critical factor in the dose size of BGG or the time of the tolerance injection.

Terres and Hughes (1959) are of the opinion that if the continued presence of antigen is required for the maintenance of tolerance, then this antigen must persist at some intracellular site where it is protected from catabolism. However, it is not necessary to postulate such a mechanism to explain the time for which tolerance persists. By making the following assumptions: half-life of BGG is 4 days (see Part I); molecular weight of BGG is 150,000; and by referring to the data in Table 10, it is possible to calculate the approximate number of molecules of BGG persisting in the mice at 4 and 6 months after the tolerance injection of 10 mgm of BGG at birth. At 4 months there will be at least $10^9$-$10^{10}$ molecules (mouse still tolerant) and at 6 months about $10^6$-$10^7$ molecules (mouse partially immune).

If the mechanism of loss of tolerance by the animal as a whole is the maturation of antibody producing cells in the absence of antigen, then no special mechanism for the protection of antigen need be postulated. If this were the case then tolerance to BSA should be lost more quickly than tolerance to BGG, as the half-life of BSA is only about 20 hours. This expectation appears to be fulfilled by the results of Terres and
Hughes (1959). They injected 12.5 mgm BSA at birth and found all mice tested were tolerant to BSA 6 weeks later but that many mice were immune when tested 11 weeks after birth. This seems to support the hypothesis that extracellular antigen has a role to play in the maintenance of the state of tolerance.

An examination is now attempted of more of the evidence used to support the hypothesis that antigen must persist for the maintenance of tolerance. In Table 7 it can be seen that doubling the amount of antigen injected at birth does not always increase the degree of tolerance, when the mice are challenged two months after the tolerance injection. Table 10 shows that 10 mgm BGG injected at birth induces tolerance which persists for at least 4 months, yet Table 11 shows that tolerance due to a course of injections totalling 10 mgm by the 4th day after birth, will barely persist for 2 months. Furthermore, a single injection of 10 mgm BGG as late as 9 days after birth (Table 8) induces at least as complete a degree of tolerance as 10 mgm injected in a course completed on the 4th day after birth, when both groups are challenged 2 months after the tolerance injections. It seems likely from these results that not only the total dose of antigen injected into the baby mouse but also the amount in the first injection determine the time for which tolerance will persist. One conclusion that can be drawn from this is that tolerance induced by a course of injections can be contrasted with tolerance induced by a single injection. Both Smith and Bridges (1958) and Terres and Hughes (1959) base part
of their arguments for the maintenance of tolerance on experiments in which the size of the initial dose of antigen was varied and the time for which tolerance persisted was then measured. The results presented in Tables 7, 8, 9, 10 and 11 appear to weaken this part of their argument. However, the results based on single tolerance-inducing injections and the time for which the resulting tolerance lasted, together with the evidence that tolerance could be made to persist by injections of antigen into the tolerant adult, do appear to support the hypothesis that the maintenance of tolerance is due to the persistence of antigen. It is thought that this criticism helps to underline the possibility that the question of antigen persistence is in fact in two parts: the intracellular persistence of antigen or antigenic derivatives; and the persistence of extracellular antigen.

Experiments were carried out to determine the effect of persisting extracellular antigen. Tolerant mice were injected with anti-BGG sera from various sources. It was hoped that this antiserum would remove any BGG in the circulation and extracirculatory body fluids. Most of the results were negative in that tolerance was not detectably decreased when the immunological state of the experimental animals was tested 2 months after the tolerance injection. In one case, however, when antiserum was injected very soon after the tolerance injection, there was a slight decrease in the subsequent degree of tolerance although this experiment was inconclusive. It is
evident that removal of extracellular antigen with antisera does not cause an immediate break from the state of tolerance. It seems likely that removal of extracellular antigen may initiate a slow recovery of the capacity to produce an immune response to BGG.

It is possible that the state of tolerance is due to the intracellular persistence of antigen, at a site fully or partially protected from catabolism. If tolerant cells containing such antigen were made to divide in the absence of extracellular antigen, so that the daughter cells were unable to replenish their "sites" with antigen, then it would be expected that the animal as a whole would slowly gain a capacity to produce antibody as the state of tolerance was lost. This was investigated in some preliminary experiments. In the first group of experiments tolerant mice were sub-lethally irradiated, and it was found that they lost their tolerance more quickly than non-irradiated mice. In a second group of experiments cells from tolerant donors were injected into lethally irradiated tolerant recipients. The amount of cell division was controlled by injecting different numbers of cells. It was found that the decrease in the degree of tolerance was greatest when the amount of cell division was greatest. These results are therefore compatible with the hypothesis that intracellular antigen causes the state of tolerance and that this antigen must be replenished from an extracellular source if the state of tolerance is to be maintained for a long time.
Experiments combining the use of X-rays and antiserum together with fresh injections of BGG have not yet been completed.

Hypotheses of the mechanism of tolerance

The current hypotheses of tolerance can be divided into two broad categories. The first category are those hypotheses which postulate that tolerance can only be induced at a critical stage during the maturation of the antibody producing cells, or that the state of tolerance is induced by large quantities of antigen; smaller quantities inducing a state of immunity. This first category could be called the "cellular physiology hypotheses". The second category of hypotheses are those concerned with the interaction between cell and antigen which results in immunological tolerance; these could be called the "mechanistic hypotheses". The Table below, summarises the various hypotheses:

<table>
<thead>
<tr>
<th>Cellular physiology hypotheses</th>
<th>Stem line</th>
<th>Paralysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adaptive Humphrey (1956)?</td>
<td>Humphrey (1956)?</td>
<td>Dixon and Maurer (1955)?</td>
</tr>
<tr>
<td>Medawar (1959)</td>
<td>Dixon and Maurer (1955)?</td>
<td>Felton (1949)?</td>
</tr>
<tr>
<td>Mechanistic hypotheses</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clonal Lederberg (1958)</td>
<td>Dixon and Maurer (1955)?</td>
<td>Felton (1949)?</td>
</tr>
</tbody>
</table>

Although this Table is a gross oversimplification, it may help in further discussion. The references in this Table refer to early hypotheses of tolerance, and indicates where these hypotheses might be fitted into this scheme.
(a) **Tolerance as an adaptive phenomenon**

It is possible to postulate that antigen might alter the genotype of the cell. Tolerance at the cellular level would probably then be an all-or-none phenomenon, and once a cell was tolerant to a particular antigen it would never lose that capacity. This hypothesis is attractive on account of its simplicity. This would be especially so when the loss of tolerance could be explained in terms of the maturation of antibody producing cells in the absence of extracellular antigen (Medawar 1959). It would not be necessary to postulate that antigen had to persist intracellularly for the tolerance of a cell to be maintained.

However, the experiments and results discussed earlier indicate that both intracellular and extracellular antigen must persist for the long term maintenance of tolerance. Furthermore, evidence from the induction of penicillinase production in *Bacillus cereus* suggests that inducing substances must persist for a state of differentiation of this nature to be maintained. This evidence also suggests that the inducer is protected from catabolism (Pollock 1950, 1952, 1953, 1958; Wainwright and Pollock 1949; Pollock and Perret 1951; and Monod 1956, 1959). It is not unlikely, therefore, that intracellularly persistent antigen in a tolerant cell, is acting in a similar way to persisting substrate (penicillin) in the adaptive synthesis of penicillinase. It seems plausible to postulate that it is not necessarily whole antigen molecules
but determinant groups which must persist at an intracellular site, for the induced state of tolerance to be maintained. The determinant group, possibly in combination with a specific receptor, might be fully or partially protected from catabolism.

It is possible to explain the loss of tolerance in terms of induced cells dividing in the absence of antigen and so diluting inducer (determinant group plus receptor) amongst daughter cells until the situation arises in which some daughter cells no longer contain inducer. These cells would then cease to be tolerant. The result of the experiments in which tolerant cells were transplanted into irradiated tolerant mice are compatible with this hypothesis. Similar results were obtained by Pollock (1958) who grew bacteria induced to form penicillinase in the absence of substrate. He found that the capacity to produce the enzyme at once, remained constant despite the great increase in the number of cells. There is a possibility that antibody producing cells mature from a stem line and at one point are capable of becoming tolerant; this will be discussed later. It is sufficient at this point to state that the results of the experiments described in this thesis which were designed to illustrate the mechanism of the loss of tolerance, are compatible with the stem line hypothesis. Both these hypotheses are compatible with Mitchison's (1959) observation that absence of extracellular antigen seemed to initiate the loss of tolerance but that the recovery of the capacity to produce a full immune response, by the animal as a
whole, was a relatively slow process. Antibody produced possibly by only a few cells, in partial tolerance may slightly speed the loss of tolerance by quickly removing the last traces of extracellular antigen.

Humphrey (1956) has suggested a direct comparison between tolerance and adaptive enzyme formation. Campbell (1957) has cited evidence to suggest that isologous (tolerated) proteins do not last for long intracellularly, whereas foreign antigenic proteins persist for detectably longer periods. It is possible that a cell contains highly specific enzymes to break down isologous material. Humphrey suggested that the induction of tolerance was in fact the induction of an adaptive enzyme to break that antigen down in the same way as isologous material. Antigenic proteins would persist for longer periods because of the lack of the enzyme. There is a considerable body of circumstantial evidence that antigen must persist for antibody production to take place. This is certainly compatible with Humphrey's hypothesis for the mechanism of acquired tolerance.

By analogy with adaptive enzyme formation in bacteria, Humphrey's hypothesis would require the persistence of antigen or antigenic derivatives, at a site within the cell. It is possible to postulate other mechanisms analogous to adaptive enzyme formation or enzyme repression, which might effect the rate of antibody production rather than the intracellular life of the antigen that stimulates the production of antibody.
The results discussed so far are compatible with the hypothesis that tolerance is an adaptive phenomenon, probably dependent on the intracellular persistence of antigen or antigenic derivatives. Cell division in the absence of extracellular antigen leads to the formation of antibody producing cells which are not tolerant. Possibly the slow catabolism of inducer without an extracellular supply of antigen to replace that which was catabolised, would have the same result. Extracellular antigen therefore is necessary for the long term maintenance of the state of tolerance.

(b) The clonal hypotheses

The clonal theory of antibody production was first postulated by Talmage (1957b). This theory was amplified by Burnet (1957; 1959) and Lederberg (1958). The clonal theory postulates that any one antibody producing cell is capable of producing two antibodies at the most. These cells arise by a process of genotypic differentiation in the embryo or possibly throughout life, or they could arise by a continuous process of mutation (Burnet 1959). Contact between either an immature antibody producing cell and antigen, or between a mature antibody producing cell and sufficient antigen will kill any cell which is genetically capable of producing antibody to that antigen.

The results of the experiment in which different numbers of cells from tolerant donors were transplanted into irradiated
tolerant recipients, showed that the loss of tolerance was directly related to the amount of cell division. These results are incompatible with the clonal theory unless it is postulated that an increase in cell division is linked to an increase in mutation. There is neither evidence nor biological analogy to support such an ad hoc hypothesis. Furthermore the evidence that antigen must persist for the state of tolerance to be maintained, is incompatible with the clonal hypothesis, unless further ad hoc hypotheses are made.

(c) The stem line hypothesis

This hypothesis can be summarised as follows: immunologically competent cells are formed by a process of division and maturation from a stem line of primitive cell types. Contact with antigen during the process of maturation induces a specific change in the cell which prevents it from producing antibody to that antigen but not to other antigens. During the development of a neonatal animal there must be a point when no mature antibody producing cells are present, but there must be a relatively large number of cells in the process of maturation. An injection of foreign antigen at this point may induce a state of tolerance, providing that this is genetically possible. If antigen is injected after the critical point, the mature antibody producing cells will react by producing antibody.

