

**Tolerance and Adjuvant Effects in Mice.**

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## Preface .

During the past twenty years the formulation of many biological concepts have been greatly influenced by the growth to prominence of new branches of mathematics, physics and biology concerned with the analysis and control of complex structures.

It has become axiomatic that structures displaying a certain degree of autonomous directive activity may profitably be considered as systems with certain inherent constraints upon their activities. These systems may in turn be subject to further constraints that determine the behaviour of other interacting systems. Such constraints may, for example, be considered as determining, by feedback control mechanisms, the size or complexity of a self-regulating system, or maintaining its physical or homeostatic stability in the face of conflicts in the external environment.

It is now evident that the immune system may be considered as part of a general system composed of discrete but often interacting entities each with inherent controls in addition to those imposed 'from above'.

One of the properties of such closely interrelated systems is that a change in one of the parameters may affect the values of others. The effects induced by changes in the variables studied do not necessarily prove a direct cause-effect relationship. The possibility exists that alterations in variables other than those under study could be responsible for the effect observed. The converse is also true. Evidence of activation or even involvement of a particular system does not necessarily imply that it is required in the production of a particular phenomenon, nor does it validate further generalisation to the involvement of this system under any conditions except those identical to the conditions under which it was initially observed.

The steps leading to the induction of antibody must involve a morass of infinitely elaborate, interdependent processes each, to a greater or lesser extent, determining the final result.

It was with due regard to the complexity of biological systems that this study was undertaken and its tentative conclusions reached. Its main concerns are the mechanisms of humoral antibody formation and tolerance induction and the effect upon these of certain bacterial adjuvants.

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## SUMMARY.

One of the main aims of this thesis was to investigate the relationship between tolerance and immunity. In order to do so the response of CBA mice to bovine serum albumin (BSA) was examined and a reproducible model system derived for the induction of tolerance to a single low dose of the antigen. The tolerant state so induced was examined with respect to the quantity, quality and class of antibodies formed.

The effect of the physical state of the antigen upon the dose threshold for tolerance induction and immunity was investigated. Attempts were made to correlate the response induced with the localisation of the antigen preparations within the lymphoreticular tissues.

The examination of the effect of the genetic background of the animals upon the response to BSA and upon the threshold for tolerance induction employed four inbred strains of mice and their  $F_1$  hybrids. Included among these strains were NZB mice which were of particular interest in view of their lymphoreticular abnormalities and their tendency to develop various autoimmune phenomena.

The relationship between tolerance and immunity was further investigated by the use of adjuvants, in particular C.parvum. The ability of these adjuvants to prevent the induction of tolerance to centrifuged BSA was examined as was the effect of C.parvum upon the lymphoreticular tissues. The findings are discussed with relation to the adjuvant effect of C.parvum and also to the haematological changes it induces, including transitory immune reactivity directed against autologous red cells.

## GENERAL INTRODUCTION

It is only since the 1950's that the biological system for the production of antibodies has come to be viewed as an integrated whole. Prior to this, the gross biochemical characteristics of this class of proteins had been defined and the nature of their specificity been shown to depend upon specific chemical groupings on the antigen. It was a main concern of early theories of antibody formation to relate this information to the in vivo morphological and cellular changes that were known to be brought about as a result of introduction into the body of a wide variety of foreign materials, and that were shown to correlate with the appearance of specific antibody in the serum.

There is now overwhelming evidence that, despite its limitations, the scheme for protein synthesis developed for relatively simple bacterial cells, is also essentially correct for the mammalian cell. Enzymic and sequential amino acid analysis of antibody molecules has supported the concept that the sequence of the amino acids, and hence the structure of the antibody molecule, has an equivalent pattern coded for in the form of relatively stable genetic material. The data which emerged has removed from general consideration those theories of antibody formation that do not have a strict genetical basis, and in addition has given new insight into the concept of antibody specificity.

In spite of these advances, one of the most elusive and least understood factors in the immune response continues to be the self-evident role of antigen in its initiation and probably also its control. It would thus seem pertinent to study the sequential fate of antigen within the body as an initial step towards understanding the processes involved in the production of antibody which are the subject of this thesis.

1. The reticuloendothelial system and the fate of antigen.

a. Historical.

The vital role of phagocytes in removing foreign substances from the tissue fluids has been appreciated since the classical studies of Metchnikoff (1905). Even before this time the phenomenon of phagocytosis had been observed by Wyssokowitach (1886). The cells, both free and fixed in tissue that were pre-~~eminent~~ in their capacity to take up vital dyes, were grouped together by Aschoff (1924) into the reticuloendothelial system, or RES. This classification has survived until recently when a new proposal was made that only the very highly phagocytic mononuclear cells and their precursors should be considered as a system, under the term mononuclear phagocytic system, or MPS (Langevoort et al., 1970). This new classification takes into better account the differences between vital dyes and suspensoids in the rapidity of their uptake and their final microscopic distribution (see Cappel, 1929). As well as being a functionally related system, the MPS includes only those cell types related to, or thought to be related to, the blood monocyte. This definition excludes dendritic, reticular, endothelial, and fibrocytic cells which were included by Aschoff in his broadest classification of the RES. As this system is not yet universally accepted, reference will be made only to the reticuloendothelial system, in spite of the fact that in certain instances only actively phagocytic cells may be involved in the processes described.

The first attempts to follow the fate of a soluble antigen in the body appears to come from Metchnikoff (1897). He used toxicity as a tracer and studied the fixation of tetanus toxin in the tissues of the chicken. He noted, however, that organs rich in blood could not be quantitated properly for their antigen content. By doing careful toxicity assays on washed organ minces, Wolf-Eisner (1908) helped to

overcome this obstacle. Rabbits were shown to concentrate tetanus antigen in the spleen, liver, bone marrow, kidney and lungs; findings later to be confirmed using a variety of techniques.

Microscopic location of antigen within the cells of the RES was first described by Sabin (1939). Injecting R-salt-azo-benzidine-azo-egg-albumin into rabbits, she used the evidence of the dye as an in vivo marker for the presence of antigen. The dark red colour was detected in the vacuoles of fixed tissue macrophages, Kupffer cells, polymorphonuclear leucocytes, monocytes, and cells lining the lymphatic vessels. Sabin also described 'shedding' of cytoplasm by dye-containing cells, which appeared to coincide with the appearance of demonstrable serum antibodies.

Of the many methods used since to investigate the fate and localisation of antigens in vivo, radioactively-labelled antigens have yielded the most complete data. This method was first used by Libby and Madison (1947) to study the distribution of  $^{32}\text{P}$ -labelled tobacco mosaic virus injected into mice. Since then, workers in this field have tended to use radioiodinated soluble antigens. The justification for supposing that the presence of label reflects the presence of undegraded or partially degraded antigen has been expounded by Campbell and Garvey (1963) and Humphrey et al. (1967).

b. Localization of antigen in vivo.

The most complete studies on the characteristics of this process come from Nossal and his co-workers in Melbourne. They used, as antigen, microgram quantities of  $^{125}\text{I}$ - or  $^{131}\text{I}$ -labelled S. adelaide flagella, either in its natural form or as purified monomeric or polymeric flagellin.

After injection into the foot pad of the rat, most of the antigen was found to pass through the draining lymph node. As little as 1-2% of the injected antigen was found at any time to be retained in the lymphoid system, the majority of antigen being excreted within the first 24 hours (Nossal et al., 1963; Ada et al., 1964). These findings are in good agreement with those of other workers in a variety of other systems (see Campbell and Garvey, 1963).

Autoradiography on the draining lymph node of the injected rats showed the majority of retained antigen to be within the medullary macrophages. A smaller quantity was associated with dendritic cells in the follicles. After a few days the label in the medullary regions was found to disappear whereas the label in the follicles diminished more gradually over a period of several weeks.

The majority of label in the medullary macrophages was enclosed within vacuoles and phagolysosomes, as judged by electron microscopy (Nossal et al., 1968 ). The much smaller quantity of antigen free in the cytoplasm of these cells was considered to be rapidly incorporated into membrane bound inclusions.

In contrast, the label in the follicles was thought to be, in the main, on the surfaces of dendritic cell processes which lay in close proximity to lymphocyte membranes. Very few labelled lymphocytes per se were found in these studies, nor was label seen associated with areas of plasmacytosis (Nossal et al., 1968a).

Although injected substances vary amongst themselves in the sites they occupy within the body, and the rate at which they are taken up, the sequence of events described above, with only a few qualitative differences, has been established as occurring after the injection of a wide variety of soluble protein antigens.

Uptake of antigens in the lymph node medulla and at the border of the red and white pulp of the spleen is a recurrent event, which is often followed by digestion of the antigen and rapid loss of label from these areas. Localization of particulate antigens and non-antigens also occurs in these areas.

Follicular localization on the other hand, appears, at least quantitatively, to show a certain specificity for antigens only. In addition, there are differences between antigens with regard to the time after injection at which follicular localization is most prominent. The majority of antigens require 3-7 days before significant levels of label are found in the follicles (McDevitt et al., 1966; Balfour and Humphrey, 1967; White et al., 1967). More rapid follicular localization has been shown to occur with homologous and heterologous IgG, homologous IgM, and with antigen-antibody complexes (Hanna et al., 1968); Herd and Ada, 1969). Localization of immunoglobulin molecules appears dependant upon the presence of an intact  $F_c$  region (Herd and Ada, 1969). The conflicting time sequences have been resolved by the accumulation of a body of evidence suggesting that participation of specific antibody is required for the follicular localization of antigen.

In both actively and passively immunised animals it has been repeatedly shown that there is an antigen specific increase in the rate and degree of both medullary and follicular uptake although the effect is most marked with respect to the latter. By contrast, in fully tolerant animals only medullary uptake is found (Balfour and Humphrey, 1967; White et al., 1967). In addition, where antibody formation is depressed by X-irradiation (Jaroslow and Nossal, 1966), anti-lymphocytic treatment (Barth et al., 1969), thoracic duct drainage (Williams and Nossal, 1966), or in germ-free and neonatal animals (Williams and

Nossal, 1966) there is a marked decrease in the efficiency of follicular uptake. A more direct approach, by Balfour and Humphrey (1967) and White et al. (1967), was to examine by fluorescent techniques the distribution of antibody around and in the germinal centres of primed animals. The site and pattern of localization of antibody in the centres was found to be similar to that already described for the antigen. The presence of antibody, capable of attaching by the  $F_c$  portion to the surface of the processes of dendritic cells, seems a likely mechanism whereby the uptake of antigen onto these cells is potentiated, either before or after antigen-antibody complexes are formed.

## 2. Follicular uptake of antigen and the immune response.

Lack of knowledge on the precise role of follicles in the production of antibody has hindered attempts to explain the role of antigen specifically trapped in them. In 1930, Hellman and White noted the appearance of Fleming's 'germinal centres' in the lymph node follicles of animals injected with foreign or toxic materials. These centres, which Hellman called 'reaction centres', also appeared in the Malphigian nodules of the spleen. Since then there has been much support based on phylogenetic, ontogenetic and kinetic evidence for the contention that these areas of the lymphoid tissue are important in the production of large quantities of antibodies.

Germinal centres are not formed in tolerant animals in response to test antigen (Cohn and Thorbecke, 1964), nor are they present in germ-free animals (Gilmstedt, 1936). In normal animals there is an appropriate time relationship between the rapid expansion of primary follicles to give germinal centres and the appearance of high titres of serum antibody (Hanna, 1965; 1966). Furthermore, specific intracellular and inter-cellular immunoglobulins have been found in a number of rabbit germinal



centres following diphtheria toxoid and ovalbumin injection (Leduc et al., 1955). Under given conditions, the secondary responses to these antigens could be limited to that lymph node draining the site of the primary injection (White et al., 1955). It is of interest that besides being antibody-producing sites, Ehrlich (1946) considered germinal centres to be also in part the place where lymphocytes returned from the blood to the lymph nodes. Follicular stimulation however, does not appear to be the first histological change associated with antibody formation. Antibody forming cells are first found in lymph node medulla and/or at the border of the red and white pulp of the spleen (Leduc et al., 1955). On the basis of this morphological evidence, it seems unlikely that specific localization of antigen in the form of antigen-antibody complexes is a prerequisite for the initiation of primary immune responses. Recent findings of IgG complexed to antigen a few hours after its injection may correct this viewpoint (Ivanyi, 1970; Yuan et al., 1970) although other roles for follicular trapping have been proposed.

Whiteet al. (1967) have suggested that antigen on dendritic cells may act as a focus for germinal centre proliferation in the chicken spleen. After antibody forming cells had disappeared from the medulla, clusters of such cells were found in germinal centres. Ada and Parish (1968) suggested that follicular localization may have a role in the promotion of tolerance and/or immunological memory, and in addition it may have an inhibitory or regulating role to play in the immune response (Jerne, 1967; Uhr and Moller, 1968). Mitchison (1969a) has suggested a role for follicular localization in increasing the effective local concentration of antigen in an area where cell migration is likely to occur.

### 3. Phagocytosis in the immune response.

#### a. Introduction.

Antibody formation has been shown in the main to be a property of cells of the lymphocytic and plasmacytic series (Attardi et al., 1959; Leduc et al., 1955), although there are a few reports that other cell types may also be involved.

Noltenius and Chahin (1969) demonstrated cells of macrophage morphology and function forming 'direct' plaques by a modification of Jerne's technique. These cells, supposedly secreting specific antibody, were found in the spleens of mice immunised against sheep red blood cells and were capable of phagocytosing india ink particles and red blood cells. In this and other similar studies (Mitsuhashi et al., 1967) the proportion of such cells was small in comparison with the number of antibody forming cells of lymphocytic character. Furthermore, the relationship of this cell type to the classical macrophage is uncertain (see p.14).

Although not generally considered as antibody forming cells macrophages are often thought to be concerned in the induction of immune responses. A priori one might expect phagocytes within the lymphoid organs in close apposition to the antibody forming cells, to be involved in first steps towards antibody production. The sinus lining macrophages of the spleen seem exquisitely situated to contact lymphocytes recirculating through this organ (Goldschneider and McGregor, 1969). Several experimental systems have demonstrated that contact of antigen with macrophages may enhance its immunogenicity. Whether or not the processes involved have any relevance to the final immune response can be considered only in relation to evidence from in vivo experiments.

b. The role of the macrophage *in vivo* and *in vitro*.

Frei et al., (1968) argued that the primary response of the rabbit to bovine serum albumin requires the intervention of the macrophage. BSA 'filtered' by passage *in vivo*, was found to lose its immunogenicity. The more particulate and hence more readily phagocytosed material seems to be removed by the passage through an animal leaving only monomeric form of the antigen which is tolerogenic. The conclusion drawn from these experiments must be taken in light of the fact that commercial BSA has often been shown to contain pyrogenic contaminants (Dvorak and Bast, 1970; Philip et al., 1966).

Gallily and Feldman (1967), amongst others, derived similar views on the role of macrophages from experiments based upon the different sensitivities of macrophages and lymphocytes to X-irradiation. Mouse peritoneal macrophages, incubated with Shigella *in vitro* and washed, were found capable of restoring agglutinin production in 550r X-irradiated hosts. Similar mice receiving bacteria alone and/or lymph node cells were found to be unreactive. Macrophages from irradiated donors were also ineffective. Mitchison (1969) showed live macrophages containing bovine serum albumin and other protein antigens to be highly immunogenic in mice. Similar transfer experiments have shown macrophages capable of conferring responsiveness to red blood cells upon neonates (Argyris, 1968). It is also of interest that macrophages of foetal or newborn animals appear capable of ingesting bacteria but not of killing them (Reade, 1968).

That macrophages may concentrate antigen and present it in a suitable form to the reactive cells is a hypothesis supported by the fact that they appear to be required for an *in vitro* immune response (Hoffman, 1970). Clusters of macrophages around presumably 'reacting' lymphocytes

have been observed in vitro (Fishman et al., 1963; Sharp and Burwell, 1960). In contrast, the presence of too many macrophages can inhibit in vitro transformation and antibody production by mouse spleen cells (Hoffman, 1970; Parkhouse and Dutton, 1966).

Opposed to the positive role of macrophages in immunogenesis, there are numerous reports in accord with the older concepts that macrophages simply destroy antigen in vivo, perhaps in doing so protecting the lymphoid cells from excessive exposure. Perkins and Makinodan (1965) showed that the ability of macrophages, which had ingested sheep red blood cells, to stimulate primed spleen cells, decreased with time after ingestion. Furthermore, in vivo stimulation of peritoneal macrophages with gelatin led to a decrease in the immune response to sheep red blood cells and aggregated human gamma globulin if these were given by the intraperitoneal route (Perkins, 1970).

c. Persistence of antigen and phagocytosis.

Protagonists for the role of the macrophage in immunogenesis have gathered a considerable amount of evidence on the intracellular fate of antigen, that suggests ways in which these cells could help to initiate an immune response.

As long ago as 1928 Wells stated that "antigenic activity is in some way related to digestive proteolysis." It is now generally accepted that the majority of materials both phagocytosed and pinocytosed by macrophages are taken up within vesicles. These move towards the nuclear region and fuse with Golgi vesicles containing hydrolases. It is within the dense granules or secondary lysosomes so formed, that digestion of the engulfed materials takes place, and only molecules of very small molecular weight appear to be released (Ehrenreich and Cohn, 1969; Cohn, 1968). What has been of interest to immunologists, however, is that

some antigen appears to remain in the macrophage, seemingly resisting degradation.

In 1951 McMaster and Kruse injected azodye conjugated to bovine gamma globulin into mice. Although most of the dye was rapidly excreted, a blue colour was evident within phagocytic cells for up to 100 days in the case of BGG and 40 days in the case HSA. This persistence of dye correlated roughly with the presence of antigenic determinants in the blood, liver, spleen and lymph nodes, as judged by the ability of extracts of these organs to induce vascular changes in the ear by the reversed passive anaphylaxis technique. Haurowitz and Crampton (1952) found radioactive label, and hence presumably antigen, associated with microsomal and mitochondrial extracts of reticuloendothelial organs (see Haurowitz, 1960). More recently, Askonas et al. (1968) showed the majority of  $^{131}\text{I}$ -labelled haemocyanin from *Maia squinado* to be digested by mouse peritoneal cells within 3-4 hours. During breakdown the label was in the main associated with phagosomes. A smaller quantity of membrane associated label, however was also found which was shown to persist for a much longer length of time.

In 1956, Garvey and Campbell isolated residual antigen from the livers of mice injected with  $^{35}\text{S}$ -labelled sulfanilo-bovine-serum-albumin. They used antibody to precipitate the antigen from liver cell homogenates, and discovered the precipitate to also contain RNA. The quantity of RNA correlated with the amount of label in the precipitates. Furthermore, antigen-RNA complexes thus obtained were 100-200 times more immunogenic than the free form of the antigen. Similar complexes could also be isolated from the animals' circulation and urine. The low molecular weight and abnormal base composition of at least certain of these RNAs has since been interpreted as precluding an informational

function (Yuan et al., 1970). Many instances have been since cited where RNA-antigen complexes, extracted from cells which have taken up the antigen, have been shown to be exceptionally immunogenic in comparison with the free form of the antigen (Askonas and Rhodes, 1965).

By contrast, a decrease in the immunogenicity of keyhole limpet haemocyanin after macrophage processing has been described (Unanue, 1969a). In fact, KLH was shown to be more immunogenic in its free form than when macrophage bound. Similar experiments, had previously shown the reverse situation to hold for Maia squinado haemocyanin which is a poorer immunogen (Unanue and Askonas, 1968a).

Recent studies by Kolsch and Mitchison (1968) have concentrated upon residual heterologous serum albumin which escapes digestion and localizes near the macrophage nucleus. This antigen, when macrophage associated, was shown to be far superior to native antigen in eliciting a primary immune response (Mitchison, 1969). The relationship between this intracellular antigen, membrane associated antigen and the antigen found complexed to RNA is unclear. Their possible role in vivo will be discussed later.

d. The role of macrophage-derived RNA.

As already indicated, the role of the RNA complexed to antigen has been taken by many workers to be non-informational in nature (see also Gottlieb et al., 1967). There is, however, evidence that an informational form of RNA may be produced by a cell in response to antigen i.e. RNA capable of transferring specific responsiveness to virgin lymphoid cells.

Fishman and colleagues in 1961 extracted RNA from peritoneal cells previously incubated with bacteriophage. These extracts were found to

induce in non-sensitised homologous lymph node cells the in vitro production of specific anti-phage antibody (Fishman, 1961; Fishman and Adler, 1963). From enzymic digestion studies these workers concluded that antigen was not an important constituent in their preparation. Similar experiments further emphasising the role of RNA were performed by Adler et al. (1966), but using peritoneal cells and lymph node cells from donor rabbits which differed in their allotypic expression. In these experiments IgM anti-phage antibody was found with the allotypic character of the peritoneal cell donor whereas the allotype of the IgG was that of the lymph node cell donor. As many workers failed to repeat this observation, it is fair to point to the difficulties involved in the antibody assay systems as well as the possible requirement for cell penetration by the RNA (see Jurezic et al., 1970). Bell and Dray (1969), however, did succeed in supporting Adler's work. RNA extracts of peritoneal exudate or lymph node cells from animals immunised against sheep red blood cells could induce lymphoid cells from non-immunised animals to produce IgM antibodies bearing the light chain genetic determinants peculiar to the RNA donor.

e. The role of macrophages in the immune response.

To cope with the body of conflicting results and conclusions drawn on the basis of both the in vivo and the in vitro experiments mentioned, it appears necessary to judge each experimental situation on its own merits.

The most obvious and most fundamental aspect of macrophage function is universally agreed to be its role as 'scavenger'. Other roles are less well agreed upon.

The investigation of highly immunogenic 'processed' antigen may lack relevance to the main inductive pathways for antibody formation.

It is difficult to validly compare the immunogenicity of cell-associated antigen or antigen-RNA complexes with that of the free native antigen. The alterations in the spatial location of antigen brought about by its altered physical form, protection from intravascular degradation, and slower excretion, as well as non-specific adjuvant effects, could all possibly give increased immunogenicity.

That antigen can persist for a considerable period within macrophages may simply be due to the inefficiency of macrophage digestion and/or heterogeneity of lysosomes or cells.

Functional heterogeneity of cell populations may in part explain the puzzling experiments of Fishman and Adler (1963). Fishman noted that only one phage particle per 1000 macrophages was necessary to induce production of an informational RNA capable of inducing a transient IgM anti-phage response in cultures of non sensitized cells. This might suggest that only a minor population of cells was responding.

Within a conventional ideology it is difficult to suggest how a messenger RNA might be produced by antigen without the participation of an antibody-like mediator. Although macrophages are thought on the whole not to be antibody producers there is evidence for cells which are functionally both macrophages and lymphocytes.

Mouse thoracic duct cells, under extreme provocation, have been demonstrated to transform into phagocytic liver cells (Boak et al., 1968). Blast transformed cells which are not susceptible to anti-macrophage serum, have been shown to stick to glass and to bind cytophilic antibody (Unanue, 1968). Established human immunoglobulin-producing cell lines contain a small percentage of actively phagocytic cells (Kammermeyer et al., 1968), as do 'mixed lymphocyte' cultures (Coulson et al., 1967 ). These highly phagocytic 'activated' cells may be an important population in the experiments of Fishman and Adler and may also account for the



reports of small numbers of 'macrophages' from primed animals which have been shown to produce 'antibody' plaques to sheep red blood cells and anti-Salmonella IgM antibody (Noltenius and Ruhl, 1969; Mitsuhashi et al., 1967).

While the role of 'informational' RNA as messenger or derepressor, acting to direct the synthesis of antibody or effector molecule, remains a possibility, it remains to be shown that the main pathways to antibody synthesis in vivo utilise these or any other special attributes of the macrophage population.

Experimentally, the role of the macrophages fixed in lymphoid organs is often simulated by the use of the peritoneal exudate cells. The justification for this lies in the seeming similarities between the two cell populations with respect to the uptake and degradation of antigen (Askonas et al., 1968) as well as the preferential localization of peritoneal macrophages in the medullary areas of the lymph node after intravenous injection (Roser, 1965; Russel and Roser, 1966). In view of the observed differences between alveolar and peritoneal macrophages (Cohn, 1968) with respect to both enzyme content and antibactericidal activity, functional differences between populations of cells and/or lysosomes may exist which would negate the use of the peritoneal macrophages as a model system. Extremely little is known about the functional attributes of the macrophage population fixed in the lymphoid organs.

In conclusion there can be no dogmatic statement concerning the function of macrophages other than that they are in the final analysis important in vivo in determining the outcome of an antigenic challenge.

#### 4. The antibody-producing cells.

##### a. Introduction.

It is now clearly established that antibody producing cells belong mainly to the lymphocytic and plasmacytic series and that they arise by differentiation and proliferation of precursor cells, which in most instances appear to be small lymphocytes. This process of differentiation can be simulated in vitro. Small lymphocytes can transform into blast cells following antigenic (Abdou and Richter, 1969; Dutton, 1966) and mitogenic (Marshall and Roberts, 1963; 1965) challenge. A similar process has been described to occur when adoptively transferred small lymphocytes are challenged in vivo (Gowans, 1966; Ford et al., 1966).

Highly differentiated cells, committed to antibody synthesis, appear restricted with respect to the class, light chain type, allotype and idiotype they produce (see Cebra, 1968; Pernis, 1967; Gell and Sell, 1965). Whether or not their antigen sensitive precursor cell also produces antibody which is of limited genic expression, is a question of great importance for the complete understanding of immunogenesis.

The presence of immunoglobulin determinants on the surface of a proportion of lymphocytes (Pernis et al., 1970), as well as the predilection of radioactively-labelled antigen for a small number of normal cells of this type (Sulitzeanu, 1968), has added strength to the hypothesis that there may exist precommitted antigen sensitive entities. This has also been suggested by the limiting dilution technique (Perkins et al., 1961) and the findings of Kennedy et al. (1966). For the production of a mouse anti-SRBC response there is a cell type in the mouse spleen which exists in limiting frequency of approximately  $10^{-5}$ . A similar number of human peripheral cells have been shown to react with

fluorescent antisera raised against the idiotypic determinants of a myeloma protein (Pernis, 1967). It seems therefore possible that there exists a population of cells that are predetermined to produce humoral antibody of one specificity against an antigen.

In a graft versus host situation, however, Simonsen (1967) found cells sensitive for the B isoantigen of chicken to exist in limiting frequency of  $10^{-1}$ . While not necessarily contradictory, these findings require resolution.

There is a simple hypothesis that direct interaction could occur between intact or 'processed' antigen and its specific antigen-sensitive cell, triggering it to proliferation and differentiation into a mature antibody forming cell. However, it has recently been conclusively shown that the immune response to an antigen may depend upon interaction between more than one population of lymphocytes, leading to the concept of the antigen-sensitive 'unit'. This concept of immunogenesis will be examined by reviewing in brief what is known about the functional aspects of the heterogeneity of lymphocytes.

b. The heterogeneity of small lymphocytes.

The developmental and functional capabilities of lymphocytes, within, and from different, sites have recently been found to show variable degrees of heterogeneity. Lymphocytes are known to differ in size, volume, electrophoretic mobility (Rubenstroth-Bauer and Lucke-Huhle, 1968) as well as cellular infrastructure and content of histocompatibility antigens (Raff, 1969; Raff and Wortis, 1970). Further divisions have also been made which, while not absolute, can divide the lymphocyte population with respect to their involvement in certain immune responses and which can be correlated in part with differences in their origin and life span.

c. The origins of lymphocytes and their competence.

There seems to be little doubt that, at least in mice, the earliest precursors of lymphocytes are derived from mesenchymal cells of the bone marrow and liver and that stem cells from the marrow continue to seed other lymphoid organs throughout the animals' life (Tyan and Cole, 1965; Zaalberg and Van der Meul, 1966; Miller, 1964; McGregor, 1968). Such conclusions are based mainly upon recolonisation of X-irradiated animals with lymphoid cells which can be traced by their chromosomal markers or by autoradiography.

During recolonisation of X-irradiated mice with mixtures of syngeneic lymphoid cells from various organs, Micklem et al. (1966) showed that donor bone marrow cells could eventually replace other cell types in the thymus, lymph nodes, spleen and bone marrow of the recipients. This property may be linked to their multipotency with regard to developing into the many cell types of the reticular system.

X-irradiated, bone marrow-reconstituted mice show complete haematopoietic recovery by 14 days and the immune system is complete by 25-30 days. Complete immune reconstitution of these mice, however, is dependent upon the presence of an intact thymus (Osoba, 1968). It appears that a certain number of bone marrow cells sequester and differentiate within this primary lymphoid organ before being released into the circulation (Ford and Micklem, 1963; Dukor et al., 1965). These 'thymus derived' cells are found in high numbers in the blood, lymph and within certain 'thymus dependant' compartments in the spleen and lymph nodes (Leuchars et al., 1966; Davies et al., 1966; Raff, 1970). Neonatal thymectomy, as might be predicted, has been shown to deplete this population of cells but leave bone marrow derived cells functional (Parrott et al., 1966). These two populations of cells, different with regard to origin, also seem to differ in their life span and their involvement in immune responses.

d. The life span of lymphocytes.

The existence of two populations of lymphocytes, differing in their life span was first suggested by Ottensen (1954). He found a biphasic fall off in the numbers of  $^{32}\text{P}$ -labelled circulating lymphocytes over an extended period of time following injections of the label into humans. Everett and others have extended this concept in the rat. Studying the uptake of radioactive markers and their disappearance in small lymphocyte populations taken from different tissues, the concept of at least two sub-populations with differing half lives was firmly established (Little et al., 1962; Caffrey et al., 1962; Everett et al., 1964, 1964a; Everett and Tyler, 1967). Despite the problems of radiation damage and label reutilisation brought about by the often necessarily long label infusion time (see Robinson et al., 1965) it became generally confirmed that the ratio of long- to short-lived lymphocytes was greater in the thoracic duct lymph than in the mesenteric node and in the spleen, with thymus and bone marrow containing but few of the long-lived variety. This rank order also parallels the ability of these populations to give a graft-versus-host reaction.

e. Recirculation of lymphoid cells.

A satisfactory explanation for the varying ratios of short- to long-lived lymphocytes in different tissues has been derived in part from studies on the recirculation of lymphocytes (Gowans, 1959; Gowans and Knight, 1964). Thoracic duct small lymphocytes, labelled in vitro with  $^3\text{H}$ -adenosine and injected into rats, were found to show a predilection for the mid- to deep-cortex of the lymph nodes and Peyers patches, and for the splenic white pulp. The passage of such cells, with no or little evident mitosis, through the post-capillary venules of the lymph node into the lymph occurred within 2-4 hours after injection.

Transport of recirculating cells through the spleen has also been described (Ford, 1969; ~~Ford~~<sup>et al.</sup>, 1966; Goldschneider and McGregor, 1968; Gowans and Knight, 1964). At present, autoradiographic studies suggest passage of cells from the marginal zone to the periarteriolar sheath via the endothelial cells of the sinus wall. The pathway from the white pulp to the blood is as yet uncertain.

Other pathways of lymphocyte travel are less well known although it seems likely that the peripheral lymph is contributed to by cells which drift through the connective tissues. The composition of the blood is also a reflection of cell types other than those derived from the thoracic duct (Yoffey, 1964).

The recirculating lymphocyte population appears to a large extent to be identical to the thymus-derived, long-lived population already described. Such a concept can be supported by the evidence that thymocytes, macrophages, large lymphocytes, bone marrow cells, Kupffer cells and generally cells with a short life span are not found, to any great extent, capable of recirculating through the transport areas of the lymph nodes and spleen, areas which correspond closely to the thymus-dependent areas already mentioned on p.18 (Goldschneider and McGregor, 1968). Furthermore, neonatal thymectomy leads to a depletion of the transport areas, a decrease in thoracic duct output and a loss of the long-lived lymphocytes, a situation which will be shown to be mimiced by other treatments.

