# SUMMARY OF THESIS ON BIOCHEMICAL ASPECTS OF ANTIBODY FORMATION PRESENTED FOR THE DEGREE OF Ph.D. UNIVERSITY OF GLASGOW : APRIL 1968

The work presented in this thesis was directed towards developing a clearer understanding of some of the biochemical aspects of antibody production. The systems employed were rabbit-anti-bovine serum albumin (BSA) for studies on the specific activity of DNA polymerase, and rat-anti-sheep red blood cells (SRBC's) for polyribosome studies.

Some rabbits were rendered tolerant to BSA by the intraperitoneal injection of this protein at birth. The establishment of tolerance was confirmed by antigen elimination tests in the mature animal. Comparative studies were made on the specific activity of DNA polymerase from the spleens and appendices of normal untreated rabbits, rabbits undergoing a primary or secondary response to BSA and rabbits previously rendered tolerant to BSA.

The results obtained indicated that in the case of the splenic polymerase activity was increased by the immunization procedures. Increased activity was observed on days $\frac{1}{2}$, 3, 4, 6 and 8 of the primary response and on days 2, 3, 4, 6 and 8 of the secondary response. In the case of the animals



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rendered tolerant to BSA enzyme activity was depressed 3, 4 and 6 days after one injection of this protein into the adult animal and 1, 4 and 6 days after a second injection. The significance of these results and their possible relationship to lack of macrophage proliferation in the secondary response and in tolerant animals was discussed.

Results from comparable studies in the appendix showed no consistent pattern but there appeared to be a decrease in the specific activity of the enzyme at various times after antigen injection. A possible role of the appendix in the immune response was considered on the basis of these results.

In view of this antigen stimulated cellular response in the spleen, studies were made on the polyribosome patterns or the spleens of control and immunized animals. A procedure was developed whereby polyribosomes could be extracted and analysed from rat spleens. No consistent differences between the polyribosomes of control and immunized rats were observed. Possible reasons for this inconsistency were discussed.

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# B I O C H E M I C A L  A S P E C T S

# O F

# A N T I B O D Y  P R O D U C T I O N

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by

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Thesis submitted for the Degree of
Doctor of Philosophy of the
University of Glasgow, Scotland.

May, 1968.

# A C K N O W L E D G E M E N T S

I should like to express sincere gratitude to Professor J. N. Davidson for permitting me to carry out this work in the Department of Biochemistry and to other members of the Department who have at one time or another advised me in this work. I am particularly indebted to Dr. T. A. Douglas and Dr. I. D. Aitken who have given me constant help and encouragement during these investigations, and to Dr. H. M. Keir for his helpful advice on the DNA Polymerase studies. The main part of this work was carried out while the author was Research Assistant to Dr. T. A. Douglas who was in receipt of an Agricultural Research Council research grant.

We now come to the important question of the significance of the toxophile groups in organs. That these are in function specially designed to seize on toxines cannot be for one moment entertained. It would not be reasonable to suppose that there were present in the organism many hundreds of atomic groups destined to unite with toxines, when the latter appeared, but in function really playing no part in the processes of normal life, and only arbitrarily brought into relation with them by the will of the investigator. It would indeed be highly superfluous, for example, for all our native animals to possess in their tissues atomic groups deliberately adapted to unite with abrin, ricin, and crotin, substances coming from the far distant tropics.

Paul Ehrlich, 1900.

# GENERAL INTRODUCTION

HISTORICAL

The concept of immunity properly began with the introduction of vaccination against cow-pox in the late 18th century (Jenner, 1798) but the first attempt to place immunology on a more secure scientific basis must be attributed to Louis Pasteur. Almost 100 years after Jenner, he showed that attenuated micro-organisms could be used for the preparation of vaccines against chicken cholera (Pasteur, 1880a) and other diseases of bacterial origin (Pasteur, Chamberland and Roux, 1881). At the same time he suggested (1880b) that in the first attack of a micro-organism on an animal some nutrient substance was consumed by the organism. On the occasion of a further attack on the same animal, the same organism would be unable to thrive and immunity would result. This was termed the "exhaustion theory." In contrast, Chauveau (1880) postulated a "retention theory" which suggested that the micro-organism left behind some substance which prevented a further attack from being successful.

However, with the elucidation of a bactericidal material in plasma, Buchner (1894) presented his hypothesis that toxins were modified and incorporated into antitoxins

in the body. This was probably the first attempt to explain the specificity of the immune response but the downfall of this theory came with the realisation that the amount of antitoxin formed was very much greater than the amount of toxin administered to the animal. Another process by which some specialised cells could attack and destroy infecting agents was being investigated by Metchnikoff (1901). This was a cellular defence mechanism termed "phagocytosis" and involved the actual ingestion of the infecting agent by the cell. This concept however failed to gain popularity until many years later.

In 1900, Ehrlich elaborated in some detail his "side-chain" theory of antibody formation. He considered that cells were fitted with "side-chains," the normal function of which was to take up foodstuffs but each of which "incidentally and by pure chance" also had the capacity to combine with a particular toxin. When this combination happened, the normal function of the "side-chain" was impaired and regeneration of the particular cells and "side-chains" involved then took place. However, according to Ehrlich, an over-compensation resulted in the production of an excess of "side-chains" which were consequently discarded into the blood thus accounting for serum antibody.

This theory explained many of the known aspects of immunity including -

(a) The specificity of antitoxins

(b) The duration of immunity (even in the absence of toxin)

(c) The excess production of antitoxin in comparison with the amount of toxin administered

(d) Failure to respond to a given toxin.

Ehrlich suggested that this last point simply implied the absence of a suitable "side-chain." He further suggested that some antibodies, particularly those directed against erythrocytes and bacteria, had 2 specificities. One of these allowed the antibodies to combine with the specific antigen and the other permitted combination with the heat labile serum factor (complement) and led to lysis of the cell involved.

Ehrlich's theories so adequately explained the known phenomena of immunity that they formed a working hypothesis for research over the ensuing 3 decades. As will be mentioned later, some modern theories of antibody formation adopt many of the principles of Ehrlich's "side-chain" theory. However, some immunologists of the early 1930's considered that the number of antibodies which an animal

could synthesize was so great as to exceed the number that would be available according to Ehrlich's "side-chain" theory, and so new hypotheses began to appear.

In the direct template theories of Breinl and Haurowitz (1930), Alexander (1931) and Mudd (1932), it was suggested that the antigenic material directed incorporation of amino acids into antibody molecules at the site of synthesis and thus ensured complementarity of configuration. These theories dictated that antibody specificity was a function of the amino acid sequence of the antibody molecule. On the other hand, Pauling (1940) suggested that the antigenic determinant served only to direct the folding of the protein chain and that the various antibody molecules differed from each other only in this respect, and not in amino acid sequence.

Burnet (1941) considered these direct template theories in detail and his criticisms of their inadequacies were as follows. The failed to account for the fact that:

1. A second or subsequent contact with an antigen causes increased antibody production (the secondary response)
2. Antibody production continues long after the antigen responsible has disappeared from the body

3. Antibody production continues in the progeny of the cells originally stimulated

4. The type of antibody produced varies with age, with the mode of administration of the antigen and from one species to another.

To overcome some of the difficulties, Burnet and Fenner (1949) proposed an indirect template theory whereby antigen modified a globulin-synthesizing enzyme resulting in the formation of an enzyme that was not originally a cell constituent. Such a proteinase would then be responsible for synthesizing the antibody globulin. These authors further suggested that a similar mechanism was involved in the destruction of the body's own aged cells but with the difference that such cells would carry distinguishing components ("self-markers") permitting their catabolism without concomitant antibody production. An important corollary to this was the prediction that exposure of foetuses to a "marker" would result in absence of an immune response to such a "marker" in adult life. This concept of "tolerance" was later confirmed experimentally (Billingham, Brent and Medawar, 1953).

In 1955, Jerne propounded some ideas that were reminiscent of those of Ehrlich of 50 years earlier. The

essential concept here was that of "natural antibodies." That is, the gamma globulin fraction of serum was considered to be heterogeneous and for every antigen there would be a gamma globulin of most closely matched complementary configuration. After combination of the antigen with the selected globulin followed by phagocytosis, synthesis of this particular globulin would then be enhanced (the "Natural Selection Theory" of antibody formation). Another theory which placed the emphasis on selection rather than instruction was the "Clonal Selection Theory" of Burnet (1959). According to this theory, extensive mutation in the relevant areas of the genes coding for gamma globulin takes place during embryonic life. This would result in a population of potential antibody forming cells each with the capacity to form a specific antibody. Also, during embryonic life, clones which had the potential to react with "self" antigenic determinants would be eliminated. In this way, self-recognition and the induction of tolerance by the administration of antigens to the neonate were explained. In the primary response where the antigen or some material derived from it encountered the appropriate clone of cells it would trigger off a sequence of events resulting in cellular proliferation, the formation of antibody-producing cells and also the formation of an

increased number of conditioned cells which do not immediately synthesize antibody but which on a further antigenic stimulus would be able to do so. This would result in the more rapid and greater production of specific antibody which is characteristic of the secondary response.

Variants of most of these theories are still in vogue and will be considered below under "Some Current Theories of Antibody Formation."

## THE TISSUES INVOLVED

The first unequivocal evidence that the spleen was a site of antibody synthesis was supplied by Fagraeus (1948) who showed that plasma cells in the red pulp of this tissue were actively involved. On the basis of fluorescent antibody studies, Leduc, Coons and Connolly (1955) concluded that the lymph nodes of hyperimmunized animals were also involved. Askonas and Humphrey (1958) investigated the ability of tissue slices to produce antibody *in vitro* and concluded that, depending on the nature of the antigen and the mode of administration, tissues as diverse as spleen, lung, bone marrow and to a lesser extent liver, were implicated in production of antibody.

More recently a considerable amount of evidence has

been amassed which implicates other lymphoid tissue in the immune response. Miller (1961) showed that thymectomy in neonatal mice resulted in depletion of the number of lymphocytes and in impairment of the immune response in the mature animal. He suggested that the thymus may be involved in producing cells to populate other lymphoid organs and also in secreting a factor which promotes lymphopoiesis in such organs. An independent but similar role has been suggested for the appendix (this is considered in some detail in the DNA polymerase section of this thesis) and for other lymphoid areas such as Peyer's patches in the intestine. In reviewing this field recently, Cooper, Gabrielsen and Good (1967) suggest that there is a thymus dependent system which is involved in cellular immunity and a second system which in birds is dependent on the Bursa of Fabricius and in mammals on the Peyer's patch type of lymphoid tissue and which is concerned with serum antibody production. Sites of antibody production (such as spleen and lymph nodes) are termed peripheral lymphoid tissues and sites of origin of the functionally diverse lymphoid cells (for example thymus, appendix) are central lymphoid organs.

## THE CELLS INVOLVED

The cellular aspect of the immune response will be considered firstly with respect to plasma cells and lymphocytes, and secondly with respect to the role of the macrophage.

In reviewing the large amount of literature on the plasma cell, Thorbecke and Benacerraf (1962) concluded that there was no doubt that plasma cells and in particular immature plasma cells were the cells actively involved in the synthesis of antibody. They further suggested that these cells arose by

reticular cell $\xrightarrow{(i)}$ haemocytoblast $\xrightarrow{(ii)}$ immature and mature plasma cells

where (i) occurred in the lymphoid tissue (haemocytoblast and large lymphocyte possibly being synonyms) and (ii) in the red pulp of the spleen. It was considered that both lymphocytes and plasma cells developed from undifferentiated reticular cells.

Nossal and Mäkelä (1962a) reached similar conclusions with some significant additions. Thus they suggested -

(a) that the antigen responsive cells may be large lymphocytes;

(b) that, after multiplication of these cells, some differentiate to plasma cells and some to resting or memory cells which they considered may appear morphologically as small lymphocytes;

(c) that antigen could also stimulate the memory cells which could then re-enter the cycle as primitive competent cells - possibly large lymphocytes.

In a review article on the lymphocyte, Yoffey (1964) pointed out that a certain proportion of lymphocytes have a very long life span and suggested that these cells were developmentally static because they had been conditioned on primary antigenic stimulation. He considered that in response to a second encounter with antigen these cells enlarged and were transformed into the large basophilic cells which appear 24 hours after antigenic challenge. Both Yoffey (1964) and Nisonoff and Thorbecke (1964) present a considerable volume of evidence that such a transformation can take place.

Mitchell and Calabresi (1964) extended this concept of "conditioned lymphocytes" and postulated that some small lymphocytes formed in a rabbit lymph node in response to a localized primary injection function as "immunological messengers." By means of these cells remote nodes receive

information which originated in the node sensitized by direct contact with antigen. Thus, if antigen was injected near a node remote to a first injection, a typical secondary response was obtained even though no antigen leakage could be detected at the height of the cellular response to the first injection.

Considerable evidence has accumulated that recirculating small lymphocytes are involved in the primary response to homografts (reviewed by Wilson and Billingham, 1967). It is considered that, when certain committed lymphocytes are stimulated, they become established in the regional lymph node, enlarge to the large pyroninophilic blast cells which are characteristic of the early part of an immune response, and that these subsequently divide to produce more committed small lymphocytes. Furthermore, Hummler et al. (1966) have shown that circulating cells producing haemolytic antibody directed against sheep red blood cells (SRBCs) showed the typical structure of small lymphocytes when examined by electron microscopy. In this connection, McGregor, McCullagh and Gowans (1967) have shown that, when rats are prevented from producing a haemolysin response to SRBCs by X-irradiation, this inability could be overcome by the administration of normal small lymphocytes. It was also suggested (Ford and Gowans, 1967) that the magnitude

of the haemolysin response depended on the concentration of lymphocytes entering the spleen at the time of administration of the antigen and that these cells which "homed" to the periarteriolar lymphoid sheath of the splenic white pulp within 3 hours of their administration to the perfusing blood, were the important cells in the ensuing immune response. Nossal (1967) has similarly emphasized the importance of lymphocyte "compartments" in the spleen and states that antigens localize in the lymphoid follicles of the white pulp within 2-4 hours of their administration.

Thus, while plasma cells are considered to be the main effector cells in antibody production (Attardi, Cohn, Horibata and Lennox, 1964), increasing importance is being attributed to the circulating small lymphocyte both as a potential precursor of such cells (via transformation to a blast cell and subsequent division) but also as an antibody producing cell in its own right.

A further potentiality of the small lymphocyte was noted by Holub (1962) who recorded that, when pure suspensions of these cells were transferred from an immunized donor and kept in Millipore diffusion chambers in the peritoneal cavity of a recipient rabbit, there was a gradual transformation of these small lymphocytes into macrophages. There have been

considerable differences of opinion in this connection but the recent report of Boak, Christie, Ford and Gowans (1968) strongly favours this concept of transformation of lymphocyte to macrophage under certain conditions. These authors initiated a graft-versus-host reaction in mice by the intravenous injection of thoracic duct small lymphocytes (98.5% pure). Eleven to 12 days after this injection, liver macrophages were isolated from the host and by use of appropriate chromosome marker techniques it was observed that 95% of those macrophages which were engaged in mitosis were of donor origin - this is, were derived from the inoculum of thoracic duct cells. However, in another set of experiments a different stimulus for macrophage proliferation was used and different results ensued. Mice were X-irradiated and then re-populated with either (1) lymph node cells and bone-marrow cells or (2) thoracic duct cells and bone-marrow cells. Host cells, lymph node cells, thoracic duct cells and bone-marrow cells all bore distinct chromosome markers for identification. Eleven to 19 days later, partial hepatectomy was used to stimulate proliferation of liver macrophages and these cells were isolated and identified. In the former case (Case 1), the majority of the dividing macrophages were

of bone-marrow origin and in the latter case (Case 2) the ratio of bone-marrow : thoracic duct : host cells as origin of the macrophages was around 8.5 : 3 : 1. In these latter experiments, however, the inoculum of lymphocytes (lymph node or thoracic duct) was very much greater than that of bone-marrow cells. Thus it was concluded that depending on the nature of the stimulus re-circulating lymphocytes and bone-marrow cells (which may have the morphology of small lymphocytes) can transform into proliferating macrophages.

Interest in the macrophage as a cell essentially involved in the mediation of the immune response was revived by the experiments of Fishman (1961). These experiments together with other evidence in favour of and against a crucial role for macrophage RNA will be considered below but observations on the cellular aspects will be introduced here.

In the experiments of Perkins and Leonard (1963), mouse peritoneal macrophages were incubated <u>in vitro</u> with RBCs from various species or with RBCs coated with serum factors to enhance phagocytosis. On microscopic examination of the macrophages it was observed that phagocytosis of the various antigens was highly selective

and could be modified by the presence of specific erythrocyte antibodies. It was concluded from these experiments that individual macrophages had the ability to recognise and ingest specific foreign material, thus fitting them for some particular role in antibody biosynthesis. Schoenberg, Mumaw, Moore and Weisberger (1964) summarized the existing evidence implicating macrophages surrounded by lymphoid cells in the immune response and recorded that in studying sections of spleen and lymph node from immunized and non-immunized rabbits, "islets" of macrophages surrounded by either plasma cells or lymphocytes were found in all cases - though in greater numbers with immunized animals. Electron microscopy revealed some areas of direct communication between the cytoplasm of macrophages and that of lymphocytic cells. Particles the size of ribosomes were seen in the cytoplasm connecting the 2 cell types though antigen could not be demonstrated in antibody producing cells.

This phenomenon was further suggested by the experiments of Nossal, Ada and Austin (1964). Twenty-four hours after the injection of labelled flagellar antigen the label was found in discrete cells in the responding lymph node and each heavily labelled centre appeared to have extensions

bearing labelled material branching out from it. It was inferred from these observations that the label was present in macrophages which possessed dendritic processes containing antigen-derived material and that when primitive cells encountered these processes they were caused to proliferate. These investigations were later developed and the conclusions confirmed by Miller and Nossal (1964).

Bartfeld and Juliar (1964) described experiments in which peripheral leukocytes from patients with rheumatoid arthritis were grown in culture for 4 days. Such patients are thought to produce macroglobulin in response to antigenic stimulation from denatured gamma globulin. Stained slides were prepared at intervals from both rheumatoid and non-rheumatoid cultures. By 2-4 days the number of small lymphocytes had decreased and there was an increase in the number of lymphoblast cells and macrophages. Also, in 6 out of 8 rheumatoid cultures but in no normal cultures organized "islets" had appeared. In some of these islets, exchange of information ("peripolesis") appeared to be taking place. Similar observations were made by McFarland and Heilman (1965) who observed that in tissue culture of peripheral leucocytes clumps of 10 or more lymphocytes were attached in radial fashion to macrophages.

They suggested that this contact may represent an alternative to cell division as a means of developing clones of antibody forming cells in lymphoid tissue after primary antigenic stimulation.

Cohen (1967) has presented evidence which further suggests that there are specific binding sites on the surface of lymphocytes receiving information from other cells. Thus, when RNA was extracted from the spleens of mice immunized against SRBCs, the RNA could confer antibody-forming capacity to normal mouse spleen cells. If this RNA was minimally digested with ribonuclease and administered to normal cells immediately before undigested "immune" RNA was given, then the conversion to antibody-producing cells was inhibited. The inhibition was specific since incubation of spleen cells with RNA (digested or non-digested) from spleens of animals immunized against one antigen did not inhibit the conversion to antibody-producing cells when RNA from animals immunized with another antigen was used. Thus it was considered that whether the active RNA was pure or an RNA-antigen complex there were specific recognition sites on the surface of the recipient lymphocytes.

Gallily and Feldman (1967) have inferred from their observations that processing of antigen by macrophages is a

necessary step in antibody production. Mouse peritoneal macrophages rendered 98% pure by culturing for 48 hours were incubated *in vitro* with Shigella antigen and were then transferred intraperitoneally to X-irradiated recipient mice. Agglutinin production was demonstrated in recipients 5 and 8 days after inoculation. However, if macrophages from an X-irradiated mouse were used, then these failed to elicit antibody production in the X-irradiated recipient. It was thus suggested that in the immune response macrophages must "process" the antigen, and that immunological unresponsiveness due to X-irradiation is a result of some effect on macrophages and not on lymphocytes.

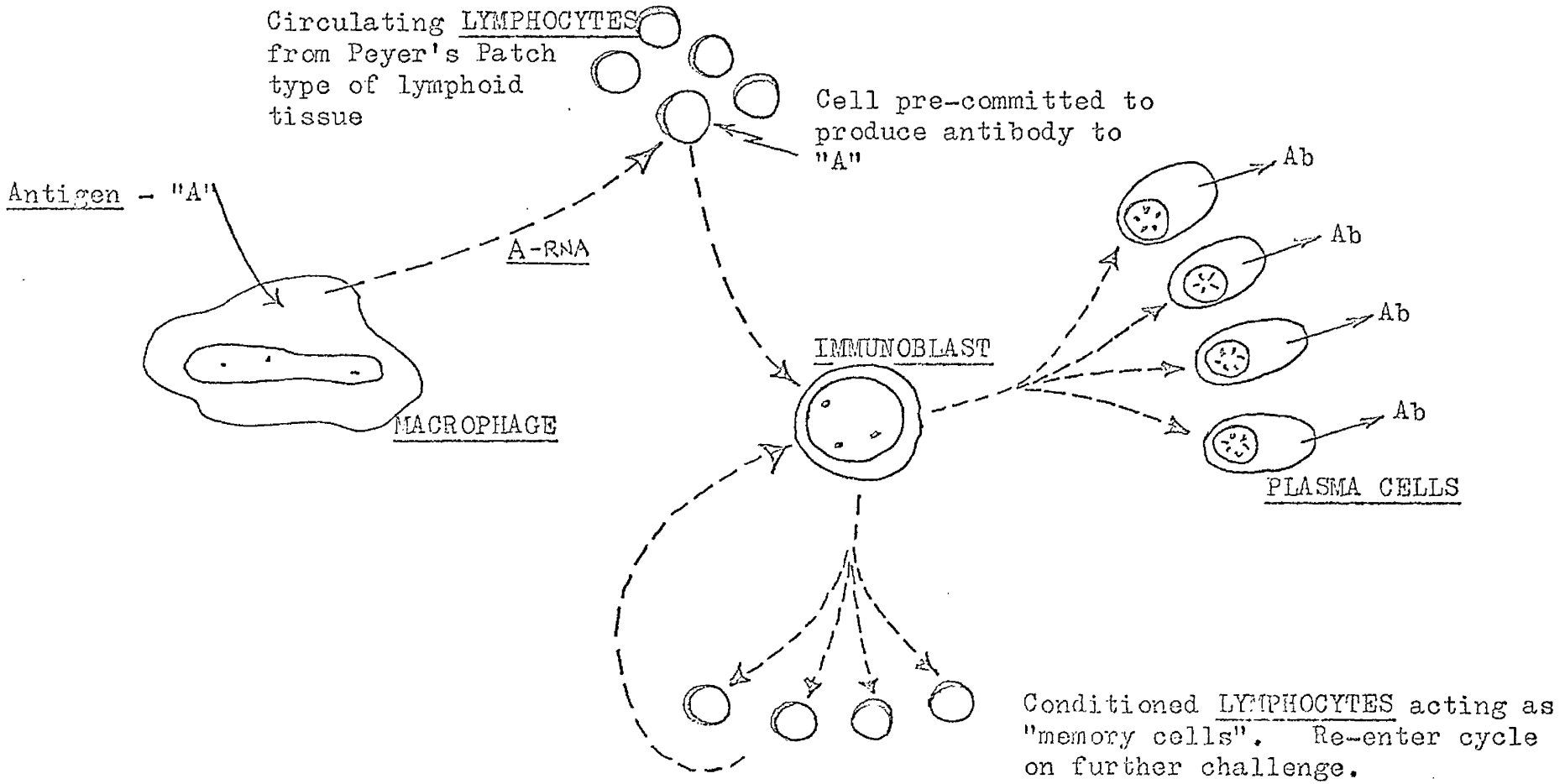
In summary, the scheme presented in Fig. 1 is postulated as a working hypothesis regarding the cells involved in the immune response.