If antibody producing cells are being formed continually from a stem line throughout the life of the animal, then it is
possible that paralysis is the same phenomenon as tolerance, at least up to a point. Before a state of true adult induced tolerance could arise, it would have to be postulated that very large doses of antigen inhibited antibody production. Pneumococcal polysaccharide paralysis might be a possible model for this first essential stage. However it is possible that paralysis to proteins as well as pneumococcal polysaccharide is a distinct phenomenon from acquired tolerance. This will be discussed later.

The stem line hypothesis was first put forward by Lederberg (1958) as part of his adaptation of the clonal theory. His hypothesis is compatible with classical histological evidence. Although the hypothesis was first put forward in connection with the clonal theory, it is equally compatible with the hypothesis that tolerance is an adaptive phenomenon.

(d) The paralysis hypotheses

From a monistic point of view, it is possible to postulate that "Felton type paralysis" and acquired immunological tolerance are one and the same phenomenon, induced by the exposure of an antibody producing cell to a certain minimum quantity of antigen. If this were so, it is possible that smaller quantities of extracellular antigen might be capable of maintaining the state of unresponsiveness.

Stark (1955) has postulated that pneumococcal polysaccharide paralysis may be due to persisting antigen catalysing
the breakdown of antibody which is continuously being produced. It has been shown that the antigen persists at an intracellular site (Coons 1954) but no proof has been forthcoming to show that antibody is produced by the paralysed cell (Sercarz and Coons 1959). The failure to paralyse adults to histocompatibility factors by injections of cells or by large skin grafts, suggests that tolerance to homografts is a separate phenomenon from paralysis. However, with regard to paralysis caused by injections of protein antigens, both in Dixon and Maurer’s (1955) experiments and in the experiments described here, it seems that there is little distinction between tolerance and paralysis.

If it is postulated that paralysis and tolerance to protein antigens are the same phenomenon, then it is only necessary to assume that it is the quantity of antigen which switches the antibody producing cell from antibody production to a state of tolerance. If, however, it seemed that the two phenomena were different, then to explain paralysis of an adult it would be necessary to assume that in the first stages intracellular antigen catalysed any antibody that was produced before a state of true tolerance came into being. For this latter hypothesis to be tenable it is necessary to accept the stem-line hypothesis or to postulate that paralysis was due to catalysis of synthesised antibody. It is difficult to reconcile such a mechanism with the necessary protection from catabolism afforded to the intracellular BGG, if such a
hypothesis is to be acceptable. Furthermore, it has been shown that large quantities of passive antibody does not break pneumococcal paralysis (Dixon, Maurer and Weigle 1955) nor protein tolerance (this thesis, Part II), yet it has also been shown that paralysis of an immune animal requires very much larger doses of antigen, or may even be impossible (Felton 1949; Part II). It seems very reasonable to suppose that tolerance and paralysis to protein antigens are in fact the same phenomenon. It would be very surprising if "tolerance" to pneumococcal polysaccharides could not be induced by minute amounts (immunising doses in an adult) of antigen injected into neonatal mice.

Tolerance to histocompatibility antigens (iso-antigens) seems to be incompatible with tolerance to serum protein antigens and paralysis to pneumococcal polysaccharide and serum protein antigens. This is one of the stronger arguments in favour of tolerance and paralysis being distinct (Billingham, Brent and Medawar 1956). However, a clear cut decision for or against such a hypothesis cannot be made until the nature of the antigenic stimulus involved in the homograft reaction has been clearly defined. Nevertheless, the evidence does indicate that there are major differences between immunologically mature and immature animals. One such difference is that very young animals do not synthesise antibodies (Freund 1930), although this may be irrelevant to the question of tolerance induction. It may simply be that the threshold concentration
of antigen required for the induction of tolerance (paralysis) is lower in a very young animal. This might explain how tolerance to histocompatibility antigens is only inducible in a neonatal animal. As far as serum protein antigens are concerned, however, it seems that on a gm. antigen/body weight basis that there is no such difference in threshold; 3 gm BGG/gm. body weight of the baby or adult CBA mouse, resulted in immunological unresponsiveness when the mice were challenged 2 months later. Here it must be pointed that BGG lacks adjuvanticity in mice (Part I). It is possible that the lack of difference between adult and neonatal animals with regard to tolerance inducing size of antigen dose, is in fact due to the absence of antibody. The presence of antibody in an adult could easily upset the equilibrium between free antigen and specific "sites" which must be occupied before a state of tolerance can be induced. This would result in larger quantities of antigen being required (per gm. body weight) in adults to induce the tolerant state.

**Acquired immunological tolerance - a working hypothesis**

The evidence discussed so far would make it appear that tolerance is an adaptive phenomenon which depends for its long term maintenance on the persistence of antigen. It is not unlikely that the state of tolerance of an antibody producing cell is due to the persistence of antigen or antigenic derivatives within that cell. Tolerance is probably an adaptive phenomenon induced by a minimum threshold concentration of
antigen, but maintained over long periods by much smaller quantities. The evidence cited in this thesis seems to rule out the clonal theory as a likely description of the mechanism of tolerance. Tolerance to histocompatibility antigens may indicate that the immunological environment in a neonatal animal differs from that in adult animals. It will probably not be possible to show whether or not tolerance to histocompatibility antigens is basically similar to tolerance to serum protein antigens, until the antigenic substance itself is isolated and used in both tolerance and paralysis experiments. The evidence that adult animals can be paralysed indicates that the stem line hypothesis is not necessary to explain the experimental facts as they are now known; paralysis of adults does not rule out the possibility that the basic tenets of the stem line hypothesis are correct.

As a working hypothesis it is postulated that all aspects of specific immunological unresponsiveness constitute the same phenomenon. The state of tolerance is an adaptive differentiational change of the antibody producing cell, induced in response to a minimum threshold concentration of antigen. The induced state is maintained by intracellular antigen or antigenic derivatives which can be replenished, as required, from extracellular antigen which may be in relatively low concentration. Using Lederberg's (1959) terminology, tolerance is an elective phenomenon in which antigen as inducer has allowed the expression of one of the facets of the cell's
genotype. The experimental facts of histocompatibility tolerance can be reconciled to this hypothesis if it is postulated that these antigens are normally cell bound and cannot normally reach a sufficiently high concentration to induce paralysis in adults but that in neonatal animals the environment is such that these antigens are released in sufficient quantities to induce tolerance. So far only living cells have been shown to induce tolerance to histocompatibility antigens.
PART III: A STUDY OF THE SECONDARY IMMUNE RESPONSE

INTRODUCTION

Hypotheses of the mechanism of antibody production can be divided into two categories: those in which it is postulated that the antibody synthesising mechanism is replicated, and those in which it is not. If a cell containing an antibody synthesising mechanism could be made to divide in the absence of antigen, then the state of the progeny would decide between the hypotheses. If all the progeny of the original cell were found to contain functioning mechanisms, as judged by their capacity to produce a secondary response, then it could be concluded that the mechanism as a whole was probably capable of replication. If, however, the mechanisms remain in only a few of the progeny or were lost altogether during cell division, then it could be concluded that at least one of the essential parts of the mechanism was incapable of replication.

Ford, Hammerton, Barnes and Loutit (1956) and Ford, Ilbery and Loutit (1957) have shown that after irradiation and transplantation of homologous or isologous tissue, most of the dividing cells in the bone marrow were of transplant origin. This evidence, taken together with the gross increase in weight of lymphoid organs during recolonisation, indicates that there is a great multiplication of at least some of the introduced cells.
In the experiments to be described, cells from mice immunised against a protein antigen were transplanted into an irradiated environment which was thought to be suitable for rapid cell division, and the rate of antibody production in the first part of the secondary response by the progeny of those cells, was measured. Large numbers of lymphoid cells were transplanted into irradiated isologous hosts, and the subsequent rate of antibody production in a secondary response was measured by the antigen-elimination technique. By altering the number of cells transplanted, it was hoped that some control had been obtained over the amount of cell division of the transplanted cells in the irradiated recipient. Similarly, an attempt was made to measure the secondary response at intervals after transplantation, during the course of recolonisation.

A protein antigen, BGG, was used. The experiments in Part I of this thesis have shown that BGG was suitable for use in mice, with the antigen-elimination technique. Furthermore the primary and secondary responses to BGG were shown to be quite distinct. Mice have a further advantage in experiments of this type in that they rarely give a primary response to an intravenous or intraperitoneal injection of BGG unless that injection is accompanied by an injection of a substance having the properties of an adjuvant (adjuvanticity (see Part I)).

MATERIALS

CBA and A and A. Sw (10) strain mice have been used in
these experiments. The stocks have been maintained by sib matings. Unless otherwise stated, cell transfers have been carried out in CBA males, to avoid any possibility of a homograft reaction due to sex linked histocompatibility genes (Eichwald and Silmser 1955, 1957; Short and Sobey 1957; Feldman 1958; and Billingham and Silvers 1958). Recipients were used when 11-12 weeks old. Donors were actively immunised with Freund's adjuvant mixture when 8-12 weeks old and their lymphoid tissue used about 6 weeks after the first immunising injection.

The antigen (BGG) was labelled with $^{131}\text{I}$ by Method 2 for use with the antigen-elimination technique. The procedure is described in Part I of this thesis. In a limited number of experiments a bacterial antigen was used; this was *Salmonella typhi H* (Burroughs Wellcome Ltd., *B. typhosum H*).

**METHODS**

**Active immunisation of donors**

Donor mice were injected sub-cutaneously with Freund's adjuvant containing 8 mgm BGG for each mouse. One month after the injection of adjuvant the mice were injected intraperitoneally with a solution containing 2 mgm BGG per mouse. Cells were transferred 15-21 days after the booster (intraperitoneal) injection.
Cell transfers

In some experiments cells were injected into lethally irradiated mice; the procedure of irradiation has been described in detail in Part II. Donor mice were killed and spleens and lymph nodes were removed by dissection, placed in Hank's solution at pH 7.2 and minced with a stainless steel rotary mincer. The suspension of cells so formed was allowed to stand for 2 minutes, so that the larger clumps of tissue could settle. The supernatant of more or less free cells was then removed by means of a pasteur pipette and put into another container. Bone marrow suspensions were prepared as follows: the heads of the long bones were cut off and Hank's solution was forced through the shafts by means of a hypodermic syringe fitted with a 26 gauge needle. By repeated recirculation of the Hank's solution a dense suspension of cells could be obtained.

Schrek's eosin technique was used to estimate the number of viable cells in the cell suspension prepared by the method just described (Schrek 1936; Schrek and Preston 1957). In all the suspensions tested, less than 15% of the cells took up the stain. The cell density of each inoculum was determined by squirting 0.025 ml into 0.975 ml of 2% acetic acid solution and by counting the number of cells in the resulting suspension. Under these conditions enucleate cells lyse. The injections were usually intravenous but were sometimes intraperitoneal. Injections of large numbers of cells were made very slowly to
avoid emboli. The time between the preparation of the cell suspension and its injection into the recipient mouse was less than 25 minutes; the cells were kept at room temperature.

**Challenging immunisation.**

Mice which had previously been injected with immunised cells (adoptive immunisation) and actively immunised and non-immunised controls, were injected with 2 mgm BGG-1151. The course of antigen elimination was followed as described in Part I. In the experiments in which the number of immunised cells in the inoculum was varied, the results obtained could be compared with the calibration experiment (Figure 8) described in Part I. This comparison could also be made in other experiments to be described in this part of the thesis (Part III).