At present it is not known to what extent the life span and behaviour of lymphocytes are linked to their origin. Neither is it known whether the phenomena described in the last two paragraphs are applicable to species other than rodents in which the majority of the experiments were performed.

f. Functional and cooperative aspects of lymphocyte subpopulations.

Clarification of the roles of subpopulations of lymphocytes has been achieved, at least in part, by work on thymectomised and bursectomised animals and on states of congenital thymic aplasia and agammaglobulinemia. Cellular and certain humoral immune responses appear to be under the influence of the thymus and to require its intact functional presence. In a similar manner, treatments which mimic neonatal thymectomy e.g. antilymphocytic serum treatment or thoracic duct drainage (Shellam, 1969a; Turk and Willoughby, 1967), leading to depression of the recirculating lymphocyte pool and depletion of the transport areas of the spleen and lymph nodes, also seem to depress cellular and certain humoral immune responses (Lance and Batchelor, 1968; Miller and Osoba, 1967). The restocking of thymus-dependent areas following such treatments appears to be a slow process dependant upon the presence of an intact thymus, but full responsiveness can be rapidly restored by the adoptive transfer of thymocytes (Martin and Miller, 1968).

It is unclear why certain immune responses appear to depend upon thymus-derived lymphocytes while others do not, but it has been further accentuated by studies initiated by Claman (1966) on immune reconstitution of unresponsive mice with various lymphoid populations.

Kennedy et al. (1965) and others found that the anti-SRBC response is not restored by the injection of thymocytes into lethally X-irradiated mice, although this was possible in neonatally thymectomised animals (Miller and Mitchell, 1968). However, when syngeneic bone marrow cells were injected along with thymus cells into the X-irradiated hosts, the response could be restored and, furthermore, the two populations acted synergistically. The ensuing haemolysin response to sheep red blood cells was rapid and often higher than the sum of the responses when either

population was used alone (Claman et al., 1966; Mitchell and Miller, 1968). A similar synergistic cooperation has been described between thymus and spleen cells for the response to bovine serum albumin in an 'inert' thymectomised-irradiated host (Taylor, 1969), between thymus and spleen in the anti-SRBC system (Claman et al., 1966a) and between bone marrow and spleen (Radovitch et al., 1968) using the same antigen.

In experiments showing cooperation between formative tissue cells, the bulk of evidence suggests that a humoral response, at least in the context of the cell transfer system, is dependent upon the presence of the bone marrow cells for the provision of the precursors of the antibody forming cells. The direct plaque-forming cells against sheep red blood cells were of this origin (Mitchell and Miller, 1968a) and the experiments of Taylor et al. (1966) suggest that  $IgG_{2a}$  is also produced by marrow cells in reconstituted animals.

The specificity of the thymus cells responses in these experiments is as yet uncertain. Taylor (1969) has shown that thymus cells taken from animals previously injected with soluble BSA, are unable to synergise effectively with bone marrow cells in reconstitution experiments. The suggestion that the thymus cells had become tolerant was weakened by the finding that the synergistic response to human serum albumin was also affected. Although thymus cells are not generally thought of as antibody forming and releasing cells, rabbit thymus cells transplanted across an allotypic barrier into a newborn animal can, in certain instances, lead to appearance of globulins of the donor expression in the serum of the recipient (Chou et al., 1967).

In graft-versus-host reactions the specific antigen sensitive cells appear to be purely thymus-dependant. X-irradiated recipients receiving thymus and bone marrow cells together produce a splenomegaly which in



part is the result of synergistic action between the two populations. However the bone marrow seems to play essentially a non-immunologic proliferative role (Hilgard, 1970).

Cooperation between thymus-dependent and non-thymus-dependent lymphocytes has been taken as an explanation for puzzling results obtained in hapten-carrier systems. Under given experimental conditions, anti-hapten secondary responses can often be shown to be best elicited if the host is primed both against the hapten and the carrier (Ovary and Benacerraf, 1963). Both cell populations are required to be normally responsive (Paul et al., 1970). Within the confines of a one cell - one antibody hypothesis such a result could be accommodated only by the cooperation of two discrete cell populations or their products of stimulation. It has been suggested that the carrier specificity resides in the thymus-derived cells (Rajewsky et al., 1969) although direct evidence is lacking.

The induction of an anti-SRBC response in vitro in spleen cells taken from at least certain strains of mice, has been shown to require at least three distinct cell types which can be separated by velocity, centrifugation, size, radiosensitivity and their ability to bind antigen (Haskillet et al., 1970; Mosier and Coppelson, 1968; Osoba, 1970). It has been repeatedly suggested that one of these cell types may be a thymus-derived population though direct evidence is lacking.

Further evidence for the interaction between cells within an existing lymphoid organ comes from experiments involving transfer of spleen cells to X-irradiated histocompatible recipients or the growth of lymphoid cells in diffusion chamber cultures. The relationship between the dose of lymphoid cells (and hence antigen-sensitive cells) and the number of antibody forming cells, has been shown to be linear over most dose ranges, although when the lymphoid cells are limiting a parabolic relationship has been demonstrated. A parabolic relationship is consistent with the

interpretation that the number of antibody-forming cells produced is dependant upon the interaction of two or more cells. The linear relationship could represent a saturation effect (Groves et al., 1969; Celeda, 1967; Bosma et al., 1968). The cell types involved in these responses are unfortunately unknown.

Such experiments support the implication derived from other indirect sources that even in fully formed lymphoid tissues there are classes of cells of varying functional involvement.

##### 5. The composite immune response.

In recent years it has become clear that there are many different ways, rather than one essential pathway, by which contact between antigen and antigen sensitive cell may be established.

Marked diminution in the antibody response to certain antigens is caused by procedures which selectively depress the thymus-derived cell population. For example whilst the responses of mice to antigens such as horse ferritin and pneumococcal polysaccharide appear to be independant of such procedures (Humphrey et al., 1964; Fahey et al., 1965) the response to bovine serum albumin is very thymus-dependant (Taylor, 1969). This distinction between antigens does not necessarily cross the species barriers. A corollary to this distinction may be the dependance of certain responses upon recirculation of cells through the lymphoid organs (Hall and Morris, 1964; Taliaferro and Taliaferro 1956; Ford and Gowans, 1967; Ford, 1968).

Further distinctions between responses to different antigens lie in their dependance upon interaction with macrophages, in differences in physicochemical nature, in the amount of natural opsonins present and other less well known variables.

As a unifying concept Mitchison (1968b) suggested that the final immune response is dependant upon the triggering of antigen-sensitive lymphocytes which bear a receptor with affinity equal to or greater than the molar concentration of the immunogen. To localise antigen, with resulting stimulation of lymphocytes, may be the main effective role of the processes just described. Under this theory there is no strict requirement for cell interaction as an essential step in an immune response but they may act as methods whereby the response can be amplified or regulated. A similar action could be envisaged to occur via released soluble factors.

It is inevitable from what has been said that each antigen, each animal, each assay must be considered to exist within each experimental system separately.

## 6. Immunological tolerance

The injection of antigen under certain circumstances, instead of leading to an immune response, may lead to an antigen specific defect in responsiveness. The lack of that particular response is usually judged by challenge with the same antigen under conditions which favour its immunogenicity. The terms tolerance, paralysis and specific unresponsiveness have been used to cover these states, their usage depending on the experimental design involved. Never the less, it has recently become popular to consider these terms synonymous, implying common basic mechanisms. In order that unjustified parallels are not drawn between the different experimental models, they shall be differentiated from one another in the introduction, although stress will be laid upon the induction of unresponsiveness to heterologous serum protein in adult animals, a basic concern of this thesis.

## I. Historical.

The phenomenon now known as acquired immunological tolerance was known many years before the realisation of its bearing on fundamental immunological mechanisms.

Wells and Osbourne (1911) and many others, in varying experimental conditions, presented results dealing with the specific suppression of an immune response by prior exposure to antigen (Glenny and Hopkins, 1924; Sulzberger, 1929; Traub, 1936; Felton and Ottinger, 1942).

The concept achieved scientific respectability as a result of Owen's (1945) study on dizygotic cattle twins. The majority of such twins are synchorial. He demonstrated some of the twins to be permanent erythrocyte chimaeras whose red cell populations were mixtures of two distinct antigenic types. Apart from an animal's own erythrocytes, genetically foreign circulating red cells could be demonstrated, often in a considerable proportion. The phenotype of these foreign cells was that of the other twin. It was suggested that the mosaicism was due to in utero exchange of primordial haematopoietic cells, which occurred via placental vascular anastomosis; the normal rejection processes being inoperative (Owen, 1945).

This suggestion was later confirmed by showing that pinch grafts between dizygotic cattle displayed anomalous behaviour (Anderson et al., 1951; Billingham et al., 1953). Only in a minority of instances were the true genetic differences between the twins revealed; in the majority of cases the grafts survived as though the animals were monozygotic. Even in those cases showing rejection the reaction was rather variable and weak.

In 1949 Burnet and Fenner advanced their hypothesis of tolerance to self. Their concept was that the immunological system becomes non-reactive to antigens with which it comes into contact in embryonic life,

the function of such a mechanism being to ensure nonresponsiveness to self components. Although not dealing with examples where suppression had been demonstrated in adult animals, it proved an excellent working hypothesis for neonatal tolerance induction, capable of being tested by the experimental introduction of antigens into animals in ovo or in utero.

## II. Tolerance induction in the neonate.

### a. Introduction.

Burnet's early attempts to support his hypothesis by the inoculation of chick embryos with flu virus, bacteriophage and human red blood cells met with little success (Burnet et al., 1950). No differences were observed in haemagglutinin or inactivation titres when treated and control chicken were challenged with the corresponding antigens at 5-8 weeks of age.

Billingham et al. (1953), using a system more analogous to Owen's bovine twins experiment, did however demonstrate what they referred to as "actively acquired tolerance." This was defined as the inability of an animal to react immunologically against foreign homologous tissues if it had been exposed to them during an early period of neonatal life. They demonstrated facilitation of skin graft survival and inhibition of specific antibody formation in mice and chicken injected in utero or in ovo with a variety of foreign tissue cells. Injection of specifically primed spleen cells led to a loss of the tolerated grafts. The phenomenon was thus shown not to depend upon antigenic transformation of the graft, to be antigen specific, and in addition the effect was demonstrated to be non-heritable.

Outside the sphere of cellular isoantigens, Buxton (1954) reported a decrease in antibody formation to Salmonella pullarum in chickens that had previously been treated with the bacteria at 15 days of age. These

experiments, however, were unconvincing due to the lack of appropriate controls. Similar results were obtained by Kerr and Robertson (1954) in calves receiving Trichomonas, although their conclusions are unconvincing due to small number of animals used. Other reports on experiments dealing with more complex and phylogenetically remote antigens were on the whole negative. <sup>et al.,</sup> Bauer/(1956) and Owen (1957) both failed to induce tolerance in rats to sheep red blood cells and Nossal's (1957) attempts to make foetal mice tolerant to flu virus were unsuccessful. Nossal in fact gave data suggesting priming of the mice.

b. Tolerance to protein antigens.

Hanan and Oyama (1954), however, did obtain results with protein antigens that were interpreted by the authors as examples of immune tolerance as predicted by Burnet. They injected 0.1 - 1.0 mg doses of alum-precipitated bovine serum albumin three times weekly, intraperitoneally, into newborn rabbits. The antigen was administered in this fashion for the first month of the experimental animals' life. After that they received intravenous injections for 114 or 186 days. When challenged with immunogenic doses of BSA and egg albumin the animals failed to produce anti-BSA precipitins and anti-BSA sensitizing antibodies for the passive anaphylaxis test or Arthus reaction. Littermate controls, which received the challenge routine only, responded positively. While most tolerant animals showed antibody responses to egg albumin the response was diminished in comparison to controls.

It has since been adequately documented that heterologous serum proteins can readily induce this form of unresponsiveness (see review by Smith, 1961). The majority of experiments have, for practical reasons, been done in rabbits although other species have proved equally amenable.

The parameters involved in tolerance induction have been extensively

surveyed and include ontogenic age of animals, dose of an antigen and the immunisation schedule (Smith, 1961). The duration of the tolerant state was found to correlate positively with the dosage when a single injection was employed and the state could be prolonged by further doses of relatively small amounts. Doses greater than 0.2 mg/day of bovine serum albumin over the first five days of a rabbit's life were found to establish unresponsiveness (Dubert and Paraf, 1957) whereas as little as 10  $\mu$ g can significantly prolong the state (Smith and Bridges, 1958).

In spite of the success of these experiments, difficulties have still been encountered with more complex antigens. Smith and Bridges (1958) found no evidence of tolerance to a variety of bacterial antigens and Gowland (1965) failed to demonstrate neonatal tolerance to Pseudomonas in rabbits injected when 1-7 days old.

c. Tolerance to self-replicating antigens.

It has, however, been suggested that where a virus is transmitted via the egg, tolerance may be induced. Rubin et al., (1962) studying avian leukaemia virus, and Traub (1936;1939) examining the spread of lympho-chorio-meningitis endemic in a small stock of mice, both suggested that if infection takes place in ovo or in utero, then the disease often took hold. If the infection took place shortly after birth the animals often became resistant. Larson (1969) showed it possible to induce tolerance to lympho-chorio-meningitis virus by experimentally infecting neonatal mice within the first 18 hours of life. When the infection treatment was postponed until 2-9 days after birth the outcome varied between complete tolerance and antibody formation with low viraemia. Solomon (1968) produced a state of partial tolerance to Salmonella gallinarum in 1-7 day old chickens as measured by agglutination test. In addition, he demonstrated that small numbers of virulent bacteria could be as effective

as large numbers of avirulent ones in inducing tolerance.

It seems possible that the ease with which tolerance could be induced in the experiments described above could be a result of the self-replicating nature of the antigens used. Differences between antigens in their tolerogenicity may possibly be due to differences in the "effective dose" of antigen or differences in their handling. A further complication is, however, that although neonates appear to be relatively poor antibody producers, they are not absolutely incompetent.

d. Immunity and tolerance in neonates.

Nossal (1959) found that foetal rats exposed to sublethal pulmonary infection with flu virus or intraperitoneal presentation of high titre flu vaccines gave evidence of immunisation. Silverstein et al. (1963) showed foetal sheep capable of responding to bacteriophage, ferritin and ovalbumin after 70 days of a 150 day gestation period. Similarly, there is evidence that newborn mice can give an immune response to allogeneic cells when these are given in doses lower than that required for tolerance induction (Howard and Michie, 1962).

The dose dependence curve of the immunological reaction for neonatal Wistar rats injected with Salmonella adelaide flagellin was described in detail by Shellam and Nossal (1968) and is of interest. Two zones of tolerance were induced by injections in the region of  $10^{-7}$   $\mu\text{g/g}$  body weight/day and  $10^{-3}$   $\mu\text{g/g}$  body weight/day. Within this range a zone of immunity was detected. Shellam (1969) also showed that  $<10^{-1}$   $\text{pg/g}$  body weight/day for two weeks and  $1\mu\text{g}-100\text{mg}$  both led to tolerance induction to polymerised flagellin, whereas doses of  $10\text{pg}-1\mu\text{g}$  under the same injection regime led to immunity. Throughout these studies partial tolerance only was demonstrated. Quantitative comparison was performed by the bacterial immobilisation test and although no antibody was produced by the neonates, they could be primed.



e. Mechanisms of neonatal tolerance.

In spite of the difficulties encountered in attempts to induce tolerance to certain antigens, and in spite of the early ontogenic maturation of responsiveness to certain antigens, it is nevertheless fair to conclude that tolerance may be more readily induced in neonates than in adult animals.

Freund (1930) first suggested that the ease of neonatal tolerance induction may be due to the relative lack of responsiveness of young animals. Mitchison (1968) found no systemic difference between neonates and adults in the dose of antigen required to produce tolerance to bovine serum albumin and lysozyme, provided that the antigen was administered to both under non-immunizing conditions. Such experiments led Dresser and Mitchison (1968) to the conclusion that the lack of an immune response in young animals, due to deficiencies of the immunologically competent cells, could account for differences between neonates and adults with regard to tolerance induction.

Other workers have suggested that defects in the immune function of reticulo-endothelial cells may account, at least in part, for the ease of tolerance induction in neonates (Mitchell and Nossal, 1966). Newborn animals tend to lack well organised phagocytic structures and although phagocytosis may occur, there still appear to be a deficiency in the processing and localisation of antigen (Reade, 1968; Williams and Nossal, 1966).

It is appealing to think that the lack of an immune response of the neonates to certain antigens may allow the display of or even facilitate tolerance induction. There is evidence for a similar situation in the adult; a parallel that has been taken to argue in favour of a common basic mechanism for tolerance induction.

### III. Tolerance in mature animals.

#### a. Historical.

Adult animals were first shown to display immune negativity with symptoms resembling those found in neonatal tolerance by Glenny and Hopkins in 1924. Studying the rate of elimination of horse anti-diphtheria toxoid serum from rabbits, they showed in two animals previously exposed to 18 or 21 ml of normal or antitoxic serum, a delay in the rapid phase of immune elimination. They suggested that the outcome might be dependent upon the ratio of antigen to antibody either present or produced, but did not rule out the possibility that "excessive stimulation might have crippled the antibody response."

Felton and Ottinger (1942) and Felton (1949) displayed the phenomenon they called paralysis which again had features resembling neonatal tolerance. They injected 0.005 - 0.5 mg of pneumococcal polysaccharide or whole pneumococci into adult white mice. In most instances a specific failure to respond to a normally immunogenic dose of 0.005 mg was reported. The polysaccharide persisted in the tissues for at least 52 weeks.

Differences in the nature of antigens, easily degradable or persisting, led to distinctions being drawn between paralysis and specifically induced unresponsiveness, in particular to natural protein antigens. At present it is advisable to consider this distinction to be of relevance to at least the kinetics if not the mechanism of tolerance induction.

#### b. Specific unresponsiveness to protein antigens.

Although Glenny and Hopkins (1924) described the induction of an unresponsive state to serum proteins, it was not until 1955 that their original observations derived support. Dixon and Maurer (1955 ; 1955a) injected rabbits for 37 days with 500mg/kg body weight/day of whole human plasma or purified bovine serum albumin. The animals were unable to

give immune elimination of the predominant antigens when challenged later. Trace amounts of antibody could, however, be detected against certain minor components of human plasma. It is of additional interest that in spite of the large dosage, the animals recovered responsiveness within six months.

Subsequent examples of selective unresponsiveness induced by large doses of protein antigens are numerous (see reviews by Smith, 1961; Dresser and Mitchison, 1968). Impetus for studies of this kind came from the work of Dresser in providing an easily reproducible model system amenable to manipulation. High speed ultra-centrifugation (Dresser, 1962a) and in vivo screening (Dresser, 1963) of bovine gamma globulin was found to remove its ability to elicit an antibody response if given to mice in soluble form. Such material was also found to readily induce tolerance, the alteration being the removal of a minor but powerful antigenic fraction that Dresser presumed to be IgA.

Using this system in CBA mice, Dresser (1962a) demonstrated that relatively low single doses of antigen, i.e. 50 - 200  $\mu$ g, were capable of giving rise to an unresponsive state. The animals were unable to respond by immune clearance to challenge with BGG in Freund's complete adjuvant. Ultracentrifuged human gamma globulin was found to be effective in C57Bl/6 mice over a wide dose range of 0.05 - 10 mg (Dietrich and Weigle, 1964). With bovine serum albumin, however, these workers found that "complete tolerance could be induced only in a small proportion of animals. The degree of tolerance was not clearly related to the dose of BSA." Close examination of the data over the dose range  $10^{-3}$  to  $10^2$  mg does, however, reveal a tendency towards partial unresponsiveness at the extremes of antigen dosage.

In this and other test systems, small doses as well as high doses of certain antigens have been described as giving rise to an unresponsive

state; additional variables are the number of injections, the form of the antigens, the route of application and the species and strain of animals used.

Mitchison showed that BSA was capable of displaying two zones of tolerance separated by a zone of immunity (Mitchison, 1964). In his model experiments for investigating the relationship between immunity and unresponsiveness he used adult CBA mice. Employing multiple injections of BSA and varying the dosage and the time, the response of treated animals and controls to challenge was measured and fitted to a double cubic surface. Four zones of dosage in time could be distinguished. More than 1 mg of BSA three times weekly led, after initial immunisation, to an unresponsive state; 0.04 - 1.0 mg led to immunisation; 1.0 - 10.0 mg also led to tolerance; less than 0.1 mg had no effect.

Further verification of the two dosage effect was later provided and attempts at similar experiments were performed using lysozyme, ovalbumin and diphtheria toxoid (Mitchison, 1968). While none of these antigens gave the low zone phenomenon, lysozyme displayed a tendency to produce unresponsiveness when given in the high dose ( $> 100 \mu\text{g}$  three times weekly). Such dose dependant responses may be compared to those derived for the response of neonates to flagellar antigens (Shellam and Nossal, 1968; Shellam, 1969).

The type of antigen used is of relevance in considering the kinetics of induction of the unresponsive state. Serum albumins and gamma globulins have been extensively used. These are found to be poor antigens in the majority of species, and techniques which reduce their immunogenicity tend to allow their tolerance inducing capacities to be observed. Ultracentrifugation and in vivo screening of bovine gamma globulin has already been mentioned. These techniques have also been exploited for the study of the responses of rabbits to BSA. It was

found that the injection of large doses of BSA into rabbits, followed by removal of their serum and injection of this BSA-containing serum into other rabbits, made tolerance induction in the latter more feasible. This was attributed to the removal of aggregated BSA by the reticulo-endothelial system of the 'filter' animals. BSA was found to give two main peaks on Sephadex G150 filtration. The first peak was presumed to be a dimer and was immunogenic while the second, presumably the monomer, readily induced unresponsiveness. Unfortunately no details were given of further biochemical characterisation of the antigens or their contaminants (Frei et al., 1965; Frei, 1969).

Injection of bovine gamma globulin into the mesenteric vein of guinea-pigs has also been used as a method of selecting for a tolerogenic population of molecules. It was found that 5, 50 and 500  $\mu\text{g}$  injected by this route regularly inhibited the normal delayed hypersensitivity response to 2.5  $\mu\text{g}$  BGG injected into a foot pad. The circulating antibody response was also diminished (Battisto and Miller, 1962).

Other procedures that reduce the immune response to antigen, tend to facilitate tolerance induction. Thoracic duct drainage prior to antigenic stimulus had this effect in rats as did pretreatment with anti-lymphocytic serum in rats and mice (Shellam, 1969 a). Following thoracic drainage only a slight non-significant depression in primary response to an optimally immunogenic dose of flagellin or polymerised flagellin was noted. However, with both treatments, the response to 100  $\mu\text{g}/\text{g}$  body weight given three times weekly for ten weeks, was suppressed. Unresponsiveness was demonstrated by the lack of response to a booster of 10  $\mu\text{g}$  polymerised flagellin two weeks after the last injection. Both treatments thus lowered the threshold for high zone tolerance induction. Results obtained in the low zone regions were equivocal due to inherent variation in the random bred Wistar rats.

#### IV. The mechanism of tolerance induction.

##### a. The cells involved.

In 1956 Billingham et al. postulated three possible routes leading to specific unresponsiveness. "Central" failure of reactivity could lead to a defect in the restricted line of normally responsive cells; "afferent" interference could affect the access of antigen to the cells; "efferent" interference could be brought about by antibody already produced.

'Central' failure of the immune response has been repeatedly suggested as the most common cause of the phenomena described above. This conclusion has been drawn mainly from adoptive transfer studies. Cells transferred from normal to tolerant animals have often been shown to be capable of an antibody response to the antigen previously used to paralyse the recipients (Mitchison, 1962a; Denman et al., 1967; but see McCullagh, 1970). Thoracic duct cells from donors made tolerant to sheep red blood cells by cyclophosphamide treatment were shown to be specifically incapable of restoring the response in neonatally thymectomised hosts (Miller and Mitchell, 1968). Forbes (1969) added normal macrophages to lymphoid cells from mice tolerant to sheep red blood cells and found no in vitro antibody formation.

Further evidence has been derived from studies of the recovery of animals from tolerance as follows.

##### b. Recovery of adults from an unresponsive state.

Early studies on the persistence of unresponsive states held suggestions that the phenomenon might be a stable condition. Such inferences, however, came from studies upon tolerance induction in neonates using pneumococcal polysaccharide (Felton, et al., 1955a) or transplan-

tation antigens (Billingham et al., 1956). Studies using antigens which are more susceptible to degradation, suggest that recovery usually takes place over a period of a few months.

Smith (1961) argued that recovery occurs when the antigen disappears from the circulation. Such a conclusion was derived from the finding that continuous repeated injections of small doses of antigen could prolong the state seemingly indefinitely. Such a hypothesis is however no longer tenable in view of the findings of Humphrey (1964a) and others (Mitchison, 1965; Dixon and Weigle, 1963). These authors all found that tolerance persisted for at least a short time after antigen could be reasonably presumed to have disappeared from the animal. These experiments tend to support the concept that, rather than maintaining cells in their unresponsive state, further antigen injections in fact operate upon new maturing cells.

More support for this hypothesis comes from the finding that the duration of the unresponsive state increases markedly with the age of the animal (Mitchison, 1965). The influence of thymectomy upon the duration of tolerance also argues for the recovery of responsiveness as being due to the appearance of a new 'virgin' population of lymphocytes.

When mice are made neonatally tolerant to bovine serum albumin, their recovery can be slowed by adult thymectomy (Taylor, 1964; Pierpaoli, 1967). The response that does occur, has been ascribed to a reaction of marrow-derived cells, acting without the cooperation of thymus-derived cells (Taylor, 1969). Further evidence for a defect induced in the thymus-derived cell population during tolerance to serum proteins comes from Isaković et al. (1965). Thymus grafts from animals tolerant to bovine gamma globulin were found to be specifically

incapable of restoring responsiveness in thymectomised and irradiated recipients. In addition, one of the best methods of producing a profound tolerant state to either normal or heated bovine gamma globulin is to inject the antigens into the thymuses of irradiated rats (Staples et al., 1966; Horiuchi and Waksman, 1968). In a cooperative system of bone marrow and thymus cells Taylor (1969) showed that irradiated (900r) and reconstituted mice had a depressed ability to respond if either of the two reconstituting populations were derived from donors given 10 mg of bovine serum albumin one day prior to transfer. The phenomenon was especially striking when thymus cells were from the injected donor.

Although it seems likely that thymus-derived cells can become tolerant with a resulting lack of responsiveness in the whole animal, tolerance of the bone marrow-derived cells also may occur. 'Thymus-independent' antigens can induce tolerance, and inability of normal thymocytes to break tolerance to sheep red blood cells has been reported (Denman et al., 1967), although the thymic inoculum was unfortunately not shown to be active in non-tolerant animals. The recovery from tolerance in the bone marrow-derived population might be expected to be relatively rapid in the absence of antigen, but at present there is little evidence available to support the idea that the duration of the tolerant state might depend upon the particular cell population involved.

c. Antigens and tolerance.

The ease with which certain serum proteins and cellular isoantigens induce tolerance in both neonatal and adult animals is striking. The reasons for this may be multiple.

It has been suggested that the final immune response of an animal may depend upon the differences between autologous structures and the



antigen. Presumably where the antigen has many determinants in common with host components, or where there are structural similarities between corresponding epitopes, the response may be weak. As already mentioned tolerance is easier to induce to 'weak' antigens (Dresser and Mitchison, 1968; Gowland, 1965). Partial support for this concept comes from a finding that smaller amounts of serum or gamma globulins were needed to induce tolerance in the chicken when turkey antigens were used rather than the phylogenetically more remote goose (Tempdis, 1964). Similarly mice become more readily tolerant to rat serum albumin than to guinea-pig, chicken or bovine albumin, as judged by immune elimination of the antigens (Dietrich, 1968).

A further reason why certain antigens appear to produce tolerance readily may lie in those physico-chemical characteristics of the antigen that allow diffusion through the body. The kinetics of tolerance induction tend to favour a hypothesis involving direct interaction of antigen with the lymphocyte. The best evidence for this comes from Mitchison (1968a). Although many reports suggested that tolerance induction within lymphoid organs may take several days (Weigle and Golub 1967), Mitchison showed by adoptive transfer that non-responsiveness of the peripheral white blood cell pool could occur within two hours, although the problems of redistribution of 'responsive' cells following antigen injection were not explored. The lack of an immune response due to the lack of phagocytosis of antigen with resulting tolerance induction has already been discussed (see Frei et al., 1968).

The relationship between the number, spatial distribution and the size of antigenic determinants as regards binding to, and perhaps perturbation of, the membrane of the antigen-sensitive cell is a relatively unknown factor in tolerance induction as, indeed, it is in antibody formation. As in antibody formation, monovalent antigens

seem to be incapable of tolerance induction, suggesting that tolerance induction is an active process with similar thermodynamic requirements to antibody formation. This aspect of tolerance induction will be discussed later.

d. The relation between tolerance and immunisation.

The relationship between specific unresponsiveness and responsiveness has often been said to hold the key to the problem of the mechanism of tolerance induction.

Mitchison (1964; 1968) demonstrated that mice receiving an extended course of multiple injections of high doses of BSA or lysozyme went through an initial phase of immunisation before passing to an unresponsive state. It seemed that no matter how high the dose above a high zone tolerance threshold, the initial steps were those of immunisation.

There have been many reports that it is possible to render animals primed to a certain antigen, tolerant to it by further injection of large doses of that antigen (Dresser, 1965). It has been argued that such experiments do not differ essentially from the induction of high zone tolerance (Dresser and Mitchison, 1968; Mitchison, 1968). The difference between high and low zone tolerance may, in a similar manner, be considered functional rather than fundamental, the high doses of antigen being required to combat that antigen 'lost' by binding to antibody. It was demonstrated that concomitant immunisation occurs even in the presence of vast antigen excess (Day and Farr, 1966). Rabbits receiving 500 mg BSA daily were found capable of antibody production which could be detected only after fractionation of the serum to detect non-absorbed anti-BSA antibodies. Such an exercise displays the difficulties in demonstrating true inhibition in the high zone as compared to "mopping up" of antibodies by excess antigen. This would be especially true in

experiments where in vivo techniques e.g. antigen clearance are used as measures of immune reactivity.

It is an attractive proposition that the requirements for tolerance induction are similar to those for immunisation. It is of some interest that the only reports of tolerance induced in vitro (to thymus dependent antigens) were done under conditions where a primary immune response can also be induced (Deiner and Armstrong, 1967; Armstrong et al., 1969; Scott and Waksman, 1969). Similarly D aminoacid polymers which are nonimmunogenic, are also incapable of producing tolerance although they can combine with antibodies produced against L aminoacid polymers (Collotti and Leskowitz, 1969).

Supra-optimal stimulation of an animal with many antigens may result in a sub-optimal response. The relationship of this phenomenon to classical tolerance is not known. It has been suggested that when the dose is above the optimal there may be restricted proliferation with ensuing exhaustive differentiation (Šterzl, 1967). The hypothesis, that the response of antigen-sensitive cells may depend upon the dose and type of antigen, receives some support from the experiments of Byers and Sercarz (1970). There was a delay in the in vitro antibody response of rabbit lymph node fragments to BSA when the cells were cultured in the presence of the antigen for three days instead of two hours. There was also a delay in the wave of proliferation of antibody-forming cells precursors. This is similar to the dose - response relationship found in the in vitro transformation of primed cells by antigen. In vivo, large doses of antigen were also shown to prolong the initial lag phase before antibody production (Modabber and Sercarz, 1970). While the relation of these phenomena to tolerance is uncertain, they suggest the need to consider tolerance as possibly involving differentiation and proliferation of antigen-sensitive cells regulated by antigen in a

manner not conducive to the production of large quantity of antibody over a long time period.

Such hypotheses suggest a reversibility of paralysis at the cellular level. There is some scant evidence to support this idea. Thoracic duct cells from normal, or tolerant, allogeneic animals can induce a high haemolysin response in host rats tolerant to sheep red blood cells. The response is brought about by host cells (McCullagh, 1970). An alternative explanation, however, in this case could be that a small newly generated population undergoes rapid multiplication.