## MOLECULAR ASPECTS

### 1. Antigens

As well as proteins and cellular structures, many other classes of compound exhibit antigenicity. Polysaccharides, nucleic acids, synthetic polypeptides can all elicit formation of specific antibody. In addition, haptenes, organic compounds of low molecular weight, can also act as antigens if complexed to carriers of one of the above classes.

FIG. 1. POSSIBLE INTER-RELATIONSHIPS AMONG THE CELLS INVOLVED IN THE IMMUNE RESPONSE




Ab = antibody

Haurowitz (1965) considered that the shape rather than the charge or polarity or any other property of the antigenic determinant was the important factor in conferring antigenicity on a compound. This conclusion was based on reports that antibodies can differentiate between o-, m- and p-isomers, between L- and D-isomers and between cis- and trans-derivatives of otherwise identical antigenic determinants. Also, antibody directed against the positively charged haptene group azophenyl-N-trimethyl-ammonium ($-N = N-C_6H_4 - N^+ (CH_3)_3$) can also combine with the uncharged azophenyl-trimethylmethane group ($-N = N-C_6H_4 - C (CH_3)_3$). The shape in each case is identical.

The experiments of Koshland and Engleberger (1963) indicated that not only did the amino acid content of different antibodies vary but, when the antibody was directed against a positively charged haptene, the difference in amino acid content in the specific antibody favoured amino acids bearing a negative charge and vice versa. Thus, while foreignness of shape may be the criterion of antigenicity, it also appears that a charged antigen can somehow direct formation of an antibody bearing an opposite charge. It was not, of course, established that the charge on the antibody

molecule is associated with the antibody combining site.

2. Antibodies

Present knowledge on the subject of immunoglobulins has been the subject of a recent essay by Porter (1967) and of an extensive review by Cohen and Milstein (1967).

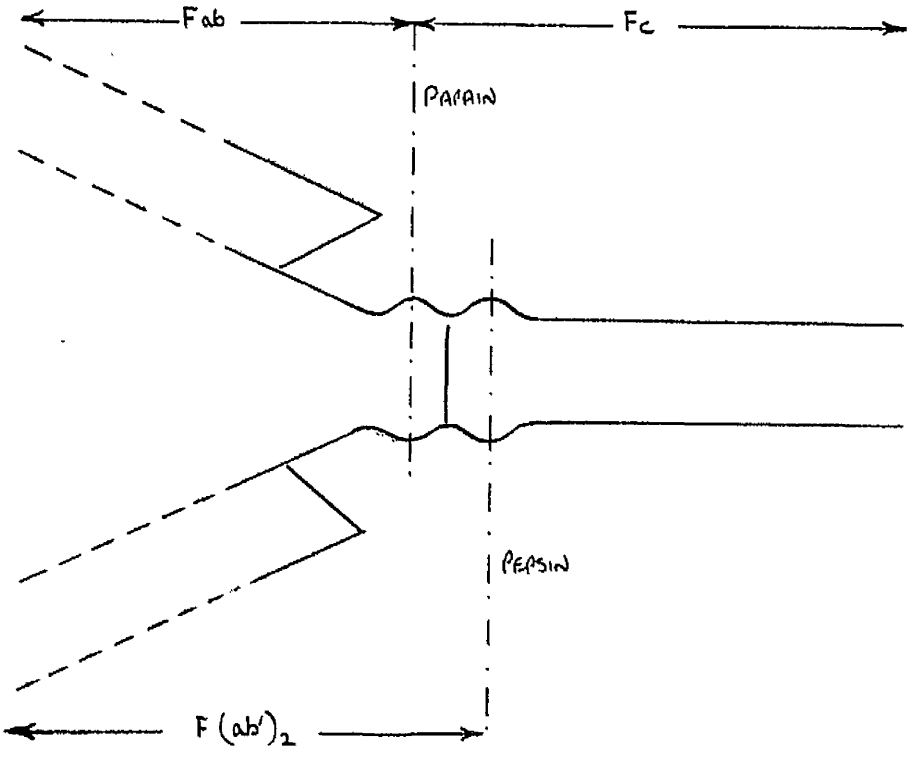
The 3 main classes of immunoglobulin are IgG (7S antibody), IgM (19S antibody) and IgA, all of which exhibit antibody activity. Molecular weights are 150,000, 900,000 and 400,000 respectively. All of the immunoglobulins are associated with carbohydrate. The structure of each is based on a 4-component unit of 2 heavy and 2 light chains held together by disulphide bonds (Fig. 2). The IgG molecule is a single unit, IgM is possibly formed from 5 units held together by disulphide bonds and IgA from 2 units which appear to dissociate readily. From electron microscopy studies it was inferred that this unit structure is Y-shaped and that the angle between the 2 arms may vary from $0^{\circ}$ to $180^{\circ}$.

Hydrolysis with papain yields 2 identical fragments (Fab) and a third fragment (Fc). Peptic hydrolysis yields a fragment (Fab')2 and small peptides from the Fc part of the molecule. The N-terminal half of the heavy chain (Fd) can be prepared from the Fab fragments.

Fig. 2 : DIAGRAMMATIC REPRESENTATION OF THE 4-CHAIN UNIT STRUCTURE OF IMMUNOGLOBULINS

Chains are linked by disulphide bridges. Broken lines indicate where variation in amino acid sequence may occur within a given chain type. Dot-dash lines indicate where proteolytic digestion takes place giving rise to the fragments indicated.

From Cohen and Milstein (1967).



Studies of these various fragments have revealed that -

(i) The antibody combining site resides in the Fab fragments

(ii) Variation in amino acid sequence is common in the N-terminal portions of heavy and light chains and may determine the three-dimensional structure of the immunoglobulin and hence antibody specificity. The amino acid sequence in the C-terminal portion is virtually constant.

(iii) Fd fragments isolated from specific antibody will bind to circulating antigen in vivo but isolated light chains do not normally possess this ability

(iv) Antibody binding capacity is enhanced when light chains from the original antibody are recombined with specific heavy chains, suggesting that the light chain does have some role in the antibody binding site

(v) Within a single class of immunoglobulin (IgG, IgM or IgA) 2 or more types of light chain have been found which differ antigenically from each other - isotypic variants

(vi) Associated with any of these types of light chain there can be a variety of heavy chain types each bearing distinct antigenic properties. These type-specific determinants can be located on the Fc or Fd parts of the heavy chains

(vii) Allotypic variants (antigenic differences between individuals of the same species) sometimes occur in both light and heavy chains (on Fc and Fd fragments) of a number of species

(viii) Idiotypic variants (antigenic specificity associated with individual antibodies) have been demonstrated in a few cases and appear to be located in the variable region of the Fab fragment.

Cohen and Milstein (1967) emphasize that isotypic and allotypic specificities alone determine the amino acid sequence of the C-terminal sections of light chains (and probably also of heavy chains) while the amino acid sequence of the N-terminal sections appears to be specific for their clone of origin and vary from one immunoglobulin to another.

The role of the various classes of immunoglobulin is not completely clear as yet. Bauer, Mathies and Stavitsky (1963) and Uhr (1964) showed that for most of the antigens studied (particulate and protein) the primary response was characterized by early production of IgM antibody. This was arrested and was followed by production of IgG antibody beginning about 7 days after the antigen injection. In the secondary response, IgG was produced earlier and in greater quantity than in the primary response and small amounts of IgM antibody could also be produced. It was suggested that the IgM mechanism did not develop immunological memory whereas the IgG system does. Bauer *et al.* (1963) postulate that the different classes of

immunoglobulin are formed in different cell types.

In this connection the experiments of Adler, Fishman and Dray (1966) are of considerable interest. The details of the experimental techniques are considered below but, in summary, rabbit peritoneal exudate cells were incubated *in vitro* with antigen, RNA was extracted and transferred to cultured fragments of non-immune rabbit lymph node tissue and antibody production ensued. When the peritoneal exudate cells and lymph node cells came from animals of different allotypes, the IgM antibody formed first in the culture contained antigenic markers characteristic of the donor of the peritoneal exudate cells, while the IgG antibody formed later had the allotypic specificity of the donor of the lymph node cells.

The immunological role of IgA is not clear. It is present in various seromucous secretions (Cohen and Milstein, 1967) in relatively high concentration. Exocrine IgA shows certain differences from serum IgA :

(a) Greater molecular weight

(b) It is associated with an antigenically distinct fragment (transport or T piece)

(c) It has a greater stability in the presence of mild reducing agents.

3. Ribonucleic Acid

As already stated, the experiments of Fishman (1961) implied that macrophage derived RNA had a crucial role in the biosynthesis of antibody. These experiments together with other evidence which suggests that RNA extracted from lymphoid tissue or from preparations of macrophages is involved in mediating the immune response will be introduced here.

In Fishman's experiments, preparations of rat lymph node cells containing not more than 5% macrophages were cultured *in vitro* and peritoneal macrophage preparations were obtained from the same animals. T2 bacteriophage was used as antigen. Either (i) T2 phage alone or (ii) macrophage alone or (iii) a washed homogenized preparation of macrophage cells pre-incubated with T2 phage was added to the lymph node cells in tissue culture. After incubation, gamma globulin was isolated from the culture medium (by salting out) and was concentrated ten-fold. Anti-T2 activity was assayed by testing the ability of T2 phage after mixing with the culture medium concentrate, to form plaques when spread over the surface of a uniform Esch. coli suspension in agar in Petri dishes. The greater the neutralizing activity of the culture fluid the lower the number of plaques.

No neutralizing activity was found when culture fluid from case (i) or (ii) was used. In case (iii) there was significant neutralization but only in 1 out of every 5 attempts. The antibody was specific for T2 phage. Other observations include :

(a) Apparently only 10 - 20% of macrophages will react with antigen.

(b) Optimal time of incubation of macrophage with T2 phage was 30 min. More than a simple absorption or ingestion appeared to be involved.

(c) The macrophages and lymph node cells must come from the same species - for example, no activity was found using rabbit macrophages and rat lymph node cells.

(d) Study of the phage-macrophage filtrates which were successful in stimulating antibody formation revealed that ribonuclease completely inhibited antibody synthesis. Control experiments showed that this was not due to any action on lymph node cells.

Fishman's tentative conclusion from these experiments was that lymph node cells in tissue culture used ribonuclease sensitive material derived from macrophages as a template for antibody production.

Mannick and Egdahl (1962), using the principle that lymph node cells of rabbits receiving a skin homograft have the potential to react against the skin of the donor rabbit, showed that a tuberculin-like skin reaction was obtained when immune lymph node cells from a recipient rabbit were injected intradermally into the donor. When non-immune lymph node cells from other rabbits were injected into the donor at a different site a skin reaction was obtained but in a fewer number of cases.

In an attempt to find the active principle, the RNA of the immune lymph node suspension was extracted and incubated in vitro with non-immune lymph node cells from a control rabbit. As control, non-immune lymph node cells incubated without RNA were used.

The suspensions, test and control, were injected intradermally into opposite sides of the back of the donor rabbit. A significant reaction was obtained in 17 out of 20 cases with the test suspension and in only 2 out of 20 with the control. Other controls, neutral lymph node cells incubated with RNA from other neutral rabbits, and the reactive RNA without the lymph node cells, both failed to produce a reaction. Further, the reaction produced by neutral cells incubated with reactive RNA was completely

specific for the donor of the skin homograft.

Fishman and Adler (1963) in the second paper of their series record experiments in which they inserted into the peritoneal cavity of rats (irradiated or non-irradiated) diffusion chambers containing various permutations of (i) RNA extracted from macrophages pre-incubated with T2 phage - RNA (M-T2), (ii) RNA from untreated macrophages - RNA (M), (iii) lymph node cells from neutral rats - LN, (iv) ribonuclease - RNase, (v) T2 phage. Their most pertinent results are summarized in Table 1.

Studies on the reactive RNA showed it to be of low molecular weight. Attempts to find antibody binding material in the RNA failed, though the possibility was not excluded that the RNA contained fragments of T2 phage complexed with it.

Friedman (1964) has similarly shown that RNA extracted from the spleens of immune mice can confer the capacity to produce specific haemolytic antibody to normal mouse spleen cells. Ribonuclease treatment of the immune donor RNA prior to incubation with normal cells, suppressed subsequent antibody formation.

One major question raised by these experiments was

TABLE 1 : From Fishman and Adler (1963)

| Group | Material in Chamber* | Antibody Response in Rats** | |
|---|---|---|---|
| | | X-irradiated | Non-irradiated |
| 1 | RNA (M-T2)+LN | 8/12 | 10/12 |
| 2. | RNA (M-T2) | 0/12 | 7/9 |
| 3 | RNA (M-T2)+LN+RNase | 0/11 | 0/11 |
| 4 | RNA (M)+LN+T2 | 0/11 | 0/11 |

* See text for details of abbreviations

** Numerator-number of rats producing antibody
Denominator - number tested

whether or not antigen was present in the RNA extracts used. Askonas and Rhodes (1965) injected [$^{131}$I-]haemocyanin intraperitoneally into mice and after 2⅓ hours collected and pooled the peritoneal cells from 20 mice. RNA was extracted by phenol and by sucrose gradient analysis the presence of macromolecular $^{131}$I was confirmed.

Some of these RNA preparations were found to be significantly immunogenic in primed recipient mice. A further important observation was that, if haemocyanin was added to the macrophage preparation immediately before RNA extraction (in amounts sufficient to give the same ratio of haemocyanin to RNA as before), then this RNA was also immunogenic in 3 out of 5 mice. From these experiments it was concluded that antigenic material was present in the reactive RNA and that the reactive RNA was not informational RNA newly formed by macrophages in response to contact with antigen. They suggested that the RNA simply permitted enhanced antigen uptake by the effector cells.

Gerughty, Rosenau and Moon (1966) have shown that antibody production can be induced in suspensions of neutral mouse spleen cells by the addition of ribosomes extracted from the spleens of immunized mice. The antigen used in these experiments was a suspension of cultured cells of mouse origin. The transferring capacity of the ribosomal

preparation was inhibited by ribonuclease. The experiments of Cohen (1967), some aspects of which have already been considered, also indicated that RNA from immune mouse spleen could confer antibody forming capacity on neutral spleen cells. Cohen recorded however that 8-12S RNA was the active form which is in contrast to the observations of Gerughty *et al.* that the reactive RNA was associated with the ribosomal fraction.

In concluding this introduction to the role of RNA in the immune response, some reference will be made to RNA metabolism in lymphoid cells.

Mitchell (1964a, 1964b) has carried out an extensive survey by autoradiography on nucleic acid and protein metabolism in lymphoid cells. She observed that the ratio of uridine uptake to leucine incorporated in 3 cell types, immunoblasts, small lymphocytes, mature plasma cells, was in the proportion 93 : 8 : 1, and concluded that in mature plasma cells protein synthesis did not depend on concomitant RNA synthesis. In studying the effect of actinomycin, it was found, using concentrations which were sufficiently high to give almost complete cessation of $^{3}$H-uridine uptake, that this caused virtually no reduction in the protein synthesis occurring in plasma cells or lymphocytes - that is, protein

synthesis appeared to be occurring in these cells without accompanying synthesis of mRNA.

A similar conclusion was reached by Miller (1964). At various time intervals after receiving a secondary stimulus of antigen, rats were injected with tritiated nucleosides. At varying times thereafter, the animals were sacrificed and autoradiographs of lymph node smears were taken. Labelled cytoplasmic RNA was detected in plasma cells 10 and 30 days after the last dose of tritiated nucleoside. Miller concluded that a stable form of cytoplasmic RNA existed in plasma cells. He suggested that this was mRNA and that by repeatedly serving as a template for antibody synthesis a single molecule of mRNA could result in production of a large number of molecules of antibody.

These results appear to conflict with those of Uhr (1963) who found that the in vitro production of antibody was inhibited by actinomycin, and of Svehag (1964) who also observed that the in vitro synthesis of IgM antibody was suppressed by the addition of actinomycin and was therefore contingent upon DNA-dependent mRNA synthesis.

## SOME CURRENT THEORIES OF ANTIBODY PRODUCTION

Although controversy still exists concerning the mechanism of antibody production, it appears to be no longer a matter of instructive versus selective theories. As Jerne (1966) recently pointed out, the question now being examined is - at what level does selection take place? For example, is it at the level of pre-existing antibody, at the cellular level or at the level of genes coding for antibody? However, this is disputed by Haurowitz who is the protagonist of the minority who still favour an instructive theory.

After considering the various stages in the biosynthesis of protein, Haurowitz (1965) postulated that the antigenic determinant complexed either with the mRNA or with the ribosomes involved in gamma globulin synthesis. The code would be translated normally along most of the length of the mRNA but, during formation of the antibody binding site, the shape of the antigenic determinant would result in rejection of the "correct" amino acids and incorporation of amino acids which would permit complementary folding of the nascent peptide chain. Haurowitz considered that the antigenic determinants persisted for many months in antibody forming tissues so the question of antibody formation in the absence

of antigen did not arise. According to his theory, the earlier and greater production of antibody in the secondary response resulted from the fact that antigen complexed to existing antibody and was more readily trapped in phagocytic cells. Thus a greater response was elicited. The question of self-recognition was considered invalid. This phenomenon, plus that of immune paralysis invoked by the injection of massive amounts of foreign protein into the mature animal, was attributed to the continuing presence of the "self" or foreign proteins in the serum.

It was paradoxically a consideration of tolerance and self-recognition that led Smithies to develop his hypothesis on the mechanism of antibody production (Smithies, 1965, 1966). He reasoned that for the organism to eliminate genes coding for undesirable antibody and to activate genes coding for desired antibody, the informational nucleic acid and the corresponding antibody product would be associated as in a virus. If the antibody coat protein of such a virus encountered the specific "antigen" in an unsuitable environment (for example, in plasma), the nucleic acid would be released and destroyed but if the contact was intracellular, replication of the virus and excess coat protein (antibody) would ensue.

Smithies' hypothesis, which involves both selective and instructive stages, can be summarized: variability in antibody structure arises by intragenic crossing over in the genes coding for immunoglobulin in one set of cells. This process would initially occur during development in early life and may occur in the thymus, but it could also occur in adult life if for any reason regeneration of the lymphoid system was required. Such genes possibly in association with genes coding for other proteins such as nucleic acid polymerase, become associated with specific antibody as the coat protein of the "antibody virus." These viruses would leave the cell where they are formed and would either reside in macrophages or in the eventual effector cell. Any viruses encountering specific "antigen" during this transfer would be eliminated and thus tolerance and self-recognition are explained. Antigen would then meet the appropriate virus either in macrophages, in which case the released nucleic acid would be passed to the effector cells, or in the effector cells themselves, and virus proliferation and antibody production would follow. During the primary response it is postulated that viral nucleic acid coding for the antibody combining site is inserted into incomplete receptor genes in the host cell. Before this stage these genes would be incapable of producing antibody

but would code only for the constant parts of the peptide chains of the antibody molecule. In the secondary response there transduced cells would be stimulated to divide and produce antibody with the same combining specificity as in the primary response but of a different class. Thus the theory also attempts to explain production of IgM antibody (characteristic of the primary response) and IgG antibody (characteristic of the secondary response).

The constancy of the C-terminal half of the immunoglobulin light chains (Bence-Jones proteins in this case) and the great variability of the N-terminal half were the main aspects considered in the theory of Dreyer and Bennet (1965). These authors examined and rejected the possibility that light chains were formed in 2 halves under the control of separate genes. They suggested that a large number of rings of nucleic acid, each coding for one particular variable portion of light chain, were stacked along the length of the chromosome. During differentiation of the immunoglobulin producing cell the common gene coding for the constant part of the light chain would pair with one of the rings. Thus a range of light chains, each having a constant C-terminal portion could be formed. This theory made no attempt to explain primary and secondary responses,

tolerance, self-recognition and IgM and IgG antibody production.

A different explanation for the observation that variation in amino acid sequence is confined to the N-terminal parts of the immunoglobulin chains was afforded by the theory of Brenner and Milstein (1966). It was assumed that a hydrolytic enzyme recognises a base sequence near the centre of the original gene coding for the chain in question and that hydrolysis takes place. Exonuclease action would then occur producing a gap in one of the strands of the DNA. It was postulated that during repair of this gap mistakes, either of base-pairing or of deletion or addition may be introduced. On division of the cell, one daughter would be of the original type while the other would contain a mutant sequence. This process could then be repeated. Eventually, on destruction of the hydrolysing or repair enzymes uniform stable clones would be produced. Again, this theory made no attempt to explain other aspects of the immune response.

One of the major difficulties inherent in forming any hypothesis on the origin of antibody diversity is that so many apparently paradoxical features have to be explained. Edelman and Gally (1967) have listed some of these :

(a) extensive variation in one half of the chain and invariance in the other half

(b) most amino acid alternatives are consistent with single base changes in the genetic code

(c) in different light chains the variable and invariable regions are of almost constant length

(d) within the variable region some invariant segments occur

(e) allotypic specificities associated with the invariant region do not randomly associate with allotypic specificities in the variable region - that is, the recombination frequency among allotypes is low

(f) most plasma cells can produce only one type of light and heavy chain.

These authors developed a theory which they considered met these requirements. In evolution, a number (50 would suffice) of genes coding for the immunoglobulin chains arose by tandem duplication. Different point mutations arose in the region of these genes corresponding to the variable section of the immunoglobulin chain. Such genes would be present in duplicated arrays such that non-identical genes lay side-by-side and during maturation of the lymphocyte somatic crossing over would occur resulting in the formation of a new gene which would appear to have arisen by point

mutation. It was postulated that mutation in the region corresponding to the invariant region would be prevented because of selection pressure during evolution.

This theory, like that of Dreyer and Bennet (1965) differs from those of Smithies (1965) and Brenner and Milstein (1966) in that antibody diversity is considered to be a result of somatic recombination of a relatively small number of genes that have arisen during evolution by mutation and natural selection. The other theories postulate only somatic variation.

For the enzyme studies reported in this thesis the choice of immune system was governed by the requirements that the antigen should be available in a pure form, that the response to the antigen should be well characterized and that induction of tolerance should be readily carried out. A system that meets these requirements is the rabbit-anti-bovine serum albumin (BSA) system. In 1954, Dixon, Maurer and Deichmiller presented quantitative observations on the primary and secondary responses of rabbits to purified BSA using immunization procedures similar to those employed here. In the primary response immune elimination of antigen began around 7 days after injection. Free antibody was detected in the serum on

the 9th day and was present in optimal concentration on the 12th day. In the secondary response immune elimination began 2 days after the injection of antigen (given approximately 9 weeks after the first). Free serum antibody was detected on the 4th day of the response and was optimal on the 7th-8th day. Dixon and Maurer (1955) reported immunological unresponsiveness in rabbits injected regularly from birth with BSA, or infused with suitably large quantities of the protein in adult life. Since these initial studies, the induction of tolerance to BSA in rabbits has been well documented (see Smith 1961 for a review). In Section 1 of this thesis, details are given with regard to injection schedules employed in initiating a primary or secondary response and in inducing tolerance. Evidence is presented that the characteristic primary and secondary responses were given and that tolerance had been produced by the methods employed.

