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THE NATURAL HISTORY OF AUTOIMMUNE DISORDERS

IN MICE AND ITS MODIFICATION BY THERAPY.

Thesis

Submitted for

the Degree of Doctor of Medicine

in the University of Otago

by

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11. PERSONAL COMMUNICATION CONCERNING USE OF 6-mercaptapurine in MICE

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CHAPTER 1

INTRODUCTION
Modern immunological concepts allow an increasing, but not yet clearly defined, status to the group of disorders which are referred to as the Autoimmune Diseases. Pride of place for acceptance under this category is usually given to systemic lupus erythematosus (S.L.E), which Dameshek (1958) has called the autoimmune disorder par excellance, with autoimmune haemolytic anaemia (Dacie, 1962) also high on the list.

One of the difficulties for investigators in this aspect of immunology, both at the purely experimental and the paraclinical levels, has been the lack of suitable experimental models in animals (Oliner et al. 1961 and Nisbet and Heslop, 1962). Hence the description by Bielschowsky, Helyer and Howie in 1959 of an inbred strain of mice, NZB/BL, which spontaneously develop an autoimmune type of haemolytic anaemia was important. Subsequently Helyer and Howie, (1961), reported hybrid mice with renal lesions analogous to lupus nephritis. A further hybrid, NZB/BL X NZW, was reported by Helyer and Howie (1963a) showing 100% of positive L.E.cell tests and development of renal changes of lupus nephritis in 8-10 months.

Widespread interest has been shown in these mice
and the original description of the features of the NZB/BL mice has been confirmed by Burnet and his co-workers (Holmes and Burnet, 1963).

Burnet (1962b) considers the detailed investigation of the NZB/BL strain of mice that develop an autoimmune type of haemolytic anaemia is important and says in his Jephcott Lecture "It should be evident that a great deal more work is called for with these animals. One might almost say that there is need to repeat all the immunological, pathological and therapeutic studies that have been made on human autoimmune disease with NZB/BL mice and almost equally all the immunological and genetic work that has been done on normal mice."

The author considers that the same could be said of the NZB/BL X NZW hybrid mice which develop a lupus type of nephritis. This statement of Burnet seems an ample justification for undertaking studies on these mice with autoimmune disease. Immunological, pathological and therapeutic studies have been carried out on the mice by the author and comparisons made with human autoimmune disorders. Particular attention has been paid to the natural history of the autoimmune processes in the New Zealand mice and to modification of such processes that can be made by therapy.
The main aim of this thesis is to study from a clinicopathological aspect the pattern of development and the nature of lesions, particularly renal ones, in NZB/BL X NZW hybrid mice. Groups of these mice were assessed clinically, weighed regularly and had frequent examinations of urine during life for protein, casts and sugar. Blood examinations for the L.E. cell test, anaemia, total leucocyte counts, as well as direct Coombs tests and some Latex antinucleoprotein tests were performed and some studies made on blood urea levels.

Full postmortem examinations were made with histological examinations, especially of kidneys and thymus, but also of liver, spleen, lymph nodes, bone marrow, pituitary, adrenal, thyroid and salivary glands, the postmortems being carried out at intervals to cover the life span of the crossbreed. The postmortem renal findings were correlated with the features observed during life. Similar observations were made on smaller numbers of the parent NZB/BL and NZW inbred strains.

At attempt was made to modify with corticosteroid therapy the disease seen in the hybrid mice. This was done in both sexes, in different age groups and at
different dosage levels. The same parameters were measured as in the control untreated animals mentioned above.

Corticosteroid therapy was also used to treat the NZB/BL mice with autoimmune haemolytic anaemia. Treatment of this strain with the antimetabolite drug 6-Mercaptopurine was also undertaken.

Before presenting the experimental studies and results it is considered relevant to review aspects of immunity and the concept of autoimmunity. Previously reported work on the NZB/BL X NZW hybrid, as well as in the parent NZB/BL strain, is then reviewed, the value of the model in immunology becoming apparent. Possible ways in which corticosteroids can be expected to modify the lesions and reactions seen in autoimmune disorders are then considered. Principles underlying special tests and methods, used extensively in the thesis, in particular the L.E. cell test and the Coombs test, are then discussed and this concludes the Introductory Chapters. A detailed description of the experiments performed and their results follows and forms the main body of the thesis. This experimental section is followed by a discussion section in which the information obtained in the project is assessed. The final section, the Appendix, contains appendices on
details of technique, tabulated experimental results and their statistical analyses.

The work involved was carried out in the Pathology Department of the University of Otago in the period February 1962 - October 1964 under the direction of Associate Professor J.B. Howie, whose sound advice, constructive criticism and continued encouragement have been most helpful to the author.
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INTRODUCTORY CHAPTERS

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CONCEPTS OF IMMUNITY AND AUTOIMMUNITY

It is proposed in this Chapter to outline the development of knowledge of immunity with a consideration of the nature of antigens and antibodies. Then factors influencing and theories concerning the production of antibodies are outlined. This leads to a discussion of the phenomena of self recognition and immunological tolerance and the development of the concept of auto-antibody production. Major reasons for the increasing interest and knowledge of autoantibody production lie in the development of suitable techniques to detect autoantibodies, their presence in a number of human diseases, numerous experimental attempts to produce or detect autoantibodies, studies of the phenomenon of immunological tolerance and the development of theories such as the clonal selection theory which seek to explain the nature of the disorders. These major reasons are expanded and provide a broad background picture of widespread interest in autoimmune disorders. Against this can be seen the suitability and usefulness of the experimental models of autoimmune disease provided by the inbred NZB/BL mice which develop an autoimmune haemolytic anaemia and by the hybrid NZB/BL X NZW mice which develop systemic lupus erythematosus.
A. THE CONCEPT OF IMMUNITY

1. Immune responses, antigens and antibodies

For many centuries man has been aware that some sort of resistance to disease could follow previous experience of that disease. The best example of this, discussed at length by Gladstone and Abraham (1962a) is seen with smallpox. Nurses for patients were often chosen from those who had previously had smallpox and variolation was practised long before Jenner introduced vaccination. In vaccination there is a good experimental illustration that a modified infection can protect against a more serious one. Also, implicit in the subsequent challenge by Jenner of the vaccinated child with material from a variola lesion without smallpox developing, is the idea of some specificity in this resistance. This was illustrated also by the living attenuated cholera and rabies vaccines of Pasteur and the first use of dead organisms in a vaccine by Theobald Smith (quoted by Gladstone and Abraham, 1962a). "Immunity" came to mean a state of resistance to infective disease, irrespective of how this was brought about, and could be innate or acquired. Immunity and circulating substances. The first unequivocal demonstration that immunity was associated
with substances contained in the blood was that of von Behring and Kitasato (quoted by Karush, 1962) when they showed in 1890 that antisera obtained from animals immunised with bacterial toxin protected normal animals from the otherwise lethal action of the toxin and that such protection was specific, tetanus antibodies providing no protection against diphtheria and vice versa (quoted by Gladstone and Abraham, 1962a).

Immune responses. In the following decade it was shown that antitoxins could be formed against non-bacterial toxins and that antibodies appeared in the circulation after the injection of non-toxic material such as sera from other animals and gradually the term "immune response" has come to cover any production of antibody to an antigen. Soon Richet recognised anaphylaxis as a deleterious effect and the Arthus phenomenon was described (Miles, 1963), these observations showing variations from the theme of immunity meaning resistance.

Antigen specificity and nature of antigens. After it was shown that antibodies act by combining with their specific antigen Ehrlich was led to propound his "lock-and-key" analogy of antibodies being specific chemical groups or side chains attached to cells, blocked by the introduction of antigen, this blocking being
followed by an excessive synthesis of antibody (Karush, 1962 and Abraham, 1962b).

However this type of explanation seemed incompatible with the development of knowledge of the specificity and multiplicity of protein antigens largely due to the work of Landsteiner with various haptens (quoted by Abrahams, 1962a). Some simple chemicals are rendered antigenic by coupling with a protein complex. Simple chemical alterations in these haptens result in different antibodies. It was shown that polar groupings which carry an electrostatic charge, the spatial arrangement of the molecule and the size were all of importance in determining antigen specificity, this relationship of immunological specificity to molecular structure being extensively reviewed by Karush, (1962). Nevertheless the actual union that occurs between antigen and antibody is not due to a specific, strong chemically active force or bond.

Specificity and nature of antibodies. The specificity of the antibody lies in the complementary spatial configuration of the reactive atoms or atomic groups (Hughes-Jones, 1963).

Antibodies were long known to be present in the globulin fraction of immune serum obtained by precipitation, with salts or alcohol, but it required
the special fractionation devised by Cohn, and electrophoresis to establish that most antibodies were gamma globulins, Fahey (1962), pointing out that additional physicochemical studies such as ultracentrifugation and immunochemical studies have shown that the gamma globulins are a heterogeneous group. This heterogeneity extends to antibody also and both Fahey (1962) and Sehon (1963) show that it is clearly established that one antigen may cause the production of several physicochemically distinct antibodies with any one of which further antigen may react specifically. This point is further emphasised by Porter (1963) who states that there are three different types of globulin antibodies, the usual 6.6S gamma globulins, macroglobulins (18S) and B2A globulins which have both common and distinct antigenic determinants, suggesting a partial identity of structure.

2. Factors influencing the nature and size of the antibody response.

Many factors are known to influence the nature and size of the antibody response. These include whether or not there has been previous exposure to the antigen i.e. the primary and secondary responses, the latter resulting in much more rapid and larger production of antibody; the frequency and timing of the antigen dosage; the
route by which it is given; the age of the animal, newborn animals having little ability to synthesise gamma globulin; nutritional factors, malnutrition may reduce the production of antibodies; non-specific stimuli may induce an antibody response, the "anamnestic reaction".

The quality of the antigen is also important and one of the requirements of a good antibody response seems to be that the antigens should be present in the body for a long time (Gladstone and Abraham, 1962b). Particulate antigens are usually more effective than soluble ones and this explains the long current use of alumina precipitated toxoids in immunisation. These probably act by delaying absorption by resulting in the formation of a local granuloma. There is evidence (White, 1963) that antibody producing cells are present at the edges of such granulomas as well as in the draining lymph nodes, with continued production of antibody. The effect is probably mainly a depot one but another factor could be the ingestion of antigen covered particulate matter by the reticuloendothelial cells subsequently allowing more intimate contact of antigen with antibody forming tissues.

Freund's adjuvant, (Freund, 1951) is a water-in-oil emulsion, usually with mycobacterial or nocardial
extracts added, which has been extensively used to enhance and prolong the antibody response. It is of particular interest to our study because it has been used successfully in endeavours to make animals react against antigens from their own tissues, as discussed later. Freund (1951) considers that though the main effect of his adjuvant is a depot one, this is not the whole explanation. He considers that the mycobacterial extracts aid the migration of inflammatory cells to the site. White (1963) draws attention to the fact that many different agents which act as adjuvants have the ability to activate the phagocytic system of the body with hyperplasia and proliferation. Some of the work of White (1963) suggests that widespread stimulation of immature and mature plasma cells in spleen, bone marrow and lymph nodes remote from the injection site can follow the addition to the water-in-oil adjuvant of a peptoglycolipid from mycobacterium tuberculosis.

The quantity of antigen is also important, having a threshold value above which however there is no very close relationship between the quantity of antigen and the amount of antibody produced. A great excess of antigen has indeed been found to inhibit the production of antibody (Gladstone and Abraham, 1962b). This may be
important in explaining why the body does not react against itself, common antigens being present in such quantities that a form of immunological paralysis and resultant self tolerance occurs.

The state of the antibody forming tissues is also relevant to the result of a given antigenic stimulation. This may be modified by some diseases such as the reticuloses and also in congenital agammaglobulinaemia. The antibody response may also be modified or prevented by treatment of the antibody forming tissues by ionising radiation, by antimitotic drugs such as 6-mercaptourine or by corticosteroids (White, 1963), the time at which these are used being critical. Possible mechanisms by which the production of antibodies is modified by corticosteroids are extensively discussed in a later chapter. Radiation, in particular, has been used extensively to provide a state of immunological tolerance where the host will tolerate grafts from other animals.

Finally, genetic factors may influence the antibody response as do species differences. In a general way individuals in any animal population will vary in their response to antigens. Occasional animals show little response to a given antigen and the progeny of such animals show similar features (Gladstone and Abraham, 1962b). Stavitsky (1961) points out that the ability of
an animal to respond to a given antigen may depend on its phenotype, and that it is possible that the type of antibody produced may depend on the genotype of the individual. This is also favoured by Fahey (1962) in discussing the heterogenicity of antibodies produced.

3. The sites of formation of antibodies

The historical aspects of development of knowledge of the actual site of antibody formation are traced by Nossal (1962) who quotes Pfeiffer and Marx in 1898 as first demonstrating that the spleen was a major site of antibody production and that this also occurred to a significant degree in lymph nodes, bone marrow and lungs. It was long considered that the reticuloendothelial cells and later that the lymphocytes were the chief antibody producers.

Fagraeus (1948a and b) however showed that it was the plasma cell series which proliferated and produced antibody in response to antigenic stimulation and she also showed that it was these cells in the red pulp and at the edge but not in the white pulp. Other evidence such as the immunofluorescent localisation of gamma globulins, studies of biosynthesis using C14 labelled amino acids, studies of plasma cells in hyperglobulinaemia, in agammaglobulinaemia and in myelomatosis support this, as does in vitro work with individual cells studying the
bacterial adherence phenomenon and also antibody production, all of which are extensively referred to by Nossal (1962a). These studies seem to leave no doubt that Fagraeus (1948a and b) was correct in her deductions (Fahey, 1962; Nossal, 1962a and b; Janeway, 1962) that gamma globulins are formed in plasmacytes, lymphoid plasma cells and in immature cells present in the lymph nodes, spleen, bone marrow and related tissues of the lymphoid system.

Stavitsky (1961) summarises the evidence that primitive cells of the lymphoid series as well as plasmacytoid series may synthesis antibody. Macrophages, large lymphocytes, haemocytoblasts and reticular cells seem to be converted to immature plasma cells which synthesise antibody and Stavitsky (1961) believes that the heterogenicity of antibody molecules produced in response to a single antigen may depend on the participation of different types of cells in the synthesis of these antibodies. Nossal (1962a), after discussing his own and others persuasive evidence for the vital role that plasma cells play in antibody formation, is equally sure that the small lymphocyte does not itself form antibody.

The types of cells just discussed have been conveniently referred to as immunologically competent cells when they "have a specific reactivity with a particular antigen or antigenic determinant" (Mackay and Burnet, 1963).
Fagraeus (1960) felt that in the present state of knowledge "neither functional aspects nor prospective behaviour of cells" could be used as a basis for nomenclature but only the actual morphological state of the cells, giving definitions of terminology for the Ciba Foundation Symposium on Cellular Immunity at which she was speaking.

Nossal (1962b) prefers because of difficulties of identification the non-committal category of large lymphocyte for cells called by others lymphoblasts, haematogenous stem cells, reticular lymphocytes, reticulum cells and blasts, excluding mast cells and macrophages as recognisable large cells in lymphoid tissue. By labelling the deoxyribonucleic acid in these cells with tritiated thymidine just prior to the induction of the secondary response he shows that these large lymphocyte type cells are the ones which produce plasma cells and thus antibody. They are what Nossal calls "memory" cells, this function of storing previous experience not residing in the plasma cells which have a limited life and cannot multiply.

4. The mode of formation of antibodies

Theories of antibody formation have been diverse and are conveniently considered (Mackay and Burnet, 1963) as either "instructive" or "selective".
The "instructive theory" followed the work of Lansteiner already mentioned and is usually also associated with the name of Pauling (Gladstone and Abraham, 1962b) who clarified ideas. It is called the direct template theory, which implies that some antigen must remain present in cells for production of antibody and the theory explains some of the ways in which an antigen could be responsible for the production of the specific combining centres of its corresponding antibody. This theory explained some of the known facts about antigen-antibody reactions but was not of much help in explaining the general biological phenomenon of immune responses.

Burnet modified the concept to an "indirect template" theory, the idea being that the antigen caused some specific genetic alteration in a cell, thus avoiding the necessity of postulating antigen persistence. Campbell and Garvey (1963) however show that antigen (or at any rate, parts of it) may indeed persist for many months or years in a way which would be consistent with the dictum "no antigen material, no antibody".

The "selective" or "elective" theory of antibody production has had its main advocate in Burnet (1959a and b) in his "clonal selection theory" and, with some modifications (Mackay and Burnet, 1963), is becoming increasingly popular. Interestingly, these authors defer
to the original patterned side chain theory of Ehrlich, already mentioned, as the first example of a selectionist theory of antibody production. The most succinct explanation of the theory is that of Mackay and Burnet (1963). "Every antigenic determinant we are ever likely to encounter is already represented in the body, not by antibody molecules but by a clone of cells capable of making that antibody molecule. When an antigen is introduced, it will make contact with a cell of the corresponding clone, presumably a lymphocyte, and by doing so stimulate it to produce, either directly or through its descendants, more globulin molecules characteristic for that cell or clone".

B. THE CONCEPT OF AUTOIMMUNITY

1. Antibodies and autoantibodies

Some of his experiments led Ehrlich to propound his axiom of "Horror Autotoxicus" (Dacie, 1962) which implies that the body will not react against its own constituents in any self destructive way. This thought was fairly general for almost half a century and is evident in the classical definition of an antibody as a substance that appears in the blood stream or body fluids in consequence of the parental injection of an antigen into the body and that reacts specifically with that antigen (Abraham, 1962a). If we allow, as discussed below, that
there are exceptions to this "instructive" definition of an antibody then Burnet (1963b) suggests that an antibody can only be defined as "a serum globulin (gamma, B2A or B2M) which reacts specifically (i.e. preferentially) with the substance being considered as antigen or haptene". His subsequent definition follows that any autoantibody is a "serum globulin which will react specifically with a substance obtainable from the animal producing the serum". Burnet stresses that such substances must not be taken out of the context of clinically, genetically or experimentally "abnormal" humans or animals. Dacie (1959) had stressed this point when he said an autoantibody must not be defined as an antibody produced by the subjects NORMAL antibody forming cells against the patients NORMAL red blood cells. In short, the use of the word "normal" in defining autoantibodies is to be avoided.

2. Self and not self

Burnet and Fenner (1949), interpreting particularly the now classic communication of Owen (1945) that dizygotic twin cattle often contain a stable mixture of each others red cells, concluded that if an embryonic animal was exposed to expendable cells from a genetically distinct race no antibody would result to this foreign antigen when the animal took on an independent existence. That their
postulate was well founded was soon apparent when Billingham et al. (1953) induced what is now referred to as "immunological tolerance" (Medawar, 1961) in mice by injecting foetal or neonatal mice with nucleated cells of a genetically distinct strain of mice. They thereby induced "a state of indifference or non-reactivity towards a substance that would normally be expected to excite an immunological response" (Medawar, 1961).

In the context of a discussion of recognition of self and not self Medawar (1956) had also said that "the reason why an animal does not react immunologically against some complex and highly differentiated constituents of its own body is because, crudely, it grew up with them and so became tolerant of what might otherwise have been their antigenic action". He could see two possible points for future trouble with this mechanism. Some antigens develop after the immunological mechanisms of the body - e.g. milk and spermatozoa, and others cannot gain access to sites of immunological response - e.g. lens and brain proteins, to allow tolerance to develop in foetal life. Later in the same discussion Burnet (1956), whose contributions have been especially valuable and fertile in this ever broadening field of immunology, pointed out that the differentiation between self and non-self must not be taken too literally. He wondered if the important division might not be between components of
expendable cells and all other material.

However Mackay and Burnet (1963) do stress that from the point of view of autoimmune disease it is a major requirement of any immunological theory that an adequate explanation of the mode of recognition of self and not self antigenic configurations can be given. Autoimmune disorders imply a failure of such a mechanism, which is most likely to be due to a disturbance of the antibody producing cells or of the mechanisms which control these cells.

C. MAJOR REASONS FOR INCREASING INTEREST IN AUTOIMMUNE DISEASES AND SOME OBSERVATIONS ON THESE DISEASES.

I. New Techniques

The upsurge in popularity of the study of autoimmunity in disease can be related to a number of factors, not the least of which has been the development of new techniques for the study of such processes over and above standard agglutination, precipitation and neutralisation methods.

The Coombs test (Coombs et al. 1945) for detecting erythrocyte fixed antibody and modified to detect free circulating antibody has led to an understanding of autoimmune components in haemolytic anaemia. Variations of agglutination techniques using tanned red cells or
inert particles such as bentonite and latex coated with a possible antigen have allowed the identification of many different autoantibodies. Those used in this thesis are reviewed in a later chapter. Other physicochemical and immunochemical advances have been mentioned in the discussion on gamma globulin. A wide variety of immunohistochemical techniques are reviewed by Beck (1963) and Holboron (1964), the most notable probably being modifications of the Coons antibody fluorescent technique and using this to trace antigen in tissue sections.

II. Observations on Diseases with a Probable Autoimmune Component.

1. The thyroid

The findings of antibodies against the self component thyroglobulin - i.e. autoantibodies in the sera of patients with Hashimoto's disease (Roitt et al., 1956) and their interpretation of this disease as the result of an autoimmune process (Doniach and Roitt, 1957) due to leak of thyroglobulin were an application of the concepts of Burnet and Fenner, plus the long current instructive theory of antigen-antibody production. Thyroglobulin was regarded as antigenic and the antibodies formed to it were regarded in their turn as causing further damage to the
thyroid parenchyma. The subsequent finding that this particular antibody was not toxic to trypsinised thyroid cells in tissue culture (Roitt and Doniach, 1958) – although another antibody is (Pulvertaft et al., 1959) – was against the aetiological significance of the thyroglobulin antibody itself. This thyroglobulin antibody may be found in patients (Owen and Smart, 1958) with thyrotoxicosis or myxoedema usually associated with localised non-progressive lymphadenoid change. Similar antibodies may be present and show an evanescent rise without any perpetuating self damage to the thyroid after surgical or viral damage to the gland (Felix-Davies, 1958). These facts also are against the neat concept of cause and continuing effects outlined above. It was soon realised that the precipitin and more sensitive tanned red cell techniques showed only one of a number of antibodies that could be present in this disease. Besides antithyroglobulin there is the cytotoxic agent noted by Pulvertaft et al. (1959). Complement-fixing antibodies are found in 90% of cases of Hashimoto’s disease and are thought due to a cytoplasmic microsomal antigen. The same workers have also shown another antibody to a colloid constituent other than thyroglobulin using the Coons fluorescent antibody technique (Roitt and Doniach, 1959).

Autoimmune thyroiditis has been dealt with at length
because of its historic place in the development of thought on autoimmune disorders and to illustrate that one must be hesitant to attribute lesions to the presence of autoantibodies in the serum (Ed., Lancet, 1961).

2. **Autoimmune type of haemolytic anaemia**

Dacie (1959) reviews the historical aspects of the development of knowledge about haemolytic anaemias from 1904 when Donath and Landsteiner described haemolysis in the sera of their patients with paroxysmal cold haemoglobinuria. A few years later Widal noted autohaemagglutination in patients with acquired haemolytic icterus and Chauffard noted an autolysin active at 37° which disappeared when his patient recovered. Dameshek and Schwartz (1940) state that the concept that haemolysins were occasionally demonstrable in acute cases of haemolytic icterus was apparently completely forgotten from about 1915. They demonstrated in two of their cases serum iso-haemolysins, at first in high titre, the unique feature of the haemolysin being its ability to haemolyse Group O cells and cells of the same group as the patients. More frequently in such cases no haemolysin could be detected and, although some workers thought that an antibody might be present, such incomplete antibodies were only demonstrated after the introduction of the Coombs test (Coombs et al., 1945). The reasons for accepting acquired
idiopathic haemolytic anaemia as an autoimmune disorder are extensively discussed by Dacie (1962) and Mackay and Burnet (1963) but, in short, hinge on the evidence of an antibody produced by the patient and demonstrably able to damage his own cells. There is no doubt that the antibody may fairly be called an autoantibody and it seems reasonable to call a disease manifesting such features an autoimmune disease.

3. Systemic lupus erythematosus as an autoimmune disorder.

Explanations of the etiology of systemic lupus erythematosus have reflected changing fashions in medicine (Ed. Lancet, 1960b), being attributed first to "toxic" then to infective causes and more recently grouped as a "collagen" disorder. Dameshek (1958) is as much responsible for the current view that it has an autoimmune basis as he was for re-directing attention to acquired haemolytic anaemias. He develops the theme that in S.L.E. there may be autoantibodies to red blood cells, platelets, leucocytes and clotting factors on the one hand and to small blood vessel constituents on the other. Dameshek also proffers as an explanation of the sex differences in incidence of S.L.E. that the female is recurrently exposed to antigenic material at the time of the menses. He also stressed that the L.E. phenomenon was only
one aspect of the disease and does not have to be present.

There has been little difficulty in accepting S.L.E. as an autoimmune disorder. Its multisystem lesions with histological evidence of fibrinoid degeneration, vasculitis, nuclear degradation and lymphoid hyperplasia, coupled with abnormal serological reactions such as the L.E. cell phenomenon and various positive antinuclear tests, false positive reaction for syphilis, autoimmune complement fixation tests and positive Coombs tests make it a good example of an "antinuclear disease" rather than a "collagen disease" (Mackey and Burnet, 1963). Whether this is a further fashion time alone will tell.

4. Other diseases with a possible autoimmune component.

Other diseases currently regarded as having autoimmune components include sedormid purpura, rheumatoid arthritis, Sjogren's disease, lupoid hepatitis, some types of male infertility, non-tuberculous Addisons disease, some types of agammaglobulinaemia, ulcerative colitis and regional enteritis, rheumatic fever, glomerulonephritis, myasthenia gravis, demyelinating forms of encephalomyelitis, amyloid disease, pernicious anaemia and sympathetic ophthalmitis, these having been reviewed by a number of authors (e.g. Steiner and Volpe 1961a, b, c; Ed. Lancet, 1961; Heslop and Simons, 1962; Mackey and Burnet, 1963).
While autoantibodies are present in many of these diseases there is seldom the clear-cut pathogenic association with cellular damage from antibody that is seen in the autoimmune haemolytic anaemias. In some instances the autoantibodies may follow tissue damage. It seems more likely, however, that a multiplicity of antibodies could be explained by a disturbance of the antibody forming tissues.

5. **Overlap of autoimmune disorders**

There has been a tendency to regard autoimmune disorders in two separate categories. Firstly, those in which the body reacts against one type of body cell (e.g. thyroid cell or erythrocyte) and those in which many systems are involved. However there has been a growing awareness, as Roitt and Doniach (1959 and 1960) point out that a patient with one of these disorders may show evidence of others. In their cases of Hashimoto's disease 10-15% showed a more widespread disturbance of immunological reactions and either gave positive complement fixation tests with saline extracts of other human organs or showed an antinuclear factor similar to that seen in lupus erythematosus. Very occasionally patients had both thyroiditis and evidence of systemic lupus erythematosus with positive L.E. tests. Such an overlap is also definite between Hashimoto's disease, disseminated lupus
erythematous, rheumatoid arthritis and Sjogren's disease in varying combinations (Ed. Lancet, 1961). These facts also favour the concept that the fundamental defect lies in the antibody forming cells or their control.

6. **Familial incidence of disorders**

Just as the one patient may show evidence of a number of different antibodies and/or clinical states so may his or her relatives, even though otherwise apparently normal. The association of a familial incidence of positive Rose-Waaler tests in relatives of patients with rheumatoid arthritis; of cases of disseminated lupus and rheumatoid arthritis in the one family; of agammaglobulinaemia patients with a high familial incidence of rheumatoid arthritis; of familial cases of systemic lupus erythematosus; of Sjogren's syndrome and of protein gammaglobulin abnormalities are referred to by Ziff (1961) and Ed. Lancet, (1961). Ziff (1961) points out that other families vary in the manifestations that a multifaceted syndrome with hereditary characters will take e.g. Marfan's syndrome. The genetic characteristics of one family will tend to modify, either bring out or suppress, certain components over others that will show more in another family group. Again a plausible interpretation of these genetic factors would be that the fundamental defect is in the antibody
forming tissues or their controlling mechanisms.

That genetic factors are present in the spontaneous autoimmune diseases seen in the inbred NZ mice and that the form the process takes shows some modification between strains has consequently aroused considerable interest.

7. **Criteria for an autoimmune component in human disease.**

Heslop and Simons (1962), reviewing this topic, list the following criteria as supporting an autoimmune component, or basis, in a given disorder:

a. the detection of autoantibodies with specific activity against self components.

b. the presence of abnormal serum globulins.

c. the clinical response to steroids may be suggestive.

d. the patient's genetic background might suggest a predisposition towards the development of autoantibodies.

e. the presence of more than one supposedly autoimmune disease in the same patient.

f. there may be an accumulation of cells believed to be immunologically competent in the vicinity of the lesions.

g. fibrinoid necrosis is sometimes seen in lesions which are believed to have an autoimmune basis.

h. the resemblance to animal experiments in which there is a known autoimmune basis implies an analogy between the two conditions.
III. Animal Models of Autoimmune Mechanisms

Suitable models would obviously be highly desirable to study the mechanisms of autoimmunity and considerable effort and ingenuity has gone into devising them. A self-reactive process has been induced by the repeated injection of homologous tissues, but usually only when an adjuvant such as Freund's adjuvant is also used.

1. Attempts to produce autoimmune haemolytic anaemia in animals.

Wagley and Castle (1949) using Freund adjuvant and injecting each of four dogs with some of its own blood found a transient positive Coombs test in one dog. Lin and Evans (1952) had a somewhat higher incidence of positive Coombs tests in rabbits, being prompted to give the injections of fresh whole blood intraperitoneally after they noted a transient positive Coombs test in a patient with a ruptured ectopic pregnancy. No anaemia developed in either series. Other workers quoted by Dacie (1962) have been equally unsuccessful in establishing any really useful model of this type of anaemia.

2. Attempts to produce an animal model of S.L.E.

Mackay and Burnet (1963) state that typical L.E. cells and lesions of S.L.E. never occur in animals inoculated with various nuclear derivatives although if these are of heterologous origin weak antibody formation may occur.
They point out that it is the rule with autoimmune conditions characterised by reactivity with accessible autoantigens that it is difficult to reproduce the disease experimentally. Steiner and Volpe (1961b) also state that the complex lesions of S.L.E. have not been reproduced experimentally, although the vasculitis and glomerulonephritis seen in this disease bear many features of experimental hypersensitivity reactions.

3. Experimental production of renal lesions.

Masugi in 1933 cited by de Wardener (1961) first showed that rabbit anti-rat kidney serum, when injected intravenously into the rat produced a renal lesion similar to that found in subacute or chronic glomerulonephritis. Schwentker and Comploier (1939) induced circulating antibodies to rabbit kidney after injections of homologous kidney using streptococcal or staphylococcal tissues as adjuvants. Steiner and Volpe (1961c) refer to other models and to the fact that an experimental form of nephrosis can be transferred in rats using appropriate lymphoid cells. Freund (1951) refers to the production of nephritis in rats inoculated with extracts of rat kidney plus his adjuvant.

The Masugi type of experimental glomerulonephritis is to be distinguished from that induced by Rich et al. (1950). Cardiovascular and renal lesions of a hypersensitivity
type developed in rabbits after injecting them with a single large dose of horse serum. Enough of this is left in the serum after antibodies to it develop for a widespread hypersensitivity reaction to follow. Hypercellularity of glomerular tufts with ischaemia develops. This type of reaction, but not the passive Masugi's condition, can be prevented by steroids, according to Thorn et al. (1954).

Recent interest also attaches to the production of a chronic glomerulonephritis by administration of amino-nucleoside. Borowsky et al. (1961) point out that such drugs have been regarded as haptens but whether the mechanism is an immune or a toxic one is not certain. This type of lesion cannot be prevented or modified by corticosteroids.

4. Experimental production of central nervous system disorders.

Freund (1951) found that rhesus monkeys could develop a disseminated form of encephalomyelitis from repeated (over 30) injections of homologous or heterologous brain material but this took up to a year to develop. With the aid of his adjuvant this allergic encephalomyelitis developed very rapidly. Rats can also be sensitised by injections of spinal cord and develop a form of allergic encephalomyelitis rather like that seen in humans. In rats this condition can be transferred using lymphocytes
(Paterson, 1960). Both corticosteroids and corticotrophin have protective effects in the development of experimental allergic encephalomyelitis if given early enough (Field and Miller, 1962).

5. Sundry experimental models

Reference has been made to experimental autoimmune thyroiditis and Heslop and Simons (1962) refer to similar experiments using lens, uveal, testicular or adrenal extracts, while Mackay and Burnet (1963) quote others.