It is obvious that the relationship between tolerance and immunisation requires further examination at the cellular level in order that the factors involved in immunogenesis be fully understood.

#### V. Factors influencing tolerance and immunity.

The number of parameters that may be involved in dictating the end result of an animal's response to an antigen, appear limitless. The size of the problem may be judged by the problem of "the animal that ruins the statistics."

The influence of psychosocial factors upon various immune responses has been studied in length. Unfortunately, although such factors as 'shock', handling, 'crowding' of animals etc. have been shown to have an influence, the outcome of such manipulations is not easily predictable, being dependant upon the type of manipulation used, the stage of the animals' life when it occurs, the dosage and intensity of the insult, the phenomenon under investigation and the means by which it is studied (see Friedman et al., 1969). The difficulties inherent in such studies mean that little basic knowledge is available on the effects of psychosocial factors although their existence is of extreme importance in experimental design.

There are, however, numerous factors which have been shown to have a reasonably predictable effect upon immune responses. Certain factors which tend to have an inhibitory effect have been mentioned in passing. It is of more relevance to this particular thesis to examine in more detail factors that enhance immune responses, in particular the non-specific influence of bacteria and bacterial products upon antibody production.

7. Non-specific stimulators of antibody formation.

a. Introduction.

An enormous list of substances is now available whose administration enhances the recipient's ability to respond to a non cross-reacting antigenic challenge (see White, 1963; Munoz, 1964; Herbert, 1964). The spectrum of substances, defined as adjuvants, is so great as to take the majority outside the scope of this thesis. In justification, it is sufficient to point to the wide range of physical characteristics attributable to these substances, their varying effects, and the likelihood that more than one mechanism of action is involved, possibly even where only a single substance is being investigated. Extrapolation between adjuvants with regard to their mechanisms of action, is also complicated by the finding that the effects noted are often dependant upon the variables involved in each particular experimental system.

For these reasons, special attention shall be reserved for those bacteria or bacterial products that act as adjuvants, and of these only the *Corynebacteriaceae* shall be considered in detail.

b. Historical.

Since the turn of the century, many physicians have remarked upon the apparent correlation between the presence of certain bacterial infections and an enhanced resistance of their patients to other non-related infectious agents and to neoplasms (see Fowler, 1969). The recent interest in non-specific immunotherapy for the treatment of patients with leukemia (Mathé, 1969; Mathé et al., 1969) is derived from these observations and receives considerable support from animal experiments.

Lewis and Loomis (1924) were among the first workers to suggest on the basis of experimental evidence that bacteria could non-specifically enhance the immune response to an antigen. The injection of sheep red blood cells into tuberculous lesions of guinea-pigs resulted in a supra-normal agglutinin titre. This work was later extended to antigens such as egg white, and horse serum with similar results (Dienes, 1927; 1936).

Freund began his classic experiments bearing in mind the above mentioned observations and the reports that petroleum jelly with lanolin (Le Moignic and Pinoy, 1916, 1916a), paraffin (Coulaude, 1935) and vaseline oil (Saenz, 1935; 1937) were all able to elevate antibody and/or hypersensitivity responses. Freund's studies on the delayed hypersensitivity to tuberculin using killed tubercle bacilli and on the adjuvant effect of water-in-oil emulsions containing these bacilli, laid the basis for the most common experimental adjuvants in use to-day (see Freund, 1956). Although administration of a mixture of an aqueous antigen solution with light mineral oil and emulsifier leads to an enhanced immune response, the addition of killed mycobacteria not only further increases the antibody and sensitisation responses but also in some way changes the characteristics of sensitisation away from an immediate type of response to a delayed one.

A wide variety of bacteria and their products have been shown capable of adjuvant action either on their own or as substitutes for mycobacteria in Freund's complete adjuvant.

Various gram-negative bacilli possess activity on their own which is believed to be associated with their endotoxin content (Stuart and Windel, 1951. Johnson et al., 1956). Khanolkar (1924) showed that Escherichia coli, Salmonella paratyphi B and pyocaneus bacillus (Pseudomonas pyocyanea /aeruginosa/) enhance antibody formation against Gaerntner's bacillus (S.enteritidis). Since then Bacillus abortus, Haemophilus influenzae, H.pertussis and certain Salmonella, Shigella and Serratia have also been shown capable of enhancing responses to a variety of immunological challenges (see Munoz, 1964).

Gram-positive cocci were first shown to enhance agglutinin formation to S.typhosa when given simultaneously to human volunteers (Clark et al., 1922). Ramon (1925) demonstrated that horses developing abscesses at the injection site of diphtheria toxoid developed higher anti-toxin titres upon hyperimmunisation than those without them. The same result could be obtained by experimental infection of the injection site with Streptococci and Staphylococci.

Gram-positive bacilli, in particular Listeria and Corynebacteria, which will be dealt separately, have also been shown, under certain conditions, to possess adjuvant activity.

#### c. Postulated mechanisms of adjuvant action.

The postulated mechanisms suggested for adjuvant action are numerous. As it is impossible to cover all of these in detail, only a few of the more commonly suggested mechanisms will be dealt with in relation to the adjuvant concerned. A few basic points, however, should be borne in mind.

The parameters involved in the study of adjuvant action are superficially similar, at least in number, to those dictating a normal immune response. The dose and the type of antigen and adjuvant as well as the route and 'timetable' of their injections have been shown to be important (Weigle, 1960; Shaw et al., 1962; Farthing and Holt, 1962; Lipton and Freund, 1953). The sex, age and nutritional status of animals are possible variables (Miles and Pirie, 1939; Axelrod and Pruzansky, 1955), as well as the species (Rice, 1947) and strain of animals used (Lewis and Loomis, 1925; Schneider, 1959). The mode of action of adjuvant is hence difficult to determine except, perhaps, within the operating experimental conditions. However, there seem to be two, mutually non exclusive, mechanisms by which adjuvants may be distinguished in accordance with their main effects.

The first involves the protection of antigen by adjuvant and its slow release. This view was first presented by Glenn et al. (1926; 1931) in their studies on alum-absorbed toxoids and was also later taken to incorporate the 'depot' action of Freund's adjuvant (Freund, 1956; White et al., 1955, 1955a ).

The second mechanism includes the more physiological rather than physical effects of adjuvants as first described by Ramon (1925) (Vide infra). Since then many inorganic and organic substances, often not chemically related, have been shown to possess adjuvant activity that is associated with the appearance of local reactions, with cell destruction, cell proliferation and often granuloma formation. The relationship of these phenomena to the stimulation of antibody forming cells within lymphoid organs and also within the granulomatous site has been discussed in detail (White et al., 1955, 1955a ).



The stimulatory and/or toxic effect of certain bacteria or bacterial products upon cells has been suggested as a possible common pathway of their adjuvant action. Hepatosplenomegaly and/or increased reticulo-endothelial activity has often been taken as a means of screening for adjuvant materials based upon this concept.

Besides increasing reticuloendothelial activity, endotoxin may alter vascular permeability (Condie et al., 1955). Endotoxin also affects liver lysosomes causing release of  $\beta$ -glucuronidase and cathepsin (Weissman and Thomas, 1962). Lysosomes from rabbits made tolerant to endotoxin or given hydrocortisone did not show this effect. These treatments also affect the ability of endotoxin to act as an adjuvant (Golub and Weigle, 1967). A further explanation for the action of endotoxin as an adjuvant related the action of Salmonella typhosa lipopolysaccharide to the 'stress symptoms' which it produced in rabbits. The responses of mice and guinea-pigs, which were fairly resistant to the endotoxin, were little affected by its adjuvant action (Kind and Johnson, 1959).

One of the most important practical and theoretical points to be considered when dealing with those adjuvants that do not have the antigen incorporated, is the timing of their administration with respect to that of the antigen. This was highlighted for endotoxin (Kind and Johnson, 1959; Taliaferro and Taliaferro, 1954). The adjuvant was found active only if given within a critical time period, at or around the time of antigen administration. This led to a suggestion that adjuvants act upon the early period of the immunisation, cutting down the induction time of antibody production. This would be consistent with the ability of many adjuvants to affect only the primary immune response to an antigen.

A further mechanism of adjuvant action postulated for B. pertussis, is based upon its well documented effects of leucocytosis and lymphocytosis

in mice (Morse, 1965; Morse and Reister, 1967). The main effect on the lymphocyte population was redistribution of these cells rather than an increased rate of multiplication. This was taken to account for the observed enlargement and hypercellularity of the paracortical areas of lymph nodes, and the hypocellularity of thymus. Neonatally thymectomised animals do not give this response (Kalpakstoglou et al., 1969).

Little is known about the physical and chemical requirements than confer upon bacterial substances the ability to act as adjuvants. The essential active constituent in mycobacteria appears to be a cell wall peptido-glycolipid (White and Marshall, 1958; White et al., 1964). The mycobacterial cell wall possesses a complex superficial network of interlacing filaments of a uniform width that are similar in form to purified peptido-glycolipids (White, 1967a,b). No such structures were found when a wide variety of other bacteria were examined, with the possible exception of Nocardia. The presence and the number of such filaments may be related to an ability of these bacteria to resist digestion by host enzymes.

It seems rather paradoxical that many substances which suppress antibody formation have been shown also capable under certain conditions of enhancing it. Dixon and McConahey (1963) found that whole body X-irradiation (400-600r), given 2-3 hours after antigen, enhanced antibody titres. Colchicine augmented antibody responses in X-irradiated and normal animals (Taliaferro and Jaroslow, 1960). Chammoughan and Schwartz (1966) showed that 6-mercaptopurine treatment five days before antigen led to an enhanced immune response.

Such experiments can be taken in conjunction with others that suggest DNA, RNA or nucleotides could enhance antibody production. Taliaferro and Jaroslow (1960) restored the haemolysin response in X-irradiated rabbits by injecting antigen with DNA or RNA digests 24 hours after

irradiation. Braun (1965) reported that injection of oligonucleotides from calf thymus led to an increase in number of plaque forming cells against sheep erythrocytes. These findings support another hypothesis that certain adjuvants may act by providing substances necessary for the control of the initial stimulatory events that lead to antibody production.

#### 8. Corynebacteria as adjuvants with special reference to C.parvum.

The Corynebacteriaceae are habitual saprophytes of the natural cavities in man and animals. Under certain conditions they may become pathogenic. While studying this pathogenicity in man, Prévot noted an intense reciprocal affinity of these bacteria to the reticuloendothelial system. Further, he noted in uncured patients a tendency to develop malignant tumors and haemopathies, features which, unlike the cases of lymphadenitis, purulence, septicaemia and metastasis, tended to distinguish the corynebacterial infections from those caused by other endogenous flora (Prévot, 1968). Although experimental injection of animals with these organisms did not induce tumors, Levaditi et al. (1965) did show live C.anaerobium to bring about a characteristic histioreticulosis associated with the accumulation of PAS + ve material in plasmods and monocytes. Since heat-killed, enzyme-digested and ultrasonicated material could give a similar response, it was suggested that there was an interaction between bacteria and the reticuloendothelial system which was not a result of the infection (Prévot, 1963). Many strains of Corynebacteria were in varying degrees able to stimulate the reticuloendothelial system of mice (Prévot and Tran Van Phi, 1964).

Attempts to isolate the "reticulostimulin" have so far proved unsuccessful. It seems to be, however, dependant upon the external

topology of the cell wall (Prévot et al., 1968). It may be of interest to follow up the reports that petroleum ether extract of C.ovis lipids given intradermally lead to an intense local reaction and cause death of phagocytes when administered on carbon (Carne, <sup>et al.</sup> 1956). The virulence of C.ovis seems to be related to its lipid content (Jolly, 1966) and the most powerful reticuloendothelial stimulators also appear the most pathogenic (Prévot, 1968).

With regard to the adjuvant effects of C.parvum, Amiel et al. (1969) showed its protective effect in mice challenged intraperitoneally with  $10^5$  isogenic leukemia cells. C.parvum in this experiment was given intravenously four days before challenge. Tumor growth in mice could also be significantly inhibited if animals were given C.parvum within two days after the sarcoma cells transfer, but protection against Ehrlich ascites tumor was established only when C.parvum was given on the same day by the same route as the tumor cells (Halpern et al., 1966). A significant delay in the onset of mammary carcinoma was found when C.parvum was given either at day -2 or between days 8-12 (Woodruff and Boak, 1966). In experiments on the resistance of syngeneic or  $F_1$  mice to AKR leukemia graft after C.parvum application, it was shown that the effect was best when it was given at day -7 but only by the same route as the cell inoculum (Lamensans et al., 1968). It is obvious that the protective effect of C.parvum is dependant upon the nature of the stimulus, as well as the route and timing of the injections and in all probability, the source of the used C.parvum preparation.

It has often been found that C.parvum is most (or only) effective when given at or near the time of tumor challenge. From a clinical view-point this is an obvious drawback. Currie and Bagshawe (1970), however, have reported favourable results in fibrosarcoma bearing mice when cyclophosphamide was administered prior to C.parvum although the

timing of the injection appeared crucial. In spite of the difficulties involved several fairly successful treatments of humans have been reported. In these cases C.parvum was used in conjunction with more conventional methods of tumor therapy (Mathé, 1969).

While there are few other reports on adjuvant activity associated with corynebacteria, C.parvum has been shown capable of substituting for mycobacteria in complete Freund's adjuvant and enhancing levels of circulating antibodies and delayed hypersensitivity to picrylated albumins in guinea-pigs (Neveu et al., 1964). Similar substitution with C.rubrum, its chloroform extracts and residues was also successful (Paronetto, 1968). The last preparation could also induce adjuvant arthritis in rats but not in mice nor in guinea-pigs (Paronetto, 1970).

The effect of C.parvum on the cellular kinetics of the response to sheep red blood cells in mice has been studied in some detail. An increase in the number of direct plaque forming cells was found if the adjuvant was given at the same time, or better four days before the antigenic challenge (Amiel et al., 1969). An increased rosette formation was demonstrated in similar experiments by Decreusefond et al., (1970). They noted that the enhanced response in C.parvum treated mice was marked by an increase in all specific rosette-forming cell types although there was an especially large increase in plasmacytes.

Associated with the injection of C.parvum into mice, various haematological sequelae have been reported. In particular it was a fall in haemoglobin level (Halpern et al., 1964), an increase in reticulocyte count (Nussenweig, 1967) and a decrease in circulating erythrocytes (Halpern and Fray, 1969). The relationship between the immunological and haematological effects of the bacteria is as yet unknown although there has been a recent report that the autoimmune manifestation of the NZB mice disease could be brought on by the administration of C.parvum (Halpern and Fray, 1969).



9. Proposed objectives of research in this thesis.

The basic objective of the work reported in this thesis was to elucidate the relationship between immunological unresponsiveness and immunological reactivity. It was hoped that a study of factors influencing the decisive initial phases of the immune response might further elucidate the mechanisms involved in initiation of these phenomena. Furthermore, such study may throw light upon the relationship of altered activity of cells to autoimmune manifestations.

In order to study the initial phases of immune responsiveness three approaches were employed. The first was chemical and physical modification of antigen resulting in alterations in its initial mode of localisation. The degree of aggregation of a protein has previously been shown to have dramatic effects upon antibody production (Dresser, 1962a; Claman, 1963; Biro and Garcia, 1965). The role of the physical state of antigen in determining unresponsiveness or reactivity appears basic to the understanding of the different mechanisms underlying these two phenomena.

The second approach involved the use of various reticulostimulating agents. C.parvum strain 10387 had previously been shown capable of preventing the induction of tolerance to bovine serum albumin in rabbits with the induction of immune responsiveness (Pinckard et al., 1967, 1967a; 1968). It was hoped to study this effect in more detail with regard to the localisation of the antigen within the lymphoid organs and the cellular disturbances caused by this and other adjuvants. In order to do this it was necessary to utilise inbred CBA mice rather than rabbits which enable the use of a larger and more uniform population.

The third approach to the study lies in the observed reticulocytosis (Nussenweig, 1967) and lowered haemoglobin level (Halpern et al., 1964)

and the increase in the number of spleen cells with reactivity directed against 'self' red blood cells (McCracken et al. 1971), that has been observed in mice treated with C.parvum. It was hoped that it would prove possible to differentiate or link the haematological disorders with the adjuvant effects of the organisms.

In order to study the antibody responses to antigens care was taken in the choice of technique. It is well established that many classical serological tests measure the secondary effects of antigen-antibody interactions. Such tests may bias their sensitivity in favour of one or more classes of antibodies. It is of particular importance in the study of adjuvants where alterations in the class of antibody has been noted (White, 1967) to use tests which are dependant upon the primary binding of antigen with antibody, and whenever possible to have information available on not only the quantity of antibody formed but also its quality.

## Materials and Methods

### 1. Animals.

#### a. Breeding and stock mice.

CBA strain inbred mice were bred by the 'traffic light' system of cousin mating from nuclei obtained from the Animals Division, Institute for Medical Research, Mill Hill, London. Three generations only were bred from each nucleus. The first generation, the progeny of the first generation and the second generation were used as stock animals or further mating pairs as the situation demanded. All other progeny were used for stock and experimental purposes only.

Mice of the New Zealand Black (NZB) strain were of stock originating from Otago Medical School, New Zealand and maintained in the Department of Surgical Science, Edinburgh, or from the progeny of breeding pairs derived from this source but bred in the Bacteriology Department Animal House. The inbred nature of this strain has been maintained by brother-sister matings for over 72 generations.

Other inbred lines of mice used - C57BL, CBA, DBA/2, and NZB x CBA, C57BL x CBA hybrids - were obtained from the Department of Surgical Science, Edinburgh.

#### b. Care of mice.

All mice were handled and cared for in accordance with the recommendations of the U.F.A.W. Handbook. Breeding mice were fed 'Oxoid' autoclaved breeding diet supplemented by bran twice weekly. Weanling mice (3-5 weeks of age) were fed a 1:1 mixture of the breeding diet and 'Oxoid' B41 experimental diet. All stock and experimental mice received the standard B41 pellet food. At all times the animals were allowed to feed and drink ad libitum.



c. Experimental groups.

Unless specifically stated to the contrary, mice of 12 - 41 weeks of age (17 to 27g body weight) were used and within any given experiment, the weight range was  $\leq$  3g and the age range  $\leq$  6 weeks.

At all times different groups within an experiment were carefully matched as to sex, weight, age and number of animals per cage and mice from different litters and generations randomised.

Wherever possible animals of one sex only were used in any given experiment and further experiments to show up the influences of sex variation performed. In particular, where peripheral cell counts were performed and alterations of in vivo phagocytosis and pinocytosis were being studied, animals of only one sex were used. These phenomena are known to show quantitative differences linked to sex (Dunn, 1954).

d. Collection of mouse sera.

Unless otherwise stated, mice were bled from the retro-orbital plexus. A hand drawn glass pipette made from tubing 4 mm external diameter and 2 mm internal diameter was used. Before use the pipette was rinsed in sterile saline containing 400 i.u. of heparin per ml and blotted dry. The blood was clotted at room temperature for 1 hour. This prevented haemolysis which tended to occur at 37° C. Following overnight incubation at 4° C the clotted blood was spun for 20,000g.min and the sera removed and stored (-20° C) till used.

2. Antigens.

Bovine serum albumin (BSA).

Immunologists have often considered serum proteins such as albumins to consist of a pure or homogeneous population of molecules. For this reason they are often preferred to more complex antigens for investigations

into the mechanics of antibody formation. Recent evidence has, however, suggested a marked degree of biochemical heterogeneity within albumin preparations (Peeters et al., 1970). Furthermore, it is now recognised that the different physical forms present in purified preparations of antigens, may vary in their immunogenic potential and in their capacity to act as test antigens for the in vitro detection of antibody. It is therefore necessary to define, to some extent, the BSA preparations used in this thesis.

a. Native BSA (BSA).

Two batches of Cohn fraction V ('Armour' - lot nos. MM2071 and NM2072) were the main sources of the BSA used in this thesis. Stock solutions were prepared in 0.85% sterile saline and stored in aliquots at  $-20^{\circ}\text{C}$  for periods of less than three months. All solutions were standardised for protein content by their absorbance at 280  $\text{m}\mu$  in a Unicam spectrophotometer, as compared to standard BSA solutions of known nitrogen content determined by the micro-Kjeldhal method (Kabat and Mayer, 1961).

This BSA is approximately 90% pure with less than 5% aggregated material. Five lines were found by immunodiffusion and immunoelectrophoresis when the preparations were tested against a potent rabbit anti-bovine serum. The main contaminant migrated as though it was a transferrin. The other contaminants were one  $\alpha$ -globulin and two  $\beta$ -globulins.

b. Centrifuged BSA (c.BSA).

c.BSA was prepared by the method of Pinckard et al. (1967). Three 4 ml quantities of BSA were spun in the cold for  $8.2 \times 10^6$  g.min in a MSE 'Superspeed 40' using a swing out rotor and with the brake off. The

top 2ml of each tube were carefully removed and used fresh. The contaminants in this preparation were qualitatively similar to those in BSA.

c. Monomer BSA (m.BSA).

Monomer BSA was prepared by filtration through Sephadex G150. The only contaminant detected by immunoelectrophoresis was the  $\alpha$ -globulin component.

d. Heated BSA (h.BSA).

Heated BSA was prepared by incubating a stock solution of BSA at 60°C for 1 hour in a water bath.

e. BSA heated at low pH (p.BSA).

The pH of a stock solution of monomer BSA (50-100 mg/ml) was lowered to 3.8 by the addition of 0.1 N HCl. After one hour at 60°C the solution was allowed to stand at 4°C for two days. The aggregates were removed by centrifugation for 5000 g.min and washed in sterile saline until the supernatants showed no visible precipitation on the addition of 10% TCA. The precipitates were then dissolved in sterile saline and the pH adjusted to 7.0. These preparations show a considerable degree of aggregation as judged by Sephadex G150 gel filtration, although 'monomer' is also present.

f. Alum-precipitated BSA (a.BSA).

Alum-precipitated BSA was prepared using the aluminium hydroxide gel 'Alhydrogel' (Seravac Labs., Maidenhead). This is a viscous homogeneous gel containing 2% Al(OH)<sub>3</sub> corresponding to 1.3% Al<sub>2</sub>O<sub>3</sub>. This

gel has a high absorption capacity for proteins, is supplied sterile and is pyrogen-free. It also has a stable uniform form (Neubert and Bethke, 1968).

A given volume of 'Alhydrogel' was added to the same or a smaller volume of stock BSA solution of concentration  $\leq$  25 mg/ml. After immediate mixing adsorption of the protein was continued by incubation at room temperature for one hour and overnight at 4°C. After spinning for 20,000g.min at 4°C the supernatant was discarded and the precipitate resuspended in sterile saline so that the final volume was identical to the original volume of 'Alhydrogel' used. Large aggregates were dispersed by repeated passage through a 19 gauge and then a 23 gauge needle.

This procedure leads to a very stable complex in which 95-100% of the BSA is bound. Repeated washing in saline leads to only an extremely small loss of bound BSA. Even with eight daily washes with 10% normal rabbit serum, over 50% of the BSA added is still bound (Pinckard, 1967; McBride - unpublished results).

g. BSA in Freund's complete adjuvant.

'Difco' Freund's complete adjuvant containing 0.05 mg Mycobacterium per ml was used. Equal volumes of BSA at the appropriate concentration and adjuvant were mixed using two syringes connected by a needle with a Luer attachment at both ends. The milky emulsion formed was tested for stability by placing a drop on the surface of water. (Herbert, 1967).

3. Radioactive iodination of BSA.

In all instances iodination of BSA was performed by the method of Hunter and Greenwood (1962) and Hunter (1967).

a. Theory.

The chemistry of radio iodination of proteins is basically that of substitution of iodine into tyrosine residues (Hughes, 1957), although under certain circumstances iodine may react with sulphhydryl groups or with tryptophan. The type and dose of substitution will therefore depend upon the availability and number of tyrosine groups in the protein. Substitution occurs when iodide is oxidised to elemental iodine in the presence of the protein.

For this oxidation the chloramine-T method of radio iodination uses a sodium salt of the N-monochloro-derivative of p-toluene sulphonamide which in aqueous solution yields hypochlorous acid. At or around the pH optimum of 7.5, cationic iodine ( $I^+$ ) is formed, the reaction then going to completion. The method is rapid, efficient and involves minimal handling risks as well as giving a high yield (% utilization of iodine) which can be 100% if the concentration of protein is sufficiently high (> 1 mg/ml) (Hunter, 1967).

As with any other labelling procedure, it is of extreme importance that the protein is altered as little as possible during iodination and that it will behave in an identical fashion to native protein in the test system. Denaturation of the protein caused by the labelling procedure, and alterations in the structure of the protein due to irradiation damage, or due to the substitution of hydrogen by iodine, must all be considered as possibly giving rise to altered biological activity. In view of the fact that 'trace labelling' of a relatively large molecular weight compound was used, such alterations in structure are probably insignificant. However, whenever it was considered to be important, the biological activity of the radioiodinated BSA was compared with that of the native form.

b. Explanatory notes.

The following procedure leads to a calculated substitution of one atom of  $^{125}I$  per 15 - 50 molecules of BSA or one atom of  $^{131}I$  per 200

molecules. Unless otherwise stated trace labelled BSA was prepared with these degrees of substitution.

Borate buffer (pH 8.4,  $\mu = 0.1$ ) was employed for labelling only when the end product was to be used in the Farr technique. Otherwise phosphate buffered saline was used (pH 7.2). Both  $^{125}\text{I}$  and  $^{131}\text{I}$  radionuclides were used. Their relative merits have been discussed in detail (Hunter, 1967).  $^{125}\text{I}$  was the radionuclide most commonly used in this thesis because of its greater half life, the better resolution obtained when using it in autoradiographic studies and the lower external radiation risks. Although the specific activity of  $^{125}\text{I}$  is lower than for  $^{131}\text{I}$  this is in part compensated for by the higher counting efficiency for the weak gamma emitter.

c. Materials.

I. Borate buffer pH 8.4;  $\mu = 0.1$

or Phosphate buffered saline pH 7.2;  $\mu = 0.2$

II. 3mCi IMS/3  $^{125}\text{I}$  in iodide form (98% iodide):

carrier free in dilute NaOH solution pH 7-9; free from reducing agent; 80-120 mCi/ml; specific activity of 4-12 Ci/mg iodine; probable isotopic abundance 25%.

or 3mc IBS/3  $^{131}\text{I}$  in iodide form; carrier free in dilute NaOH solution pH 8-10; free from reducing agent; 200 mCi/ml; specific activity of 20 Ci/mg iodine; isotopic abundance of about 15%.

Data and material supplied by The Radiochemical centre, Amersham, Buckinghamshire.

III. Chloramine-T (BDH) 0.5 mg/ml aliquots in distilled water, stored at  $-20^{\circ}\text{C}$  till used.

IV. BSA Stock solution 20 mg/ml were prepared as described earlier.

d. Procedure.

Normal safety precautions for the handling of high activity samples were adhered to throughout. 0.25 ml of the appropriate buffer was added to the solution containing the radionuclide. With continuous stirring, to this was added 0.25 ml of 2% BSA solution (5 mg) immediately followed by 0.25 ml (125  $\mu$ g) Chloramine-T. After 1-2 min the reaction mixture and washings were transferred to a 1" dialysis sack, the final volume being approximately 4 ml. All additions and manipulations were performed using 1 ml sterile disposable syringes and needles. Dialysis was carried out in the cold (4°C) for 2-3 days against 5-6 changes of the appropriate buffer.

After this period of time chloramine-T and unreacted iodine were to a large extent removed, the preparation being approximately 98-99% precipitable by 10% TCA.

The yield (% utilization of iodine) using this method was over 80% with borate buffer and over 90% when phosphate buffered saline was used.

Note:

Chloramine-T is an oxidising agent and a small excess of sodium metabisulphite is often added to bring the reaction to a close, reducing both the Chloramine-T and also any unreacted iodine to iodide. The small quantities of Chloramine-T and the degree of substitution required as well as the fact that unwanted materials were removed by dialysis made a reducing agent unnecessary.

#### 4. Scintillation counting

##### a. Theory.

The passage of a charged particle across a solid or liquid phosphor leads to a transfer of the energy of the particle to the phosphor in the form of molecular excitation or ionisation. A portion of this energy will be re-emitted naturally as small flashes of visible or ultra-violet light i.e. scintillations. Scintillation counters can utilise either solid or liquid phosphors. Soft  $\beta$  emitters (e.g.  $^3\text{H}$ ,  $^{14}\text{C}$ ,  $^{35}\text{S}$ ) use liquid or gel phosphors and the source is brought into intimate contact with the detector, usually organic phosphors in suitable solvents. In this way the geometrical efficiency of the arrangement is at a maximum and self absorption within the source reduced to a minimum. In gamma ray spectrometry the phosphor of choice is normally an alkali metal iodide activated with a trace of thallium.

The scintillations produced in the phosphor as the charged particles traverse it, are absorbed by the photocathode of a photomultiplier tube that then emits electrons, the flow of which is amplified after passing through many anodes, finally emerging as a single pulse that can be recorded on a scaler.

Radionuclides produce characteristically different energies of radiation. As each photon may produce a pulse, these also will vary in magnitude. Discrimination between pulses of different magnitudes or 'heights' can be achieved by means of a pulse height analyser. This device can be adjusted by means of 'threshold' and 'window voltage settings' to accept only pulses within a selected 'channel'. Through this channel only pulses emanating from photons with a given energy will flow and be recorded. This enables pulses specific for the radionuclide being used to be selected for at the expense of extraneous 'noise'.



The efficiency in counting a radioactive preparation is measured by the number of counts recorded per number of disintegrations times 100. This figure often falls short of 100% for a variety of reasons dependant on the source, the detector and their relationship, as well as the operating conditions.

The distribution of the recorded pulses, like that of the disintegrations of the radioactive nuclide, naturally follows a Poisson form. The error in a single determination is thus the square root of the total number of counts collected ( $n$ ). The standard proportional error being  $n/n^2$  or  $1/n$ , a measurement which can be easily reduced to any required dimensions by counting for a sufficient length of time. However, the number of pulses determined has at least two components. One is specific for a given nuclide and the other contributed to by 'background noise'. Since two count rate determinations must be made to arrive at the net count rate, the standard errors of the differences will be compounded of the individual errors. If the total counts and time corresponding to count rate  $A$ , are represented by  $N_a$  and  $t_a$  respectively, and of  $B$ ,  $N_b$  and  $t_b$ , with standard errors of  $a$  and  $b$  then:

$$(A \pm a) \pm (B \pm b) = A \pm B \pm (a^2 + b^2)$$

or

$$(A \pm a) \pm (B \pm b) = A \pm B \pm \frac{A}{t_a} + \frac{B}{t_b}$$

#### b. Equipment.

A Nuclear Enterprises "Gamma-Matic" Mark 1-A Scintillation Spectrometer. The "Gamma-Matic" possesses a 2" NaI, thallium-activated, counting well.

c. Procedure.

The E.H.T. voltage and the amplifiers were set in order to minimise fluctuations due to changes in ambient temperature and minor voltage fluctuations, and to minimise photomultiplier 'noise'.

The pulse height analyser was adjusted so as to include the main energy peaks of the radionuclide to be counted with minimal background noise. In practical terms a background of 1-3 c/sec was sufficiently easy to obtain with any of the nuclides used.

All samples were counted such that the probable counting error was less than 10%, and normally less than 1%.

All samples for comparison, were counted with the same geometry.

Care was taken that the activity of all samples were within the resolving limits of the counter i.e. 'dead' time. ( $< 5 \times 10^5$  c/sec).

5. The ammonium sulphate method for the detection of anti-BSA antibodies.

This technique was performed by the method of Farr (1958) and Minden and Farr (1967).

a. Theory.

The Farr technique gives a measure of the capacity of an antisera to combine with those macromolecular antigens soluble in 50% saturated ammonium sulphate (SAS/2), the method being capable of detecting both precipitating and non-precipitating antigen-antibody complexes.