The two most apparent sequels to the administration of an antigen to an animal are the fairly extensive cellular reaction in the responding lymphoid tissue and the production of circulating antibody. If the former phenomenon involves synthesis of DNA as most reports indicate (see Section 2), then in this connection some

other effects of antigen injection might be expected. Certainly, if it is a valid concept that antigen stimulates the proliferation of primitive conditioned cells (e.g. lymphocytes) to form a clone of antibody-forming cells (Burnet, 1959), then it would be expected that in the responding tissue there would be an increase in the specific activity of DNA nucleotidyltransferase - EC 2.7.7.7 (DNA polymerase). This is one of the enzymes involved in the biosynthesis of DNA.

In this thesis, results are presented from comparative studies on the specific activity of this enzyme in the spleen and appendices of control unimmunized animals, and of rabbits killed during the primary or secondary immune response. As a further control, some rabbits were rendered tolerant to the antigen and when mature they were challenged with 1 or 2 injections of the same protein. The activity of the enzyme in the spleen and appendices of such rabbits was determined. These results are presented and discussed in Section 2.

If the production of circulating antibody is analogous to the biosynthesis of other proteins then in this context also antigen injection would have many direct and indirect consequences. Thus, it might be predicted that in the

responding tissue the specific activity of DNA-dependent RNA nucleotidyltransferase would increase; that more mRNA would be formed than in the non-antibody forming tissue; that GTP synthesis and breakdown would be enhanced; that there would be an increase in the proportion of heavy polyribosomes and so on. The last of these predictions was chosen for detailed investigation and in Section 3 the results of the investigations are presented and discussed. In these studies the rat-anti-sheep red blood cell system was employed.

Some of the points raised in this General Introduction are further considered together with the material presented in the three Sections, in the General Discussion.

# S E C T I O N 1

## S E R O L O G Y

## INTRODUCTION

The aim of the serological experiments reported in this Section was not primarily to investigate the nature of the response to BSA since this is already well known, but to ascertain that the methods used were eliciting characteristic primary and secondary responses. Also, it was necessary to establish that "tolerant" rabbits were in fact unresponsive to antigen administered in adult life.

Since the concentration of antibody present in many of the sera tested was expected to be quite low (for example, in the early part of both primary and secondary responses) and since it was required to ensure the absence of antibody in the sera of "tolerant" animals, the serological procedures adopted were sensitive ones, able to detect very small quantities of circulating antibody.

## MATERIALS AND METHODS

### Animals

In the majority of the experiments, 3-5 month old male and female New Zealand white rabbits were used.

### Antigens

2% or 10% (w/v) solutions in 0.15M saline of crystalline bovine serum albumin (Armour Pharmaceuticals, Eastbourne, England) and pig gamma globulin (Pentex Incorporated, Kankakee, Illinois, U.S.A.) were the antigens used.

### Antisera

(a) Raised for Analytical Purposes. On days 1 and 14, rabbits received 2 x 0.5 ml. intramuscular injections of bovine normal serum (BNS) or pig normal serum (PNS) emulsified in Freund's Complete Adjuvant (Difco, Detroit, Michigan). On day 28, they were given intraperitoneal injections of 1.5 ml. of 1 : 3 dilutions (in 0.15M NaCl) of BNS and PNS respectively. One day later, the respective antigen was administered intravenously (0.15 ml. of 1 : 3 dilution) and the antisera collected after 6 days.

(b) Raised during Experimentation. Rabbits referred to below were used in the DNA polymerase studies (Section 2). In all cases (except for "tolerance-inducing" injections),

blood samples were taken for serological studies prior to each injection and prior to the death of the animals.

(i) Primary Response Group : (Group $1^{o}$). In some experiments, rabbits were killed on days $\frac{1}{2}$, 1, 2, 3, 4, 6 and 8 after a single intravenous injection of antigen (30 mg./Kg.). Uninjected rabbits served as controls. In later experiments, rabbits were killed $\frac{1}{2}$, 3 and 6 days after 1 injection of BSA (intravenous, 30 mg./Kg.) and 3 and 6 days after 1 intravenous injection of sterile 0.15M NaCl (1 ml./Kg.).

(ii) Secondary Response Group : (Group $2^{o}$). Each rabbit received 2 intravenous injections of BSA (30 mg./Kg.) separated by a period of 8 weeks. Rabbits were killed on days $\frac{1}{2}$, 1, 2, 3, 4, 6 and 8 after the second injection. Uninjected rabbits served as controls. As with Group $1^{o}$, later experiments were carried out where rabbits were killed on days $\frac{1}{2}$, 3 and 6 of the secondary response to BSA and 3 and 6 days after a second intravenous injection of sterile 0.15M NaCl.

(iii) Tolerant Group : (Group T). 100 mg. of BSA was administered intraperitoneally to each rabbit 2 days after birth. Five weeks later, each rabbit received a further injection of BSA (100 mg. intravenously) and at 3 months

they were randomly allocated to Group $1^{o}$ or $2^{o}$ for further treatment.

Immunoelectrophoretic Analysis

Analyses were performed on a micro-scale using the apparatus produced by L.K.B. (Stockholm, Sweden). The procedure was as described by Hirschfeld (1960).

Trace-labelling of Antigens

This was according to the method of McFarlane (1958). $^{131}I$ was obtained from the Radiochemical Centre, Amersham, England. The specific activity of the proteins was adjusted to approximately 5 µc./mg. of protein by the addition of unlabelled homologous protein.

Serological Procedures

(a) Antigen Elimination Tests.

This was essentially as described by Talmage, Dixon, Bukantz and Dammin (1951) except that the antigen dose was 30 mg./Kg. body weight.

(b) The Farr Test.

Principle : The antigen binding capacity test (ABC) of Farr (1958) involves interaction of antibody to BSA with $^{131}I$ trace-labelled BSA. Since the antibody molecule is a gamma globulin, it is precipitated at half-saturation with ammonium sulphate and any [$^{131}I$]BSA bound to antibody

is concomitantly precipitated. The quantity of antibody present is then proportional to the radioactivity detected in the precipitate so long as antibody is not present in excess.

Procedure : The test was carried out as described by Campbell, Garvey, Cremer and Sussdorf (1964). Final washed antigen-antibody precipitates were dissolved in 4.5 ml. of 0.2N NaOH and the radioactivity determined. Non-specific precipitable activity (N) was measured by treating 1.0 ml. of 1 : 10 normal rabbit serum plus 0.5 ml. of $[^{131}I]$BSA exactly as the test sera were treated.

Calculation : Total precipitable activity (T) was measured by treating 1.0 ml. of 1 : 10 normal rabbit serum plus 0.5 ml. of $[^{131}I]$BSA with 20% (w/v) trichloroacetic acid. The precipitate obtained was dissolved in 4.5 ml. of 0.2N NaOH and the radioactivity estimated. If S is the radioactivity in the dissolved sediment in a test, then the percent antigen bound to antibody in that test is given by

$$\frac{S - N}{T - N} \times 100$$

The ABC value of an antiserum was taken to be the reciprocal of that dilution of antiserum which precipitated 33% of the antigen present. This is the $ABC_{33}$ value. In practice,

this value was approximated by graphing the percent antigen bound to antibody at various antiserum dilutions against the reciprocal of the dilution plotted on a log scale. The point at which 33% of the radioactivity was precipitated was then noted.

(c) The Indirect Haemagglutination Test.

Principle. Formalinized red blood cells are first rendered adhesive by treatment with tannic acid and then coated with BSA. Treated cells are then incubated in the presence of various dilutions of the test serum. The presence of antibody is indicated by the degree of agglutination of the cells as determined by the sedimentation pattern in the individual test.

Red Blood Cells. Formalinized chick red blood cells - FRBCs were supplied by Difco (Detroit, Michigan).

Tanning and Coating Cells. The standard procedure described by Herbert (1967) was adopted except that tannic acid at a concentration of 1 : 5,000 was used.

Titrations. Progressive two-fold dilutions of test sera were made in 0.15M NaCl containing 0.5% normal rabbit serum. For the test, 0.3 ml. of serum dilution and 2 drops of cells were mixed together in the cups of plastic haemagglutination trays, and haemagglutination patterns

read after 2-3 hours at room temperature.

Scoring. Scoring of the patterns obtained was according to Stavitsky (1954). The last sediment pattern showing an incomplete ring round the smooth mat of cells ("2+" by Stavitsky's notation) was considered to be the end point.

## R E S U L T S

### Purity of Antigens

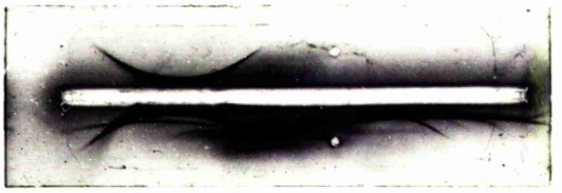
Both the BSA preparation and the PGG preparation used in these investigations showed up as single precipitin lines on analysis by immunoelectrophoresis (Fig. 1 : 1).

### Serological Procedures

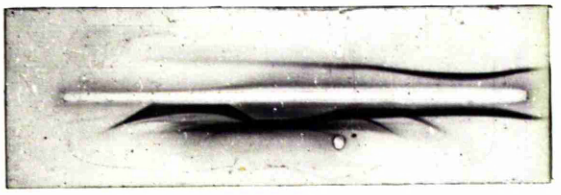
(a) Antigen Elimination Tests. The elimination of $[^{131}I]$ BSA and $[^{131}I]$ PGG from the circulation of rabbits "tolerant" to BSA and of $[^{131}I]$ BSA from the circulation of 2 non-tolerant rabbits is shown as a function of time in Fig. 1 : 2 and the main findings are presented in Table 1 : 1. In none of the 4 "tolerant" rabbits which received a single injection of $[^{131}I]$-BSA was there a change in the rate of elimination of the protein after the initial equilibration. On the other hand the $[^{131}I]$ PGG in the 2 rabbits "tolerant" to BSA and the $[^{131}I]$ BSA in the non-tolerant animals both showed an increase in the rate of elimination beginning between 7½ and 9 days after injection of the antigen.

In the 2 "tolerant" rabbits given a second injection of $[^{131}I]$ BSA a constant rate of elimination of the protein was again observed. Immune elimination of a

FIG. 1 : 1. IMMUNOELECTROPHORETIC ANALYSIS OF ANTIGENS




(a)

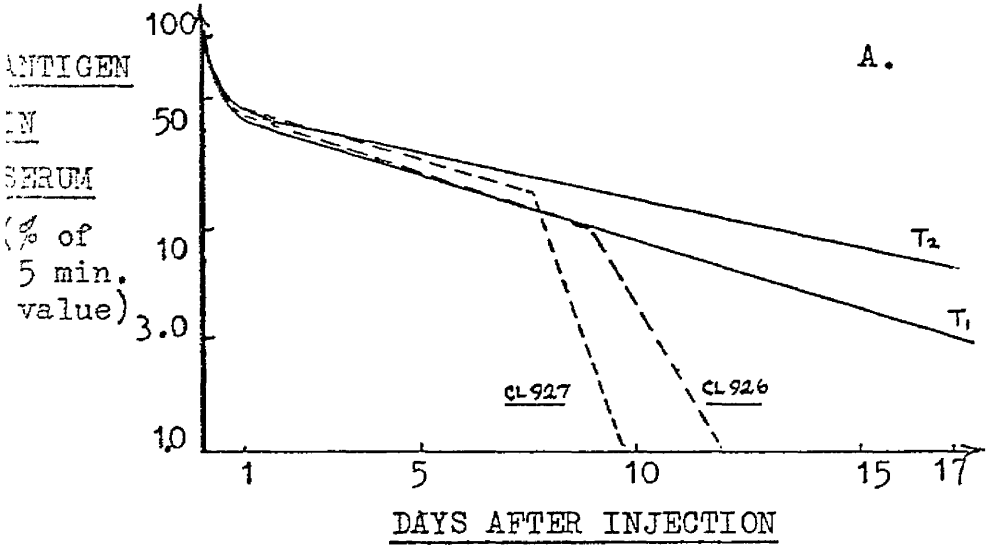


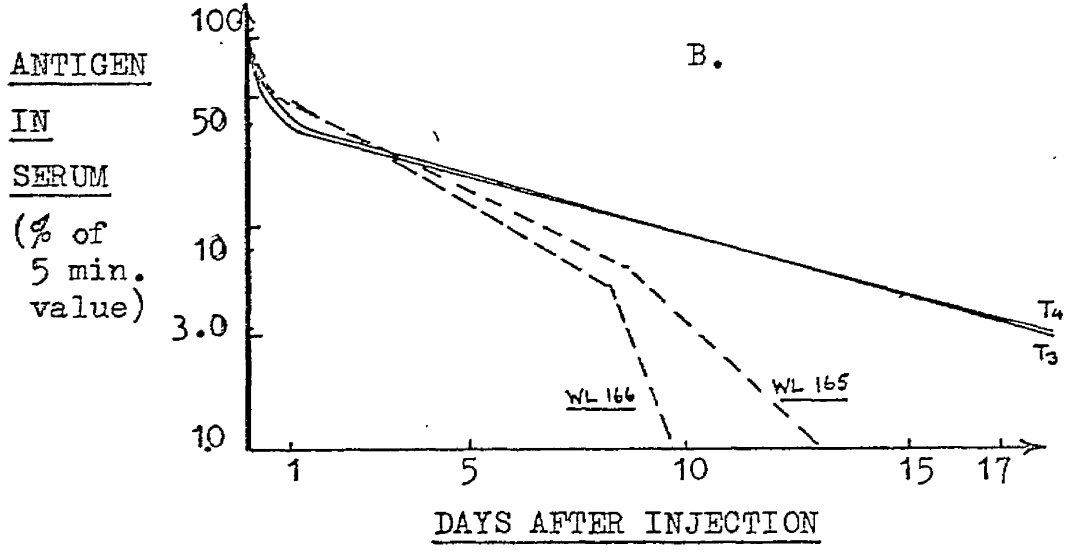

(b)

(a) BSA (top) and BNS (bottom) using rabbit-anti-BNS as antiserum.

(b) PGG (top) and PNS (bottom) using rabbit-anti-PNS as antiserum.

FIG. 1 : 2. ANTIGEN ELIMINATION TESTS IN TOLERANT AND NON-TOLERANT ANIMALS




A. Elimination of [$^{131}$I] BSA in tolerant rabbits ($T_1$, $T_2$) and in normal rabbits (CL 926, CL 927).

B. Elimination of [$^{131}$I] BSA in tolerant rabbits ($T_3$, $T_4$) and of [$^{131}$I] PGG in rabbits tolerant to BSA (WL 165, WL 166).

In A and B rabbits received 1 injection of trace-labelled antigen.

C. As for B but rabbits received 2 injections of the respective antigens, separsted by 8 weeks

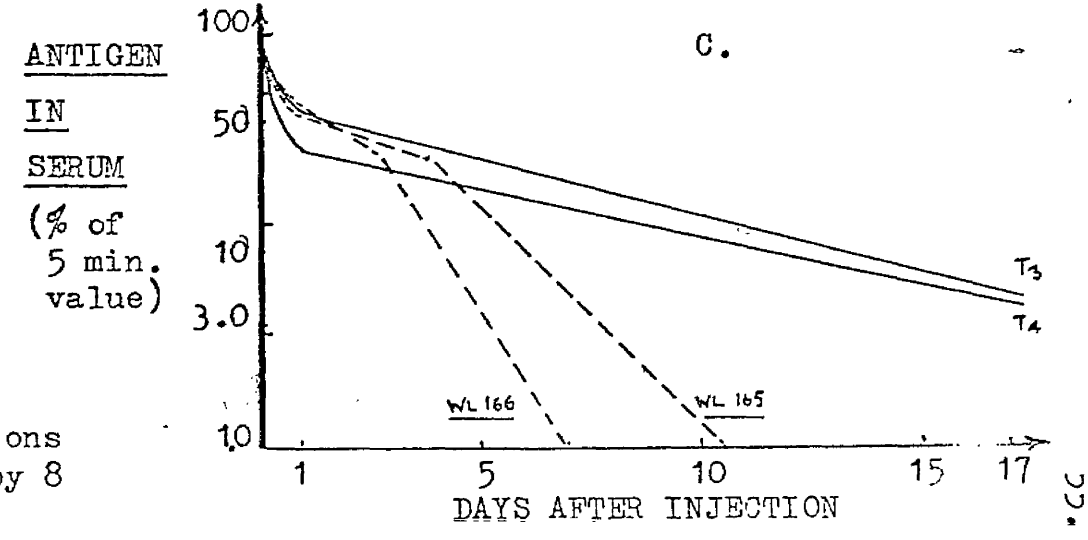


TABLE 1 : 1. SUMMARY OF ANTIGEN ELIMINATION STUDIES

| Rabbit Number | Immune Status* | Change in Elimination Rate occurring at | Half-life during Non-immune Elimination | Parent Antigen Remaining in Serum at 24 hrs |
|---|---|---|---|---|
| $T_1$ | Tol. "1°" BSA | - | 4.5 days | 32 |
| $T_2$ | Tol. "1°" BSA | - | 6.3 days | 35 |
| $T_3$ | Tol. "1°" BSA | - | 5.0 days | 36 |
| $T_4$ | Tol. "1°" BSA | - | 5.1 days | 29 |
| CL 926 | - 1° BSA | 8.7 days | 4.3 days | 36 |
| CL 927 | - 1° BSA | 7.7 days | 4.0 days | 43 |
| WL 165 | Tol. 1° PGG | 8.7 days | 2.6 days | 47 |
| WL 166 | Tol. 1° PGG | 8.2 days | 2.1 days | 49 |
| $T_3$ | Tol. "2°" BSA | - | 5.4 days | 36 |
| $T_4$ | Tol. "2°" BSA | - | 5.9 days | 25 |
| WL 165 | Tol. 2° PGG | 4.1 days | 4.2 days | 33 |
| WL 166 | Tol. 2° PGG | 3-4 days (?) | ? | 41 |

* Tol. = rabbit from tolerant group (group T - Material and Methods).

1° = rabbit received 1 injection of $^{131}I$ labelled antigen

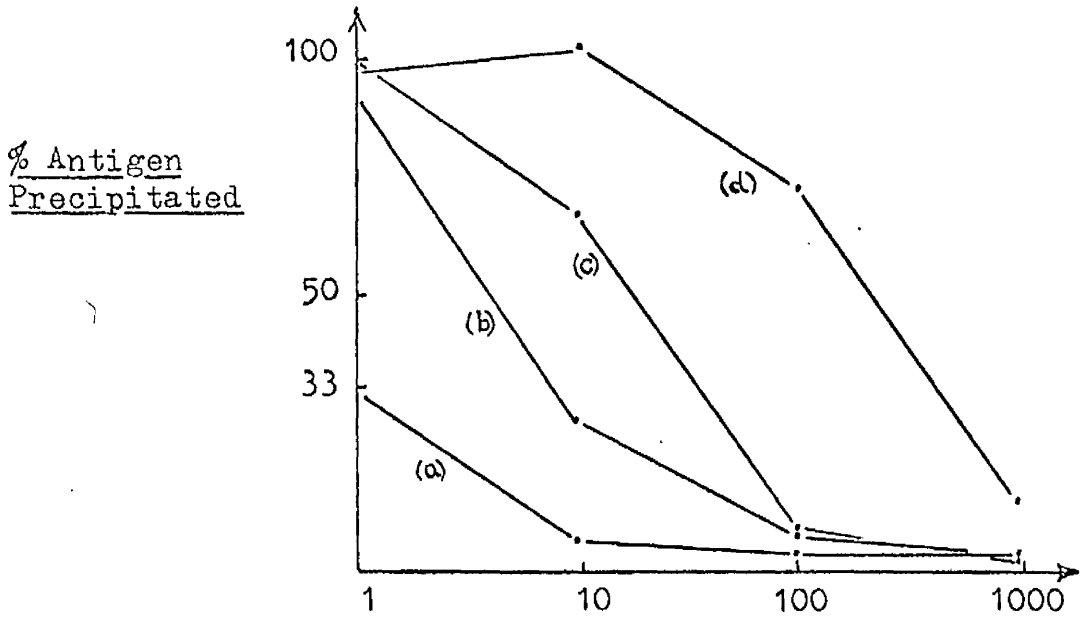
2° = rabbit received 2 injections of $^{131}I$ labelled antigen, separated by 8 weeks.

second dose of PGG began earlier (about 4 days after antigen) and was more dramatic than in the primary response.

(b) The Farr Test. The procedure for determining the $ABC_{33}$ value of an antiserum is illustrated for 4 antisera in Fig. 1 : 3. Serum from rabbit Number 33, taken 8 weeks after a primary injection of antigen showed an $ABC_{33}$ value of 1 and, when taken 8 days after a second injection, the value was 9. Rabbits 25 and 35, killed 8 and 4 days respectively after a second injection of antigen, had respective $ABC_{33}$ values of 50 and 600. The $ABC_{33}$ values for the sera found to contain antibody are presented in Table 1 : 2.

By this test serum antibody was never detected before immunization nor during the first 8 days following primary antigenic stimulation nor in samples taken prior to the fourth day of the secondary response. $ABC_{33}$ values of serum from different animals at identical times after immunization showed considerable variation. Thus, 8 weeks after primary antigenic stimulation, values ranged between 1 and 9; on the fourth day of the secondary response, values of 7 and 600 were found in different rabbits; and on the 8th day of the secondary response, $ABC_{33}$ values of 9 and 50 were found.

FIG. 1 : 3. ILLUSTRATION OF PROCEDURE USED TO FIND $ABC_{33}$ VALUE




(a) Rabbit no. 33 - 8 weeks after primary injection.

(b) Rabbit no. 33 - 8 days after secondary injection.

(c) Rabbit no. 25 - 8 days after secondary injection.

(d) Rabbit no. 35 - 4 days after secondary injection.

TABLE 1 : 2. RESULTS OF FARR TEST ON POSITIVE SERA

| Rabbit Number | Immunological Status * | % [$^{131}$I]-BSA precipitated at Dilution | | | | $ADC_{33}$ |
|---|---|---|---|---|---|---|
| | | 1:1 | 1:10 | 1:100 | 1:1000 | |
| 27 | 8 weeks ($1^{o}$) | 66 | 7 | 3 | 3 | 5 |
| 28 | " | 71 | 5 | 4 | 4 | 5 |
| 29 | " | 93 | 28 | 5 | 5 | 9 |
| 30 | " | 21 | 4 | 4 | 4 | 1 |
| 31 | " | 92 | 8 | 5 | 4 | 8 |
| 33 | " | 33 | 6 | 4 | 5 | 2 |
| 37 | " | 90 | 13 | 6 | 5 | 8 |
| 39 | " | 76 | 7 | 5 | 4 | 6 |
| 42 | " | 25 | 4 | 3 | 2 | 1 |
| 27 | 4 days ($2^{o}$) | 89 | 3 | 2 | 2 | 7 |
| 35 | " | 92 | 96 | 70 | 12 | 600 |
| 26 | 6 days ($2^{o}$) | 98 | 81 | 76 | 8 | 600 |
| 34 | " | 92 | 91 | 56 | 9 | 500 |
| 25 | 8 days ($2^{o}$) | 94 | 65 | 7 | 3 | 50 |
| 33 | " | 86 | 28 | 7 | 4 | 9 |

* 8 weeks ($1^{o}$) indicates that blood sample was collected 8 weeks after a single injection of antigen.