**Bacterial agglutinin titration**

Bacterial agglutinin titres were estimated by the method used by Mitchison (1956, 1957). Serum was prepared from the blood of mice thought to contain agglutinins and from control mice which as far as was known had had no previous contact with Salmonella antigens. Serial (X2) dilutions of the serum were made in saline (0.9% NaCl). The total volume of antibody solution was 0.1 ml. To this was added 0.1 ml of the bacterial suspension. The tubes were thoroughly shaken to mix the serum and the bacteria, and the tubes were then left overnight at room temperature. The agglutinin titre was defined as the minimum concentration of antiserum which would cause macroscopic
Figure 31.
The effect of the interval between primary and secondary antigen injection. The numbers on the figure represent months between injection of Freund's adjuvant containing BGG and the secondary antigen injection.
agglutination patterns. As the concentration of antiserum equals minus the log to the base 2 of the tube number, the tube number itself makes a convenient method of expressing the titre, loosely called the "log titre".

EXPERIMENTS AND RESULTS

1. Persistence of the actively acquired secondary response

Three groups of mice were actively immunised with Freund's adjuvant containing BOG, which was prepared and injected in the usual way. One group of mice was injected with BGG-\textsuperscript{131} one month after the injection of adjuvant; the second group was injected with labelled BOG 2 months later; and the third group 4 months later. The rate of antibody production in the first part of a secondary response was seen to decline with increasing time between the adjuvant injection and rechallenge with labelled antigen. The results are illustrated in Figure 31. The decline in response may be compared with that obtained after adoptive immunisation.

2. Adoptive secondary response due to cells from different organs

The rate of antibody production in a secondary response by about the same number of cells from different organs was measured in irradiated recipients. Twelve immunised CBA males were killed and cell suspensions prepared from their spleens, bone marrow and lymph nodes. Thirty 3 month old CBA males were given 950 r. of X-irradiation. Five of these mice received $75 \times 10^6$ immunised spleen cells (i.e. spleen cells from donors
Figure 32.

Four month old CBA males; no response. The recipient mice were injected with 75 million spleen cells from non-immunised donors, immediately after 950 r. A similar negative response was obtained after adoptive immunisation with cells from immunised donors, when the cells were kept at 49.5°C for 20 mins. before injection. BGG-I\textsuperscript{131} injected 6 weeks after adoptive immunisation.
immunised against BGG by the prescribed method) by intravenous injection; three received $76 \times 10^6$ immunised lymph node cells intraperitoneally plus $0.7 \times 10^6$ non-immunised bone marrow cells intravenously (the small amount of bone marrow cells was injected as therapy against radiation death). An attempt was made to inject some mice intravenously with lymph-node cells but the mice died presumably due to clumping of cells in the suspension causing emboli. Six irradiated mice received $78 \times 10^6$ immunised spleen cells which had been maintained at $49.5^\circ\mathrm{C}$ for 20 minutes to kill the cells (Billingham, Brent and Medawar 1956); these mice also received $0.7 \times 10^6$ non-immunised bone marrow cells, all the injections being made intravenously. Finally four mice received an intravenous injection of $72 \times 10^6$ non-immunised spleen cells.

Six weeks after the cell transfers, the recipient mice, plus five non-immunised and non-irradiated mice, were challenged by an intravenous injection of $2 \text{mgm BGG-}^{131}$ and the elimination of the labelled antigen was followed for twelve days. The elimination of antigen in mice receiving heat killed immunised spleen cells, those receiving non-immunised spleen cells and those which had been neither irradiated nor previously immunised, were indistinguishable, showing no signs of an immune response (for example see Figure 32). The mice which received living, immunised lymphoid cells all showed a definite secondary response. The number of cells from each tissue was roughly the same but the response obtained from spleen cells was much
Adoptive secondary response in irradiated recipients (950 r.) injected with 74 million spleen cells intravenously, 6 weeks before injection of BGG-I$_{131}$. Same immunised donors used for the experiments illustrated in figures 34, 35 and 36.
Figure 34.

As figure 33, but 76 million lymph-node cells.
Figure 35.

As figure 33, but 68 million bone marrow cells.
Figure 36.

Combination of the means of figures 33-35 and the results of the passive immunisation calibration experiment, (figure 8).
Figure 37.
The effect of adoptive immunisation of irradiated CBA mice by two injection routes; intravenous and intraperitoneal.
greater than that obtained from bone marrow. The results are illustrated in Figures 33, 34, 35 and 36.

In a further experiment, aliquots of cells (55 \times 10^6) from another pool of immunised spleen cells were injected intra-venously into one group of irradiated mice and by the intraperitoneal route into another group. The recipients were challenged with BGG-\textsuperscript{131} 4 weeks later. A slightly greater response was obtained from those mice which were injected with cells by the intraperitoneal route. It therefore seems unlikely, that even if the slightly lower response of the mice injected with immunised lymph-node cells intraperitoneally was significant (see Figure 37) when compared with the response in mice receiving the same number of immunised spleen cells, that this difference could be due to the difference in the injection route. It is possible that the lower response found with lymph-node cells was due to the presence of the small number of non-immunised bone marrow cells. In Experiment 8 (below) it is shown that higher proportions of non-immunised cells mixed with immunised cells in an inoculum do decrease the response, but not by as much as the difference between the response obtained from spleen cells and from lymph-node cells. It therefore appears likely that the difference in response between spleen and lymph-node cells is a difference reflecting the two different tissues (in these experimental conditions).

It is possible to estimate the rate of antibody production by the same number of cells from different organs by comparing
Figure 38.
The effect of adoptive immunisation of non-irradiated recipients. Isologous transfer of cells. Numbers indicate millions of immunised spleen cells.
the results obtained in these experiments with those illustrated in Figure 8 (Part I). It can be seen that cell for cell spleen makes antibody in a secondary response about 8 times faster than bone marrow and nearly twice as fast as lymph-node cells.

3. **An adoptive secondary response in non-irradiated recipients**

Antibody production in an adoptive secondary response was measured in non-irradiated isologous and homologous recipients. CBA males which had been immunised in the usual way were killed and a suspension made of their spleen cells. Sixty million of these cells were injected intravenously into each of five CBA males and a further twenty million cells into each of a further two recipients. Sixty million of these cells were also injected intravenously into each of five A strain females. The recipients were challenged with BGG-\(\text{I}^{131}\) only 3 weeks after the cell transfers.

There was a marked secondary response in the CBA male recipients receiving 60 x \(10^6\) immunised cells but only a slight response in those receiving only 20 x \(10^5\) cells (see Figure 38). The response was much less than that obtained in lethally irradiated recipients receiving the same number of cells. The non-irradiated mice were challenged only 3 weeks after transplantation whereas the irradiated mice were challenged 4 to 8 weeks after transplantation. If both groups had been challenged at 6 weeks, it seems very likely, both from subsequent experiments and from the previous experiment (No. 1) in
Figure 39.
Adoptive immunisation of A strain mice with 60 million immunised CBA spleen cells. Recipients not irradiated. Half-life of BGG-I\textsuperscript{131} within control range (i.e. 3.2-3.8 days).
which actively acquired immunity diminished, that the difference would have been much greater. There was no detectable response whatsoever in the A strain mice which had been injected with immunised CBA cells (Figure 39). The injected CBA cells were probably destroyed in a homograft reaction (Mitchison 1957). If this is so, then this result confirms that obtained when heat killed spleen cells were injected into irradiated recipients. This result probably shows that the antibody synthesising mechanism is not transferred from the injected cells into the cells of the host. Cell-fixed and cell-free passive antibody transferred with the cells is shown not to influence the secondary response to a significant degree, although such an influence might be expected from Jerne's (1955) natural selection theory of antibody production.

4. An adoptive secondary response in sub-lethally irradiated mice

A similar experiment to that described in the previous section (Experiment 3) was carried out in sub-lethally irradiated (500 r.) CBA and A Sw mice. Fifty million CBA (male) immunised spleen cells were transplanted into five CBA recipients and three A Sw (male) recipients. The mice were challenged by an injection of 2 mgm BGG-\textsuperscript{131}I five weeks later.

A large secondary response was observed in the CBA recipients. This response seemed as great as that which would be obtained by the same number of cells transplanted into lethally irradiated recipients, although no direct controls were
Figure 40.
The effect of adoptive immunisation of sublethally irradiated CBA and A Sw mice (500 r.) with immunised cells from CBA donors.
carried out to verify this point. There was no detectable response in the three A Sw mice (see Figure 40). It seems likely that sufficient of the A Sw lymphoid tissue was undamaged by the irradiation to promote a homograft reaction against the immunised CBA cells. This result therefore confirms the results described in the previous sections.

5. **Primary response to bacterial antigen**

In subsequent experiments it was important to know when an irradiated mouse injected with lymphoid tissue would be more or less completely recolonised by the transplanted cells. The experiment described in this section was designed to answer that question on the basis of the recovery of the capacity to produce a primary response. The most reliable primary response appeared to be that elicited by bacterial antigens. *Salmonella typhi H* was chosen, as the titration of agglutinating antibody to this antigen has been found to be simple and reliable.

CBA mice were given 950 r. of X-irradiation and were then injected intravenously with $20 \times 10^6$ CBA spleen cells from untreated donors. The recipients were divided at random into four groups of five mice in each group. The first group were challenged with 0.2 ml of a standard suspension of *S. typhi H* one day after the transplantation of cells, the second group was challenged 8 days after transplantation, the third group 15 days later and the fourth group 25 days after transplantation. The mice were bled on the 1st, 3rd, 8th, 15th and 22nd days.
This diagram illustrates the experiment designed to show the recovery of the capacity to produce a primary response after transfer of normal cells (20 million CBA spleen) into lethally irradiated CBA recipients. The numbers represent the days between irradiation and cell transfers and challenge with 0.2 ml of a standard suspension of *Salmonella typhi H*. 

**Figure 41.**
after the injection of the bacteria. The agglutinin titre was measured.

At 8 days after antigen injection there is a marked difference between the four groups. At 15 days after the antigen injection mice which were injected with antigen 8 or more days after transplantation showed similar titres of antibody. The group of mice which were challenged only one day after the transplantation showed a marked inhibition of response even 22 days afterwards. Figure 41 illustrates this result.

As judged by the agglutinin titre at 8 days after antigen injection, it seems that the capacity to produce a primary response has largely recovered by a month after transplantation of 20 million cells. It is possible that the agglutinin titres at day 15 after antigen injection, are similar because antigen is stimulating the division of immunologically competent cells. It seems very likely that the titre at day 8 is a measure of the multiplication of immunologically competent cells in the irradiated host.

6. The effect of inoculum size

Antibody production in a secondary response was measured in lethally irradiated recipients six weeks after the transplantation of different numbers of immunised cells. Twenty CBA males were actively immunised against BCG in the usual way. They were killed 16 days after the last immunising injection
Figure 42.
The effect of inoculum size on the adoptive secondary response, 6 weeks after cell transfer. Each block represents the range of response in 5 CBA mice. Numbers represent millions of cells from a single pool.
Figure 43.

Combination of the means of the experiment illustrated in figure 42, with the means of the passive immunisation calibration experiment (figure 8).
and a pooled spleen cell suspension prepared. Recipient CBA males, three months old, were given 950 r. of X-irradiation. Five of these irradiated mice were injected intravenously with $74 \times 10^6$ cells, five received $37 \times 10^6$ cells, five received $18.5 \times 10^6$ cells and five received $9 \times 10^6$ cells. The four groups of mice were challenged six weeks later and the elimination of labelled antigen was followed for 10 days.

The results show that the response is graded according to the number of cells injected into the recipient mice. Those mice which received $74 \times 10^6$ cells gave the greatest response. Those which received $9 \times 10^6$ cells gave the least response, whilst the other groups were graded in between, giving responses appropriate to the number of cells injected. The results are illustrated in Figure 42.