The test is based upon the principle that radioiodinated BSA is soluble in SAS/2 whereas gamma globulin is not; any \* antigen bound to antibody will therefore be precipitated. \*Antigen not bound will remain in the supernatant. When measured in antigen excess, soluble antigen-antibody complexes are precipitated by SAS/2 and the amount of \* antigen

bound per ml of serum can be calculated. This amount is known as the antigen binding capacity (ABC) of the serum at the particular antigen concentration used. The formation and dissociation of antigen-antibody complexes is markedly inhibited once the SAS has been added, the results therefore are likely to be a true reflection of the amount of bound antigen at a given point in time.

By measuring the ABC values at more than one antigen concentration it is possible to derive a figure based on qualitative differences between antibody populations. This manoeuvre detects the presence or absence of unoccupied antibody combining sites when antigen is in excess. If most of the available antibody combining sites are bound, an increase of antigen concentration does not increase the ABC value of the antiserum. In practise, as the antigen concentration increases, the ABC value also tends to increase to a maximum value, but never decreases. As a general rule, a marked discrepancy in ABC values at two given antigen concentrations indicates the presence of 'non-avid' antibody, normally a population with a rapid dissociation rate (Minden and Farr, 1967).

It can be seen that the Farr technique is one of the few primary techniques capable of quantitating the direct binding of antigen with antibody. Techniques which rely on secondary or tertiary features of the combination of antigen and antibody have long been criticised as being possibly misleading. The results are dependent upon a series of events which often, if not always, solely involve, or are more efficiently performed by, one particular class of antibody; a result of biological capacities of immunoglobulins other than antigen binding. Results obtained from these techniques can thus be easily biased by the presence of a minority population of antibodies.

b. Reagents.

I. Borate buffer pH 8.3 - 8.5,  $\mu = 0.1$

6.184 g boric acid  
9.536 g borax (sodium tetraborate)  
4.384 g sodium chloride  
H<sub>2</sub>O to 1000 ml

After the addition of SAS this buffer maintains pH of 7.9.

II. Normal rabbit serum (NRS)

A one in ten dilution in borate buffer for antisera dilution.

A one in 100 dilution in borate buffer for \*I-BSA dilution.

III. Saturated ammonium sulphate - (SAS), Mayand Baker Ltd.

Specific gravity 1.233 to 1.248.

Stored at 4°C with crystals remaining to prevent supersaturation.

Kept at 4°C before and after addition.

IV. 50% saturated ammonium sulphate (SAS/2) in borate buffer.

SAS diluted 1:1 with borate buffer.

Kept at 4°C throughout.

V. \*I-BSA

The concentration of stock \*I-BSA was carefully measured by reading in an SP500 Unicam spectrophotometer at 280 m $\mu$  and compared with a stock BSA preparation of approximately the same concentration which had previously been standardised by the micro-Kjeldhal method for nitrogen determination. An aliquot of the stock \*I-BSA solution was diluted volumetrically with 1:100 NRS in borate buffer to the required dilution for the test. The normal rabbit serum is necessary to minimise adsorption of the \*I-BSA to the glass and also to prevent denaturation of the antigen by the effects of dilution. The antigen concentrations

most commonly employed were  $0.1 \mu\text{g N}$  and  $0.01 \mu\text{g N}$  added in equal volumes to 0.5 ml antiserum (see note 1, p.71 ).

VI. Trichloroacetic acid (TCA) - 20% (w/v) aqueous solution.

c. Procedure.

Unless otherwise stated volumetric procedures were used as well as standard procedures to eliminate carry over during diluting.

I. Antiserum dilutions.

All antisera were initially diluted 1:10 in borate buffer to give a convenient standard volume. From this four-fold serial dilutions were made employing normal rabbit sera (1:10) as diluent (see note II, p.71 ).

II. Experimental tubes.

For each antigen concentration 0.5 ml aliquots of the antisera dilutions were transferred in duplicate to 75 x 12 mm glass tubes.

III. Control tubes.

For each antigen dilution the following control were used:

- a) 4 "control" tubes received 0.5 ml of 1:10 NRS instead of the antiserum.
- b) 4 "TCA" control tubes received 0.5 ml of the same 1:10 NRS.
- c) 4 "AgAdd" tubes which receive only the\* antigen.

IV. Addition of the \*I-BSA.

\*I-BSA at the appropriate antigen concentration was added to all control and experimental tubes. Each tube received  $0.5 \pm 0.01$  ml from an automatic syringe with valve and cannula attachment (Turner & Co. Ltd. London).

V. After shaking, all tubes were incubated at  $4^{\circ}\text{C}$  overnight.

- VI. With all reagents kept at  $4^{\circ}\text{C}$  all experimental tubes and the 4 "control" tubes (Procedure IIIa) received 1.0 ml of SAS and were immediately shaken by a rotating mixer. The SAS was added by means of an automatic syringe, carefully tested for accuracy of delivery. Control tubes labelled "TCA" controls received 1.0 ml of 20% TCA instead of SAS and the contents were immediately mixed.
- VII. After incubation at  $4^{\circ}\text{C}$  for 30 min all tubes were spun for 4,500 g.min in an MSE refrigerated ( $4^{\circ}\text{C}$ ) centrifuge.
- VIII. After centrifugation the supernatants were discarded and the tubes, containing white precipitates, blotted by inversion onto absorbent paper.
- IX. The "TCA" control tubes are ready for counting.
- X. All other tubes received 3 ml SAS/2 by automatic syringe and the precipitates well resuspended. The tubes were again incubated at  $4^{\circ}\text{C}$  for 30 min before recentrifugation ( $4^{\circ}\text{C}$ ), removal of supernatants and blotting (see note III, p.71 ).
- XI. Counting:
- All tubes were counted as described elsewhere (p.62 ).

d. Calculations.

The "Ag Add" control tubes are a measure of the amount of radioactivity added to all tubes. The "TCA" control tubes give a measure of the amount of this radioactivity bound to protein and hence

precipitable by antibody. In all cases the \* antigen was not used whenever the "TCA" control was less than 98% of the "Ag Add" value. In fact, in the majority of cases "TCA" control values were greater than "Ag Add" values owing to the different geometry of the two samples.

Because the geometry of the experimental tubes and "control" tubes are equivalent to those of the "TCA" controls, and because these tubes give a better estimate of the radioactivity bound to the antigen the average of these tubes was taken as the amount of radioactivity capable of precipitation by anti-BSA antibody.

In calculating the antigen binding capacity of an antiserum, account must be taken of the small quantity of non-antibody bound, non-specifically trapped radioactivity present in the precipitates. Correction for this value is obtained from the "control" control tubes. The number of counts in these tubes are normally about 5-10% of those found in the "TCA" control tubes. The amount of radioactivity bound specifically to antibody in experimental tubes (exp) can be calculated as a percentage of that capable of being bound by the formula:-

$$\% \text{ antigen bound} = \frac{\text{exp} - \text{cont}}{\text{TCA} - \text{cont}} \times 100 \quad (\text{see note IV, p.71} )$$

This value was calculated for the average of each pair of experimental tubes. Within the limits of 30% to 80% this value of percentage of antigen bound by antibody is directly proportional to the log reciprocal of the antiserum dilution. Furthermore, between 10% and 30% there is almost direct proportionality with the reciprocal of the antiserum dilution. These values are approximate and vary slightly between antisera. Quantitative comparison of antisera is therefore not easy nor very meaningful using purely the percent of \* antigen bound. Instead comparisons are made in the region of antigen excess. Where two points lie in the region of 30-80% bound, the dilution of antiserum is calculated which would bind 33% of the total available antigen. Where there

is direct proportionality the antigen binding capacity can be calculated directly. For comparative purposes the antigen-binding capacity (ABC) is expressed as  $\mu\text{g N}^* \text{BSA bound per ml of undiluted serum.}$

Where it was possible to measure ABC values by both methods good agreement was found in nearly all cases. Calculations of per cent bindings and ABC values were carried out using the Edinburgh Regional Computing Centre's KDF9 or 360/50 computers (see appendix 1).

e. Statistical analysis of results.

Initial inspection of data from animals given the same treatment showed that the ABC values do not follow a normal distribution making comparison of treatments difficult. A near normal distribution was however, derived by logarithmic transformation of the data. Sera showing no antigen binding capacity were treated as positive with an ABC value of 0.01. This manoeuvre led to little or no alteration in the geometric mean of the majority of groups. Results for groups of mice with different treatments were compared by the 'student's' t-test. Before doing this their variances were tested for equality by the variance-ratio (F) test. Where it was decided that the true variances of the two samples were probably the same, then the t-test was performed. Where a significant difference was found in their variances a modified test was performed (Bailey, 1959). Initial calculations were carried out with the assistance of the personnel and PDP9 computer in the Department of Social Medicine, Edinburgh.

f. The effect of antigen dilution.

The effect of alterations in  $^*$  antigen concentration upon the ABC values has been taken as a measure of qualitative differences in the



antibody binding. This dilution effect can be conveniently expressed as a ratio of the ABC values at two antigen concentrations, i.e. ABC at low antigen concentration divided by ABC at high antigen concentration times 100. In this work, unless otherwise stated, the antigen concentrations used were  $0.1 \mu\text{g N}$  and  $0.01 \mu\text{g N}$  added.

g. Notes on the Farr technique.

I. The low antigen concentrations,  $0.1 \mu\text{g N}$  and  $0.01 \mu\text{g N}$ , most commonly used in the work reported have been shown extremely sensitive in the detection of low levels of antibody (Pinckard et al., 1968), and are hence very valuable in studies involving tolerance.

II. It is desirable to dilute the test sera in autologous serum, but with mice this is impractical. Preliminary experiments showed that the use of normal rabbit sera caused only a very slight loss in accuracy when compared to autologous sera.

III. Washing of precipitates in SAS/2 may be omitted from the procedure (Mitchison, 1964). Washing does lead to a lowered 'control' and experimental value and a slightly lower ABC value although the resulting slight increase in sensitivity of the technique is probably compensated for by the loss in time and increase in variation brought about by the washing. For the sake of consistency of method, washing of precipitates was included in the technique used in this thesis.

IV. The original formulation (Farr, 1958) of the per cent of antigen specifically bound by antibody took into consideration the fact that amount of non-antibody bound radioactivity in the precipitate would be proportional to the amount of non-antibody bound  $^*I$ -BSA in the super-

natant bound, and not to the total amount of test antigen added. The formula derived was based on the assumption that the ratio of counts in the control precipitate to supernatant would equal the ratio of non-antibody bound counts in the experimental precipitate to supernatant. This ratio, when multiplied by the number of counts in the experimental supernatant, gave the non-antibody bound counts in the precipitate. This could be subtracted from the number of counts in the experimental precipitate to give specifically bound counts. These were expressed as a percentage of counts added. Subsequently a new method of calculation evolved that was based on the fact that  $^{*}I$ -BSA in the experimental supernatant is directly proportional to the total amount of non-antibody bound  $^{*}I$ -BSA in a given mixture (Minden and Farr, 1967). This method had the advantage of simplicity and was expressed as:

$$\% \text{ bound} = 100 - \frac{\text{Experimental supernatant counts}}{\text{'Control' supernatant counts}}$$

This in fact can easily be further simplified to involve precipitate counts:

$$\frac{\text{Exp} - \text{'Control'}}{\text{TCA} - \text{'Control'}} \times 100\%$$

the formula expressed on p.69

All these formulas give identical results although the last is the easiest to use and does not require correction for a constant background.

#### 6. Agglutination of sheep red blood cells.

Sera were heat-inactivated ( $56^{\circ}\text{C}$  for 30 min). 0.05 ml volumes were used to make doubling dilutions with phosphate buffered saline (pH 7.2;  $\mu = 0.2$ ) in 'Falcon' microtitre plates. 0.05 ml of saline or 0.02 M 2-mercaptoethanol was added to each well and the plates incubated overnight at  $4^{\circ}\text{C}$ . 0.05 ml of a 0.4% suspension of washed sheep cells

was added to each well. The plates were incubated at 37°C for 1 hour and room temperature for two hours before the end points were established.

## 7. Bacterial Adjuvants.

### a. Corynebacterium parvum NCTC 10387

C.parvum 10387 was obtained from the National Collection of Type Cultures, Colindale, London. This strain was originally obtained from Professeur Prevot in Paris by Dr. H. Seeliger, Bonn, Germany in 1954. The American Type Culture Collection obtained it from Dr. Seeliger and typed it as C. parvum strain ATCC 12930. Colindale accepted it from them as C.parvum. Correspondence with Professor Raynaud and Professor Prevot in Paris, however, cast doubts on its identity. These workers consider it more likely to be a non-lysogenic strain of C.granulosum. In view of the difficulties inherent in the classification of anaerobic corynebacteria, for the purposes of this thesis it will still be considered as C.parvum 10387.

### Culture of C.parvum 10387.

To avoid the problems of contamination of the bacterial suspension with determinants cross-reacting with BSA, the organism was grown in a bovine-free media of minced cooked-meat infusion broth.

Minced cooked-meat was prepared with 500 grams of sheep heart, trimmed free of all connective tissue and finely minced. This was boiled for 20 min in 500 ml of distilled water with 1.5 ml 1N NaOH added. The resulting suspension was filtered through muslin and the solids blotted dry.

Infusion Broth was prepared from 500 g of lean sheep meat minced and mixed with 1 litre of distilled water containing 5 g NaCl. This was kept at 4°C for 24 hr and then boiled for 15 min. The resulting suspension was filtered through muslin and then filter paper and the pH of the filtrate adjusted to 7.4 with 1N NaOH.

The minced cooked heart was distributed in 100 ml and 500 ml bottles to a depth of 1"-2". The sheep meat infusion broth was added till the bottles were 80% full before sealing and autoclaving. Seitz-filtered glucose was then added (10% w/v) until the final concentration was 1%. The bottles were loosely sealed and steamed for 20 min.

A freeze dried culture of C.parvum was added to the 100 ml bottles which were then incubated anaerobically for 48 hr at 37°C. Following a purity check by Gram stain 25 ml of the starter cultures was added to the 500 ml bottles of media. These were incubated for a further 3 days at 37°C. The resulting bacterial suspension was tested for purity by plating on blood agar and Gram staining. The final bacterial suspension was filtered through eight layers of muslin and spun down at 4,500 g.min in an MSE "Major" refrigerated centrifuge (4°C), then resuspended and washed six times in sterile saline. Before use the bacteria were killed by heat (70°C/60 min), counted microscopically and dry weights measured by dessication. They were stored at -20°C until used.

b. SF 16.

The diphtheroid SF 16 was originally isolated from the synovial membranes and fluid of a patient with rheumatoid arthritis by Drs. Stewart, Alexander and Prof. Duthie in the Northern General Hospital, Edinburgh. This strain is typical in its morphological, cultural and biochemical characteristics of 60% of diphtheroids isolated from patients with this disease by these workers (Stewart et al., 1969; Stewart unpublished 1970).

Culture of SF 16.

The freeze-dried bacteria obtained from Dr. Stewart were grown on bovine-free infusion broth agar (see above) in 10% CO<sub>2</sub>. After incubation at 37°C for 72 hours the bacteria were washed off the surface of the agar with sterile saline, spun at 4,500 g.min (4°C) and washed six times in sterile 0.85% saline. The bacteria were killed by heating at 70°C for 60 min before being counted and weighed dry. They were stored at -20°C before use.

- c. Corynebacterium anaerobium was a kind gift from Prof. Prévot, Institut Pasteur, Paris. The suspension was killed and preserved in 1% formalin. Immediately before use the suspension was washed six times in sterile 0.85% saline.
- d. Bordetella pertussis, Lot 74432, was obtained from Burroughs Wellcome & Co., London, in the form of pertussis vaccine B.P. in 0.01% thiomersal as preservative. Again the suspension was washed six times in sterile saline before use.

8. Cellular studies.

- a. The preparation of mouse spleen cell suspensions.

Materials.

- 1) Minimal Eagles Medium (MEM), Burroughs Wellcome & Co., London.  
Made to single strength in distilled water and to contain 5 ml of a 4.4% sodium bicarbonate solution per 100 ml.
- 2) Loose fitting ground glass homogenisers.
- 3) Metal sieve 0.5 mm mesh size.

### Method.

The spleens were collected and all subsequent procedures were done in the cold. Each organ was cut into three pieces and the individual cells homogenised free, using a loose-fitting homogeniser containing sufficient MEM to prevent air bubbles being trapped. The cells and washings were transferred to universal containers and allowed to stand for 3 min to allow the separation of large particles. The cell suspension was passed through a previously moistened wire sieve. This procedure leads to a consistently high yield of spleen cells with viabilities of  $> 95\%$  as judged by the trypan blue dye exclusion test (final concentration 1:2000 w/v). Where necessary sterile procedures were performed.

#### b. Column separation of spleen cells.

Many methods have been used in attempts to specifically deplete leucocyte populations of one or more morphologically, or functionally, distinct type of cell. The most common method employs the use of glass bead columns to separate cells that adhere to glass from those that do not. Although most commonly used for the separation of peripheral white blood cells, Plotz and Talal (1967) and Shortman (1966) have used the technique for rodent spleen and lymph node cells respectively. The method to be described is a modification of that of Plotz and Talal (1967) and is based on the observation that the presence of divalent cations seems important in the attachment of certain cells to glass. The mechanism is unknown.

This method has been shown to achieve a degree of separation of two main populations of cells. One depleted of mononuclear cells and granulocytes and the other enriched in these cell types as well as in cells actively forming antibody.

Materials.

- 1) Hank's tissue culture medium was prepared either:-
  - a) without divalent cations (H);
  - b) with divalent cations  $\text{Ca}^{++}$  0.0018M,  $\text{Mg}^{++}$  0.001M and 10% calf serum (HSC).
  - c) without divalent cations and with 10% calf serum and 0.01M EDTA (HSV).
  
- 2) Glass columns - 15" x  $\frac{3}{4}$ " - three-quarters full with glass beads 2 mm diameter and washed for 10 min in concentrated nitric acid followed by 20-30 volumes of distilled water. Air was drawn through the column overnight to dry it. The capacity of the column was about 25 ml.
  
- 3) Spleen cell suspensions were prepared as described previously p.75 .

Method:

The column was equilibrated with HSC at 37°C. Cell suspensions from three spleens were pooled and suspended in 5 ml of H medium. An equal volume of HSC was added and the mixture incubated for 20 min at 37°C. Cells and washings were added to the column with the outflow clamped and left at room temperature for 10 min. Cells were eluted and collected in 40 ml fractions, 90 ml of HSC and 75 ml of HSV being passed through. The final fraction was collected by passing a further 25 ml of HSV through after the column had run dry. This was the only time the fluid level was allowed to drop below the level of the beads. The flow rate throughout was approximately 4 ml/min. The cells were centrifuged at 500g for 7 min before resuspending to the required volume. Viable and total cell counts were performed at this stage. The first and last (5th) fractions only were used in the experimental work to be described.

## 9. Histological techniques

All histological techniques were performed according to standard methods collated by Humanson (1967).

Fixation of organs for sectioning was carried out using 10% formalin in 0.85% saline or phosphate buffered saline or employing corrosive sublimate ( $\text{HgCl}_2$ ). Where autoradiography was to be performed the fixative of choice was 10% formalin in saline.

After dehydration in alcohols and clearing in toluene the tissues were infiltrated with paraffin (56-58° grade) and embedded in the same.

Sections 5  $\mu$  thick were cut, floated and warmed onto glass slides lightly smeared with 'Tissue-Tac' (Dodge Reagents Inc.).

Staining of sections depended mainly on Harris's Hematoxylin I (progressive method), counterstained with eosin. Pyronin-methyl green staining for nucleic acids was found useful where autoradiography was done. Bacteria were stained for by the Brown and Brenn method.

Sections were mounted in D.P.X. (BDH).

## 10. Autoradiographic sections

### Materials:

- 1) Ilford K5 Nuclear Track Emulsion in gel form. Stored at 4°C.
- 2) ID 19 Developer - Ilford Ltd., used at 20°C in full strength.
- 3) Ilford 'Hypam' rapid fixer (liquid).

### Method:

Sections were taken to water before coating with emulsion. The emulsion was melted by diluting the shredded gel with 2 parts of water at 50°C with very gentle stirring. The slides were coated and the excess emulsion allowed to run off. The slides were air dried in a



horizontal position before being stored at 4°C. Throughout the process care was taken to avoid exposure of the emulsion to any light other than minimal exposure to a brown safety light. After the requisite exposure time (3-4 weeks for  $^{125}\text{I}$  and  $^3\text{H}$ ) the autoradiographic sections were developed (5 min with intermittent agitation), fixed and washed. The slides were stained and mounted in D.P.X.

During examination care was taken to take into account the natural background grains and distinguish this from 'specific' grains. It was found convenient to study sections serially in order that grains could be established beyond reasonable doubt as being the result of specific disintegrations.

#### 11. Carbon clearance:

The clearance of colloidal carbon from the circulation has been long established as one of the methods of choice for quantitative estimation of the granuloplectic activity of the reticulo-endothelial system. Although the kinetics of phagocytosis have been shown to depend upon a variety of factors like particle size, type of particle, concentration of particles as well as other more complex variables, this method is still used widely, in particular in comparative experiments. The method is that of Biozzi et al. (1953).

#### Materials:

- 1) Carbon - 'Pelikan' shellac-free india ink, Gunther Wagner Ltd. (C11/1431a). This is a suspension of particles in fish glue with a small amount of phenol added as preservative. The particle size is approximately  $250^{\circ}\text{A}$ .

- 2) Calibrated bleeding pipettes.
- 3) 0.1%  $\text{Na}_2\text{CO}_3$ .
- 4) 2% gelatin solution in distilled water neutralised with ammonia.

Method:

The carbon suspension was spun at 5,000 r.p.m./15 min to remove large particles. The dry weight of carbon in the suspension was estimated and a 15 mg/ml suspension was made in 1% gelatin as stabiliser. This was kept at 37°C until injected in 5 mg quantities into mice via the lateral tail vein. Mice were repeatedly bled for 0.05 ml from the retro-orbital plexus over a period of 30 min and the blood lysed in 4 ml of 0.1%  $\text{Na}_2\text{CO}_3$ . The concentration of carbon was estimated by readings obtained at 660  $\text{m}\mu$  in a Unicam SP 500 Spectrophotometer with the red photocell. Blanks were prepared from blood collected from uninjected animals. The log concentration of carbon, estimated by reference to a standard curve, was plotted against time. A straight line equation could thus be derived. The slope of the line was calculated by the method of least mean squares. This gives the value of K (the phagocytic index) which is a measure of the rate of clearance of carbon, or the granuloplectic activity of the reticuloendothelial system. The animals used in these experiments were within a very narrow weight range and no correction was necessary for animal and organ weights.

12. Estimation of haemoglobin.

The method was that of Halpern et al. (1964). 0.05 ml of blood from the retro-orbital plexus was lysed in 4 ml of 0.1%  $\text{Na}_2\text{CO}_3$  and immediately mixed. The released haemoglobin was read spectrophoto-

metrically at 540 m $\mu$ . Experimental results were expressed as a percentage of control animals, saline injected and bled contemporaneously.

13. Peripheral white blood cell counts.

0.05 ml of blood was collected into 0.5 ml 1% acetic acid and the white cells counted in a standard Nauerberg counting chamber until the standard proportional error was  $\leq 1\%$ .

14.  $^3\text{H}$ -thymidine incorporation in vivo.

Cells actively synthesising DNA were labelled by a 'flash' labelling procedure. 20  $\mu\text{Ci}$   $^3\text{H}$ -thymidine TRA 61 (Thymidine-6-T (n) in aqueous sterile solution 5000 mCi/mM - Radiochemicals Centre, Amersham) was injected intravenously into the lateral tail vein of mice which were killed 15 min later by cervical dislocation. The organs were removed and processed for autoradiography.

15. Acid phosphatase assay.

Materials.

- 1) acetate buffer pH 5.0, 0.4 M.
- 2) p-nitrophenylphosphate 5 mM solution
- 3) absolute ethanol
- 4) 0.25 M NaOH solution

Method.

Cells were sonicated at 20 kilo cycles/sec for 10 minutes. To 0.35 ml of the sonicate was added 0.1 ml of buffer and 0.05 ml of

substrate. After incubation at 37°C for 30 min the reaction was stopped by the addition of 2 ml absolute ethanol. 0.2 ml of sodium hydroxide was added and the released p-nitrophenol read spectrophotometrically at 410 m $\mu$  against a blank where 0.35 ml of 0.29 M sucrose was used in place of the enzyme preparation.

16. Splenectomy.

Mice were anaesthetised by Nembutal and ether. The spleens were removed and the blood vessels to the spleen sealed by cauterisation. The small incision wound was closed with a single metal clip. Full sterile precautions were used throughout.

## RESULTS

### 1. The immune response and the induction of tolerance to BSA in mice.

The initial aim of this work was to investigate the response of mice to BSA, in particular with regard to the induction of tolerance by low doses of antigen.

Although the response of mice to multiple injections of BSA is well characterised (Mitchison, 1964), their response to a single injection is less well known. In spite of the inconsistency in the results of Dietrich and Weigle (1964) in their attempts to make mice tolerant with a single injection of native BSA, it was decided to use single injections of ultracentrifuged BSA to induce tolerance, if possible, as this would give the simplest experimental system.

Preliminary experiments were performed to examine the relationship between the dose of soluble ultracentrifuged BSA (c.BSA) and the response of CBA mice, as judged by their response to a challenge with highly immunogenic alum-precipitated BSA. Having shown that tolerance could be induced by microgram doses of antigen, more information concerning the induction of this state was derived by examining the class and affinity of antibody produced, the effect of the physical state of the antigen and the ability of different strains of mice to become tolerant. The final aim of these experiments was to gain information that, when taken in conjunction with the studies done with C.parvum, would enable some of the more important parameters in tolerance induction to be elucidated as a step towards simulation of the basic mechanisms involved.

Antigen-binding capacities of antisera were routinely measured against antigen concentrations of  $0.01 \gamma N^* I\text{-BSA}$  and  $0.1 \gamma N^* I\text{-BSA}$ .

Table 1

The response of CBA mice to varying doses of c.BSA.

Group	Treatment day - 20	Challenge day 0	ABC day 12		ABC day 20	
			0.10 $\gamma$ N <sup>a</sup>	0.01 $\gamma$ N	0.10 $\gamma$ N	0.01 $\gamma$ N
1	10 mg c.BSA	5 mg a.BSA	3.60 (4) <sup>c</sup>	0.69 (5)	1.09 (4)	0.98 (4)
2	5 mg c.BSA	5 mg a.BSA	5.63 (4) <sup>b</sup>	0.49 (6)	4.14 (5)	1.47 (5)
3	2 mg c.BSA	5 mg a.BSA	0.30 (5)	0.20 (5)	2.26 (5)	0.45 (5)
4	0.05mg c.BSA	5 mg a.BSA	0.00 (3)	0.00 (3)	0.06 (6)	0.04 (7)
5	Saline	5 mg a.BSA	0.44 (6)	0.08 (6)	5.26 (4)	1.03 (4)

<sup>a</sup> \* BSA concentration added in test

<sup>b</sup> number of sera tested per group

<sup>c</sup> geometric mean of ABC values for a group

The use of the lower concentration ( $3.6 \times 10^{-10}$  M) of the antigen is known to yield the most sensitive reproducible assay for low levels of antibody (Pinckard et al., 1968a). At this level the minimal binding capacity of the antisera is in fact being measured (Minden and Farr, 1967) and it might be expected that the high affinity antibody was being preferentially detected. This problem can be overcome by also measuring the ABC values of the antisera at a higher antigen concentration. Additional information concerning the immune response is gained by this manoeuvre as the effect of antigen dilution on the ABC value represents a measure of the quality of the antibodies being detected. In fact, for the experiments performed, the quantity of anti-BSA antibody present in an antisera was found to be adequately represented by the ABC value measured at  $0.1 \gamma$  N or  $0.01 \gamma$  N <sup>\*</sup>I-BSA no matter what concentration of the antigen was used. In many cases only the results achieved with the latter concentration will be quoted and the effect of dilution of antigen will be referred to where appropriate.

All injections were given by the intraperitoneal route to CBA mice unless otherwise stated.

a. The response of mice to varying doses of centrifuged BSA (c.BSA).

The response of CBA mice to varying single doses of c.BSA (day -20) was tested by their ability to respond to a challenge with 5 mg of a.BSA given twenty days later (day 0). The antigen-binding capacities of sera taken at 12 and 20 days after challenge were measured at two antigen concentrations (Table 1).

Results:

Because of the small numbers of mice in this preliminary experiment, the results do not warrant full statistical analysis. However, mice in

Table 2

The induction of tolerance to BSA in CBA mice.

Experiment	Group	TREATMENT DAY - 20	CHALLENGE DAY 0	ABC DAY 10	ABC DAY 20	t-TEST <sup>c</sup>
I	1	200 $\mu$ g c.BSA	5 mg a.BSA	a 0.02	(3)	P < 0.01
	2	50 $\mu$ g c.BSA	5 mg "	b 0.01	(11)	P < 0.01
	3	10 $\mu$ g c.BSA	5 mg "	0.02	(8)	P < 0.01
	4	Saline	5 mg "	0.04	(5)	
II	5	10 $\mu$ g c.BSA	5 mg BSA in FCA+ 5 mg a.BSA	0.01	(6)	P < 0.01
	6	Saline	"	0.01	(4)	

a ABC at 0.01  $\gamma$  N \* BSA - geometric mean of ABC values for a group

b number of sera tested per group

c significance of difference between ABC values for experimental and control groups at day 20



group 4 receiving 50  $\mu$ g of c.BSA seem to be rendered hyporesponsive when compared to control group 5 ( $P < 0.01$ ). In addition, by inspection, 5 mg of c.BSA is shown to be about the optimal priming dose for antigen given in this form (group 2) as judged by the early high ABC values of sera in this group.

b. The induction of tolerance to BSA in CBA mice.

In order to reaffirm that low zone tolerance was being induced following the injection of  $\mu$ g doses of c.BSA, a further experiment was performed that was, in addition, designed to examine the effect of potential variables such as dose of c.BSA, the route of injection, the sex of the animals, their body weight and the strength of the challenge dose.

Centrifuged BSA was found to induce a similar degree of unresponsiveness in male and female CBA mice, weighing 16-18 or 25-27 g, whether injected intravenously or intraperitoneally. As these variables were found to be unimportant the results were pooled and can be seen in Table 2 as a function of the dose injected and time after the challenge. Doses of 10, 50 and 200  $\mu$ g of c.BSA (groups 3, 2 and 1) were all capable of inducing a profound hyporesponsive state in CBA mice as compared with the normal responsiveness of the saline treated controls of group 4 ( $P < 0.01$ ). In addition, it can be seen from the results for groups 5 and 6 that challenge with antigen in complete Freund's adjuvant resulted in a general increase in circulating antibody titres in controls but the degree of tolerance induced was not affected.

Comments:

In the light of these results it was possible to adopt a standard regime for tolerance induction. A profound state of hyporesponsiveness to BSA could be induced by doses of 10-200  $\mu$ g of centrifuged antigen

Table 3

The effect of physical state of the antigen upon tolerance induction.