6. Autoantibodies and delayed hypersensitivity in experimental models.

In both the thyroid and central nervous system experiments circulating autoantibodies develop. However, even in high titre these do not cause lesions when injected into normal animals; lesions can follow the transfer of living lymph node cells from immunised animals (Ed. Lancet, 1961). These facts suggest that the lesions resulting from auto-sensitisation are related to an immune response of the delayed hypersensitivity type mediated by lymphocytic series cells, also that the serological abnormalities noted are products of the disease rather than aetiologically significant.

This last conclusion is not so clear-cut with autoimmune renal disease and some would disagree mildly with it (Steiner and Volpe, 1961c), whereas others also
particularly experienced in the field (Muehrcke, 1962) would completely disagree with it. Muehrcke would explain the glomerular lesions seen in post streptococcal glomerulonephritis as resulting from the combination of antibody with renal glomerular basement membrane protein, the complex combining with complement. The product of this amalgamation he considers initiates the inflammatory process. Pollak, Pirani and Kark (1961a) regard the karyorrhexis and haematoxyphil bodies of lupus nephritis as the result of a gammaglobulin antibody reacting at glomerular level with antigen. The view that they are due to an in vivo L.E. factor effect is not widely held.

7. Homograft immunity

The homograft immunity of normal animals (Burnet, 1961) is considered to reside in the lymphocytes of the host, as seen in the host versus graft reaction. An immune response on the part of the host to the foreign antigens of the tissue or cell graft is followed by rejection of the graft. This occurs more rapidly with second grafts from the same donor. This transplantation immunity seems a very good example of the body's ability to recognise "not self" antigens. However transplantation tolerance (Hasek et al. 1961) can occur if the host is immunologically tolerant, as at birth - this presumably being related to the immaturity of the antibody forming
tissues at that time. Medawar (1962) stresses his belief that tolerance is a delaying of the capacity to react to an antigen and that this postponement continues indefinitely only in the continued presence of antigen. There is obviously a subtle distinction between this postponement of the ability to react and an actual paralysis of such ability due to excess of antigen in an adult animal, as already mentioned. Further study of these phenomena may well add to the understanding of breakdown in self tolerance that is evident in the autoimmune disorders.

Stetson (1963) discusses the balance of evidence for a pathogenic effect between isoantibodies and cellular hypersensitivity mechanisms in the homograft reaction and states a good case for the cytotoxicity of some antibodies before concluding that there has been a decade of preoccupation with the lymphocyte and delayed hypersensitivity where the central role may well be the conventional antibodies.

8. Graft versus immunologically tolerant host reactions.

The reaction of graft versus immunologically tolerant host, as seen in the Simonsen phenomenon and in the development of runt disease, is generally considered to be due to a delayed type of sensitivity reaction
associated with the lymphocytes of the graft (Cock and Simonsen, 1958; Nisbet and Heslop, 1962).

In the Simonsen phenomenon (Simonsen, 1957) suspension of spleen cells or leucocytes from adult fowls when injected intravenously into 18-day chick embryos cause a severe haemolytic anaemia with strongly positive Coombs tests and fatal within two weeks of hatching (Cock and Simonsen, 1958).

Runt disease was induced in mice by Oliner et al. (1961) who injected into parental strains tissues from F₁ hybrids; the parental strain did not tolerate the hybrid tissue which contained foreign antigens but the hybrid graft tissue did not react against the parental host. Most of these parental mice developed a hunched position, with narrowed palpebral fissures and ruffled fur, the general appearances being regarded by Helyer and Howie (1963b) as similar to those seen in the NZB/BL mice. Oliner, Schwartz and Dameshek (1961) showed that their "runts" had a high mortality, associated with anaemia, leucopenia, thrombocytopenia and marked splenomegaly. The anaemia was accompanied by positive Coombs tests, shortened red cell survival time and these authors were able to elute a gamma globulin antibody from the red cells and show that it was specific to the contralateral parental strain. No positive L.E.cell tests were observed.
Dameshek (1960) also draws attention to what is now commonly called homologous or secondary disease where the immunological mechanisms of the host are destroyed by irradiation and bone marrow grafts are then given for survival. He points out that in these circumstances features similar to runt disease occur in man with weight loss, skin and bowel disturbances, severe anaemia and leucopenia with evidence of effects against several systems (Ed. Lancet, 1960).

Although it is generally believed that these three phenomena are examples of graft cells reacting against host cells, the exact mechanisms are not known and some authorities (Loutit, 1962) favour the view that runt disease is a manifestation of lymphoid aplasia on the part of the host animal. The parallels of these immunological models to human autoimmune disease are not clear, although some authors, such as Oliner et al. (1961), consider them close.

9. **Spontaneous autoimmune diseases in animals**

No satisfactory naturally occurring models exist apart from the NZ inbred strains and hybrids.

A strain of blue minks, the *Aleutian mink*, has been described in which there is a high incidence of hepatitis and renal disease, with widespread vasculitis accompanied by the deposition of fibrinoid material.
These animals have extensive lymphoid infiltration of many organs, large numbers of plasma cells and develop hypergammaglobulinaemia. This syndrome, which is called Aleutian disease (Wagner, 1963), has been shown to be transmissible by filter passing agents in genetically susceptible animals. No definite autoantibodies have been demonstrated. Wagner (1963) prefers in the meantime to regard Aleutian disease as a connective tissue disorder, but feels that an underlying autoimmune disorder triggered by a virus infection is possibly the basic mechanism. His claim that this spontaneous disease may prove a useful model for studying pathogenesis is a reasonable one.

A preliminary report from Lewis, Schwartz and Gilmore (1964) indicates that they have seen isolated cases of an autoimmune type of haemolytic anaemia and a type of systemic lupus in dogs.

The dearth of recognised naturally occurring autoimmune disorders in animals is apparent. Against the previously described contrived experimental models it is obvious that in this field the description of the New Zealand inbred and hybrid strains of mice was a major event. These strains will be considered in detail in the next chapter but it should be noted here that they provide models of the two disorders most widely accepted in man as autoimmune in origin; also that they provide
these models of haemolytic anaemia and S.L.E. where experimental efforts have been unable to do so.

IV. The Clonal Theory and Autoimmunity

In discussing this theory, as most other authors have done, the terminology and phraseology introduced by Burnet are extensively used.

1. The importance of host factors in autoimmune disorders.

In the light of the increasing recognition of the role of genetic factors in diseases of autoimmunity the continual stressing by Burnet (1959 a and b and 1961) of the importance of host factors, especially the genetics of populations of immunologically competent cells in the patient or experimental host assume even more significance. He feels that in immunologically based drug reactions such as Sedormid purpura any platelets will have a haptene effect with the drug - it is the host that determines whether a thrombocytopenia develops.

In experimentally produced replicas of autoimmune disease due to reactions against normally inaccessible antigens - e.g. Hashimoto's disease (Witebsky and Rose, 1956) Freund's adjuvant is needed. This adjuvant Burnet considers causes some weakness of immunologic status and gives the immunologically modified cells the "aggressiveness" to populate the thyroid. In the group of autoimmune
diseases against common body antigens he sees no place now for the concept that a normal body element can become antigenic when it is chemically modified by toxin, virus or drug. Cells are the important thing and antibodies are merely an easily measured circulating index of their activity. He defines an "immunologically competent cell (I.C.C.) as one that differs from other morphologically and physiologically similar cells by specific reactivity with a particular antigen or antigenic determinant."
The nature of the reaction is immaterial - "to elaborate soluble agents that raise blood pressure, contract muscle, cause cells to proliferate or die."

2. Clonal theory

Burnet then develops his concept of immunologic homeostasis. If cells immunologically reactive against self need to be eliminated during late embryonic life then they also need to be in postnatal life. These are called "forbidden clones" and their regulation is "immunological homeostasis". A clone can be defined as a group of individuals of like genetic constitution obtained by asexual reproduction from a single original individual (Richardson, 1961).

Burnet (1962a) considers that all specificity of antibodies or of immunologically competent cells is genetically determined and that those which develop with
specificity for self components are eliminated in foetal life, perhaps mainly in the thymus or under thymic influence. Other groups of cells carrying primary immunological patterns are formed, perhaps in or under control of the thymus and populate the lymphoid tissue of the spleen, lymph nodes and submucosae. Here, already preadapted to a certain line of immunological competence, they are committed to this line by proliferation following contact with an antigenic determinant, usually exogenous. He envisages somatic mutation in the stem cells - the mutants being the parents of different clones - as having an important role in widening the group patterns of reactivity that are primarily available.

It is pointed out (Ed. Brit. Med.J., 1962) that the theory is not generally accepted; also it denies the whole concept of the earlier "instructive" idea that cells had to be educated to react specifically to an antigen by means of some sort of contact with the specific antigen.

3. The thymus and the theory

Much of the work of Miller (1963) shows that the thymus has a humoral effect on the antibody forming lymphoid tissue. Burnet (1962c) summarises his studies of its abnormalities in the NZB/BL mice and in patients with myasthenia gravis. His clinical colleagues have felt sufficiently sure of the importance of the thymus in
autoimmune disorders to have recently reported the first instance of thymectomy for treatment of a severe and intractable case of S.L.E. (Mackay et al. 1963). However by this stage the gland was atrophic due to steroid therapy and the patient showed no clear benefit from the operation.

4. The theory and the disorders

In terms of this theory, then, autoimmune disease would be due to mutant cells giving rise to forbidden clones, or failure of the body, perhaps at thymic level, to eliminate such clones. It would not be due to the earlier idea of a change rendering antigenic a self component in a mature host. All biological variation is a product of genetics and environment (Olsen, 1962) and currently it is the genetic factors which are regarded as the more significant in the etiology of autoimmune disorders, a concept which is strikingly supported by the occurrence of such disorders in genetically related strains of NZ mice.

V. Morbid Anatomical Recognition of Autoimmune Disease

Steiner and Volpe (1961a) stress the fact that there are no strict criteria for the recognition of such diseases. In different situations microscopically similar round cells and plasma cells are taken to have
different meanings. In the disorders we are considering, infiltration of the target organ such as the kidney by immunologically competent cells may be aetiologically important and be followed by tissue destruction and replacement. On the other hand, similar groups of cells in another site, say in relation to a gastric ulcer, would be considered by most as of no pathogenic significance. Steiner and Volpe (1961a) say that the only morbid anatomical evidences of actual autodestruction are erythrophagocytosis and the haematoxyphil body.

Thymic lesions are reported by Burnet (1962c) in the NZB/BL strain of mice and in the thymus of patients with myasthenia gravis. This description introduces a further dimension into the morbid anatomical assessment of these disorders. He notes that the thymus may show enlargement of the medulla with germinal centres, incomplete lymph follicles and accumulations of plasma cells and mast cells, i.e. "an immunological type of lymphocyte proliferation" (Ed. Brit. Med. J., 1963a). As already discussed, Burnet uses this to support his concept of the central role of the thymus in immunological homeostasis, and of the prime importance of disturbances of the thymus in the causation of autoimmune disease.

VI. Viruses and Autoimmune Disorders

Hotchin (1962) following, as he points out,
observations of Burnet and Fenner (1949) has reopened the whole question of viruses in the etiology of autoimmune disorders by describing the effects of lymphocytic choriomeningitis L.C.M. infection in neonatally infected mice who are immunologically tolerant of this infection. Adult mice first infected develop liver and spleen enlargement, with yellowish discoloration of the latter. Histologically they have a lymphocytic meningo-encephalitis and myelitis, severe hepatitis with lymphocytic infiltration of the necrotic areas, interstitial pancreatitis, splenic hyperplasia, swollen hyperplastic lymph nodes, lymphocytic infiltration of salivary glands, testes, endocardium, pericardium and lung, as well as occasionally of synovium. Inoculation of newborn animals sometimes produced runting and death, or an immune tolerance with persistent tolerant infection. In this state, after 10-12 months, a delayed hypersensitivity type of reaction was sometimes induced with weight loss, ruffled fur, blepharitis and hunched position, loss of hair, degenerative skin changes and some general resemblance to the younger runts. Histologically there was hepatitis with round cell infiltration, splenic hyperplasia and haemosiderin deposition, suppurative pyelonephritis and the presence of some amyloid and S.L.E.-like renal lesions. To what extent these lesions are similar to those
of the NZB mice and NZB/BL X NZW hybrid mice will be of major interest, as will attempts to find a possible viral aetiologica.l agent in these mice.

D. OBSERVATIONS AND GENERAL CONCLUSIONS

The enthusiasm of the claim by Helyer and Howie (1963a) that the NZB/BL X NZW hybrid mice they were describing would prove invaluable in research into the nature and therapy of systemic lupus erythematosus was matched by that with which Burnet (1963) predicted the autoimmune, genetic and aetiologic problems that may be solved by future study of the NZB/BL mice. Against the background information discussed in this chapter and the considerations of the previous limited and unsatisfactory experimental models it will be realised that the claims were realistic ones. Indeed Miles (1963), after stating that the outstanding problem in the presumed autoimmune disorders is to find the reasons for the body's evident response to its own antigens, adds "the solution of the problem in man may be greatly advanced by the analysis in the mouse of the syndromes which, prima facie, appear to be analogous to the human disease."

The present study is aimed at collecting more information about these NZ strains of mice and at seeing
if the natural course can be altered by using drugs, particularly corticosteroids. Of the various factors discussed in this chapter such drugs might be expected to exert beneficial effect on autoimmune processes by a depressant action on antibody production, on antibody antigen combination or on inflammatory reactions resulting from such combinations. The possible modes of action of these drugs and the problems involved in their use will be discussed in more detail after the mice themselves have been described.
CHAPTER 3

THE STRAINS OF NEW ZEALAND INBRED MICE

A. HISTORICAL

B. NZB/BL MICE

1. Reported features of NZB/BL mice
2. Reported modifications of basic pattern of disease of NZB/BL mice.

C. NZB/BL X NZW HYBRID MICE

1. Reported features of NZB/BL X NZW hybrid mice
2. Reported modifications of natural history of NZB/BL X NZW hybrid mice

D. FURTHER REPORTED INVESTIGATIONS OF THE DISEASE PROCESSES IN NZB/BL MICE AND NZB/BL X NZW HYBRID MICE.

E. FURTHER OPINIONS ON THE NATURE OF THE DISEASE PROCESS IN NZB/BL X NZW HYBRID MICE.

F. CONCLUSION
THE STRAINS OF NEW ZEALAND INBRED MICE

A. HISTORICAL

Mr Hall of the Animal Department of the University of Otago Medical School brought some mice to New Zealand in the 1930's from the laboratories of the Imperial Cancer Research Fund, Mill Hill, London. He used these as a basis for a mixed colony which has been maintained by him ever since in his Animal Department of the University of Otago Medical School. In 1948 Bielschowsky and Bielschowsky (1956) chose a pair of mice with agouti coats from this colony with the intention of establishing an inbred colony with agouti coats. In the event, two inbred strains were established from this original pair by selecting litter mates for coat colour in the 7th generation when some brothers and sisters produced black coats (Bielschowsky et al. 1959). One strain continued developing with selection for an agouti coat and it was noted that there was obesity in this strain. After 10 generations of brother-sister mating the coat colour was uniform and Bielschowsky and Bielschowsky (1956) then bred for the characteristic of obesity, giving their interesting NZO strain showing hereditary obesity.

The other strain had a black coat (NZB/BL) Fig.1 and this strain, after 11 generations of brother-sister mating, were seen to die with jaundice and hepatosplenomegaly.
FIG. 1. NZB/BL mouse.

FIG. 2. NZW mouse.

FIG. 3. NZB/BL X NZW hybrid.
which was later characterised by Bielschowsky, Helyer and Howie (1959) as haemolytic in type. After 20 generations some of this strain reverted to the care of Mr Hall in 1953. They were maintained as a closed colony by brother-sister mating. It is of some interest that the strain which has now passed its 50th generation has been maintained as two separate colonies since the 20th generation - one in the Animal Department and one in the Cancer Research Department - each colony having apparently identical haematological features. This favours a genetic background rather than any extrinsic cause of the syndrome seen.

Other inbred strains have been developed from the original Mill Hill stock and two are of interest to us. Both have also been established by brother-sister mating for coat colour. The first, established by Dr. Marianne Bielschowsky, is the NZY (piebald) strain which sometimes get ovarian and pituitary tumours. The NZW (white) strain, Fig. 2, was established in 1954 by Mr Hall and is now past 30 generations. It is to be stressed that both of these strains do derive from the original stock and must have some genetic relationship to the NZB/BL strain, albeit distant now.

By crossbreeding the NZB/BL with the NZY/BL strain Helyer and Howie (1961) produced the NZB/BL X NZY/BL
hybrid in which they reported a 25% incidence of positive L.E. tests in animals all of whom developed renal lesions suggestive of systemic lupus erythematosus.

Later, by crossbreeding the NZB/BL with the NZW strain Helyer and Howie (1963a) developed the NZB/BL X NZW = NZBW hybrid, Fig. 3, in which they reported a 100% incidence of positive L.E. tests by the time of death and a more acute form of renal disease, causing renal failure in 8-10 months, compared to 18-24 months in the previously reported NZB/BL X NZY/BL hybrid. It is stressed that these are hybrids, not inbred strains.

B. NZB/BL MICE

Information concerning the NZB/BL strain has been expanded by Helyer and Howie (1963b) and by Burnet and his co-workers (Holmes et al. 1961; Burnet, 1962a, b, c; Holmes and Burnet, 1963). The following is a summary outline of the main haematological and morbid anatomical features which have so far been reported by these two groups of workers in the NZB/BL (= NZB) strain.

1. Reported features of NZB/BL mice

NZB mice show no early clinical sign of anaemia. After 9-12 months they tend to become hunched with narrowed palpebral fissures, partly lose their fur and may become jaundiced later. Hepatosplenomegaly can be
detected. They develop a positive Coombs test usually between the ages of 4-8 months and free circulating antibody becomes detectable between 3 and 15 months. At first their anaemia is usually mild with haematocrit about 40% but this tends to decrease slowly. The reticulocyte count may be very high and the red blood cells show variable degrees of anisocytosis and polychromasia. They occasionally give positive L.E. cell tests. Burnet (1963b) quotes Norrins as finding 30-40% positive antinuclear factor tests in old mice of the strain.

The spleen may be considerably enlarged, contains an excess of haemosiderin and of erythropoietic tissue and may also show an increase in lymphoid tissue with plasma cells and Russell bodies. The liver is enlarged, often showing areas of necrosis in the older animals. Haemosiderin is plentiful but extramedullary erythropoiesis not so common. The gallbladder may contain pigment stones. The bone marrow is often hyperplastic.

The serological findings appear to clearly establish the autoimmune nature of the haemolytic anaemia. As stated, the Coombs test becomes positive between the age of 4-8 months. Free circulating antibody is present by the ficin technique and may reach titres of 1/2000. It can also be demonstrated by the albumin technique, but at lower titres. The antibody can be eluted from the red
blood cells and the eluted antibody and the free circulating antibody have the same serological characteristics. A weak complete antibody can also be demonstrated in many older animals. This sometimes causes spontaneous agglutination. The features are very like those in the human disease counterpart.

Lymphadenopathy, sometimes marked, is related to numerous plasma cells in the medullary cords and to myeloid metaplasia. Sometimes haemosiderin and erythrocytes are present in phagocytes. Lymphoid infiltrates in many organs, especially kidneys and lungs have been noted.

**Renal lesions** seen have been endothelial proliferation, hyaline thickening of the basement membrane, sometimes with a wire looping effect, sometimes with fibrinoid necrosis in the tuft. The latter results have had some resemblance to amyloid deposits but not the staining reaction of amyloid. Some tubules contain haemosiderin and in others casts have been noted and marked tubular dilatation. Fibrinoid has also been seen in small renal vessels and the striking perivascular infiltrates with round cells have already been mentioned.

Burnet has described **thymic lesions** which he sees in 95% of NZB mice over 8 months of age with proliferative appearances in the expanded medulla giving the appearance
of germinal centres similar to those often seen in the spleen and lymph nodes of these mice.

2. **Modifications of basic patterns of disease of NZB/BL mice.**

Various modifications of the course of the disease have been achieved. Thus Helyer and Howie (1963b) found that splenectomy in older more affected mice was rapidly fatal with profound anaemia. However in young mice before the onset of overt disease the severity of this was damped down although there was no definite effect on longevity. They were also able to induce temporary improvement in clinical state, anaemia, splenomegaly and an accompanying fall in antibody levels in animals treated with adrenocorticotrophic hormone (ACTH). Perhaps their observation that spleen weights were least in their female breeding animals is due to a corticosteroid effect during pregnancy.

Holmes et al., (1961) by transfer of spleen cells from older affected to younger not yet affected NZB animals were able to induce positive Coombs tests earlier in these young animals, some of the tests becoming positive in a week and persisting. They interpret their findings as showing that forbidden clones of cells develop with age, i.e. the donor spleen cells, and can be demonstrated by passage. This could mean either that
there is a general weakness of immunological homeostasis allowing clones to flourish that in the normal animal would be eliminated, or that the self destructive clones that arise are intrinsically more difficult to control. While these authors may well be correct in stating that they have transferred living clones from the older animals, an alternative explanation of their results would be that the dose of 'same as self' antigen they have given to the younger animals has broken down their genetically weak immunological mechanisms somewhat earlier than usual. They produce evidence against this interpretation. The extensive use that Burnet has made of the findings in these mice, including the thymic findings, in formulating some of his concepts of autoimmunity and in extending the clonal theory has already been indicated.

The effects of thymectomy on the NZB/BL strain seem to have provided the only point of real difference in the observations of the Australian and New Zealand workers. Burnet considers that thymectomy delays the onset of the disease, whereas Helyer and Howie state that few NZB/BL animals survive wasting disease from thymectomy long enough to develop positive tests.

Bielschowsky and Bielschowsky (1962) by painting NZB/BL mice with the carcinogenic agent aminofluorene
induced transplantable lymphoid tumours in this low incidence tumour strain. They conclude that the lymphoid tissues of this strain are abnormally susceptible to carcinogenic agents, which may reflect some instability in homeostatic mechanisms.

C. NZB/BL X NZW HYBRID MICE

1. Reported features of NZB/BL X NZW hybrids

Helyer and Howie (1963a) described the NZB/BL X NZW hybrid mice as having a 100% incidence of positive L.E. cell tests and as dying of renal failure when 8–10 months of age. Their kidneys may be enlarged and dusky pink, or contracted, pale and granular. They showed hypercellularity and thickening of capillary walls of the glomeruli with wire loop formation. Fibrinoid necrosis involving the basement membrane and cellular elements of the glomerulus were sometimes noted and hyaline thrombi and occasional haematoxylin bodies were seen. Tubular damage with hyaline cast formation was noted. Fibrinoid necrosis was sometimes also seen in the small renal vessels and these vessels were always surrounded with striking lymphoid deposits. The fibrinoid vascular lesions were also found in the spleen, lymph nodes, stomach, thyroid and myocardium. They considered these renal lesions were more like those of the nephritis of systemic lupus erythematosus than were those of the NZB mouse.
2. Reported modifications of natural history of NZB/BL × NZW hybrid mice

Helyer and Howie (1963c) found that this hybrid withstood neonatal thymectomy satisfactorily but developed their renal failure more rapidly, often by 20 weeks, with histological features of an active lupus nephritis. An extension of this work was that neonatal transfer of a normal thymus to this strain did not prevent the development of the lupus nephritis.

D. FURTHER REPORTED INVESTIGATIONS OF THE DISEASE PROCESSES IN BOTH NZB/BL AND NZB/BL × NZW HYBRID MICE

Helyer and Howie (1963a) transferred at birth neonatal thymuses from NZB/BL mice and NZBW hybrids into mice of a completely unrelated strain (CBA/T6) which had been neonatally thymectomised. The CBA/T6 mice developed a glomerulitis, showing endothelial proliferation, some basement membrane thickening and fibrinoid change. In addition they became uraemic and had positive L.E.cell tests, positive Latex tests, positive Coombs tests and positive ficin tests. They seemed, then, to have developed an S.L.E. type of illness. Helyer and Howie point out that this could have been related to transfer of thymic cells which populated or modified the host's CBA/T6 lymphoid tissue or modified it in a humoral manner.
An alternative explanation would be that some aetiological viral factor had been transferred. A further explanation could be that the lesions and serological abnormalities in the CBA/T6 animals represented some form of runt disease, although the plan of the experiment is such as to minimise the chances of a host versus graft reaction by using neonatally thymectomised mice and the chances of a graft versus host reaction are minimised by using neonatal thymus as donor material.

Extensive breeding experiments have been carried out with these strains to try to clarify the genetics of the situation. Howie (1964) notes that seven separate groups of hybrids have been bred from the NZB/BL strain by mating with other inbred strains and have developed evidence of autoimmune disease, the nature of the disease depending on the non-autoimmune parent. No evidence of sex linkage or milk factor transmission was found. The age of onset varied considerably and there were differences between the sexes. In two of the hybrids autoimmune disease has been found in the F2 generation, suggesting that the tendency to get the disease is inherited as a dominant factor. Steps are being taken to elucidate any relationship of transplanted viral infection in the strain to the disorders.

Burnet (1962a) has also commented on the genetic
background involved and says that his original idea that
the disease in the NZB mice was related to a single
recessive gene had become untenable and that to explain
some of his experiences with crossbreeding and back-
crossing the mice he considered that some modifier genes
must be present in the NZB animals.

E. FURTHER OPINIONS ON THE NATURE OF THE DISEASE PROCESS
IN THE NZB/BL X NZW HYBRID MICE

Lupus nephritis or not?

It is of importance to indicate here that some of
the main authors on S.L.E. in recent years, namely
Muehrcke, Kark, Pirani and Pollak (1957) and Pollak et al.
(1961a), have seen sections of the kidneys from hybrid
NZB/BL X NZW animals. Associate Professor J.B. Howie,
visiting America, has shown histological sections to
Drs. V.E. Pollak, C.L. Pirani and R.M. Kark, while
Dr. V.E. Pollak, visiting New Zealand, has seen further
material in the Pathology Research Department from
Associate Professor J.B. Howie and Dr. S.J. Helyer. The
American workers agree that the lesions in the mice are
remarkably similar to those in their human cases of S.L.E.
and accept them as an example of lupus nephritis. It
is of interest to note that they have been provided with
the nucleus of a colony of their own, by courtesy of the
New Zealand workers and Mr Hall of the Animal Department.
CONCLUSION

The inbred NZB/BL mice described by Bielschowsky, Helyer and Howie (1959) and the NZB/BL X NZW hybrid mice originally described by Helyer and Howie (1963a) present many features of autoimmune disorder which are being extensively explored both in New Zealand and Australia. It should be noted that mice have now been sent to many parts of the world by Mr Hall, Associate Professor Howie and Dr Helyer. Considerable clinical and clinicopathological and morbid anatomical information has been reported concerning the NZB/BL strain.

It was with a background of the above information and with the help of the facilities and practical knowledge available from Associate Professor Howie and Dr Helyer that the main basis of this thesis was aimed to collect more information on the times at which the various lesions arose in the hybrid NZB/BL X NZW animals, on their rate of progress and whether this could be predicted from a consideration of laboratory findings other than postmortem ones. Also whether the lesions or the clinicopathological test could be modified by therapy.

It also seemed desirable to make some observations on the NZW strain itself, about which nothing has been published to date.
Some studies were made on the NZB/BL animals in the direction of therapeutic modification of their disease by corticosteroids and by antimetabolites.

In addition, the opportunity was taken of studying the thymuses to see if lesions similar to those reported by Burnet could be seen in the NZB/BL animals and, in particular, whether any thymic abnormalities occurred in the NZB/BL X NZW hybrid.
CHAPTER 4
CORTICOSTEROIDS IN AUTOIMMUNE DISORDERS

A. EFFECTS OF CORTICOSTEROID DRUGS
1. The anti-inflammatory effects of corticosteroids.
2. Effect of corticosteroids on antigen antibody mechanisms.
3. Summary of relevant modes of action
4. Renal effects of corticosteroids
5. Other effects of corticosteroids

B. CORTICOSTEROID DRUGS USED IN THERAPY
1. Range of corticosteroids currently available
2. Relative status and potency of clinically useful hydrocortisone-like steroids
3. Drug selected

C. CORTICOSTEROID THERAPY IN AUTOIMMUNE DISORDERS
1. Treatment of autoimmune haemolytic anaemia with corticosteroids
2. Treatment of systemic lupus erythematosus with corticosteroids
3. Proposed drug therapy in lupus nephritis in hybrid mice

D. CONCLUSION
CORTICOSTEROIDS IN AUTOIMMUNE DISORDERS

Hench et al. (1949) first used corticosteroids and adrenocorticotrophin to treat patients with rheumatoid arthritis. They clearly showed that the effect was a suppressive one and they introduced the idea of a high initial dosage fairly rapidly reduced. They showed the rapidity of suppression of symptoms, signs and evidence of inflammation such as temperature and sedimentation rate; equally clearly they showed the rapidity of relapse after cessation of short treatment. They speculated on other arthritides, including that of disseminated lupus erythematosus having a relationship to rheumatoid arthritis and considered that other conditions might benefit from the hydroxycorticosteroids when more of these were available. Although in relatively few cases of rheumatoid arthritis is corticosteroid therapy considered indicated today this therapy is often indicated in other diseases, of particular interest to us being the management of acquired haemolytic anaemia (Ed. Lancet, 1962a) and of systemic lupus erythematosus (M.R.C.Report, 1961). In this chapter it is proposed to review the effects of corticosteroid drugs, to discuss the different drugs available for use and, finally, to consider the use of such drugs in autoimmune disorders.
A.  **EFFECTS OF CORTICOSTEROID DRUGS**

These are variously classified but they can be summarized under three headings, modified from Cope (1961): (i) electrolyte effects, the tendency to sodium retention and to enhanced potassium loss, these being quite independent of any anti-inflammatory or antirheumatic action. Corticosteroids having this effect predominantly are often referred to as mineralo-corticoids. (ii) androgenic effects. (iii) the hydrocortisone effects, also called glucocorticoid effects. Of these the most interesting to us is the anti-inflammatory action.

Much of the experimental work on the effects of corticosteroids has been performed on mice. A review of these effects, particularly on responses seen in general pathology is relevant to the proposal to use these drugs in autoimmune disorders in this species.

1. **The anti-inflammatory effects of corticosteroids**

The ability of animals to respond to stress is considerably modified by an excess or deficit of adreno-corticosteroid hormones (Thorn et al., 1954). This is particularly so in terms of the inflammatory response, in both its local and general manifestations, and in repair.
Effect on repair

Spain et al. (1950) showed that in wounds of mice treated with cortisone there was a lack of exudate and of fibrin, cellular elements were diminished, there was little new capillary formation and sparse fibroblastic proliferation. Epithelialisation was slightly delayed. They also showed that the phagocytic activity of the reticuloendothelial system can be markedly diminished by cortisone.

Effects on phagocytosis

Benacerraf et al. (1954) showed that this was not so much an inhibition of phagocytosis in the first place as a marked inhibition of the return of normal phagocytic activity, a similar result to that obtained by blockade with India ink preparations. They interpreted this as due to an inhibition of cell proliferation by the cortisone they had used, as they obtained an identical effect with a cytotoxic nitrogen mustard preparation. Robinson (1956) showed in cortisone treated rabbits injected with pneumococci that even though these bacteria were phagocytosed by cells of the reticuloendothelial system, they were not destroyed.

Effects on fibrosis

Magarey and Gough (1952) showed that the cellular response and the fibrosis that usually followed the intra-
peritoneal injection of quartz into mice was considerably reduced by treatment with cortisone. Such fibrosis could be prevented, but once it had occurred it was not reduced by cortisone (Curran, 1952).

**Effects in inflammatory responses**

Dougherty (1952), using histamine as an irritant in adrenalectomised mice, found that the intensity of the inflammatory response was inversely proportional to the local concentration of corticosteroid. Larger than normal amounts of corticosteroid inhibited the proliferation of fibroblasts and the invasion of phagocytic cells into the area. Duke-Elder and Ashton (1951) drew attention to the effects of cortisone in reducing capillary permeability where this is increased by inflammation but not affecting normal capillary permeability. This effect was elucidated in detail by Ebert and Wisler (1951) who showed in the ear chamber technique that capillary and arteriolar tone is increased by cortisone, endothelial swelling is reduced, capillary permeability to plasma proteins is decreased and the cellular exudate modified.