The rate of antibody production in an adoptive secondary response is proportional to the number of cells transplanted. This can clearly be seen in Figure 43, where the results obtained in this experiment are compared with those obtained in the passive immunisation calibration experiment (Part I, Figure 8). It appears that a two fold reduction in the number of cells transplanted results in roughly a two fold reduction in the rate of antibody production six weeks later in an adoptive secondary response.

7. Effect of time of challenge

The rate of antibody production in an adoptive secondary
The effect of the time between adoptive immunisation and injection of BGG-I\textsubscript{131} into the recipients (950 r.). Each block represents the range of response in from 4 to 7 mice. Day 22 omitted to avoid confusion. The numbers represent days between cell transfer and injection of labelled antigen.

Figure 44.
Figure 45.
Combined means of figure 44 together with the means of the passive immunisation calibration experiment.
response was measured in lethally irradiated recipients at intervals after the transplantation of the same number of immunised spleen cells. The experiments were divided into three groups, different numbers of immunised cells and different times of challenge being used in each group.

In the first group of experiments, twelve immunised CBA males were killed and a pool of their spleen cells prepared. Forty-five CBA males, three months old, were irradiated with 950 r. of X-irradiation. They were each then injected intravenously with 30 million of the immunised spleen cells. The 45 recipients were then divided at random into one group of 10 mice and five groups of 7 mice in each group. The group of 10 mice was challenged with BGG-I\(^{131}\) one day after the transplantation of cells; of this group 5 mice died during the course of bleedings which followed. A group of 7 mice was challenged at 8 days after transplantation; of this group 4 mice died subsequently. The remaining 4 groups of 7 mice, were challenged at 15, 22, 29 and 43 days after transplantation. There were no radiation deaths in the last four groups. The death of mice in the groups challenged one and eight days after irradiation and transplantation was probably due to aggravation by bleeding of the effects of irradiation.

In a second group of experiments, larger numbers of cells were used and the time between transplantation and challenge was greater. Eight CBA males were irradiated and injected with \(75 \times 10^6\) immunised spleen cells intravenously. Five of
Figure 46.

As figure 44, but a larger number of cells (75 million instead of 30) and a longer time. Numbers represent weeks.
Figure 47.
Similar to figures 44 and 46, but 34 million cells. Numbers represent days (0 is 30 mins.). Each line is the mean of the responses obtained in five mice.
these mice were challenged 6 weeks later and the remaining 3 mice were challenged 4 weeks after that, that is, 10 weeks after the transplantation of the immunised cells.

In the third group of experiments, the irradiated recipients were injected with $34 \times 10^5$ immunised spleen cells and challenged at 30 minutes after transplantation and at 2 days, 4 days and 8 days after transplantation. The elimination of labelled antigen was followed for only 8 days in this experiment.

In all the experimental groups there was a progressive decrease in the secondary response with increasing time since the transplantation of cells. In the first group the fall in response was seen to be appreciable by the 8th day. By the 43rd day the response was very small indeed (see Figures 44 and 45). In the second group, in which larger numbers of cells were used, the response was still relatively large 10 weeks after transplantation but even so it was considerably less than at only 6 weeks after transplantation (see Figure 46). In the third group of experiments, a similar progressive decrease was observed, even within 48 hours of adoptive immunisation (Figure 47). It seems possible to conclude that the capacity for an adoptive secondary response progressively decreases during re-colonisation of the irradiated recipient. It also seems unlikely that this decrease is due to a homograft reaction due to segregation within the inbred line, as the decrease is detectable within 48 hours. The possible relationship between
cell division and the decreasing capacity to produce a secondary response will be discussed.

8. **The effect of mixing cells from immunised and non-immunised donors**

This experiment was very similar to the previous experiment in which the effect of inoculum size was investigated (Experiment 6). Four groups of irradiated mice were injected with $100 \times 10^6$ spleen cells by the intravenous route. This number of cells consisted of varying proportions of cells from a pool of immunised spleen cells and from a pool of non-immunised spleen cells. The first group of 5 irradiated recipients were injected with $100 \times 10^6$ non-immunised spleen cells. The second group were injected with $85 \times 10^6$ non-immunised cells plus $15 \times 10^6$ immunised cells. The third group received $50 \times 10^6$ non-immunised cells plus $50 \times 10^6$ immunised cells. The fourth group received $100 \times 10^6$ immunised cells. The recipients were challenged by an intravenous injection of 2 mgm BGG-131 8 weeks after the adoptive immunisation.

Unfortunately several mice died due to the effects of irradiation. A great deal of significance cannot be placed on these rather limited results, which, however, tend to show a graded response according to the number of immunised cells transferred, in much the same way as was shown in the previous experiment (No. 6), albeit there is a quantitative difference. In this experiment a two fold decrease in the number of immunised
Figure 48.
The effect of mixing cells from immunised and not immunised donors, in different proportions. Isologous transfers of cells into irradiated recipients (950 r.). The numbers and proportions of cells are indicated on the diagram.
cells resulted in a four-fold decrease in the rate of antibody production in the subsequent adoptive secondary response (Figure 48). In the previous experiment the same decrease in the number of immunised cells resulted in a two-fold decrease in the rate of antibody production. The difference in the decrease in rate of antibody production observed in the two experiments must therefore be due to the presence of non-immunised cells in the inoculum used in the experiments described in this section. Mixing equal numbers of immunised and non-immunised cells appears to halve the rate of antibody production by the immunised cells. It appears that there is competition for something by both types of cell and that the immunised cells are at an equal or less disadvantage than the non-immunised cells. The response obtained in the group of mice injected with only 15 million immunised cells plus 85 million non-immunised cells, was too small for an accurate estimate to be made of the rate of antibody production.

9. The effect of passive antibody on an adoptive secondary response

Fifteen CBA mice were irradiated (950 r.) and were then adoptively immunised with $14 \times 10^6$ spleen cells. These mice were then divided at random into three groups. Nine weeks later, the first group of mice were injected with 0.2 ml of CBA anti-BGG serum, the second group with 0.05 ml and the third group were retained as controls. One hour after the injection of antiserum, the mice were injected with 2 mgm BGG-$^{131}$.
Figure 49.
The effect of passive isologous antibody on the adoptive secondary response. CBA mice injected with 14 million immunised spleen cells two months previously. One group injected with 0.2 ml antiserum (ca. 1 mgm Ab protein/ml). Another group received 0.05 ml. C represents the control which received no antibody. Mean responses from 5 mice.
Figure 49 illustrates the slightly different responses obtained. It can be seen that the passive antibody very slightly increased the rate of antibody production in the secondary adoptive response, and also that a four fold difference in the amount of passive antibody resulted in much less than a two fold difference in the rate of antibody production. This result therefore makes it unlikely that the results of Experiment 5 (inoculum size) could be due to differences in the amount of passive antibody transferred with the cells.

DISCUSSION

In order to draw a conclusion from the results described in this part (Part III) of the thesis, it is necessary to assume that immunised cells can and do divide in the irradiated environment. The validity of this assumption is discussed first. If the assumption proves acceptable then it can be concluded that cell division causes no increase in a subsequent secondary response. This result is therefore compatible with those hypotheses which require that antigen must persist for a secondary response to take place.

Evidence that immunised cells divide in the irradiated recipients

There is no direct evidence that immunised cells divide, although a limited amount of evidence suggests that this is a likely occurrence.

The experiments described in Experiment 5 (Part III) and
the experiments of Makinodan, Gengozian and Congdon (1956) can be interpreted as showing that immunologically competent cells divide in an irradiated environment. Makinodan et al. injected equal numbers of isologous bone marrow cells into lethally irradiated mice. Groups of these mice were injected with heterologous (sheep) erythrocytes at intervals afterwards and the primary response to this antigen was then measured. When compared with non-irradiated controls, the primary response in mice injected a day after irradiation and transplantation of cells was markedly depressed. The primary response increased when the interval between transplantation and injection of antigen was increased, so that by one month after the transfer of cells the response had recovered to a level greater than 50% of that in the controls. When rat erythrocytes were used the rate of recovery of the capacity to produce a primary response was apparently slower. This result is fundamentally the same as that described earlier in this thesis (Experiment 5) in which Salmonella typhi H was used as the antigen. The results obtained using the bacterial antigen are complementary to those obtained by Mitchison (1956, 1957) whose results showed that cells producing antibody in a secondary response to a bacterial antigen recolonised irradiated hosts and could be detected by their continued production of antibody.

Ford, Hammerton, Barnes and Loutit (1956) and Ford, Ilbery and Loutit (1957) have shown that lethally irradiated mice injected with histologically identifiable bone marrow cells
later contained dividing cells of donor origin in their bone marrow tissues. Grabar, Courcon, Ilbery, Loutit and Merrill (1951) have shown that rat lymphoid cells are functional when injected into lethally irradiated mice, as judged by their ability to produce rat serum proteins. Makinodan (1956) and Anderson (1957) have shown that circulating rat cells are present in a similar situation. Furthermore, it has often been shown that the erythrocytes in some of these rat/mouse chimaeras are of rat origin. It seems from these results that homologous and closely related heterologous myeloid and lymphoid cells are capable of division and also of carrying out their normal physiological function when transplanted into irradiated hosts. It therefore seems very likely that these conclusions can be extended to isologous transplantations.

Urso and Congdon (1957) have measured the total number of bone marrow cells, relative number of circulating leucocytes and spleen weights at intervals after lethal irradiation and transplantation of different numbers of isologous bone marrow cells. For instance, they showed that after an injection of 12 million cells into lethally irradiated mice, the nucleated bone marrow cell count fell initially by a considerable amount but that the number of cells increased to more than 50% of that in the non-irradiated controls, in about a week. The numbers of circulating leucocytes also fell but took about a fortnight to reach 50% of that found in controls. Spleen weights also decreased immediately after treatment and took about a week to
recover to the same weight as the controls but afterwards became heavier and showed a considerably greater variation in weight than did the controls. Urso and Congdon also showed that recovery was quicker if the number of cells transplanted was increased.

To summarise so far, it seems that mouse and rat lymphoid cells can multiply in irradiated mice. The evidence also suggests that there is a great amount of cell division by the transplanted cells in recolonising the irradiated mouse. It seems from the results described in Experiment 5 and those of Makinodan et al. (1956) and Urso and Congdon (1957) that cell number recovers faster than the capacity to produce a primary response. It is possible that this is due to immunologically competent cells dividing more slowly than other types of lymphoid cells.

Although it can be seen that both lymphoid cells as a whole and immunologically competent cells in particular, divide in irradiated hosts, it has not been shown directly that immunised cells are equally capable of division as non-immunised cells. The results of Leduc, Coons and Connolly (1955) may possibly be interpreted as showing that immunised cells can and do divide in non-irradiated animals. It is quite possible, however, that the groups of cells producing antibody in a secondary response were in fact due to the division of immunisable cells during the induction period and not due to the division of cells after they had been induced to produce a
secondary response on subsequent contact with antigen. Yet another interpretation of their results is that the observed groups of antibody synthesising cells in a secondary response are due to migration of immunised cells into areas of lymphoid organs most suitable for the production of antibody. The results of Coons et al. (1956) are therefore compatible with the hypothesis that immunised cells can divide both in non-irradiated and irradiated environments. Unfortunately, from the point of view of this discussion they do not rule out several other explanations which are incompatible with this hypothesis.