Experiment	Group	TREATMENT DAY -20	CHALLENGE DAY 0	ABC DAY 8	ABC DAY 20	ABC DAY 30	t-TEST <sup>c</sup>		P
							Difference between groups		
I	1	50 $\mu$ g BSA	5 mg a.BSA	not done	0.25 (5) <sup>a</sup>	0.73 (5)	1 - 2	0.1 < P < 0.3	
	2	50 $\mu$ g c.BSA	5 mg a.BSA	not done	0.14 (8)	0.26 (8)	2 - 3	P < 0.001	
	3	saline	5 mg a.BSA	not done	2.83 (8)	7.70 (8)	-	-	
II	4	50 $\mu$ g h.BSA	200 $\mu$ g BSA	0.01 (3)	0.04 (6)	not done	-	-	
	5	50 $\mu$ g p.BSA	200 $\mu$ g BSA	0.48 (6)	0.27 (6)	not done	-	-	
	6	50 $\mu$ g c.BSA	200 $\mu$ g BSA	0.00 (6)	0.00 (6)	not done	-	-	
	7	saline	200 $\mu$ g BSA	0.00 (6)	0.00 (6)	not done	-	-	
III	8	50 $\mu$ g <sup>125</sup> I-c.BSA	5 mg a.BSA	not done	0.81 (8)	not done	8 - 9	not significant	
	9	50 $\mu$ g c.BSA	5 mg a.BSA	not done	0.62 (5)	not done	9 - 10	P < 0.01	
	10	saline	5 mg a.BSA	not done	2.68 (8)	not done	-	-	

a ABC at 0.01  $\gamma$  N \* BSA - geometric mean of ABC values for a group

b number of sera tested per group

c significance of difference between ABC values at day 20 and day 30

given intraperitoneally (day -20) to CBA mice of either sex and 16-27 g body weight. A dose of 50  $\mu$ g c.BSA was chosen for use in the majority of further experiments. The tolerant state was tested for by a challenge with 5 mg a.BSA given twenty days later (day 0). The test sera were obtained at approximately day +10, +20 and +30 after this challenge. This schedule was used in future experiments.

c. The effect of the physical state of the antigen upon tolerance induction.

Recent evidence has suggested a marked degree of biochemical heterogeneity in albumin preparations (Peeters et al., 1970). Furthermore, it is recognised that different physical forms of an antigen may vary in their immunological potential. The following experiments were performed in order to investigate the effects that the physical state of the antigen might have upon tolerance induction.

The capacity of different physical forms of soluble BSA in tolerance induction was tested in the dose range already shown to be optimal for c.BSA. The results are presented in Table 3.

Results:

Soluble 'native' BSA (BSA) was found to be capable of inducing tolerance (group 1) and although the state was not quite as profound as that induced by c.BSA (group 2) the difference was not statistically significant ( $0.1 < P < 0.3$ ).

Mildly denaturated heated BSA (group 4) resulted in priming of 50% of animals as judged by the response to 200  $\mu$ g soluble BSA. This challenge by itself results in no detectable antibody formation in normal or tolerant animals (groups 7, 6) BSA, which had been heated at low pH and the residual monomer removed (pBSA), primed all mice for a good secondary response to 200  $\mu$ g of soluble BSA (group 5).

Table 4

The response of CBA mice to p.BSA.

Group	Treatment day -20	Challenge day 0	ABC - day 8	
			* p.BSA	* BSA
1	50 $\mu$ g p.BSA	200 $\mu$ g BSA	1.85 <sup>a</sup> (6) <sup>b</sup>	0.48 (6)
2	4.5 mg p.BSA	400 $\mu$ g p.BSA	25.08 (pool) <sup>c</sup>	6.60 (pool)
3	4.5 mg c.BSA	400 $\mu$ g c.BSA	1.05 (pool)	0.22 (pool)

a ABC at 0.01  $\gamma$  N \* antigen added

b number of sera tested per group

c pooled sera from six mice

Included in Table 3 is a study on the effect of routine radioiodination upon the capacity of BSA to induce tolerance (groups 8, 9, 10). The low levels of substitution routinely used in this thesis did not affect this biological activity of the molecule, a fact of considerable importance to later studies.

The ABC values of sera for group 5 (Table 3) were tested not only with \*BSA in the Farr test but also against the same concentration of \*p.BSA. The results can be seen in Table 4 for sera taken at day 8 (group 1). The ABC values were much higher when the aggregated test antigen was used. Similar results were obtained whether c.BSA or p.BSA was used to immunise the mice (groups 2, 3).

The reason why 4 to 5 fold higher ABC values were obtained when \*p.BSA is used as test antigen are at present under investigation. The consistency of the increase, irrespective of the ABC value, obtained with \*p.BSA allows valid conclusions to be drawn from experiments where only \*BSA is the test antigen. Furthermore, in order to obtain a reproducible assay system with \*p.BSA it is necessary to lower the concentration of ammonium sulphate in the Farr test to 43%, with a resulting loss of precipitation of a proportion of the soluble antigen-antibody complexes. These considerations, together with the difficulties involved in preparing a suitable aggregated \*p.BSA preparation, makes the use of \*p.BSA unsuitable for routine assay work at present and as a result \*BSA only was used routinely in this thesis.

#### Comments:

The results presented show that the response of mice to 50  $\mu$ g of BSA is dependent upon the physical state of the antigen. The greater the degree of aggregation the less the degree of tolerance induced and the greater the degree of priming.

Table 5

Tolerance induction to BSA in different strains of mice.

Group	Strain	ABC day 20			ABC day 30		
		Test <sup>c</sup>	Control <sup>d</sup>	t-test <sup>e</sup>	Test	Control	t-test
1	CBA	0.35 <sup>a</sup> ± 0.11	2.11 <sup>b</sup> ± 0.45 (9)	P < 0.001	1.11 (2)	3.73 <sup>±</sup> 0.21 (6)	not done
2	C57B1	0.02 <sup>±</sup> 0.01	0.04 <sup>±</sup> 0.03 (8)	0.1 < P < 0.2	0.03 <sup>±</sup> 0.02 (4)	0.08 <sup>±</sup> 0.04 (4)	0.2 < P < 0.4
3	CBA x C57B1	0.40 <sup>±</sup> 0.18	1.24 <sup>±</sup> 0.18 (6)	P < 0.1	0.43 <sup>±</sup> 0.32 (4)	1.86 <sup>±</sup> 0.41 (4)	P < 0.1
4	NZ B	3.02 <sup>±</sup> 0.84	7.34 <sup>±</sup> 1.02 (10)	P < 0.02	7.72 <sup>±</sup> 2.20 (9)	12.33 <sup>±</sup> 1.70 (8)	0.1 < P < 0.2
5	NZ B x CBA	1.56 <sup>±</sup> 0.43	6.86 <sup>±</sup> 1.10 (8)	P < 0.001	3.28 <sup>±</sup> 0.75 (9)	16.34 <sup>±</sup> 4.90 (7)	P < 0.001
6	DBA/2	0.04 <sup>±</sup> 0.02	0.49 <sup>±</sup> 0.20 (7)	P < 0.002	0.05 <sup>±</sup> 0.08 (3)	0.83 <sup>±</sup> 0.25 (6)	P < 0.001

a ABC Geometric mean <sup>±</sup> standard error - at 0.01  $\gamma$  N \*BSA. b number of sera tested per group.

c 50  $\mu$ g c.BSA at day -20; 5 mg a.BSA at day 0; sera taken at day 20 and 30.

d saline at day -20; 5 mg a.BSA at day 0; sera taken at day 20 and 30.

e significance of differences between ABC values for control and test groups.

d. Tolerance induction in different strains of mice.

Marked strain differences are known to exist in the immune responsiveness of mice to given antigens and/or antigenic determinants (see p.112). Few synchronous studies, however, have been performed on the strain requirements for tolerance induction, although on the basis of empirical reasoning, a common threshold for tolerance induction has been suggested (Dresser and Mitchison, 1968). It seemed important to examine different inbred mouse strains with respect to the induction of tolerance and immunity in order that the relationship between these two effects could be examined.

It was of particular interest to include New Zealand Black (NZB) mice in this experiment because of their lymphoreticular abnormalities and their proclivity towards autoimmune phenomena. These features are shared to a lesser degree by the (NZB x CBA)  $F_1$  hybrids also examined. Other mouse strains included in the study were C57B1, CBA, their  $F_1$  hybrids and DBA/2.

Inter- and intra-strain weight variation were large (up to 6g) although no correlation was observed between the final ABC values and body weight.

All mice, with the exception of the NZB strain, were 3-5 months old. NZB mice ranged from 3 to 10 months of age and were either Coomb's negative or Coomb's positive. No external features of severe illness were apparent in any of the mice.

Results and Comments:

The results in Table 5 show marked strain differences in the response to 5 mg a.BSA ('control' columns). It is interesting that (CBA x C57B1)  $F_1$  hybrids display responsiveness intermediate between those of their parents whereas (NZB x CBA)  $F_1$  hybrids seem to take on the parental responsiveness of NZB.

In contrast to this marked variation in the 'control' responses, all strains seemed to become tolerant to a lesser or greater degree ('test' columns). The significance values in certain cases are doubtful probably because of the low level of normal responsiveness in the appropriate 'control' mice.

Of particular interest in these results is the observation that NZB mice, although becoming tolerant to a certain degree, did not seem to display the same sensitivity to tolerance induction as the equally responsive (NZB x CBA)  $F_1$  hybrids. The response of NZB mice did not correlate in any way with sex, age or weight nor with the presence of Coomb's positivity in the experimental animals.

e. Observations upon the quality of antibodies and tolerance.

It is well established that an immune response is characterised by a change in the affinity of the antibody formed (Eisen and Siskind, 1964). It is often assumed that this occurs by the preferential stimulation of high affinity antibody producing cells which parallels the decrease in available antigen. By extrapolation it might be suggested that only high affinity clones would become tolerant to low doses of antigen.

This hypothesis was tested using as an index the effect of antigen dilution upon the binding capacity of the sera. The dilution effect was expressed as the ratio of the values of the sera measured at antigen concentrations of  $0.01\gamma N$  and  $0.1\gamma N$  \* BSA added. This fraction is expressed as a percentage. A low percentage is normally taken as being indicative of the presence of an antibody population with a high dissociation rate (Minden and Farr, 1967; see p.65 ).



Table 6

The quality of antibodies produced in tolerant animals.

Experiment	Group	Treatment day -20	Challenge day 0	Effect of dilution <sup>a</sup> day 12	t-test <sup>b</sup> day 12	Effect of dilution day 20
I	1	10 mg c.BSA	5 mg a.BSA	26.1 (4) <sup>c</sup>	P < 0.01	26.7 (4)
	2	50 μg c.BSA	5 mg a.BSA	17.7 (6)	not significant	21.8 (6)
	3	saline	5 mg a.BSA	17.1 (6)		21.8 (6)
II	4	150 μg c.BSA	5 mg a.BSA	19.5 (6)	not significant	29.4 (6)
	5	saline	5 mg a.BSA	20.8 (6)		32.73 (6)

a ABC at 0.01γ N \*BSA / ABC at 0.1γ N \*BSA x 100%.

b significance of differences between the dilution index for experimental and control groups.

c number of sera tested per group.

**Results:**

Table 6 shows the effect of antigen dilution upon the antigen-binding capacities of sera from primed and 'tolerant' CBA mice, and 'non-tolerant' control mice treated in the standard manner with BSA. ABC values for these groups were significantly different ( $P < 0.001$ ).

Although the error in these calculations is high, it can easily be seen that there is the expected tendency for higher affinity antibodies to be formed later in the immune response. 'Tolerant' sera (compare day 12 and day 20) (groups 2,4), however, show indices insignificantly different from 'control' sera (groups 3,5) whereas animals that have been primed display high affinity antibodies soon after challenge (groups 1; day 12;  $P < 0.01$ ).

**Comments:**

These experiments suggest, but do not conclusively prove, that tolerance to BSA in mice is mainly characterised by a decrease in the quantity of antibodies produced rather than by a change in their affinity. They also suggest that there is little or no priming effect following tolerance to low doses of BSA.

f. The class of antibodies produced in the tolerant animals.

Possible changes in the class of antibodies produced following challenge with a.BSA that might be the result of immune deviation caused by prior exposure to soluble BSA were investigated. Radioimmuno-electrophoresis was performed on 'tolerant' and 'non-tolerant' sera taken 12 and 20 days after challenge. The developing sera were rabbit anti-mouse gamma globulin (RAMG) and rabbit anti-mouse whole serum (RAMS). Four immunoelectrophoretic lines developed with RAMG that were known to be associated with antibody activity (Plate 1a).

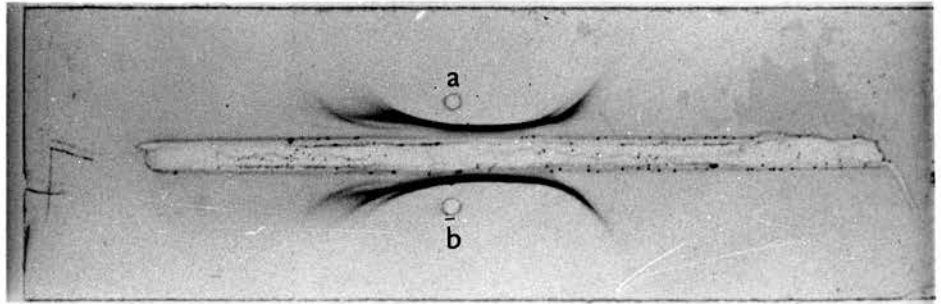


Plate 1a. Immunoelectrophoresis of sera from tolerant mice.

Well a. - 'non-tolerant' control sera, day 20.

Well b. - 'tolerant' sera, day 20.

Trough - rabbit anti-mouse gamma globulin.



Plate 1b. Autoradiography showing the binding of <sup>125</sup>I-BSA to the immunoelectrophoretic lines in Plate 1a.

Autoradiography after exposure to  $^{125}\text{I}$ -BSA showed that the anti-BSA antibody in sera from tolerant and non-tolerant mice was of one class of immunoglobulin tentatively assigned  $\gamma\text{G}_1$  on the basis of immunoelectrophoretic behaviour. The only differences between 'tolerant' and 'non-tolerant' sera lay in the density of the lines developed following exposure to  $^{125}\text{I}$ -BSA (Plate 1b).

Table 7

The effect of varying doses of C. parvum upon tolerance induction to BSA.

Group	Dose of <u>C. parvum</u> <sup>c</sup> day -24	TREATMENT day -20	CHALLENGE day 0	ABC day 10	ABC day 20
1	$8.0 \times 10^8$ organisms	50 $\mu$ g c.BSA	5 mg a.BSA	0.03 <sup>a</sup> (8) <sup>b</sup>	1.50 (7)
2	$3.0 \times 10^8$ organisms	50 $\mu$ g c.BSA	5 mg a.BSA	0.02 (8)	1.06 (3)
3	$0.8 \times 10^8$ organisms	50 $\mu$ g c.BSA	5 mg a.BSA	0.03 (8)	0.34 (15)
4	saline	50 $\mu$ g c.BSA	5 mg a.BSA	0.00 (5)	0.27 (11)
5	saline	saline	5 mg a.BSA	0.16 (6)	2.00 (12)

a ABC at  $0.01 \gamma$  N \*BSA - geometric mean of ABC values for a group

b number of sera tested per group

c C. parvum injected i.p.

Table 8

The effect of timing and route of administration of C. parvum upon tolerance induction.

Experiment	Group	Administration of $5 \times 10^8$ C. parvum		Tolerance induction		day 12		day 20	
		Route	Time before/after c.BSA	day -20	day 0	ABC	t-test <sup>c</sup>	ABC	t-test <sup>c</sup>
I	1	i.p.	8 days before	100 $\mu$ g c.BSA	5mg a.BSA	not done	-	1.22 <sup>a</sup>	P<0.001
	2	i.p.	4 days before	100 $\mu$ g c.BSA	5mg a.BSA	not done	-	1.26	P<0.001
	3	i.v.	4 days before	100 $\mu$ g c.BSA	5mg a.BSA	not done	-	2.10	P<0.02
	4	tolerant control		100 $\mu$ g c.BSA	5mg a.BSA	not done	-	0.29	(8)
	5	non-tolerant control		saline	5mg a.BSA	Not done	-	2.67	(8) P<0.001
II	6	i.p.	4 days before	100 $\mu$ g c.BSA	5mg a.BSA	1.18	(6) P<0.01	4.85	(6) P<0.001
	7	i.p.	1 hr before	100 $\mu$ g c.BSA	5mg a.BSA	1.13	(6) P<0.01	7.30	(6) P<0.001
	8	i.p.	4 days after	100 $\mu$ g c.BSA	5mg a.BSA	0.40	(6) P<0.05	2.32	(6) P<0.05
	9	tolerant control		100 $\mu$ g c.BSA	5mg a.BSA	0.23	(6)	0.90	(6)
	10	non-tolerant control		saline	5mg a.BSA	0.55	(6) P<0.05	4.34	(6) P<0.01

a ABC at 0.01  $\gamma$  N \*BSA - geometric mean of ABC values for a group. b number of sera tested per group.

c significance of differences between ABC values for experimental groups and tolerant control.

2. The effect of bacterial adjuvants upon the induction of tolerance to BSA.

Various adjuvants have been reported to be able to prevent the tolerance induction to certain antigens in mice. C.parvum (strain 10387) had already been shown to possess this ability in rabbits (Pinckard et al., 1968) and the following experiments were performed in an attempt to study in detail the action of this adjuvant.

For comparative and aetiological purposes other agents were also used. These were C.anaerobium obtained from the Institut Pasteur, Bordetella pertussis - a well documented adjuvant, and SF16- a strain of diphtheroids isolated by Steward et al. (1969) from the synovial membranes and fluid of a patient with rheumatoid arthritis.

The standard regime for tolerance induction was used in CBA mice.

a. The effect of C.parvum upon tolerance induction.

Varying doses of C.parvum were given four days (day - 24) before the tolerance inducing regime in a preliminary experiment to test the ability of the organism to prevent the induction of tolerance to c.BSA in CBA mice. The results are expressed in Table 7. A dose of  $8 \times 10^7$  bacteria was found to be minimally effective (group 3). When doses greater than  $10^9$  bacteria were given there was slight evidence of toxicity with certain preparations as judged by visible signs of illness in the injected mice. For these reasons, doses of  $3 - 10 \times 10^8$  bacteria were chosen for future experiments.

Table 8 shows the effect of timing and the route of C.parvum administration. There was no difference in the ability of C.parvum to prevent induction of tolerance to BSA whether the adjuvant was given eight days (group 1), four days (groups 2,6) or one hour (group 7) before the tolerance-inducing regime. The effect was less marked if adjuvant was

Table 2

The effect of different bacterial adjuvants upon tolerance induction.

Group	Adjuvant	Tolerance induction		day 12		day 20	
		day -20	day 0	ABC	t-test <sup>c</sup>	ABC	t-test
1	6x10 <sup>8</sup> organisms i.p. <u>C. parvum</u> 4 days before c.BSA	50 $\mu$ g c.BSA	5mg a.BSA	0.98 <sup>a</sup> b (7)	P<0.001	3.23 (7)	P<0.01
2	<u>C. anaerobium</u> " " "	50 $\mu$ g c.BSA	5mg a.BSA	0.88 (8)	P<0.001	2.11 (6)	P<0.02
3	SF 16 4 days before c.BSA	50 $\mu$ g c.BSA	5mg a.BSA	1.91 (8)	P<0.001	4.13 (6)	P<0.01
4	tolerant control	50 $\mu$ g c.BSA	5mg a.BSA	0.13 (8)		0.25 (8)	
5	non-tolerant control	saline	5mg a.BSA	2.82 (8)	P<0.001	7.69 (7)	P<0.001

a ABC at 0.01  $\gamma$  N \* BSA - geometric mean of ABC values for a group

b number of sera tested per group

c significance of differences between ABC values for experimental groups and tolerant control



given four days after c.BSA (group 8). When C.parvum was given intravenously the overall effect seemed to be enhanced (group 3) although the variance of this group was high and therefore significance of the results was diminished.

Incorporated into the design of these experiments was the effect of sex of the animals which was found not to be an important variable. In addition, control experiments showed that C.parvum had no obvious effect upon the responsiveness of animals to challenge with 5 mg a.BSA 24 days after the adjuvant treatment.

Comments:

C.parvum can prevent the induction of lowzone tolerance to c.BSA in CBA mice of either sex if given up to eight days before, or the same day as c.BSA injection. This effect was not as marked if the adjuvant was given four days after the antigen.

For convenience and reproducibility of results, the intraperitoneal injection of  $3-10 \times 10^8$  C.parvum organisms four days before c.BSA treatment was considered a suitable system for further experiments.

b. The effect of SF16, C.anaerobium and B.pertussis on the induction of tolerance.

Preliminary experiments showed that C.anaerobium and SF16 had a similar effect to C.parvum on the prevention of tolerance induction, with a similar lower threshold of effectiveness ( $2.0 \times 10^8$  organisms). The results of a comparative experiment upon the effect of these agents are presented in Table 9. Both agents show similar effects to those obtained with C.parvum, in preventing the induction of tolerance ( $P < 0.001$ ; day 20).

Table 10

The effect of *B.pertussis* and *C.parvum* upon tolerance induction.

Group	Adjuvant		Tolerance induction		day 12		day 20	
	Organism	Time before c.BSA	day -20	day 0	ABC <sup>a</sup>	t-test <sup>b</sup>	ABC	t-test
1	<u>C.parvum</u> <sup>c</sup>	4 days	100 µg c.BSA	5mg a.BSA	1.18	P<0.01	4.85	P<0.01
2	<u>B.pertussis</u> <sup>d</sup>	4 days	100 µg c.BSA	5mg a.BSA	0.21	P<0.1	0.84	P<0.1
3	<u>C.parvum</u>	1 hour	100 µg c.BSA	5mg a.BSA	1.13	P<0.01	7.33	P<0.001
4	<u>B.pertussis</u>	1 hour	100 µg c.BSA	5mg a.BSA	0.89	P<0.01	3.71	P<0.01
5	tolerant control		100 µg c.BSA	5mg a.BSA	0.23		0.90	
6	non-tolerant control		saline	5mg a.BSA	0.55	P<0.01	4.34	P<0.01

a ABC at 0.01 γ N \* BSA - geometric mean of ABC values of 6 sera for each group

b significance of differences between ABC values for experimental groups and tolerant controls

c 6 x 10<sup>8</sup> organisms C.parvum injected i.p.

d 4 x 10<sup>9</sup> organisms B.pertussis injected i.p.

The effect of B.pertussis upon the induction of tolerance is summarised in Table 10. The dose chosen had been shown previously to give an adjuvant effect in other systems. B.pertussis appears to be more effective if given one hour rather than four days before c.BSA (groups 4, 2).

Comments:

SF16, C.anaerobium and B.pertussis have, like C.parvum, been shown to be capable of preventing the induction of tolerance to c.BSA in CBA mice. In view of the difficulties inherent in comparing bacteria by numbers or weight, when the active principle cannot be assessed, it would be invalid to judge the relative effectiveness of these agents from the results presented.

c. The effect of C.parvum upon responsiveness to low doses of c.BSA.

The experimental results presented in Table 8 showed a relatively high early rise in ABC values of sera from C.parvum treated animals undergoing the tolerance inducing schedule (groups 6,7; day 12). It has been already shown (section 1a) that this response is typical of animals that have been primed. In order to show that priming occurred following the initial injection of c.BSA, even though no serum antibody could be detected, the effect of C.parvum upon the response was examined:

1. by the response of animals to challenge with a second sub-immunogenic dose of BSA,
2. with relation to the quality of anti-BSA antibody formed,
3. with regard to the class of anti-BSA antibody produced.

Table 11

The effect of bacterial adjuvants upon responsiveness to low doses of c.BSA.

Group	Adjuvant		Tolerance induction		day 12	day 20
	Organism	Time before c.BSA	day -20	day 0	ABC <sup>a</sup>	ABC
1	<u>C.parvum</u> <sup>b</sup>	4 days	100 $\mu$ g c.BSA	200 $\mu$ g BSA	0.20	0.15
2	<u>B.pertussis</u> <sup>c</sup>	1 hour	100 $\mu$ g c.BSA	200 $\mu$ g BSA	1.86	2.11
3	tolerant control		100 $\mu$ g c.BSA	200 $\mu$ g BSA	0.00	0.00
4	non-tolerant control		saline	200 $\mu$ g BSA	0.00	0.00

a ABC at 0.01  $\gamma$  N \*BSA - geometric mean of ABC values for six sera per group

b  $6 \times 10^8$  organisms C.parvum injected i.p.

c  $4 \times 10^9$  organisms B.pertussis injected i.p.

Table 12

The quality of antibody produced in adjuvant treated CBA mice.

Experiment	Group	TREATMENT			EFFECT OF DILUTION <sup>a</sup>		EFFECT OF DILUTION	
		Day -24	Day -20	Day 0	Day 12	t-test <sup>b</sup>	Day 20	t-test
I	1	<u>C.parvum</u> <sup>c</sup>	100 $\mu$ g c.BSA	5mg a.BSA	31.2	P<0.01	34.1	P<0.05
	2	saline	100 $\mu$ g c.BSA	5mg a.BSA	20.8	not significant	32.7	not significant
	3	saline	saline	5mg a.BSA	19.5		29.4	
II	4	<u>C.parvum</u>	100 $\mu$ g c.BSA	5mg a.BSA	25.3	P<0.01	28.6	not significant
	5	saline	100 $\mu$ g c.BSA <sup>d</sup> <u>B.pertussis</u>	5 mg a.BSA	25.6	P<0.01	30.2	not significant
	6	saline	100 $\mu$ g c.BSA	5 mg a.BSA	15.5	not significant	25.6	not significant
	7	saline	saline	5mg a.BSA	16.5		28.9	

a. ABC at 0.01  $\gamma$  N<sup>\*</sup> BSA / ABC at 0.1  $\gamma$  N<sup>\*</sup> BSA x 100%

b. six sera tested in each group

c. 5 x 10<sup>8</sup> organisms C.parvum injected i.p.

d. 2 x 10<sup>9</sup> organisms B.pertussis injected i.p. 1 hr before c.BSA

## Results:

As can be seen from Table 11, 200  $\mu$ g of BSA given at day 0, which in itself did not produce an immune response in normal or supposedly tolerant animals (groups 4,3), did show that C.parvum and B.pertussis treated animals undergoing a tolerance induction schedule, became primed by the tolerance-inducing c.BSA (groups 1,2).

The significantly higher affinity of antibodies produced by adjuvant treated animals early in the response to challenge with a.BSA supported the concept that they were primed (Table 12; groups 1,4,5).

Radioimmuno-electrophoretic patterns of sera from animals treated with C.parvum and undergoing the standard tolerance inducing regime were compared with those from tolerant and non-tolerant animals. Autoradiography showed binding of  $^{125}\text{I}$ -labelled BSA by 'tolerant' and 'non-tolerant' sera to a gamma line which was tentatively considered as being  $\gamma^G_1$ . Only sera from C.parvum treated animals bound antigen in a  $\gamma_2$  line as well as  $\gamma_1$  and only then if the sera were taken twenty days but not twelve days after challenge. According to its position the  $\gamma_2$  line was tentatively designed as being  $\gamma^G_{2b}$  (Plate 2).

## Comments:

C.parvum pretreatment led to the priming of mice that received a tolerogenic dose of c.BSA. In addition, the features of the response to later challenge were altered such that the classes of antibodies produced were different.

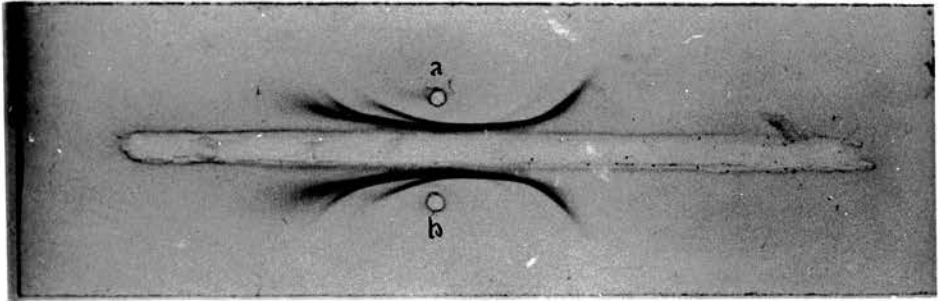


Plate 2a. Immunoelectrophoresis of sera taken 20 days after challenge with a.BSA. Well a. - sera from mice pretreated with C.parvum and undergone a tolerance inducing schedule. Well b. - 'tolerant' control sera. Trough - rabbit anti-mouse gamma globulin.

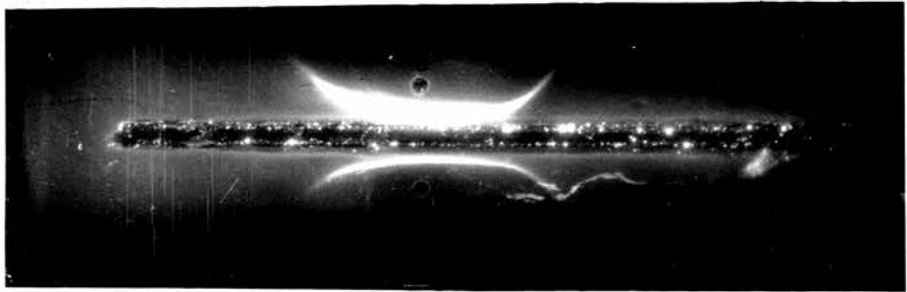


Plate 2b. Autoradiography showing the binding of <sup>125</sup>I-BSA to the immunoelectrophoretic lines in Plate 2a.

Table 13

The effect of *C. parvum* upon organ weight.<sup>a</sup>

ORGAN	NUMBER OF EXPERIMENTS	TOTAL NUMBER OF ANIMALS	% INCREASE IN WEIGHT (individual experiments)				
spleen	5	55	35	36	50	76	119
liver	4	51	4	6	19	21	
thymus	2	29	30	35			
lymph node	1	20	23				

a organs weighed 4 days after injection of  $4 - 8 \times 10^8$  organisms.



3. The effects of adjuvants upon lymphoreticular tissues.

C.parvum has been shown to be capable of preventing the induction of tolerance to c.BSA in CBA mice. In order to examine the mechanism of adjuvant action, it seemed relevant to look at the effects upon the lymphoreticular tissues brought about by these organisms, in particular over the first days following injection.

a. The effect of C.parvum upon organ weight.

The weights of various organs four days after C.parvum injection ( $4-8 \times 10^8$  organisms) are given in Table 13. All animals were carefully matched with respect to body weight which did not change following adjuvant injection.

A consistent significant increase in the weight of spleens from adjuvant injected animals over the saline treated controls was noted. An increase in size of the livers also followed C.parvum injection although this effect was rather variable. Significant increases in thymus weight and cervical lymph node weight were also found four days after injection ( $P < 0.01$ ).

b. The effect of C.parvum upon peripheral white blood cell counts.

There was an initial drop in the circulating white cell count following the injection of standard doses of C.parvum (Fig. 1). The level reached a minimum value some hours after injection, however, values obtained 5 and 8 days after injection were not significantly different from the controls. The decrease appeared to be mainly due to a fall in the numbers of circulating lymphocytes.

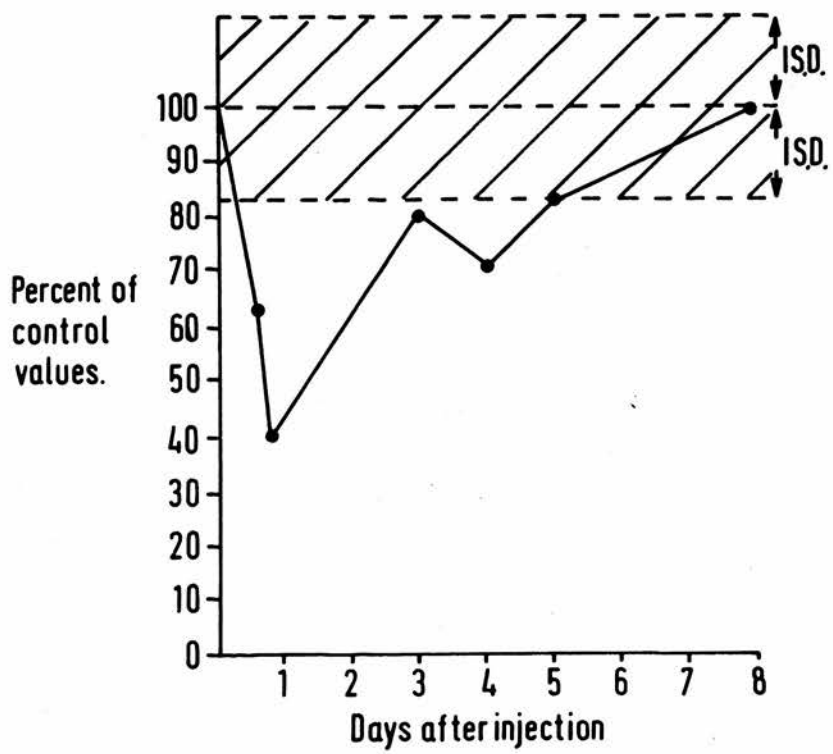


Figure 1. The effect of *C. parvum* upon peripheral white cell counts.