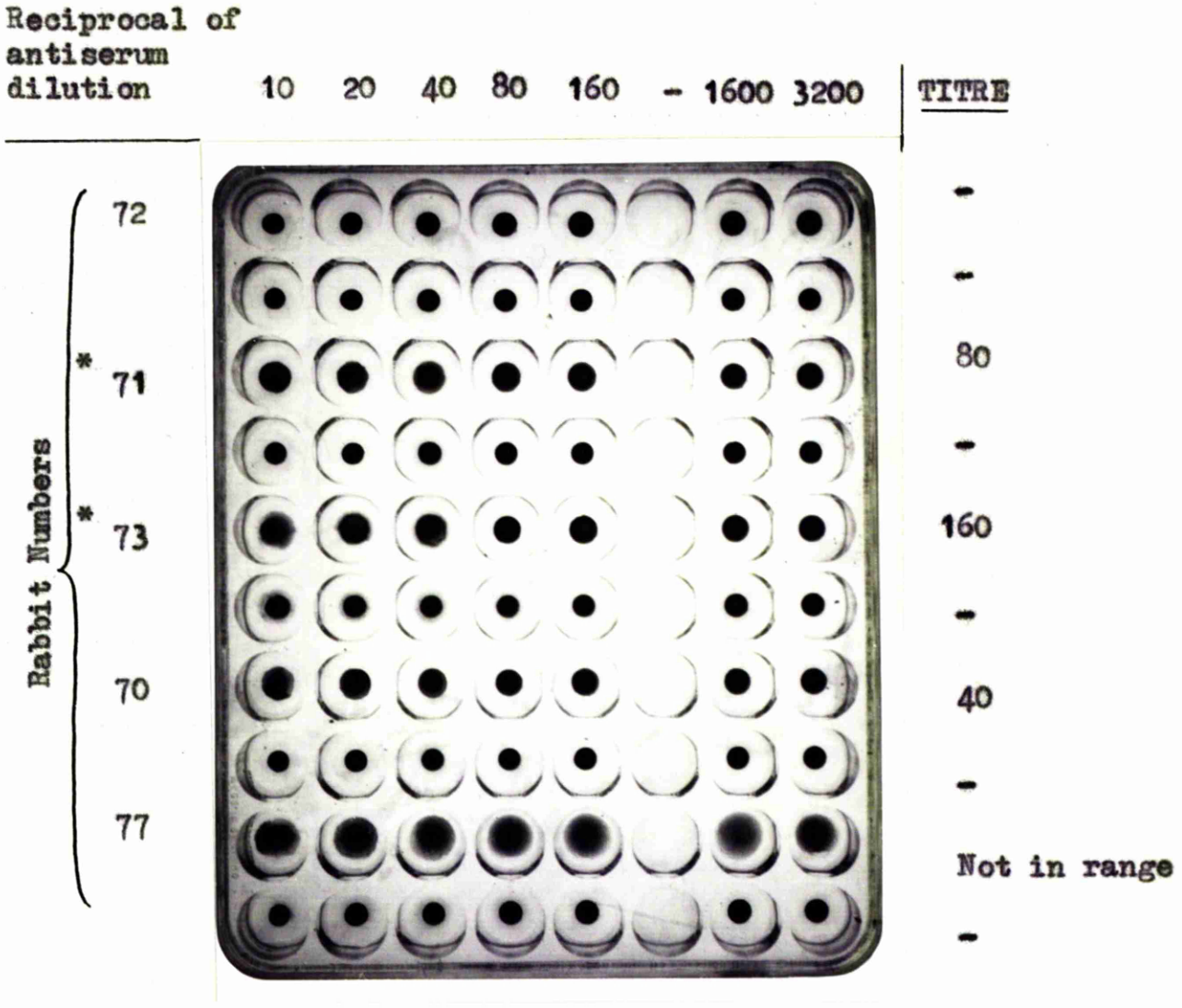
6 days ($2^{o}$) indicates that blood sample was collected 6 days after a second injection of antigen.

(c) Indirect Haemagglutination Test. This test was used in the later experiments and in determining whether or not antibody could be detected in the serum of rabbits "tolerant" to BSA. Fig. 1 : 4 shows the patterns obtained at the end of a typical assay.

Antibody to BSA was never detected in serum taken from unimmunized animals (whether pre-injection samples or serum from saline injected controls), in animals killed during the first 6 days of the primary response, nor in animals killed before day 3 of the secondary response. Furthermore, no antibody was found in any of the serum samples taken from tolerant animals.

As Table 1 : 3 indicates, antibody was present in some sera taken 8 weeks after a first injection of BSA, in 1 case 3 days after a second injection and in all cases in the rabbits killed on the sixth day of the secondary response. There was again considerable animal to animal variation in results. Titres found 8 weeks after a primary injection of BSA ranged from zero (rabbit Number 72) to 640 (rabbit number 71) while on the sixth day of the secondary responses values as different as 3,200 (rabbit Number 79) and $2 \times 10^5$ (rabbit Number 77) were observed.

FIG. 1 : 4. PATTERNS OBTAINED AFTER A TYPICAL INDIRECT HAEMAGGLUTINATION TEST FOR ANTIBODY




The first of each pair of rows contained "test" cells plus the antiserum dilution, while the second contained control cells (not coated with antigen) plus the antiserum dilution.

* Blood sample taken 8 weeks after primary injection of BSA.

TABLE 1 : 3. RESULTS OF INDIRECT HAEMAGGLUTINATION TEST ON SERA FROM SECONDARY RESPONSE GROUP OF RABBITS

| | Time after second injection of BSA | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 12 hours | | | | 3 days | | | | 6 days | | | |
| Rabbit Number | 73 | 74 | 81 | 82 | 69 | 70 | 71 | 72 | 77 | 78 | 79 | 80 |
| Antibody Titre | -(160) | -(160) | -(ND) | -(ND) | -(160) | 40(640) | -(80) | -(-) | $2x10^5$(80) | $10^5$(80) | 3,200(320) | $10^5$(80) |

Values in parenthesis are titres just prior to the second injection of BSA (i.e. 8 weeks after a primary injection).

ND - Not done-

# D I S C U S S I O N

## GROUP 1°

Consistent with the findings of Dixon *et al.* (1954) were the observations that immune elimination of BSA began between the 7th and 8th day (Table 1 : 1) and that no free antibody was detected during the first 8 days of the primary response. The presence of serum antibody 8 weeks after primary inoculation (Tables 1 : 2 and 1 : 3) is in accord with the observations of Richter and Haurowitz (1960) and Richter, Zimmerman and Haurowitz (1964) who have shown that antibody is still present in serum more than a year after a single intravenous injection of BSA.

## GROUP 2°

The occurrence of free serum antibody on the 4th day of the secondary response and of highest antibody titre on the 6th day (Tables 1 : 2 and 1 : 3) is again consistent with the observations of Dixon *et al.* (1954). Thus it appears that the immunization procedures used in these studies result in characteristic primary and secondary responses to BSA.

GROUP T

The facts that -

(i) no immune elimination of BSA was detected in any of the 4 rabbits given 1 injection of [$^{131}$I] BSA nor in the 2 given 2 injections (Fig. 1 : 2 and Table 1 : 1)

and

(ii) no free antibody was detected in the serum of rabbits of Group T

is taken as evidence that in Group T as a whole tolerance had been induced. It is of course possible that tolerance had not been established in some of the rabbits killed during the first 8 days of the "primary response" or the first 4 days of the "secondary response" since free antibody would not normally be detected in the sera of non-tolerant animals at these times.

While the main findings of this Section are that characteristic primary and secondary responses and specific immune tolerance resulted from the corresponding experimental procedures adopted in these investigations, a number of other interesting features emerged from the serological tests carried out.

1. Antigen Elimination Studies

The percentage of the radioactivity remaining in the circulation 24 hours after antigen injection (Table 1 : 1) reflects the degree of equilibration between the intra and extra vascular spaces and the extent of phagocytosis of the $^{131}I$-labelled protein. Nossal and Ada (1964) have shown that in mice injected with a protein to which they have previously been immunized or rendered tolerant, the degree of phagocytosis of the protein in the responding lymph node tissue is very much greater over the first few hours than that occurring in normal mice injected with the same protein. That is, macrophages from tolerant or immunized mice have a greatly enhanced ability to recognise and ingest the foreign protein. Thus it was of interest to find that elimination of BSA over the first 24 hours was greater in the tolerant rabbits (average per cent BSA remaining was 33% - Table 1 : 1) than in the normal animals (average of 40% remaining) and that elimination of PGG was greater over the first 24 hours of the secondary response (average of 37% remaining) than of the primary response (48% remaining). In the case of the 2 rabbits given a challenge injection of [$^{131}I$-]PGG, the presence of anti-PGG

in their serum prior to this injection may also have contributed to the greater elimination of the antigen than occurred during the first 24 hours of the primary response.

Another point of interest in Table 1 : 1 is that there appears to be an inverse relationship between the percent antigen remaining in the serum at 24 hours and the ensuing half-life of the protein during the non-immune phase of elimination. Thus, for example, comparing average values, 33% of [$^{131}$I] BSA remained in the serum of tolerant rabbits given 1 injection of the protein and the half-life thereafter was 5.2 days. In non-tolerant rabbits given 1 injection of the antigen the percent antigen remaining at 24 hours was greater (40%) but the ensuing half-life was lower (4.1 days). Rabbit Number WL 165 is a more striking example. Twenty-four hours after 1 injection of [$^{131}$I] PGG, 47% of the antigen remained in the serum and the half-life during non-immune elimination was 1.6 days. In the same animal, 24 hours after a second injection only 37% of the antigen was present in the serum but the half-life thereafter was 4.2 days.

In view of the findings of Nossal and Ada (1964) and results to be presented in Section 2 of this thesis, it is suggested that this inverse relationship can be explained

in the following way. In the early stages of the primary response, phagocytosis of the antigen occurs to only a limited extent but proliferation of macrophages takes place. (Jandl, Files, Barnett and MacDonald (1965) present evidence that suggests that this may occur). There is thus a subsequent enhancement in the rate of elimination of the antigen from the serum.

The macrophages of immune and tolerant animals however can readily ingest the foreign protein of which they have had prior experience and this obviates the need for macrophage proliferation. Thus, in comparison with the primary response, antigen elimination occurs more rapidly over the first 24 hours but at a slightly slower rate thereafter.

A test for this hypothesis would be to examine the effect of a mitotic inhibitor (for example, colchicine) on the half-life of BSA in tolerant rabbits and during the non-immune phase of elimination in primary and secondary response rabbits. According to the hypothesis presented above, a mitotic inhibitor would have no effect on the non-immune antigen elimination pattern in tolerant and secondary response animals. In primary response animals it is predicted that the percent antigen remaining at 24

hours would not be affected but that the half-life of the protein would be higher than in animals not treated with an inhibitor.

A further feature which emerges from Table 1 : 1 is that the half-life of PGG over the non-immune phase of elimination was considerably shorter than that of BSA in the primary response group (average of 2 days 9 hours compared with 4 days 3 hours). In general it appears that elimination of gamma globulins occurs more rapidly than elimination of serum albumins (Dixon et al., 1954) probably because their greater molecular weight renders them more susceptible to phagocytosis.

However, in contrast to the situation which holds in the case of bovine gamma globulin (Dixon et al., 1954) the change in the elimination rate of PGG in the primary response corresponds to the change in elimination rate of BSA (latent period of just over 8 days in both cases - Table 1 : 1). The shorter latent period which is characteristic of the secondary response was also evident.

2. The Farr Test

Since the antigen elimination studies indicated that detectable antibody production did not begin until the 8th day of the primary response and around the 4th day of the

secondary response it was not surprising that the Farr Test failed to detect antibody before these times (Table 1 : 2). The considerable scatter in $ABC_{33}$ values of serum samples taken from different animals at identical stages of immunization reflects the animal to animal variation that is a recognised feature of the immune response (see, for example, Crowle, 1961).

Pinckard, McBride and Weir (1967) have recently suggested that when the Farr Test is carried out using $[^{131}I]$ BSA at a concentration of 1 µg.N/ml. (as was used here) not all the 19S antibody will be detected. These authors have found that for optimal detection of 19S antibody a BSA concentration of 0.001 - 0.01 µg.N/ml. should be used. However, 19S antibody appears to be involved only in the early part of the primary response (for example, Bauer et al., 1963) so this possible limitation of the Farr Test as used here does not invalidate the overall results. It is possible that some 19S serum antibody was present 8 days after a primary injection of BSA but was not detected since conditions were not optimal.

3. The Indirect Haemagglutination Test

As can be seen from Table 1 : 3 the indirect haemagglutination test proved a more sensitive means of

detecting antibody than the Farr Test. Thus, in the secondary response, titres up to 200,000 were observed 6 days after BSA injection as compared with a maximum $ABC_{33}$ value of 600 in the Farr Test.

A possible relationship between variation in antibody titre in rabbits given identical courses of immunization (Tables 1 : 2 and 1 : 3) and variation in the cellular response in these animals is discussed in Section 2.

# S E C T I O N  2

## DEOXYRIBONUCLEIC ACID NUCLEOTIDYLTRANSFERASE

## INTRODUCTION

While the necessity for cellular proliferation during the immune response has been disputed, immunologists using a variety of experimental techniques and immunological systems have observed that antigenic stimulation of lymphoid cells normally results in DNA synthesis and cell division.

In a system employing spleen cells transferred to X-irradiated isologous recipient mice, Capalbo, Makinodan and Gude (1962) observed that secondary antigenic stimulation of these cells resulted in a 2-fold reduction in their mean generation time - from about 24 hours to about 12 hours. Also, a 4-fold increase in cell number was observed over the first day of the secondary response. These investigations were extended by Urso and Makinodan (1963) who investigated the response in lymphoid cells undergoing a secondary immune response to bovine serum albumin. These cells were cultured in diffusion chambers in X-irradiated recipients. With this system, proliferation was observed to begin shortly after the first day and to comtinue up to the fourth day. Again a 2-fold reduction in the generation time of the responding cells was observed.

Similar cell transfer studies have indicated that cellular proliferation is involved in the primary response. Gengozian (1964) examined the mitotic activity and tritiated thymidine incorporation of lymph node cells cultured in diffusion chambers in X-irradiated mice. Primary antigenic stimulation of these cells resulted in DNA synthesis and cellular proliferation beginning about the fourth day of culture.

These conclusions conflict with the observations of Roberts, Dixon and Weigle (1957) and Neil and Dixon (1959). Suspensions of lymph node cells from rabbits immunized against BSA were injected subcutaneously and intramuscularly into X-irradiated unimmunized recipient rabbits. Immediately following the transfer, BSA was administered intravenously to the recipient. Over the first 5 days of the ensuing secondary response the authors observed no mitotic activity among the donor cells at the injection site and they concluded that morphologic transition rather than mitotic activity was involved in this secondary response system.

The use of drugs has also led to conflicting conclusions on the need for DNA synthesis and mitotic division in the immune response. Sterzl (1961) used a

system whereby normal rabbit spleen cells were incubated *in vitro* with antigen and then injected intraperitoneally into 3-5 day old rabbits. Thereafter, mitotic inhibitors were injected daily for 5 days and the effect on antibody production was noted. While some purine antagonists completely inhibited the primary immune response, the majority of pyrimidine antagonists and inhibitors of mitotic division appeared to have no effect on antibody production. The author concluded that the formation of a cell clone by cellular proliferation was not a necessary prerequisite to antibody production. However, Merritt and Johnson (1965) found that administration of 5-fluoro-2-deoxyuridine (a specific inhibitor of DNA synthesis) to mice, could cause the abolishing of the primary response to bovine gamma globulin. The timing of drug administration was critical. When the drug was given one hour before or up to 14 hours after the antigen, the immune response was enhanced, but if administration was 18 hours or more after antigen then the response was abolished. Thus, in this system it appeared that DNA synthesis was required and that it normally began around 18 hours after antigen injection.

Using autoradiography, a number of workers have investigated DNA synthesis in lymphoid cells undergoing an

immune response by measuring uptake of tritiated thymidine. Nossal and coworkers concluded that antigen-stimulated cell division occurred in the responding lymphoid tissue in the primary response (Nossal, Mitchell and MacDonald, 1963) and also in the secondary response (Nossal and Mäkelä, 1962b and Mäkelä and Nossal, 1962). It has also been emphasised that re-utilisation of label may represent a serious hazard to interpretations of results obtained by this technique. (Mitchell, MacDonald and Nossal, 1963). Cohen and Talmage, (1965) also found by autoradiography that antibody forming cells (detected by fluorescein staining) in the secondary response had been engaged in DNA synthesis in the earlier part of the response. In contrast to these results, Tannenberg (1967) found that, when mice undergoing a primary haemolysin response were infused continuously with tritiated thymidine from the time of immunization to the time of sacrifice 4 days later, 19S antibody synthesising cells were not derived by antigen-stimulated cell proliferation. The cells were considered not to have been engaged in DNA synthesis since no incorporation of thymidine could be detected by autoradiography.

Further evidence that DNA synthesis or cell division must occur before antibody synthesis will take place has

been provided by *in vitro* studies. Spleen cells from rabbits previously immunized against either a soluble antigen (Dutton and Eady, 1964; Harris and Littleton, 1966) or SRBCs (Richardson and Dutton, 1964) were challenged *in vitro* with the same antigen. In all cases DNA synthesis was greater than in control cells from unimmunized animals.

A situation which has features in common with the immune response is that of regenerating liver. In this system a specific stimulus (partial hepatectomy) results in cellular proliferation accompanied by an increase in the specific activity of DNA polymerase. See, for example, Fausto and Van Lancker (1965).

The work reported in this Section concerns comparative studies on the specific activity of DNA polymerase in the spleens and appendices of rabbits. The animals used were either unimmunized rabbits as controls, rabbits undergoing a primary or secondary response to BSA, or rabbits rendered tolerant to BSA (as described in Section 1) and challenged with 1 or 2 injections of this protein in adult life.

MATERIALS AND METHODS

Details regarding animals, antigens used, injection and bleeding schedules and induction of tolerance are given under Materials and Methods in the Serology Section. In summary, rabbits were killed by an intravenous injection of euthatal (May and Baker, Dagenham, England) at various intervals after a primary or secondary immunisation with BSA. Saline-injected rabbits were used as controls. Some studies were done on animals rendered tolerant to BSA and killed after a primary or secondary challenge with this protein.

Preparation of Enzyme : Immediately after death of the rabbit, the spleen and appendix were removed. The mucosal coat of the appendix was separated from the muscle layer and stored at $-70^{\circ}$ until required. The spleen was used immediately for preparation of DNA polymerase. The following operations, based on the method of Keir (1962) were carried out at $0^{\circ}$.

The tissue was homogenised in 10 vol. of a tris buffer (0.01M tris-HCL, pH 8.0 containing 0.001M EDTA, 0.005M 2-mercaptoethanol and 0.002 M $MgSO_4$) in a Potter-Elvehjem homogeniser using 6 excursions of the pestle. This was sufficient to disrupt the osmotically swollen cells with minimal nuclear damage.

The cell homogenate was then centrifuged for 1 hour at 105,000 g in a Spinco Model L2 Ultracentrifuge. To 6.0 ml. of the supernatant was added solid KCl to 0.15 M and then 0.2 N acetic acid was added dropwise with constant stirring until the pH had fallen to 5.0. The solution was stirred for a further 3 min. then centrifuged for 5 min. at 1,000 g. The centrifuge tube was carefully drained and the precipitate reconstituted in 2.5 ml. of tris buffer (as above plus 0.15 M KCl). To obtain a homogeneous preparation, it was necessary to homogenise the suspension gently.

The protein concentration of this pH 5.0 precipitate fraction was measured by the method of Lowry, Rosebrough, Farr and Randall (1951) and the fraction was immediately assayed for enzymic activity.

Assay of Enzyme : Assay of the pH 5.0 fraction for polymerase activity was based on the method of Grey et al. (1960).

The reaction volume was 0.25 ml., composed of 5.5 µmoles of tris-HCL (pH 8.0), 1.0 µmole of $MgSO_4$, 15.0 µmoles of KCl, 0.12 µmoles of EDTA, 3.5 µmoles of 2-mercaptoethanol, 200 µg. of calf thymus DNA (previously denatured by heating at 100° for 10 minutes and then cooled rapidly to 0°), 50 mµmoles each of dATP, dCTP, dGTP and ($\alpha$ - $^{32}$P)dTTP, the appropriate volume of pH 5.0 precipitate fraction which contained

100-150 µg. of protein, and distilled water.

The ($\alpha$ - $^{32}$P)dTTP was synthesised as described by Gray et al. (1960) or obtained from International Chemical and Nuclear Corporation (City of Industry, California, U.S.A.). Its specific activity in assays was 1-15 x $10^6$ counts/min./µmole.

Incubation was at 37°. At 30, 60 and 90 min. after incubation was begun, a 0.05 ml. sample was removed from each reaction vessel and pipetted on to an appropriately numbered filter paper disc (Whatman No. 1, 2.5 cm. dia.). Each disc had previously been coated with 0.05 ml. of a 0.2% (w/v) solution of BSA to aid precipitation.

The discs were passed through 4 washes of ice-cold 5% (w/v) trichloroacetic acid (15 ml./disc, 10 min./wash) containing 0.15 mM sodium pyrophosphate, then dried after final passes through absolute alcohol and ether.

Radioactivity due to DNA precipitated on to the paper discs was estimated on a Nuclear Chicago gas-flow counter.

In a few preliminary experiments, certain of these procedures were not followed - thus

(i) Control rabbits were not injected with saline

(ii) The tissues were not weighed

(iii) The tissues were homogenised in 10 ml. of tris buffer irrespective of weight, rather than in 10 vol.

(iv) The pH 5.0 precipitate fraction was not reconstituted in a standard volume of tris buffer.

Calculation : In the later experiments where the procedures for extracting and assaying the enzyme were standardised, the activity of the polymerase could be expressed per g. of tissue or per whole tissue using conversion factors calculated as follows :

"a" = weight of whole tissue in g.

"b" = µg. of protein in 0.02 ml. of pH 5.0 precipitate fraction

"c" = mµmoles ($\alpha$ - $^{32}$P)dTMP incorporated per mg. of protein per incubation period.

1. Volume after homogenizing = 11a ml.

2. 10 ml. removed for centrifuging, i.e. $\frac{10}{11a}$ of total.