**Competition between cells in the inoculum**

Immunised cells in the inoculum could possibly be at a selective disadvantage when compared with non-immunised cells in the inoculum, or in the host. However, after lethal doses of irradiation it seems unlikely that the host cells would be in any way able to compete with transplanted cells. Non-immunised cells might be able to compete more successfully than immunised cells in either of two ways: firstly, the advantage of non-immunised cells could be due to an incapacity of immunised cells to divide at the same rate as non-immunised cells, whilst in an irradiated environment; secondly, the disadvantage could be due to immunised cells competing unsuccessfully with non-immunised cells for "space" in lymphoid organs of the host.
If the selective disadvantage was merely a matter of slower rates of division, then the interpretation of the experiments designed to show the effect of challenge time in terms of replicating or non-replicating antibody synthesising mechanisms, would not be affected. If the immunised cells divided at all and the antibody synthesising mechanism was replicable, then an increase in the rate of antibody production would be expected. As will be shown later in this discussion, if a hypothesis requiring a replicating antibody synthesising mechanism is to be retained, it will have to be postulated that the selective disadvantage results in no division of immunised cells, or to their death.

Lymphoid cells may only be able to produce antibody successfully whilst in the environment of a lymphoid organ. One interpretation of the results described earlier in this part of the thesis, could be that immunised cells compete unsuccessfully with non-immunised cells for places in that environment. When cells from an immunised donor are transplanted into irradiated hosts, the secondary response is subsequently found to be greater than if the same number of cells had been transplanted into non-irradiated hosts. This is shown when Experiments 2, 6 and 7 are compared with Experiment 3 described in this thesis (Part III). The irradiation of the host kills off most of the host lymphoid cells thereby decreasing the amount of competition between the host cells and the introduced cells. However, the competition
between host (non-immunised) cells and transplanted (immunised) cells cannot have been completely one sided, as it was shown that immunised cells injected into a non-irradiated host did produce some antibody in a secondary response. Based on these criteria it is evident that the irradiated environment is more of an advantage than a disadvantage to transplanted cells. Similar results were obtained by Mitchison (1956, 1957) who adoptively immunised mice against Salmonella typhi I. He found that much greater agglutinin titres were obtained when immunised cells were transferred into irradiated hosts than when the same number of cells were transferred into non-irradiated hosts.

None of the experiments described so far have shown whether there is any competition between immunised and non-immunised cells in the inoculum, as opposed to competition between host cells and transplanted cells. Experiment 8 was designed to test the effect of mixing cells from immunised and non-immunised donors. By comparison with Experiment 6 it can be seen that the four fold difference in rate of antibody production that was observed, must be due to the non-immunised cells that were mixed with the immunised cells in the inoculum. The results suggest that the chance of an immunised cell finding a suitable niche was about the same as the chance of a non-immunised cell finding the same niche. It seems that the decrease in the rate of antibody production is due to the product of the decrease in total number of immunised cells and the decreasing
proportion of immunised cells to total number of cells. If immunised cells were at a marked disadvantage then the equation would be affected by a constant which would be greater than one. However, in the conditions of this experiment, this constant appears to be approximately one. This could be summarised as follows:

\[ R = \left( n_1 - n_2 \right) \cdot \left( p_1 - p_2 \right) \cdot \frac{1}{K} \cdot \frac{1}{T} \]

Where \( R \) is the rate of antibody production in a subsequent adoptive secondary response; \( n \) is the total number of immunised cells in the inoculum; \( p \) is the proportion of immunised cells to total cells; and \( K \) is the "disadvantage constant" of immunised cells. \( T \) is the time between adoptive immunisation and challenge. When \( K = 1 \), the immunised cells will not be at a disadvantage compared to non-immunised cells. This conclusion can be no more than a suggestion in view of the rather preliminary nature of the experiment.

In some of these experiments (see Figures 38 and 43) it can be seen that competition has led to a lower rate of antibody production. As has already been pointed out, this could be due to immunised cells not finding a suitable niche where they could produce antibody successfully. The cells unable to find a niche might either produce small amounts of antibody, or produce no antibody at all because of their dedifferentiation or death.
Interpretation of results

The literature cited so far makes it clear that there is no evidence that immunised cells do not divide in an irradiated environment. In some respects the irradiated environment may be more favourable for cell division or for the continued existence of immunised cells. It seems likely, therefore, that the transplanted immunised cells divide in the irradiated hosts. This assumption must provisionally be accepted if the following interpretation of the results obtained in the experiments described in this part of the thesis, is to be made.

It has been assumed that the smaller the number of cells in the inoculum the more these cells will have to divide in order to completely recolonise the irradiated host. The experiments of Urso and Congdon (1957) showed that on the basis of numbers of bone marrow cells, numbers of circulating leucocytes and on spleen weights, that there was a ceiling to the total number of cells in the lymphoid tissues of a mouse. If there was, on a rough estimation, 1000-2000 million lymphoid and myeloid cells in an adult mouse, it will be seen that 9 million cells would have to divide about 7 times and 74 million cells only about 4 times to fill the space occupied by the estimated number of cells; providing the space was vacated as a result of irradiation of the host. The evidence discussed earlier suggested that an irradiated mouse is almost completely recolonised one month after irradiation and transplantation. Some of the graphs presented by Urso and Congdon make it clear
that the rate of multiplication of the transplanted cells falls off as the mouse becomes more and more fully recolonised. In the experiment designed to show the effect of inoculum size the mice were challenged 6 weeks after irradiation and transplantation of immunised cells. At this time it would be expected that the immunised cells in the mice injected with 9 million cells would have completely recolonised their hosts and in doing so have divided about 3 times more than the cells in the mice injected with 74 million cells. The results clearly show that the rate of antibody production in a secondary response was proportional to the number of cells injected into the irradiated hosts. It is therefore clear that the capacity to produce a secondary response did not increase in proportion to the expected amount of cell division.

A hypothesis requiring a replicating antibody synthesising mechanism could be retained if it was postulated that the immunised cells divided at a constant rate during the 6 week period. It seems more likely that the rate of division would be proportional to the overall rate of division, which in turn seems to be related to the amount of recovery that has taken place (Urso and Congdon 1957). Furthermore, the experiments of Makinodan et al. (1956) and Experiment 5 show that mice are probably completely recolonised by a month after transplantation, if not earlier (Urso and Congdon 1957). It would therefore appear that by 6 weeks after transplantation the transplanted cells would have completely recolonised the host and the rate of
division correspondingly fallen off. The rate of cell division would probably decline earliest in those mice receiving the largest inoculum. At 6 weeks after transplantation, the total number of cells resulting from the original inoculum would be approximately the same in all the recipients irrespective of the original inoculum size.

The experiments designed to show the effect of the time of challenge of irradiated mice injected with immunised cells, confirmed the previous experiment. The response was in fact found to decline steadily from the time of transplantation. The steady decline in this capacity seems to imply that part of the antibody synthesising mechanism was being lost. Controls in which isologous antibody was injected showed that the decline was not due to loss of antibody; antibody very slightly increased the secondary response, but a four fold increase in passive antibody only increased the rate of antibody by about 1.2 fold. This was confirmed in a serial transfer experiment in which there was no response in the second stage recipients, although in a control experiment there was a secondary response in first stage recipients. The experiment (No. 1) which shows that the secondary response declines even after active immunisation, shows that the declining capacity for a secondary response to a protein antigen is confined to neither the irradiated environment nor to adoptive immunity in mice.

The results described in this part of the thesis can therefore be interpreted as evidence that the antibody
synthesising mechanism does not replicate. Hypotheses requiring a replicating mechanism might be rescued by postulating that in the conditions of these experiments the immunised cells are unable to divide, that they die, or that they are unable to replicate their antibody synthesising mechanisms.

**Theories of antibody production**

Replicating and non-replicating antibody synthesising mechanisms have already been mentioned several times. It is possible to divide the hypotheses of the mechanism of antibody production into other categories. Lederberg (1959) has defined two categories, "elective" and "instructive", based on whether the information required for antibody specificity is genetic or acquired. Elective theories propose that the information necessary for antibody specificity comes from the cell itself. Antigen in such a situation would merely switch on the production of the appropriate antibody. Instructive hypotheses on the other hand, propose that the necessary information is directly acquired from the antigen. The replicating/non-replicating classification can be combined with the elective/instructive classification. This is shown in the table below:
The clonal theory was first postulated by Talmage (1957) who discussed reasons for discarding Jerne's (1955) natural selection theory. The clonal theory has since been enlarged and amplified by Burnet (1957, 1959) and by Lederberg (1959) who has considered some of the genetic implications of this theory. The other hypotheses which require a replicating antibody synthesising mechanism were postulated by Burnet (1953) and by Schweet and Owen (1957). Burnet postulated the
existence of templates which were copies of the antigen and that these "genocopies" were capable of replication and the formation of antibodies. Schweet and Owen (1957) postulated that antigen entered the nucleus and there caused specific changes in the genetic material (DNA) of the antibody producing cells, which were heritable in the usual way. If the experimental conclusions of this section of the thesis are accepted, then all the theories discussed in this paragraph can be eliminated as being untenable. These experiments can help to decide in favour of the non-replicating hypotheses, but cannot help in a decision between elective and instructive hypotheses.

The evidence that persistence of antigen is required for a secondary response to take place

It has already been pointed out in the general introduction that the phases of antibody production can be oversimplified into two parts: the inductive phase and the phase of production and release of antibody into the circulation. It was also pointed out that a study of the secondary response might help to elucidate the mechanism of induction. The evidence discussed in the previous section suggests that the induced state of an antibody producing cell which enables that cell to synthesise a particular antibody is "non-replicating". As antibody production is in the first instance initiated by antigen, it seems reasonable to postulate that antigen or antigenic derivatives might persist to maintain the induced state. Such a hypothesis would be compatible with the evidence that the
antibody synthesising mechanism is non-replicating.

There is a considerable body of evidence to show that some antigens can persist for considerable lengths of time within an animal. For instance, Felton (1949) has shown that pneumococcal polysaccharides can persist in paralysed mice for many months. Heidelberger (1953) has shown that the same antigen can persist through an immune response. McMaster and Kruse (1951) and McMaster, Kruse, Sturm and Edwards (1954) showed that protein antigens were still detectable in mice 14 weeks and in rabbits 8 weeks after an immune response to these antigens had disappeared. Other workers have shown that some of these antigens persist intracellularly. Kaplan, Coons and Deans (1950) have shown this using the fluorescent antibody technique. Erickson, Armen and Libby (1953) have demonstrated the persistence of Tobacco Mosaic Virus intracellularly for up to 15 days after injection of the antigen into mice, by using electron microscope techniques. Haurowitz and Crampton (1952) have labelled protein antigens with radioactive isotopes such as $\text{I}^{131}$ and detected the radioactivity in liver up to 50 days after injection. In earlier experiments Haurowitz, Crampton, and Sowinski (1951) and Crampton and Haurowitz (1950) claimed that radioactivity, representing antigen, was first associated with the microsome fraction of the cells of the liver but that within a few hours the activity was then mostly associated with the mitochondria and the nuclei of the cells.

The main assumptions of all Burnet's later theories
(Burnet 1949, 1953, 1957, 1959) of antibody production has been the unlikelihood that antigen could persist for a matter of several years. Two well known examples of long lasting immunity were the persistence of immunity to measles for 65 years (Panum 1847) and to yellow fever for 75 years (Sawyer 1931). Burnet, Freeman, Jackson and Lush (1941) postulated that the long lasting immunity was due to virus persisting as a subclinical infection or in some latent form. Landsteiner (1946) put forward a similar hypothesis. The experiments of Traub (1936, 1939) may possibly show that the failure of mice to produce antibody to choriomeningitis virus may have been due to the development of latency by the virus. Smadel, Levy, Diercks and Cameron (1952) have shown that scrub typhus rickettsiae may persist for up to 12 months and in some instances when antibody has reached low titres, the rickettsiae may cease to be latent and disease symptoms may re-occur (Brill's disease). Similarly, Parker, Menon, Meridith and Woodward (1954) showed that the rickettsiae of Rocky Mountain Spotted Fever could persist for about a year. The phenomenon of recurrent Herpes and the rise and fall of circulating antibodies to the virus, is well known (Scott 1957). It seems reasonable therefore, to reject the argument of Burnet and Fenner (1949) and to postulate that long lasting immunity to viruses is due to latency of the virus, which persists in cells where it is unable to stimulate the production of antibody until, as the result of a stimulus, it loses its latency and is able to restimulate antibody production. In the case of latent Herpes it seems that the virus survives in
dermal cells, unless there is a cut or abrasion which can stimulate the release virus and start a fresh cycle of infection, antibody production and further latency of the virus, followed by the antibody level slowly declining, eventually enabling another cycle of events to take place.