**Effect in connective tissue cells**

That the non-phagocytic cells of the connective tissue such as the fibroblast are affected by cortisone has been noted and Asboe-Hansen (1952) who, besides referring to the fact that fibroblasts actually become
smaller and more pyknotic, shows that there is a decrease in numbers and an alteration in granularity of mast cells in mice treated with large doses of cortisone. He thinks this suggestive of a decrease in production of hyaluronic acid. Jennings and Florey (1954) review the strong evidence that cortisone inhibits the synthesis of sulphate-containing mesenchymal mucins in various tissues, although they show that it does not have this effect on epithelial mucins in the colon of the mouse.

**Adverse effect in infection**

These aspects of modification of the inflammatory response by corticosteroids are probably largely responsible for the detrimental effect of these drugs on the resistance of animals to infections. Tuberculosis (Hart and Rees, 1950), enterococcal (Gledhill and Rees, 1952), brucella abortus (Haskins et al. 1959), corynebacterial pseudotuberculous (Antapol, 1950), pneumococcal and influenzal viral (Kass et al., 1954), poliomyelitis viral (Shwartzman et al. 1950) and coxsackie viral (Kilbourne and Horsfall, 1951) are but a few of many examples of infections in mice that are markedly enhanced in animals receiving corticosteroid therapy.

There are a few experimental infections where cortisone exerts a beneficial effect in mice, such as those due to histoplasma (Baum et al. 1954) and Plasmodium
berghei (Singer, 1954). That modifications of the local inflammatory response may be beneficial when combined with appropriate antibiotics is well established in clinical medicine, for example in tuberculosis (Ed. Brit. Med.J., 1960c, Ed. Lancet, 1962c). The rapid relief of severe systemic symptoms of fever or toxaemia is also useful in a number of infections (Ed. Lancet, 1960a).

With these reservations it must be remembered that the effect on resistance to infection is more usually disastrous to the mouse, as shown by at least a thousandfold increase in susceptibility of cortisone treated mice to brucella abortus (Haskins et al. 1959) and the conversion of adult mice into susceptible hosts for coxsackie virus (Kilbourne and Horsfall, 1951). These authors were prompted to comment that "it is a matter of great interest that the administration of a chemically defined substance may convert a state of apparent complete insusceptibility to one of marked susceptibility to a viral infection".

**Beneficial effect in inflammatory conditions**

Apart from this generally harmful effect to the animal body in the local inflammatory response to infections, inflammation due to unknown and to allergic causes is generally modified in a beneficial manner (Cope, 1959) and this fact obviously forms the basis of the widespread use of these drugs.
2. **Effect of corticosteroids on antigen antibody mechanisms**

Studies have been made on modifications by corticosteroids of the various types of antigen antibody reaction, of circulating antibody levels and of the antibody forming tissues in an attempt to elucidate their mode of action and of benefit.

**Lympholytic and thymolytic effects**

White and Dougherty (1944) described the striking degenerative changes due to adrenal cortical hormones that occurred in mice in the lymphocytes of lymph nodes, spleen, thymic cortex and Peyer's patches. They noted depletion and oedema of lymphoid tissue with pyknosis and fragmentation of lymphocytes. A slight rise of serum proteins was considered by them to be due to antibody release from lymphoid cells into the circulation and they suggested this as an explanation of the anamnestic reaction (Dougherty et al. 1944 and 1945). These latter observations on serum proteins have not been confirmed by others. However, these authors drew attention to the lymphopenia that follows the use of these hormones and attributed it to a failure of delivery of lymphocytes into the circulation (Dougherty and White, 1944). Dougherty (1952) showed that anaphylaxis and the Arthus phenomenon in mice could be considerably modified by systemic use of cortisone; also
that the amount of hormone needed to suppress antibody formation was far greater than that needed to diminish allergic inflammation. His experiments showed that the corticosteroids did not influence the reactions of hypersensitivity solely by an effect on antigen-antibody mechanisms but also, if not largely, by their anti-inflammatory action.

**Effects on hypersensitivity reactions, immediate or delayed**

Although there are species differences, in mice as stated above, the Arthus phenomenon (Germuth and Ottinger, 1950), and anaphylaxis (Nelson et al. 1950) can be modified by cortisone. Other evidence for a modification of antibody reaction is that of modification of the delayed type of bacterial hypersensitivity reaction (Long and Favour, 1950), the prolongation of life of second set skin homografts (Krohn, 1954) and the protective effect in experimental allergic encephalomyelitis (Field and Miller, 1962), reactions which probably mainly have a cellular mechanism.

**Effects on circulating antibody levels and formation of antibodies**

Fischel et al. (1951) showed that cortisone administration impaired the rise in titre of circulating antibodies but not the rate at which administered antibody
disappeared. White (1963) points out that the biological half life of homologous antibody passively transferred is not shortened by cortisone, which indicates its main effect on antibodies is on their production. These same authors, Bjorneboe et al. (1951) also drew attention to the fact that clinically in several of the diseases, including S.L.E., associated with hyperglobulinaemia, a fall in level of serum globulin was usually noted with cortisone therapy, rather than the rise White and Dougherty (1944) had noted; also Fischel (1950) had shown that with the lymphopenia associated with this therapy in animals there may be a drop in antibody level, as measured by using simple antigens and immunochemical estimation of the corresponding antibody levels. Bjorneboe, Fischel and Stoerk (1951) considered that there was actual destruction of circulating antibody in steroid treated animals but in view of the observation of White (1963) mentioned above, this seems doubtful. Berglund (1956a) showed a lower level was particularly so if cortisone was administered for some days before the provocative antigen and (Berglund 1956b) that there was a dosage effect in the ability of cortisone to cause a reduction of antibody levels when continued after the stimulating antigen. The overall opinion (Ed. Lancet 1960a) is, then, of an impairment of antibody synthesis in cortisone treated animals.
Mode of action of cortisone on antibody forming tissues

The mechanism by which the antibody forming tissues are affected has generally been considered an antimitotic one, although Heilman and Kendall (1944) thought that the effect of cortisone on a type of spontaneous lymphosarcoma they were studying in mice was due to its catabolic effect to a degree which resulted in the death of malignant lymphoid cells. They realised this was not a universal phenomenon with tumours, mainly applying to certain lymphoid tumours, though also observed for rhabdomyosarcoma in C3H mice by Higgins et al. (1950). Indeed Foley (1952) showed that strain specific tumours could be transferred to alien mice given large doses of cortisone. Presumably this is due to breakdown of the host's immunological defences.

Other actions of large doses of steroids that have been interpreted as cytotoxic and causing degeneration are the production of foetal abnormalities, such as cleft palate, in pregnant mice (Fraser and Fainstat, 1951) and the recent reports of cataracts in patients on large doses of corticosteroids for a prolonged time (Black et al. 1960). Inhibition of cell division has been noted also in mouse epidermal cells (Green and Ghadially, 1951; Bullough, 1952); hepatic cells (Roberts et al. 1952);
fibroblasts in tissue cultures (Holden et al. 1951) and endothelial cells (Ashton and Cook, 1951).

**Effects on myeloid tissue**

Quittner et al. (1951) point out that bone marrow cell proliferation in the mouse is not inhibited by large doses of cortisone. Although they observed the well known effects in the peripheral blood of marked lymphopenia, marked eosinopenia and a slight reduction in polymorphonuclear leucocytes, marrow smears showed an active marrow with an increase in myeloid-erythroid ratio. This was due to an absolute increase in the number of myeloid cells which they suggested could be due to an inhibition of release into the circulation. It is of considerable interest that Weir and Brenner (1959) reported permanent granulocytosis, like leukaemia, in some mice treated with large prolonged doses of steroids, particularly when the therapy is intermittent. A reversible but protracted granulocytosis has also been noted in some children treated with prednisone in large doses (Harris and Vandergrift, 1960). Rosenthal et al. (1950) concluded that eosinophils undergo increased peripheral destruction rather than an inhibition of production in the bone marrow.

**Effects on renal lesions**

With particular reference to experimentally produced renal lesions Thorn et al. (1954) quote evidence that
cortisone will block actively induced nephrotoxic nephritis (e.g. due to horse serum) where it interferes with antibody production and probably tissue reactivity, but it will not modify the passively induced disease using kidney antiserum. However these authors believed that the clinical benefit of cortisone has little relationship to antibody inhibition, except in a few specific disorders such as acquired haemolytic anaemia. They consider the main effects are anti-inflammatory at local levels.

**Intimate mode of action of cortisone**

A cellular level of action rather than impairment of antibody synthesis probably explains the anti-inflammatory effect of cortisone, not only in infection but also in collagen disorders and hypersensitivity reactions (Ed. Lancet 1960a). There has been recent speculation on the "intimate action of steroid hormones" (Ed. Lancet 1962d and following correspondence) with the prevalent view that they are co-factors essential to enzymatic reactions both at a nuclear and cellular level. White (1963) mentions recent evidence that cortisone can stabilise the lysosomal membranes of some cells. As it is thought that antigens are manipulated by intracellular digestive enzymes, this observation may have some bearing on the modus operandi of cortisone in interfering with antibody production.
3. Summery of relevant modes of action

The consensus of opinion, then, has been that the main actions of corticosteroids are local and are modifications of the inflammatory response. Nevertheless the considerable evidence of destruction of and modification of antibody forming cells already mentioned assumes more significance in terms of the clonal theories of Burnet than in terms of older instructive theories of antigen-antibody production. The lympholytic effect of corticosteroids will tend to destroy clones of cells, including those which are producing autoantibody, or perhaps more important, the marked and long known thymolytic and lympholytic effects (Wells and Kendall, 1940a, b) may have a considerable therapeutic significance if the thymus does occupy the central role in immunological homeostasis envisaged by Burnet (1962). From the above review it is concluded that there is little evidence of any direct modification of antigen-antibody reactions by corticosteroids.

4. Renal effects of corticosteroids

While corticosteroids may and do modify autoimmune processes it must be remembered, with particular reference to hoped for benefit to renal lesions, that cortisone may cause kidney lesions. Rich et al. (1950) showed
that A.C.T.H. and cortisone prevent hypersensitivity lesions developing to horse serum in the rabbit. However, cortisone but not A.C.T.H. caused dilatation of glomerular capillary loops, local necrosis of cells of loops and the formation of large masses in the tufts "recalling the focal hyaline masses in the tufts of diabetics and some cases of D.L.E." All of their rabbits showed glycosuria. These findings have been confirmed and extended by McLean et al. (1951), Bloodworth and Hamuri (1955) and Wilens and Stumpf (1955). Moran et al. (1962), using the electron microscope, showed that the resemblance to the diabetic kidney was close but there were differences. They also showed that most of the rabbits who developed glycosuria also had moderate albuminuria. Fortunately this severe renal effect of cortisone seems peculiar to the rabbit and may be related to severe lipemia that is caused in this species (Wilens and Stumpf, 1955c). Nevertheless in any study such as that planned here, where steroids are to be used to treat or prevent renal lesions, it is important to check whether any glycosuria develops. Also to check in the final analysis whether histologically there is an ill effect on the kidneys from the steroid.

Similar considerations will apply to the use of cytotoxic antimetabolites such as 6 mercaptopurine,
reference having been made to the induction of a glomerulonephritic type of lesion by the antimetabolite aminonucleoside. It would also apply to the use of amphotericin B, which often causes renal tubular damage, interstitial tissue damage and calcification (Wertlake et al. 1963).

5. Other effects of corticosteroids

There are many of these (Cope, 1961) most of which can be regarded as side effects. The most important are the glycogenic, the catabolic or more correctly, anti-anabolic effects, a tendency for fat deposition, the general effects such as euphoria or mental change, and the inhibition of the patients own adrenals. This last has been shown to be due to inhibition of corticotrophin production rather than a direct effect on the adrenal gland (Ferriman and Page, 1960 and Treadwell et al. 1963).

In a study involving long term use of corticosteroids it is necessary to consider the dangers and side effects of such therapy. This is done in the experimental part of the thesis. Nearly all patients receiving 75-100 mg. of cortisone per day will develop some side effect or other (Spence, 1958). If they already have diabetes or infection, whether obvious or latent, these may be aggravated, as may hypertension, congestive heart failure, osteoporosis, peptic ulcer or a psychosis. The dosage
required for precipitation of side effects will vary in the individual and there is also a variation with sex, females being on the whole more prone to side effects (Fernandez-Herlihy, 1960), especially when postmenopausal (Ed. Brit. Med. J., 1957).

Apart from factors such as these variations in the exact corticosteroid used will influence the type of side effect. The considerations leading to the choice of a drug suitable for the experimental investigation of mice are given in the chapter on the major experimental procedures employed.

B. CORTICOSTEROID DRUGS USED IN THERAPY

1. Range of corticosteroids currently available

Structural formulae of these are shown in the accompanying figure, Fig. 4. Hydrocortisone and cortisone are considered interchangeable within the body. Because of their salt retaining properties, large doses over long periods are not practicable. The introduction of the extra double bond into ring A of these two steroids to give prednisolone and prednisone causes an increase in potency which is accompanied by a reduction in effect on salt and water metabolism and is a desirable modification where the therapeutic use is to obtain maximal "anti-inflammatory" action as in the type of study proposed here.
FIG. 4. Structural formulae of corticosteroids (modified from Lederle brochure on triamcinolone, 'Ledercort').

FIG. 5. Shows relative effects and dosage of main corticosteroids (slightly modified from Cope, 1959).

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Glycogen Deposition</th>
<th>Electrolyte Excretion</th>
<th>Eosinopenia</th>
<th>Anti-rheumatic</th>
<th>Granuloma Inhibition</th>
<th>Relative Dosage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrocortisone (F)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Cortisone (E)</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>1</td>
<td>130</td>
</tr>
<tr>
<td>Prednisolone ((\Delta) F)</td>
<td>4</td>
<td>1</td>
<td>3</td>
<td>4</td>
<td>20</td>
<td>25</td>
</tr>
<tr>
<td>Prednisone ((\Delta) (\varepsilon))</td>
<td>3</td>
<td>1</td>
<td>3</td>
<td>3</td>
<td>16</td>
<td>30</td>
</tr>
<tr>
<td>Aldosterone</td>
<td>0.3</td>
<td>50</td>
<td>–</td>
<td>0</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td>9(\alpha)-Fluorohydrocortisone (9(\alpha)-FF)</td>
<td>20</td>
<td>50</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>5</td>
</tr>
<tr>
<td>(\Delta)(\alpha)-9(\alpha)-Fluorohydrocortisone</td>
<td>20</td>
<td>50</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>3</td>
</tr>
<tr>
<td>2-Methyl-9(\alpha)-fluorohydrocortisone</td>
<td>10</td>
<td>200</td>
<td>2</td>
<td>–</td>
<td>40</td>
<td>–</td>
</tr>
<tr>
<td>Methylprednisolone</td>
<td>10</td>
<td>–</td>
<td>3</td>
<td>4</td>
<td>–</td>
<td>20</td>
</tr>
<tr>
<td>Triamcinolone ((\Delta)(\alpha)-9(\alpha)-fluoro-16-hydroxy-F)</td>
<td>30</td>
<td>Nil</td>
<td>3</td>
<td>4</td>
<td>10</td>
<td>25</td>
</tr>
<tr>
<td>Dexamethasone ((\Delta)(\alpha)-9(\alpha)-fluoro-16a-methyl-F)</td>
<td>17</td>
<td>“</td>
<td>15</td>
<td>28</td>
<td>190</td>
<td>4</td>
</tr>
<tr>
<td>Betamethasone ((\Delta)(\alpha)-9(\alpha)-fluoro-16(\beta)-methyl-F)</td>
<td>“</td>
<td>“</td>
<td>30</td>
<td>“</td>
<td>“</td>
<td>&lt;4</td>
</tr>
</tbody>
</table>
The introduction of a fluorine radical at C9 increases potency more. Further addition of a hydroxyl group at Cl6 (-OH) gives triamcinolone with almost complete elimination of the undesirable sodium retaining effects (Ed. Brit. Med. J., 1959a). In fact the suggestion in this editorial is that there is a naturally-occurring adrenal steroid with such a Cl6 hydroxyl group which has a water and salt losing effect, contrasted with the reverse effects of aldosterone.

If instead of a Cl6 hydroxyl group a Cl6 methyl (CH₃) group is added to 9 fluoroprednisolone, an even more potent anti-inflammatory effect is obtained. If the methyl group is in the delta position with respect to Cl6 the drug is dexamethasone, if in the beta position, betamethasone, the corticosteroid selected for this study.

Any of the corticosteroids mentioned can be prepared free, as an alcohol or esterified. If the ester is a phosphate or hemisuccinate the solubility, especially in water, is markedly increased. Latham and Mason (1962) point out that all the corticosteroids readily available for oral use prior to that date had been relatively insoluble. It is this fact which raises difficulties in the long term oral use of a corticosteroid drug in experimental animals.
2. **Relative status and potency of clinically useful hydrocortisone-like steroids**

A table of the potency of these drugs slightly modified from Cope (1959) shows in a relative way the dosages that are effective in man (Fig.5).

Church (1962) regarded betamethasone phosphate as equivalent to 1.25 mg. of betamethasone alcohol. Betamethasone alcohol, 1 mg. is equivalent in anti-inflammatory action to 8 mg. prednisolone and 37 mg. of cortisone according to Glyn and Fox (1961). This figure of an eightfold increase of potency over prednisone is also given in Todays Drugs (1961). In terms of betamethasone phosphate this would equal a tenfold difference. Wilkinson (1961) also states there is a tenfold difference between betamethasone and prednisolone. Toogood (1962) considered betamethasone to be 10-15 times more active on a weight for weight basis than prednisone when used in treating asthmatic patients.

Of dexamethasone, Cope (1959) commented that it offered the best clinical prospects in that it had very high antirheumatic activity with a negligible sodium retaining effect. However it has become more usual (Cope, 1961) to stress the fact that increased potency is of little importance unless there is a dissociation of undesirable glucocorticoid side effects from anti-
inflammatory action. On these grounds triamcinolone has lost favour as it causes weight loss, potassium loss and an unusual type of myopathy (Dubois, 1958), this latter being noted rarely in dexamethasone and prednisolone treated patients (Golding and Begg, 1960). Dubois (1960) found dexamethasone less predictable from individual to individual and more prone to cause Cushingoid features than prednisteroids.

Betamethasone may fulfill the prediction of Cope (1959) for dexamethasone. Betamethasone is accepted as being marginally more potent than dexamethasone in its anti-inflammatory action (Glyn and Fox, 1961; Church, 1962) and as having less effect on salt retention than the prednisteroids (Today's Drugs, 1961). Church (1962), using an oral solution of the ester, betamethasone phosphate, found it causes less dyspepsia and less ulcer aggravation than previous corticosteroids, it being considered that the increased solubility and ease of absorption avoided high local concentrations of corticosteroid in the gastric mucosa. Wilkinson (1961) had also felt that there was less dyspepsia with this drug than its predecessors.

Much of the differences in opinion re efficiency compared to side effects relate to different assessments of comparable dosage levels. Bailey et al. (1961) now
attribute much of the reported differences as due to variations in solubility. They show higher levels of triamcinolone absorbed from the gut and excreted in the urine than of oral prednisolone (and they infer of cortisone and dexamethasone). They show this difference is avoided if soluble prednisolone phosphate is injected. Oral use of more soluble drugs such as the phosphates should avoid this difficulty of absorption.

3. Drug selected

Betamethasone phosphate has the greatest anti-inflammatory effect of the steroids already described. In addition, being synthesised from hecogenin obtained from the sisal plant waste (Ed. Lancet, 1962b) it is cheap (Today's Drugs, 1961). It is highly soluble in water (Church, 1962) as a pleasant practically tasteless solution (personal observation), properties which lend themselves to ease of oral administration to mice over long periods of time. It therefore was selected and its detailed mode of handling is discussed later.

C. CORTICOSTEROID THERAPY IN AUTOIMMUNE DISORDERS

1. Treatment of autoimmune haemolytic anaemia with corticosteroids

There is a striking unanimity of opinion that the main treatment of autoimmune haemolytic anaemia is
corticosteroid therapy (Rose, 1954; Dacie, 1960b and 1962; Wintrobe, 1961; Ed. Lancet 1962a; Britton, 1963; Mackay and Burnet, 1963). While there are some differences of opinion as to the best preparation, the most used drugs are the prednisteroids which must initially be used in high doses, the Ed. Lancet (1962a) stating that the smallest effective initial dosage for an adult is 60 mg. daily of prednisolone. Rose (1954) and Wintrobe (1961) stress that dosage must be judged by the amount necessary to produce the desired result, the latter stressing that very high dosage being necessary on occasions initially. The desired result is to raise the haemoglobin to 10-12 g/100 ml. and a falling reticulocyte count after an initial rise sometimes, Dacie (1960b) points out that it is seldom possible to bring about clinical cure and that one should be content with the haemoglobin level mentioned, reducing the steroid dosage as rapidly as possible to a minimum level which will maintain this balance between blood destruction and formation. Dacie (1962) and Wintrobe (1961) note that although the direct antiglobulin test may become less positive with the steroid therapy, it does not usually become negative. All of these authors agree that therapy may have to be continued over months, or even years, which may raise considerable problems with
steroid complications. Wintrobe (1961) states that 70-90% of cases respond to corticosteroid therapy. Mackay and Burnet (1963) feel that any condition with a substantial autoimmune basis will show some improvement with the steroid dosage mentioned above. While agreeing that long term treatment has to be undertaken in some cases, and mentioning it lasting over five years, these authors are obviously reluctant to embark on therapy for over three months because of the side effects.

2. Treatment of systemic lupus erythematosus with corticosteroids

The Medical Research Council Report (1961) on the treatment of systemic lupus erythematosus ends with the observation that at that time, in the absence of a superior therapeutic agent, it seems imperative that patients ill with systemic lupus erythematosus should be treated vigourously with steroid hormones. It was already generally accepted that corticosteroids relieved the symptoms and lessened the lesions of the disease (for example Baehr and Levitt, 1954; Beck, 1955; Hill, 1957; Snyder, 1960 and Larson, 1960) but all of these authors believe that when renal lesions are present they do not benefit and, indeed, continue to progress while the patient is on therapy. The Medical Research Council Report is not so sure of this. However, Dubois (1956 and
1960); Holman (1960) and particularly Pollak et al. (1959 and 1961a and b) were convinced that this was a matter of dosage.

These latter authors showed that if there was renal involvement large doses of steroids must be given from the beginning of treatment and continued for at least six months. When this was done using an average of 47.5 mg. of prednisone per day (approximately 200 mg. of cortisone) they found that 9/16 patients with lupus glomerulonephritis were still surviving to an average of 34 months at the time of their report, whereas of a previous but otherwise similar group of ten patients treated with an average of 50 mg. of cortisone per day there were no survivors, the average age of death being 13.8 months. Theirs is the first really convincing evidence of real benefit, with biopsies to prove it, in the glomerulonephritic cases of S.L.E. They introduce a new concept into steroid therapy. All of the previous authors cited and the Ed. Lancet 1960b, although prepared to use any initial dose - e.g. 4000 mg. daily of cortisone - to control a crisis, were anxious to reduce the dose as soon as possible, as is the correct approach in autoimmune haemolytic anaemias, as discussed in the previous section and, indeed, in any other form of suppressive therapy. The clinical state and improvement in anaemia, leucopenia,
thrombocytopenia, sedimentation rate, L.E.cell tests etc. were used as guides to enable the lowest possible maintenance dose to be gauged. However, Pollak et al. consider that if there is definite evidence of glomerulonephritis the dosage must not be reduced below 40 mg. prednisone/day for six months.

These authors also showed that the difference in their two groups of patients was not due to prednisone being used in the second series, rather than cortisone, because patients on lower doses of prednisone showed the same mortality as their earlier group on cortisone. Nevertheless, Larson (1961) in his extensive monograph, in view of the doubt that then existed as to the efficacy of currently used corticosteroids on renal lesions in S.L.E., suggested that newer drugs with more anti-inflammatory action might be of more definite benefit. Fernandez-Herlihy (1960), although aware of the work of Pollak et al. stated that it was not yet known whether long term treatment with corticosteroids would alter the course or the prognosis of chronic systemic lupus erythematosus.

3. Proposed drug therapy in lupus nephritis in hybrid mice

These major differences in opinion about the ability of corticosteroids to really benefit the
glomerulonephritis of patients with S.L.E. existed at the time this thesis was planned. It seemed of considerable interest to see if these drugs had any effect on the renal lesions of the hybrid mice which develop a glomerulonephritis associated with positive L.E. cell tests, which Helyer and Howie (1963a) have described as a lupus nephritis. Such treatment has not been attempted before. To do so a satisfactory drug to use over a long period was needed and the selection of betamethasone phosphate has been discussed. The dosage scheme is discussed later.

CONCLUSION

Betamethasone phosphate is a potent corticosteroid drug with marked anti-inflammatory effects and ability to depress antibody formation.

Because of its water solubility it should provide a convenient method of administering corticosteroids to mice over prolonged periods. Corticosteroid drugs form the mainstay of treatment of autoimmune haemolytic anaemia in man and induce remissions. While they are used extensively in treatment of patients with S.L.E., there is no universal agreement as to their benefit on the lupus nephritis lesions. It would be of considerable interest to test the effects of these drugs on the autoimmune
type of haemolytic anaemia seen in the NZB/BL mice and, in particular, to test whether corticosteroids modify the lupus nephritis type of lesions seen in the NZB/BL X NZW hybrid mice.
CHAPTER 5

REVIEW OF SPECIAL PROCEDURES USED IN THE THESIS

I THE LUPUS ERYTHEMATOSUS CELL PHENOMENON

II LATEX AGGLUTINATION TESTS

III THE COOMBS TEST

IV THE FICIN ANTIBODY TEST

V STATISTICAL METHODS

VI HISTOPATHOLOGICAL STAINS

VII DOSAGE OF CORTICOSTEROID THERAPY IN MICE
REVIEW OF SPECIAL PROCEDURES USED IN THE THESIS

A number of special tests, both cytological and serological, have been used as indicators of disease processes in this thesis. It is proposed in this chapter to consider their historical background, as well as their significance and some of the difficulties which can arise in their evaluation. This applies to the L.E.cell test, the latex nucleoprotein test, the Coombs test and the ficin antibody test, and unless their inclusion is relevant to the reviews, details of the techniques are given in the Appendix.

In addition, the general principles involved in the statistical analyses used are discussed. The interpretation of the histopathological stains used is also discussed, but the methods used are detailed in the Appendix. Finally, an assessment is made of a probably suitable dosage of corticosteroid for long term use in the mice, based on reported trials.
I. THE LUPUS ERYTHEMATOSUS CELL PHENOMENON

Review of L.E. cell phenomenon

The description by Hargraves et al. (1948) of L.E. cells in bone marrow preparations from patients with disseminated lupus erythematosus has proved to be a very useful contribution to medicine. It was soon realised that the changes observed were in vitro ones and could be induced in normal marrow cells in the presence of plasma from patients with S.L.E. Essential components appeared to be (1) a lytic factor; (2) a source of nucleoprotein to react with the lytic factor and (3) viable phagocytic leucocytes (Zimmer and Hargraves, 1952).

Subsequent investigations of the clinical and experimental conditions in which L.E. cells may be seen have been well reviewed by Wilkinson and Sacker (1957) and Bywaters and Scott (1960). The factor from the plasma has been found in all body fluids, exudates and in the urine of patients with S.L.E., and crosses the human placenta. It has been shown (Lee et al. 1950; Holman, 1960) both electrophoretically and ultracentrifugally to be a gamma globulin and as such it can be entirely inactivated and quantitatively precipitated by antiserum to normal human globulin. It will keep indefinitely if frozen.
The nucleoprotein substrate can comprise dead cell nuclei of any avian, mammalian or reptilian species tested (Ed. Brit. Med. J. 1960a). The fact that dead nuclei are required was shown by Snapper and Nathan (1955) and explains why the phenomenon has rarely been noted in vivo - and then only in moribund patients or where blood has been drawn to make smears after prolonged venous obstruction (Ogryzlo, 1956).

Nucleoprotein of one or more lobes of a polymorphonuclear leucocyte is affected by an influx of gamma globulin L.E. factor, the nuclei becoming larger, losing their chromatin pattern and developing a homogeneous purplish staining appearance about the time that the cell membrane ruptures (Wilkinson and Sacker, 1957). The L.E. body thus formed may then be ingested by a living phagocytic cell, usually a neutrophil polymorphonuclear, but occasionally an eosinophil or basophil leucocyte, myelocyte or monocyte, to produce the classical L.E. cell.

Positive Feulgen and weak methyl green staining of the L.E. bodies was generally interpreted as evidence of depolymerisation of the desoxyribonucleic acid of the substrate nuclei by the L.E. factor. The change is not a depolymerisation, Godman and Deitch (1957a and b) having shown that the L.E. factor displaces histones from the substrate nucleoprotein and Holman (1960) has
further shown that this reaction can be inhibited by saturating the nucleoprotein with basic substances e.g. histones or protamine. It is of interest that similar inhibition was achieved by antimalarial drugs such as atabrine which are sometimes used in the therapy of S.L.E.

Criteria of L.E. cell

Variations in criteria of the phenomenon must explain, partly at least, the varied incidence of positive findings in cases of S.L.E. and of "false" positive results in other disorders. The more rigid the criteria the less likely are really typical cells to be found in diseases other than S.L.E. It would now be generally accepted that a classical L.E. cell in Romanowsky stained films is usually a rather bloated neutrophil polymorphonuclear leucocyte with the nucleus displaced to the periphery by a pale purple, homogeneous spherical inclusion which shows no chromatin pattern and no surrounding membrane. These inclusions or L.E. bodies vary in size and occasionally are multiple. Extracellular L.E. bodies, often surrounded by polymorphonuclear leucocytes to form "rosettes", are sometimes seen, but if no definite L.E. cells are present then the L.E. test must be considered negative, although the finding is suspicious.

A clear distinction must be made from nucleo-
phagocytosis where a phagocytic leucocyte has ingested a free nucleus with subsequent blurring of the chromatin pattern of the latter by enzymatic changes. This is the Tart cell which Hargraves et al. (1948) clearly distinguished in their original paper. Erythrophagocytosis can also cause confusion if the strict criteria for the identification of L.E. cells are not followed. These latter phagocytic phenomena are not considered necessarily related to systemic lupus erythematosus. The above are the criteria for L.E. cells adopted in this thesis.

Implications and associations of the L.E. factor

The effects of this factor on nuclei and the fact that, like most known antibodies in humans it was a 7S gamma globulin (Holman, 1960) lent support to the idea that the L.E. factor is an autoantibody to some part of the nucleoprotein. This was strengthened by evidence of other autoantibodies. Hill (1957) in his Lumleian lectures drew attention to the occurrence of haemolytic anaemia with positive Coombs test, leucopenia, thrombocytopenia and abnormalities in gamma globulins which he considered were related to false positive tests for syphilis, positive Rose-Waaler tests, circulating anticoagulants and cold agglutinins in some patients with S.L.E. This theme was further developed by Dameshek
(1958) who pointed out that there were sometimes antibodies to prothrombin and antihaemophilic globulin and speculated on autoantibodies to small blood vessel constituents. Subsequently in this disease there has arisen evidence of antibodies to isolated cell nuclei and various constituents of cell nuclei such as deoxyribonucleic acid, deoxyribonucleoprotein and histone which have been demonstrated by a variety of techniques. Chief among these have been complement fixation, precipitation reactions, latex agglutinations, tanned cell agglutination, Coombs' consumption test, passive cutaneous anaphylaxis and also fluorescent antibody techniques. By this last technique Beck (1961) and Beck et al. (1962) have demonstrated antinucleolar antibodies as well as antidesoxyribonucleoprotein. Antibodies to isolated nucleoli and to cytoplasmic microsomes have also been identified (Ed. Brit. Med. J., 1960a). This editorial stresses that most workers regard the antibody phenomena they observe as indicating an unusual state of the immune-antibody-producing system, rather than a cause of the disease itself. Experimental passive transfer of positive L.E. test sera in animals and man has not produced any recognisable damage. Such experiments are reviewed by Clark et al. (1963) who obtained similar negative findings using rats rendered
immunologically tolerant of human proteins.

Antinuclear serum factor is present in almost every case of systemic lupus and it is not identical with the L.E. factor (Annot. Lancet, 1961) as it is found in other autoimmune disorders such as autoimmune thyroiditis in which L.E. tests are negative. Neither is there any direct correlation between globulin causing positive direct Coombs tests and positive L.E. tests in patients with disseminated lupus (Wilkinson, 1957).

Pathogenic significance of L.E. factor

With reference to the L.E. factor and any causative role in S.L.E. it has already been noted above that L.E. cells are rarely seen in vivo and that the L.E. factor crosses the placenta (Beck and Rowell, 1963) but does not cause damage to the foetus (Larson, 1960). The latter author also points out that growing cells in tissue cultures are not affected by high titres of L.E. factor. The phenomenon tends to occur late in the disease (Steiner and Volpe, 1961(b)). In addition the titre is not proportional to the severity of the disease (Harvey et al. 1954) although it must be admitted that the test is not a very sensitive one (Beck and Rowell, 1963).