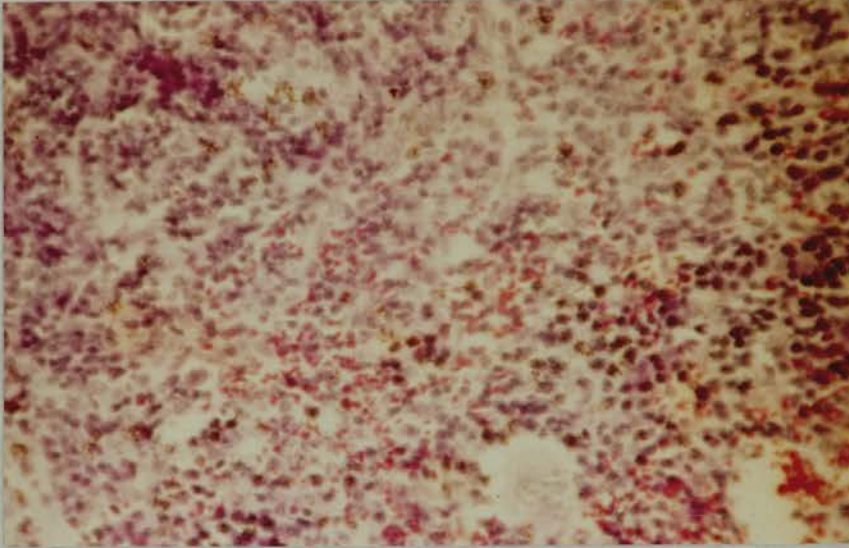


Plate 3. The incorporation of <sup>3</sup>H-thymidine into cells in the spleen  
of C. parvum - treated animals, x310.

c. Histological examination of the effects of *C.parvum*.

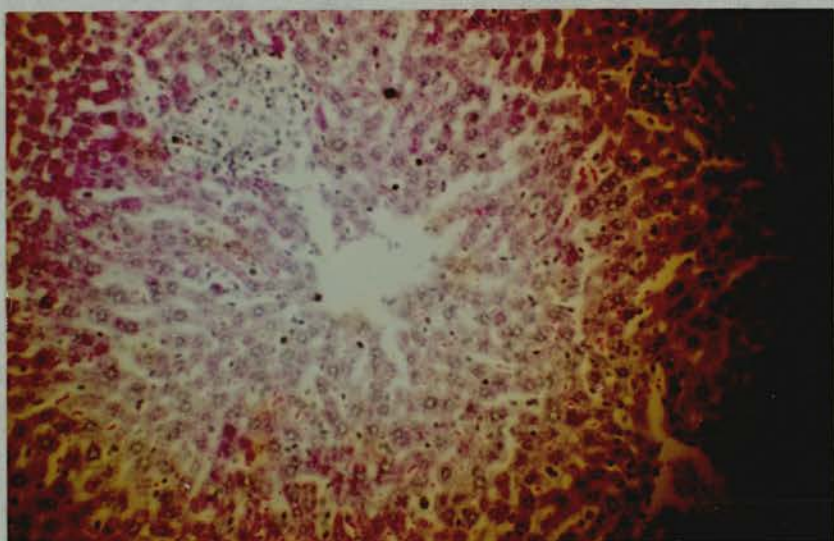
Various organs taken from CBA mice treated four days beforehand with *C.parvum* were studied histologically and compared with normal controls. Animals received  $1 \mu\text{Ci/g}$  body weight of  $^3\text{H}$ -thymidine intravenously 15 minutes before sacrifice. Sections of various organs were examined for the presence of cells that had taken up the DNA precursor.

In comparison with the controls, *C.parvum* treated animals had intensely active spleens with expanded white and red pulp. A marked degree of extramedullary haematopoiesis occurred and numerous megakaryocytic cells were present. The general hyperplastic response was most marked in the perifollicular regions and it was (Plate 3) in this area that the most profound increase in thymidine uptake occurred. An increase in the number of labelled cells could be seen also in the red and white pulp.

The livers of *C.parvum* treated animals had numerous granulomata with mononuclear cell infiltration, in particular around the portal tracts. Few infiltrating cells were found to have incorporated  $^3\text{H}$ -thymidine. There was a striking increase in the number of both reticuloendothelial and parenchymal cells that had incorporated labelled thymidine, although the former appeared to be mostly involved (Plate 4a, 4b).

The most obvious changes in the cervical lymph nodes were connected with hypercellularity and expansion of the paracortical areas. On histological evidence this did not appear to be completely due to proliferation of existing cells although studies on the uptake of  $^3\text{H}$ -thymidine were not performed. A very marked medullary plasmacytosis was also often found.

a.



b.

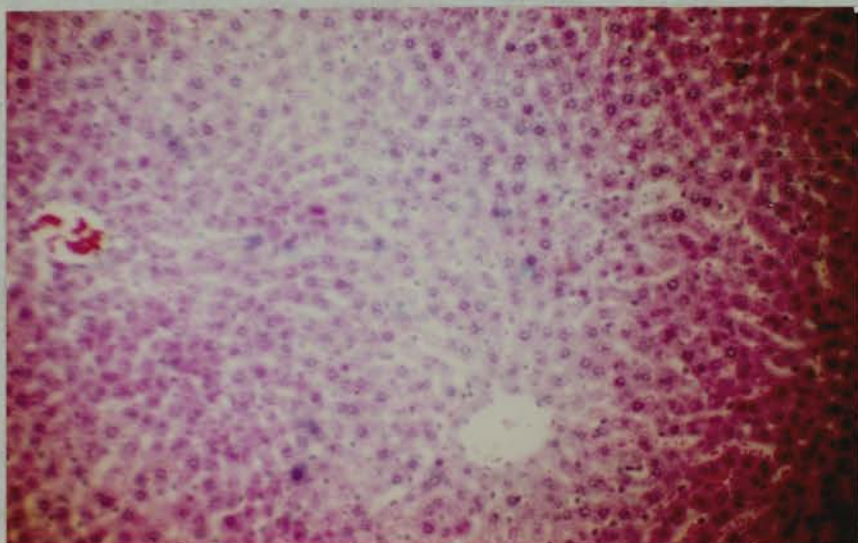


Plate 4. The incorporation of  $^3\text{H}$ -thymidine into cells in the liver of

a. C. parvum-treated mice

b. control mice  
x125

A considerable apparent increase in the number of reticular cells and a general increase in the cellularity of the cortex in the thymuses of C.parvum treated mice was found although the study of  $^3\text{H}$ -thymidine uptake was not performed.

The changes following C.parvum administration will be considered further in connection with the localisation of antigen within these organs.

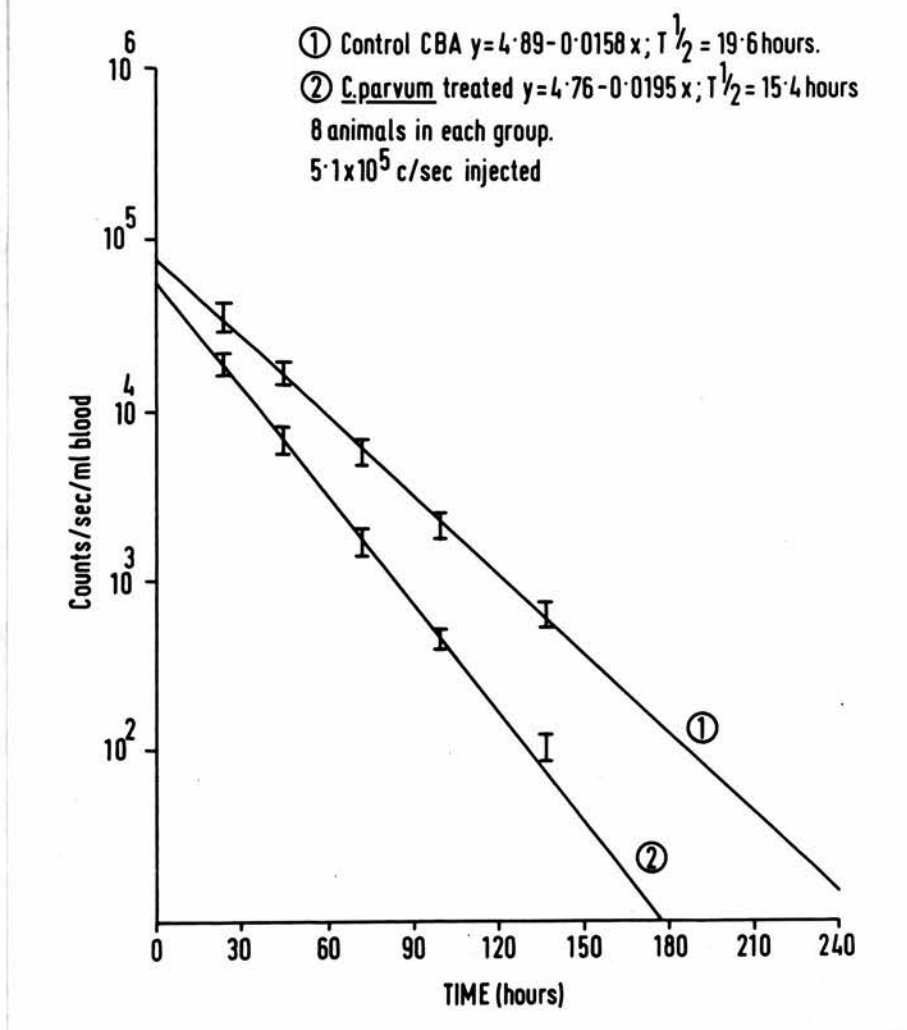


Figure 2a. The clearance of 50 µg c.BSA from the circulation of C. parvum treated mice.

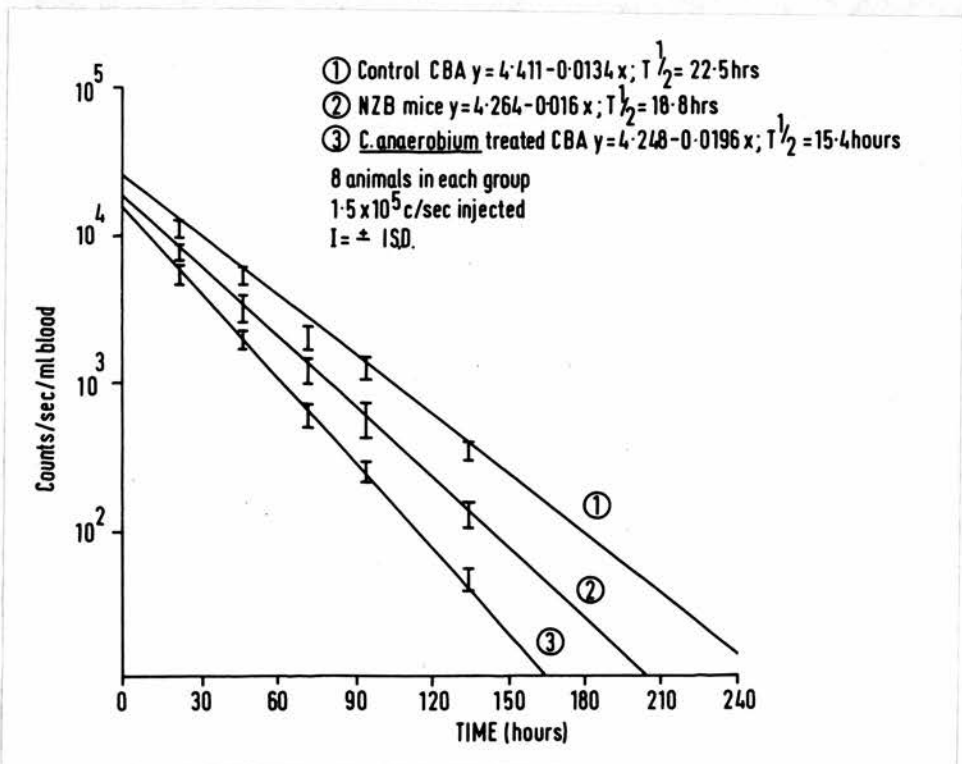


Figure 2b. The clearance of 50 µg c.BSA from the circulation of C. anserobium treated mice.

4. The effect of adjuvants upon the localisation and clearance of BSA.

The clearance of 50  $\mu\text{g}$  of  $^{125}\text{I}$ -c.BSA from the circulation and its localisation within the lymphoid tissues was examined in order to study the mechanism whereby the C.parvum injection altered the balance between tolerance induction and immunisation.

a. The clearance of BSA from the circulation.

CBA mice were injected with C.parvum or C.anaerobium ( $6 \times 10^8$  organisms) and four days later tested for their ability to clear from the circulation 50  $\mu\text{g}$  of  $^{125}\text{I}$ -c.BSA injected intravenously. The mice were bled every 24 hours. The amount of radioactivity precipitated from the blood samples by 10% TCA was plotted on a log scale against the time after injection. The results are presented in Figures 2a, 2b. The equations for the best straight lines derived by the method of least mean squares and the half life of the c.BSA for each treatment are also given. Adjuvant treated mice showed faster non-immune clearance of the injected antigen than did the controls. The highly significant differences were corroborated by similar experiments done with smaller numbers of animals. It is of interest that NZB mice displayed accelerated clearance when compared to CBA controls (Figure 2b).

By extrapolation to time zero ( $x = 0$ ) it is possible to arrive at a figure that represents the concentration of c.BSA that would be present if the antigen was evenly distributed throughout the fluid volume of the mouse. Having this information, and knowing the exact amount of antigen injected, it is possible to determine the volume of fluid the antigen is being distributed in. Although all mice were closely matched with respect to body weight and all received the same dose of the same preparation of c.BSA, the experimental animals show a 27 - 31% decrease



Table 14

The gross localisation of \*BSA in tissues.

Group	Treatment		Counts / sec / mg tissue <sup>b</sup> (day 1)				
	day - 4	day 0	Spleen	Liver	Thymus	Cervical lymph nodes	Kidney
1	<u>C. parvum</u>	50 $\mu$ g * c.BSA <sup>a</sup>	8.2	7.3	17.3	9.8	14.3
2	-	50 $\mu$ g * c.BSA	9.3	7.7	14.0	10.4	12.0
3	-	50 $\mu$ g * h.BSA	5.8 <sup>oo</sup>	6.0 <sup>*</sup>	5.0 <sup>oo</sup>	8.7	7.0

a 6.7 x 10<sup>5</sup> counts / sec / 50  $\mu$ g BSA injected i.p.

b mean values of 7 animals per each group

\* t-test with group 2 - P < 0.05

oo t-test with group 2 - P < 0.001.

in the amount of antigen per ml of blood at zero time. Therefore C.parvum or C.anaerobium injection appears to result either in an increase in the volume throughout which the antigen is being distributed or in an initial rapid disappearance of antigen from the circulation soon after its injection.

b. Gross localisation of BSA.

In two independent experiments the organ distribution of 50  $\mu$ g  $^{125}$ I-c.BSA given intraperitoneally was examined in C.parvum pretreated and normal control mice. Another group received 50  $\mu$ g of heat-aggregated  $^{125}$ I-BSA. The radioactivity detected in the liver, spleen, thymus, cervical lymph nodes, kidney and blood 28 hours after injection was taken as a measure of the amount of antigen retained. The pooled results of these experiments are expressed in Table 14.

Total counts in the spleens and thymuses of C.parvum treated mice (group 1) were significantly increased over the control group 2 but not if the results were expressed as counts/mg of tissue. No other significant differences were found between these two groups.

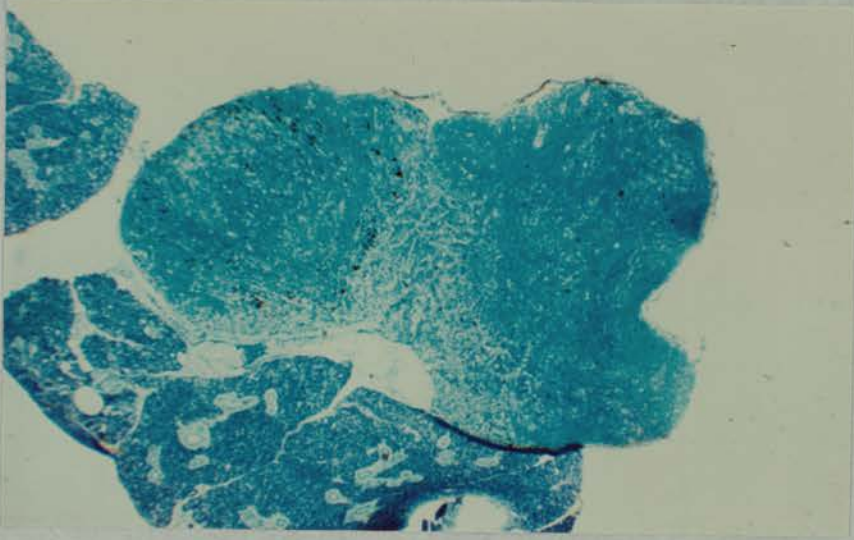
Heat-aggregated BSA showed statistically significant differences from c.BSA in its localisation in the spleen, liver and thymus (group 3). The effect was most marked in the thymus where the total organ counts were only 36 % of control values. No significant differences in the organ weights between groups 2 and 3 were found.

c. Autoradiographic examination of the localisation of  $^{125}$ I-BSA in tissues.

Tissues examined in section b. were processed for autoradiography.

Centrifuged BSA given to normal mice did not localise in any particular area within the lymphoid organs, with the exception of a

a.



b.

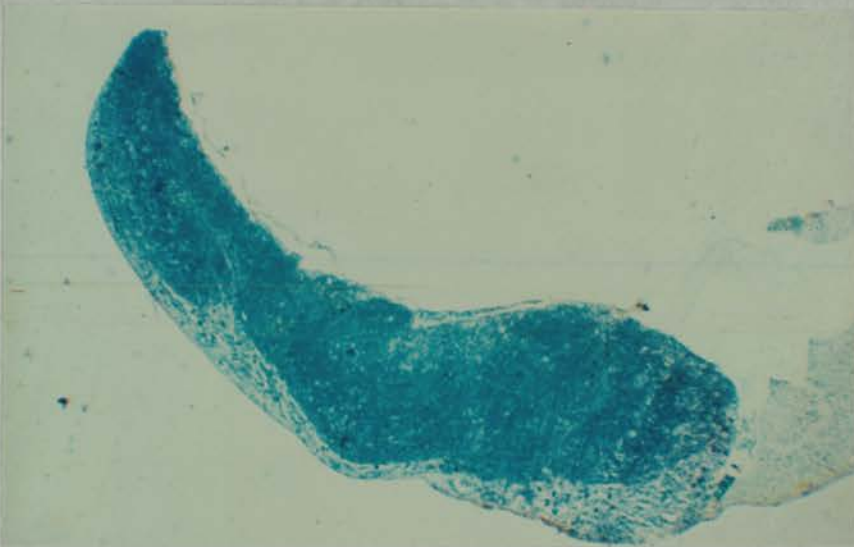


Plate 5. Autoradiography on a section of cervical lymph node from a mouse pretreated with *C. parvum* (a.) and a control mouse (b.) injected with 50  $\mu$ g c.BSA.

x 50

slight degree of uptake by medullary macrophages in the lymph node and the red pulp of the spleen. In the C.parvum treated animals  $^{125}\text{I-c.BSA}$  uptake was increased, in particular in the cervical lymph nodes (Plates 5a, 5b). There was no germinal centre localisation similar to that found in primed animals.

The distribution of heat-aggregated BSA within the spleen and lymph node was similar to c.BSA but with a slight increase in the quantity localised within macrophages (Plate 5c). Mice receiving h.BSA did not display the expected reduction in grain counts in thymus, but in no group was any apparent localisation of antigen noted within thymic cells.

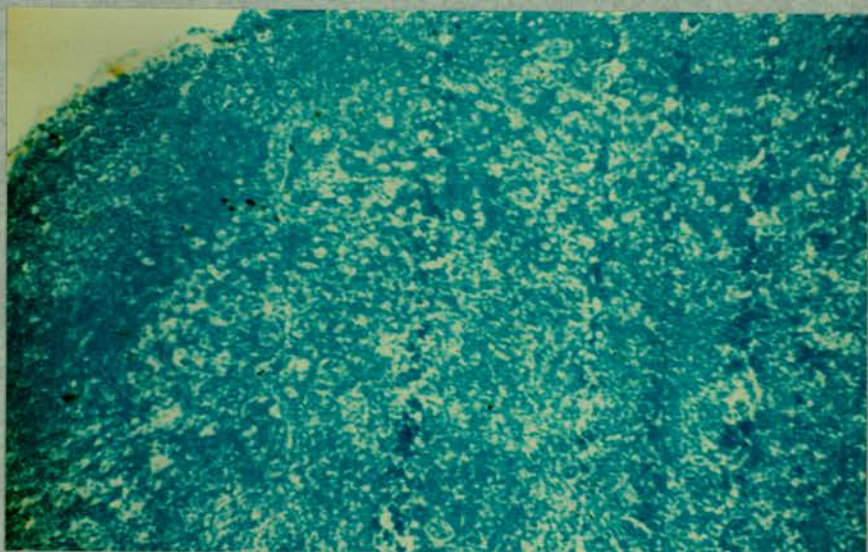


Plate 5c. Autoradiography on a section of cervical lymph node from  
a mouse injected with 50  $\mu$ g h.BSA, (x125).

Table 15

The effect of C. parvum upon carbon clearance.

Experiment	TREATMENT	Equation for carbon clearance <sup>b</sup>
1	<u>C. parvum</u> <sup>a</sup> Day-4; 5.5mg carbon Day 0 saline Day-4; 5.5mg carbon Day 0	$y = 2.3115 - 0.0197x$ <sup>c</sup> (3) $y = 2.4054 - 0.0077x$ (3)
2	<u>C. parvum</u> Day-4; 5.5mg carbon Day 0 saline Day-4; 5.5mg carbon Day 0	$y = 1.9312 - 0.0360x$ (4) $y = 2.1451 - 0.0139x$ (4)

a  $6 \times 10^8$  organisms i.p.

b Derived by the method of least mean squares;  $y = \log \text{mg carbon}/100 \text{ ml blood}$ ,  
 $x = \text{time in min (0 - 30 min)}$

c number of mice per group

5. The effect of C.parvum upon the localisation and clearance of carbon.

a. The clearance of carbon from the circulation

The clearance of colloidal carbon in C.parvum treated and control mice was examined in order to study and verify the effect of C.parvum upon reticuloendothelial activity. Female mice were injected with 5.5 mg of carbon intravenously four days after C.parvum treatment and then bled at four minute intervals for 30 minutes. The results of two such experiments are expressed in Table 15 as the equation for the straight lines derived from plotting the log of concentration of carbon (mg/100 ml blood) against time. The method of least squares was used to derive equations.

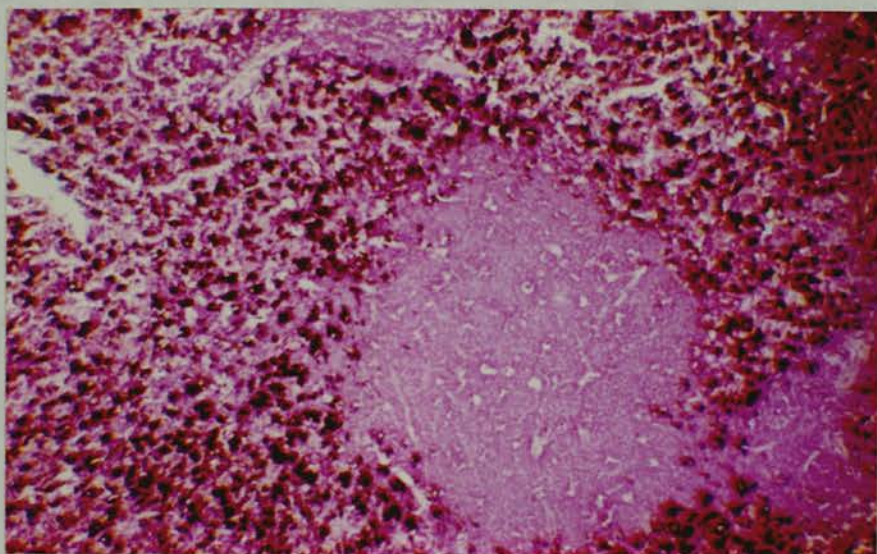
Results:

There are two obvious points. The first is that carbon is cleared more rapidly from the circulation of C.parvum treated animals than controls. This was true for all animals tested. Secondly, in groups of animals closely matched by weight and sex, by extrapolation to time zero, it can be seen that the volume of fluid through which the carbon is distributed appears to be greater in C.parvum treated mice. Similar results were obtained when the clearance was studied 30 - 120 minutes after injection.

b. Histological examination of carbon localisation.

Control mice and mice that had received C.parvum 4 days previously were sacrificed 4 hours after the intravenous injection of 5.5 mg of carbon and their spleens and livers processed for histological examination.

a.



b.

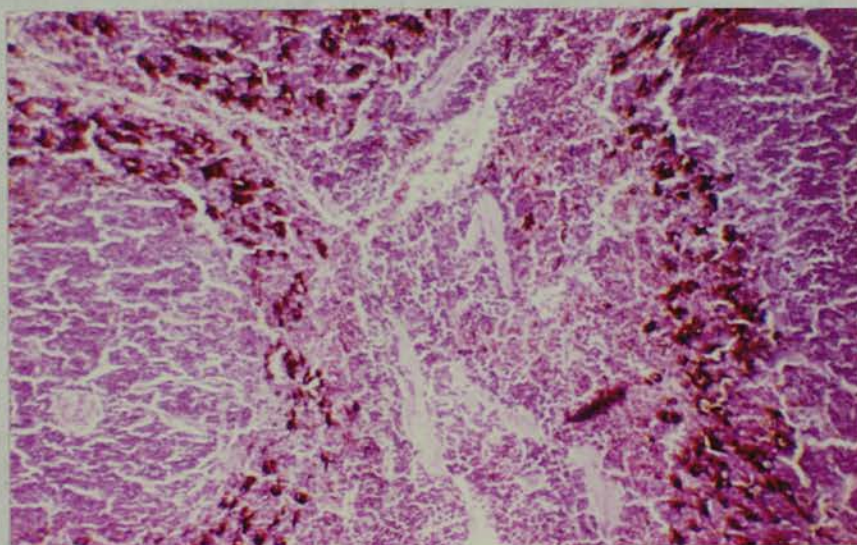


Plate 6. The localisation of carbon in the spleens of G. parvum pretreated (a.) and control (b.) mice, (x 125 ).



In control animals carbon was found localised in the marginal zone and in the macrophages of the red pulp of the spleen. In C. parvum treated mice there was a marked increase in the uptake in red pulp (Plates 6a, 6b).

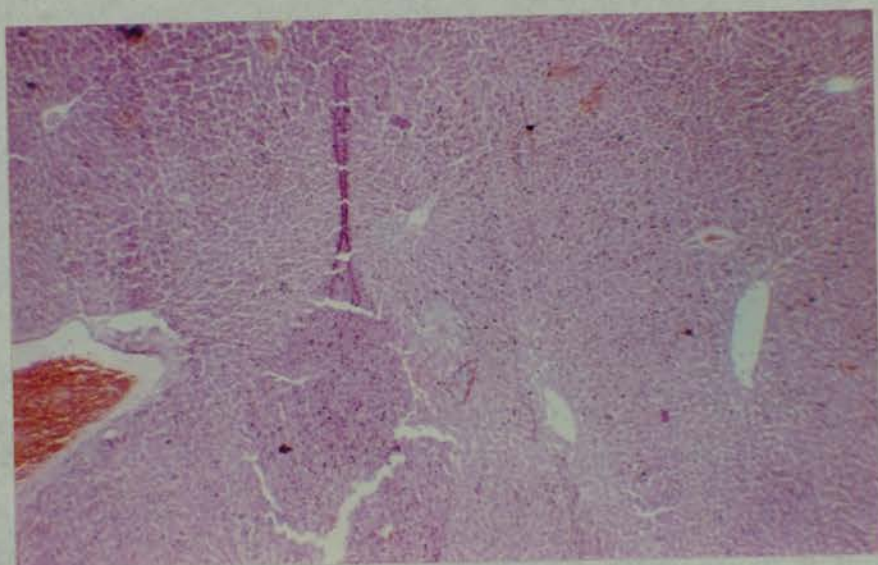
An increase in the number of histiocytes containing carbon in the livers of experimental animals was also found (Plates 7a, 7b). Few cells in the granulomas found in C. parvum treated animals had taken up the carbon.

6. The activity of macrophages treated with C. parvum in vitro.

As C. parvum was found to affect the uptake of c.BSA in vivo, attempts were made to reproduce this effect in vitro. The assay system for activity of macrophages was modified from the work of Ehrenreich and Cohn (1967). The time course of  $^{125}\text{I}$ -BSA uptake was studied in this laboratory by Mrs. A. McCracken. The amount of  $^{*}\text{BSA}$  taken up by washed peritoneal macrophages adhering to glass was found to increase with time over the first 24 hours of culture.

The acidphosphatase level, often taken as a measure of macrophage activity, was increased in peritoneal macrophages taken from animals that received C. parvum four days before hand (164% of control). However, no change could be detected in the uptake and retention of  $^{*}\text{BSA}$  by these macrophages at any time during the culture period (up to 48 hr). Similarly normal macrophages incubated in presence of C. parvum ( $10^6$  -  $10^7$  organisms per chamber) failed to show any enhanced uptake of  $^{125}\text{I}$ -BSA.

a.



b.

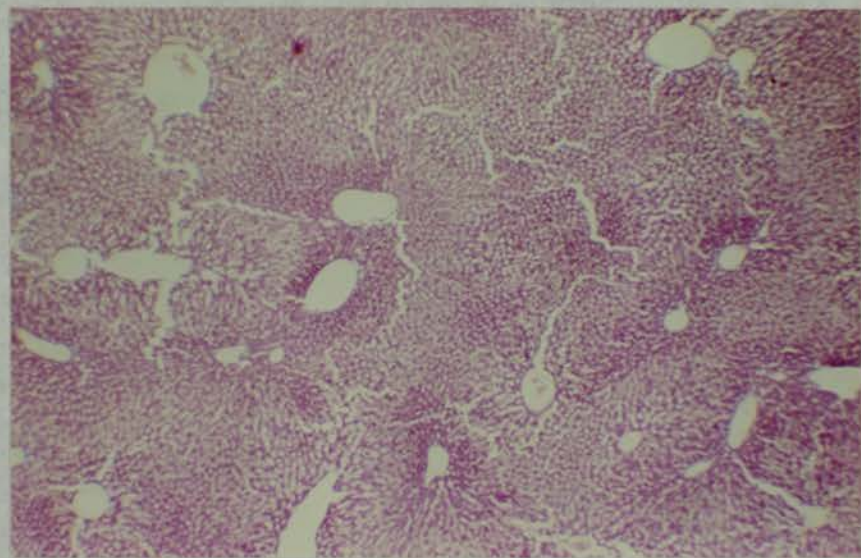


Plate 7. The localisation of carbon in the livers of C. parvum pretreated (a.) and control (b.) mice, (x125).

Table 16

The effect of spleen cells from *C. parvum* treated donors upon tolerance induction.