Heidelberger (1953) has shown that immunity to pneumococcal polysaccharides may last for many years in humans. He also states that these polysaccharides are resistant to all mammalian enzymes. It is probably this lack of suitable enzymes for its catabolism, which allows the polysaccharide to persist, in situations where antigenic proteins would be expected to be broken down. This could well be the reason for the continued production of anti-pneumococcal antibodies over a period of many months, whereas anti-protein antibodies are produced at maximum rates for only a day or two.

Libby and Madison (1947) and Erickson, Armen and Libby (1953) have shown that the primary response declines with the disappearance of antigen (TMV). The persistence of the capacity to produce a secondary response may in fact be correlated with the persistence of antigenic determinant groups (haptens), rather than whole antigen molecules. Ingraham (1951a, 1951b) has shown that Sulphur$^{35}$ labelled sulphanilic acid coupled to BGG can be used as a hapten. The sulphanilic acid is not metabolised and Ingraham was able to show that the label was largely eliminated from liver and spleen with a relatively short half-life. However, a significant proportion
was retained in both liver and spleen and was eliminated with a very long half-life of about 40 days. He also showed that if the mice had been previously immunised the half-life in the liver was not markedly affected, whereas there was a significant increase in the half-life of the labelled hapten in the spleen. The hypothesis that it is hapten which persists is further supported by the early experiments of Topley (1930) who showed that 24 hours after being taken up by spleen cells, bacteria had apparently lost their antigenicity. More recently, similar experiments have been carried out by Walsh and Smith (1951), Fagreus and Grabar (1953) and Harris, Harris and Farber (1954). The experiments described in this thesis (Part III), Experiments 2, 3 and 4, confirm this. The time of radiation sensitivity of antibody producing cells suggests that the first stage of induction takes place within a few hours of the entry of antigen into the cell (Taliaferro and Taliaferro 1951). Simple chemicals cannot be haptenic unless coupled onto a molecule of large molecular weight (Landsteiner 1946). It follows, that if hapten must persist for the induced state to persist, then hapten must be transferred from the antigenic molecule to a receptor or site within the cell, as the first step in the process of induction of antibody formation.

It can be concluded that antigen can persist intracellularly, in some cases for a matter of years. The continued production of antibody over long periods seems to be positively related to the persistence of whole antigen in some
It is quite possible that the maintenance of the capacity to produce a secondary response (maintenance of the induced state) is similarly related to the persistence of hapten. It can be postulated that this hapten, persisting within the cell, perhaps in combination with a specific site, induces a physiological state which enables the cell to produce specific antibody.

If hapten must persist to maintain the induced state, it is possible that whole antigen is required to stimulate antibody synthesis in the induced cells. This would imply that antigen was bifunctional in the overall stimulation of antibody production. The plausibility of this concept is shown by two phenomena: firstly, there is the failure in certain instances of an animal to produce any antibody after a single injection of antigen, but these animals may show a typical secondary response when a second injection of antigen is made (Burnet 1959); secondly, there is the phenomenon of the non-specific secondary response. It seems that induction and production are separate phenomena, each stimulated by antigen in a different way.

The failure of antibody production after a first injection of antigen shows that antibody production cannot take place until induced cells are present in the animal. The production of antibody after a second injection shows that production normally takes place in response to an injection of antigen. The failure to produce antibody in a primary response would be
due to all the antigen having been metabolised before the phase of induction was complete. It would have to be postulated that in a primary response in which there was production of antibody, sufficient antigen was left over to stimulate the phase of production.

A secondary response could be defined as the increase in circulating antibody soon after stimulation, in an animal which has previously shown a primary response. A specific secondary response is elicited by a second injection of the same antigen used to elicit the primary response; such a secondary response usually results in a rapid and large increase in the amount of circulating antibody. A non-specific secondary response can be defined as a rise in circulating antibody to the antigen used to elicit the primary response, which is elicited by unrelated antigen or some other unrelated stimulus. The presence of a non-specific secondary response probably indicates that the stimulation of antibody production is a separate phenomenon from induction.

Prolonged bleeding may constitute a non-specific stimulus to antibody production. Several earlier workers claimed that the titre of bacterial agglutinins rose in immunised animals after prolonged bleeding, although other workers were unable to obtain similar results. More recently, Barr and Glenny (1952) showed that antitoxin titre was maintained in an immunised horse after prolonged bleeding. They showed that antitoxin must have been synthesised, at an increased rate, together with
other serum proteins, to replace those lost during bleeding. Monaco (1939) had obtained similar results in rabbits injected with typhoid vaccine 4 months previously, by subjecting the animals to low oxygen tension. This stimulus caused the production of all serum proteins including antibody. In both the experiments of Barr and Glenny and those of Monaco, the titre relative to the concentration of other serum proteins did not increase.

Wahl (1938) reviews examples of non-specific secondary responses elicited by injections of proteins; the rise in antibody titre was in all cases smaller and more irregular than that in a secondary response stimulated by an injection of specific antigen. Tsukahara (1921) and Boyse (1959) showed similar phenomena in the rise in bacterial and erythrocyte agglutinins.

It is possible that the rise in circulating antibody in a non-specific secondary response is merely due to the release of preformed antibody into the circulation. White and Dougherty (1944), Dougherty, White and Chase (1944), and Chase, White and Dougherty (1946) have shown that adrenal cortical hormones caused the dissolution of leucocytes with the release of antibody. Garvey and Campbell (1957) have shown that a small amount of antibody released during a specific secondary response was synthesised during the preceding primary response. Most of the antibody released into the circulation in a specific secondary response, however, is probably synthesised de novo in
response to the injection of antigen. This has been shown by the incorporation of radioactively labelled amino acids into the antibody molecules (Green and Anker 1951; Humphrey and McFarlane 1952; Bulman and Campbell 1953; Taliaferro and Taliaferro 1957; and Askonas, Simkin and Work 1957). Richter and Haurowitz (1960) have shown that even the first antibody released in a secondary response to a protein antigen is probably nearly all synthesised de novo. A study of the incorporation of amino acids into antibody during plasmapheresis has not been made. Until such an experiment is carried out it will not be possible to prove that a non-specific secondary response is due to synthesis of antibody rather than release of stored antibody. The maintenance of antibody titre after plasmapheresis is in favour of the hypothesis that antibody is synthesised in a non-specific secondary response.

To summarise, it can be stated that there is evidence that at least hapten must persist for the induced state to be maintained. Continuing antibody production can be related to the persistence of unmetabolised antigen. It is not unlikely that antigen has two roles to play in the stimulation of an antibody response: firstly, antigen may transfer determinant groups or haptens to specific sites within the antibody producing cell, thereby inducing within that cell a capacity to produce a specific antibody; secondly, antigen molecules may stimulate induced cells to synthesise specific antibody.
Evidence against the "instructive/non-replicating" category of hypothesis

It has already been concluded that all the hypotheses requiring a replicating antibody synthesising mechanism can be discarded. It can be seen in the Table, earlier in this discussion, that the non-replicating hypotheses can be subdivided into two categories: those which postulate that antigen acts in an instructive role and those which postulate that antigen acts in an elective role.

Ehrlich's (1900, 1906) "side-chain theory" was the first theory of the mechanism of antibody production. This theory, which would now be categorised as elective, required that every antibody specificity was predetermined and therefore that there was strict genetic control of antibody specificity. The early experiments of Landsteiner (see Landsteiner 1946, for review) showed that antibodies could be produced to a wide range of simple chemicals (normally not found in the body) when used as haptens. These antibodies were highly specific. In view of these findings, Breinl and Haurowitz (1930), Alexander (1932) and Mudd (1932) all independently postulated that the antigen acted as a template forming the specific site of the antibody. Pauling (1940) amplified this hypothesis to include the then current hypotheses of protein synthesis. The Haurowitz-Pauling template hypothesis has been very popular, possibly because at first sight it appears to be a monistic concept. Of more biological appeal was the fact that it was not necessary to
postulate that each antibody producing cell possessed a seemingly infinite store of information necessary to produce every conceivable antibody specificity.

However, the Haurowitz-Pauling template hypothesis is not now compatible with current hypotheses of the mechanism of protein synthesis. There is also evidence that antibody specificity is not determined secondarily, as was suggested by Pauling and Campbell (1942), after the gamma globulin molecule was formed (Heidelberger, Treffers, Schoenheimer, Ratner and Rittenberg 1942; Haurowitz, Schwerin and Tunc 1946). It is generally believed that protein molecular morphology is determined by amino acid sequence (Bailey and Sanger 1951) and furthermore, that the amino acid sequence is determined by the arrangement of base pairs in ribose nucleic acid molecules (Crick 1958). Except by postulating that antibody is synthesised by a unique mechanism, it is hard to see how the Haurowitz-Pauling template hypothesis can be retained.

Talmage (1957) has presented evidence to suggest that the Haurowitz-Pauling template hypothesis is unlikely on kinetic grounds. Calculations based on the experiments of Topley (1930) show that 1 molecule of antigen gave rise to enough antibody to agglutinate 600 bacteria (Hooker and Boyd 1931). This is about $1 \times 10^5$ antibody molecules/second/bacterium injected (Jerne 1955), which is about 1 antibody molecule/second/antigen molecule assuming that the entire bacterium was composed of antigen molecules and that all the bacteria
injected were utilised in antibody production. Similar calculations can be made from the experiments of Talmage, Freter and Thomson (1958) who used sheep erythrocytes as the antigen. They calculated that if all antibody was produced within 48 hours, that one antibody molecule would have to be produced every 8 seconds for every Forssman site injected into the rabbits. However, only about 1% of the antigen goes to the spleen (Ingraham 1955) where it gives rise to 90% of the antibody (Taliaferro and Taliaferro 1950). It follows that the rate of antibody production must be nearer 10 molecules/second/template. Furthermore, it seems unlikely, from experiments with labelled antigens, that more than a small proportion of the antigen which goes to the spleen, is in fact taken up by antibody producing cells. Most antigen seems to be taken up by reticulo-endothelial cells which have not been shown to produce antibodies. It follows that the rate of antibody production would have to be greater than 10 molecules of antibody/second/template. Available evidence is that protein synthesis is much slower than this figure (Borsook 1956). This conclusion is therefore incompatible with the Haurowitz-Pauling template hypothesis.

Further evidence against the template hypothesis comes from the experiments of Talmage, Baker and Akeson (1954) who showed that the release of antibody by antigen in a complex mixture under physiological conditions, took minutes rather than seconds. Jerne (1951) has shown that antibody released
in a secondary response is more avid than antibody produced in a primary response, whereas the specificity of the antibody appears to decrease in a secondary response. Similar results have been obtained by Farr (1958). Antibody production is faster in a secondary response than in a primary response. If the template hypothesis was tenable it would be expected that the avid antibody would be released more slowly than the less avid antibody produced in a primary response.

To summarise, it can be stated that the great weight of evidence is not compatible with the hypothesis that antigen or determinant groups act as morphological templates around which the complementariness of the antibody molecule is formed. It seems that the instructive role of antigen is most unlikely in the formation of antibody.