German (1958) was able to produce haematoxyphil bodies in the kidneys of rabbits and guinea pigs by injection into the abdominal aorta of L.E. cell preparations
from patients with disseminated lupus erythematosus. This however does not indicate any pathogenic significance in the L.E. factor itself in vivo.

The L.E. factor lacks species specificity of antigen and identical factors have not been obtained in animals by injection of nuclear materials. However such materials are known to be poor antigens. After drawing attention to this point Larson (1960) goes on to say that there is no evidence that the serum factors found in patients with disseminated lupus erythematosus are not antibodies and that "the search for analogous factors in animals may prove difficult." They have since been described in mice by Helyer and Howie (1961) and in the NZB/BL X NZW mice, with which this thesis is mainly concerned (Helyer and Howie, 1963a).

**Discussion of method of L.E. cell test preparations**

Helyer and Howie (1961) made their L.E. test preparations by obtaining 1 ml. of blood from anaesthetised animals by percutaneous cardiac puncture prior to postmortem. This blood was defibrinated and incubated at 37°C for one hour. It was then centrifuged at 3000 r.p.m. for twenty minutes and films from the buffy coat were stained by May–Grunewald Giemsa techniques. As this method was impracticable for repeated use in the one animal, as well as rather time consuming, they have for several
years used a much simpler method which they find gives satisfactory results (Helyer and Howie, 1963c).

One heparinised capillary tube of blood is obtained from the mouse tail, sealed by heat at one end and stood vertically for one to two hours at room temperature. It is then centrifuged for five minutes, a film made of the buffy coat and stained by the May-Grunewald Giemsa technique (Appendix No.2). Mudrik et al. (1961) have described and verified in their S.L.E. cases the reliability of a rather similar technique in which, after spinning the heparinised tube of blood, they resuspend the buffy coat in the plasma. They did this with a stylet and claim it gives the little necessary trauma to the leucocytes (Snapper and Nathan, 1958), as well as bringing them into intimate contact with the L.E. factor in the plasma. Holman (1960) notes that the reaction takes place in the thermal range 18°-45°C. The time factor was explored by Wilkinson (1957) who showed that, of varying times, in 2½ hours the number of L.E. cells per 1000 polymorphs was maximal and degenerative changes in leucocytes inconspicuous. In practice bleeding batches of animals, by the time the blood was collected, stood for the hour, centrifuged, haematocrit measured and L.E. preparation made, approximately two hours had usually elapsed.
L.E. test method used

The films prepared by the above microhaematocrit method were smeared evenly with immersion oil before being examined under the 16 mm. objective for at least five minutes before a negative report was given. Special attention was paid to the edges and tail of the film (Wilkinson, 1957; Bywaters and Scott, 1960) where the L.E. cells tend to accumulate and also to any clumps of cells or areas where there was suspicious extracellular material. The criteria for a positive test previously outlined were strictly followed. The presence of one typical L.E. cell was recorded as a positive test. Whereas in the L.E. tests from humans a search for at least ten minutes is made, this was not considered practical in view of the large number to be examined. It was considered that any disadvantage in the lesser time would be offset by repeated examinations at intervals throughout the animal's life. All tests were viewed without reference to whether the previous test had been positive or negative.

Specificity of L.E. cell test

Concerning false negative tests, which may be due to lack of complement, Haserick (1951) estimated that at least 5-10% of cases of S.L.E., undoubted on other grounds, had negative L.E. tests. Hill (1957) as well
as well as Wilkinson and Sacker (1957) give similar figures. Dubois (1960) in some 400 cases found only 80% with positive tests, and this was after repeated examinations.

Hill (1957) doubted that there are any real false positives, considering a positive test as tantamount to a diagnosis of systemic lupus erythematosus. However, Hijmans et al. (1958) represent current opinion when they accept a small proportion of otherwise indistinguishable cases of rheumatoid arthritis as having positive L.E. tests. Mackay et al. (1959) have described cases of juvenile cirrhosis associated with positive L.E. tests but no other evidence of disseminated lupus erythematosus. Mackay and Burnet (1963) stress the difficulty of interpreting such a positive test associated with one other abnormality e.g. skin or liver changes, but no multisystem disease. These authors are reluctant to accept a diagnosis of S.L.E. if repeated L.E. tests are negative.

Sensitivity of L.E. cell test

Most of the above authors stress this need for repeated examinations before deciding that the L.E. cell test is negative. This immediately implies a lack of reproducibility intrinsic in the test or in the examination of the slides or else a fluctuation in the level of L.E. factor in the serum. Because in the normal
differential leucocyte count in mice polymorphonuclear leucocytes are much less frequent than they are in man (Small, 1941), it seems likely that in mice the test would be even more variable and less reproducible than it is in man.

Positive L.E. tests and drugs

The hypotensive drug hydralazine can induce a reversible syndrome akin to rheumatoid arthritis or S.L.E. with occasional positive L.E. tests (Dunstan et al. 1954). This type of toxicity was induced in dogs by Comens (1956) but not in rabbits (Wilkinson, 1957). Wilkinson also points out that similar claims have been made concerning penicillin, phenylbutazone, hydantoin and antitetanus serum. He feels that many reports do not distinguish the Tert cell from the L.E. cell. Subsequently Benton et al. (1962) add the anticonvulsant trimethadione as causing a fatal case with renal changes of S.L.E. at postmortem and they discuss the syndrome of S.L.E. due to phenylhydantoin, reversible by drug withdrawal. Peterson and Good (1962) refer to four further cases due to hydantoin and one to a long acting sulphonamide. All five children developed signs and symptoms suggesting S.L.E. and showed the L.E. cell phenomenon. All showed recovery, including reversal of renal biopsy changes and of the L.E. cell phenomenon when their drugs were stopped.
Whereas these authors believe that these children are genetically predisposed to react in such a manner to the drug, Hill (1957) would say they are really primarily variants of S.L.E. Benton et al. (1962), however, regard this as unlikely on the grounds that in some series hydralazine induced its systemic lupus-like syndrome in up to 10% of hypertensives. It seems unlikely to these authors that this percentage of any group of hypertensives suffer from a masked form of S.L.E. However in terms of clonal theories it is not so unlikely. These drugs may produce the syndrome of S.L.E. by altering fundamental homeostasis in the control of forbidden clones.

The effect of corticosteroids on L.E. phenomenon in man

There is considerable evidence that L.E. cells become less common or even disappear in adequately treated cases of S.L.E. (Dubois, 1956; Hill, 1957; Wilkinson, 1957).

L.E. phenomenon in animals

Workers in experimental autoimmune disorders such as Oliner et al. (1961) have diligently sought but never found the L.E. phenomenon in different strains of mice with runt disease. As a refinement to their technique they added extra complement. They point out that mice have low levels of complement and that it has long been
known that in vitro immunological reactions involving mouse tissues require the addition of complement. Holman (1960) had shown that complement was fixed during the reaction of L.E. factor with nucleoprotein, although Lee et al. (1950) felt that complement was not necessary to demonstrate the L.E. phenomenon in man.

Apart from the unconfirmed induction of the phenomenon by hydralazine already referred to, the only descriptions of L.E. cells in preparations from animals of which the author is aware are those of Helyer and Howie (1961 and 1963a and b) confirmed by Holmes and Burnet (1963) in the NZB animals.

CONCLUSION

The L.E. phenomenon is a useful sign in investigating autoimmune disorders. It is not generally regarded as important in pathogenesis. It would be of value to obtain more knowledge of the test during the life span of NZB/BL X NZW hybrid mice with particular reference to the age at which the test became positive, whether it fluctuated, how it correlated with Coombs tests or with renal lesions and whether it was influenced by steroid therapy.
II. **LATEX AGGLUTINATION TESTS**

A number of tests are available to detect antibody, all based on antigen-antibody reactions that produce agglutination or flocculation of otherwise inert particles. Goodman and Bozicevich (1964) trace the historical background of these tests to the observation of Nicolle in 1898 that finely divided talc would form clumps in the presence of watery extracts of *Escherichia coli* and antisera to *E. coli*. It was later found that this could occur in high dilutions and that other particles such as collodion could be used, antigen being adsorbed on the particle and the "sensitised" particles used to detect antibody. However difficulties in the preparation of particles and a tendency toward non-specific agglutination prevented extensive use of this method of antibody detection.

**Erythrocyte particles.**

Erythrocytes provide a uniform particle size and are a useful indicator of agglutination, provided the surface can be satisfactorily coated with antigen. Tannic acid treated erythrocytes were successfully used for this purpose by Boyden (1951). The current status and limitations of haemagglutination and haemagglutination inhibition reactions using various treatments of the red
blood cells are extensively reviewed by Stavitsky (1964). He states that they are extremely sensitive, easy to perform and have a wide applicability. However, they are occasionally non-specific and it is unwise to rely exclusively upon them as to the nature of an antibody.

**Bentonite particles**

Bentonite is a special type of aluminium silicate clay with a large surface area and many negative charges which repel each other and keep the flakes suspended in water. It was these properties which led Bozicevich during the 1950's to employ it for absorption of antigens in flocculation tests, for example to detect rheumatoid factor, antibodies to desoxyribonucleic acid in patients with S.L.E., thyroglobulin antibodies, as well as autoantibodies in experimentally induced autoimmune disease (Goodman and Bozicevich, 1964). These authors have found bentonite considerably less sensitive than the red cell techniques in detecting antibodies to protein antigens but equally as sensitive if the antigen is a polysaccharide.

**Latex particles**

Singer and Plotz (1956) first used these particles coated with gamma globulin to detect rheumatoid factor, an antibody to gamma globulin, in the sera of patients
with rheumatoid arthritis. They are negatively charged spherical particles usually 0.8 - 1.1 µ in diameter and have since been coated with a variety of antigens and used, for example, in serological tests for S.L.E., chronic thyroiditis, estimation of growth hormone and diagnosis of some infections such as histoplasmosis. A styrene monomer is polymerised to form a colloid suspension in water, this being referred to as latex particles (Goodman and Bozicevich, 1964). The test is far more convenient than those using tanned cells, although probably a little less sensitive.

In the latex agglutination test for D.L.E. (L.E. test, Hyland) which was used in parts of this thesis the makers inform that the particles are coated with desoxyribonucleoprotein obtained from calf thymus. Lewis (1960) traces the background work leading up to the development of this test in which agglutination of the coated particles results from an antibody to nucleoprotein that is present in the sera of many patients with S.L.E.

**Antinuclear antibody tests in S.L.E.**

It is generally agreed that the sera of patients suffering from S.L.E. contain a series of substances which react with nuclear components and can be detected by a variety of immunological methods of which the latex
agglutination tests are just one. Others mentioned by Seligmann (1964) are antiglobulin consumption tests, fluorescent antibody tests, precipitation in a liquid medium or gel, complement fixation, passive cutaneous anaphylaxis, the L.E. test itself, as well as the tanned erythrocyte and bentonite tests already discussed. He favours a fluorescent antinuclear test as the screening test that should detect all cases of S.L.E. He finds the bentonite test, using particles coated with desoxyribonucleic acid, more often positive than the Latex test using particles covered with nucleoprotein. Nevertheless, this Latex test is positive in 70-80% of patients with S.L.E. in an active phase.

**Significance of antinucleoprotein antibody in S.L.E.**

Whether the antibody has any pathogenic significance is still unknown, although Seligmann (1964) believes that it has. It is not known whether the antibody can enter living cells in vivo. He makes the interesting suggestion that even if not primarily responsible for tissue damage, the antibody could form complexes with nuclear components from cells undergoing physiological disintegration, such complexes being deposited say in glomerular capillary walls and causing some of the histological damage. These antinuclear tests are not specific for S.L.E., occurring sometimes
for example in rheumatoid arthritis. High titres, however, are more likely in S.L.E.

**CONCLUSION**

As the latex agglutination test was available, having been first used in the mice by Dr. A. Sharard, the opportunity was taken to make some observations on the incidence of positive results, especially in the hybrid mice with S.L.E. and to see if the incidence was modified by steroid therapy, which all authors agree occurs in properly treated human cases.
The description of their antiglobulin test, often called the Coombs test, by Coombs, Mourant and Race (1945) was the result of a few years of rapidly increasing knowledge of haemolytic processes following the first description of the Rh blood group and the recognition of its importance in haemolytic disease of the newborn. In this condition free circulating antibody could sometimes be demonstrated in the serum of the mothers of affected babies using the then current ordinary saline techniques, but some serum which would be expected to contain anti Rh did not appear to do so. This proved to be because it was present in what is now regarded as an "incomplete" form and its presence was demonstrated by a "blocking" effect. Thus if Rh positive cells were suspended in a serum suspected of containing this complete or blocking anti D they were subsequently no longer agglutinable by ordinary saline acting anti D, and this blocking was specific in this example for the blood group antigen D, (Mollison, 1947).

The complete or saline acting antibody was regarded as bivalent and consequently leading to agglutination in vitro, whereas the incomplete or blocking antibody was regarded as monovalent. It proved to be much the commoner in haemolytic disorders and the more important. When both
sorts of specific antibody are present the incomplete one will be preferentially absorbed by cells with the appropriate antigen. It was later shown that this "incomplete" antibody would, in fact, cause agglutination in appropriate media, that usually used to test the system being 20% bovine albumin.

This fact also probably explains the mode of action of the incomplete antibodies which coat the erythrocytes in autoimmune haemolytic anaemia. They cause small agglutinates in protein media which are readily trapped "in backwaters of the circulation, particularly in the spleen. The cells comprising the agglutinates probably become spherocytic and ultimately undergo lysis or are phagocytosed by reticulo-endothelial cells," Dacie (1960b).

Dacie also prefers considerable evidence that the spleen is the main site of red cell destruction in these cases, including radiochromium studies.

Autoantibody specificity

In humans the type of autoantibody found usually has been regarded as lacking specificity, although there may be a relationship with the Rh systems and occasionally it is specific against "e", the commonest Rh antigen. This has led to speculations about the antibody being directed against some fundamental precursor blood group substance (Dacie, 1959, 1960b and 1962b). Dacie (1960b)
feels that the very "imperfection" of the antibodies formed and the fact that they are active against nearly all humans red blood cells argues for the primary disturbance being one of the antibody forming tissues, as would fit in with the theories of Burnet, rather than due to alterations of the red cell antigens in the patient.

Principles of the Coombs test

The Coombs test makes use of the fact that these antibodies are gamma globulins which, when they are coating erythrocytes, will cause agglutination in the presence of an anti-gamma globulin. This last can be obtained by repeated injections of human serum or gamma globulin into rabbits or goats and treatment of the serum produced to remove any Anti A, Anti B or non-specific antihuman factors by using A, B and O cells. One is left with a potent antiserum to human globulins and it can be shown that the ability to agglutinate red blood cells coated with incomplete antibodies is due to the anti-gamma globulin fraction because it can be neutralised by adding gamma globulin to the Coombs serum but not other globulins (Dacie, 1962).

Although the gamma globulin of an animal comprises many specific antibodies which must depend on variations in the gamma globulin molecules (Abrahams, 1962) these different components of the gamma globulin fraction do not
behave differently antigenically when injected into other species. It is on this point that the main usefulness of the Coombs test hinges. It will detect a large variety of incomplete antibodies, many of which can be shown to have specificity when tested with antigens from the same species. Thus, apart from helping to elucidate the mechanisms of Rh sensitisation the historical aspects of which are reviewed by Dacie (1962) and Race and Sanger (1962), the latter authors also point out that it has demonstrated the existence of the Kell system of blood groups and has revealed practically all the subsequently discovered blood group systems.

**Significance of Coombs test**

The principles and background of this method have been described in detail because the demonstration of erythrocyte fixed incomplete autoantibodies by the direct Coombs test and of free circulating antibody by an enzyme technique are fundamental steps in the diagnosis of an autoimmune type of haemolytic anaemia (Dacie, 1964). The ability to do this and, in addition to extract the antibody from eluates of affected red cells, have been the bases on which Helyer and Howie (1963b) and Holmes and Burnet (1963) have called the haemolytic anaemia of NZB/BL mice autoimmune in nature.

Dacie (1960b and 1962) regards a positive direct
antiglobulin test as a *sine qua non* for the diagnosis of autoimmune haemolytic anaemia. Free circulating antibodies are not found as often as fixed ones, percentages varying in different series up to approximately two thirds in his own series. Dacie also observes that the free antibody is detected more frequently in the more severe cases. Dacie (1962) quotes Evans and Weiser (1957) as pointing out that the level of antibody in the plasma depends on the rate of combination with erythrocytes receptors, on rate of production with spill-over into the plasma if production is rapid, and on its release from destroyed cells. It must also depend on the rate of gamma globulin turnover. These factors are variable so there is no close correlation between fixed and free circulatory antibody. Dacie (1960b) notes that, although the degree of positivity of the test does not correlate closely with the degree of haemolysis, nevertheless in a given patient the test lessens in strength as the patient improves.

A positive direct Coombs test obviously means that the red cell is coated by a protein that can cause agglutination in the presence of the Coombs serum. This is usually a warm incomplete antibody, reacting best at 37°C., with agglutination being inhibited by adding small amounts of gamma globulin to the Coombs reagent.
Sometimes there are mixed gamma and non-gamma globulin antibodies and sometimes just macro-globulin antibodies of the cold variety (Dacie, 1960b).

Dacie (1960a) does stress that a positive direct antiglobulin test does not necessarily mean that a patient is suffering from autoimmune haemolytic anaemia. Positive results may also be obtained if blood is refrigerated when an incomplete cold non-gamma globulin antibody usually present in human bloods is absorbed by the red cells. Also, positive results will be obtained if the test is left too long before reading. Silica in improperly clean glass can cause positive results. Rarely normal cells give a positive result.

Not uncommonly blood from patients with S.L.E., rheumatoid arthritis, leukaemia, myelosclerosis, sarcoidosis or aplastic anaemia may produce positive tests. These may be related to non-antibody globulins attached to the cell surface. At least they are sometimes not inhibited by gamma globulin and Dacie states that there may be no evidence of red cell destruction.

At the same time Dacie (1960a) lists the three main causes of false negative results as impotent antiserum, failure to wash the red cells properly free of surrounding plasma or serum and, finally, using the
Coombs serum at an inappropriate dilution.

Method

The main technical points to avoid the errors mentioned here are referred to in the description of the technique itself in Appendix No. 2, p. 11. The test was carried out by a conventional slide technique on the day of bleeding, using a positive and a negative control on each occasion.

CONCLUSION

The direct Coombs test is usually a sensitive indicator of the presence of a gamma globulin antibody on the red cell surface. As such it should provide a useful indicator for the assessment of changes in such antibody levels with age or therapy in NZB/BL mice and offer a point for comparison with human cases of A.I.H.A. It would be useful to obtain more information about the behaviour of the test in the hybrid mice with lupus nephritis.
IV. THE FICIN ANTIBODY TEST

Enzyme treatment of red blood cells

About the same time as Coombs et al. (1945) developed their method of demonstrating incomplete antibodies, Pickles (see Pickles, 1949) who was following an observation of Burnet, found that cells previously exposed to a filtrate of a broth culture of vibrio cholera would show specific agglutination with incomplete Anti D sera and later showed that a similar result could be obtained by treating the cells enzymatically with trypsin. Pickles doubts if the exact mechanism of these two methods is the same but some structural alteration in the envelope of the red cell renders it agglutinable by specific "incomplete" antibodies.

The fact that this alteration of cell surface permits agglutination in an otherwise "incomplete" system is taken by Humphrey and White (1963) as strong evidence against the idea that incomplete antibodies are univalent. They consider them bivalent and the phenomenon suggests to them that the specific receptors on the red cell are in a surface pit or hole so that combination with one antibody valency bond makes the other inaccessible. Enzyme pre-treatment of the erythrocyte alters the surface stereochemically in some way that this
second valency bond becomes accessible with agglutination following.

Free circulating antibodies can also be detected by using an indirect Coombs technique in which known normal cells are incubated in serum from a patient suspected of having such antibodies. These cells are then tested with Coombs antiglobulin serum, agglutination indicating that the cells are now coated with a gamma globulin antibody. However the enzyme treated erythrocyte technique mentioned above has proved a more sensitive way of detecting incomplete antibodies (Dacie, 1962).

**Significance of positive ficin tests**

Of 45 patients with positive direct Coombs tests, both the indirect Coombs test and the enzyme test were positive in 20 patients and the enzyme test positive in a further 14 patients, both tests being negative in 11 patients (Dacie, 1964). Dacie actually uses trypsin but notes that papain, ficin or bromelin can be used. These figures in no way imply that the direct Coombs test (which was positive in the whole group of 45 patients) is more sensitive than the enzyme technique. They show rather that in a high proportion (72% in the 1964 series of Dacie) free circulating incomplete antibody is present. Dacie regards this as in
equilibrium with the erythrocyte fixed antibody.

Further information as to the relative sensitivity of the tests available is that Marrack (1963) states that the Coombs test is a very sensitive one and detects antibody levels of about 54 μg/ml. which is much less than can be detected by direct agglutination of unsensitised A or B cells but nevertheless is about four times less sensitive than the various enzyme techniques.

Antibodies that are only detectable by an enzyme technique may be imperfect and may have little clinical importance in humans, according to Dacie (1964).

**CONCLUSION**

The ficin antibody test is a sensitive method of demonstrating free circulating antibody and as such it should be useful for further studies on the NZB/BL mice with haemolytic anaemia and on the NZB/BL X NZW hybrids.
V. STATISTICAL METHODS

Hill (1961) defines these as "methods specifically adapted to the elucidation of quantitative data affected by a multiplicity of causes." Lees (1962) takes statistics to mean "the science of sampling, not merely of counting figures". The complicated analysis to which statistical data are often subjected give form and order that were not obvious in the original figures but while so doing their precision is dependant on the accuracy of the original data (Tippett, 1943). It is obvious as he points out that, granted this accuracy and applying orthodox analyses, no statistical results can be reached that are not implicit in the data.

Standard deviation

In animal experiments technical difficulties vary and variations due to the material used are considerably reduced by using inbred strains. Although the crossbred mice forming the main subject of study in this thesis are derived from two highly inbred strains, it could be expected that individual mice in the F1 generation studied might show considerable variation from mouse to mouse. The given characteristic of the population being measured (i.e. the parameter) would show a range of distribution. The arithmetical mean, the
sum of numerical data divided by the number of animals, gives a general idea of the average value of the parameter being considered but no indication of the range above and below this average in which the findings in any given animal are likely to lie (Bailey, 1959). Many characteristics in animals have a symmetrical distribution about the mean forming a "normal" or Gaussian curve (Hill, 1961). It is useful to give a measure of this variation and that employed by statisticians is the standard deviation which is a measure of the scatter of observations around their mean. The standard deviation gives the limits on each side of the mean within which two thirds of the results or observations lie and outside of which one third lie (Coward, 1947). There is only a 5% chance that a measurement which follows a normal distribution will differ from the mean by more than three times the standard deviation (Hill, 1961).

**Standard error**

The standard deviation is only a result obtained in a sample of a theoretically very large number (a universe) of animals and such a result may differ more or less from the real value for that universe. The amount by which it does so is indicated by the standard error (S.E.) of the mean which is going to depend on the range of original results (reflected in the standard
deviation) and the size of the sample (thus $S.E. = \frac{S.D.}{\sqrt{n}}$).

It will be noted that to halve the inaccuracy of an average result four times the number of animals must be used (Coward, 1947). The standard error of the mean is really an indication of the precision of the mean. The standard error of the whole strain of mice is unlikely to differ from that found in a sample of reasonable size by more than plus or minus twice this standard error (Hill, 1961).

In some of the experiments that follow statistics have been used, stating a mean figure ± its standard error in untreated animals. Then the mean of treated animals can be compared with this figure to see if there is any statistically significant difference in the means between otherwise similar groups.

"t" Test

Hill (1961) points out that where numbers in the sample are small, then the observed standard deviation may not be a good measure of the standard deviation for the whole strain also that, with small numbers, the ratio of the differences between the means of samples to their standard error will not be distributed evenly like a normal curve but has what is referred to as a "t" distribution. To test the significance of any difference between samples of less
than 30 the "t" Test is applied (Hill, 1961). This observed "t" value is coupled with the number of values independently contributing to it and the level of significance read from tables.

The "t" Test is also particularly applicable when observations which are paired in some way are being examined, as in comparing figures in the one group before and after treatment (Hill, 1961) and consequently is applied in some of the experiments in this thesis. The test is applied to the mean of the differences between pairs to see whether such a mean is significantly greater or less than zero.

Other statistical methods have been used, such as the deduction of the likely equation that represents a trend over a period of time and allows graphic representation of results obtained. For these, as for all the statistical analyses, the author is greatly indebted to Mr. G.F. Spears of the Preventive Medicine Department.

Application

A practicable planned "statistical" experiment needs previous knowledge of the qualitative relations involved and a knowledge to within a fairly narrow range of the quantitative results to be expected (Lees, 1962). Such knowledge was available for the NZB/BL strain but
not for the NZB/BL X NZW crossbreed. Some of the information obtained in the earlier experiments with these hybrids, as later reported, enabled a more closely planned approach in some of the further experiments described. In particular the use of initially matched pairs in both the NZB/BL and NZB/BL X NZW later experiments enabled a more clear-cut view of the effects of steroid treatment to be obtained.

**Level of significance**

Herdan (1955) points out that in comparing two groups of observations we must think of certain definite characteristics of the disease; we cannot compare the disease as such in the two groups. We can use for comparison the outcome, the duration, the number and severity of the complications or, as is used in the following experiments, certain specific characteristics. Whatever criteria we use in a therapeutic trial, to assess the effects of therapy the "Null Hypothesis" is really used viz. that no significant difference is expected between groups of similar animals. If significant differences i.e. differences likely to have arisen by chance in less than one per twenty cases (the conventional level of significance is usually taken as P 0.05) are obtained, and other factors are considered equal, then it is reasonable to infer that the difference is due to the
treatment. Lees (1962) states that there are few exceptions to the rule that a treatment which is not obviously and convincingly effective is ineffective, so no claim is made in this thesis for a treatment being effective unless this is unequivocally so.

Throughout the thesis the level of significance, $P$, will be written before the figure which it is less than and after any figure which it is greater than, e.g. $P 0.05$ indicates that $P$ is less than $1/20$; $0.01 P 0.05$ indicates that $P$ is greater than $1/100$ and less than $1/20$. 

VI. HISTOPATHOLOGICAL STAINS

The main tissue stains used in various parts of the thesis were Haematoxylin-eosin (HE), Periodic Acid Schiff (PAS), latterly with Alcian Blue (PAS/AB), Masson Trichrome, Martius Scarlet Blue (MSB) and the Unna-Pappenheim stains. The methods are detailed in the Appendix No. 6. A few general observations are made on these stains with particular reference to the kidney.

HE stain

With the HE stain the fibrinoid material in the glomeruli was brightly eosinophilic, sometimes rather granular and with a slightly orange tinge.

PAS stains

PAS stains glycogen and polysaccharides magenta to purple colours, basement membranes in the kidney being a reddish-purple in colour. This reaction is a true histochemical one and the intensity of colour is directly proportional to the quantity of reactive 1:2, glycol material in the tissues (Lynch et al. 1963). This fact prompted an estimate of the amount of PAS positive material in the tuft in some of the later experiments using large doses of steroids where diabetogenic effects were encountered. In this case one might expect an increase in PAS positive material and such indeed was
found although fibrinoid which is also PAS positive was much less in amount in these mice. PAS in connective tissue stains polymerised sugars which are bound to proteins i.e. glycoproteins (hence PAS positive basement membranes).

True acid mucopolysaccharides are PAS negative but these connective tissue polysaccharides will stain blue with Alcian Blue. PAS is therefore often usefully combined with Alcian Blue as a stain. Epithelial mucins stain shades of blue to purple, while the neutral polysaccharides stain with the PAS alone and are reddish-purple in colour (Lynch, 1963). One of the greatest uses of the stain was in studying the thymus. Because of their content of heparin, which is an acid mucopolysaccharide, mast cells in both medulla and cortex stain a striking bluish-green colour with PAS-Alcian Blue, while the granules of the frequent large cortical cells described by Metcalf and Ishidate (1962) stain reddish.

In the mouse the glomerular basement membrane (Fig. 7) is a purple-red colour and the cytoplasm of the tuft contains varying amounts of red material, often apparently granular, sometimes smudgy, occasionally on the edge of the basement membrane, but usually this was clear at its edge. Nevertheless the assessment of
FIG. 6. Kidney showing patchy glomerular basement membrane thickening; PAS positive material in cells of proximal tubules but not in the cells of the two tubules containing PAS positive casts in a 39 week old NZB/BL X NZW female hybrid mouse (aged 39 weeks). (PAS X258).

FIG. 7. Glomerulus showing patchy nature of early basement membrane thickening. NZB/BL X NZW female hybrid aged 35 weeks. (PAS X444).
basement membrane thickness in a reproducible way was found particularly difficult. The tubular basement membrane is red. The distal tubular cytoplasm is a very pale pink and one can clearly see the basal striations in these cells. Part of the proximal tubule, including its descending part, has much red PAS positive material concentrated near the lumen, the rest of the cell being paler but not nearly as pale as the distal tubules. The proximal part of the proximal convoluted tubules has a more deeply staining red cytoplasm and still has some, but much less, PAS positive material at the luminal edge.

When casts are present they are usually homogeneous but not infrequently will stain varying shades of red, blue or purple, suggesting that their composition is by no means uniform. A similar conclusion concerning the casts was obtained with the Masson's trichrome and the Martius-Scarlet-Blue stains. A further point about the casts was that they were never seen in the proximal tubules containing this cytoplasmic PAS positive material but in the more distal tubules (see Fig.6). In mice with advanced renal lesions there are often large rounded deposits of PAS positive material in the proximal tubular cytoplasm, rather than the evenly distributed effect seen in normal tubules.
Masson's Trichrome stain

This stain has been found useful by Drs. Helyer and Howie for identifying fibrinoid which it stains a bright red, not unlike the colour it stains red blood corpuscles so that in the glomerulus, with minor degrees of fibrinoid change, it has to be carefully established that the bright red colour is not due to red blood cells. The basement membranes of glomeruli and tubules stain a definite but light blue. Cytoplasm itself is a dull red colour, dense in the proximal convoluted tubules and much lighter in distal and collecting tubules. In the proximal convoluted tubular cells (Fig.8) a pale blue staining material is present at the luminal edge of the cells, presumably the same material that is PAS positive. This blue staining suggests this is a mucin (Lynch et al. 1963; Culling, 1957) which is consistent with the PAS staining. As noted above, some of the casts seem to contain similar staining material. With this stain nuclei are dark and a point of some usefulness was that the endothelial nuclei are much darker than the epithelial ones, which, apart from the relationship to the basement membrane, helped in establishing the origin of hypercellularity in the glomerular tuft (Fig.9).

Martius-Scarlet-Blue (MSB) stain

This is classed as a variation of the trichrome

FIG. 9. Glomerulus showing marked hypercellularity clearly endothelial in most areas because of relationship to slightly thickened basement membrane. This thickening is irregular in distribution, mainly affecting peripheral loops. Fibrinoid also present. NZB/BL X NZW female hybrid aged 35 weeks. (Masson's X660).
staining methods for fibrin by Lendrum et al. (1962). They envisage fibrinoid as comprising a fibrinous exudate, the meshes of the fibrin network varying in size, depending on the density of the tissue into which the exudate has occurred. Globulin and other components of the exudate will determine some of the staining properties of the "fibrinoid". In the case of intracytoplasmic or intravascular deposits the fibrillary nature of the fibrin is not obvious, the meshwork being compressed. It is the size of this meshwork which determines the molecule size of dyes that enter and stain the material. They state that with this stain the most recent fibrin as seen in some vessels at postmortem is yellow, like erythrocytes, but fibrin that has been formed for a definite but short time is strongly red. In the hypertensive and diabetic kidney they trace the development of such red fibrinoid deposits, then fading red with pale blue appearing, then deposits that are hyaline, blue and acellular and finally blue intercapillary masses containing nuclei - the Kimmelstiel-Wilson glomerulus. Their thesis is that fibrinoid is fibrin primarily, that it changes its staining reactions with age and that it finally gives staining reactions akin to collagen, which also stains a deep blue with the MSB stain. The possibility of exploring this tissue sequence arises with the NZBW mice.
under study. It is also considered by the author that such changes with time might explain some of the apparent differences between the more rapidly developing renal lesions seen in the NZBW mice and the definite but less rapidly developing changes in the nephritis of the NZB/BL mice.