Group	Treatment	Tolerance induction			day 12		day 20	
		day -20	day 0	ABC	t-test	ABC	t-test	
1	1st fraction of normal cells <sup>c</sup> 1 hr before c.BSA	100 µg c.BSA	5mg a.BSA	0.08 <sup>a</sup>	(5)	0.5 < P < 0.6	0.62	(5) 0.6 < P < 0.7
2	5th fraction of normal cells <sup>c</sup> 1 hr before c.BSA	100 µg c.BSA	5mg a.BSA	0.13	(6)	0.5 < P < 0.6	0.62	(6) 0.7 > P > 0.6
3	1st fraction <i>C. parvum</i> treated cells 1 hr before c.BSA	100 µg c.BSA	5mg a.BSA	0.23	(6)	P < 0.001	1.28	(6) P < 0.05
4	5th fraction <i>C. parvum</i> treated cells 1 hr before c.BSA	100 µg c.BSA	5mg a.BSA	0.26	(6)	P < 0.05	1.15	(6) P < 0.2
5	6 x 10 <sup>8</sup> <i>C. parvum</i> organisms 4 days before c.BSA	100 µg c.BSA	5mg a.BSA	0.32	(6)	P < 0.001	2.32	(5) P < 0.001
6	tolerant control	100 µg c.BSA	5mg a.BSA	0.05	(5)		0.55	(4)
7	non-tolerant control	saline	5mg a.BSA	0.59	(6)	P < 0.001	4.12	(6) P < 0.001

a ABC at 0.01 γ N \* BSA - geometric mean of ABC values for a group

b number of sera tested per group

c 0.8 x 10<sup>6</sup> cells injected i.v.

7. The effect of *C.parvum* treated spleen cell populations upon tolerance induction.

The following experiment was designed to test the ability of spleen cells to transfer the adjuvant effect of *C.parvum*. Populations of cells that adhered to and that did not adhere to glass were distinguished in order that the activities of the two populations could be examined separately. A modification of the method of Plotz and Talal (1967) was used to separate the cell populations on glass bead columns.

Donor mice received either  $6 \times 10^8$  *C.parvum* organisms or saline intravenously four days before sacrifice. The spleens were removed, homogenised and the non-adherent (first) and adherent (fifth) cell fractions separated. Recipient mice were injected intravenously with  $0.8 \times 10^6$  cells from one or other of these fractions 24 hours before the start of the standard tolerance inducing regime.

Results:

The results of this experiment are presented in Table 16. The recipients of normal spleen cells (groups 1,2) became almost as hyporesponsive as tolerant control animals ( $P > 0.5$ ). In contrast, recipients of spleen cells from *C.parvum* treated donors, irrespective of whether an adherent or non-adherent cell population was injected, show a significant lack of tolerant state ( $P < 0.05$ ; day 12; groups 3,4).

The results of acid phosphatase assays on  $2.1 \times 10^5$  cells of the different cell populations injected are in Table 17. Although the *C.parvum* treatment caused an increase in acid phosphatase levels of both adherent and non-adherent cell populations, a more substantial increase was observed in the fifth cell fraction.

Table 17

Acid phosphatase assay on  $2.1 \times 10^5$  spleen cells.

<u>C. parvum</u> treated spleens		Control spleens	
Fraction	OD <sub>410</sub> m $\mu$	Fraction	OD <sub>410</sub> m $\mu$
1st non-adherent	0.37 <sup>a</sup> (4)	1st non-adherent	0.27 (4)
5th adherent	1.01 (4)	5th adherent	0.36 (4)

a mean of four assays per group

Table 18

The effect of cell culture supernatants upon tolerance induction.

Group	TREATMENT	day 12		day 22	
		ABC <sup>a</sup>	t-test	ABC	t-test
1	'C.parvum' supernatant <sup>c</sup> 1 hr before tolerance regime <sup>b</sup>	0.14	not significant	0.94	not significant
2	control 1 <sup>d</sup> supernatant <sup>c</sup> 1 hr before tolerance regime	0.10	not significant	0.85	not significant
3	'B.pertussis' " 1 hr before tolerance regime	0.15	not significant	0.63	not significant
4	control 2 supernatant <sup>c</sup> 1 hr before tolerance regime	0.23	not significant	0.85	not significant
5	6x10 <sup>8</sup> C.parvum four days before tolerance regime	1.18	P < 0.001	4.85	P < 0.001
6	tolerant control <sup>b</sup>	0.23		0.87	
7	non-tolerant control	0.55	P < 0.001	4.34	P < 0.001

a ABC at 0.01  $\gamma$  N \*BSA - geometric mean of 6 sera

b 100  $\mu$ g c.BSA at day -20; 5mg a.BSA at day 0

c 1 ml of supernatant injected i.p. 1 hour before c.BSA

d 1 ml of 'reconstituted' control supernatant

## 8. Attempts to detect the release of lymphokines following C.parvum

Recent studies suggested that the interaction of phytohaemagglutinin with normal lymphoid cells, or of antigen with specifically sensitised lymphoid cells, could result in release of biologically active molecules (see Lawrence and Landy, 1969). Those reported include a macrophage-inhibiting factor, mitogenic factor, transfer factor, interferon, an inhibitor of DNA synthesis and a cytotoxic factor. It was thought possible that the release of active soluble factors may in part be responsible for the adjuvant effect of C.parvum.

Salmonella endotoxin has recently been shown capable of stimulating the incorporation of <sup>3</sup>H-thymidine into 'normal' mouse spleen cells (Peavy et al., 1970).

My attempts to induce in vitro transformation of both mouse spleen cells and human peripheral leucocytes with C.parvum were unsuccessful. Furthermore, cell-free and bacteria-free supernatants, taken from cultures of spleen cells incubated with and without C.parvum or B.pertussis ( $10^8$  organisms per spleen) for 4 or 24 hours at 37°C were unable to prevent the induction of tolerance of mice to c.BSA injected one hour later (Table 18, groups 1,3). Control animals (groups 2,4) received 1 ml of supernatant from normal spleen cell cultures to which the appropriate number of bacteria had been added 10 minutes before the supernatant was removed. The supernatant was cleared of cells and bacteria by centrifugation.

9. The effect of splenectomy upon the adjuvant effect of *C. parvum*.

These experiments were performed with the aim of studying the impact of splenectomy upon the adjuvant effect of *C. parvum*.

The response to SRBC was examined since, in mice, this is known to be dependant upon the presence of the spleen (Perkins, 1970).

Experimental CFE mice were injected with *C. parvum* and four days later with  $5 \times 10^7$  SRBC. The splenectomy or sham splenectomy was performed one day before adjuvant injection, five days before the administration of the SRBC.

Results:

The results of this experiment are summarised in Table 19. Splenectomy led to the expected decrease in response to the antigen, (groups 3 and 4). The adjuvant effect of *C. parvum* was noticeable, (groups 4 and 2). Splenectomy did not completely abolish the adjuvant effect, (groups 1 and 3).



Table 19

The effect of splenectomy upon the response to SRBC, and the adjuvant effect of C. parvum.

Treatment from day 0			Agglutination titres	
day 0	day 1	day 5	day 13	day 23
1. splenectomised	<u>C. parvum</u> <sup>a</sup>	$5 \times 10^7$ SRBC <sup>e</sup>	$3.0^b - 0.4^c(6)^d$	6.0 - 0.6 (6)
2. sham splenectomy	<u>C. parvum</u>	$5 \times 10^7$ SRBC	5.2 - 0.3 (6)	8.6 - 0.1 (6)
3. splenectomy	saline	$5 \times 10^7$ SRBC	1.2 - 0.2 (5)	2.3 - 0.7 (5)
4. sham splenectomy	saline	$5 \times 10^7$ SRBC	2.5 - 0.2 (5)	4.0 - 0.6 (5)

a.  $4 \times 10^9$  organisms C. parvum i.p.

b. mean of  $\log x$ .

c. standard error of the mean.

d. number of CFE male mice in a group.

e. injected i.p.

## Discussion

In this thesis the response of mice to bovine serum albumin has been described with particular emphasis upon the development of a model system for the induction of tolerance by a single dose of the antigen. The tolerant state and certain of the factors influencing its induction have been studied in detail. Considerable attention was given to the effect of the 'physical' state of the antigen as well as to the effect of different genetic background of the experimental animals upon the process of tolerance induction. The effect of bacterial adjuvants, and C.parvum in particular, upon the induction of tolerance in the experimental system studied has been described. The results of each of these studies shall be discussed individually with reference to the present state of available knowledge.

### Bovine serum albumin as an antigen in mice.

The well documented finding that BSA is a poor immunogen in mice (Dresser and Mitchison, 1968) has been confirmed in this study. Centrifuged BSA (c.BSA) induced a profound degree of low zone tolerance in CBA and other mouse strains when given in saline in doses of 10 - 200  $\mu$ g. Higher doses (2-5mg) appeared to result in simultaneous existence of tolerance and immunity.

Although low zone tolerance to BSA in mice has been reported to follow multiple injections of the antigen (Mitchison, 1964) there are few reports that the state can be induced by a single injection. The experiments of Dietrich and Weigle (1964) on mice, using single doses of BSA suggested that "tolerance could be induced only in a small proportion of animals" and that "the degree of tolerance was not clearly related to

the dose of BSA." Dietrich (1968) later reported that tolerance, as judged by immune clearance of antigen, could be induced in C57Bl/6 mice by i.p. injection of 1 or 10 mg BSA but not by 10 or 100  $\mu$ g. These dosage requirements do not agree with those presented in this thesis. However, doses of 1 - 100  $\mu$ g of BSA have recently been reported to induce tolerance in CFA mice (Thorbecke, 1969), a result which is in agreement with those reported in this thesis. It is possible that the indirect immune elimination method of antibody assay used by Dietrich (1968) is responsible for the conflicting reports.

The single doses of BSA found to induce low zone tolerance in mice are strikingly similar to those used by Mitchison (1968) for multiple injections. This supports the assumption of Dresser and Mitchison (1968) that the dose per one injection rather than the total dose injected should be used to evaluate the threshold of tolerance induction to serum proteins.

Mitchison (1968) reported that the induction of tolerance with microgram quantities of BSA required weeks of thrice weekly injections. From this observation he concluded that the rate of tolerance induction is slow in comparison with the rate of elimination of the antigen from the circulation ( $T_{1/2} \approx 19$ hr). The results expressed in this thesis do not fully support this hypothesis for the following reasons.

A similar degree of partial tolerance to BSA can be induced in the absence of detectable priming, over a ten fold dose range of single antigen injections. A second injection of BSA makes the tolerant state more profound if this is given ten days after the first injection but not if injected along with or 24 hours after the first injection (McBride; unpublished). A tentative explanation may be that not all antigen-sensitive cells are made tolerant by the first injection, possibly because of their inaccessibility to antigen, and that having, in time, become accessible they can be made tolerant by a second injection of antigen. Another possibility that also requires investigation is that recovery of a 'virgin' cell population may occur during the time interval before challenge.

The effect of the physical state of BSA upon tolerance and immunity.

The response of CBA mice to BSA given in different physical forms, displayed their capacity to discriminate between 'aggregated' and 'non-aggregated' material.

Bovine serum albumin that was denatured by heat and by low pH (p.BSA) with non-aggregated material removed, was found to be highly immunogenic in doses that led to tolerance induction when centrifuged material was used. In contrast, h.BSA prepared simply by heating a saline solution of BSA, was shown to prime only 50% of the animals injected in the same dose range. Native BSA was only slightly less tolerogenic than centrifuged BSA (c.BSA).

The removal of aggregated materials from purified serum proteins has become a standard method for enhancing their ability to produce tolerance. 'Aggregate-free' preparations of bovine and human gamma globulins, prepared by ultracentrifugation or in vivo 'filtration', have been found tolerogenic in mice (Dresser, 1962a; Golub and Weigle, 1969). Similar conclusions have been reported on experiments employing other species (Battisto and Miller, 1962; Biro and Garcia, 1965; Pinckard et al., 1968).

Frei et al. (1965; 1968) attempted to relate the presence of phagocytosible aggregates in BSA to the antigenicity of the native material. BSA prepared by passage of native antigen through a rabbit was found to be capable of inducing tolerance in other rabbits. The native antigen and also 'dimer' BSA, separated from monomer by gel filtration, induced immunity.

Enhanced phagocytosis of antigen that could occur as the number of the aggregates in the BSA preparations increases, could account for the results reported in this thesis. Autoradiographic studies on the localisation of c.BSA and h.BSA supported this hypothesis. The gross

localisation of antigen in tissues 24 hours after i.p. injection also showed differences between c.BSA and h.BSA. The finding of slightly less h.BSA than c.BSA in the spleen and livers of injected mice may be the result of phagocytosis of aggregates by the macrophages of the peritoneal cavity. Of particular interest, however, was the finding that there was on average 50 to 75% less h.BSA than c.BSA localised in the thymus of injected mice.

The role of the thymus, or cells derived from the thymus, in the induction of tolerance has recently received considerable support (Taylor, 1969; Pierpaoli, 1967). Aggregated BGG has been shown capable of inducing tolerance in X-irradiated rats only if it was injected directly into shielded thymus but not by any other route (Horiuchi and Waksman, 1968). The inability of aggregated BSA to penetrate the thymus following i.p. injection could result in the escape of this population of cells, some of which are undoubtedly capable of responding to BSA.

The centrifuged (c.BSA) and heated BSA (h.BSA) used in the experiments described in this thesis have been shown to have different effects upon a lysosomal enzyme content of mouse spleen cells. The acid phosphatase level was increased 48 hours after an injection of 50  $\mu$ g c.BSA whereas an injection of h.BSA led to a decrease (Jacob and Weir, 1971). Fractionation of spleen cells suggested that these changes occurred within the lymphocyte population (Jacob, 1971). The early proliferative response of certain lymphoid cells to thymus-dependant antigens (Davies et al., 1966) suggests that it may be thymus-derived cell population that is reacting differently to the two physical forms of BSA. Aggregated HGG can stimulate the in vitro transformation of primed rabbit spleen cells to a significantly higher degree than aggregate-free HGG (Abdou and Richter, 1970).

It is therefore possible that differences in the relative abilities of 'aggregated' and 'non-aggregated' BSA to directly stimulate lymphocytes, or to directly make them tolerant, could account in part for the results reported here. It is, however, difficult to avoid the conclusion that, in the present model, antigen handling plays a vital role in determining whether tolerance or immunity is induced. It is at present not known if steps subsequent to antigen handling are also involved.

The finding that the aggregated BSA used in these experiments seems to possess determinants that are not normally found on the native molecule (McBride, unpublished) also requires further examination. Native BSA is relatively incapable of inhibiting the binding of <sup>\*</sup>p.BSA by anti-BSA antibodies, suggesting the presence of 'denaturation' sites on the aggregated molecule. Antibodies that bind these sites appear to follow the injection of native or aggregated BSA. It is possible that it is the antibody directed against these sites that accounts for the higher ABC values obtained when aggregated <sup>\*</sup>BSA is used as the test antigen in the Farr assay. Similar findings have been reported by other workers using different techniques (Peters and Goetzl, 1969). The relationship of these 'denaturation' sites to the immunogenicity of the molecule is at present not known.

#### Strain differences in the immune response and induction of tolerance to BSA.

The induction of tolerance in different strains of mice was undertaken in order to investigate whether the low zone threshold for tolerance induction to BSA existed independent of the physiologic and genetic background of the strain, and independent of the normal response of the strain to that antigen.

a. The immune response to a.BSA.

As expected, differences were found in the normal responses of six inbred strains of mice to a standard dose of a.BSA. While the presence of subclinical infections and other environmental influences can not be disregarded, it seems likely, especially in view of the responses of the  $F_1$  hybrids tested, that the different levels of responsiveness of the strains can be attributed to genetic factors.

The immune response to an antigen is a complex process which must involve genetic control at several levels. The ability to respond to a specific antigen, however, can be selected for by breeding experiments (Sobey et al., 1966; Hardy and Rowley, 1968). Furthermore, immune responsiveness of mice and guinea pigs to certain synthetic polypeptides was shown to be under the control of several autosomal genes (McDevitt and Benacerraf, 1969). The genetic control of the response to more complex antigens is less well documented.

Marked differences were shown to exist in the strength of the responses of five inbred mouse strains to egg albumin adsorbed on alum (Fink and Quinn, 1953). The production of natural anti-SRBC agglutinins in two inbred mouse strains, their  $F_1$  hybrids and reciprocal backcrosses appeared to be under a dominant quantitative control (Stern et al., 1956). Similar results were obtained in a study of the number of anti-sheep erythrocyte plaque-forming cells produced in different inbred mouse strains (Playfair, 1968). By contrast, other reports suggest a lack of genetic control of the response to complex antigens (McDevitt and Sela, 1965).

The genetically controlled differences in the specificity of antibodies produced in mice to branch chain aminoacid polymers have been interpreted as concerning the nature of the antigenic determinant to which the animal makes its most vigorous response (Mozes et al., 1969).

Furthermore, genetically controlled differences in the fine specificity of antibodies to one haptenic group has been reported (Paul et al., 1970a).

It has been suggested that these fine genetically controlled features of the response may often be masked when there are a large number of different antigenic determinants against which the whole response is directed (McDevitt and Sela, 1965), resulting in an apparent lack of genetic influence.

If, as seems likely, the differences between the responses in the strains tested, are genetically based, one must ask why the differences are so obvious. McDevitt and Sela (1965) could find no differences between the responses of C57Bl and CBA mice to BSA given in complete Freund's adjuvant. The discrepancy between their results and those reported in this thesis may be due to a dose or adjuvant effect, but the following should also be considered. In this work the low antigen concentrations used to test for serum antibodies could be preferentially detecting high affinity antibodies. These antibodies might be expected to more readily display the fine differences in specificity for antigen that other workers have reported show genetically controlled strain variations. Marked differences were found in the affinity of antibodies produced by the different strains of mice used in this thesis, as measured by the effect of antigen dilution. Although only a few animals were tested in each group, the results suggested that those strains for which high ABC were found also produced high affinity antibody (McBride, unpublished). Further support for this hypothesis comes from recent experiments that show that the response of mice to ovalbumin adsorbed on alum appears to be linked to H2 histocompatibility locus, provided only low doses of ovalbumin are injected (Vaz et al., 1970). Low doses of antigen preferentially stimulate high affinity antibodies (Eisen and



Siskind, 1964) and these might be expected more readily to show differences between strains because of their high degree of specificity.

By contrast to the differences between strains in their response to a.BSA, all strains, with the possible exception of NZB mice appeared to become tolerant following a single injection of 50  $\mu$ g c.BSA. Where results of limited statistical significance were obtained this appeared to be due to the rather small numbers of animals in the groups and the low levels of antibodies produced in the control animals.

b. Tolerance induction.

Few synchronous studies are available on the strain requirements for tolerance induction. Inbred mouse strains have been shown to differ in the dose of ultracentrifuged HGG required to produce high zone tolerance (Golub and Weigle, 1969). This variation, however, seems to be dependant upon the presence of trace amounts of aggregates in the antigen preparations, and hence presumably upon the efficiency with which the strains process these aggregates. On the basis of empirical argument it has been suggested that the low zone threshold for tolerance induction, in the absence of an immune response, is relatively constant for different species and strains of animals (Dresser and Mitchison, 1968), an antigen concentration of  $10^{-8}$  M to  $10^{-10}$  M being most efficient. This hypothesis is strongly supported by the data in this thesis,  $10^{-7}$  M c.BSA inducing a degree of tolerance in six inbred mouse strains. It appears that the actual thresholds of immunisation are also similar in the strains studied, and that the variation in antibody titres is the result of events subsequent to those necessary for the triggering of the response. As a corollary, it could be inferred that those genetic factors that govern the immune response are not important in the state of tolerance, and that the pathways of

induction are different for the two processes. It is tempting to consider the uptake or non-uptake of antigen by reticuloendothelial cells as being the factor determining the eventual outcome of challenge.

c. The response of NZB mice to BSA.

Of particular interest in this study were the responses of the NZB and (NZB x CBA)  $F_1$  mice to BSA. Mice of NZB strain spontaneously develop immunopathological disorders of an autoimmune nature. They have a high incidence, with age, of Coomb's positive haemolytic anaemia (Bielchowsky et al., 1959). Both NZB mice and their hybrids also show a varying but high incidence of such generalised disorders as thymic and renal lesions (Burnet and Holmes, 1962; Mellors, 1965), serum anti-nuclear factors (Norins and Holmes, 1964), glomerulonephritis (Howie and Helyer, 1968) and malignant lymphomas. The degree to which these phenomena are expressed varies markedly with the age of the animal, its sex and the line studied. Associated phenomena include abnormal plasma cell proliferation and macroglobulinemia (East et al., 1965; de Vries and Hijmans, 1967).

No conclusive evidence exists for an initiating mechanism of the autoimmune processes. Both intrinsic genetic defects involving abnormal proliferation of autoreactive clones (Burnet, 1962a) or thymic epithelia (de Vries and Hijmans, 1966), and extrinsic viral-like factors (East and de Souza, 1966) have been implicated as causative agents.

Fundamental to understanding the genesis of the NZB mouse disease is to know whether the defect in these mice operates specifically in the self-recognition system or if it affects the immunologic performance of the animal as a whole. It was in order to investigate the latter of these alternatives that the NZB and (NZB x CBA)  $F_1$  mice were included among the strains undergoing the tolerance inducing schedule.

NZB mice and their  $F_1$  hybrids responded best of all the strains tested to challenge with a.BSA. The degree of tolerance produced in NZB mice after prior treatment with c.BSA was markedly less than with the other strains. In particular, the degree of tolerance produced in (NZB x CBA)  $F_1$  mice was much greater than in the NZB strain alone. In both 'tolerant' and 'non-tolerant' pooled NZB sera the effect of dilution of antigen upon the antigen binding capacities was less marked than in sera from the other strains (McBride, unpublished). This seems to suggest that the affinity of the antibodies produced in these mice is high.

Recent reports have suggested that the response of mice to 2, 4, 6 - trinitrophenyl hapten (Rathburn and Hildemann, 1970) and ovalbumin (Vaz et al., 1970) are regulated by the products of alleles at the H2 locus of mice. In this respect it is interesting to compare the poor response of DBA/2 mice with that of NZB strain, as these are histocompatible at the main H2 locus.

The characteristics of the response of NZB mice to BSA are suggestive of influences which do not appear to be present in the response of mice from non-autoimmune strains. This conclusion has been supported by work undertaken in other laboratories. Early development of immune reactivity in NZB mice has been described using SRBC as a test system (Playfair, 1968; Evans et al., 1968), but not when pig or chicken erythrocytes were used (Playfair, 1968). Hyper-reactivity of young adult NZB mice to SRBC (Deiner, 1966; Morton and Siegel, 1969), to egg albumin and bovine gamma globulin (Staples and Talal, 1969) and BSA in complete Freund's adjuvant (Cerottini et al., 1969) has been reported. In contrast, old overtly autoimmune NZB mice showed depressed primary but not secondary responsiveness to SRBC (Deiner, 1966) and young adult mice showed no hyper-reactivity when challenged with keyhole limpet

haemocyanin. In addition to the studies reported in this thesis NZB mice have recently been shown relatively refractive in their ability to be made tolerant to BGG (Staples and Talal, 1969).

In the view of the number of studies dealing with responsiveness of NZB mice it is surprising that there is so little data concerning the in vivo fate of the antigens used. Very recently Morton and Siegel (1970) reported that the clearance of colloidal carbon and technetium-99m sulphur colloid from the circulation of old NZB mice was faster than that observed in the young animals and Balb/c mice. The reticuloendothelial activity correlated with the state of splenomegaly. In this study young Coombs negative and Coombs positive NZB mice showed an accelerated rate of clearance of a tolerogenic dose of  $^{125}\text{I}$ -BSA as compared to the CBA strain. Furthermore, by extrapolation to time zero it was found that NZB mice had a higher plasma volume as compared to weight-matched CBA mice or there was a very rapid initial clearance of antigen from the circulation. The ability of NZB mice peritoneal macrophages to take up  $^{125}\text{I}$ -BSA was superior to that of macrophages from CBA animals during the early stages of tissue culture (McBride and McCracken, unpublished), and Balb/c mice (Whyte, unpublished). This finding seems to be related to the age of the animal from which the macrophages are taken. The abnormal responsiveness of the NZB mice may therefore be associated with abnormalities in the reticuloendothelial activity of this strain. These could be associated with their persistent viral infection (East and de Souza, 1966). Differences in the handling and processing of c.BSA or trace amounts of aggregates in the antigen preparations may result in the prevention of tolerance induction. Another possibility, however, is that tolerance is difficult to induce because of a defect in the thymus-derived lymphocyte population that appears to exist in the NZB strain (Chaffer et al., 1970). In any event it seems that the

immunological performance of these mice is abnormal in aspects of their response other than the production of autoantibodies.

The quality of antibodies produced in tolerant animals.

Since the antigen binding capacity of an antiserum is known to be influenced by the relative avidity of the antibody present, the effect of antigen dilution upon the ABC value was determined using two concentrations of antigen. This provided information upon the quality of the antibodies being produced by tolerant animals.

The results presented in this thesis show that the quality of binding of BSA by anti-BSA antibody is similar in both 'tolerant' and control sera following challenge with a.BSA. Furthermore, the 'effect of dilution' ratio increased to the same degree in both tolerant and control animals with time during the immune response.

An immune response is characterised by a change in the affinity of the antibody formed, from low, early after immunisation, to high late in the response (Eisen and Siskind, 1964). This can be interpreted as the display of a mechanism based on thermodynamically driven selection of cell populations participating in immunologic reactions. It is assumed that as the antigen concentration decreases, only those cells capable of synthesising antibody of high affinity can capture the antigen. This results in their preferential stimulation and proliferation. By extrapolation it might be suggested that only high affinity cells would become tolerant to low doses of antigen if direct interaction of antigen with lymphocytes was necessary also for this process to occur. This hypothesis has received some degree of experimental support. Rabbits made partially tolerant by neonatal injection of DNP and a carrier gave rise to anti-hapten antibodies of low affinity for DNP- $\epsilon$ -lysine after challenge (Theis and Siskind, 1968).

The similarities between the antibody affinities of sera from tolerant and non-tolerant mice shown in this thesis do not support the simple hypothesis that only high affinity clones are affected during tolerance induction to BSA. This may be due to the complexity of the antigenic form where the heterogenous array of anti-BSA antibodies for the many antigenic determinants on one molecule may mask the effect. On the other hand the difficulties inherent in considering tolerance in simple thermodynamic terms should not be minimised. The presence of only low affinity antibodies in the serum of tolerant animals following challenge, described by Theis and Siskind (1968), may be explained as follows. The specificity of much of anti-DNP antibody is not only associated with the 'immunodominant' hapten molecule but also with the carrier. As a result the affinity of the antibodies observed may depend upon the test antigen used. Changes in the specificity of the antibodies or the class of antibodies produced by tolerant animals could lead to the observation of low affinity antibodies in these animals. The same result could also be obtained by the selective suppression or the removal by antigen of high affinity antibodies that could be the result of a certain degree of priming during the initial stages of tolerance induction.

#### The effect of adjuvants upon tolerance induction.

The effect of bacterial adjuvants upon the response of mice to BSA was examined at the low antigen doses in order to further elucidate the factors that govern the induction of tolerance or immunity.

C.parvum, C.anaerobium, SF16 and B.pertussis have been shown to be capable of preventing the induction of low zone tolerance to BSA in CBA mice. Instead of tolerance a degree of priming is produced that was detected by the qualitative and quantitative aspects of the response

after further challenge. The sex of the animals had little effect upon the results of these experiments though the dose of adjuvant and the time of its injection were found to be crucial. The importance of these variables has been reported in other similar systems.

Claman (1963) demonstrated that mice could respond rather than become tolerant to 1.9 mg BGG if they were injected two hours previously with 100 mg of S.typhosa endotoxin. Similar results were obtained using BCG or actinomycin D as adjuvants (Dresser, 1962; Claman and Bronsky, 1965).

Adjuvants of this type have often been shown to be effective only if they are given at a time when they may reasonably be thought of as influencing the initial stages of a response. The mechanisms by which they work, however, are obscure.

The fate of a tolerogenic dose of BSA in normal and C.parvum treated animals has been studied quite extensively in this thesis. The conclusions as to the effect of C.parvum treatment may be expressed as follows:

- a. BSA is catabolised faster during non-immune elimination of the antigen from the circulation.
- b. BSA appears to be distributed throughout a greater plasma volume in C.parvum treated animals.
- c. There is no obvious difference in the gross distribution of c.BSA in the lymphoid organs.
- d. In normal animals the c.BSA was diffusely scattered throughout the lymphoid organs. In C.parvum pretreated animals antigen was, in addition, concentrated by the reticuloendothelial cells in the medulla and marginal zone of the lymph node and spleen.

In addition to altering the distribution of injected antigen, C.parvum also causes alterations in the circulation of lymphoid cells and in the haematological picture. Before considering in greater detail the adjuvant action of this organism it is necessary to evaluate the role of these other effects.

Changes in lymphoid organs following C.parvum administration.

Two features of C.parvum administration that have been shown to occur a few days after injection are lymph node and thymic hyperplasia.

Marked hypercellularity and expansion of the paracortical regions of cervical lymph nodes was found to follow i.p. administration of C.parvum. The number of blast cells was increased in these areas. These changes are similar to those observed after an injection of antigenic and non-antigenic adjuvants (Taub et al., 1970). These workers stressed the similarity of this response to that following local stimulation of contact hypersensitivity (Oort and Turk, 1965). This response is thought to be thymus dependent (Parrott and de Souza, 1966). Other changes in the lymph node include a generalised reticuloendothelial hyperplasia and marked medullary plasmacytosis. The former may be reflected in the increased uptake of c.BSA already mentioned.

Thymic hyperplasia can be found in animals that received C.parvum four days before sacrifice. Histological examination revealed little alterations in the medulla to cortex ratio and the only consistent finding was reticulum cell hyperplasia with generalised hypercellularity. Unfortunately, uptake of  $^3\text{H}$ -thymidine by dividing cells was not examined.

Results of experiments by other workers, which employed adjuvants other than C.parvum, should be mentioned. Peripheral blood lymphocytosis and splenomegaly, accompanied by a profound decrease in the number of lymphocytes in the thymus and peripheral lymph nodes, followed the i.v. injection of pertussis organisms into mice (Morse, 1965). On the other hand, Taub et al., (1970) observed that B.pertussis injection led to an influx of cells into the thymus dependant, paracortical areas of the lymph node draining the site of injection. The seemingly conflicting results obtained with C.parvum and B.pertussis warrant a full comparative



investigation of the effects of these adjuvants although it seems probable that with both agents, changes in the recirculating lymphocyte pool after adjuvant injection could account for the lymphocytosis, leucopenia and alterations in thymic, lymph node and splenic cellularity.

Haematological changes following administration of *C.parvum*.

Although the normal cytokinetics of the RES are little understood, it is known that its size and filtering ability is increased following the injection of bacterial endotoxin (Biozzi et al., 1955), methyl cellulose (Palmer et al., 1953), zymosan (Gorstein and Benacerraf, 1960) and many other substances of varying chemical composition. Furthermore, repeated injections of these substances have, on occasion, been shown to cause splenomegaly and anaemia with haematological changes resembling hypersplenism in man (Motulsky et al., 1958). Similar changes, with the induction of a transitory state of anaemia, can be shown to follow a single or a small number of injections of *C.parvum*. Other workers have noted decreased haemoglobin levels (Halpern et al., 1964), lowered circulating red cell counts (Halpern and Fray, 1969), and reticulocytosis (Nussenweig, 1967). These findings have been verified and extended by published (McCracken et al., 1971) as well as unpublished work carried out in this department. A summary of these changes are shown in Fig.3. Certain of the effects are found only inconsistently and hence a rough guide is also given as to the frequency of their occurrence following the injection of  $4 - 6 \times 10^8$  organisms. The reason for the irregular expression of some of these effects may lie in the rapid adaptation to anaemia that rodents appear to be capable of by virtue of their high capacity for splenic extramedullary haematopoiesis (Schooley, 1970).