Evidence for the "elective/non-replicating" category of hypothesis

Of the four categories of hypothesis listed in the Table earlier in the discussion, the category of "elective/non-replicating" hypotheses are less well defined than the other groups of hypotheses. This is especially so when the possible molecular mechanisms involved in antibody synthesis are considered. Basically these hypotheses are merely re-statements of the experimental facts: namely that antigen entering the antibody producing cell induces a change in the cell which results after a time in the production of antibody. The antigen can be said to elect from all the antibody producing
capabilities of the cell, the one particular mechanism required to produce the specific antibody against that antigen.

The possible analogy between adaptive enzyme formation in bacteria and antibody production will now be considered. Monod (1959) and Pappenheimer, Scharff and Uhr (1959) have reviewed the analogy between these phenomena. The evidence that such an analogy is good can be briefly described as follows:

1. Both an adaptive enzyme and an antibody are synthesised de novo in response to exposure of the cell to inducer; antigen for antibody production (Taliaferro and Talmage 1955; and Green and Anker 1955) or substrate for adaptive enzyme formation (Monod, Pappenheimer and Cohen-Bazire 1952; Monod and Cohn 1953; Hogness, Cohn and Monod 1955; Rotman and Speigelman 1954).

2. Gamma Globulin specificity remains the same for different antibody specificities. A similar phenomenon has been described for adaptive enzymes by Cohn and Torriani (1952, 1953), who showed that a protein closely related to β-galactosidase existed in E. coli before induction of enzyme synthesis. It is evident that this protein (P₂) is no more a precursor of β-galactosidase than is normal gamma globulin a precursor of antibody.

3. A single molecule of inducer (antigen) may initiate the production of many molecules of product. The evidence for this with respect to antibody production has already been discussed. It has been shown to be true in relationship to
penicillinase production in *Bacillus cereus* by Pollock (1953); Pollock and Torriani (1953); and Pollock, Torriani and Tridgell (1954).

(4) Both adaptive enzymes and antibodies are large protein molecules, possessing a degree of complementariness for simple chemicals. For instance, Tanenbaum, Mage and Beiser (1959) have used several inducing substances as haptens. Antisera were prepared in this way to protocatechuic acid and B-galactoside. They then tested the cross-reaction of several analogous substances by means of the hapten-inhibition test. It was shown that the degree of haptenic cross-reaction closely paralleled the degree of cross-reaction of these substances in inducing the formation of B-galactosidase. This suggests that the method by which the information, embodied in the hapten or substrate (or substrate analogue), is utilised by the cell is basically the same for both antibody production and for adaptive enzyme formation. These results are compatible with the hypothesis that hapten combines with a specific site within the cell as the first step in the phase of induction.

One of the chief arguments against the validity of the analogy between adaptive enzyme formation and antibody production, is that there is a wide range of possible antibody specificities, whereas there is a limited number of adaptive enzymes known in bacteria. It is sometimes argued that an antibody producing cell could not possibly contain enough genetic material to control the specificity of every known antibody. This type of
argument has been used to support the clonal theory. Goebel and Avery (1929) and Goebel, Avery and Babers (1934) have shown that a single rabbit can produce antibodies to the following substances when they have been conjugated onto a protein: β-galactoside, α-galactoside, β-glucoside and α-glucoside. However, E. coli (strain ML) will only produce β-galactosidase. Furthermore it has been shown that a single mutational event can result in strains of bacteria which will not produce one carbohydrate but may produce another (Monod 1956). A one-gene one-enzyme hypothesis is more or less compatible with the experimental facts of adaptive enzyme formation. There is no basis at all for a one-gene one-antibody hypothesis.

A further argument against the analogy between antibody production and adaptive enzyme formation, is that enzymes are homogeneous whereas antibodies are heterogeneous, even with regard to their binding of haptens. Cohn and Monod (1953) and Cohn (1959) have claimed to have demonstrated the homogeneity of induced enzymes, which appears to be independent of whether or not a substrate or an analogue was used to induce the formation of the enzyme. The heterogeneity of proteins in general has been reviewed by Colvin, Smith and Cook (1954) and Hill, Kimmel and Smith (1959). The homogeneity of adaptive enzymes is, however, based on functional rather than physical criteria. The functional heterogeneity of antibodies has been estimated by their binding of haptens by Pauling, Pressman and Grossberg (1944); Pauling and Pressman (1945) and Karush (1956),
who showed that the variation in free energy of binding followed a normal (gaussian) distribution. The heterogeneity of antibody has been reviewed by Talmage (1957a); Owen (1954) and Wurmsen and Filitti-Wurmsen (1957). Foster (1957) has enlarged on Jerne's (1951) findings that differences in avidity exist between antibody produced in a primary and a secondary response, by showing that difference in lytic capacity can exist, even though the sera may have the same agglutinating power.

It seems that the analogy between adaptive enzymes and antibodies is useful. The two mechanisms probably differ in complexity, which might account for many of the anomalies observed between the two phenomena.

The genetic basis of antibody production

It has already been pointed out that one of the chief criticisms of the analogy between adaptive enzyme formation and antibody production is that there is a precise genetic basis for the specificity of the former, but not for the latter. It is argued that an antibody producing cell could not possibly contain enough information to synthesise all possible antibody specificities. The work of Landsteiner (1946) lent weight to this hypothesis when it was shown that artificial haptens could elicit specific antibodies. This line of argument can be weakened if the argument of Haurowitz (1956) is accepted, that less than 50,000 and possibly only 10,000 distinct antibody
specificities would be enough to give all the known antibodies. Furthermore, Talmage (1959) has pointed out that an economy could be effected in the amount of genetic information necessary, if there was some mechanism whereby the necessary genetic information was coded. Finally, there is one clearly defined case in which an anti-hapten antibody cross-reacted with a naturally occurring antigen (Kabat, 1937). This might dispose of any argument which held that elective hypotheses are impossible because artificial haptens do not occur naturally.

There is a considerable amount of evidence that the capacity to produce high or low titres of antibody is highly heritable. This has been demonstrated on the basis of resistance to tuberculosis by Wright and Lewis (1921) in guinea pigs; Webster (1933a, 1933b, 1937) in mice; and by Lurie and Zappasodi (1939) in rabbits. Study of variability in antibody response in inbred lines has shown that there is a genetic basis for much of the variability of response. This was shown in guinea pigs by Lewis and Loomis (1923); in rabbits by Lurie (1938, 1941); and in mice by Gorer and Schultz (1938), Davidson and Stern (1949) and Fink and Quinn (1953). Scheibel (1943) selected for and against the ability to produce antitoxin to a standard dose of diptheria toxin, and claimed that there was a single gene control over the ability to produce antitoxin. Similar claims by Kleczkowska and Kleczkowski (1939), who used small doses of human serum in rabbits, seem to be without factual basis. Chase (1941) has shown that susceptibility to 2:4 di-
nitrochlorobenzene is highly heritable in guinea pigs. Sang and Sobey (1953); Sobey (1954); Sobey and Adams (1955); Sobey, Adams, and Claringbold (1956); and Sobey and Adams (1959) have all demonstrated the heritability of the antibody response. It can be concluded that genetic control of the antibody response is indisputable. It is not clear from any of the evidence, however, that this genetic control extends to antibody specificity. The experiments of Schneibel (1943) and Chase (1941) are certainly compatible with the hypothesis that there is strict genetic control over antibody specificity.

It seems that there is a fundamental difference between the mechanism of adaptive enzyme formation and antibody production, especially with regard to the genetic control of these mechanisms. The fact that such a difference exists by no means rules out the possibility that there is a strict, though complex, genetic control over antibody specificity. As a tentative speculation it is reasonable to suppose that if a zygote contains all the possible potentialities of all the different cells in an adult animal, that the immature antibody producing cell can similarly contain all the potentialities for producing all the different antibodies that the animal as a whole can produce. *A priori*, it does not seem necessary to postulate that because there are so many possible antibodies that an instructive hypothesis or a clonal hypothesis are necessary.
Conclusion

The results described in this part of the thesis together with much of the evidence mentioned in the discussion, are not incompatible with the hypothesis that antibody production is an adaptive phenomenon. The analogy between adaptive enzyme formation in bacteria and antibody production appears to be reasonable, although there are differences of complexity between the two phenomena.
GENERAL DISCUSSION

Antibody synthesis has been compared with induced (adaptive) enzyme synthesis in bacteria by Monod (1959) and by Pappenheimer, Scharff and Uhr (1959), while Needham (1955) and Cohn (1958) have made comparisons between differentiation seen in animal cells and differentiation of a population of bacteria, with respect to their synthesis of an induced enzyme. Pollock (1958) has even compared the "dedifferentiation" (modulation) of mammalian cells grown in vitro with dedifferentiation of a population of bacteria. It therefore seems that there is some basis for considering antibody synthesis as an aspect of cellular differentiation in the widest sense.

In the most general terms, cellular differentiation can be defined as the cellular changes through which a cell passes in becoming specialised to carry out a particular function. Here, differentiation is considered as a process rather than the end product of a process. Experiment and consequently theory has concentrated on examples from the fields of embryogenesis and morphogenesis; for instance the series of changes between zygote and muscle cell, or zygote and nerve cell.

The capacity of a cell to produce antibody can be regarded as the final stage in a process of differentiation which results in a cell that is capable of producing a particular antibody. In this way antigen (determinant group or hapten) can be represented as the specific inducer of the series of
changes through which the "immature" antibody producing cell passes in becoming capable of producing the appropriate antibody.

There are several well known examples of relatively simple chemical substances acting as inducers of differentiation processes. For example, secondary sexual characters can be induced to develop by sex hormones, or overall growth rates affected by thyroxine (see Burrows 1949; and White, Handler, Smith and Stetten 1954). Morphogenetic changes in slime moulds seem to be induced by a complex of relatively simple chemicals diffusing from some of the cells (Bonner 1952; Sussman 1958). Substances diffusing from cell to cell may be responsible for embryonic induction (evocation) (see Holtfreter 1948; Yamada 1958). It is possible that some substances inducing embryonic or morphogenetic changes may be macromolecular in character, and can only pass from one cell to another if there is continuity of the cytoplasm (Grobstein 1956; Weiss 1953), although Grobstein and Dalton (1957) and Niu (1956) have shown that some of these substances are of low molecular weight.

Hammerling (1953) has shown that in the unicellular alga Acetabularia, the morphology of the "hat" is controlled by the nucleus. After removal of the "hat", macromolecular substances diffuse from the nucleus and are thought to affect the specific enzymes which control the morphology of the "hat". It has been suggested that these substances are ribonucleoproteins (RNA). If this were so, and RNA was the template responsible for the
synthesis of enzymes and other proteins (Crick 1958) necessary for the regeneration of the "hat", then it could be argued that the RNA was not the inducer, but was in fact the first product of the differentiated state. It seems possible that the macromolecular substances presumed to pass from cell to cell by Grobstein (1956) are also templates which synthesise enzymes. It therefore follows, on this basis, that induction is the process which stimulates the formation of templates by the nucleus. Observed differentiational changes would therefore be mediated through enzymes synthesised by these templates. Certain differentiational "changes" may be able to pass from one cell to another if templates can pass from cell to cell. The spread of melanin forming capacity in guinea-pig skin, observed by Billingham and Medawar (1948) may be an example of this.

If differentiation is the result of a specific switching on of template synthesis, then it would not be necessary to postulate that differentiation involved changes in the genetic material, as postulated by Morgan (1934). The experiments of King and Briggs (1956) may support Morgan's hypothesis. Their experiments are not conclusive however, as cytoplasm was transferred with the nuclei, in their nuclear transfer experiments. The earlier work of Spemann (1938) gave results which were incompatible with Morgan's hypothesis. Nuclear differentiation is well known to take place in *Ascaris sp.* (see Mather 1948), in which the two chromosomes of the zygote break up into fragments
in some of the cells of the embryo. However, it does not appear that this process involves mutational like changes. Cytological studies of polytene chromosomes in diptera have shown that the banding is the same in the cells of different tissues (see Gall 1958). However, it has been shown that Balbiani Rings (puffs) may be associated with different bands at different times in the development of a diptera larva. It has been suggested that the puffs represent specific syntheses by the genetic material. If the substances being synthesised were RNA templates, then it could be postulated that induction was a process affecting this synthesis.