With the MSB stain the basement membrane of the glomeruli appears a greenish-blue; that of the tubules more a pale blue. The glomerular cytoplasm is a pale green. The material already discussed in the luminal aspect of the proximal convoluted tubules is a pale blue, deeper more distally. Nuclei are very pale with this stain. Fibrinoid in glomeruli and arteriolar wall appears an orange to orange-red in colour, and as already noted, collagen is a light royal blue. Lendrum et al. (1962) note that some Russell bodies stain with this stain and some of the granules of renal tubule cells in cases with albuminuria; this presumably also depending on relative molecular size. With this stain, as with the Masson's Trichrome stain, the granules of the juxta glomerular apparatus can be clearly seen but in both cases the colour is slightly different to fibrinoid. Nevertheless, considerable caution is required in interpreting minor degrees of fibrinoid change near the hilum of the glomerulus because of confusion with these granules. This stain was found convenient and reproducible.
Unna-Pappenheim stain. Methyl Green-Pyronin stain

The methyl green is considered specific for desoxyribonucleic acid (DNA) by many and the pyronin for ribonucleic acid (RNA) which it stains a pale red (Culling, 1957) to pink colour. Holmes and Burnet (1963) have placed considerable stress on the presence of pyronin positive cells in groups of round cells in the NZB/BL mice, especially in the thymic medulla. Some observations on the thymus are made which include the use of this stain. Mast cells in the thymus are well stained, the granules appearing almost black. The stain was also used in some mice on lymph node and splenic sections, as well as renal sections.
VII. DOSAGE OF CORTICOSTEROID THERAPY IN MICE

Helyer and Howie (1963b) have previously noted that the effect of adrenocorticotrophic hormone (A.C.T.H.) was beneficial to the haemolytic anaemia of NZB/BL mice. The use of this drug would not be practicable in large numbers of animals for a prolonged time. Response varies greatly in different patients, resistance often develops, batches vary and for correct control repeated measurements of urinary 17 hydroxycorticosteroids (Ed. Brit. Med.J. 1957) are needed. Also, though still having its advocates in the long term therapy of rheumatoid arthritis, it has not yet been claimed to have the same benefits claimed for prednisone in renal cases of S.L.E.

In a short preliminary trial using injections of cortisone acetate (0.125 mg./day in adult NZB/BL mice) it proved difficult, using a mantoux syringe for accurate measurement of dosage, to avoid some leakage of the drug. The mice became rather irritable and nervous when handled when on daily cortisone injections. The method obviously was inappropriate to large numbers of animals for a prolonged time.

One of the main problems to be solved before this thesis could be properly launched was to arrive at a suitable dose of a corticosteroid that itself was suitable for long term administration to mice. The
considerations underlying this are now discussed.

**Review of dosages of corticosteroids in mice**

Most of the animal work referred to in the section on corticosteroids has been done over a short time using large doses usually of cortisone, and as Florey (1962) points out, many workers have been carrying out experiments on "cortisone poisoning", rather than cortisone therapy. This would apply to some of his own work (Jennings and Florey, 1954 and Roberts, Florey and Johlik, 1952) where doses of 2.8 mg. and 2.25 mg. of cortisone per day were used over periods of up to six days. Even on the latter dose 20 gm. mice became ill and often died in six days. Doses of 1.25 mg., or over usually caused foetal abnormalities when given to mice in early pregnancy by Fraser and Fainstat (1951).

The prolonged effect of a single injection of cortisone was demonstrated by some protective effect for up to 96 hours. In assessing the effect of massive doses of cortisone on the peripheral blood and bone marrow of the mouse Quittner et al. (1951), using a daily 2.5 mg. dose of cortisone per mouse, point out that small deposits of cortisone usually remain at the injection site for five days and sometimes up to nine days. This would favour a cumulative effect of cortisone by injection. Antapol (1950), a co-author of the previous
paper, points out that this type of dose is equivalent to 50 times the then currently used largest cortisone dosage in humans i.e. 100 mg/kg/mouse Cr 2 mg./kg./man

Using adrenalectomised mice Wragg and Spiers (1952) showed that eosinopenia due to cortisone varied between strains and was more predictable in males. Highly sensitive strains responded significantly to 1 microgram of cortisone and maximally to 6 micrograms, whereas the most resistant responded to 3-6 micrograms but needed almost 0.1 mg. to attain maximal eosinopenia.

With reference to the antimitotic effects of cortisone, Heilman and Kendall (1944) had suppressed a particular mouse lymphosarcoma using 0.3 mg. cortisone orally daily. Green and Gladially (1951) showed that a similar or larger dosage would inhibit cell division in the epithelium of the mouse ear and Bullough (1952) also noted an effect on epidermal activity over a dosage ranging upwards from 0.1 mg. Dougherty and White (1944) showed a marked lymphopenic effect within three hours of a single 0.25 mg. dosage. Baum et al. (1954) using 0.2 mg./day for ten days found an increased susceptibility to acute infections.

Mouse resistance to an infection such as tuberculosis was markedly diminished by Hart and Rees (1950) using 0.5 mg. cortisone on six days a week for fourteen days.
and 0.25 mg. daily for twenty five days. They point out that these are 12 and 6 times the doses then currently used in human diseases - their mouse dosage being equivalent to approximately 25 and 12.5 mg./kg./ mouse/daily dose. Gladhill and Rees (1952) following up observations made in the previous experiment showed that these mice often developed enterococcal infections with liver damage. This was not so with 0.2 mg./day but was with 0.3 mg. or more. They also noted that one fifth of the dosage used by Hart and Rees (1950) did not decrease resistance to tuberculosis. This dose of cortisone, however, will still have a definite effect on the inflammatory reaction with depression of fibrosis, as shown by Curran (1952). He used 0.1 mg./day also basing his dosage on Hart and Rees (1950) for up to eleven weeks after an initial dose of 0.4 mg. for two weeks.

**Evaluation of corticosteroid dosages**

It therefore seemed logical to select a dose of cortisone for mice larger than that which always gave maximal eosinopenic response - 0.1 mg. (Wragg and Spiers, 1952), which would still affect the inflammatory response - 0.1 mg. (Curran, 1952), which did not make mice much more susceptible to infections - 0.1-0.2 mg. (Gladhill and Rees, 1952) and which therefore might be continued
for a long period. A dose of 0.125 mg. of cortisone/mouse appears to fulfil these criteria. Such a dosage also gives partial protection against anaphylactic shock in mice (Nelson et al. 1950). An anti-inflammatory ratio of approximately 30/1 for betamethasone phosphate, compared to cortisone, as previously discussed and shown in Fig. 5, would suggest a dosage of 4 micrograms of betamethasone phosphate as being appropriate for therapeutic long-term effect in adult mice.

Atkinson (1962) considered that a dose of 1-2 micrograms of betamethasone would be indicated for long-term therapy. Using a thymic involution assay method (Weaver and Atkinson, 1955) for betamethasone (Atkinson, 1962) considered the new drug 5 times as effective as prednisolone and 10-20 times as effective as hydrocortisone. This thymolytic effect in terms of the thesis of Burnet (1962a) may be highly significant. Calculating from an indicated cortisone dosage of 0.125 mg./mouse a dosage of 5-10 micrograms of betamethasone would be suggested. Atkinson had noted an antianabolic effect in all mice using over 10 micrograms for 20 gm. mice and advised against any higher dosage than this. On the other hand, as stated, he found that depending on the strain 2-4 micrograms produced measurable thymic involution (Atkinson, 1962) and this could be given in divided doses.
CONCLUSION

A dose of cortisone of 0.125 mg./day is predicted as suitable for mice for long term therapy. Converting this to betamethasone in terms of anti-inflammatory effect this is equivalent to 4 micrograms a day. Divided doses of 2-4 micrograms a day have shown to give a measurable thymic involution in two days. A dose of 2-4 micrograms betamethasone phosphate per day seemed reasonable to aim at whether judged from the anti-inflammatory or thymolytic effects of the drug.
SECTION II

EXPERIMENTAL SECTION
SECTION II

EXPERIMENTAL SECTION

Chapter

6  INTRODUCTORY CHAPTER TO EXPERIMENTAL SECTION

7-11  A. EXPERIMENTAL SECTION A
       The NZB/BL X NZW hybrid mouse

12  B. EXPERIMENTAL SECTION B
       The influence of betamethasone phosphate on
       NZB/BL mice

13  C. EXPERIMENTAL SECTION C
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14-17  D. EXPERIMENTAL SECTION D
       Sundry
       14  Side effects of betamethasone therapy
           in mice.
       15  The thymus.
       16  The NZB/BL strain of inbred mice.
       17  The NZW strain of inbred mice.
CHAPTER 6

INTRODUCTORY CHAPTER TO EXPERIMENTAL SECTION
INTRODUCTORY CHAPTER TO EXPERIMENTAL SECTION

Introduction

The direction of the experimental work embodied in this thesis has already been indicated. It falls naturally into several main categories which, however, does not necessarily represent the chronological order in which the work was performed. These categories have determined the arrangement of the presentation of results, which is outlined below. After that, brief mention is made of the handling of the mice, the obtaining of specimens and the methods used for various tests, these being common to all sections of the thesis. Details of the actual methods and techniques are given in the Appendix.

GENERAL ARRANGEMENT OF RESULTS IN THE EXPERIMENTAL SECTION OF THE THESIS.

A. The NZB/BL x NZW hybrids

This comprises the bulk of the project. Results are tabulated in Appendix No.8, p.48 et seq. Tables 1-71.

1. The design of three experiments in which these mice were used is discussed, covering different age patterns.

2. Basic trends in untreated hybrid animals are evaluated in detail drawing on the information from these three experiments -
a. Clinicopathological findings
b. Renal findings
c. Non-renal findings

3. Different dosage levels of betamethasone phosphate are detailed. After each dosage scheme the changes noted in treated groups of hybrid mice with three different dosage schemes are separately compared with the relevant untreated groups.

B. The influence of betamethasone phosphate on NZB/BL mice. Results are tabulated in Appendix No. 8, Tables 72-75.

1. The design of the experiment is described.
2. Evaluation of the effect of various dosage schedules is compared with observed and previously described basic trends in these mice.

C. The influence of 6-mercaptopurine on NZB/BL mice.

1. The rationale and design of the experiment described.
2. The effect on elderly NZB/BL animals is described.
3. The combined effects of betamethasone and 6 mercaptopurine are described on elderly NZB/BL mice.
4. The effect of 6-mercaptopurine on young NZB/BL mice is described. Results are tabulated in Appendix No. 8, Tables 76-86.
D. General chapters are included on side effects of corticosteroid therapy; on the thymus gland; on the NZB/BL mouse and on the NZW mouse.

Finally, in Section III of the thesis a general discussion is undertaken.

GENERAL PROCEDURES ADOPTED

It is now proposed to briefly indicate the pattern of dealing with the mice and their investigations. Procedures underlined are detailed in the appropriate appendices.

GENERAL CARE

The methods of housing, feeding, watering, marking, weekly weighing, inspecting, dusting and hygiene of the mice are outlined in the appendix on general care of the animals (Appendix No.1, p.1). The handling of betamethasone phosphate and some estimations of fluid balance in the mice are described in Appendix No.4, p.25.

HAEMATOLOGICAL INVESTIGATIONS (Appendix No.2, p.5)

Blood collection during life was from the tail vein after a razor cut. The detailed method of warming the animals that is essential for successful bleeding of large numbers is detailed in Appendix No.2, p.6. As
it was seldom possible to bleed more than 24 animals at a time the bleeding programmes sometimes extended over two weeks in the early stages. Prior to postmortem blood was obtained directly from the heart. Heparinised capillary tubes were used for the microhaematocrit method of P.C.V. estimation. Ordinary 0.02 ml. white cell pipettes were used for white blood cell counts taking blood directly from the tail cut, as was done with a drop of blood for a blood film for the differential leucocyte count.

The author co-operated with Associate Professor J.B. Howie in the production of a Coombs reagent. The direct Coombs test was performed on the day of bleeding using the washed cells from the microhaematocrit tube. Serum was kept at -10°C for ficin tests for free circulating antibody which were usually performed in batches, when convenient. The latex tests were done on the same day as the bleed and, although the L.E. test preparations by the method already discussed were made on the day of bleeding, they were not examined until convenient.

URINARY AND RENAL FUNCTION INVESTIGATIONS (Appendix No.3, p.14).

For regular urine collection the mice were placed on metal pans and could often be induced to urinate. They usually also pass some urine while being anaesthetised.
prior to postmortem examination. Albustix were introduced to test for albuminuria and Clinistix for glycosuria, while narrow range pH papers were used to test the urinary pH.

For urine microscopy a drop of urine picked up in a microhaematocrit tube was stood vertically to sediment for 12-24 hours and the lower-most drop examined. On a few occasions the fluid intake and urinary output of some of the mice was checked.

For blood urea examinations 0.1 ml. of blood or serum was used, if obtainable, in a standard urease method. The blood was usually measured directly from the tail vein, but sometimes less than this volume was all that it was practicable to obtain, allowing for other requirements. Some blood sugar estimations were made on the AutoAnalyzer.

POSTMORTEM TECHNIQUES (Appendix No.5, p.32)

The mice were killed with ether anaesthesia and then dissected by a standard postmortem technique, the detailed appendix of this referring also to the histological fixatives used, to the method of weighing of organs and to other tissues retained for co-operative study.

Microscopic examinations were carried out on some samples of most major organs but in particular on the kidneys where any lesions found were graded + to 4+,
depending on whether they were mild, moderate, severe or very severe in a scheme which is detailed in the section on the kidney. In the major part of the project the findings were recorded in a chart which is duplicated in Appendix No.7, p.42.

**Histopathological stains** used are detailed in Appendix No.6, p.36.
EXPERIMENTAL SECTION A

THE NZB/BL X NZW HYBRID MICE

Chapter

7 Introductory description of experiments with NZB/BL X NZW hybrid mice

8 Some clinical, haematological and chemical pathological basic trends in NZB/BL X NZW hybrid mice

9 Renal morbid anatomical and histopathological findings in NZB/BL X NZW hybrid mice

10 Non-renal morbid anatomical and histopathological findings in NZB/BL X NZW hybrid mice

11 Influence on the renal and other lesions in NZB/BL X NZW hybrid mice of the corticosteroid drug betamethasone phosphate
CHAPTER 7

INTRODUCTORY DESCRIPTION OF EXPERIMENTS

WITH NZB/BL X NZW HYBRID MICE
INTRODUCTORY DESCRIPTION OF EXPERIMENTS WITH NZB/BL X NZW HYBRID MICE

Three major groups of hybrid animals were studied.

GENERAL OUTLINE OF FIRST EXPERIMENT

A total of 153 animals were studied in the first experiment which is referred to as Experiment CT (cortisone therapy). These comprised 16 NZB/BL, 18 NZW and 119 NZB/BL X NZW (NZBW) hybrids.

This group of 119 NZBW animals was supplied by the Animal Research Farm of the University of Otago. They were labelled individually as depicted in the diagram in Appendix No.1. Their identity was retained throughout the experiment by repeated marking with a picric paint every month or so. Mice with NZB/BL father and an NZW mother were designated CW (cross-white mother); those with an NZB/BL mother and NZW father were designated CBW (cross-black mother, white father). This was to enable an assessment of any differences in the hybrid animals dependant on the direction of the cross. At 10 weeks of age 42 animals (= Group I) were withdrawn and placed on steroid therapy and at 22 weeks a further 27 (= Group II). These are discussed in detail later. Completely untreated animals are designated as Group III.

Groups of 16 NZB/BL mice were available and 18 NZW
mice bred at the same time as the crossbred animals. It was proposed to examine these in the same manner as the crossbreed i.e. to use them as a type of control animal.

The sex, birth range and numbers in each of the three strains are tabulated (Table 1, Appendix No.8, p.51). It will be noted that all of the 153 mice were within 7 weeks of each other in date of birth and that 77 of the 119 NZBW mice were born within 2 weeks of each other. From NZBW, NZB/BL and NZW groups postmortems were performed at fairly regular intervals to cover the life span of NZBW mice. At first the untreated NZBW animals were selected at random but after 6 months it was sometimes necessary to kill animals showing abnormalities.

The reported (Helyer and Howie, 1963a) survival time of 8 to 10 months of these NZBW hybrid animals is longer than their first impressions (Helyer and Howie, 1962) of 6 to 8 months. An attempt to anticipate renal lesions about this age explains the timing of mouse postmortems shown in Table 2 which are concentrated in the 20th to 40th weeks of age groups (Table 2, Appendix No.8, p.52). The postmortems were planned to show any sex differences or differences depending on the direction of cross in the parents of the NZBW mice. The
main aim was to obtain the pattern of early lesions, particularly renal ones, in these mice. The mice were bled regularly to obtain blood for white blood counts, haematocrit, L.E.cell phenomenon and Coombs test, as well as occasional ficin tests. Serum was obtained for protein analyses in collaboration with Dr. A. Sharard and for testing for antinuclear factors. Measurements of blood urea levels were performed on a number of animals. The last animal in the series was killed at 63 weeks of age. Over the last 7 months of the experiment the urine of every surviving animal was tested for albumin and examined microscopically at approximately weekly intervals and on occasion tested for sugar and pH. The time spent on this urine collection allowed adequate clinical appraisal over and above that of weekly weighing of each mouse. At the time this thesis was undertaken mice of the NZB/BL strain or the NZB/BL X NZW hybrid had not been tested for blood urea levels, urinalysis or urine microscopy. The L.E.cell test and Coombs test had not been studied at intervals during life and the time of onset of renal and other lesions not established. Information on these points seemed worthy of study.

It is planned to correlate as much as possible of the haematological and chemical pathological information listed above with the detailed morbid anatomical findings at postmortem examination. Later the attempts made to alter
these features by betamethasone therapy are discussed.

GENERAL OUTLINE OF SECOND EXPERIMENT

In the next major experiment (BT experiment) with the NZB/BL X NZW hybrids 34 female mice were taken at the age of 20 to 26 weeks (mean age 21.7 ± 0.5 weeks) and all were bled, tests being carried out as in the previous experiment for P.C.V., Coombs test and L.E. cell test with, in addition, blood urea examinations and latex antinuclear tests. Half of these mice were left untreated and were followed over the succeeding 3 months being weighed regularly and all of the tests mentioned being repeated after 3, 7.5, 10.5 and 12.5 weeks before the surviving mice were killed in the 13th-14th week of intensive observation, when their ages ranged from 33 to 40 weeks. As well as the blood tests mentioned, regular urine examinations were carried out for albumin, pH and sugar and some blood sugar estimations were made at the time of death.

GENERAL OUTLINE OF THIRD EXPERIMENT

A further younger group of 32 female NZBW hybrid mice (ECT experiment) were studied aged 7 to 26 weeks, particular attention being paid to their exact age to show 8 animals aged 7 to 8 weeks and 4 animals at each
of the exact ages of 11, 14, 17, 20, 23 and 26 weeks. These were not studied during their life span as this age period had already been covered in the first series. Prior to postmortem, however, haematocrit, Coombs test, latex test, and blood urea examinations were performed. Urines were examined microscopically and tested for albumin and sugar, several concurrent blood sugar estimations also being made. These mice were randomly selected from others their own age from a larger colony and should provide a picture of the average time of development of lesions, in particular the histopathologically studied renal and thymic ones. The further extensive histological examination of other organs carried out in the earlier two series was not extended to this group but spleen weights were studied to compare them with the other age groups.

CONCLUSION

In retrospect the first experiment gives considerable clinicopathological information about untreated NZB/BL X NZW hybrid mice, especially prior to and during the development of renal lesions but it gives insufficient histological information about the time of this, as nearly all animals had at least mild renal lesions when killed. As a prospective study the second experiment gives
considerably more information about the clinicopathological status during the development of renal lesions and then the histological picture of moderate to severe renal lesions. Utilising the knowledge gained from the first experiment the third experiment was planned to give information about the time of onset of renal lesions and more clinicopathological information about the early part of the life of these mice.

In reporting the results it is therefore proposed to combine the information from all of the untreated hybrid NZBW mice studied in these three experiments to give the main features seen in these hybrids. This will form a baseline against which the effects of various steroid dosages used can be assessed.
CHAPTER 8

SOME CLINICAL, HAEMATOLOGICAL AND CHEMICAL PATHOLOGICAL BASIC TRENDS IN NZB/BL X NZW HYBRID MICE

I. BODY WEIGHTS

II. HAEMATOCRIT RESULTS

III. TOTAL LEUCOCYTE COUNT RESULTS

IV. COOMBS TEST RESULTS

V. FREE CIRCULATING ANTIBODY RESULTS

VI. L.E. CELL TEST RESULTS

VII. LATEX ANTINUCLEOPROTEIN TEST RESULTS

VIII. BLOOD UREA RESULTS

IX. URINARY ALBUMIN TEST RESULTS

X. URINE MICROSCOPY RESULTS

XI. URINARY AND BLOOD SUGAR TEST RESULTS

XII. URINARY pH

XIII. OBSERVATIONS ON FEATURES SUGGESTING THE NEPHROTIC SYNDROME IN MICE WITH LUPUS NEPHRITIS
SOME CLINICAL, HAEMATOLOGICAL AND CHEMICAL PATHOLOGICAL BASIC TRENDS IN NZB/BL X NZW HYBRID MICE

It is proposed in this chapter to describe the results of the various features studied during life in the hybrid animals of both sexes, occasional reference being made to findings in the NZB/BL and NZW animals studied where this is relevant. Body weights were followed every week in the animals but have been analysed with particular reference to the ages at which therapy was commenced in treated animals. These weights in untreated animals are described in the first section. In the sections of this chapter that follow results are reported of total leucocyte counts, haematocrit, Coombs tests, ficin tests, L.E.cell tests, latex antinuclear tests, blood urea levels, urine albumin, urine microscopy, urine sugar and urinary pH. Finally a note is made on the occurrence of a nephrotic type of syndrome in the mice.

The detailed results are tabulated in the appropriate tables in Appendix No.8, p.48 et seq. with statistical analyses, where undertaken, recorded in Appendix No.9, p.162 et seq.
I. BODY WEIGHTS IN NZB/BL X NZW HYBRIDS

The hybrid animals were weighed weekly and these weights have been analysed to show some of the main features at different ages. The results are recorded in Tables 8-13 in Appendix No.8, p.58 and the statistical analyses in Appendix No.9, Stat. tables 1-17, p.164.

MALES

Aged 10 weeks

Untreated CBW males had a mean weight of $30.4 \pm 0.4$ g. (S.E.)

" CW " " " " " 30.5 ± 0.3 g.

" CBW+CW " " " " " 30.5 ± 0.2 g.

There is no evidence of any difference in weights between CBW and CW males at this age.

Aged 22 weeks

Untreated CBW males had a mean weight of $36.5 \pm 0.3$ g.

" CW " " " " " 34.4 ± 0.7 g.

The difference between these groups is statistically significant $0.01 < P < 0.05$ but the magnitude of difference is less than 2 g.

Untreated CBW and CW males weighed $35.5 \pm 0.4$ g.

Aged 28 weeks

CBW males had a mean weight of

" CW " " " " " 34.2 ± 0.9 g.

The difference between these groups is not really significant $0.04 < P < 0.05$. 


CONCLUSION

Male NZB/BL X NZW hybrid mice had attained their adult weight by about 22 weeks of age or before. There were no major differences in weights dependent on the direction of the crossbreed. In this experiment adult male mice with an NZB mother and an NZW father tended to weigh slightly more than mice of the reverse crossbreed.

FEMALES

Aged 10 weeks
Untreated CBW females had a mean weight of 24.9 ± 0.5 g.
  " CW    "    "    "    "    " 23.6 ± 0.7 g.
  " CBW+CW "    "    "    "    " 24.2 ± 0.4 g.
There is no evidence of difference in weight between CBW and CW females at this age.

Aged 22 weeks
Untreated CBW females had a mean weight of 31.3 ± 1.2 g.
  " CW    "    "    "    "    " 31.3 ± 1.1 g.
  " CBW+CW "    "    "    "    " 31.3 ± 0.7 g.
There is no difference in weight between CBW and CW females at this age.

Aged 28 weeks
Untreated CBW females had a mean weight of 33.7 ± 2.7 g.
  " CW    "    "    "    "    " 31.0 ± 1.8 g.
  " CBW+CW "    "    "    "    " 32.1 ± 1.5 g.
There is no significant difference between CBW and CW females at this age.
These observations on the weights of female hybrids were confirmed by considering those untreated animals from the second experiment with a mean weight of 29.7 ± 0.6 when their mean age was 21.7 ± 0.4 weeks (Appendix No. 8, Table 58, p. 124; Appendix No. 9, Stat. Table 40, p. 204).

Individual weights can vary considerably in small samples as is shown in the ECT Tables where animals aged 7-8 weeks varied from 17 to 28 g., the heaviest animal being one of the youngest. (Appendix No. 8, Tables 69-71, p. 140).

When uraemia developed it was common for the weight to fall, often profoundly. This is shown in e.g. Animal F45 in the BT Table whose weight dropped from 29.5 g. to 20 g. in 2.5 weeks during which time the blood urea rose (Appendix No. 8, Table 58, p. 124).

CONCLUSION

Female NZB/BL X NZW hybrid mice have attained their adult weight by about 22 weeks of age or earlier; females weigh 3-6 g. less than males of the same age. There are no significant differences in weights dependent on the direction of the crossbreed, but what difference there is shows the same trend as in males, animals with an NZB/BL mother being on the average slightly heavier than those with an NZW mother. When uraemia develops the weight falls.
II. HAIEMATOCRIT RESULTS IN NZB/BL X NZW HYBRIDS

MALES

The haematocrit (P.C.V. or Hb.) of young and adult males normally ranged from 45-50% (Appendix No.8, Tables 32-33, p.82). There was a tendency for it to drop in the older mice. In the males 6 mice had levels of 42 or below in the last bleed prior to postmortem. These showed renal lesions varying in severity from 1 with +, 2 with 2+ and 3 with 3+. Two of the animals with lesser affected kidneys had + Coombs tests. The 3 with the worst renal lesions had blood urea of 81, 90, 94 mg./100 ml. and negative Coombs tests. The slight fall in P.C.V. could be related to the renal disease or to the positive Coombs tests.

FEMALES

The young animals had P.C.V's like the males of over 45% (Appendix No.8, Tables 34-35, p.84). In the first experiment 5 showed P.C.V's dropping to under 42%, and, although the total numbers of animals were less than the males and the females were younger than their male counterparts, the fall was more definite, levels of 36, 38, 39, 40 and 40% being noted. Three of these animals were uraemic and, although the other 2 had blood urea levels of 54 and 65 mg.%, they also had 3+ renal lesions and
neither of these animals had positive Coombs tests. It seems quite likely that the anaemia is in some way related to the renal disease.

In a later experiment the mean haematocrit of 17 untreated females at 21.7 weeks of age was 44.1 ± 0.5. One of these animals was found dead with severe renal lesions and another became uraemic with P.C.V. dropping to 24% prior to death. The mean of this untreated group had fallen to 39.6 ± 1.4 at a mean age of 35 weeks and whether the uraemic animal is included or not the fall from 21 to 35 weeks is statistically significant (0.02 P 0.05, if the uraemic animal is excluded). It is established later that this fall corresponds to the time of development of florid renal lesions (Appendix No.8, Table 60, p.127; Appendix No.9, Stat. Table 43, p.208).

**CONCLUSION**

NZB/BL X NZW hybrid animals may show a fall in haematocrit which in the females in this project occurred when severe renal lesions were present but before the animals had become frankly uraemic. When uraemia develops the haematocrit falls even further.
III. TOTAL LEUCOCYTE COUNT RESULTS IN NZB/BL X NZW HYBRIDS

The results of the total leucocyte counts as performed from the tail, and not the heart, are recorded in the Tables 14-19 in Appendix No.8, p.64, and are extensively analysed in the Stat. Tables 26-37, Appendix No.9, p.189. It is proposed first to report the counts in untreated animals at different ages in each sex and to see if there are any differences between the CBW and CW crossbreeds. It has already been indicated that the actual timing of the counts and the mode of analysis are more concerned with the effects of therapy, as discussed later.

MALE MICE

No significant differences were noted at any age tested from 10 weeks to 9 months between CBW or CW male mice of the same age group. Mean figures obtained for male mice are tabulated below -

<table>
<thead>
<tr>
<th>Age</th>
<th>No. of mice</th>
<th>Mean W.B.C.</th>
<th>S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 weeks</td>
<td>65</td>
<td>10,015</td>
<td>257</td>
</tr>
<tr>
<td>av. 22 &quot;</td>
<td>41</td>
<td>8,634</td>
<td>460</td>
</tr>
<tr>
<td>av. 28 &quot;</td>
<td>CBW (10)</td>
<td>8,850</td>
<td>527</td>
</tr>
<tr>
<td></td>
<td>CW (7)</td>
<td>7,500</td>
<td>463</td>
</tr>
<tr>
<td>av. 32 &quot;</td>
<td>12</td>
<td>5,667</td>
<td>355</td>
</tr>
<tr>
<td>av. 9 months</td>
<td>10</td>
<td>5,350</td>
<td>387</td>
</tr>
</tbody>
</table>
It can be seen that there is a steady fall in total W.B.C. in male mice over the period of observation.

FEMALE MICE

A similar trend in untreated female mice was seen. Also in this sex at no age was there a significant difference between the CBW and CW animals. The counts in females regularly tended to be slightly lower than in males of the same age.

<table>
<thead>
<tr>
<th>Age</th>
<th>No. of mice (female)</th>
<th>Mean W.B.C.</th>
<th>S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 weeks</td>
<td>44</td>
<td>8,468</td>
<td>267</td>
</tr>
<tr>
<td>av. 22</td>
<td>22</td>
<td>8,136</td>
<td>525</td>
</tr>
<tr>
<td>av. 28</td>
<td>9</td>
<td>7,056</td>
<td>680</td>
</tr>
<tr>
<td>av. 32</td>
<td>6</td>
<td>5,417</td>
<td>473</td>
</tr>
<tr>
<td>av. 9 months</td>
<td>4</td>
<td>4,375</td>
<td>590</td>
</tr>
</tbody>
</table>

Total leucocyte counts from tail vein compared with blood from heart

Prior to postmortem in the early parts of the experiment blood for the W.B.C. was often collected by intracardiac puncture. It was noticed that figures from here were lower than from previous tail vein bleeds. To see if this was consistent, 15 pairs of animals were bled from both sites to examine the extent of this difference (Appendix No.8, Table 59, p.125 and Appendix No.9, Stat. Table 42, p.207). The difference was a consistent and a striking one (P 0.001) counts from the heart in each
case being about one half that from the tail. The discrepancy may be due to a technical fault in that from the heart the blood is first aspirated in a syringe, then onto a dry slide, then into a W.B.C. pipette, in contrast to the direct tail technique.

**CONCLUSION**

Total leucocyte counts tend to fall with age in both sexes of NZB/BL X NZW hybrid mice. No difference was present dependent on the direction of the crossbreed. Blood collected from the heart has lower total leucocyte counts than that from the tail and so the two cannot be compared.
IV. COOMBS TEST RESULTS ON NZB/BL X NZW HYBRIDS

FEMALE HYBRIDS

In the third experiment with 32 animals, none of the 20 animals tested under the age of 20 weeks had positive tests. In the older 12 animals there was one positive test at 20 weeks and one equivocal, a further 1/4 equivocal test at 23 weeks and 0/4 positive at 26 weeks (ECT Tables 69-71, Appendix No.8, p.141).

In the second experiment there were 4 positive and one equivocal test in 34 females of mean age 21.7 ± 0.4 weeks and these included the youngest animal, aged 19.5 weeks, in which a positive Coombs test was noted. Of the 17 negative animals studied for a further 3 months who were untreated only one positive in the 30th week and one equivocal test in the 24th week resulted, although the test was repeated on four occasions. These positive results were transitory (Appendix No.8, Table 63, p.130).

The test was not available in the early part of the first experiment when the reagent was being prepared. When introduced one positive and 2 equivocal results showed in 9 untreated females ranging in age from 25-28 weeks. In subsequent bleeds a few more became positive so that 3/9 positive plus one equivocal result had occurred in animals up to 35 weeks of age (Tables 28 and 29, Appendix No.8, p.78).
In all of the females discussed the test tended to fluctuate, varying from positive to negative over periods as short as three weeks. In no case was a level of over 2+ recorded and usually only +. It does appear that the more frequently one repeated the test the higher the incidence of positive findings.