3. 6.0 ml. of supernatant used to prepare pH 5.0 precipitate fraction i.e. $\frac{6}{10} \times \frac{10}{11a}$ or $\frac{6}{11a}$ of total

4. Precipitate reconstituted in 2.5 ml. i.e. $b \times \frac{2.5}{0.02}$ µg. of protein in 2.5 ml.

This 2.5 ml. came from $\frac{6}{11a}$ of whole tissue

Thus there are $b \times \frac{2.5}{0.02} \times \frac{11a}{6}$ ug. of "pH 5.0 precipitate" protein in whole tissue i.e. 229.2 ab µg.
or 0.23 ab mg. of protein

Now 1.0 mg. of protein incorporates c mµmoles of dTMP

so <u>activity in whole tissue is c x 0.23 ab</u>

and <u>activity per gm. of tissue is c x 0.23 b</u>.

RESULTS

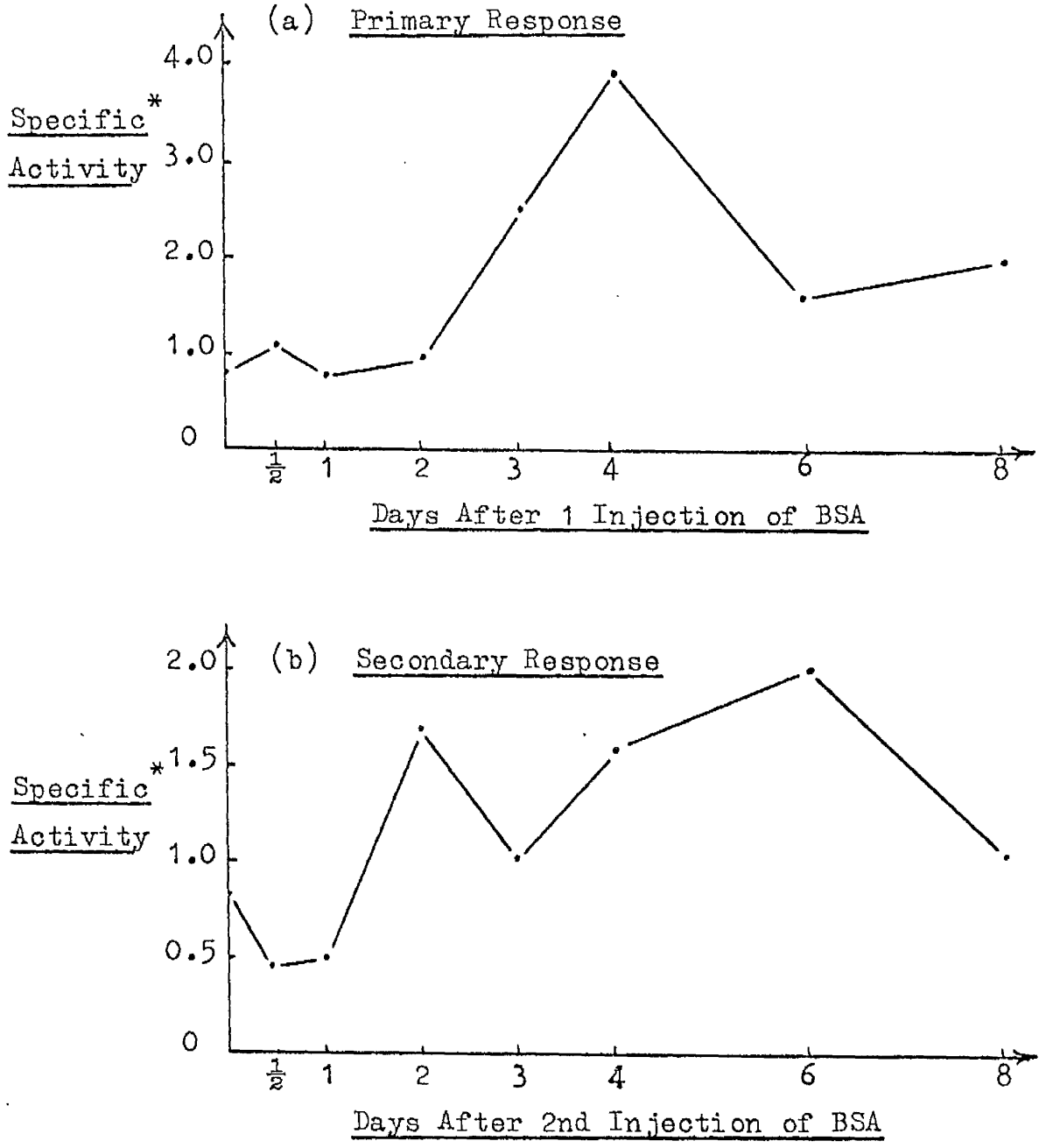
In presenting these results the term "specific activity" is used unless stated otherwise, to indicate the number of mμmoles of dTMP incorporated into DNA per mg. of reconstituted pH 5.0 precipitate protein in a 60 min. incubation. The results will be presented under (1) Preliminary Studies - in which the procedures adopted were slightly different (as outlined in the Materials and Methods Section) from those used in (2) later Studies.

1. PRELIMINARY STUDIES

(a) Non-tolerant Animals : The specific activity of DNA polymerase extracted from 4 normal rabbit spleens was found to be 1.02, 0.72, 0.80 and 0.70 - average 0.81. The effect of a single or second injection of BSA on the activity of the enzyme is shown as a function of time in Fig. 2 : 1. Each point except the zero time uninjected control respresents one rabbit. This figure indicates that the specific activity of the spleen polymerase was higher on days $\frac{1}{2}$, 3, 4, 6 and 8 after a single injection of BSA and on days 2, 3, 4, 6 and 8 after a second injection.

In further experiments it was observed that following a single injection of BSA the specific activity of the polymerase had the following values :

FIG. 2 : 1. EFFECT OF ANTIGEN ADMINISTRATION ON SPECIFIC ACTIVITY OF DNA POLYMERASE IN RABBIT SPLEEN




* Specific Activity in mμmoles of $[^{32}P]$ dTMP incorporated per mg. of protein.

| | |
|---|---|
| On day ½ | 1.82, 0.83 |
| Day 3 | 1.95, 1.43 |
| Day 6 | 2.43, 1.13 |

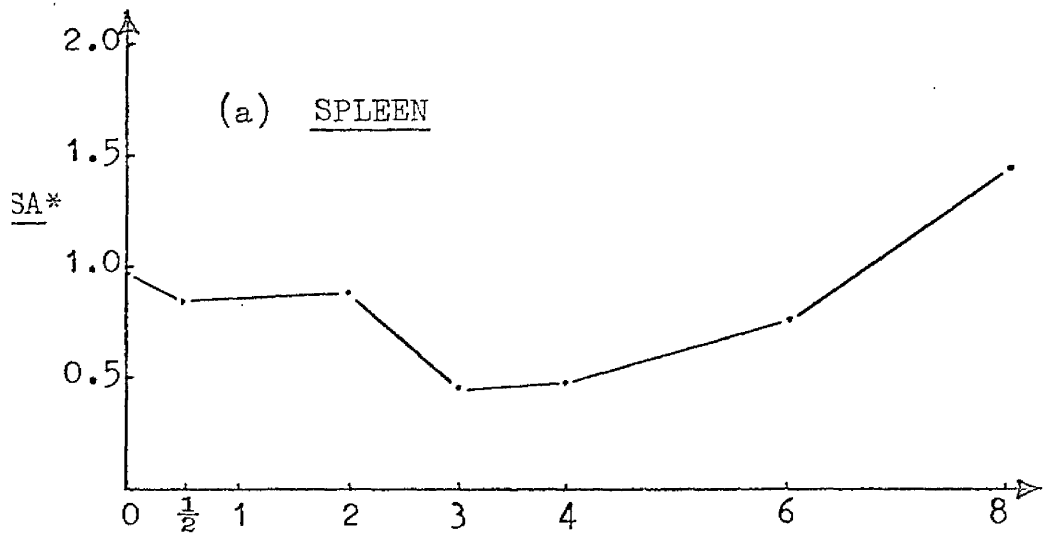
After a second injection the values were :

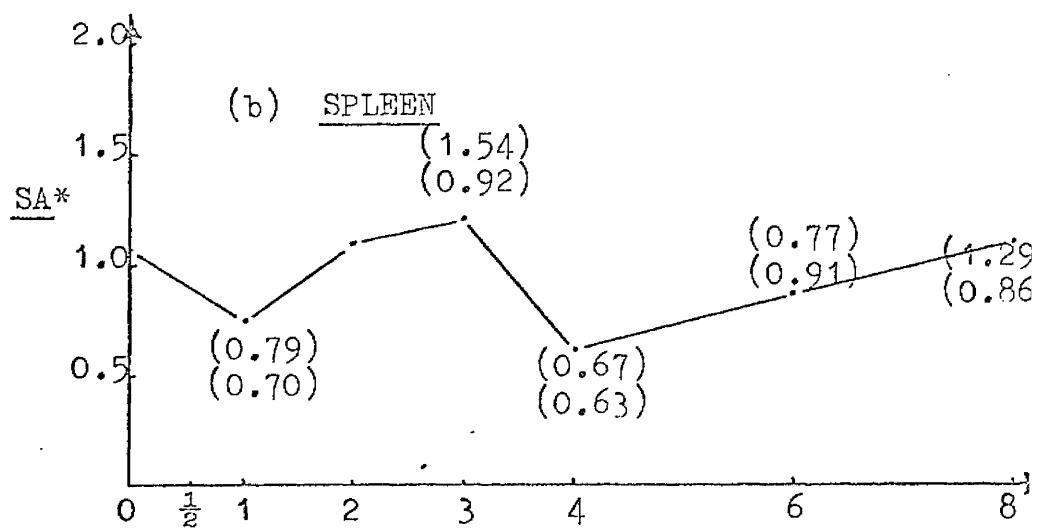
| | |
|---|---|
| Day 4 | 1.30 |
| Day 6 | 1.74 |

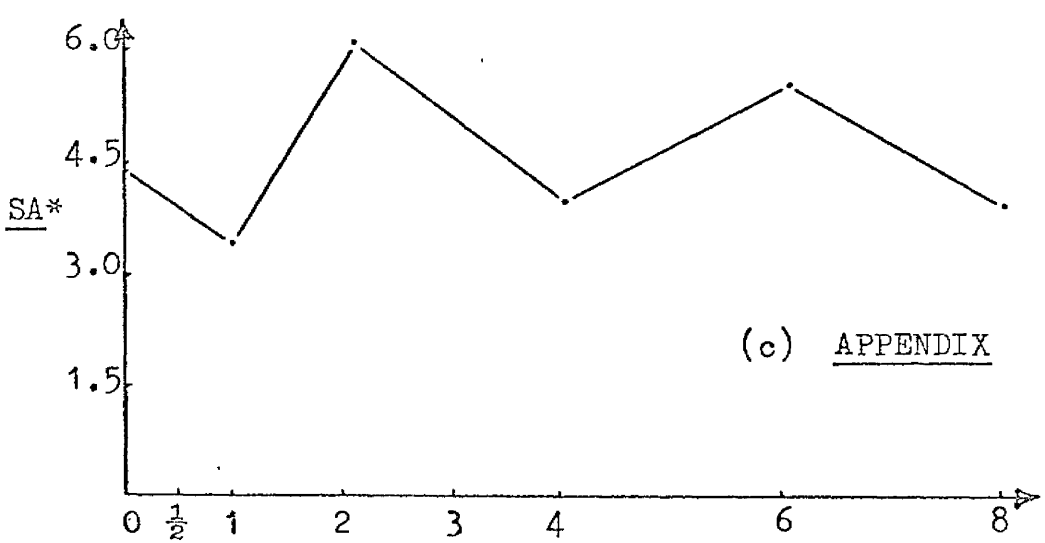
(b) Tolerant Animals : The specific activities of DNA polymerase in the spleens and appendices of rabbits tolerant to BSA and killed at various times after 1 or 2 injections of this antigen are indicated in Fig. 2 : 2. In some cases, 2 rabbits were killed at the same time after injection of BSA. In these instances the average specific activity was taken and the individual values noted beside the relevant point on the graph.

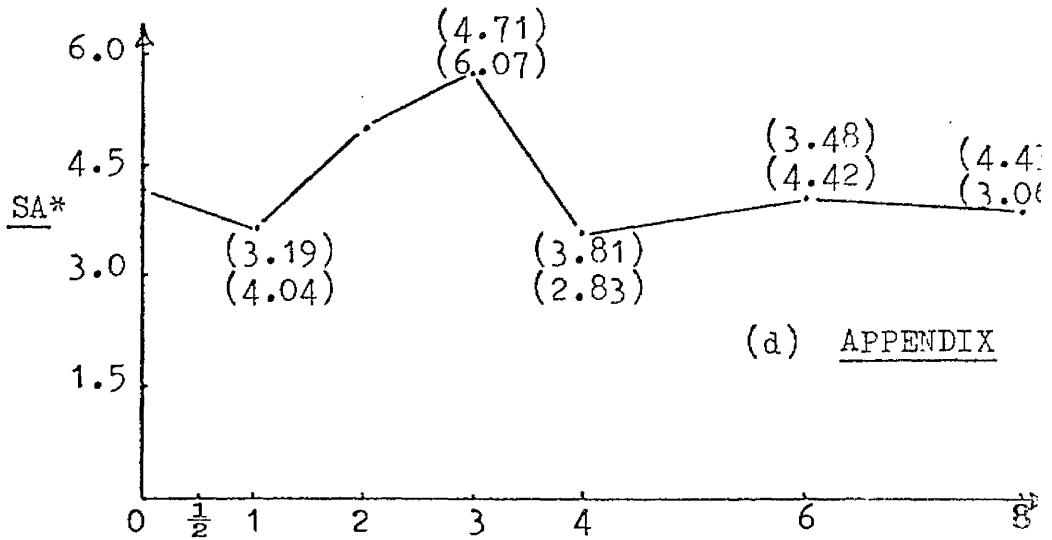
These graphs show that the spleen polymerase activity was higher than the control value 8 days after 1 injection of antigen and in 1 rabbit 3 days after a second injection. Decreases in activity were observed 3, 4 and 6 days after 1 injection and 1, 4 and 6 days after a second injection. The polymerase activity was greater in the appendix than in the spleen but there was considerable variation in the results. Activities higher than controls were observed on days 2 and 6 of the "primary response" and in 1 case on day 3 of the

FIG. 2 : 2. EFFECT OF ADMINISTRATION OF BSA ON THE SPECIFIC ACTIVITY OF DNA POLYMERASE IN THE SPLEENS AND APPENDICES OF RABBITS TOLERANT TO BSA




* SA = Specific Activity in mμmoles $[^{32}P]$dTMP incorporated per mg. of protein.

"secondary response." Reduced activities were noted 1 day after 1 injection of antigen and in some cases on days 1, 4, 6 and 8 after a second injection.

2. LATER STUDIES

Further estimations were made of the DNA polymerase activity in the spleens and appendices of normal rabbits and of rabbits killed on days $\frac{1}{2}$, 3 or 6 after 1 or 2 injections of BSA and on days 3 or 6 after 1 or 2 injections of saline. Table 2 : 1 shows the weights of these animals and of the tissues removed from them. This table can be summarised :

1. SPLEEN. With the exceptions of rabbits 90 and 76 and to a lesser extent 89 and 91, there was little difference between the weights of the control spleens. There was more scatter in the spleen weights of rabbits injected with BSA, the highest average in both the primary and the secondary response groups having occurred 3 days after antigen injection.

2. APPENDIX. Although there was considerable variation in the individual tissue weights, the average appendix weight for the majority of the rabbit groups was close to 1.85 g./Kg. The exceptions were the uninjected controls, the rabbits killed 6 days after a single injection of saline and the group

TABLE 2 : 1. WEIGHTS OF RABBITS AND TISSUES USED IN LATER EXPERIMENTS

| Rabbit Number | Weight at Death (Kg) | Days After* BSA | Days After* Saline | Tissue Weight (g./Kg.)** Spleen | | Appendix | | Thymus | |
|---|---|---|---|---|---|---|---|---|---|
| **EXPERIMENT 1** | | | | | | | | | |
| 96 | 2.05 | 3(1°) | – | 0.42 | | 1.81 | | 1.28 | |
| 97 | 2.25 | 3(1°) | – | 0.50 | (0.46) | 1.66 | (1.97) | 1.58 | (1.46) |
| 98 | 2.00 | 3(1°) | – | 0.46 | | 2.44 | | 1.53 | |
| 93 | 2.35 | 6(1°) | – | 0.29 | | 1.68 | | 1.28 | |
| 94 | 2.30 | 6(1°) | – | 0.61 | (0.44) | 2.35 | (1.90) | 2.39 | (1.70) |
| 95 | 2.45 | 6(1°) | – | 0.43 | | 1.67 | | 1.42 | |
| 99 | 2.55 | – | 6(1°) | 0.41 | (0.40) | 2.52 | (2.33) | 1.41 | (1.26) |
| 100 | 2.68 | – | 6(1°) | 0.38 | | 2.23 | | 1.11 | |
| **EXPERIMENT 2** | | | | | | | | | |
| 85 | 2.04 | 3(1°) | – | 1.03 | | 2.33 | | – | |
| 86 | 1.95 | 3(1°) | – | 0.45 | | 1.94 | | 1.30 | |
| 87 | 2.04 | 3(1°) | – | 0.29 | (0.56) | 1.23 | (1.84) | 0.83 | (1.00) |
| 88 | 1.98 | 3(1°) | – | 0.45 | | 1.85 | | 0.88 | |
| 89 | 2.30 | – | 3(1°) | 0.54 | (0.76) | 2.02 | (1.79) | 1.14 | 1.45 (~~0.97~~) |
| 90 | 2.07 | – | 3(1°) | 0.98 | | 1.56 | | 1.76 | |
| **EXPERIMENT 3** | | | | | | | | | |
| 73 | 3.40 | 3(2°) | – | 0.27 | | 1.82 | | 1.50 | |
| 74 | 3.28 | 3(2°) | – | 0.29 | (0.44) | 1.59 | (1.90) | 0.64 | (1.06) |
| 81 | 3.53 | 3(2°) | – | 0.45 | | 2.28 | | 1.45 | |
| 82 | 3.45 | 3(2°) | – | 0.73 | | 1.92 | | 0.63 | |
| 91 | 2.60 | No injection | | 0.56 | (0.51) | 2.68 | (2.44) | 1.35 | (1.59) |
| 92 | 2.95 | No injection | | 0.46 | | 2.17 | | 1.83 | |

continued .....

* (1°) = one injection
(2°) = two injections separated by 8 weeks

** Figures in parenthesis are average tissue weights per group.

TABLE 2 : 1 (continued). Weights of Rabbits and Tissues used in Later Experiments.

| Rabbit Number | Weight at Death (Kg) | Days After* BSA | Days After* Saline | Tissue Weight (g./Kg.)** Spleen | | Appendix | | Thymus | |
|---|---|---|---|---|---|---|---|---|---|
| EXPERIMENT 4 | | | | | | | | | |
| 69 | 2.87 | 3(2°) | - | 0.68 | | 1.86 | | 1.18 | |
| 70 | 2.96 | 3(2°) | - | 1.42 | (0.85) | 1.56 | (1.84) | 0.73 | (0.99) |
| 71 | 2.93 | 3(2°) | - | 0.69 | | 1.91 | | 1.18 | |
| 72 | 2.76 | 3(2°) | - | 0.59 | | 2.01 | | 0.85 | |
| 75 | 3.29 | - | 3(2°) | 0.43 | (0.34) | 1.57 | (1.67) | 0.51 | (0.71) |
| 76 | 3.50 | - | 3(2°) | 0.25 | | 1.77 | | 0.91 | |
| EXPERIMENT 5 | | | | | | | | | |
| 77 | 3.80 | 6(2°) | - | 0.33 | | 1.65 | | 0.37 | |
| 78 | 3.62 | 6(2°) | - | 0.69 | (0.52) | 0.75 | (1.29) | 0.51 | (0.55) |
| 79 | 3.43 | 6(2°) | - | 0.66 | | 1.28 | | 0.48 | |
| 80 | 2.98 | 6(2°) | - | 0.38 | | 1.49 | | 0.84 | |
| 83 | 3.22 | - | 6(2°) | 0.47 | (0.45) | 1.33 | (1.85) | 0.71 | (0.67) |
| 84 | 3.60 | - | 6(2°) | 0.43 | | 2.36 | | 0.62 | |

* (1°) = one injection
(2°) = two injections separated by 8 weeks

** Figures in parenthesis are average tissue weights per group.

killed 6 days after a second injection of BSA.

3. THYMUS. With one exception, the average thymus weight for each group of animals killed after a single injection of BSA or saline was higher than that of the corresponding group killed after a second injection. The rabbits killed 3 days after a single injection of BSA and those killed 3 days after a second injection have, on average, the same thymus weights. The average values are higher than the corresponding controls ½ day and 6 days after 1 injection of BSA and 3 days after a second injection, and lower 6 days after a second injection.

From the value of the specific activity expressed per mg. of protein, the activity per g. of tissue and per whole tissue was computed as indicated under Materials and Methods. These results are shown in Table 2 : 2.

Since these assays were done at different times, reagents of varying ages had to be used. In particular, the use of fresh rather than ageing preparations of nucleoside triphosphates could affect the result of enzyme assays and for this reason controls were included for each assay. This effect also makes comparison between assays less valid.

TABLE 2 : 2. SPECIFIC ACTIVITY OF DNA POLYMERASE FROM THE SPLEENS AND APPENDICES OF CONTROL AND IMMUNIZED RABBITS

| Expt. | Rabbit Number | Days After* | | Specific Activity (mμmoles of dTMP incorporated into DNA | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Spleen | | | Appendix | | |
| | | BSA | Saline | per mg. protein | per g. tissue | per whole tissue | per mg. protein | per g. tissue | per whole tissue |
| 1 | 96 | ½(1°) | – | 2.76 | 36.83 | 32.04 | 6.27 | 56.2 | 209.2 |
| | 97 | ½(1°) | – | 2.68 | 33.89 | 38.30 | 3.96 | 30.0 | 112.1 |
| | 98 | ½(1°) | – | 2.05 | 26.88 | 24.46 | 10.48 | 103.6 | 505.8 |
| | 93 | 6(1°) | – | 1.21 | 15.60 | 10.45 | 4.32 | 50.7 | 200.2 |
| | 94 | 6(1°) | – | 2.90 | 50.11 | 70.15 | 8.84 | 71.2 | 385.0 |
| | 95 | 6(1°) | – | 2.67 | 36.85 | 38.69 | 8.44 | 67.9 | 277.2 |
| | 99 | – | 6(1°) | 2.04 | 26.75 | 28.09 | 8.31 | 76.4 | 491.6 |
| | 100 | – | 6(1°) | 1.39 | 28.77 | 29.63 | 7.75 | 76.6 | 458.3 |
| 2 | 85 | 3(1°) | – | 3.94 | 67.98 | 142.75 | 8.08 | 133.8 | 635.6 |
| | 86 | 3(1°) | – | 1.79 | 30.07 | 26.46 | 4.61 | 82.7 | 312.6 |
| | 87 | 3(1°) | – | 2.88 | 39.75 | 23.85 | 5.84 | 116.9 | 292.2 |
| | 88 | 3(1°) | – | 3.78 | 66.93 | 59.57 | 8.26 | 136.8 | 502.0 |
| | 89 | – | 3(1°) | 2.18 | 42.12 | 52.23 | 7.94 | 142.5 | 662.4 |
| | 90 | – | 3(1°) | 3.18 | 32.91 | 67.13 | 9.59 | 163.2 | 527.2 |

continued .....