Early hypotheses of the mechanism of adaptive enzyme synthesis have been discussed by Pollock (1953), Monod (1956) and Pollock and Mandelstram (1958). All these theories took pains to eliminate the possibility that adaptation was due to a process of deinhibition (specific inhibition of an inhibitor) (Vogel 1957). However, Pardee, Jacob and Monod (1959) have presented evidence in support of Vogel's hypothesis in the case of the adaptive synthesis of β-galactosidase in *E. coli*. Monod and Cohen-Bazire (1953), Cohn, Cohen and Monod (1953) and Vogel (1957) showed that the presence of an amino acid which was being synthesised by a series of enzyme catalysed steps could inhibit the synthesis of one of the enzymes necessary for its synthesis. This biochemical example of feedback control has been called 'enzyme repression'. It seems possible that the amino acid combines with a specific "receptor" to form the "repressor" which interferes with the synthesis of the enzyme, possibly by
interfering with template synthesis at the level of the gene. Vogel (1957) postulated that an adaptive process could be due to the repression of a repressor normally present in the cell. The experimental evidence of Pardee et al. (1959) shows that the experimental facts of β-galactosidase formation in E. coli are compatible with Vogel's hypothesis. The hypothesis has been expanded by Szilard (1960).

Vogel (1958) drew a parallel between cellular differentiation and enzyme repression in bacteria. Szilard has extended this to antibody production. It is possible to state this hypothesis of differentiation in general terms. It can be postulated that most of the template synthesising potentialities of the nucleus are normally repressed, by complete repressors synthesised by the nucleus. Constitutive formation of an enzyme or antibody could be due to a genetic absence of this repressor. The nucleus is also postulated to synthesize specific receptors (template plus specific site?) which can combine with substrates (haptens or inducers) to form repressors of the formation of the repressor of template synthesis.

If differentiation can be interpreted in terms of a repression model, then the stability of a differentiated state can be interpreted in terms of cycles of induced syntheses (Hinshelwood 1952, 1953; Pollock 1953, 1958; and Buttung, unpublished). The stable states might be due to the inducer being a by-product of one of the syntheses that it induces. Once the cycle of events has started, it would tend to continue
if in equilibrium with other processes in the cell. Stability might be a result of endogenous inducers, whilst change to another differentiated state might be due to inducers of exogenous origin. It is relatively simple to remove exogenous inducers, for instance in vitro; this may account for modulations of mammalian or bird cells grown in vitro. The dedifferentiation of bacteria, with respect to their capacity to produce an induced enzyme would also come into this category (Pollock 1958).

It is possible that certain differentiated states are more basic than others. For example, an erythrocyte is quite incapable of any differentiational change, yet a connective tissue cell may have two stable modulations, the fibroblast and the macrophage (Bloom 1937). Such a modulation, or possibly also of mammalian cells in vitro, might represent two stable equilibria in the synthetic mechanisms of the cell. Heat shocks can initiate changes from one stable state to another in Paramecium aurelia (Beale 1954). It is possible that an equilibrium of enzyme catalysed syntheses might shift from one stable state to another as a result of templates passing into the cell and synthesising enzymes. This might be another explanation of the phenomenon observed by Grobstein (1956) and Billingham and Medawar (1948). In such a system the template would be a "modulator" rather than an "inducer". The experimental facts of antibody production are compatible with the hypothesis that antigen (determinant groups) act as true inducers causing the production of a new specificity, rather than
upsetting the balance of the overall synthesis of antibodies by the cell, which would be expected if antibody production was the result of a modulation. Acquired immunological tolerance as well as antibody production can be interpreted in terms of repression and derepression phenomena by postulating that antibody production is stimulated by a uni-site receptor and tolerance by a multi-site receptor.

One might consider that in evolutionary terms, antibody production is an adaptation to inducers of foreign origin. Selection for versatility has led to a system which can respond to inducers which the system has never met before at any time in its evolution, although it may have come in contact with structural analogues. Tolerance may have evolved as a specific mechanism to prevent antibody production to an animal's own proteins or polysaccharides. Such a tolerance mechanism would be elective and it would be expected from this, that the genetic basis of the inducibility of tolerance would differ from the genetic basis of the inducibility of antibody production.

It was once thought that adaptive enzyme formation was a relatively simple mechanism. It is now realised that the mechanism is extremely complex. It seems therefore, by analogy, that hypotheses of antibody formation and tolerance, already growing more complex, must become even more so, in order to be compatible with the growing volume of relevant experimental facts. It seems reasonable to state that in the future, basically simple hypotheses of antibody formation, such as the
Haurowitz-Pauling template hypothesis, or even the clonal hypothesis, will not be of much value.
Part I

1. Bovine gamma globulin (BGG) has been successfully labelled with iodine$^{131}$ by Method 2, a modification of the method of Wormal.

2. BGG-$I^{131}$ was eliminated from the circulation of CBA mice with a half-life of about 4.0 days.

3. BSA-$I^{131}$ and HSA-$I^{131}$ were eliminated from the circulation of CBA mice with a half-life of about 20 hours.

4. The antigen-elimination technique using BGG-$I^{131}$ could detect a wide range of antibody responses in CBA mice. The technique was made quantitative in passive immunisation experiments.

5. BGG did not elicit an immune response in CBA mice unless accompanied by a non-specific stimulus. BGG is said to lack "adjuvanticity" in CBA mice.

6. Denatured BGG, precipitated BGG or BGG incorporated in an emulsion (Freund's adjuvant), successfully immunised CBA mice.

7. An intravenous (iv) injection of untreated BGG could elicit an immune response if a subcutaneous injection of an adjuvant substance was made at the same time.
8. A wide range of lipids and lipidophilic substances were found to have adjuvanticity.

9. There was no relationship between Reticulo-Endothelial System (RES) stimulants and adjuvanticity.

10. Possible mechanisms of adjuvant action were discussed.

11. The relationship between adjuvanticity and auto-immunity was briefly discussed.

Part II

12. Tolerance was induced to BGG by injections of relatively small quantities of BGG into neonatal CBA mice.

13. The long term maintenance of tolerance was shown to be related to the persistence of extracellular antigen.

14. Cell division in the absence of antigen was shown to decrease the degree of tolerance in a mouse. This probably shows that tolerant state of a cell is induced by intracellular antigen.

15. Paralysis was induced in adult mice, by relatively large doses of antigen. Irradiation of the mouse increases the degree of paralysis obtained with a given dose of antigen.

16. The relationship between tolerance and paralysis was discussed. It was concluded that they are probably aspects of the same phenomenon.
17. Tolerance (= paralysis) is thought to be an induced state, induced by a minimum threshold concentration of antigen.

18. An analogy between adaptive enzyme formation (enzyme repression) and tolerance was briefly discussed.

Part III

19. The evidence that antibody producing cells divide in the irradiated environment was discussed. The assumption that they do was accepted as a basis for further discussion.

20. It follows that the experiments show that cell division does not increase the rate of antibody synthesis in a subsequent secondary response. Such an increase would have been expected if the Clonal Hypothesis was tenable. The results are therefore compatible with hypotheses which postulate that:

   (a) antigen must persist for a secondary response to take place;
   (b) antibody synthesising mechanisms are non-replicating.

21. The evidence that antigen persists in order that a secondary response can take place, was discussed.

22. The evidence against the Haurowitz-Pauling template hypothesis was discussed. Such evidence is kinetic, based on the rate of antibody synthesis per molecule of antigen taken up by the antibody producing cells. This hypothesis was also
considered to be incompatible with current hypotheses of protein synthesis.

23. The evidence that antibody production is an "elective" mechanism was discussed.

24. The parallel between adaptive enzyme formation (enzyme repression) and antibody formation was discussed. The genetic basis of antibody production was also mentioned.

25. It was concluded that antibody production is an adaptive phenomenon with similarities to well known examples of cellular differentiation. It was thought that there is a precise but complex genetic control of antibody specificity.
POSSIBLE LINES FOR FURTHER RESEARCH

Some of the conclusions arrived at in this thesis have led to the formulation of various working hypotheses. Several workers have postulated mechanisms of antibody production, based on analogies with adaptive enzyme formation or enzyme repression. It is now possible to design experiments which can help to decide whether or not any of these working hypotheses are compatible with the facts, as defined in these experiments. The possible experiments can most easily be summarised as a series of questions.

I - Concerning adjuvanticity

What are the cytological effects of non-ionic detergent adjuvants? Do they stimulate the maturation of plasma cells? Do they stimulate cell division in any particular cell type? Do they increase pinocytosis in any cell type?

Is cell division a result of induction of the capacity to produce antibody, or is cell division a prerequisite of inducibility?

Do adjuvants, other than those containing mycobacteria, induce delayed hypersensitivity?

How does antigen stimulate the production of antibody in a secondary response?

How many auto-immune diseases can be related to the gain of adjuvanticity by tissues which do not normally have
adjuvanticity nor have induced tolerance? Can any naturally occurring auto-immune diseases be related to a metabolic disorder involving any lipid or lipidophilic substance?

II - Concerning tolerance

What is the nature of the genetic control of the inducibility of tolerance?

What is the relationship between tolerance and antibody production as far as quantity of antigen is concerned? Can tolerance be explained in terms of a multi-site receptor (repressor) and antibody production in terms of a uni-site receptor?

Is tolerance an immunological phenomenon? In a state of tolerance, do highly specific enzymes break down tolerated substances before they can reach the antibody inducing site? Can tolerance be explained as a specific inhibition of part of the mechanism of the production of a particular antibody?

Can histocompatibility antigenic substances be extracted from tissues in a soluble form and used to 'paralyse' adult animals? Is the environment of a neonatal animal favourable for the release of histocompatibility substance? How far can the ease of induction of tolerance in young animals be related to the failure of these animals to produce antibodies?

Does the neonatal environment, as suggested by Dixon and Weigle (1957) prevent the phase of induction of otherwise
mature cells? Does an adult environment induce a state of differentiation which allows specific induction of antibody production to take place? If so, is the embryo environment similar to an in vitro environment? What is the factor present in an adult which allows induction to take place? Is it a chemical or contact with certain neighbouring cells?

III - Concerning antibody production

What is the effect of a long term and intensive selection for and against antibody production to one purified antigenic substance? Is the resulting difference in level of antibody production related to the production of antibody to any other antigen? Does such a long term selection affect any other detectable character of the animal? Can the genetics of antibody production be explained in terms of a wide spectrum of specificities, each with its own receptor, a wide band of which is activated even by a simple hapten?

What is the rate of turnover of templates (RNA)?

What is the rate of increase in antibody production in a primary response? Can this acceleration of antibody production be related to RNA (template) synthesis?

Is the rate of antibody production in a secondary response always constant, or is there an acceleration at a time normally associated with the acceleration of antibody production in a primary response?
Do antibody producing cells normally only produce one specificity of antibody? Is this because the period of inducibility in any one cell is of short duration? (Nossal and Lederberg 1958, and Attardi, Cohn, Horibata and Lennox 1959.)

What is the relationship of antibody specificity to gamma-globulin specificity? Are the two parts of the molecule (Porter 1959) synthesised separately? Can different types of antibody (Askonas, Humphrey and Porter 1956) be related to differences in the gamma-globulin part of the molecule, and can these differences be clearly related to different cell types?
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