MALE HYBRIDS

When the test was introduced 2/16 untreated males aged 25 and 28 weeks had positive tests, subsequently a further 3 became positive with one equivocal test. It will be remembered that in this series the number tested was being steadily reduced due to elective post-mortems. Of 5 animals surviving over the age of 40 weeks, 3 had positive tests (Tables 26 and 27, Appendix No.8, p.76). In only one male animal did the test remain persistently positive, sometimes at the level of 3+ (CW male 58 in Table 27, Appendix No.8, p.77). Throughout this time the mouse was not anaemic, with P.C.V. ranging from 45-50%. Thus, although this mouse also had a positive ficin test, there is no evidence that haemolysis was occurring, if it was it was well compensated.

Coombs test and haematocrit

Average haematocrit (Appendix No.8, Tables 32-35, p.82) in males with positive Coombs tests was 44% and
in females 42%. Allowing for the fact that those animals surviving long enough to get positive Coombs tests also tended to have renal disease, it is clear that there is no strong evidence for or against haemolysis. Some of these animals had higher haematocrits than their fellows with negative Coombs tests.

It has not been established whether the positive Coombs test in the hybrid animals is due to the same antibody as occurs in the NZB/BL animals. If it follows the pattern of human S.L.E. it may not be the same type of warm autoantibody that is usually found in cases of A.I.H.A.

**NZW animals**

No positive results were noted in the 10 NZW mice tested, although an occasional equivocal result was seen (Table 30, Appendix No.8, p.80).

**NZB/BL animals**

As would be expected 6/7 NZB/BL animals in the first experiment had positive Coombs tests (Table 31, Appendix No.8, p.81). This serves to control the hybrid results. It is also noted that in the NZB/BL mice once it is definitely positive, the test tends to remain so, apart from perhaps one negative fluctuation and then it usually progresses in severity. This expected finding is also a control one showing that the usual failure of
of the hybrid NZBW tests to increase is a real one. The Coombs test is further explored in later experiments with NZB/BL mice.

CONCLUSION

Moderate numbers of NZB/BL X NZW hybrid animals of both sexes have positive Coombs tests. These were first noted in females in the 20th week of age. The actual incidence of positive tests varies with the frequency of testing because they fluctuate in intensity and between positive and negative. They are never very strong, nor are they associated with severe anaemia in the few females studied up to 40 and males up to 62 weeks of age.
V. FREE CIRCULATING ANTIBODY RESULTS IN NZB/BL X NZW HYBRIDS.

The ficin technique to estimate free circulating erythrocyte autoantibody was used in parallel with the Coombs test measuring fixed erythrocyte autoantibody in the first experiment. The ficin test was performed on two occasions in the NZB/BL X NZW hybrids, once at about 26 weeks of age and again at 39-40 weeks of age. As the points are general ones all of the NZBW animals tested in the first experiment are presented together.

RESULTS

In the NZB/BL X NZW hybrid on the first test occasion there were 69 tests, 42 males and 27 females, showing positive (+) agglutination in 4 and an equivocal (±) result in one. Of these 5 tests 3 corresponded with positive Coombs tests and 2 did not. When retested some 14 weeks later there were 22 NZBW animals still in the experiment and these included 5 positive and 2 equivocal results, 5 of these 7 results corresponding to animals who showed positive Coombs tests at some stage (Appendix No.8, Tables 26-29, p.76). Only one of these positive tests was a strong one (visual agglutination) and corresponded to a 2+ Coombs result. This CBW female mouse may well have had a haemolytic process as its P.C.V. dropped to 37% when the blood urea was normal at 38 mg %.
It should be noted that only 2 of the animals with initially positive tests were still alive for the second test and both of these still showed just (+) agglutination. The titre of free circulating antibody had not increased which is in contrast to what one sees in NZB/BL animals. All of the NZB/BL animals tested changed from negative to positive between the two ficin tests. None of the NZW animals did so (Appendix No.8, Tables 30-31, p.80).

CONCLUSION

There is a weak circulating erythrocyte autoantibody in a small number of NZB/BL X NZW hybrids. It is not present as frequently in a free state, as judged by the ficin technique, as it is in a fixed state, as judged by the Coombs technique.
VI. L.E. CELL TEST RESULTS IN NZB/BL X NZW HYBRIDS

The programme of bleeding the mice has already been outlined, the criteria adopted and the method of examining the slides, including a search for five minutes. In this part of the experiment over 600 L.E. cell test slides were examined by the author, including the NZB/BL and NZW tests, as well as the hybrid ones. These are tabulated in L.E. Tables (Appendix No.8, Tables 20-25, p.70).

HYBRID MALES

In 60 NZBW males aged about 10 weeks there were no positive tests. In 37, aged about 20 weeks there were no positive tests. There was 1/16 positive test in untreated males at 26 weeks. By 32 weeks 3 more out of 11 untreated animals had become positive but no more in the few animals examined up to one year of age.

HYBRID FEMALES

At 10 weeks all 44 females had negative tests. At 20 weeks all 21 untreated animals had negative tests. The first 2 positive tests were in animals of 27 and 32 weeks out of 12 untreated surviving around this age. Other animals becoming positive at some time brought the total female L.E. positive cases to 5/11 untreated animals
tested over the age of 27 weeks. The range of ages in the small groups surviving at any one bleed towards the end of the experiment makes it difficult to put these positive tests in perspective. However they represent almost 1/2 of the animals still left surviving after 27 weeks, the time of the first positive test being noted.

In the second experiment 3/34 animals tested at mean age of 21.7 weeks had positive tests and these animals were all aged 20–21 weeks (Appendix No.8, Table 61, p.128). Of 15 untreated animals retested at 25 weeks of age and 29 weeks of age no positive tests were found but 7/15 were positive at 32 weeks. This confirms the view that positive tests are distinctly uncommon before the age of 30 weeks. Subsequently one further test was positive so that over half of the female hybrid animals will show positive tests by the age of 35 weeks if tested repeatedly.

Subsequent pattern of positive L.E. cell tests in hybrid animals

It can be seen from the L.E. Tables that the subsequent tests of an animal showing a positive result tend to vary. It may be an isolated event followed by a series of negatives. It may remain strongly positive at the subsequent bleed. It may be positive, revert to negative over periods of up to several months and then become positive again and remain so. This could equally well
reflect the insensitivity of the test or a real fluctuation in strength of L.E. factor.

**Inbred strains**

No positives were found in the 77 tests done on the **17 NZW animals** or in the 45 tests done on **12 NZB/BL animals**. While it is noted that these tests were not performed on many of these animals in the older age groups because of the design of the experiment, these negative tests in the two different strains do point to the significance of the positive tests in the NZB/BL X NZW hybrids.

**Correlation with Coombs tests**

When an attempt is made to correlate the 9 positive L.E. cell test results in the first experiment with Coombs tests it is found that of the 8 animals in which the Coombs test was performed 2 had positive Coombs tests on one or more occasions, 4 had negative tests and 2 had equivocal tests on one occasion. Those that were positive were not necessarily so at the same time as their L.E. cell test was positive. As discussed under Coombs tests, many animals had positive Coombs tests without positive L.E. cell tests and this obviously also applied to all the NZB/BL animals. It is concluded that there is no close correlation between the occurrence of positive L.E. cell tests and positive Coombs tests in the NZBW hybrids.
The relationship of positive L.E. cell tests to severity of renal lesions in this experiment is discussed after the renal lesions are described.

The effect of temperature on the L.E. cell test

The effect of temperature on the L.E. cell test was investigated in a group of 16 NZBW female animals aged 32 weeks by carrying out the standard preparation of L.E. films as already described at room temperature and simultaneously duplicating the procedure, except for the incubation which was done at 37°C. The number of positives on this particular occasion was 6/16 under the usual room temperature conditions, compared to 3/16 in the tests carried out at 37°C. Other conditions such as viewing time were as similar as possible and the quality of the preparations was reasonably good on this occasion. All three positive at 37°C were positive in the room temperature test.

Practically the only variations in quality of the preparations encountered was an insufficient quantity of white blood cells present on the film. This could be expected more often in the steroid treated animals where the total leucocyte count was lowered. Some films were of poor quality and a few films were quite unsatisfactory. The relatively smaller numbers of polymorphonuclear leucocytes compared with lymphocytes in the circulating
blood of mice may reduce the chances of the L.E. phenomenon occurring compared to human blood. However, polymorphonuclear leucocytes are relatively more common in the steroid treated animals so this would favour the production of the phenomenon, if the L.E. factor were present.

**Summary of L.E. cell test results**

The L.E. cell test used is an insensitive one, subject to temperature variations, variations in the quality of the films and variations between positive and negative tests in the same animal. Although positive results are occasionally seen in NZB/BL X NZW female hybrids as early as 20 weeks of age they are uncommon before 30 weeks. By 9 months of age over half the females will show positive tests if these are repeated. Few positive tests were seen in males in the age groups studied, so by inference from previous reports of Helyer and Howie (1963a) they must become positive much later in males. No correlation was found with the incidence of positive Coombs tests. No positive results were seen in the NZB/BL and NZW mice studied.
VII. LATEX ANTINUCLEOPROTEIN TEST RESULTS IN NZB/BL X NZW HYBRIDS

FEMALE HYBRIDS

In a group of 34 NZB/BL X NZW female hybrids tested at mean age 21.7 ± 0.4 weeks there were 6 positive tests (17%), 2 quite strongly so. Of 17 of these animals with negative tests 3/16 were positive 3.5 weeks later, 8/16 10.5 weeks later and 14/16 were positive at some stage over the 3 months of observation. Apart from minor fluctuations when weak, once the test became positive it tended to remain so and to become stronger. Allowing that this was a group of 17 selected to have negative tests at 21.7 weeks there is thus a very high chance that animals of mean age 35 weeks will have a positive test (Appendix No.8, Table 62, p.129).

The 3/17 negative tests are of interest. One animal died with severe renal disease at 24 weeks with a negative latex test on the one occasion it was tested. The 2 other animals both had severely affected kidneys (3+). On the other hand, some animals with a minor or moderate degree of renal involvement had positive tests. Even the frankly uraemic animal in this series (F45, Table 62, Appendix No.8) had its first + positive test just before death, although throughout the three month period it had been noted to have albuminuria and presumed renal damage.
These figures show that severe renal disease may be present in the hybrid females without positive latex tests.

The fairly high percentage of positive tests when first performed in this series (17%) at an age (21.7 weeks) when renal lesions are just developing suggests that some latex tests may be positive before the renal lesions develop. However when this point was explored in the 32 females killed at 3 weekly intervals up to the age of 26 weeks, no positive latex tests were found in animals with definitely normal kidneys. Again, of the 32 animals in this young series, 6 had renal abnormalities that ranged from + to 4+, no positive latex tests were found, further supporting the conclusion in the previous paragraph that renal disease, sometimes severe, may be present with a negative latex test.

CONCLUSION

A high proportion of female NZB/BL X NZW female hybrids develop positive latex antinucleoprotein tests by the mean age of 35 weeks. Animals dying of uraemia prior to this age may have negative tests. There is no definite evidence of positive tests occurring before the onset of renal lesions.
VIII. BLOOD UREA RESULTS IN NZB/BL X NZW HYBRIDS

In this section the range of blood urea levels seen is discussed and then observations are made on the development of uraemia and on the correlation between urinary abnormalities and blood urea levels.

1. BLOOD UREA RANGES

NZB/BL X NZW hybrids

To study the range of blood urea levels seen in NZBW hybrids, 35 female animals aged 5 months were bled on 4 occasions, over a period of 3 months. The 170 blood urea values obtained are tabulated in Table 4 (Appendix No.8, p.54). To assess the normal range one animal in whom the level rose to 280 and 1000 mg. in the last two bleeds has been excluded. Three initially high readings were repeated and found within the normal range. Three low values of 4, 4 and 7 mg. were unacceptable as judged by ordinary levels in previous and later tests. These 6 estimations were therefore excluded.

The observed range was 12-80 mg./100 in treated and untreated animals, no difference being noted in the age group studied. If we now take as the "normal" range that in which 95% of the total number of observations lie (Stewart and Dunlop, 1962) the range is 16-76 mg.%. The method is not a particularly accurate one with an
acceptable error of ±10% (Varley, 1962) under ordinary laboratory conditions where blood is measured from a container, probably even greater when it has to be accurately measured directly from the tail and also when the volume (0.05 - 0.1 ml.) is, perforce, less than that usually used in the method (0.2 ml.). If we take ±10% as the range of variation expected then all (not just 95%) of the observations fall within the stated normal range ±10%. Probably the range of variation is even greater.

This applies to female hybrid mice ranging in age from their 20th to 40th week. It is only at the last bleed when the mice ranged from 32-40 weeks of age that the whole batch of results is in the upper part of the range, viz. 32-76 mg./100 ml.

A further approach, using a different group of animals, to the question of normal blood ureas is to consider the 31/32 female animals aged 7-26 weeks in the ECT Tables (Tables 69-71, Appendix No.8, p.141) that were not uraemic and which at postmortem did not have severe renal lesions. Here the range of observed blood urea values was 20-74 mg./100 ml. which corresponds with that stated above. Within this range, however, the 12 animals aged 7-11 weeks had urea levels of 20-38 mg./100 ml., all but 2 being below 30 mg./100 ml., whereas all of the
19 older animals lay between 30 and 74 mg. %, all but 2 between 30 and 57 mg. %.

**Variations in urea results**

The actual blood urea level varied from animal to animal at the same age and also in the same animal, where there were quite wide variations from time to time and even when repeated within a few days of each other. As with any biochemical test (Varley, 1962) a result that does not fit in with those before it or with the clinical status bears repeating before undue emphasis is placed on it. The opportunity to do this is not present if a sample is taken only at postmortem so in the latter part of these experiments the animals to be killed were bled on the previous few days so that an abnormal result could be rechecked.

**NZB/BL mice**

NZB/BL male animals aged exactly 8 months had blood ureas ranging from 32-56 (with an average of 46) mg./100 ml. in a group of 16 animals. Combined with a further similar sized group bled at 8 and 9 months a blood urea level range from 20-66 mg. was obtained in the NZB/BL animals.

**Blood urea ranges in mice**

Holmes and Burnet (1963) note a range of
50–80 mg./100 ml. in the NZB/BL mice, while Meier et al. (1962) state that mice have a normal blood urea level of 40–60 mg./100 ml. Certainly in the range of 60–80 mg. it is difficult to evaluate the significance of a result as these tend to fluctuate, partly due to the poor accuracy of the method. The upper limit of the range is the important one obviously. It tends to be higher with age, as indicated above, but variations are such that this cannot be relied on in the individual case. An upper level of 66 mg./100 ml. in NZB/BL animals and 74 mg./100 ml. in the hybrid animals seems reasonable from this study. Unfortunately, however, the method only gave clear-cut evidence of abnormality when there was a steady rise in the level. This was a late phenomenon almost always well anticipated by other abnormal findings. Nevertheless, despite its earlier fluctuations and inconsistency, it was a relentlessly progressive rise once the urea level was clearly out of the normal range. Fig. 10 shows the high levels to which the urea may rapidly rise in the last few weeks of life in both NZB/BL X NZW hybrids and NZB/BL animals.

2. OBSERVATIONS ON THE RATE OF ONSET OF URÆMIA

Three of the matched female hybrid mice aged 6½ months were chosen as approaching the age at which renal failure would be expected and their blood urea levels
were followed on 8 occasions over the next 8 weeks until the condition of one was such that the trio had to be killed.

Results are tabulated in Table 5 (Appendix No.8, p.55) and the findings for one animal are shown in Fig.10. When arranged in such table form it is obvious that the 3 results on one day (11.12.63) were too high, probably by a factor of 2X. If we omit this day the table shows the way blood urea levels may fluctuate on repeated testing about the normal range before a fairly rapid rise in CW female 12 to a level of 300 mg./100 ml. in less than 2 weeks from a normal reading. Also the table shows that there may be quite marked albuminuria in the presence of a normal blood urea (CW female 12 and 13). Subsequent experience has shown this to be the usual occurrence.

The rapid rise of blood urea which was associated in CW female 12 with listlessness and weight loss is shown graphically (Fig.10). Similar rates of rise are shown in one 19 month old NZB/BL animal which was in a pilot group tested and in another NZBW female animal observed in a later experiment but shown in this graph to illustrate this trend.
FIG. 10. shows rapidity of terminal rise in blood urea level when observed at frequent intervals.

FIG. 11. shows pattern of albuminuria with fairly rapid increase to high levels.
3. OBSERVATIONS ON BLOOD UREA LEVELS AND URINARY ABNORMALITIES.

MALES

Groups of 5 NZBW and 2 NZW male animals showing urinary changes were tested for blood urea levels about the same age as the above female NZBW mice. The results with the urine findings that prompted the blood urea testing are shown in Table 6 (Appendix No.8, p.56). This shows that mice may have consistent or variable mild to moderate albuminuria with normal blood urea levels. This applies to NZW male mice as well as NZBW male mice. One of each group had shown definite microscopic abnormality 4 weeks before the blood urea examination so that the finding of many casts is not necessarily an immediately ominous sign, especially in males.

FEMALES

Table 7 (Appendix No.8, p.57) shows a further group of 8 NZBW animals with blood urea and urinary findings at ages 7-8 months. They are females who were chosen both with and without urine abnormalities. There is no absolute correlation between abnormal urinary findings and raised blood urea. One animal, CW female 18, showed a transient rise in blood urea to 128 mg.% with a subsequent drop into the normal range. This animal
had up to 300 mg./100 ml. of albumin in the urine and histologically had severe active renal lesions.

One further animal showed a single transient blood urea level of 120 mg.% (CW female 38 in Table 7) with moderate glomerular hypercellularity and fibrinoid at postmortem. If this urea level was correct then this was the only animal seen with a normal urine and a raised blood urea. No animal was seen with a persistently raised blood urea and a normal urine if the urine had been tested repeatedly. In contrast, CW female 23 had persistent severe albuminuria and an equally consistent normal blood urea. This pattern was seen in later experiments also with heavy albuminuria prior to any rise in blood urea, such urea rise being preterminal.

CONCLUSION

In NZB/BL X NZW hybrid female animals that were not frankly uraemic blood urea levels ranging from 16-76 mg./100 ml. were noted. Levels tended to be in the lower part of this range in animals under the age of 4 months, to lie in the middle part of the range in the 4-6 month group. In an individual animal, however, unless the urea was clearly rising out of the range
variations in readings within the range were not helpful. Once the level does rise out of the range it continues to rise steadily and in a few weeks female animals become markedly uraemic and have to be killed. Albuminuria, sometimes with urinary microscopic abnormalities, was almost always present if the blood urea was raised.
IX. **URINARY ALBUMIN TEST RESULTS IN NZB/BL X NZW HYBRIDS.**

Studies on the urinary albumin levels were aimed at seeing if proteinuria developed in the NZB/BL X NZW hybrids, together with the characteristics of such proteinuria. These include the degree of proteinuria, its time of onset, constancy or fluctuation in intensity, and whether it was more prominent in females or males. It was also desired to correlate the proteinuria with any observed microscopic urinary abnormalities.

**Method**

An extensive screening procedure was carried out over the last 8 months of the first experiment. By the beginning of this time all animals were 5½ - 7 months of age. Several observations are included from the other experiments but these were less extensive.

Tables 38-43, Appendix No. 8, pp. 88-102, show the results of approximately weekly testing of urines for albumin and the microscopic findings if these were performed at the time. These weekly tests were continued until the death of the animal.
RESULTS

1. CHARACTERISTICS OF THE ALBUMINURIA

Preliminary and subsequent testing on younger animals had indicated that up to 30 mg. of albumin/100 ml. of urine was often found, and also noted in stock animals. Up to and including 30 mg. has been regarded therefore as normal and 30+ as of equivocal importance.

At the time the regular testing was begun 26 females were surviving. Of these one, aged 6½ months, already showing 1000+ mg. of proteinuria, 3 more were showing 100+ mg., 2 showing 100 mg. and 3 30+ mg. i.e. 9/26 showed a possibly significant abnormality and 6/26 a probably significant abnormality.

By contrast, of 40 males of the same age, none showed the highest levels of albuminuria, one showed 100+ mg., 3 showed 100 mg. and 6 showed 30+ mg. i.e. 10/40 showed a possibly significant abnormality and 4/40 a probably significant abnormality.

Subsequent testing confirms this first impression, showing a more abrupt increase on the part of the females than the males. Fig.11 shows typical patterns of albuminuria seen in individual females and males respectively over a period of 6-12 weeks.
prior to death. The animals were killed before being moribund so this is not the full extent of albuminuria. The pattern in the female tends to rise more rapidly and to higher levels than in the males. Indeed only one test of 1000+ mg. was ever recorded in a male and this on only one occasion, whereas 7 different females in the first experiment and some in later experiments showed these high levels. This, however, may only reflect the earlier onset of disease in females and the lack of severely affected males in this series. In 2/7 of the females the level was repeated, in the one case over 4 successive weeks and in the other some 6 weeks after the first recording of 1000+ mg. (CW female 17 in the Graph).

One of the only 2 females still alive at 9 months of age in the first experiment had had no urinary abnormality whatsoever. Much longer living males had occasional records of 30+ mg. for albumin or occasional casts would be noted. In the male urinary tables a tendency can be seen every few weeks for an intermittent increase in albuminuria.

In the second experiment (Table 64, Appendix No.8, p.131) of 34 female NZBW animals tested at the mean age 21.5 weeks, 3/34 had moderate to marked albuminuria and these comprised 2 animals aged 20 weeks
with 100 mg. of albuminuria and one aged 22.5 weeks with 300 mg. Subsequently at 24 weeks 1/15 further untreated animals had developed albuminuria, at 32 weeks 5/15 and at 35 weeks 10/15. This underestimates the incidence of albuminuria because of the selection at 21.5 weeks. It is of some interest that this last group included 4 animals who fairly abruptly showed levels of 300 mg. and this included the 3 oldest animals whose actual ages ranged from 37.5-39 weeks. Again some fluctuation was present in the amount of albuminuria.

In the final experiment where female animals were studied in groups of 4 none of the 28 animals tested up to, and including the age of 23 weeks, showed more than 30+ mg. of albuminuria. However at 26 weeks 2/4 showed 100 mg. or over. This trend is similar to that seen in the previous experiments (Tables 69-71, Appendix No.8, p.141).

2. ALBUMIN AND FORMED ELEMENTS IN THE URINE

Tables 38-43 (Appendix No.8, p.88) also show the relationship between observed urinary protein levels and observed microscopic findings in the urinary sediment. Examples can be seen in all groups of animals of normal or slightly raised urinary albumin levels with
definite microscopic abnormalities, also of gross proteinuria with no casts or just an occasional hyaline cast. All the same, any microscopic abnormality, apart from an occasional cast, either hyaline or granular, tends to be associated with a definite albuminuria though the reverse is by no means the case. It is for this reason and the numbers involved that after the first 2 complete urinary screenings urine showing 30 or less mg. of albumin were not examined microscopically in the succeeding 2 months though they were for the last 4 months of the experiment by which time the number of experimental animals had been reduced to more manageable levels.

3. SUMMARY OF GENERAL PATTERN OF ALBUMINURIA

Levels over 30 mg./100 ml. were not found in animals tested below the age of 20 weeks by which time 9% of female animals showed levels of 100 mg. or over. The incidence of high tests increased fairly steadily in females over the following weeks, approximately one quarter of animals having these levels by the age of 26 weeks, at least one third by the age of 32 weeks with a more rapid rise so that at least two third had shown these higher levels of albumin by the age of 36 weeks.
Once the level of 200 mg. was repeated it tended to remain elevated. Levels of 300 mg. rarely dropped below 100 mg. again and such a drop was only transitory. The rate of rise of urine albumin levels was over weeks rather than months. Levels of 1000 mg.+ were sometimes seen but this was unusual.

The onset of albuminuria was less clear-cut and later in males. Although males were studied for some 20 weeks longer than females, they seldom showed the high levels of albuminuria noted in the females.

High levels of albuminuria may occur without the presence of formed elements in the urine but the reverse is unusual.
X. URINE MICROSCOPY RESULTS IN NZB/BL X NZW HYBRIDS

The urines of many of the NZB/BL X NZW hybrids which were regularly tested for albumin were also examined microscopically, the method being detailed with some experimental justification for it in Appendix No.3, p.14.

RESULTS (Tables 38-43, Appendix No.8, p.88).

Urinary casts were frequently found in the more severely affected animals and could be present in large numbers, 20+ per low power field. Their correlation with renal lesions is discussed later.

The urinary casts seen were quite similar in morphology, though smaller, to those seen in human renal disorders. Finely granular casts were frequently noted, as were hyaline casts, especially in fresh urine. Large broad casts were common in the later stages. Cellular casts were not as common as granular ones but the distinction was not always clear-cut (Fig.30). Casts were occasionally bile stained in NZB/BL animals. The casts were not doubly refractile with polarised light. No doubly refractile lipid bodies were found in the urine after examination of any slightly suspicious droplets. Cylindroids were common in the urine,
especially in older male animals. They appeared from the renal sections to be formed in the distal and collecting tubules.

Red blood cells were rarely seen in the urine and when they were it was, with one exception, in females, so could well have been related to cyclical hormone changes. White blood cells were occasionally seen but it is difficult to separate them with certainty from tubular epithelial cells (Page et al. 1960). White blood cells were not seen in clumps, and they usually are at some stage, if they are significant of urinary tract infection (Page et al. 1960). These cells are not further discussed in this study.

Crystals were often seen and these had the appearances of calcium oxalate, triple phosphate and uric acid crystals. They were doubly refractile using polarised light. Amorphous deposits of urates were also seen.
XI. URINARY AND BLOOD SUGAR TEST RESULTS IN NZB/BL X NZW HYBRIDS.

Glycosuria and blood sugar levels

With occasional testing of urines it was at first thought that glycosuria never occurred in the hybrids. However if animals were tested very frequently the Clinistix on occasions did show a faint blue and rarely a deep blue colouring, indicating glycosuria. This was found using different bottles of Clinistix. The faint trace has been noted occasionally from 11 weeks of age and the positive tests from 14 weeks. It may merely mean that the Clinistix is too sensitive for mice and detects minute traces of glucose. In another colony of 81 hybrids recently tested, 22 showed faint traces of glucose. Six of a colony of 75 NZB/BL mice also showed it but no frank glycosuria.

To explore the point further, some blood sugars were determined on the AutoAnalyzer. Of 4 animals aged 26 weeks (ECT Tables 69-71, Appendix No.8, p.141) who showed a trace of sugar at the time, one had had a definite positive test. The blood sugars determined at the same time as a faint urinary trace of sugar was detected were 153, 168, 153 and 144 mg./100 ml. A further 12 females of mean age 35 weeks had random
blood sugars ranging from 102-168 mg./100 ml. but no consistent pattern was evident between blood level of sugar and presence or absence of a faint trace of urinary glucose (B.T. BW Table 65, Appendix No.8, p.132) which had been present at some time in 5 of these 12 animals tested on 4 different occasions.

CONCLUSION

Repeated testing shows faint traces of urinary sugar and rarely definite glycosuria in a moderate number of hybrid animals. There is no clear correlation with blood sugar levels. The mechanism involved in this glycosuria is worthy of further study.
XII. URINARY pH

This was retested on 3 occasions over a 3 month period in a group of female mice (B.T. BW Table 66, Appendix No.8, p.134). The range at 21.7 weeks was pH 5.6-7.1; at 30 weeks it was 5.8-7.1 and at 34 weeks it was 5.9-6.4, with no definite pattern evident in the group or for individual animals followed over this time.
XIII. OBSERVATIONS ON FEATURES SUGGESTING THE NEPHROTIC SYNDROME IN NZB/BL X NZW HYBRID MICE WITH LUPUS NEPHRITIS

One untreated female mouse in the first main experiment had ascites and bilateral pleural effusions at postmortem. During life an excessive weight gain (4 g, in one week) had been commented on at the 8th, the 11th week of age and again at the 19th week when it was noted clinically that the abdomen was swollen. This persisted until the postmortem in the 31st week of life. Whether the first two spurts are significant is not obvious because neither took the mouse to an untoward weight (over 36 g, for a female of this strain is quite unusual). The final spurt of weight gain which brought the mouse to over 40 g, in the 19th week was certainly unusual and probable ascites was present from that time until the postmortem 11 weeks later. Proteinuria generally 100 mg.+ was observed but there is no measurement of the volume of urine secreted or the albumin content of the urine prior to the putative date of onset of ascites.

The ascitic fluid itself was tested with an Albustix strip and showed a low level of protein (100 mg./100 ml.) indicating that the effusion in the serous cavities was a transudate.
postmortem blood urea was 142 mg./100 ml. and this correlated well with a urea level in the ascitic fluid of 141 mg./100 ml. The renal lesions were graded 3+ for severity and 2+ for activity but no special features were detected to indicate why this mouse developed ascites. In particular, with the light microscope, the basement membrane seemed no different to other mice. It is also to be noted that from the main urine chart (Mouse CBW F.16, Table 40, Appendix No.8, p.96) that microscopic examination of the urine on 6 occasions showed 1-4 casts/L.P.F. on one occasion but was normal on the other 5 tests. L.E.cell tests at 10, 20 and 26 weeks were negative as was the Coombs test at 26 weeks of age.

In another group of 34 female NZBW mice one other female mouse, unsuspected clinically of having ascites, and weighing 30 g., was found dead in the 23rd week of life with pleural and peritoneal effusions as well as pale kidneys which, unfortunately, were autolytic. This animal had a negative L.E. test, latex test and Coombs test 2 weeks before death, at which time the blood urea level was 42 mg./100 ml. and the P.C.V. 46 vol.%. Heavy albuminuria was not noted on the one test performed.
CONCLUSION

A nephrotic type of syndrome was seen in 1/80 female mice but not seen in 66 males. A further female mouse probably had a similar syndrome, making a total of 2.5% for females. This may be present from 4 months of age judging by excessive weight gain.
CHAPTER 9

RENAL MORBID ANATOMICAL AND HISTOPATHOLOGICAL FINDINGS IN NZB/BL X NZW HYBRID MICE

GENERAL

EXPERIMENTAL

A. RENAL FINDINGS IN NZB/BL X NZW HYBRID MICE

B. METHOD OF ASSESSMENT OF RENAL LESIONS

C. CHRONOLOGY OF DEVELOPMENT OF RENAL LESIONS IN NZB/BL X NZW HYBRID MICE

D. CORRELATION WITH RENAL LESIONS
   1. L.E. cell tests.
   2. Coombs tests.
   3. Urinary findings (i) Albuminuria (ii) Casts
   4. Macroscopic descriptions

E. SUMMARY OF RENAL FINDINGS IN NZB/BL X NZW HYBRID MICE
The general anatomy of the mouse kidney will be outlined in this chapter then (A) a description given of the lesions seen in the hybrid animals after which (B) a method of assessing these lesions is described; (C) the development of the renal lesions is traced and the pattern of disease in the two sexes reported. (D) An attempt is made to correlate some of the clinicopathological findings with the microscopic renal lesions. This is done with the (1) L.E.cell test; (2) Coombs test; (3) albuminuria and casts and (4) the macroscopic renal findings. Finally, (E) a summary of the findings is given.
GENERAL OBSERVATIONS ON THE MOUSE KIDNEY

A median section of a mouse kidney shows the cortex containing mostly convoluted tubules, as well as the glomeruli, and the pyramidal medulla comprising straight tubules radially arranged with medullary rays projecting from the base of the pyramid into the cortex. The apex of the medullary pyramid is a single papilla projecting into the funnel-shaped renal pelvis (Fig.12). The nephron has a similar structure to that of man (Snell, 1941). Bloom and Fawcett (1962) note that in the normal glomerulus (Fig.14) epithelial nuclei, which are larger and paler, are ten times as common as the smaller darker endothelial nuclei. The site of an nucleus is by no means always obvious in relation to a capillary lumen. Estimations of hypercellularity are completely dependent on similar thicknesses of sections, not easy to achieve at 3 μ with this particular tissue. It was early in this experiment noted that the epithelial lining of Bowman's capsule varies in height, being flat in females and more cuboidal in males, as previously indicated by Snell (1941). The difference is such that one can sometimes tell the sex of a mouse from the height of the cells of Bowman's capsule. Another difference from
FIG. 12. Mouse kidney showing single renal papilla projecting into funnel-shaped renal pelvis. (HE X22).

FIG. 13. Three affected kidneys from NZB/BL X NZW female hybrid animals aged 26 weeks showing varying degrees of pallor.
man is that it is much easier to identify the cells of the juxta glomerular apparatus in the mice because their granules are prominent.