* 1° = one injection

2° = two injections

TABLE 2 : 2 (continued)

| Expt. | Rabbit Number | Days After* | | Specific Activity (mumoles of dTMP incorporated into DNA | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Spleen | | | Appendix | | |
| | | BSA | Saline | per mg. protein | per g. tissue | per whole tissue | per mg. protein | per g. tissue | per whole tissue |
| 3 | 73 | $\frac{1}{2}(2^{\circ})$ | - | 1.13 | 17.67 | 16.26 | 4.07 | 90.8 | 563.0 |
| | 74 | $\frac{1}{2}(2^{\circ})$ | - | 1.46 | 18.91 | 17.40 | 2.63 | 49.0 | 255.3 |
| | 81 | $\frac{1}{2}(2^{\circ})$ | - | 2.29 | 40.03 | 63.64 | 7.21 | 109.5 | 881.1 |
| | 82 | $\frac{1}{2}(2^{\circ})$ | - | 2.10 | 24.63 | 62.08 | 5.10 | 83.3 | 550.5 |
| | 91 | No injection | | 1.78 | 33.16 | 48.08 | 5.46 | 91.7 | 639.0 |
| | 92 | No injection | | 3.05 | 49.11 | 66.31 | 6.70 | 108.0 | 690.4 |
| 4 | 69 | $3(2^{\circ})$ | - | 2.54 | 32.13 | 62.97 | 6.86 | 131.0 | 699.3 |
| | 70 | $3(2^{\circ})$ | - | 3.35 | 57.76 | 236.81 | 5.07 | 89.8 | 413.9 |
| | 71 | $3(2^{\circ})$ | - | 1.74 | 27.62 | 56.06 | 11.86 | 231.9 | 1296.1 |
| | 72 | $3(2^{\circ})$ | - | 4.25 | 74.30 | 120.36 | 8.46 | 145.9 | 808.5 |
| | 75 | - | $3(2^{\circ})$ | 0.71 | 13.06 | 18.42 | 6.14 | 115.8 | 597.5 |
| | 76 | - | $3(2^{\circ})$ | 1.28 | 25.02 | 22.27 | 9.09 | 163.1 | 1011.1 |
| 5 | 77 | $6(2^{\circ})$ | - | 2.90 | 46.70 | 59.31 | 4.35 | 107.1 | 672.3 |
| | 78 | $6(2^{\circ})$ | - | 1.44 | 21.86 | 54.65 | 4.76 | 95.3 | 259.2 |
| | 79 | $6(2^{\circ})$ | - | 0.75 | 19.32 | 43.47 | 3.55 | 62.0 | 273.0 |
| | 80 | $6(2^{\circ})$ | - | 2.45 | 41.13 | 46.06 | 3.51 | 63.8 | 282.5 |
| | 83 | - | $6(2^{\circ})$ | 1.38 | 26.98 | 41.01 | 3.29 | 75.7 | 324.6 |
| | 84 | - | $6(2^{\circ})$ | 1.53 | 25.68 | 39.81 | 4.28 | 94.5 | 803.3 |

* $1^{\circ}$ = one injection

$2^{\circ}$ = two injections

The results presented in Table 2 : 2 are summarised below:

1. SPLEEN

(a) NORMAL RABBITS (91 and 92): The specific activities were higher than in the early experiments. There was closer agreement between the 2 tissues when the enzyme activity was expressed per whole tissue rather than per mg. of protein.

(b) PRIMARY RESPONSE: At all 3 time intervals studied, the average specific activity was higher than the average of the corresponding controls although in most cases there was considerable animal to animal variation.

(c) SECONDARY RESPONSE: The average specific activity compared to the corresponding controls was lower at $\frac{1}{2}$ day but higher at days 3 and 6. The increase at day 3 was particularly consistent. When activities per whole tissue were compared, the increase was found to be more consistent at day 6 and more pronounced at day 3.

2. APPENDIX

(a) NORMAL RABBITS (91 and 92): There was fairly close agreemnnt between the 2 values especially when comparison was made of activity expressed per whole tissue. Again polymerase activity in the appendix was found to be considerably greater than in the spleen.

(b) PRIMARY RESPONSE: There was considerable animal to animal variation making it inappropriate to compare the test values with the control.

(c) SECONDARY RESPONSE: The average value at ½ day was lower than the corresponding control but again there was considerable variation in the results. Polymerase activity in days 3 and 6 were virtually the same in the appendices of antigen injected and saline injected rabbits.

In order to facilitate comparison of the specific activities of DNA polymerase found in these later experiments with the values found earlier, Table 2 : 3 was compiled. In effect, the control for each assay was corrected to unity and the values for the antigen stimulated animals were correspondingly adjusted.

This table shows that increases in DNA polymerase activity were consistently found in the spleens of non-tolerant rabbits killed ½, 3 and 6 days after a single injection of antigen and 3 and 6 days after a second injection. Values were depressed ½ day after a second injection of BSA. In the appendix, values were lower than the normal value of unity at all time intervals studied in the primary response, and also 12 hours after a second injection of antigen, but normal on the third and sixth days

TABLE 2 : 3. COMPARISON BETWEEN EXPERIMENTS OF SPECIFIC ACTIVITY OF DNA POLYMERASE FROM SPLEENS AND APPENDICES OF CONTROL AND IMMUNIZED RABBITS

| Immune Status \ Time after BSA | Ratio:- Specific activity of DNA polymerase / Specific activity (averaged) of corresponding controls | | | | | |
|---|---|---|---|---|---|---|
| | Spleen | | | Appendix | | |
| | 12 hrs. | 3 days | 6 days | 12 hrs. | 3 days | 6 days |
| Normal - | 1.26 | 1.99 | 1.95 | - | - | - |
| | 2.25 | 2.41 | 3.00 | - | - | - |
| | 1.02 | 1.76 | 1.40 | - | - | - |
| Primary | 1.60 (96)* | 1.47 (85) | 0.70 (93) | 0.78 (96) | 0.92 (85) | 0.54 (93) |
| Response | 1.56 (97) | 0.67 (86) | 1.69 (94) | 0.49 (97) | 0.53 (86) | 1.10 (94) |
| | 1.19 (98) | 1.07 (87) | 1.55 (95) | 1.31 (98) | 0.67 (87) | 1.05 (95) |
| | - | 1.41 (88) | - | - | 0.94 (88) | - |
| | 1.48 ave. | 1.63 ave. | 1.72 ave. | 0.86 ave. | 0.77 ave. | 0.90 ave. |
| Tolerant - "Primary Response" | 0.87 | 0.47 | 0.79 | - | - | 1.28 |

continued ...

* Figures in parenthesis refer to rabbit numbers - see Tables 2 : 1 and 2 : 2

TABLE 2 : 3 (continued). Comparison between experiments of specific activity of DNA polymerase from spleens and appendices of control and immunized rabbits.

| Immune Status \ Time after BSA | Ratio:- Specific activity of DNA polymerase / Specific activity (averaged) of corresponding controls | | | | | |
|---|---|---|---|---|---|---|
| | Spleen | | | Appendix | | |
| | 12 hrs. | 3 days | 6 days | 12 hrs. | 3 days | 6 days |
| Normal - Secondary Response | 0.54 | 1.22 | 2.44 | - | - | - |
| | - | - | 2.16 | - | - | - |
| | 0.47 (73)* | 2.54 (69) | 1.99 (77) | 0.67 (73) | 0.90 (69) | 1.15 (77) |
| | 0.60 (74) | 3.35 (70) | 0.99 (78) | 0.43 (74) | 0.67 (70) | 1.26 (78) |
| | 0.95 (81) | 1.74 (71) | 0.51 (79) | 1.19 (81) | 1.56 (71) | 0.94 (79) |
| | 0.87 (82) | 4.25 (72) | 1.68 (80) | 0.84 (82) | 1.11 (72) | 0.93 (80) |
| | 0.69 ave. | 2.62 ave. | 1.63 ave. | 0.78 ave. | 1.06 ave. | 1.07 ave. |
| Tolerant "Secondary Response" | - | 1.43 | 0.71 | - | 1.10 | 0.80 |
| | - | 0.85 | 0.84 | - | 1.55 | 1.03 |
| | - | 1.14 ave. | 0.78 ave. | - | 1.33 ave. | 0.92 ave. |

* Figures in parenthesis refer to rabbit numbers - see Tables 2 : 1 and 2 : 2

of the secondary response.

In the spleen of tolerant rabbits, values were depressed at all times in both the "primary" and the "secondary" responses with the exception of 1 rabbit killed on the third day of the "secondary response." Increased activity was found in the appendix on the sixth day of the "primary response" and in one case on the third day of the "secondary response", and a decrease was observed in 1 rabbit killed 6 days after a second injection of antigen.

To indicate possible differences in properties of the various enzyme preparations, the specific activities were also found for 30 min. and 90 min. incubation times. By subtraction, the amount of dTMP incorporated into DNA over 3 successive 30 min. time intervals was found and Table 2 : 4 incorporates these results for the instances where the specific activity of the spleen enzyme from non-tolerant rabbits was particularly high (above 3.0 per 60 min.) and particularly low (below 1.4 per 60 min.).

In the first group, with the exception of rabbit 70, the enzyme activity was lower over the first 30 min. incubation than over the other 2 incubation periods and in 2 cases activity was highest over the third time interval. In the second group, the pattern was not greatly different. With the exceptions of rabbits 75, 76 and 79, activity was highest in the 30-60 min. incubation period.

TABLE 2 : 4. THE SPECIFIC ACTIVITY OF DNA POLYMERASE OVER 3 SUCCESSIVE 30 MIN. INCUBATION PERIODS IN THE CASES WHERE THE SPECIFIC ACTIVITY PER 60 MIN. WAS PARTICULARLY HIGH (ABOVE) OR PARTICULARLY LOW (BELOW)

| Rabbit Number | Days After* | | Specific Activity (mμmoles TMP incorporated per mg. protein) | | |
|---|---|---|---|---|---|
| | BSA | Saline | 0–30 min. Incubation | 30–60 min. Incubation | 60–90 min. Incubation |
| 92 | No injection | | 1.31 | 1.74 | 2.40 |
| 90 | - | 3($1^{o}$) | 1.27 | 1.91 | 1.47 |
| 85 | 3($1^{o}$) | - | 1.70 | 2.24 | 1.97 |
| 88 | 3($1^{o}$) | - | 1.55 | 2.23 | 2.32 |
| 70 | 3($2^{o}$) | - | 1.69 | 1.66 | 1.49 |
| 72 | 3($2^{o}$) | - | 1.98 | 2.27 | - |
| 100 | - | 6($1^{o}$) | 0.52 | 0.87 | 0.61 |
| 75 | - | 3($2^{o}$) | 0.41 | 0.30 | 0.36 |
| 76 | - | 3($2^{o}$) | 0.66 | 0.62 | 0.60 |
| 83 | - | 6($2^{o}$) | 0.61 | 0.77 | 0.60 |
| 93 | 6($1^{o}$) | - | 0.34 | 0.87 | 0.54 |
| 73 | 3($2^{o}$) | - | 0.48 | 0.65 | 0.60 |
| 79 | 6($2^{o}$) | - | 0.43 | 0.32 | - |

* $1^{o}$ = one injection.

$2^{o}$ = two injections separated by 8 weeks.

# DISCUSSION

## A. TISSUE WEIGHTS

Variation in the weight of lymphoid tissues taken from control animals can be due to a number of factors. Thus the blood content of the spleen at the time of killing has considerable bearing on the weight of this tissue; the weight of the thymus bears an inverse relationship to the age of the rabbit; and thirdly, the weight of the lymphoid tissues in general is dependent on the immunological status of the animal at the time of killing. The tissue weight variation which was evident in a number of cases indicates a lack of uniformity in the condition of the rabbits used. This was one of the major shortcomings of these experiments and could only be overcome by using rabbits of more closely matched age and weight raised under germ-free conditions and by perfusing the lymphoid tissues immediately after the death of the animals.

The greater irregularity in the tissue weights of the immunised animals (Table 2 : 1) reflects the observation (noted earlier) that in different animals there is considerable variation in response to a given antigen administered under comparable conditions.

Conclusions which can be drawn with some degree of confidence regarding antigen induced changes in the weight

of lymphoid tissues are that there was an increase in spleen weight 3 days after a second injection of BSA and a decrease in appendix weight 6 days after a second injection. Wissler, Fitch and La Via (1960) have recorded that rats undergoing a primary response to a particulate antigen had maximum spleen weight 2 days before serum antibody concentration was optimal so an increase in spleen weight on the third day of the secondary response to BSA is in accord with the occurrence of optimal concentration of anti-BSA antibody found on day 6 by Dixon et al. (1954). The weight decrease in the appendix on the sixth day of the secondary response may reflect the possible role this tissue has in the immune response in the rabbit. This is discussed more fully below (B - 1(b) and 3(b)).

## B. DNA POLYMERASE STUDIES

### 1. NORMAL RABBITS

(a) SPLEEN. The average value for the specific activity of DNA polymerase in the pH 5.0 precipitate fraction of rabbit spleen (2.42 in the later experiments) compares favourably with the value of 0.81 recorded by Shepherd (1965). A combination of factors, general improvement in technique, greater speed in carrying out the manipulations involved and the use of purer preparations of reagents explains the

greater activity obtained for the spleen enzyme in the second series of experiments compared with that obtained in the first (2.42 compared with 0.81).

(b) APPENDIX. The average specific activity of DNA polymerase extracted from normal rabbit appendices was 6.08 compared with the value of 8.96 reported by Shepherd (1965). This value is considerably greater than that recorded for the spleen indicating more extensive cell proliferation in the appendix. In part this greater enzyme activity may be due to the fact that in comparison with the spleen a greater proportion of the cells in the mucosa of the appendix are lymphoid (there are comparatively few red blood cells) but it also reflects a growing conviction that the appendix has an important role in the immune response in the rabbit.

Ford and Gowans (1967) have recently suggested that potentially antigen responsive cells are not fixed in the tissue which will mediate the immune response but that the important cells in this respect are the lymphocytes in the circulating blood at the time of antigen administration. Nossal (1967) also points out that in mice the antigen responsive cells are not fixed and states that they are probably derived from bone marrow and circulate with the morphology of small lymphocytes in lymph and blood.

In the rabbit it appears probable that the appendix is a source of these small lymphocytes. Sussdorf (1960) has shown that, although the haemolysin response in the rabbit is mediated solely by the spleen, X-irradiation of the animal with shielding of the spleen alone results in a diminution of the response. However, X-irradiation with shielding of the appendix alone did not cause a reduction in the haemolysin response.

Sutherland, Archer and Good (1964) have shown that neonatal appendectomy depresses antibody production in rabbits challenged antigenically 9 weeks later, to the same extent as does neonatal thymectomy. Furthermore, combined neonatal appendectomy-thymectomy resulted in a much more severe depression of antibody production than did splenectomy-thymectomy.

Thus, while the appendix may not be the site of antibody synthesis and while injection of antigen does not result in any consistent changes in DNA polymerase activity in the appendix (Table 2 : 2), nevertheless the considerable DNA synthesising activity which occurs in this tissue may have direct relevance to the events occurring in the spleen subsequent to antigen injection.

2. PRIMARY RESPONSE

(a) SPLEEN. It has already been stated that different animals respond to varying degrees to a given antigen administered under comparable conditions. As will be considered more fully under Section C of this Discussion, this observation may in part be the explanation for the considerable scatter in the levels of enzyme activity recorded for both spleen and appendix at various stages of the immune response.

However, in spite of this scatter it is clear from Table 2 : 3 that the specific activity of the enzyme has increased on days $\frac{1}{3}$, 3 and 6 after the intravenous injection of BSA. On the basis of the preliminary studies (Fig. 2 : 1a), activity is also high on days 4 and 8 but normal on days 1 and 2 of the primary response. This effect of BSA injection on DNA polymerase activity can be compared with the effect of injection of SRBCs on thymidine kinase activity in mouse spleen, reported recently by Raska and Cohen (1967). These authors record that kinase activity rises sharply over the first 8 hours of the haemolysin response and this is followed by a less dramatic increase reaching a peak at 2 days. On the basis of the results reported here, this biphasic pattern with its earlier

increases in enzymic activity might have been predicted since dTTP is a prerequisite for DNA synthesis and since the response to a particulate antigen is more rapid than the response to a soluble antigen (Taliaferro, Taliaferro and Jaroslow, 1964).

Craddock, Winkelstein, Matsuyuki and Lawrence (1967) on measuring the uptake of tritiated thymidine into the DNA of the spleen of rats undergoing a primary response to SRBCs, recorded an increase in DNA synthesis within 24 hours followed by a more marked increase which reached a peak on the fourth day after antigen injection.

A third approach again leading to conclusions which corroborate the findings reported here was that of Wissler et al. (1960). These authors found that in the spleen of rats undergoing a primary immune response to a particulate antigen, mitosis began at 36 hours and reached a maximum between day 3 and day 4.

Jandl, Files, Barnett and MacDonald (1965) have recorded that, when rats were injected with heated autologous erythrocytes, macrophage proliferation occurred at about 24 hours. This could be the explanation for the increase in DNA polymerase activity found 12 hours after a primary injection of BSA, for the initial rise in thymidine kinase

activity reported by Raska and Cohen (1967) and for the early increase in DNA synthesis reported by Craddock et al. (1967).

In the case of tolerant rabbits killed after a primary injection of BSA (Fig. 2 : 2a), the 12-hour increase in splenic polymerase activity is absent and there is a decrease in enzyme activity in the animals killed 3, 4 and 6 days after injection. If macrophage proliferation is taken to be the cause of the early increase in DNA polymerase activity in non-tolerant rabbits, then the absence of this increase in tolerant rabbits is of some interest. Harris (1967) has shown that peritoneal macrophages from a tolerant rabbit were able to ingest the antigen to which it was tolerant and, when incubated with normal rabbit spleen cells, could mediate an immunological response in vitro. Nossal and Ada (1964), avoiding the limitations of interpretation associated with the use of peritoneal macrophages, have shown that lymph node macrophages from a tolerant rat could recognise and ingest the antigen to which the rat was tolerant. Further, they reached the surprising conclusion that immune rats and tolerant rats showed a very much greater ability to phagocytose the antigen at the periphery of the lymphoid follicles than did control

rats responding to a first injection of antigen.

Thus it appears that, while replication of macrophages may be a necessary step in the primary response in non-tolerant rabbits, macrophages from a tolerant animal have no need to replicate on challenge since they have a sufficiently enhanced ability to recognise and ingest the antigen to which the animal was tolerant.

(b) APPENDIX. The specific activity of DNA polymerase in the appendix appears to decrease 12 hours after a first injection of antigen. There is a further and more consistent decrease at 3 days and a shift back to normal values at 6 days (Table 2 : 3). Where activity was expressed per whole tissue, these decreases were more pronounced in all cases.

On the basis of these findings and the earlier discussion, it is suggested that 12 hours after antigen dividing lymphocytes have begun departing from the tissue. Thus the proportion of less rapidly dividing cells has increased and there is a concomitant decrease in the specific activity of the recovered enzyme. The weight of the tissue, and hence the enzymic activity per whole tissue (Table 2 : 2), has also decreased. The same situation holds, perhaps on a slightly magnified scale, at 3 days. By the sixth day after BSA, there is regeneration of lymphoid cells within

the appendix (either by proliferation of existing cells or by arrival of cells from bone marrow or thymus), resulting in an increase in the specific activity of the polymerase. The tissue weight is still low so enzyme activity per whole tissue is lower than the control value.

In the case of the tolerant animals, no clear pattern emerged (Fig. 2 : 2c) and insufficient studies were done to allow conclusions to be drawn. It is possible that the figures recorded represent a scatter of normal values.

3. SECONDARY RESPONSE

(a) SPLEEN. Compared with the primary response, the secondary response is characterised by a shorter latent period and an earlier and higher peak titre of serum antibody (Dixon et al., 1954). Thus the earlier rise in polymerase activity occurring between days 1 and 2 (Fig. 2 : 1b) and the more pronounced increase seen at day 3 (Table 2 : 3) might have been predicted.

In contrast to the primary response group there was a depression in polymerase activity at 12 hours in the secondary response animals. As already stated, Nossal and Ada (1964) have shown that macrophages from an animal given a second injection of antigen have a greatly enhanced ability to ingest the antigen than do their primary response

counterparts. Thus it is suggested that as in the case of the tolerant "primary response" rabbits replication of macrophages is not required. The significance of the observed decrease in polymerase activity will be discussed later (D).

Autoradiographic evidence has led Nossal and Mäkelä (1962b) to the guarded conclusion that antigen stimulated DNA synthesis in rat lymph node cells has begun by 24 hours and is optimal between days 3 and 4 of the secondary response, which shows close agreement with the pattern of DNA polymerase activity found in these studies. The results presented here can also be compared with the findings of Urso and Makinodan (1963). Diffusion chambers containing rabbit spleen cells undergoing a secondary response to BSA were implanted into X-irradiated recipients and the authors reported that the mitotic index of the cells began to rise on the first day after antigenic stimulation and reached a peak on the third day. Dutton and Eady (1964) reported that in a system involving the in vitro addition of a protein antigen to primed rabbit spleen cells uptake of tritiated thymidine into DNA began to increase markedly 20 hours after antigen and was still rising when the experiment was stopped 50 hours after antigen addition.

In contrast to these findings, Cohen and Talmage (1965) concluded that from cell transfer studies involving mouse spleen cells responding to a second injection of a particulate antigen, DNA synthesis in cells whose progeny produced antibody began within 5 hours and had ceased within 48 hours. The latter figure was arrived at on the basis of the following observation. Primed mice were given a challenge injection of bovine gamma globulin. After 48 hours the spleens were removed and single cell suspensions incubated for 2 hours in a medium containing tritiated thymidine. The cells were washed and transferred into a neutral host. After a further 24 hours, 107 antibody containing cells in the recipient's spleen were identified and since none was found to be labelled it was assumed that DNA synthesis had stopped by 48 hours. However, as Hale, Cooper and Milton (1965) have pointed out, studies based on the uptake of tritiated thymidine involve many assumptions, some of which are open to question. In particular, the proportion of proliferating cells actually in S phase (the DNA synthesising phase) in a 2-hour period cannot be more than one-third and may be as low as one-sixth if the observations of Hale et al. (1965) on proliferating blood leucocytes are applicable and assuming that the generation time of immature

plasma cells is about 12 hours (Capalbo et al., 1962). Furthermore, if cell division with a concomitant increase in unlabelled cells does occur, as the results of this section suggest, then failure to observe labelled antibody forming cells on the third day of the secondary response is not surprising. It should also be noted that these studies were concerned only with cells actually producing antibody and, as Biozzi, Stiffel, Mouton, Bouthillier and Decreusefond (1968) have shown, cellular multiplication is not restricted to this group of cells.