**Figure 3. Haematological changes 4-14 days after C.parvum.**

<u>Effect</u>	<u>Frequency of occurrence (1-5)</u>
1. hepatosplenomegaly	5
2. lowered total red cell counts	3
3. lowered haemoglobin levels	4
4. increased reticulocyte counts	4
5. increased plasma volume (?)	5
6. leucopenia	5
7. Coombs positivity	2
8. rosettes against 'self' red cells	4
9. plague forming cells against 'self' red cells	4

4-8x10<sup>8</sup> organisms injected i.p.

Hypersplenism is a wide spread syndrome which occurs in many disorders associated with splenomegaly. These include, amongst others, infectious diseases such as malaria, tuberculosis and leishmaniasis; the myeloproliferative and lymphoproliferative diseases; the reticulo-endothelioses; certain haemolytic diseases such as thalassaemia, spherocytic anaemia, and even some instances in which the hypersplenism must be labelled primary because no specific cause is found. The characteristics of hypersplenism are enlarged spleen, anaemia, leucopenia or thrombocytopenia. The mechanism whereby the anaemia occurs often appears to be related to sequestration of circulating blood cells within an enlarged splenic pool (Bagshawe, 1970). It is possible that here there is excessive red cell breakdown and it has been suggested that this may occur by increased phagocytosis, antibody mediated destruction, changes in the surface lipids of red cells that increase the tendency to sequestration in the spleen, or an increased glucose consumption that could lead to a deficit in a necessary metabolic substrate. The mechanism whereby the leucopenia is brought about is unknown.

The similarities between the haematological changes in hypersplenism and those following administration of C.parvum to mice have not yet been fully evaluated although they are striking. It is necessary to consider the consequences of these changes with respect to the lymphoproliferative and adjuvant effects of this organism and also with respect to the finding that lymphoid cells from the spleens of C.parvum treated animals can form rosettes and produce haemolytic plaques with activity directed against autologous red cells (McCracken et al., 1971; McBride, unpublished).

The proliferative response of the spleen and liver to *C.parvum*  
and haemolysis.

There is evidence to suggest that particulate materials can increase the size and filtering ability of the RES. The reactions which follow the injection of these materials (Marshall and White, 1950; Wissler et al., 1957; Kelly et al., 1960, 1962) have in general also been shown to follow damage to autogenous red cells (Jandl et al., 1965). A marked increase in the number of dividing cells in the liver was demonstrated four days after *C.parvum* injection by 'flash' labelling of DNA with <sup>3</sup>H-thymidine. Both parenchymal and reticuloendothelial cells were involved although the increased mitotic activity was most striking in the latter population. Kelly and her co-workers (1960; 1962) noted that foreign particles can stimulate division of hepatic reticuloendothelial cells and *C.parvum* can be found to be present within these cells four days after injection (McBride, unpublished). The nature of the stimulus for parenchymal cell division is obscure though it is likely that it contributes to the hepatomegaly often found in treated animals. One possible explanation is that it is a 'work' hypertrophy. The liver has been shown to be the main source of purine supply for bone marrow cells and possibly also cells in the periphery (Lajtha and Vane, 1958). The liver may expand to fulfil increased requirements of these cells. Most of DNA synthesis in the spleen of normal mice seems to be associated with the medium to large mononuclear cells in the marginal zone and in the red pulp, with light but frequently labelled cells also in germinal centres. This is in a good agreement with the findings of other workers (Jandl et al., 1965). Four days after administration of *C.parvum* there is a marked increase in the number of labelled cells in the perifollicular areas and the red pulp.

The importance of cells in the marginal zone of the spleen with respect to the uptake of antigens and the subsequent immune response has often been inferred. Snook (1964) showed that carbon and saccharated iron oxide first appear localised in the marginal zone before moving outwards, to be found a few hours after injection mainly in the macrophages of the red pulp. A similar location of carbon has been shown in this study to be present four hours after injection. In C.parvum treated animals carbon was found to localise less in the cells of the perifollicular region and more in the red pulp. This altered distribution may be due to an increase in number and also activity of the cells in these regions but may also be contributed to by alterations in the flow of blood through the spleen.

The proliferative response in the perifollicular areas presumably occurs as a response to the lodgement of C.parvum within reticuloendothelial cells. On the other hand it may also be contributed to by the uptake of damaged autologous cells. Jandl et al. (1965) demonstrated that chronic haemolysis could give rise to a very similar picture of reticuloendothelial hyperplasia without any toxic, foreign or immunological mechanisms being apparently involved.

#### Anti-self reactivity in C.parvum treated animals.

C.parvum treated animals have been shown to possess many haematological disorders (see Fig.3). The anaemic state is transitory, first being in evidence several days after injection of the organism and disappearing 2-3 weeks later. It is accompanied by an increase in the number of rosette and direct plaque-forming cells with reactivity directed against autologous or syngeneic red cells (Holton and McBride, unpublished; McCracken et al., 1971). It is of interest that C.parvum injected into

NZB mice gives rise to an exceptionally large increase in the number of cells forming rosettes with autologous red cells (Holton and McBride, unpublished), and that several injections of the adjuvant can result in the early onset of Coomb's positivity in these mice (Halpern and Fray, 1969). It was also of interest to observe that SF16, could give rise to anti-self reactivity similar to that found with C.parvum in CBA mice (McCracken et al., 1971). SF16 is a strain of diphtheroid-like organisms isolated from the synovial fluid of a patient with rheumatoid arthritis (Stewart et al., 1969). This condition is often associated with an unexplained state of anaemia that has the characteristics of the anaemia associated with chronic infection (Alexander and Duthie, 1962). At present, however, it is a matter of speculation whether this type of autoimmune response is of pathogenic significance.

The diversity of possible autoimmune mechanisms has recently become clear. Alteration in antigenic structure i.e. the conversion of a given cell or unit from 'self' to 'non-self' with resultant autoantigenicity, can be considered as one possible basic mechanism. Another mechanism could be centered around alterations in some or all cells comprising the immunocyte complex.

Attempts to show that C.parvum and SF16 share common determinants with, or attach to, or destroy mouse red cells, have so far proved negative (McCracken et al., 1971). As a result the hypothesis that the anti-self reactivity has its basis in the abnormal lymphoid proliferation induced by C.parvum has been strengthened, although proof is difficult to obtain.

Preliminary results suggest that splenomegaly following C.parvum injection cannot per se result in increased anti-self red cell activity, even if anaemia is detectable. Methyl cellulose, B.pertussis, Mycoplasma and BCG give rise to negligible alterations in the numbers of

spleen cells with reactivity directed against self (McCracken, unpublished; Young and McBride, unpublished). It would be of interest to examine the effect of phenylhydrazine which is known to be capable of experimentally inducing autoimmune haemolytic anaemia in mice (Morton and Siegel, 1970). At present, however, it seems that the trigger for this reaction may not be sequestration of red cells in an enlarged splenic pool, nor enhanced phagocytosis of these cells but may lie at the lymphoid cell level.

There are many clinical examples where an intimate relationship has been found between autoimmune disease and leukaemic lymphoproliferative processes e.g. macroglobulinemia, cold haemagglutinin disease and chronic lymphocytic leukemia. Furthermore, the induction of experimental autoimmune disease in animals almost invariably involves the use of Freund's complete adjuvant, a powerful lymphoproliferative agent.

The destruction of lymphoid cells capable of reacting against self antigens has been postulated to occur during foetal life (Burnet, 1959). By extrapolation escape of cells from this process has been suggested as a mechanism whereby 'abnormal' clones might persist and give rise to autoimmune phenomena. It also seems possible that loss of tolerance to self might be the result of alterations in the immune reactivity of normal lymphoid cells. In the same way that C.parvum alters the threshold for the induction of immunity to low doses of BSA, it might also alter the threshold of immunity to 'self' antigens that are normally tolerated. Many 'self' antigens must exist in quantities that are not sufficient to normally induce tolerance or immunity. An example is the idiotypic determinants on the antibody molecules. Anti-idiotypic antibody can be induced in mice by the provision of a 'helper' thymus-derived population (Iverson, 1970; Iverson and Dresser, 1970). It also seems possible that lymphoid cells could exist that produce anti-'self' antibodies,

perhaps of restricted biological activity, but which can be stimulated to induce transient autoimmune phenomena. Bussard and Hannoun (1962) reported that lymph node cells from rabbits primed with SRBC produced isospecific and autospecific red cell antibodies as well as the expected anti-SRBC antibodies. A background level of anti-liver mitochondrial antibodies of the IgM class exists in rats and other species. They are normally present in a steady state and give rise to no autoimmune disease but they can be stimulated following damage caused by  $\text{CCl}_4$  (Weir 1961, 1963).

There is now a considerable body of opinion that stresses the involvement of the thymus in immunological diseases and in view of the thymic changes that result from C. parvum administration this aspect of the subject cannot be ignored.

NZB mice show thymic lesions characterised by expansion of the medulla with germinal centre formation (Burnet and Holmes, 1962). These observations, combined with the knowledge of the thymic influence on immune responses in general, led other workers to search for such lesions in other autoimmune phenomena. In humans germinal or 'reaction' centres in the thymus have been reported in rheumatoid arthritis, endocrinopathies, hypogammaglobulinemia and aplastic anaemia (Miller, 1963) and has long been known for myasthenia gravis (Castlemann and Norris, 1949). These findings led to the concept that the occurrence of germinal centres in the thymus represented a characteristic reaction found in autoimmune states and, in other words, the anatomic evidence of "forbidden clones" (Burnet, 1962). There is, however, little evidence to show that these changes are of primary importance to the disease states and not part of a generalised hyperplasia. De Vries and Hijmans (1967) considered them to be of the latter nature and drew attention to other changes in the thymus of NZB and related autoimmune mouse strains. These changes involved hyperplasia of the large epithelial cells that seem to be



involved in the formation of Hassal's corpuscles, followed by their hypoplasia and subsequent deletion. The relationship of these phenomena to the disease processes is uncertain.

Experimental studies suggest that the presence of the thymus in NZB mice is not necessary for the onset of a positive Coomb's test (Howie and Helyer, 1966). Furthermore, the presence of a thymus graft from a normal strain did not prevent the subsequent disease state although this graft, in turn, developed lesions similar to those developed in the NZB thymus (Holmes and Burnet, 1964).

It seems likely that the thymus is not directly concerned with the initiation of an autoimmune disease at least in NZB mice. A normal controlling function which is disturbed in autoimmune states may, however, be present. Neonatal thymectomy results in an increased incidence of Coomb's positivity (de Vries et al., 1964; Sutherland et al., 1965), anti-nuclear factor (Brezin et al., 1965) and anti-self reactivity of lymphoid tissues in a GVH reaction (de Vries et al., 1964).

In addition to controlling immune responses the thymus may also play a role in the control of haematopoiesis. Neonatal thymectomy in the opossum resulted in an abnormal persistence and increase in myeloid cells, particularly erythroblasts (Miller et al., 1965). Thymic explants have a beneficial effect upon the growth of all cell elements of the spleen (Auerbach, 1963; Metcalf, 1964). Thymus cells have been shown to work synergistically with bone marrow cells in the recovery of erythropoiesis in heavily X-irradiated F<sub>1</sub> recipients (Goodman and Shinpock, 1968).

Alterations in myeloid tissues are also often found associated with immunological disorders and, like the thymic changes, may be characteristic of autoimmune reactions although the inter-relationship of these phenomena has received little attention. It has recently been suggested

that the defect in NZB mice might be associated with an abnormally large stem cell pool, allowing reactivity against self to be expressed by virtue of the relative lack of competition by foreign antigens and haematopoietic requirements for precursor cells (Morton and Siegel, 1969). Furthermore it was postulated that mycobacterial adjuvants might increase the size of this pool (Siegel and Morton, 1967). The results obtained in this thesis suggest that further examination of the phenomena at the stem level may be of value.

#### The adjuvant effect of *C.parvum*.

There appears to be many possible modes of action by which *C.parvum* could exhibit its adjuvant activity. Which of these effects are crucial may depend upon the system under investigation.

From a teleological point of view, optimal stimulation of an immune response could be facilitated by the trapping of an antigen-reactive population of cells within a site, or close to site where there is already enhanced localisation of antigen (Balfour and Humphrey, 1967; Goldschneider and McGregor, 1968). Increased trapping of c.BSA by reticuloendothelial cells might be considered as being responsible for altering the threshold of immunity to this antigen and preventing tolerance induction. However, there is an increasing amount of evidence that the initial stages of an immune response are characterised by an altered distribution of reactive cells (Abdou and Richter, 1969), and *C.parvum* might facilitate this process. While the experimental observations in this thesis are consistent with the occurrence of both these processes, it has not been possible to conclusively prove that one or both effects are responsible for the adjuvant action with BSA.

In spite of the differences in in vivo localisation of c.BSA between *C.parvum* treated and control mice, no enhanced in vitro uptake of  $^{125}\text{I}$ -BSA

by macrophages from C.parvum treated animals was found, nor by macrophages incubated with the organisms. Unanue et al. (1969) showed that the retention and catabolism of haemocyanin (MSH) by macrophages in vitro was similar whether or not B.pertussis was present. They did however show that macrophages incubated with the adjuvant could transfer the adjuvant effect, whether the antigen was or was not associated with the same macrophage population. Lymph node cells treated with the adjuvant in vitro were ineffective in transferring adjuvanticity. These workers postulated that the adjuvant effect of B.pertussis was, at least in part, brought about by its ability to labilise lysosomal membranes, a capacity shared by many adjuvants (Spitznagel and Allison, 1970).

The explanation for the redistribution of circulating reactive cells that appears to follow adjuvant injection, is not clear. Attempts to show a direct effect of C.parvum upon lymphoid cells in vitro by inducing transformation or the release of biologically active substances, proved negative. However, the hypothesis that mechanisms such as these are in fact operative in trapping and perhaps stimulating lymphoid cells remains attractive.

In this thesis attempts to transfer the adjuvant effect of C.parvum demonstrated that the induction of tolerance to BSA could be prevented by the transfer of populations of spleen cells from adjuvant treated animals whether or not the cells adhere to glass and independent of their acid phosphatase content. Transfer of bacteria is unlikely to have accounted for this effect. This experiment suggests that the adjuvant effect of C.parvum may not be solely attributable to increased reticulo-endothelial activity and the system deserves further investigation from this viewpoint.

Little information is available to support or deny association between the haematological changes induced by C.parvum and the adjuvant effects. Splenectomy, which is known to cure many of the haematological defects associated with hypersplenism (Tumen, 1970), did not completely abolish the adjuvant effect of C.parvum found following the intraperitoneal injection of SRBC. However, the effect of splenectomy upon the haematological changes induced by C.parvum remains at present unexamined.

It is not yet known whether or not the autoimmune reactivity found in C.parvum treated animals is also a manifestation of its adjuvant effect. The presence of anaemia itself does not seem sufficient for this reactivity to develop as methyl cellulose injection which caused splenomegaly and anaemia, did not lead to an increase in the number of rosette-forming cells against syngeneic red cells. Neither is reticulo-endothelial stimulation apparently sufficient, as a few other known reticuloendothelial stimulating agents were relatively ineffective. However, it still remains to be shown that the disturbances are primarily at the lymphocyte level. There is a good evidence for suggestion that there is a relationship between the myeloid and lymphoid cell systems (Yoffey, 1964; Wu et al., 1968). One of the modes of action of mycobacterial adjuvant (Siegel and Morton, 1967), and perhaps also C.parvum, might be to increase the size of the pluripotential stem cell pool. Although Albright and Makinodan (1965) and Doria (1968) have reported that erythropoiesis is generally associated with a decreased level of antibody formation, it would be of interest to consider the haematological disturbances as being due to an adjuvant action at this level of organisation.

CONCLUSIONS.

The main findings of this thesis are as follows.

1. Ultracentrifuged native BSA (c.BSA) is a poor immunogen in CBA mice.
2. Single low doses (10 - 150  $\mu$ g) of centrifuged BSA can readily induce a state of hyporesponsiveness in CBA mice as judged by their impaired response to challenge with alum-precipitated BSA (a.BSA).
3. The class and quality of the antibodies formed in tolerant mice following challenge is similar to those found in non-tolerant controls.
4. Four different inbred strains and two F<sub>1</sub> hybrid mouse populations differ quantitatively in their response to immunogenic alum-precipitated BSA.
5. In contrast, these different populations of mice, with the possible exception of NZB strain, become all tolerant if given 50  $\mu$ g of centrifuged BSA.
6. Of the groups of mice tested, NZB and (NZB x CBA) F<sub>1</sub> mice respond best to alum-precipitated BSA. In addition, NZB mice are relatively refractory to tolerance induction.
7. The use of aggregated BSA in the place of tolerogenic centrifuged BSA switches the response of CBA strain from tolerance towards immunity.
8. A tolerogenic dose of centrifuged BSA is not localised to any extent by the reticuloendothelial system.
9. The same dose of aggregated BSA (h.BSA) is found localised to a greater extent than centrifuged BSA within macrophages.
10. Treatment with C.parvum, C.anaerobium, SF 16 or B.pertussis is capable of preventing the induction of tolerance to centrifuged BSA in CBA mice. This effect depends upon the dose and timing of adjuvant administration.

11. C.parvum pre-treated mice show an accelerated rate of non-immune clearance of centrifuged BSA and an enhanced localisation of the antigen within the cells of the reticuloendothelial system.

These main conclusions, along with the results of other experiments, are discussed with respect to proposed mechanisms of tolerance induction and immunity and their control. The adjuvant action of C.parvum is discussed with relation to the effects of the bacteria upon various cell populations and in regard to the haematological changes that follow injection, including the increase in the number of spleen cells with reactivity directed against autologous red cells.

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LIST OF ABBREVIATIONS.

ABC	antigen binding capacity
AgAdd	antigen added
BCG	Bacille-Calmette-Guerin vaccine
BGG	bovine gamma globulin
BSA	bovine serum albumin
a.BSA	alum-precipitated bovine serum albumin
c.BSA	centrifuged bovine serum albumin
h.BSA	heated bovine serum albumin
p.BSA	bovine serum albumin heated at low pH
D	dextrorotary
DNP	dinitrophenyl
EDTA	ethylenediamine tetracetic acid
F <sub>1</sub>	first hybrid generation
FCA	Freund's complete adjuvant
GVH	graft-versus-host
H-2	histocompatibility locus No.2
HGG	human gamma globulin
HSA	human serum albumin
KLH	keyhole limpet haemocyanin
L	levorotary
MSH	<u>Maia squinado</u> haemocyanin



NRS	normal rabbit serum
NZB	New Zealand Black
PAS	periodic acid-Schiff reaction
PFC	plaque-forming cell(s)
PHA	phytohaemagglutinin
RES	reticuloendothelial system
RNA	ribonucleic acid
SAS	saturated ammonium sulphate
SRBC	sheep red blood cell(s)
TCA	trichloroacetic acid

g	gram(s)
kg	kilogram(s)
mg	milligram(s)
$\mu\text{g}$ or $\gamma$	microgram(s)

hr	hour(s)
min	minute(s)
sec	second(s)

M	molar
mM	millimolar
mCi	millicurie(s)
ml	millilitre(s)

S.D. standard deviation

S.E. standard error

$\mu$  ionic strength

P	probability level
%	per cent
w/v	weight per volume
*BSA	radioactive preparation
$^{125}_{\text{I}}$	mass number of nuclide
r	roentegen
g	gravitational acceleration
$^{\circ}\text{C}$	degree Celsius
$\gamma^{\text{G}}, \gamma^{\text{M}}$ etc.	symbol of gammaglobulins
+ve	positive
<u>et al.</u>	et alii (and collaborators)
i.e.	id est (that is)
i.p.	intraperitoneal(ly)
i.v.	intravenous(ly)
p.	page

Appendix 1.

Flow Diagram for Farr results:

ABC = number of sets of data for each serum - 1 or 2.

TCA = TCA 'control' counts.

CONT = 'control' counts

sets = number of sets of dilutions for each sera.

D (1:dil) = dilutions.

Bin 1 and Bin 3 = original 'experimental' counts, sets 1 and 2.

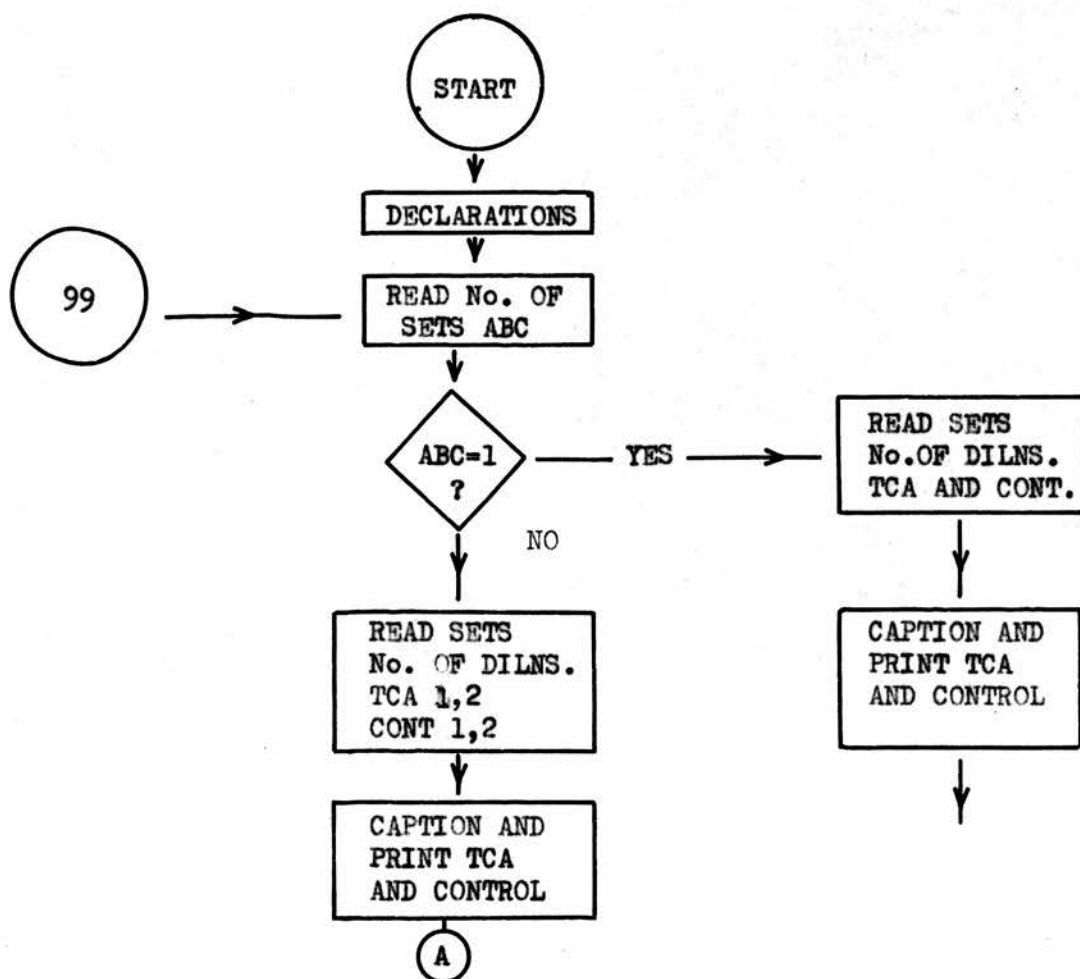
Bin 2 and Bin 4 = % binding.

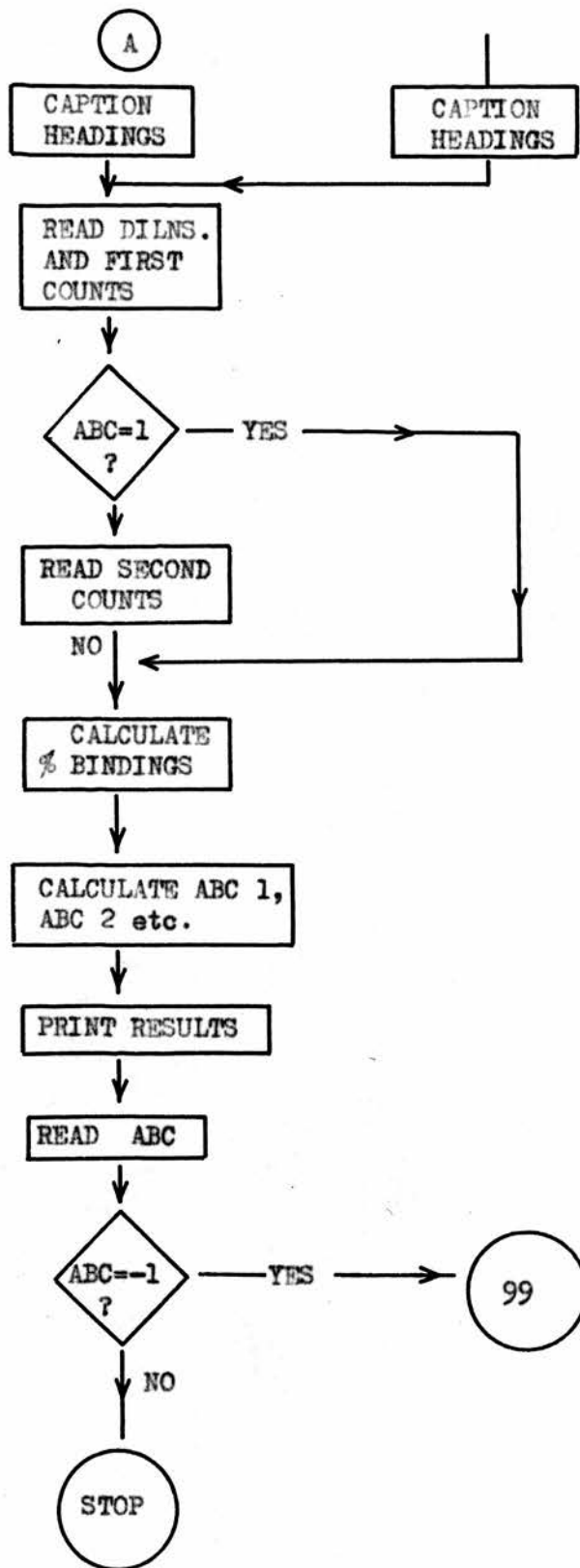
ABC 1 = ABC - 33 values for first set.

ABC 2 = direct ABC values for first set.

ABC 3 = ABC - 33 values for second set.

ABC 4 = direct ABC values for second set.





The interpretation of the program for obtaining percent bindings, ABC - 33 and direct ABC values, written for the 360/50 Edinburgh University computer (IMP compiler), is included in the pocket at the back of this thesis.

```

1  %LIST
2  |IMM 001/00000 MCBRIDE FARR RESULTS
3  %LIST
4  %BEGIN
5  %REALSLONG
6  %INTEGERFN ELAPSED TIME
7  %RESULT=1
8  %END

9  %INTEGER ABC,TCA1,TCA2,CONT1,CONT2,SETS,DIL,I,Z
10 %REAL Y
11 99: READ (ABC)
12 |NO OF SETS OF RESULTS
13 NEWPAGE
14 %IF ABC = 1 %THEN -> 2
15 READ(SETS);READ(DIL);READ(TCA1);READ(TCA2);READ(CONT1);READ(CONT2)
16 |SETS=NO OF SETS OF DIL: DIL = NO OF DIL PER SET
17 NEWLINES (3)
18 %PRINTTEXT'TCA '
19 PRINT(TCA1,4,0)
20 PRINT(TCA2,4,0)
21 NEWLINE
22 %PRINTTEXT'CONT '
23 PRINT(CONT1,4,0)
24 PRINT(CONT2,4,0)
25 NEWLINES (3)
26 %PRINTTEXT'
27'
28' DIL COUNTS BIN ABC1 ABC2 COUNTS'
29 %PRINTTEXT' BIN ABC1 ABC2
30'
31'
32 -> 1
33 2: READ(SETS);READ(DIL);READ(TCA1);READ(CONT1)
34 NEWLINES (3)
35 %PRINTTEXT'TCA '
36 PRINT(TCA1,4,0)
37 NEWLINE
38 %PRINTTEXT'CONT '
39 PRINT(CONT1,4,0)
40 NEWLINES (3)
41 %PRINTTEXT'
42'
43' DIL COUNTS BIN ABC1 ABC2
44'
45'
46 1: %BEGIN
47 %REALARRAY D(1:DIL),BIN1,BIN2,BIN3,BIN4,ABC1,ABC2,ABC3,ABC4 %C
48 (1:SETS*DIL)
49 |BIN1 AND 3 REFER TO SET 1: 1 CONTAINING ORIGINAL COUNT %C
50 3 CONTAINING % BINDING : 2 + 4 TO 2*ND SET OF DATA.
51 %CYCLE I = 1,1,DIL
52 READ (D(I))
53 %REPEAT
54 %CYCLE I = 1,1, SETS*DIL
55 READ(BIN1(I))
56 %IF ABC = 1 %THEN -> 3
57 READ(BIN2(I))
58 3: %REPEAT
59 %CYCLE I = 1,1,SETS*DIL
60 %IF BIN1(I)=0 %THEN ->4
61 BIN3(I)=((BIN1(I)-CONT1)/(TCA1-CONT1))*100
62 4: %IF BIN1(I)<=0 %OR BIN3(I)<=0 %THEN BIN3(I)=0
63 %IF ABC = 1 %THEN ->6
64 %IF BIN2(I)=0 %THEN ->5
65 BIN4(I)=((BIN2(I)-CONT2)/(TCA2-CONT2))*100
66 5: %IF BIN2(I)<=0 %OR BIN4(I)<=0 %THEN BIN4(I)=0
67 6: %REPEAT
68 Z = 0
69 %CYCLE I = 1,1,SETS*DIL
70 Z = Z + 1
71 %IF Z=DIL %THEN ->12
72 %IF BIN3(I)=0 %AND BIN3(I+1)=0 %THEN ->12
73 Y=(LOG(D(Z+1))-LOG(D(Z)))*(BIN3(I)-33)/(BIN3(I)-BIN3(I+1))
74 %IF Y>7 %THEN ->888
75 |*VR
76 ABC1(I)=EXP(Y)*D(Z)*0.066
77 %IF ABC1(I)<0.001 %THEN ABC1(I)=0
78 ->13
79 888: ABC1(I)=100
80 ->13
81 12: ABC1(I)=0
82 13: ABC2(I)=BIN3(I)*D(Z)*0.002
83 8: %IF ABC= 1 %THEN ->10
84 %IF DIL=Z %THEN ->14
85 %IF BIN4(I)=0 %AND BIN4(I+1)=0 %THEN ->14
86 Y=(LOG(D(Z+1))-LOG(D(Z)))*(BIN4(I)-33)/(BIN4(I)-BIN4(I+1))
87 %IF Y>7 %THEN ->887
88 |*VR
89 ABC3(I)= EXP(Y)*D(Z)*0.0066
90 %IF ABC3(I)<0.001 %THEN ABC3(I)=0
91 ->15
92 887: ABC3(I)=100
93 ->15
94 14: ABC3(I)=0
95 15: ABC4(I)=BIN4(I)*D(Z)*0.0002
96 10: PRINT(D(Z),4,1)
97 PRINT(BIN1(I),5,0)
98 PRINT(BIN3(I),3,2)
99 PRINT(ABC1(I),6,3)
100 PRINT(ABC2(I),3,3)
101 %IF ABC=1 %THEN -> 11
102 PRINT(BIN2(I),5,0)
103 PRINT(BIN4(I),3,2)
104 PRINT(ABC3(I),6,1)
105 PRINT(ABC4(I),3,3)
106 11: %IF DIL=Z %THEN NEWLINE
107 %IF Z=DIL %THEN Z=0
108 NEWLINE
109 %REPEAT
110 %END
111 READ (ABC)
112 %IF ABC=-1 %THEN ->99 ;| NEXT SET OF SERUM
113 %ENDOFPROGRAM

```

128 STATEMENTS COMPILED SUCCESSFULLY  
CODE OCCUPIES 5264 BYTES GLAP 380 + 0 BYTES DIAG TABLES 164 BYTES TOTAL 5880 BYTES

OBJECT PROGRAM FILE COMPLETE