The interstitial tissues of the mouse kidney are inconspicuous. Helyer and Howie (1963b), as well as Holmes and Burnet (1963), have described the groups of round cells, including plasma cells, that are seen around the hilar vessels of the NZB/BL animal and also extending into the kidney around the vessels (Fig. 39). Helyer and Howie (1963a) reported similar changes in the NZBW hybrids. Holmes and Burnet (1963) noted that some lymphoid changes occur with age in all mice. A moderate sized hilar collection can be seen in a microphotograph in the volume edited by Snell (1941) on normal mouse histology but the lymphoid cells do not receive comment.

(A) RENAL FINDINGS IN NZB/BL X NZW HYBRID MICE

I. MACROSCOPIC FINDINGS

The kidneys in severely affected animals became pale and swollen and in older animals sometimes contracted and granular. In one female animal found dead at 24 weeks of age they were swollen, pale and haemorrhagic. Fig. 13 shows pallor in a kidney from a female mouse aged exactly 26 weeks. These are the
FIG. 14. Normal glomerulus. Most of the nuclei are pale and epithelial. Note dark endothelial nuclei in interstitial tissue. (HE X1044).

FIG. 15. Glomerulus showing moderate but definite diffuse endothelial hypercellularity. NZB/BL X NZW female aged 27 weeks. (HE X512).

FIG. 16. Glomerulus showing slight increase in cellularity, which is endothelial in origin. Same kidney as Fig. 14. NZB/BL X NZW female aged 17 weeks. (HE X1044).
earliest definite colour changes noted, they became increasingly common in females in the 30-40 week age group but were rare in males examined.

Kidney weights

FEMALES

In the 7-8 week age group the average weight was 235 mg. (body weight 21 g.); kidney weights tended to increase with age until at adult ages of 20 weeks and weight 32 g. the average kidney weight was 295 mg., these being histologically within normal limits. It is clear from Tables 69-71, Appendix No.8, p.140 that kidney weight is roughly proportional to body weight (the body weight g. x 9-12 = kidney weight in mg.). This relationship breaks down when uraemic animals are observed where, with weight loss and large kidneys, a factor of 15-20 is more appropriate (Appendix No.8, PM 311, Table 71, p.144 and mouse F45 in Tables 58-59, p.124). In the group of adult females studied in the first (CT) experiment and covering a wide range of renal involvement the mean kidney weight was 360.5 ± 24.5 mg. (Tables 10 and 11, Appendix No.8, p.60 and Stat. Table 25, Appendix No.9, p.188). In the experiment where the untreated hybrids were of mean age 35 weeks the mean kidney weight was 355 ± 12.2 mg. (Appendix No.8,
FIG. 17. Renal cortex showing glomerular hypercellularity in oldest (63 weeks) NZB/BL X NZW male hybrid examined. (HE X270).

FIG. 18. Glomerulus showing considerable hypercellularity, many of the dark nuclei being endothelial; fibrinoid and early wire loop changes also present. Same animal as Fig. 17. (HE X924).
It was found, then, that in adults the kidney weight is greater in older adult female animals. In the first experiment the kidney weights were also analysed to see if there was any significant difference dependent on the direction of the hybrid cross. None was found (Stat. Table 25, Appendix No.9, p.188).

**MALES**

In the adult males of the first experiment the mean kidney weight was $480.5 \pm 15$ mg. Again no significant difference was found dependent on the direction of the crossbreed (Tables 8 and 9, Appendix No.8, p.58 and Stat. Table 25, Appendix No.9, p.188).

**II. MICROSCOPIC FINDINGS**

The figures are selected to illustrate points. A given figure often shows a number of features of the disease process apart from those indicated in the caption.

**GLOMERULI**

One of the earliest and usually the earliest renal lesion detected was a hypercellularity of the glomeruli (Figs. 15 and 16). This was often striking under a low power of the microscope (Fig.17). The excessive number of nuclei were usually judged to be
FIG. 19. Glomerulus showing slightly beaded nature of fibrinoid material adjacent to basement membrane in loop at top of glomerulus and in cytoplasm just above centre of the same glomerulus. NZB/BL X NZW female hybrid aged 27 weeks. (Massons X936).

FIG. 20. Glomeruli showing extensive fibrinoid change close to basement membrane. NZB/BL X NZW female hybrid aged 35 weeks. (MSB X660).
endothelial (Fig. 18) but in some cases epithelial nuclei also seemed increased in numbers. This hypercellularity was general in a given glomerulus. Only in the earliest stages did it seem patchy in the kidney, usually being present throughout, although by no means equally in all glomeruli in a given kidney.

Pyknosis was common soon after hypercellularity developed in the glomeruli. Karyorrhexis was less common and no haematoxyphil bodies were seen in this series. Fibrinoid was another striking glomerular abnormality seen. It was recognisable usually soon after, occasionally before the hypercellularity. While clearly adjacent to and/or involving the basement membrane in some cases (Fig. 20), it was much more often seen in the cytoplasm of the glomerular tuft. Typical glomerular fibrinoid tended to increase up to the lesions that were moderately severe (e.g. Fig. 39) but was less marked in some of the very severe lesions. This strongly eosinophilic material appears fragmented or granular under high powers of the microscope (Fig. 19). Sometimes the appearance was smudgy if there was plenty of it. The fibrinoid material stained strongly positive with P.A.S. and bright red with Masson's trichrome. It was not possible to estimate minor degrees of this change without the special stains.
FIG. 21. Glomeruli showing apparent loss of axial basement membrane with patchy thickening of peripheral basement membrane in enlarged tufts. Very little PAS positive material in cytoplasm of tufts. Note commencing granularity of PAS positive tubular cytoplasm material. NZB/BL X NZW female aged 34 weeks. (PAS X444).

FIG. 22. Glomeruli showing usual basement membrane pattern with early thickening present. (PAS X444).
In later experiments the Martius-Skarlet Blue stain was found equally as good, or better, than the Masson's trichrome stain.

**Basement membrane thickening** developed to start with, usually in a patchy manner in a given glomerulus (Fig. 22). It usually increased in the more severe lesions and became marked and extensive. "Wireloops", due to rigid, thickened glomerular capillary walls resembling loops of bent wire were common in the moderately severely affected animals. They were less evident in the very severely affected animals, although there is a good example in Fig. 27. These loops were usually only clearly recognisable on the edges of the glomeruli. In many enlarged proliferated glomeruli (Fig. 21) the capillary basement membrane appeared unfolded and stretched out, the more central, axial, areas being free of basement membrane.

Early the capillary lumen might be dilated but later many capillaries were narrowed or obliterated due to the increased nuclei and thickening of the capillary wall (Figs. 23 and 24).

Areas of **local hypercellularity** were very common (Fig. 23). They seemed to precede areas of **local necrosis** of the glomerular tuft. These were common and various stages of development of this lesion were seen.
FIG. 23. Renal cortex showing large hyperplastic glomeruli with lobulation, considerable hypercellularity, pyknosis, obliteration of capillary lumina and some fibrinoid. NZB/BL X NZW female hybrid aged 35 weeks. (HE X180).

FIG. 24. Glomerulus showing lobulation, tendency of nuclei to clump and earliest capsular adhesion. Same kidney as Fig. 23. (HE X660).
They comprised areas of capillary obliteration, nuclear proliferation and clumping with some pyknosis. Fibrinoid change was also noted in some of these areas and on occasions a few polymorphonuclear leucocytes, but these last were quite unusual.

Capillary obliteration was also sometimes caused by hyaline thrombi. These rounded or cylindrical, strongly eosinophilic, homogeneous structures (Fig. 25) were moderately P.A.S. positive but did not give the same staining reactions as the fibrinoid material. They occurred mainly in the more severely affected cases. While they usually affected most of the glomerulus they could be confined to a part of the glomerular tuft (Fig. 26).

As these changes proceeded within the glomeruli the whole glomerulus showed an increase in size. This was usually more or less uniform in the kidney with an allowance for the varied diameters from sections of glomeruli at different levels (Fig. 23). The size could become several times normal in some of the severe cases (Fig. 21). In considering size, allowance has to be made for the fact that the deeper juxta glomerular glomeruli are larger at all ages. These deeper glomeruli are often those first affected and more severely affected.
FIG. 25. Glomerulus showing extensive hyaline thrombus formation and patchy moderate basement membrane thickening. Some adjacent tubules are moderately dilated and granular PAS droplets are seen in the tubular lining cytoplasm. NZB/BL X NZW female hybrid aged 35 weeks. (PAS X660).

FIG. 26. Glomerulus showing local homogeneous hyaline thrombus material and more diffuse, granular PAS positive material in tuft cytoplasm. NZB/BL X NZW female hybrid aged 35 weeks. (PAS X660).
Lobulation of the glomerular tuft, Fig. 24, was frequently observed and could often be related to areas of local hypercellularity as can be seen in many of the illustrations, and to local necrosis. As the disease progressed adhesions sometimes occurred between tuft and capsule (Figs. 24, 27 and 28).

In the severely affected glomeruli fibrosis and scarring were evident (Figs. 27 and 28) and sometimes the glomeruli were quite disorganised, even merging into the interstitial tissue (Fig. 27). Other glomeruli in such a kidney would show some, but seldom striking proliferation of capsular epithelium, often with proteinaceous material in Bowman's space and fibrin-like material in relation to the basement membrane of the parietal epithelium (Fig. 27). Variable degrees of adjacent pericapsular hypercellularity would be evident (Fig. 28). Typical epithelial crescents were not seen in the few severely affected kidneys examined.

TUBULES

The earliest definite change noticed in the tubules was a granularity and clumping of P.A.S. positive material in the cytoplasm of the proximal convoluted tubules. This caused a striking difference from the homogeneous P.A.S. positive material normally noted in
FIG. 27. Kidney shows grossly disorganised glomeruli, but at varying stages of the lupus nephritic process. Wire loops, local areas of hypercellularity, necrosis and fibrinoid, adhesions, capsular and interstitial changes are present. Uraemic female NZB/BL X NZW hybrid aged 32 weeks. HE X396.

the luminal aspect of the proximal tubular epithelial cells (Fig. 25). The larger of these globules often gave similar staining reactions to the glomerular fibrinoid with the M.S.B. stain. With few exceptions the tubular changes only occurred when the glomerular lesions were well established (Fig. 29). Tubular dilatation then developed and this could be present without much cast formation, especially probably in the more acute cases (Fig. 31). Later cast formation became marked (Fig. 32). The casts showed a multiplicity of sizes, types and staining abilities. Some were long and broad, others small; some were obviously hyaline, others granular or even cellular. A striking feature with all stains was the marked variation in staining between different casts and, indeed, within the same cast. No foreign body reactions were noted in the interstitial tissue, even with the most disorganised of tubules. As far as could be ascertained the casts were always sited in the distal convoluted or collecting tubules (Fig. 29). When glomerular damage was extensive and tubular dilatation considerable the cortex, like the medulla, could come to look like a tubular region (Fig. 33). Red blood cells and leucocytes were not recognised in the capsular or tubular lumens.
FIG. 29. Renal cortex showing early tubular dilatation and cast formation in the paler distal tubules. Also shows large glomeruli with patchy basement membrane thickening and unfolding of the basement membrane. NZB/BL X NZW female hybrid aged 35 weeks. (PAS X184).

FIG. 30. Cast in urine suggesting that granular nature of many casts probably has a cellular basis. (X1080).
FIG. 31. Kidney showing extensive glomerular involvement with marked tubular dilatation with little cast formation. Uraemic NZB/BL X NZW female hybrid aged 26 weeks. HE X175.

FIG. 32. Kidney showing extensive glomerular involvement with marked tubular cast formation. More uraemic NZB/BL X NZW female hybrid aged 32 weeks. HE X175.
FIG. 33. Kidney showing gross disorganisation of cortex and numerous casts especially in tubules of medulla. Note perivascular parenchymal lymphoid tissue. 32 weeks old NZB/BL X NZW female hybrid. HE X67.
INTERSTITIAL TISSUE

Although some mice showed prominence of endothelial nuclei the first clear-cut abnormality in the interstitial tissue was an increase in such tissue (Fig. 34). This was never definite until glomerular damage was well established and could seldom be confidently recognised in kidneys in which the tubules did not already show change. Apart from endothelial nuclei increase there was some lymphoid tissue increase, sometimes pyronin positive (Figs. 35 and 36). Fibrinoid material could sometimes be seen in the interstitial tissue of severely affected kidneys and sometimes in relation to tubular basement membrane. Fibrous tissue was recognisably increased, particularly with relation to markedly damaged glomeruli.

VASCULAR CHANGES

Fibrinoid changes were seen in the walls of afferent arterioles, sometimes in the interlobular arteries (Fig. 36) and occasionally in larger arteries. Such changes were usual only in severe lesions so were infrequent in this series.

LYMPHOID CELL AGGREGATES

Hilar lymphoid aggregates, both perivascular and peripelvic in distribution, occurred in all affected
FIG. 34. Renal cortex with glomeruli graded 2-3+ and also showing earliest evidence of increased interstitial tissue. NZB/BL X NZW female mouse aged 35 weeks. (HE X384).

FIG. 35. Renal cortex showing a moderate increase in interstitial tissue especially periglomerular. The predominantly perivascular nature of the round cells is seen. NZB/BL X NZW hybrid aged 35 weeks. (HE X184).
FIG. 36. Kidney showing smudgy fibrinoid, identifiable with HE stain, in small artery near centre. There is an increase in interstitial tissue in which many of the round cells were pyronin positive. NZB/BL X NZW female hybrid aged 35 weeks (overall grading 3+ for severity and 3+ for activity).

FIG. 37. Corticomedullary region of kidney showing mass of vessels distended with lymphocytes in lymphoid tissue and Russell bodies at edge of photomicrograph. NZB/BL X NZW female hybrid aged 35 weeks. (MSB X288).
hybrid mice. They increase in size to become quite large in older animals. Definite large masses could be present without many pyronin positive cells being noted. In older animals, however, they were abundant, although Russell bodies (Fig. 37) were rare. These masses of lymphoid cells occasionally showed reaction centres (Fig. 38a). In other cases vessels were stuffed with lymphocytes (Fig. 37). These illustrations also show the way tubular renal parenchyma was replaced by these proliferating masses of lymphoid tissue.

Apparently similar groups of lymphoid cells (Fig. 33), more clearly perivascular in their distribution, were always present in affected kidneys (Figs. 35 and 38b). The diffuse increase in lymphoid cells that was seen in the interstitial tissue has already been noted in Fig. 36.

OVERALL TYPE AND DISTRIBUTION OF LESIONS

This can be stated invariably histologically to appear as a glomerulitis and only when this is well established are tubular and interstitial changes clear-cut and the lesions enter the category of a glomerulonephritis. Thus in the 16 hybrid females of mean age 35 weeks half had a definite glomerulonephritis and the others a glomerulitis. If anything, this underestimates the incidence of the glomerulonephritis by this age because this group
FIG. 38a. Corticomedullary region kidney showing area of lymphoid tissue with pale reaction centre. Note compression of tubules and close relationship to affected glomerulus NZB/BL X NZW female. (HE X184).

FIG. 38b. Renal cortex showing periarterial parenchymal collection of lymphoid tissue including many plasma cells. 55 week old NZB female mouse (Parenchymal 2-3+). HE X384.
was chosen to be normal at 21 weeks.

In the glomerulus at the earliest stages the lesion may be local, parts of the glomerulus appearing normal but the hypercellularity is soon considered diffuse with local more marked areas. Also the affected glomeruli may have a focal distribution. This is reflected at a moderate to severe stage by some glomeruli still looking fairly normal, although not absolutely normal.

CONCLUSION

The renal lesions of glomerulitis and glomerulonephritis reported show marked similarity to those described in human cases of S.L.E. by many authors, especially Muehrcke, Kark, Pirani and Pollak (1957) which group, as already mentioned, consider the mouse lesions akin to those they have seen in humans.
212.
(B)

METH01J OF ASSESS1VIENT OF RENAL LESIONS FOUND

It was decided to introduce a systematic method
of recording changes and their severity so all the
kidney sections in the first experiment were re-examined
and graded from 0 to 4
mentioned above.

"+"

for each of the parameters

An expanded and modified table of the

sort used by Muehrcke et al. (1957) was devised with the
help of a personal communication from Dr Pollak which is reproduced as Appendix No.7 (p.42).

A 4+ grade was taken

as the severest sort, as seen in some of the uraemic

..
I,

animals (e.g. Figs.31-33), with a gradation down to
just definite lesions recorded as +.

In general these

grades correspond to very severe, severe, moderate and
mild, + including any definite unequivocal lesions.
Some intermediate examples are shown in Figs.39a and
39b.

Many of the descriptive terms used in the table

are self explanatory and all are used in their generally
accepted meaning.

Where there is confusion the usage

as discussed by Muehrcke et al. (1957) is followed.
Such a method of recording the results allows an
assessment to be made of the overall severity and overall
activity of the lesions,which is necessary to interpret
the chronological development of the lesions, as well
as to compare treated with untreated animals.


FIG. 39a. Glomerulus showing early lobulation, hypercellularity with some clumping of nuclei and pyknosis, capillary narrowing and some fibrinoid in 30 weeks old NZB/BL X NZW female hybrid animal. Preponderance of such glomeruli would be graded 2+ for activity and severity but other less affected glomeruli led to a 2+, + grading in this particular animal. (HE X612).

FIG. 39b. Glomerulus showing local necrosis areas with fibrinoid 3+, hypercellularity and pyknosis 2+ and very close relationship to masses of lymphocytes and plasma cells which are replacing tubular structures. NZB/BL X NZW female hybrid aged 35 weeks. (HE X426).
Overall severity of lesions was estimated by considering the total damage in the main anatomical subdivisions described above viz. glomeruli, tubules, interstitial tissue and blood vessel changes but not including the lymphoid masses in this estimate.

Overall activity was assessed as 0-4 as recommended by Muehrcke et al. (1957) and Pollak et al. (1961a) who state a convincing case for their belief that the glomerular changes of karyorrhexis (which includes pyknosis) local necrosis, hyaline thrombi, haematoxyphil bodies and endothelial hypercellularity, coupled with fibrinoid change and any periglomerular cellular infiltration give the best guide to activity of lupus renal lesions. Their periglomerular cellular infiltration is covered by the heading of a diffuse interstitial increase in lymphoid tissue.

Having made this assessment of overall severity and overall activity if lesions were present their type and distribution could usually be classified as glomerulitis or glomerulonephritis where there was tubular and interstitial damage in addition to glomerular change. In either case it was of interest to note whether they were primarily membranous, proliferative and membranous or proliferative; whether they were local or diffuse within the affected glomeruli and, finally, whether affected glomeruli had a focal or generalised distribution throughout
the section.

Using this method of assessment the chronological development of the lesions in both sexes is next reported, with particular reference to severity and activity, hypercellularity and fibrinoid, basement membrane thickening and collections of lymphoid tissue.

(C) CHRONOLOGY OF DEVELOPMENT OF RENAL LESIONS IN NZB/BL X NZW HYBRID MICE

1. FEMALES

SEVERITY AND ACTIVITY OF RENAL LESIONS

In 20 female animals examined under the age of 4 months no abnormalities were found. Occasionally some difficulty was experienced in deciding whether there was early hypercellularity. Of 5 animals examined at age 17 weeks, one had definite but slight hypercellularity and fibrinoid and again in one other the observations were equivocal. In the 4 animals examined at 20 weeks, 3 showed equivocal hypercellularity, one of these showed minimal evidence of fibrinoid and the 4th also showed minimal fibrinoid but did not appear hypercellular. One out of 3 animals aged 21 weeks had definite but not severe lesions, while at 23 weeks 2 out of 4 had definite + lesions, while the other 2 were equivocal (ECT Tables 69-71, Appendix No.8, p.140).
At exactly 26 weeks the 4 animals examined had unequivocal renal lesions. These varied in severity from + to 4+ and likewise in activity, the most abnormal animal having a blood urea of 124 mg. %, the other 3 blood ureas being normal. These 4 animals illustrate the range of abnormality that can be seen at exactly the same age in these hybrid mice. Two of the mice had large hyperplastic glomeruli and showed tubular damage with dilatation and granularity of tubular cytoplasm, as well as many casts, especially in the most severely affected animal. The other 2 showed no tubular damage and of these one had impressive hypercellularity of the tuft and a little fibrinoid, whereas the other had more fibrinoid and less hypercellularity. Pyknosis was present in all but also showed these variations.

Beyond this age all female mice had abnormal kidneys. These tended to become increasingly so but at any given age, as described above, the range of variations is great. However over the age of 30 weeks females with minimal definite involvement (i.e. +) were not seen, the range being from 2+ - 4+. The variation seen in the second experiment illustrates these points further, where in a group of 17 females with 16 surviving to a mean age of 35 weeks, abnormalities varied from + to 4+; in these animals 2 had +, 8 had 2+, 5 had 3+ and 2 had 4+ severity
of renal lesions. Within these groups the 2 least affected and the 2 most affected happened to be the youngest animals. It should be pointed out here that this group of 17 was especially selected at 21 weeks to give animals with no abnormal findings so the overall pattern is of lesser severity than in an unselected group (BT BW renal histology Tables 67-68, Appendix No.8, p.136).

Where the point was studied in the first experiment the trends outlined were similar and there seemed no real difference between the hybrid groups dependent on the direction of the cross, although the numbers in each group are small (Table 51, Appendix No.8, p.116).

GLOMERULI

Glomerular hypercellularity and fibrinoid change

The hypercellularity can be detected before fibrinoid. It may be the first lesion any time after 16 weeks of age (Figs. 15 and 16). Both fibrinoid and hypercellularity contribute towards the estimate of activity of lesions, as well as to severity. Consequently the general pattern (Table 53, Appendix No.8, p.118) shows the same trend as the previous Table 51, showing overall severity and activity of renal lesions themselves, in the female hybrids of the first experiment.

The main point from the Tables is that both
parameters tend to increase steadily with the degree of renal involvement. However this is partly deceptive because of the lack of 4+ severity animals in the study. In such cases hypercellularity may lessen and fibrinoid alter so that, even if it still looks the same in H&E sections, it does not give the usual special staining reactions. Hypercellularity tends to occur a little earlier than fibrinoid; it may become very marked and remain a feature of severe lesions. Fibrinoid is also marked. It lessens in the most severe lesions, but few of these were studied in this thesis.

Pyknosis of the tuft nuclei was common soon after hypercellularity could first be identified, especially in the females.

Areas of local necrosis in the glomerular tuft could be recognised in any severely affected kidneys and so were seen as early as 26 weeks of age in females severely affected at that age.

Glomerular basement membrane thickening

This tended to become evident between the 18th and 28th weeks but nearer the later age. Thus 10/44 females had no thickening and these were all under 27 weeks of age. Females were likely to get more thickening and more rapidly than the males. Five females in the first experiment, but only one male showed 3+ - 4+ degrees of
basement membrane thickening and these mice ranged in age from 27–42 weeks, so again this was a lesion that came on about the same age but increased more rapidly and was likely to become severe several months earlier in females than in males (CT Composite Tables 44–47, Appendix No.8, p.104).

**TUBULES**

Most of the tubular changes seen have already been mentioned. Tubular dilatation and cast formation are common, being always gross in an animal graded as 4+ and usually quite marked in one graded as 3+. Tubular lesions are in general only recognised well after glomerular lesions. In uraemic animals the tubular dilatation may be very marked. Sometimes the tubules are definitely moderately dilated in a non-uraemic animal.

**VASCULAR CHANGES**

Arterial and arteriolar damage have been noted but are mainly a feature of more advanced lesions in older animals. Thus fibrinoid change was noted in 4/16 of the affected females of mean age 35 weeks.

**LYMPHOID CELL AGGREGATES**

Hilar lymphoid aggregates were beginning to accumulate about 17 weeks of age in some animals and by 20
weeks were quite definite in 4/4 female animals examined (ECT Tables 69-71, Appendix No.8, p.141). One of these animals was the first to show parenchymal perivascular collections. By 23 and 26 weeks the hilar collections had become quite marked. In the first experiment an occasional female up to 24 weeks of age showed little or no evidence of these cells, but apart from these exceptions, after the age of 20 weeks these accumulations became prominent and tended to be more so in the older animals (Tables 54-55, Appendix No.8, p.119). They were more marked than in NZB/BL animals of similar age and were scanty in NZW animals of similar age (CT Composite Tables 48-49, Appendix No.8, p.112).

The tendency, then, is for the hilar and parenchymal collections of lymphoid cells to increase with age and/or with severity of the disease. The increase may precede or exceed the renal lesions as there are examples in the series of animals with a 2+ degree of hilar and of parenchymal lymphoid tissue having minimal lesions. Also in the pale kidney illustrated (Fig.13) from a 26 weeks old animal, although the renal lesions are 4+, the hilar and parenchymal collections of lymphoid tissue were scarcely 2+ in grading, considerably less prominent than in other less affected animals the same age. Nevertheless no examples were seen of definite renal
glomerular abnormalities in which there was clearly no increase in lymphoid tissue.

2. MALES

SEVERITY AND ACTIVITY OF RENAL LESIONS

When severity and activity of renal lesions were charted for age groups in the males examined in the first experiment it was found that minor abnormalities, + or equivocal were seen in the 18 week plus age group but there were none of 2+ severity or activity until the 27th, 28th and 29th weeks. No lesion of 3+ severity was seen until after 30 weeks and even from 30 until 64 weeks lesions of 2+ severity were as likely to occur (6/11) as were 3+ lesions and no 4+ severity lesions were seen in these males. A further point is that even as late as 58 weeks of age the male lesion might show little (+) activity. The pattern in the male hybrids shows no difference dependent on the direction of the crossbreeding (Table 50, Appendix No.8, p.115).

GLomeruli

Glomerular hypercellularity tends to occur earlier than fibrinoid and has a similar time sequence to the above (Table 52, Appendix No.8, p.117). In one animal aged 63 weeks the hypercellularity was the striking abnormality (Fig.18). How long it had been present is not known.
Basement membrane thickening was absent in 9/26 males and these 9 were all under 28 weeks of age. It was first noticed, as in the females, between 18 and 28 weeks of age. Only one male (aged 38 weeks) had a 3+ degree of basement membrane thickening. There were 8 males examined over this age with less than 3+ thickening and these included a 48 week old animal with + thickening and a 63 week old animal with 2+ thickening (CT Tables 44-45, Appendix No.8, p.104).

LYMPHOID CELL AGGREGATES

Groups of hilar lymphoid cells were not prominent in 4/5 male mice examined between 16-26 weeks and in 2/6 examined at 24-28 weeks. Subsequent to this age they were always present. In this respect they were about 8 weeks later than the females, though as with all parameters examined, variations from mouse to mouse are such that only general rules can be noted (Table 54, Appendix No.8, p.119.)

(D) CORRELATIONS WITH RENAL LESION

The results that follow represent a correlation of findings tabulated under previously considered headings in Appendix No.8 and correlated with the renal lesions. Most of the information has been co-ordinated
1. **Correlation of positive L.E. cell tests with renal lesions.**

**Hybrid males**

In the 25 untreated males tested in the first experiment 5 had positive L.E. cell tests on at least one occasion. Two of these showed 3+ severity or activity of renal lesions and the others showed 2+. None showed less. However there were 7 equally or more severely affected male animals than this, so the test will not predict all of the moderately severely affected animals. (CT Tables 44-45, Appendix No.8, p.104).

**Hybrid females**

In the hybrid females in the first experiment 5/18 animals untreated and of all ages up to 40 weeks had positive L.E. cell tests. Of the 5, 4 had 3+ renal lesions and one had 4+ lesions. Only one other animal in the 18 had this severity of renal lesion with a negative L.E. cell test. In the later experiment where 16 animals had the test performed regularly and survived to a mean age of 35 weeks, 8/16 had positive tests on at least one occasion, mostly at about 32 weeks of age. Of these 5 tests corresponded to 3+ or 4+ renal lesions, 2 tests to 2+ renal lesions and the 8th mouse had + renal lesions. Two of the mice with 3+ renal lesions had
negative tests. The conclusion in the females is that animals with the more severe renal lesions tend to be the ones that have the positive tests (CT Tables 46-47, Appendix No.8, p.109).

A more important correlation, however, in both is with the time at which the test became positive. They were rarely positive in animals under 30 weeks of age. No positives were seen in the repeated large number of tests performed in young animals before the time at which renal lesions have subsequently been shown to develop so that the test is not useful to indicate onset of the disease process itself. Neither can its absence be taken to indicate that the disease is not already severe or active. The establishment of these points is of considerable importance in planning the stage at which therapeutic measures should be commenced in any future trials.

The ages at postmortem of animals in the first experiment in which these positive tests were found is also of interest. Even with the females, with the exception of a 27 and a 33 week old animal, all were 37-42 weeks and the males 38-50 weeks. The female grouping is such that one can say that the younger severely affected animals are less likely to have positive cell tests than
the older severely affected animals. The reason for this cannot be stated. It probably reflects the disease process but a variable factor is that the older animals had more L.E. tests performed and this may explain why they were more likely to have positives found.

2. **Correlation of severity and activity of renal lesions with incidence of positive Coombs tests**

In the untreated males 6/16 tested had positive Coombs tests but the renal lesions in these varied greatly in severity. It was not possible to say whether the Coombs test was ever positive before renal lesions develop because it was not available when the youngest animals in the first series were killed.

Similarly in the females, although 3/9 females tested in the first experiment had positive or doubtful positive Coombs tests at some time, there was no clear relationship to severity of disease; 3 of the severely affected animals having negative tests. Usually when positive Coombs tests were found at some time, there was at least a 2+ severity of renal lesions. In the 32 animals tested later from 7 to 26 weeks, one aged 20 weeks had a positive Coombs test and equivocal renal lesions but otherwise all animals with normal kidneys had negative Coombs tests and so did the youngest animals with definite lesions (ECT Tables 69-71, Appendix No.8,p.140).
There is no close correlation between Coombs tests and severity and activity of renal lesions. There may be a correlation between positive Coombs tests and the presence of renal lesions.

3. Correlation of renal changes with urinary findings

These findings are recorded in the CT Composite Tables 44-47, Appendix No.8, p.104, where there is a summary of the findings in 40 males and 41 females from the first experiment, studied adequately from the urinary aspect by repeated weekly examination over periods of up to 6 months and recorded in detail in Tables 38-41 in Appendix No.8, p.88.

(i) Albuminuria

The degree of basement membrane thickening correlated well with severe albuminuria, the 5 female and one male animal in the first experiment with 3+ or 4+ thickening having albuminuria to the extent of 300 mg./100 ml. or over.

It also correlates well with overall severity of renal lesions, these 6 animals having 3+ or 4+ severity of renal lesions.

At this point it is appropriate to note that 12 other animals, including one with a nephrotic syndrome, had 300 mg./100 ml. or more of albuminuria with lesser degrees of basement membrane thickening than 3+, so that
although animals with marked basement membrane thickening
do have heavy albuminuria, others with similar albuminuria
do not. Nevertheless, 9 out of this further 12 had
overall severity of lesions classed as 3+ or 4+.
Combining the above figures albuminuria of 300 mg./100 ml.
or more in 15 out of 18 (83%) animals meant the renal
lesions were severe. This good correlation of
albuminuria over 300 mg./100 ml. is with renal lesion
severity, as well as basement membrane thickening.
Analysing this further, it is found that of 12 females
showing albuminuria of over 100 mg./100 ml. 11 had 3+
renal lesion severity and/or activity and the 12th had 2+.
On the other hand, all 10 males showing this level of
albuminuria had 2+ or over severity and activity of
renal lesions. There is then a good positive correlation
and animals that have 100 mg. levels of albuminuria can
be expected to have 2+ renal lesions if males and
3+ if females. However looking at the question in reverse,
there were 7 males and 11 females showing 2+ or over
activity and severity who had minimal albuminuria.

Albuminuria at levels of under 100 mg./100 ml.
may or may not be associated with renal lesions. Levels
repeated at over 100 mg. can be taken to show that renal
lesions of 2+ or over will be present.
(ii) Casts

The correlation with renal findings was considered in the first CT experiment (Composite Tables 44-49, Appendix No.8, p.104). In 7 different animals the observation of large numbers (over 20) of urinary casts, mainly granular, per low power field was made. Three of these were females and here the lesions were 2+ to 3+ in severity and, combined with a further 4 females who showed 3+ renal severity and 5-20 granular casts per low power field, one may say that 6/7 mice showing these large numbers of casts will have severe renal lesions.

With the males only 1/9 showing apparently similar urinary findings had 3+ severity of renal lesions, 4/9 had 2+ and 4/9 + severity. The urinary examination was not necessarily the most recent, but the most positive one, so at first sight we need not expect a very close correlation between the observed urinary maximum finding and the severity of renal lesions. However, if the lesions are steadily progressive there should be a good correlation and we would expect more similarity between the sexes. The differences could be due to the male lesions tending to fluctuate more than the steadily progressive female ones, or because they are progressive, the female lesions may represent a stage
beyond that at which maximal numbers of casts reach the bladder from dilated tubules. This appears unlikely, although in the few uraemic animals studied, urine was scanty and so were casts in the last few days of life. Neither can we simply equate the casts present in urine during life with the glomerulonephritis as odd intercurrent infections, particularly with dehydration or fever, may have caused cast formation in some males without any striking renal lesions.