As in the "primary response," studies in tolerant rabbits did not produce the same changes in polymerase activity as did studies in non-tolerant animals (Fig. 2 : 2b). In only 2 cases (1 rabbit killed on the third day and 1 on the eighth day of the "secondary response") was polymerase activity higher than the control value, while rabbits killed on days 1, 4 and 6 of the "secondary response" showed spleen polymerase activity considerably lower than the control. The significance of the decrease will be discussed later (D). It is tentatively suggested that the increase in polymerase activity in 2 cases may be a result of a break in tolerance or to the introduction of some contaminating antigenic material during the injection of BSA. Further studies would be required to clarify this point.

(b) APPENDIX. There was again more variation in the results from the appendix studies than from the spleen studies (Tables 2 : 2 and 2 : 3). The average specific activity of the enzyme, and the average activity expressed per g. of tissue was lower than the corresponding control 12 hours after the second injection of antigen but equal to the control value by the third and sixth days. The tissue weight however had considerably fallen by the sixth day. It is again suggested on the basis of the earlier discussion that dividing cells leave the appendix soon after the introduction of antigen and that this continues at least until the sixth day of the secondary response. By the third day, however, regeneration of cells within the tissue occurs. Thus the specific activity of the DNA polymerase has recovered by the third and sixth days but activity per whole tissue is still low (Table 2 : 2).

From the studies in the tolerant rabbits (Fig. 2 : 2d) it appears that some migration of dividing cells from the appendix may have occurred by the first day of the "secondary response" (decrease in specific activity of the enzyme) and that regeneration was occurring on the third day (increase in specific activity of the enzyme). However, it is clear that in view of the considerable scatter in

these results insufficient studies have been carried out to allow any definite conclusions to be drawn.

## C. DNA POLYMERASE ACTIVITY AND SEROLOGY

It has already been suggested that variation in DNA polymerase activity from one rabbit spleen to another may reflect the animal to animal variation in the extensiveness of the immune response. This could be readily checked in the later experiments only in the case of rabbits killed 6 days after a second injection of BSA since circulating antibody is not detectable at the other time intervals. Table 2 : 5 shows the relation between serum antibody titre (see Serology Section) and polymerase activity and it can be seen that on the basis of this limited comparison low polymerase activity is accompanied by low antibody production and high polymerase activity by high antibody production.

It is tempting to conclude from these observations that where abnormally low spleen DNA polymerase activities were obtained (e.g. rabbit numbers 86 and 93 - Table 2 : 3) these rabbits were not giving a good response to BSA and that the opposite was the case where high activities were observed (e.g. rabbits 70 and 72 - Table 2 : 3).

TABLE 5 : RELATION BETWEEN ANTIBODY TITRE AND DNA POLYMERASE ACTIVITY IN 4 RABBITS KILLED ON THE SIXTH DAY OF THE SECONDARY RESPONSE TO BSA

| Rabbit Number | Titre of * Serum Antibody | Specific Activity of DNA Polymerase |
|---|---|---|
| 77 | 200,000 | 1.99 |
| 78 | 100,000 | 0.99 |
| 79 | 3,200 | 0.51 |
| 80 | 100,000 | 1.68 |

* Titres are the reciprocals of the highest serum dilution to agglutinate tanned chick RBC coated with BSA. (See Serology Section).

## D. THE MEANING OF CHANGES IN DNA POLYMERASE ACTIVITY

It is clear from Table 2 : 4 that incorporation of dTMP into DNA does not occur at a uniform rate over the 90 min. incubation period. This emphasizes the fact that the *in vitro* assay of the enzyme is complicated by a number of factors:

(a) Destruction of the enzyme during incubation at $37^{o}$;

(b) Presence of other competing enzymes - e.g. nucleases and phosphatases compete respectively with the polymerase for the DNA template and the triphosphates;

(c) Possible presence of an enzyme activator or inhibitor in the preparation.

The reaction catalysed can be represented by the equation -

dATP + dCTP + dGTP + dTTP + heat-denatured DNA

$\downarrow$

double-stranded DNA + pyrophosphate.

Interference due to phosphatases in the enzyme preparation is minimised by using an excess of nucleoside triphosphates, but the concurrent action of nucleases is a matter for greater concern. With the exception of rabbits 75 and 79 where the spleen enzyme activity was abnormally

low (Table 2 : 4), the incorporation of dTMP into DNA was generally lower over the first 30 min. incubation period than over the second and third periods and in view of this it is suggested that the reaction being followed is essentially a balance between the action of DNA polymerase and endonuclease. During the first 30 min., while the polymerase is catalysing the synthesis of labelled double-stranded DNA, endonuclease is catalysing the breakdown of the single-stranded template. In so doing it promotes the formation of a larger number of DNA templates of lower molecular weight but still capable of functioning as template DNA. Thus synthesis of double-stranded DNA is enhanced over the 30-60 min. incubation period and, depending on the relative proportions of the 2 enzymes, over the 60-90 min, incubation period.

The most obvious interpretation of the observation that the specific activity of DNA polymerase has increased in some cases after the injection of antigen is that the proportion of the enzyme to other proteins in the pH 5.0 precipitate fraction has increased. Dutton and Eady (1964) have recorded that in the early part of the secondary response <u>in vitro</u> approximately 1% of rabbit spleen cells are stimulated by antigen to synthesize DNA and this agrees fairly well with the figure of 2% found by Harris and

Littleton (1966) for spleen cells examined on the second day of the secondary response. If further cell division means that by the third day 5% of the rabbit spleen cells are synthesizing DNA in response to the antigenic stimulus a 2.62 fold increase in DNA polymerase activity in the whole spleen (Table 2 : 3) means that there is a $\frac{(2.62 - 1.00)}{5}$ x 100 or 32-fold increase in the activity of the enzyme in the cells actually responding to the antigen. An increase of this magnitude appears to be inconsistent with the observation of Capalbo et al. (1962) that during the latent and log phases of the secondary response the generation time of the responding cells is decreased only 2-fold, from 24 hours to 12 hours.

It is tempting to speculate that, while the enhanced activity of DNA polymerase reported here may in part reflect replication of the complete complement of the DNA of the responding cells and the subsequent mitoses of these cells, it may also indicate replication of a particular gene coding for antibody structure. The need for such a phenomenon to explain the rapidity of the immune response has been emphasized by Smithies (1965) and invoked by him in proposing a mechanism for the primary and secondary responses.

The decrease in polymerase activity in the spleens of rabbits killed 12 hours after a second injection of antigen and in many cases in the tolerant rabbits can be explained in the following way. Twelve hours after a challenge injection of antigen there is considerable localisation of the BSA in the spleen and an increase in the enzymes catabolizing the antigen. Thus in the pH 5.0 precipitate fraction there is no increase in the proportion of DNA polymerase (as discussed earlier) but an increase in non-polymerase protein. This results in an artificially low value for the specific activity of the enzyme.

In the tolerant animals the same phenomenon may account for the depression observed in the early part of the "primary response" and on day 1 of the "secondary response" but fails to account for the subsequent falls on days 3, 4 and 6 of the "primary response" and days 4 and 6 of the "secondary response." That these depressions occur where there are marked increases in the non-tolerant rabbits (with the exception of the third day of the "secondary response") leads to the speculation that in tolerant rabbits antigen derived material not only fails to provoke an increase in DNA polymerase activity but actually causes inhibition of the enzyme in the tolerant

cells (the existence of which has been postulated by McGregor *et al.*, 1967). Such an inhibitor would, after homogenising and if present in sufficient concentration, exert an influence on the measured specific activity of the polymerase extracted from the whole tissue.

Thus an animal would be tolerant to an antigen because in the cells committed to this antigen DNA synthesis and the subsequent maturation of the cell is inhibited. Macrophage mediated inhibition of the antigen-provoked increase in DNA synthesis has already been recorded by Parkhouse and Dutton (1966) although these investigations did not involve tolerant animals.

# S E C T I O N 3

## POLYRIBOSOMES

INTRODUCTION

When this aspect of these studies was begun, considerable evidence had been presented which implicated ribosomal aggregates - or polyribosomes - in the biosynthesis of proteins in bacteria (for example, Gilbert, 1963; Schlessinger, 1963), in plants (Marcus and Feeley, 1965) and in animals (for example, Marks, Burka and Schlessinger, 1962; Gierer, 1963; Howell, Loeb and Tomkins, 1964). However, there was a paucity of information on polyribosomes in lymphoid tissue and what few reports there were presented conflicting results.

Thus, Stenzel, Phillips, Thompson and Rubin (1964) concluded that the functional ribosomes in rabbit spleen cells responding to protein antigens were monomers (single ribosomes) and dimers (aggregates of 2 ribosomes linked by mRNA). Their investigations appeared to rule out ribonuclease action on larger aggregates of ribosomes. Pagoulatos (1964) prepared polyribosome pellets from the spleens of normal and immunized rabbits. He did not attempt to resolve these into constituent ribosomal aggregates but observed that when these preparations were

incubated in the presence of an ATP regenerating system and a soluble cell-free fraction from rat liver, incorporation of labelled amino acids into acid precipitable material took place. This occurred to a much greater extent when polyribosome pellets from immunized rabbits were used and led Pagoulatos to the conclusion that the spleen of the immunized animal was much richer in active ribosomes than the normal spleen.

Manner and Gould (1965) found single ribosomes in the lymph nodes of normal rats but in immunized animals they were able to extract material which they considered corresponded to polyribosomes. However, this material was extremely resistent to ribonuclease but could readily be disaggregated to single ribosomes by proteolytic enzymes. These authors concluded that polypeptide or protein was involved in linking the individual ribosomes of "immune" rat lymph node tissue.

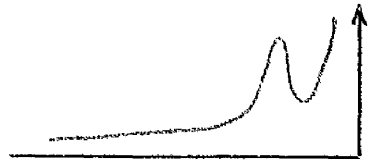
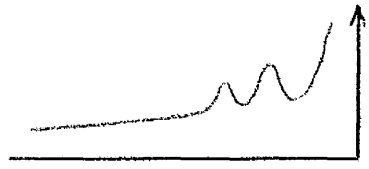
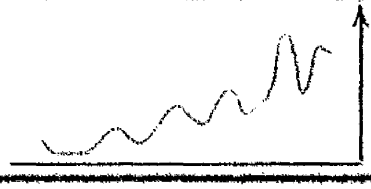
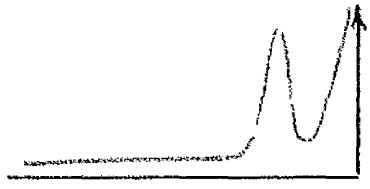
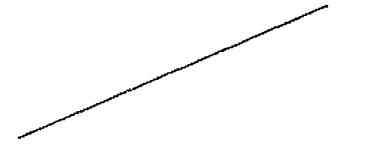
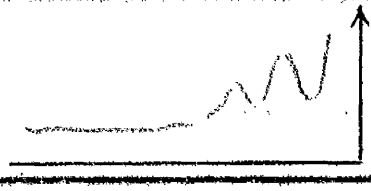
Resolution of rabbit spleen polyribosomes into 4 components was recorded by Voss, Bauer and Finnerty (1965) but here again the proportion of heavy polyribosomes was small. Incorporation of labelled uracil and leucine was greater in lymphoid cells from immunized than from non-immunized animals. Scharff and Uhr (1965) and Tawde,

Scharff and Uhr (1966) failed to resolve lymph node cell polyribosomes from immunized rabbits but showed that this was a result of excessive ribonuclease action. When ribonuclease activity was inhibited by addition of large amounts of HeLa cell cytoplasm nascent protein synthesis was found to be associated with polyribosomes.

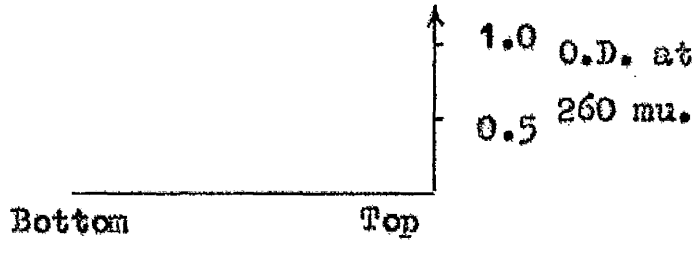
Norton, Lewis and Ziff (1965) recorded that in rabbit lymph nodes polyribosomal size increased with progressive immunization. Thus, for example, in rabbits which had received 1 injection of typhoid O antigen, labelled amino acids were incorporated into protein almost exclusively on single ribosomes. However, after 4 injections incorporation was largely associated with heavy polyribosomes - aggregates of 5-10 ribosomes. These observations applied to synthesis of both 19S and 7S antibody. La Via, Vatter and Northup (1966) resolved rat spleen polyribosomes into monomers, dimers and a small proportion of aggregates of 3 ribosomes and concluded that the H and L chains of antibody were synthesized in fragments largely on the monomers and dimers.

Thus it can be seen from this literature survey, which is summarized in Table 3 : 1, that the studies which had

TABLE 3 : 1. SUMMARY OF LITERATURE SURVEY OF LYMPHOID CELL POLYRIBOSOMES UP TO SUMMER 1966

| Authors | Tissue | Pattern* | Conclusion |
|---|---|---|---|
| Stenzel et al. 1964 | Rabbit spleen |  | Antibody synthesis on monomers and dimers. |
| Manner and Gould, 1965 | Rat lymph node |  | Polyribosomes bound by peptide or protein |
| Voss et al. 1965 | Rabbit lymph node |  | Antibody synthesis on ribosomal aggregates (not monomers) |
| Scharff and Uhr, 1965 | Rabbit lymph node |  | Antibody synthesis on polyribosomes (peak activity on aggregates of 8 ribosomes) |
| Norton et al. 1965 | Rabbit lymph node |  | Antibody synthesis on polyribosomes. Aggregates of 5-10 ribosomes. |
| La Via et al. 1966 | Rat Spleen |  | Antibody synthesis on monomers, dimers and a little on trimers. |

*




Fraction from Density Gradient

been carried out on lymphoid cell polyribosomes had produced conflicting results. In particular, few of the techniques employed had permitted good resolution of the polyribosome population possibly because of ribonuclease activity and this was in all probability one of the major causes of the confusion.

The aim of the investigations reported here was to determine if polyribosomes could be extracted from lymphoid tissue and to characterise the extraction procedure. It was also intended to determine the effect of administration of antigen on the polyribosome patterns obtained from the responding tissue.

## MATERIALS AND METHODS

### Animals

Rabbits. Rabbits were 3-5 month old New Zealand Whites of both sexes.

Rats. Albino male rats (weighing 145-155 g.) were used. The animals were starved overnight prior to killing. Free access to water was allowed at all times.

### Antigen

The antigen used was sheep red blood cells - SRBCs (Burroughs Wellcome and Co., London). The cells, which were supplied in Alsever solution, were washed 3 times and then made up as a 1% (v/v) suspension in 0.15M NaCl. They were warmed to 37° just prior to injection.

### Immunization

Rats to be injected were anaesthetized using trilene (ICI). Each rat was injected intravenously with 1.0 ml. of the antigen preparation and killed after 3 or 6 days.

### Preparation and Resolution of Polyribosome Pellets

The method employed was based on that of Wettstein, Staehelin and Noll (1963). Sucrose concentrations used in

this technique were made up in a buffer consisting of 0.05M tris-HCl. (pH 7.6), 0.025M KCl. and 0.005M $MgCl_2$. Sodium deoxycholate (DOC) was made up as a 10% (w/v) solution in 0.05M tris-HCl. (pH 8.0), 0.025M KCl. The polyribosome pellet was suspended in a solution composed of 0.005M tris-HCl. (pH 7.6), 0.025M KCl., 0.005M $MgCl_2$ referred to as TKM.

Rabbits were anaesthetized by the intravenous injection of nembutal (Abbott Laboratories, Kent) and killed by exsanguination. Rats were stunned and killed by decapitation. The following operations were carried out at $4^{\circ}$. Immediately after the death of the animal, the tissue (liver or spleen) was removed, trimmed, rinsed in 0.25M sucrose, blotted dry and weighed. Two vol. of 0.375M sucrose and, in the case of the spleen 3-7 vol. of 0.25M sucrose, were added. The tissue was homogenized gently using a Potter-Elvehjem homogenizer with 4 excursions of the pestle.

After homogenizing, the suspension was centrifuged (10 min., $1^{\circ}$, 15,000 g) in a Spinco model L2 ultracentrifuge to remove mitochondria and cell debris. Six ml. was

transferred from the middle of this post-mitochondrial supernatant (PMS) to a 10 ml. measuring cylinder and 0.9 ml. of the DOC solution added. The cylinder was inverted 3 times and 3.15 ml. of this DOC-treated PMS was layered on to a discontinuous sucrose gradient (3.15 ml. of 2.0M sucrose and 3.15 ml. of 0.5M sucrose) contained in cellulose nitrate centrifuge tubes. Using these quantities, 2 polyribosome pellets could be obtained from each 6.0 ml. of PMS. After centrifugation (4 hours, 1°, 160,000 g), the supernatant was decanted and the surface of the polyribosome pellet was washed carefully with 0.2 ml. of TKM. The TKM was decanted. Liver polyribosome pellets were reconstituted in a further 0.15 ml. of TKM, and spleen polyribosome pellets in 0.2 ml. of TKM. The suspended polyribosomes were resolved by carefully layering 0.04 - 0.05 ml. on to a 15% - 34% linear sucrose gradient and centrifuging in an SW 50 rotor in a Spinco model L2 (30 min., 1°, 200,000 g).

Monitoring of Gradient

After the density gradient centrifugation step, the tube was transferred to a perspex holder, the base punctured and 45% sucrose containing methylene blue as marker was

pumped in, using a Watson-Marlow flow inducer (Watson-Marlow Ltd., Bucks.). In this way the contents of the tube were forced upwards through connecting tubing to a flow-cell in a Beckman DB spectrophotometer which in turn was linked to a recorder. This arrangement, which permitted continuous monitoring of the optical density of the gradient at 260 mμ., is outlined schematically in Fig. 3 : 1. The flow-cell was designed by Dr. T. A. Douglas and is shown in diagrammatic form in Fig. 3 : 2.

Analysis of Patterns

The patterns were analysed by dropping perpendiculars from the troughs between the peaks to a base-line, and expressing the area under each peak (calculated using a compensatory planimeter) as a percentage of the total area.

FIG. 3 : 1. DIAGRAMMATIC REPRESENTATION OF METHOD USED TO MONITOR SUCROSE DENSITY GRADIENTS

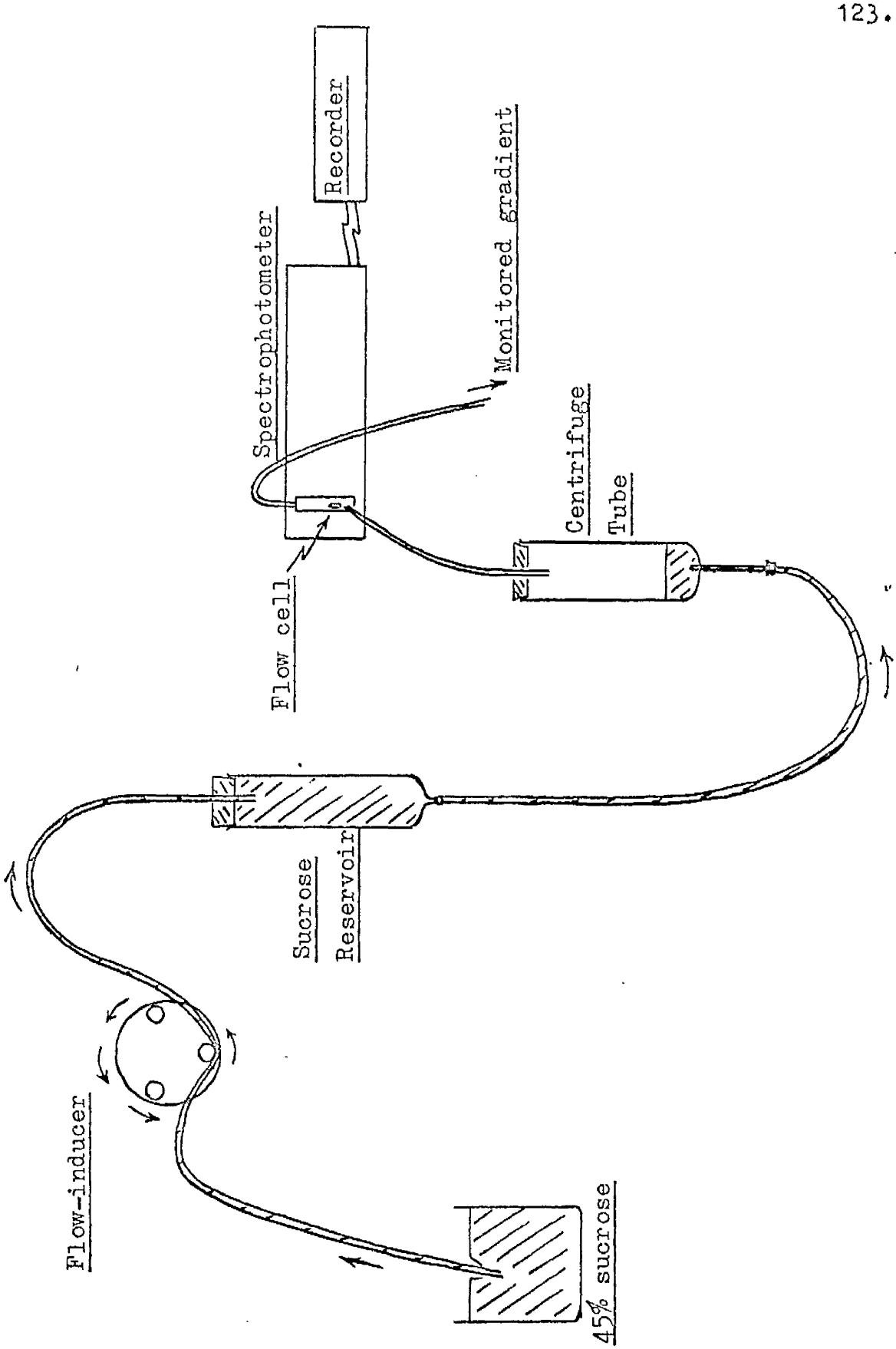
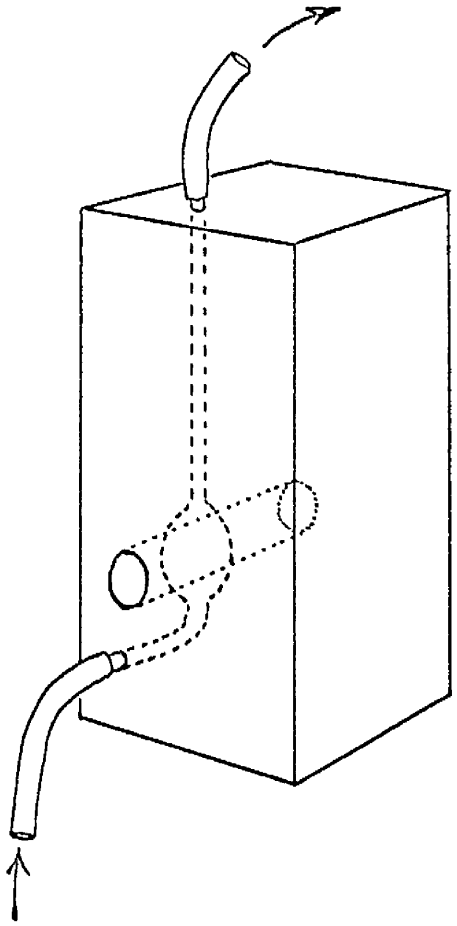