Of the total 16 animals showing a marked degree of cast formation at some stage, only 2 did not have tubular casts at postmortem and these were both males. The other 14 showed a range of readings for numbers of histological casts up to very many only in those with severe renal lesions. As stated above, most of the urinary casts were granular and this corresponds with the fact that 10/16 did show a considerable multiplicity of casts in the histological section. Once again those that did show this multiplicity of casts histologically were those that had the most severely affected kidneys.

Seven of the 12 animals showing lesser numbers of urinary casts, 1-4 per low power field, had 2+ or greater severity of renal lesions and 5 of this 7 had 2+ numbers of casts in the histological sections. The remaining 5/12, however, showed minimal or no lesions, so
seeing a few casts is not going to help in deciding whether renal lesions are present or not.

We should next consider those 39 animals whose urines showed no or only an occasional granular cast. Of 21 males, 8 showed 2+ or more of renal lesion severity without convincing urinary casts. While the 3 animals in this group with 3+ severity of lesions did regularly show occasional granular casts, one of them died with uraemia without ever showing more casts than this. It was only in these 3 rather old male animals that there was any more than an occasional cast in the histological material when these had not been seen in the urine. This is evidence that negative urinary findings usually reflect the true state of affairs in the tubules. With the 18 females there were 9 showing 2+ and 5 showing 3+ renal lesion severity but no urinary casts. Only 3 of the 18 had a moderate number of casts histologically.

The overall conclusion here was that if no casts were seen on repeated examination there were not likely to be many casts histologically. However this was no guide as to the actual presence of moderately severe renal lesions.

A further check on the reliability of the urine examination is to take the animals in which histologically the grading was 2+ or more for numbers of casts. There
were 17 such kidneys, 8/11 female urines showed definite casts at one of the three higher levels discussed above, as did 4/6 male urines. In 4/17 occasional casts were seen and in 1/17 no urinary casts were seen. Of the 6 animals with 3+ casts histologically, 5 had been noted to have many urinary casts and the 6th had 1-4 per low power field. These figures confirm the general proposition that the urinary findings of casts correlate with the renal findings.

4. Evaluation of kidney macroscopic descriptions

Gross changes, sometimes more than one change in the same kidney, have been recorded in the CT Composite Tables (Tables 44-47, Appendix No.8, p.104) as swollen, as pale or white, as granular or as showing punctate red glomarular surface markings, particularly obvious with the hand lens. This last change was noted in 5 males with histological lesions ranging from 0 to 2+ in severity and activity and in 6 females, 3 over 3+ in severity and/or activity. No obvious usefulness arises from comment on this point.

On 15 occasions the kidney was noted as pale or white (4 males and 11 females). In 3/4 males and 9/11 females these kidneys showed 3+ severity and/or activity so the kidney pallor was a significant observation.
On 8 occasions kidneys were said to be swollen. In 4 males no severe lesion was present. Indeed, in retrospect, looking at the weights it would have been more correct to say that these were big kidneys, rather than "swollen" ones.

In the only 2 females said to have swollen kidneys there was severe disease present.

Likewise, in the only 3 kidneys (all female) in the NZEW mice recorded as granular all were severely affected, as could be expected.

Thus out of a total of 37 observations of variation in kidney appearances, 22 referred to female kidneys, and of them 17 observations corresponded to severe renal disease, whereas only 5 of the 15 observations on male kidneys reflected severe disease. In reverse it was found that quite severe disease was not excluded by an apparently normal gross appearance although in making this statement the author's relative inexperience with mouse kidneys in general must be allowed.

(E) SUMMARY OF RENAL FINDINGS IN NZB/BL X NZW HYBRID MICE

A few of the NZB/BL X NZW hybrid mice that develop a form of nephritis show renal lesions from the 18th week, an occasional female mouse dying by the 24th week. The number showing early lesions increases until
between the 23rd and 26th week nearly all females have recognisable glomerular lesions.

Many of the histological features and the presence of positive L.E. cell tests indicate that this is a lupus nephritis. The earliest recognisable lesions are glomerular hypercellularity, probably mainly endothelial, and slightly later fibrinoid is seen in the cytoplasm and also in proximity to the basement membrane. These are followed by an increase in size of the glomeruli, lobulation of the glomerular tuft, local areas of necrosis and some nuclear pyknosis. Hyaline thrombi are sometimes seen. Basement membrane thickening and wire loops develop and later changes occur in the tubules about the time that glomerular adhesions and pericapsular changes are occurring. The progress of these lesions is fairly rapid in the females, and even if they approach 40 weeks of age without succumbing, nearly all will have severe renal lesions.

In most of the male hybrids the time of onset of recognisable renal lesions is probably much the same but the rate of progress of these is certainly considerably slower.

Groups of lymphoid cells accumulate, first in the renal hilum and then also in the renal parenchyma with a perivascular distribution. These collections of
cells are recognisable a few weeks before the renal lesions and, like the renal lesions, they increase with age but not necessarily in close correlation with the renal lesions.

Positive L.E. cell tests are uncommon below 30 weeks of age, even in the occasional severely affected animals. They are much commoner in the more severely affected animals over the age of 30 weeks.

Albuminuria of over 100 mg./100 ml. of urine is usually, and of over 300 mg./100 ml. of urine, almost always associated with severe renal lesions. Likewise, if many casts are found in the urine, the renal lesions will be severe. Nevertheless moderate and sometimes severe renal lesions can be present without these degrees of urinary abnormality.
CHAPTER 10

NON-RENAL MORBID ANATOMICAL AND HISTOPATHOLOGICAL FINDINGS IN NZB/BL X NZW HYBRID MICE

I. SPLEEN
II. LIVER
III. THE LUNGS
IV. THE HEART
V. THYROID
VI. SALIVARY GLANDS
VII. PANCREAS
VIII. ADRENALS
IX. PITUITARY
X. LYMPH NODES
XI. BONE MARROW
NON-RENAL MORBID ANATOMICAL AND HISTOPATHOLOGICAL
FINDINGS IN NZB/BL X NZW HYBRID MICE

General morbid anatomical and histopathological features and findings in the hybrid animals are reported in this chapter based on examinations of the spleen, liver, lung, heart, salivary glands, pancreas, thyroid, adrenals, pituitary, lymph nodes and bone marrow in samples of animals. As with the parameters studied in previous chapters some reference is made to findings in untreated NZB/BL and NZW mice where this is relevant. It must be remembered that few animals with very severe lupus nephritis were examined so the results reported can illustrate certain aspects seen but do not give a complete picture of findings in these hybrid mice.
I. THE SPLEEN

The mouse spleen (Snell, 1941) is slightly curved in its longitudinal aspect and wedge-shaped in cross section. It is usually divided into three approximately equal parts by the branches of the main splenic artery. The main differences to the human organ are that there are usually not so many red cells in the red pulp. As this latter contains many lymphocytes as well as reticular cells and macrophages, it is not so clearly demarcated from the perivascular white pulp, as is the case in humans. Haemosiderin pigment is not uncommon in the macrophages. In addition, megakaryocytes, myelocytes and erythroblasts, as well as plasma cells, are present to a varying degree. Snell (1941) also points out that the Malpighian bodies around the central arteries sometimes show "germinal centres".

NZB/BL X NZW hybrid females

Spleen weights in both the first (Tables 44-47, Appendix No.8, p.103) and third experiments (Tables 69-71, Appendix No.8, p.140) showed a striking increase in weight with age. This tended to occur at about 6 months of age. Animals 4-8 weeks old had spleen weights of 50-75 mg. In the 28 animals studied in groups of 4 up to the age of 23 weeks the average spleen weights varied from
75 to 89 mg., the range being from 65-105 mg. There was an abrupt rise in the average spleen weight of the next 4 mice killed at age 26 weeks, viz. 176 mg., the actual weights being 95, 130, 190 and 320 mg. Reference to Fig. 40 shows this variation in size and it is of considerable interest that the animal with the smallest spleen had the white pale kidneys and was uraemic. Its body weight had fallen by one third of that of its fellows so the spleen may earlier have been heavier. The other 3 spleens are distinctly above the weights seen prior to this age.

In the CT experiment the mean weight of the spleen for hybrid females was $173.2 \pm 27.4$ mg., and this represents a scatter of ages between 17 and 40 weeks (Tables 10 and 11, Appendix No. 8, p. 60 and Stat. Table 23, Appendix No. 9, p. 186). Although the pattern was definitely similar to that described above, an occasional animal 9 months of age had a spleen of apparently normal size e.g. CWF 38 in CT Composite Table with a spleen weighing 100 mg. With an older age group of 32-39 weeks the mean spleen weight was $192 \pm 21.5$ mg., occasional animals again being well below or above this range (Table 59, Appendix No. 8, p. 125 and Stat. Table 41, Appendix No. 9, p. 206). Thus the spleens of animals around 40 weeks of age would often be three times that of
animals aged 20 weeks. The gross appearance altered from a pale pink, almost transparent organ weighing 50-75 mg. in 4-8 week old animals, to a massive 250-340 mg. organ in some of the females with very prominent white pulp easily visible to the naked eye (Fig.41) in animals in the 35-40 week age group in both sexes. A betamethasone treated spleen, shown in Fig.42 for convenience, is markedly reduced in size.

When spleen weights are compared with total leucocyte counts in the mice averaging 35 weeks of age (Table 59, Appendix No.8, p.125) there is no close correlation, nor are there any total leucocyte counts suggestive of leukaemia. Of 8 bone marrows examined in this group, none was leukaemic.

If spleen weights are compared between the CBW and CW animals there was no evidence of any difference dependent on the direction of cross of the hybrid (Stat. Tables 21-23, Appendix No.9, p.184).

**Hybrid males**

Here the adult males had spleen weights of 121.5 mg. ± 9.0. The NZW males had spleen mean weight of 68.5 mg. ± 5.5 so the hybrids were considerably heavier. The spleen weights in hybrid males were lower in weight than the females but like the females they tended to increase in weight with age. As with the
FIG. 40. Shows variation in kidney colour and splenic size in four NZB/BL X NZW female hybrids aged 26 weeks. (X2).

FIG. 41. Spleens at age 26 week from previous figure. Cross section shows masses of lymphoid tissue, in this instance more prominent in the animal with the more normal kidneys. (X10).

FIG. 42. Spleen of 35 week old NZB/BL X NZW female hybrid treated with large doses of betamethasone, showing marked depletion of spleen size and lymphoid tissue compared with either of above. (HE X64).
females there was no difference in spleen weights depending on the direction of the cross (Tables 8 and 9, Appendix No. 8, p. 58 and Stat. Table 20, Appendix No. 9, p. 183.)

**Histologically** the large size of untreated spleens was due to a marked increase in lymphoid tissue in these mice. Although sometimes evident earlier (Fig. 41) this could usually be definitely appreciated from about 30 weeks of age in the female, at least 2 months before it was recorded in the males. Small lymphocytes were not prominent in the white pulp but sheets of larger lymphocytes (Figs. 43-44) and often plasma cells spread out and occupied the site of the red pulp. These changes were seen in 7/24 males and 6/18 females examined in the first experiment but there was a clear relationship to age, as they were seen in all of the females examined in the later experiment with mean age 35 weeks.

Germinal centres were uncommon before 20 weeks of age but increasingly common thereafter.

This good correlation of increasing spleen weight and increasing lymphoid tissue in the spleen of the NZBW animals with the ages of the animals obscures the significance of a correlation between these splenic changes and renal lesions such as a mass of lymphoid tissue in the kidney and the degree of activity of the renal lesions,
FIG. 43. Spleen showing large proliferated masses of lymphoid tissue. Normal malpighian corpuscles have almost disappeared. Erythroblasts and megakaryocytes still present beneath capsule and in scattered groups in red pulp. Female hybrid mouse aged 39 weeks in whom the spleen weighed 340 mg. Renal activity 3+, 2+. (HE X66).

FIG. 44. Spleen showing lesser degree of similar process to previous figure.
as these also are correlated with age. It is important to point out that none of the animals recorded as definitely showing an increase of lymphoid tissue in the spleen was free of renal lesions. As shown in the photograph the reverse did not always hold as there was clear evidence that lymphoid tissue could be increased in the kidneys, and the kidneys be diseased, before the spleen was impressively larger. Splenic megakaryocytes and myeloerythroblastic tissue were much less prominent in these mice than in the NZB/BL animals and became less marked as splenic size increased, such tissue coming to lie mainly in the subcapsular region.

**NZB/BL mice**

NZB/BL spleen weights, when arranged in order of age, also tended to show a rise with age as has been previously noted by Helyer and Howie (1963b). These authors described the increase in haemosiderin in the macrophages, the increased erythropoietic activity in the spleen and the fact that many plasma cells were present in the older animals. This haemosiderin increase is usually detectable from about the age of 28 weeks. Although lymphoid tissue was increased in both NZB/BL and NZBW spleens the resulting appearances were different because of the concurrent increase in erythropoietic tissue in the NZB/BL mice, which gave a much more pleomorphic
histological picture in this strain.

One NZB/BL mouse which had histological evidence of bronchopneumonia showed a marked increase in polymorphonuclear leucocytes in the splenic sinuses.

NZW mice

NZW male spleen weights lay in the narrow range of 58-75 mg. in 10 animals that were well when killed. Two animals who had dropped rapidly in weight, had spleens below this range (42 and 55 mg.) and one who was a dysentery suspect had a spleen weight of 125 mg. The mean weight for the NZW males was $68.5 \pm 5.5$ mg. That of the 4 females in the series, as in the NZBW and the NZB/BL strains, was higher at $98.5 \pm 3.5$ mg. (range 90-105 mg.). In contrast to the hybrid, however, it is clear from these ranges that there was no significant progression of spleen weight with age (male age range 24-56 weeks) in the males and, indeed, in the 4 females, the 2 highest weights were in the 2 youngest animals (Table 48, Appendix No.8, p.112 and Stat. Table 39, Appendix No.9, p.202). Although female numbers are small in the 3 strains examined, spleens were heavier in females than in males.

Histologically megakaryocytes and erythropoietic tissue were present in these NZW spleens but were not striking and did not increase. Germinal centres occurred
in a few of the mice over 30 weeks of age. In the females, although the weight did not increase with age, there did seem to be more lymphoid tissue and plasma cells in the older mice, though not of the same order as seen in the NZBW mice.

In this strain the most striking feature seen was in 2 male NZW mice aged 34 and 40 weeks with spleens of normal size in which there was replacement of the red pulp by an eosinophilic hyaline acellular material which was also seen in the liver and suggestive of amyloid disease. It was also noted in the adrenals. This material gave atypical amyloid staining reactions and is described in more detail in Chapter 17 on the NZW mouse.

CONCLUSION

The mean weights of spleens in both the male and female hybrids was considerably higher than weights seen in NZW animals. In the hybrids weight tended to increase with age, particularly at the age of 6 months and onwards and was considerably greater in females at the ages studied. This increase in weight was associated with a large increase in lymphoid cells in the spleen. Accumulation of such cells in the kidney may antedate histologically recognisable accumulations in the spleen. The presence of an amyloid-like material was noted in the spleens of two NZW male animals.
II. LIVER

NZB/BL X NZW hybrids

The general appearance of the liver seldom caused comment, except for minor degrees of pallor. Weights in 22 adult untreated NZBW male animals ranged from 1160-2040 mg, with an average of 1655 mg. In 16 adult untreated females the range was 1170-1960 mg, with an average of 1460 mg. (Composite Tables 44-47, Appendix No.8, p.103). On the whole heavier animals had heavier livers, though the correlation was not close. The sex difference could also be due to the females being lighter. As already noted, parasitic cysts were seen in a few livers.

Histologically the mouse liver is basically similar to the human, although the lobular pattern is less definite and the outline of the large polygonal hepatic cells is often indistinct (Snell, 1941).

In the NZB/BL X NZW hybrids, along or around interlobular vessels, sometimes lying in the angle of bifurcation of such a vessel, at other times clearly also close to a bile duct, collections of round cells were sometimes seen. In no case were these very striking.

MALES

In the males they were seen in 2/22 untreated
animals and these were older, in their 48th and 63rd week respectively. Both of these had moderately severe and active renal lesions but with no more than usual aggregations of lymphoid tissue in the kidney. The hepatic perivascular change may be present earlier because it was seen in 1/7 early and moderately dosed steroid treated animals examined in the 25th week of life.

FEMALES

Apparently similar lesions were noted in 7/16 untreated NZBW female animals examined. There were slight aggregations in 3 animals aged 9, 11 and 13 weeks, before any renal lesions were present, and more definite in 4 others aged 31-39 weeks. All of these latter had moderately severe and active renal lesions. Similar lesions were present in most of the later group of untreated females examined aged 35 weeks. In neither male nor female can one say whether the correlation was with age or general activity of the autoimmune disorder. No necrotic liver lesions such as have been noted in the NZB/BL mice by Dr. Helyer and Associate Professor Howie were seen in these NZBW animals, but it must be remembered that few animals with very severe disease were examined.

NZB/BL animals

Of the NZB/BL livers examined histologically 2/6
males (43 and 55 weeks) had small collections of cells and 3/6 females aged 25, 38 and 55 weeks.

**NZW animals**

Only 1/13 (aged 26 weeks) of the NZW male animals showed this change and not the older animals nor any of the 4 NZW female animals examined.

A striking change was seen in the liver of 2 NZW male animals (34 and 40 weeks). A pale eosinophilic extravascular material lying between cords of liver cells and similar to that seen in the spleen of the same 2 animals was present. These findings are discussed and illustrated in Chapter 17 on the NZW mouse as suggestive of amyloid disease.

**CONCLUSION**

The conclusion was reached that parenchymal hepatic lesions are not a feature of the NZBW mice at the ages studied. Perivascular collections of round cells are sometimes present, more often in the females and in animals over 6-7 months of age. The presence of an amyloid-like material was noted in the livers of 2 NZW male animals.
III. THE LUNGS

Histologically some minor differences from the human are noted by Snell (1941). In the mouse the alveolar wall contains varying numbers of lymphocytes and occasionally granular leucocytes. He also notes that the pulmonary veins in the lung contain cardiac muscle in their walls. In the CT experiment 67 lungs from the 3 strains were examined histologically using H.E. stain and a sample of the lungs from some of the later experiments contributes to the overall picture.

NZB/BL X NZW hybrids

Peribronchial and/or periarterial masses of round cells could be seen both near the hilum but also radiating out with bronchial and pulmonary arterial branches (Fig.46). These were mainly lymphoid cells but usually plasma cells were also evident. They were first identified in some female mice as early as the 21st week and in females between 30 and 40 weeks they are very common. They were clearly recognised in most of the NZBW males between 38 and 63 weeks. The masses could come to occupy well over half of a lung section.

A further feature noted was the unexpected presence of a peribronchial alveolar inflammatory exudate containing polymorphonuclear leucocytes and macrophages.
FIG. 45. Myocardium showing moderate diffuse interstitial myocarditis in an NZB/BL X NZW male hybrid aged 47 weeks. (HE).

FIG. 46. Lung showing moderate peribronchial, perivascular and parenchymatous collection of round cells in NZB/BL X NZW female hybrid. Renal grading 3+, 2+. (HE X21).

FIG. 47. Thyroid gland showing small focal collections of round cells in NZB/BL X NZW female hybrid aged 39 weeks. Renal grading 3+, 2+. (HE X172).
in moderate numbers of the mice. Bronchopneumonic processes were seen in both sexes in both inbred strains and especially in the hybrids and were found in some of the elective postmortem animals, so were by no means confined to moribund or uraemic animals (Fig. 48). It was usually associated with an impressive lymphoid infiltration of the lung, as described above, but this was not always so. In one female NZBW animal there was histological evidence of an accompanying tracheitis with no convincing lymphoid infiltration of the lung (Fig. 49). These acute inflammatory changes were common in the untreated animals. Thus in a sample of 6 lungs from untreated females aged 32-38 weeks in the BT experiment, 2 showed this bronchopneumonic process over and above the lymphoid change and it is illustrated later.

The lungs from an NZBW mouse dying uraemic showed alveoli containing considerable oedema fluid.

**NZB/BL mice**

Similar groups of lymphoid cells have been described by Holmes and Burnet (1963) in the NZB/BL mice and were seen in several of the older NZB/BL animals. In both the NZB/BL strain and the hybrids, if the collections are large, they are seen as small white nodules macroscopically.
FIG. 48. Lung shows extensive peribronchial lymphoid collections. There is a diffuse increase in round cells in the bronchial wall and the lumen contains acute inflammatory cells. NZB/BL X NZW female hybrid aged 34 weeks. (HE X120).

FIG. 49. Trachea showing lymphocytes and plasma cells in lamina propria, a few acute inflammatory cells in the lumen and traversing the ciliated respiratory epithelium. (HE X300).
NZW mice

NZW mice histologically showed some small peribronchial and perivascular collections of round cells but none were seen as striking as those in the NZB/BL and NZBW strains.

CONCLUSION

It has been found that the lungs of NZB/BL X NZW mice of both sexes, especially those over 30 weeks of age, showed large peribronchial and perivascular collections of round cells, including plasma cells. Bronchopneumonic changes were not uncommon in the older hybrid mice of this crossbreed.
IV. THE HEART

The structure of this organ in mice is very similar to that in humans. The mean heart weights of females was $163 \pm 4.8$ mg. at the mean age of 35 weeks (Table 59, Appendix No.8, p.125 and Stat. Table 41, Appendix No.9, p.205). The weight in these hybrids was rather higher than in NZW females, mean weight $137.5 \pm 6.0$ mg. (Stat. Table 39, Appendix No.9, p.202). Heart weights are further discussed in relationship to the possibility of hypertension in considering the complications of steroid therapy. No significant difference was noted between males or females dependent on the direction of the crossbreed (Stat. Table 24, Appendix No.9, p.187).

**NZB/BL X NZW hybrids**

Histologically changes were seen in some of the 86 hearts examined. In the NZBW animals minor degrees of perivascular aggregations of round cells, mainly lymphocytes, were noted in 2 females and 1 male mouse in the 26-30 week age group. In 2 males (42, 47 weeks) there was in addition a marked patchy replacement of myocardial fibres with fibrous tissue, in some areas extensively involving the subendocardial tissues (Fig.45).
These lesions were probably the result of a myocarditis. The only early lesion noted was a small patch of myocardial necrosis in an 18 week old male animal. The possibility that the patchy fibrosis was due to occlusive vascular disease has not been excluded. No evidence of this was seen in any vessels in multiple sections from one block of affected myocardium. However further blocks were not examined.

In 2 of a sample of 9 animals from the later female group with a mean age of 35 weeks there were similar perivascular groups of round cells scattered through the myocardium, and in the one of these 2 animals which was uraemic there was, in addition, patchy interstitial fibrosis extending to the endocardium.

The only other cardiac lesion noted was a thickening of the mitral valve with prominence of endocardium and an increase in round cells with occasional Anitschow type of myocytes in the base of the valve and the valve ring. This lesion was seen in an untreated NZBW male animal aged 24 weeks which had minimal renal lesions but had lost weight rapidly before being killed. There was naked eye and microscopic evidence of bronchopneumonia so the cardiac lesions may have been related to that. A section at this level is largely fortuitous and the incidence of valvular endocarditis in
these hybrids remains unknown.

NZB/BL animals

Two examples of the perivascular round cell aggregations were seen in these animals aged 55 and 85 weeks but none were noted in NZW animals.

CONCLUSION

In this series in both sexes perivascular collections of round cells were occasionally found in the hearts of NZB/BL X NZW hybrid mice and interstitial fibrosis was also noted.
V. THYROID

The thyroid gland in the mouse has two lateral paratracheal lobes and a narrow isthmus. It comprises varying sized follicles lined by cuboidal epithelium and containing eosinophilic colloid material. As the gland is not easy to see at postmortem the whole trachea was blocked, but using this method there were many failures to obtain thyroid tissue in the first sections. Further examination of such blocks has not been undertaken.

There was only one gross thyroid abnormality in 153 postmortems. This was a large pale tumour 0.5 cm. in diameter arising low down in the gland of a 55 week old NZB/BL female animal (Fig.50a). Histologically this comprised uniform large neoplastic epithelial cells, in a few places arranged in acini and forming colloid, but with evidence of invasion (Fig.50b). It is considered to be an adenocarcinoma of the thyroid gland.

Of sections examined from 35 different animals in this study the general histological picture was remarkably uniform. In one male NZEW animal in its 47th week there were perivascular collections of lymphoid tissue and the colloid was depleted. Similar collections of cells were seen in one NZBW female animal
FIG. 50a. Thyroid adenocarcinoma on trachea in 55 weeks old NZB/BL female. (HE X21.6).

FIG. 50b. Thyroid showing invasive edge of poorly differentiated adenocarcinoma in NZB/BL female mouse aged 55 weeks. (HE X228).
aged 39 weeks and would just warrant the use of the term *thyroiditis* (see Fig. 47).

Colloid depletion was noted in one NZB/BL animal and one NZW animal. This latter animal had rapidly lost weight before death and, although the cause of this was not obvious, it suggests that the depletion is related to stress. One other NZW male animal killed in its 50th week showed an anatomical variation with thyroid vesicles apparently in the tracheal wall.

**Parathyroid glands**

These were observed in 5/35 of the thyroid sections observed and in each case had the general normal pattern described by Snell (1941).
VI. SALIVARY GLANDS

These glands were examined for the first time in these strains because of their occasional involvement in some possibly autoimmune disorders in man e.g. Sjogren's syndrome (Mackay and Burnet, 1964). The arrangement and structure of these glands differs in mouse and man and the following facts are based on the description of Snell (1941). The two large lobes of the submaxillary glands overlap in the midventral line of the neck. Closely applied to their lateral surfaces are the sublingual glands. The parotid glands lie ventrolaterally in the neck and extend as far as the shoulders. The submaxillary gland alveoli comprise special serous cells, the sublingual alveoli are mucous secreting, while the parotid alveoli are also serous secreting, the cells containing supranuclear eosinophilic zymozen granules.

No macroscopic abnormality was seen in 50 glands examined from the hybrids and the 2 strains. Microscopically one usually had obtained varying proportions of these 3 glands and quite frequently a lymph node between 2 of the glands. Follicles in these nodes usually showed reactive centres.
FIG. 51. Salivary gland of NZB/BL X NZW hybrid mouse showing some lymphoid infiltration. (HE X600).

FIG. 52. Pancreas showing lymphoid tissue aggregate in close relationship to islets. NZB/BL X NZW hybrid female animal aged 35 weeks. (HE X187).
**NZB/BL X NZW hybrids**

In the NZBW animals 11/26 salivary glands examined showed perivascular aggregations of round cells, usually lymphocytes, sometimes with plasma cells. These were present in 6 females and 5 males examined. They were first seen in the animals at 26–27 weeks, were more striking and occurred earlier in the females, as well as being more frequent in this sex. Of these 6 female mice with the salivary gland lesion, 5 had a marked renal increase in lymphoid tissue and 4/6 had severely affected kidneys. The salivary changes were not seen in male or female mice with normal kidneys. Whether the parotid parenchyma is destroyed by any active process or just compressed is not clear but the latter seemed the more likely, with one exception (Fig. 51).

**NZB/BL animals**

In the NZB/BL animals 2/9 showed similar salivary lesions but not so severely.

**NZW animals**

None of 14 NZW salivary glands examined showed prominent perivascular collections of round cells.
CONCLUSIONS

NZB/W hybrid mice, when they have developed renal lesions, often also have prominent perivascular collections of round cells in the salivary glands.
VII. PANCREAS

In the mouse the pancreas comprises many irregularly shaped lobes of varying size which lie in the mesentery of the duodenum and extend across to the spleen (Snell, 1941). A few sections of pancreas were examined.

Histologically the architecture of the gland is similar to the human, except that the islets are more prominent in mice.

NZB/BL X NZW hybrids

In the first experiment in 1/8 NZBW males and 1/2 females examined perivascular collections of lymphoid tissue were noted in the pancreas without any parenchymal pancreatic lesion. These pancreatic lesions were seen in animals that had multiple lesions elsewhere e.g. the male animal had myocardial and thyroid, as well as pulmonary, reticuloendothelial and severe renal lesions. It seems, then, that in the pancreas perivascular collections of lymphoid tissue may be present in older animals, especially those severely affected in other organs.

In the later experiment, of a sample of 8 pancreases examined from the female hybrids of mean age 35 weeks, 4 showed moderate perivascular infiltrations
of round cells (Fig. 52). In close proximity to many
of the glands large lymphoid follicles or lymph
nodes were seen, evidence of the widespread lymphoid
hyperplasia in these animals.

**NZB/BL animals**

In the NZB/BL animals one 55 week old female
animal had similar perivascular lesions in the pancreas
to those seen in the NZBW animals. This animal also
showed marked collections of lymphoid tissue in liver,
lung and parotid glands. Foci of erythropoietic
tissue were noted in the pancreas of an NZB/BL male
of 55 weeks.

**NZW animals**

These groups of lymphoid tissue in the pancreas
were not seen in 4 NZW animals examined.

CONCLUSION

Perivascular collections of round cells and
prominent pancreatic lymph nodes were seen in at least
half of the NZBW female animals aged 8–9 months.
VIII. ADRENALS

Macroscopically these are small, smooth surfaced spherical glands weighing about 3 mg. in adult mice.

In the mouse these glands have the same basic pattern as in humans with cortex divided into zones and surrounding the medulla. However the zona reticularis in adult male mice is even less obvious than in man and often cannot be recognised. It is a wide zone in virgin female mice until they are past their useful breeding age. Apparently this special reticular or "X" zone (Snell, 1941) varies in different strains. It was present in females of the 3 strains examined here and it is usually possible to say what sex of animal the adrenals have come from.

Satisfactory adrenal gland sections were examined from 61 animals comprising 25 male and 14 female untreated NZBW animals, 5 male and 6 female NZB/BL animals and 7 male and 4 female NZW animals.

The large epithelial cells with vesicular nuclei in the zona fasciculata showed some variation in the foaminess of their cytoplasm. Probably lipid depletion was related to stress in that it was noted as striking in only 2 male NZBW animals with signs of bronchopneumonia. Occasionally in older mice, especially
those of the NZB/BL strain, there was some thickening of the capsule with fibrous tissue which extended into the outer layers of the cortex and in which there was an excess of round cells.

One of the 2 NZW male animals in which the "amyloid like" hepatic and splenic deposits have been described had a similar hyaline change in the inner zone of the cortex.
IX. PITUITARY

No macroscopic abnormalities were noted in the pituitary glands. Histologically (Snell, 1941) the gland shows 3 distinct parts viz. the pars distalis comprising cords and alveolar groups of epithelial cells with a fine connective tissue stroma; the well developed pars intermedia contains many polygonal cells similar to the pars distalis and long spindle shaped cells with darkly staining nuclei which may be endothelial, and the pars nervosa containing mainly neuroglial elements. The pars intermedia and nervosa are in intimate contact and are separated from the pars distalis by the residual lumen of Rathke's pouch.

A total of 72 pituitary sections were satisfactory for examination comprising those from 26 untreated male NZBW, 18 untreated female NZBW, 12 NZB/BL and 16 NZW mice. The proportions of chromophobe to chromophil cells showed some variations but these were not striking. Only H.E. stains were used. Basophils were not prominent. No definite abnormalities were noted in any of the pituates examined. An epithelial lined cyst noted in one elderly NZB/BL animal's pituitary is apparently a finding that is occasionally recorded (Snell, 1941).
X. LYMPH NODES

In the older hybrid animals these were often moderately, and sometimes markedly enlarged by hyperplasia of lymphoid tissue. Reaction centres were common. Sheets of pyronin positive cells, often clearly plasma cells could be identified, although Russell bodies were not easily found, as they are in NZB/BL animals. Plasma cell formation seemed particularly common in the mediastinal nodes. That the lymph nodes were enlarged was supported by the frequency with which nodes would be seen in close relationship to kidney, pancreas, thymus and salivary glands in the sections of these organs. Occasionally the vessels in the node were distended with small lymphocytes. Although a few very large nodes were seen, no evidence of capsular invasion was found. Mast cells were particularly uncommon in lymph nodes and this was useful with the Unna Pappenheim stain for distinguishing lymph node from atrophic thymus, which contains mast cells.
XI. BONE MARROW

No bone marrow abnormalities were noted in a moderate number of marrows examined from the NZB/BL X NZW hybrids. In particular, erythropoiesis was less striking than in the NZB/BL mice.