FIG. 3 : 2. DIAGRAMMATIC REPRESENTATION OF FLOW CELL



## RESULTS

In presenting these results, the following terms and abbreviations are used:

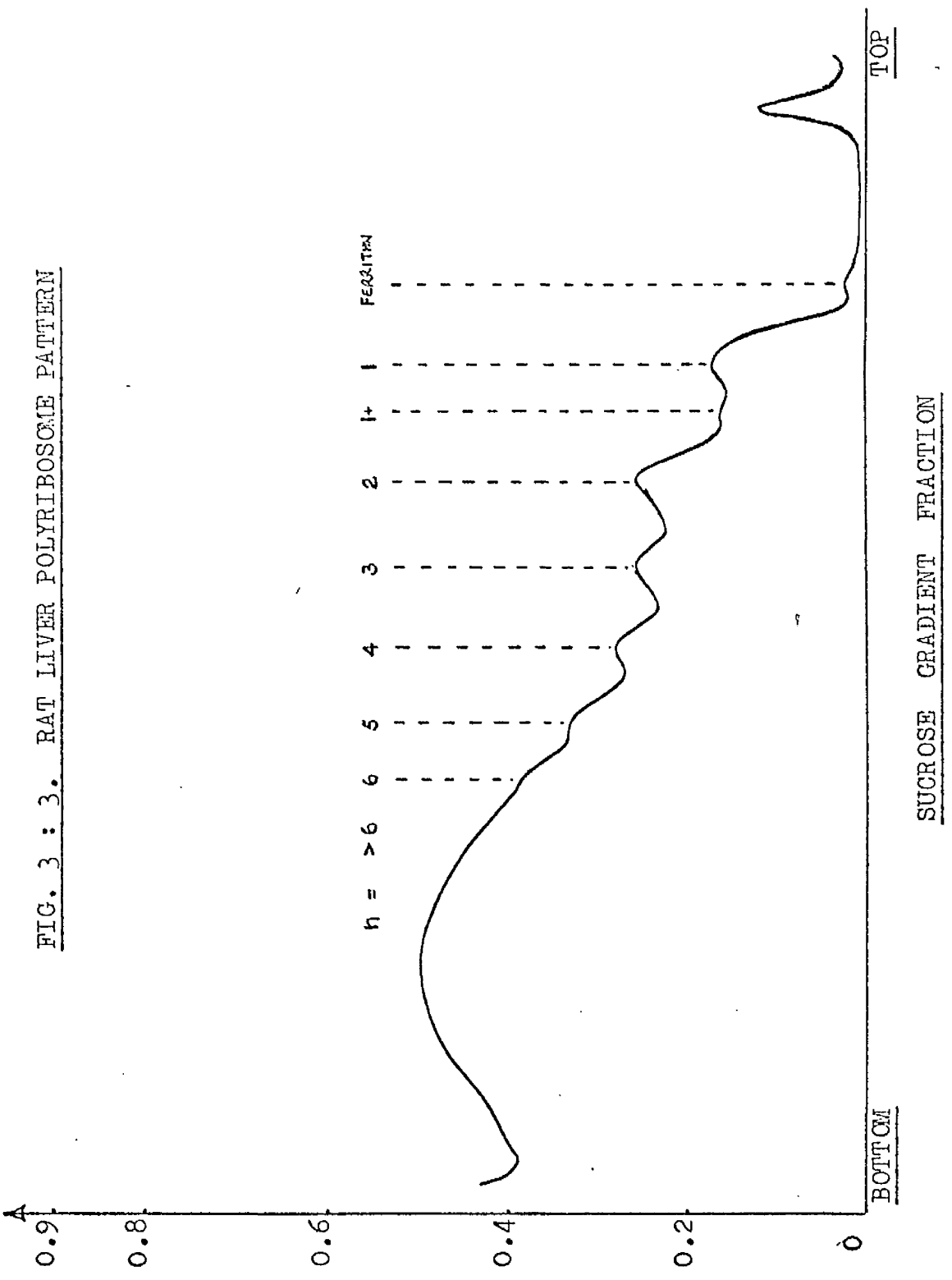
n = 1 (monomers), n = 2 (dimers), n = 3 (trimers), n = 4 (tetramers), n = 5 (pentamers) and n = 6 (hexamers) are terms used respectively for single ribosomes and aggregates of 2, 3, 4, 5 and 6 ribosomes attached to a single molecule of messenger RNA (mRNA). Also, n = 1+ is used to describe the material sedimenting between monomers and dimers on a sucrose density gradient and the term "heavy polyribosomes" is taken to refer to aggregates of more than 5 ribosomes.

### Rat Liver Polyribosomes

The pattern obtained on density gradient analysis of rat liver polyribosomes is shown in Fig. 3 : 3. The pattern closely resembles that reported by Wettstein *et al.* (1963) for rat liver polyribosomes and labelling of the peaks is based on the findings of these authors.

It can be seen from Fig. 3 : 3 that there is a large proportion of heavy polyribosomes in rat liver (sedimenting in the lower third of the tube). Ribosome aggregates up to pentamers could be identified. The pre-monomer material absorbed at 320 mμ. and was designated "ferritin."

FIG. 3 : 3. RAT LIVER POLYRIBOSOME PATTERN



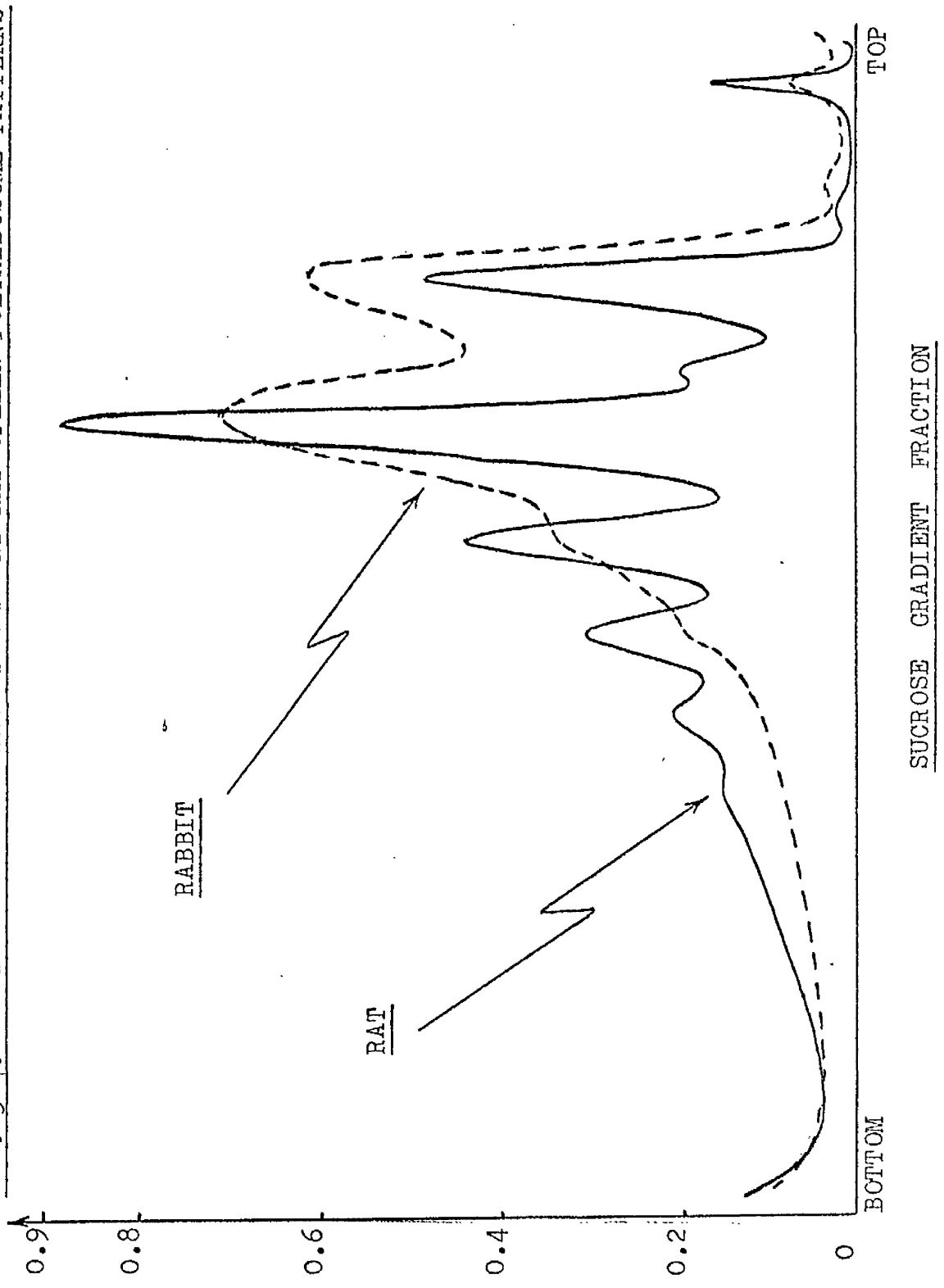
This material was observed consistently in both liver and spleen preparations.

Comparison of Patterns from Rabbit Spleen and Rat Spleen

Rabbit and rat spleens were employed to determine whether the procedures used for the preparation and analysis of rat liver polyribosomes would permit isolation of lymphoid tissue polyribosomes. On analysis of the pellets prepared from these tissues the patterns presented in Fig. 3 : 4 were found. With rabbit spleen there was a large proportion of monomer, slightly less dimer, a small amount of trimer material and a very low proportion of heavier polyribosomes. In contrast better resolution was obtained using rat spleen and there was a considerably greater proportion of heavy polyribosomes. For this reason subsequent studies were carried out using rat spleen tissue.

It was consistently observed that there was a greater preponderance of dimers than of any other aggregates in the polyribosome population of rat spleens. There was slightly less monomer material and the proportion of the remainder of the ribosomal aggregates varied from animal to animal.

FIG. 3:4. COMPARISON OF RABBIT SPLEEN AND RAT SPLEEN POLYRIBOSOME PATTERNS



The Effect of Dilution during Preparation of Polyribosomes

In view of the smaller size of the spleen, it was necessary to use 9 vol. of sucrose (2 vol. of 0.375M and 7 vol. of 0.25M) at the homogenizing stage to give sufficient material for centrifugation. For studies in liver polyribosomes, the tissue was homogenized in 2 vol. of 0.375M sucrose. To check any possible effect of the dilution in the case of the spleens, the following experiment was carried out. Spleens from 2 rats were finely minced together. Part was homogenized in 2 vol. of sucrose and part in 9 vol. Polyribosomes were prepared and analysed as before.

It was found that dilution of the tissue with 9 vol. of sucrose resulted in a lower proportion of heavy polyribosomes in the polyribosome pellet than did dilution with 3 vol. This effect was further investigated following the experimental plan shown in Fig. 3 : 5.

The results of this experiment are presented in Table 3 : 2. Comparison of Patterns 1 and 2 shows that a greater proportion of heavy polyribosomes was extracted when the tissue was homogenized in 3 vol. and not subsequently diluted to 10 (31.3% compared with 26.3%). In parallel with this, the proportion of lower ribosomal aggregates

FIG. 3 : 5. PLAN OF EXPERIMENT DESIGNED TO INVESTIGATE THE DECREASE IN PROPORTION OF HEAVY POLYRIBOSOMES WITH INCREASE IN DILUTION OF TISSUE

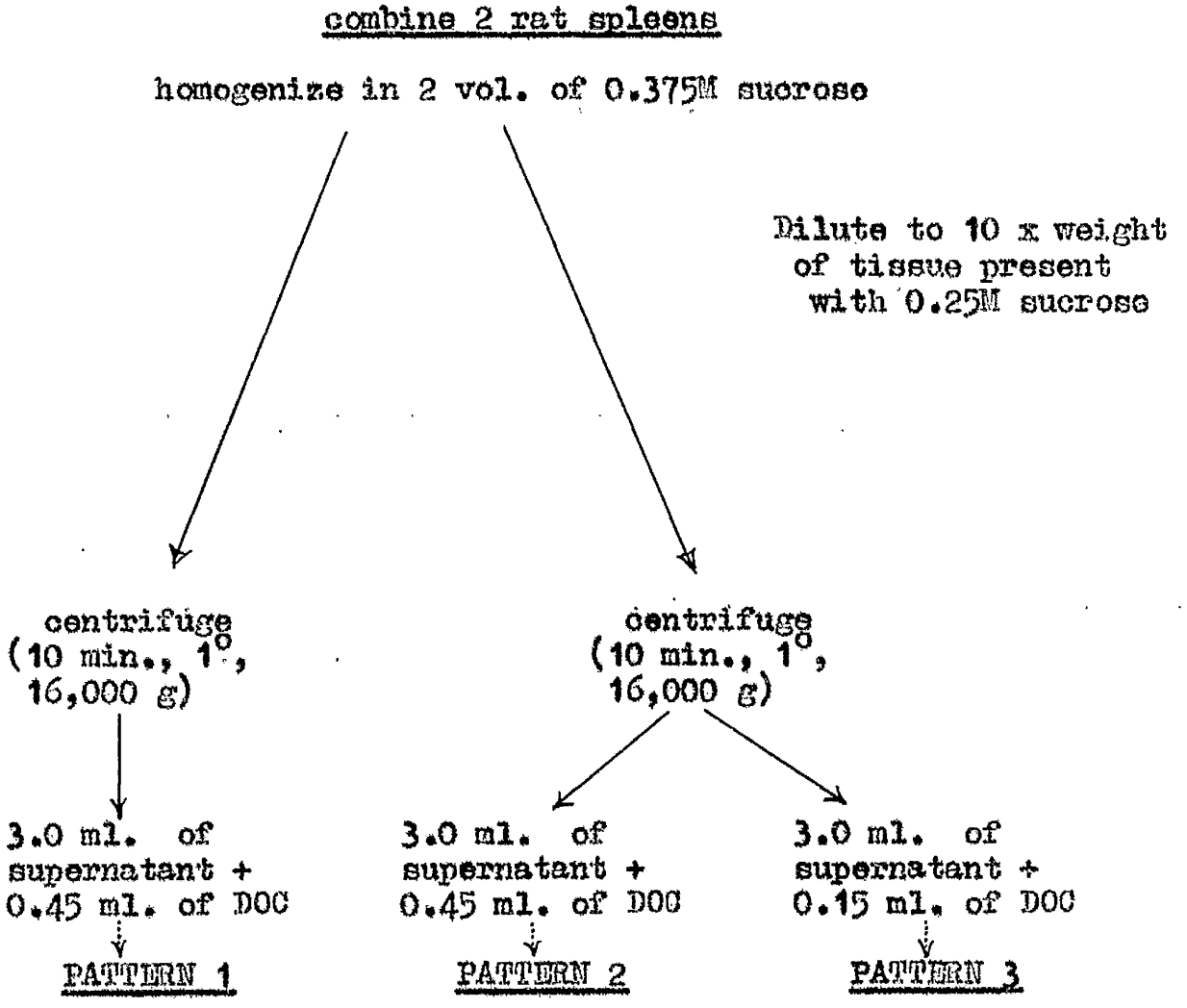


TABLE 3 : 2. EFFECT OF DILUTION DURING EXTRACTION ON DISTRIBUTION OF RIBOSOMAL AGGREGATES IN RAT SPLEEN POLYRIBOSOME PREPARATIONS

| Pattern | % of Polyribosomes Present As | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | n=1 | n=1½ | n=2+1½ | n=3 | n=4 | n=5 | n=6 | n=>5 |
| 1 | 13.7 | - | 20.8 | 11.3 | 12.0 | 10.7 | 7.3 | 31.3 |
| 2 | 14.1 | 2.2 | 22.5 | 13.9 | 12.4 | 10.0 | 7.1 | 26.3 |
| 3 | 15.4 | 5.4 | 24.3 | 15.1 | 13.2 | 10.4 | 7.0 | 20.9 |

For procedures used to obtain Patterns, see Fig. 3 : 5

extracted was greater when the homogenate was diluted to 10 vol. Thus loss of some heavy polyribosomes in the diluted preparation was not due to increased physical shearing during homogenizing since some loss occurred even when dilution took place after homogenizing. The loss was more marked when the DOC : RNA ratio was made the same in both diluted and undiluted preparations (Patterns 1 and 3). Comparison of these patterns indicates that the loss of heavy polyribosomal material resulting from dilution of the homogenized tissue is not due to a difference in the DOC : RNA ratio. It is also apparent that in the diluted preparations (Patterns 2 and 3) recovery of heavy polyribosomes was greater at the higher DOC concentration (26.3% compared with 20.9%). In all subsequent experiments the spleens were homogenized in 5 vol. of sucrose (2 vol. of 0.375M and 3 vol. of 0.25M) without further dilution. This total volume was necessary to give sufficient material for the centrifugation steps.

Location of Monomers in Rat Spleen Polyribosome Pattern

In order to locate the monomer peak a rat spleen polyribosome pellet was prepared and the pattern from a 0.04 ml. sample was obtained. To the remainder of the reconstituted polyribosomes was added 0.2 ml. of TKM

containing ribonuclease (10 µg./ml.). The tube was incubated at 37° for 5 min. then 0.04 ml. was removed and a ribosome pattern prepared as before. The patterns obtained are compared in Fig. 3 : 6 (page 135). After treatment with ribonuclease there was an increase in material absorbing at 260 mµ. at the top of the gradient and also in the monomer region. There was still some dimer material present but most of the larger aggregates had been degraded.

Effect of Immunization on Polyribosome Patterns

Polyribosome patterns were prepared and analysed from 3 groups of rats:

(a) Control, un-injected

(b) Killed 3 days after an injection of SRBCs

(c) Killed 6 days after an injection of SRBCs.

Tables 3 : 3a, 3b and 3c (pages 136, 137 and 138) show the distribution of ribosomal aggregates in the individual animals and as average values for each group and also incorporate the weight of each spleen.

It can be seen from these Tables that, while a high proportion of heavy polyribosomes could consistently be extracted from rat spleens, there was considerable animal

to animal variation as is evidenced by the high standard deviations. Using this system, no significant changes in the spleen polyribosome patterns resulted from antigen administration.

Also evident from these Tables was the fact that there was considerable variation in the spleen weights of both normal and stimulated rats.

FIG. 3 : 6. THE EFFECT OF RIBONUCLEASE ON RAT SPLEEN POLYRIBOSOMES

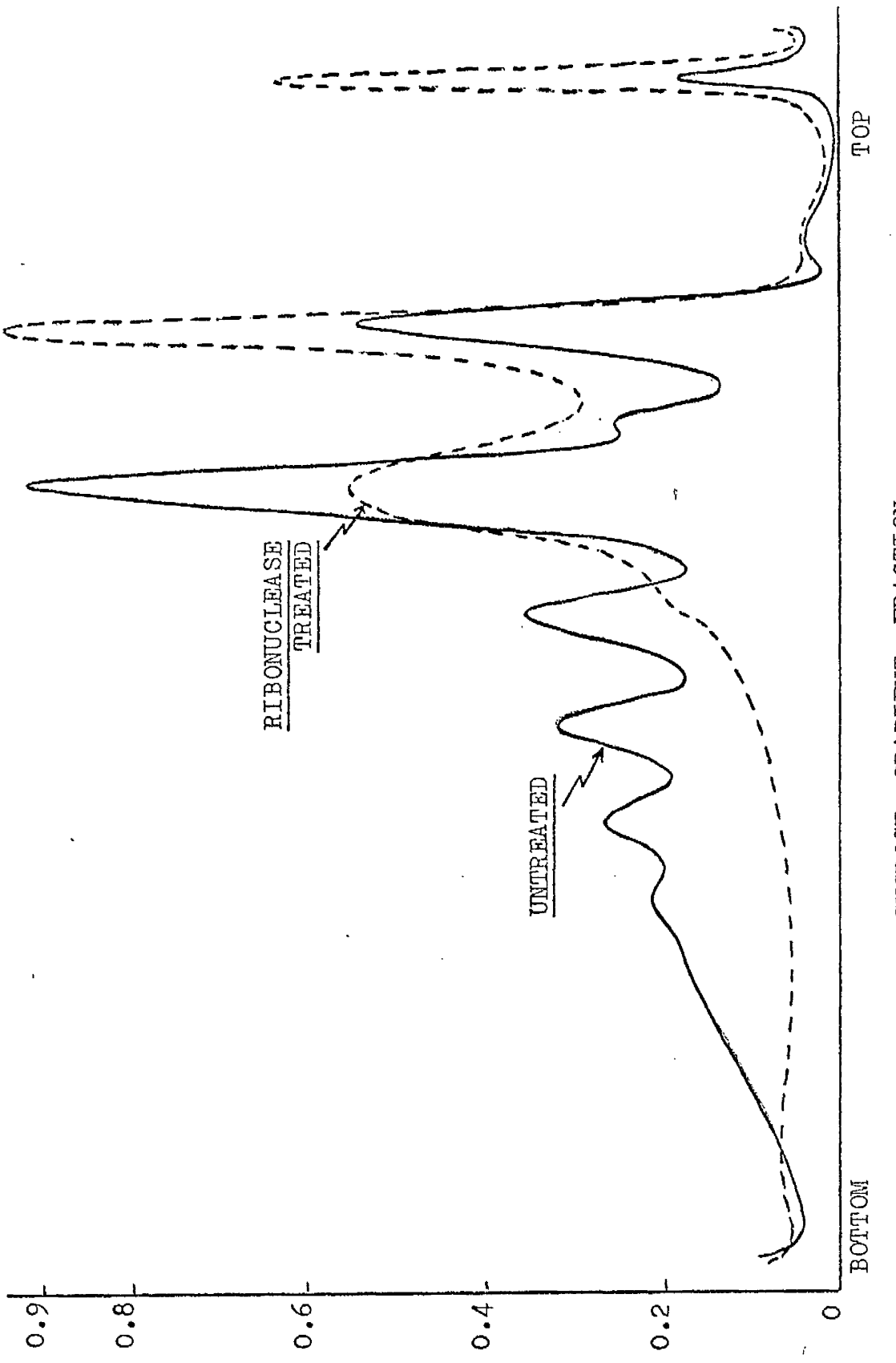


TABLE 3 : 3a : DISTRIBUTION OF RIBOSOMAL AGGREGATES IN POLYRIBOSOME PELLETS EXTRACTED FROM 11 NORMAL RAT SPLEENS

| | Percent of Total Polyribosomes Present as | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Rat | n=1 | n=1½ | n=2+1½* | n=3 | n=4 | n=5 | n=6 | n=>5 ** | Spleen Weight (g.) |
| 1 | 15 | - | 34 | 14 | 12 | 8 | 5 | 16 | 1.08 |
| 2 | 17 | - | 36 | 18 | 11 | 6 | - | 10 | 1.53 |
| 3 | 17 | - | 36 | 18 | 10 | 9 | - | 8 | 1.83 |
| 4 | 13 | 4 | 27 | 12 | 11 | 10 | 8 | 27 | 0.92 |
| 5 | 17 | - | 36 | 17 | 11 | 9 | - | 10 | 1.83 |
| 6 | 12 | 5 | 28 | 8 | 9 | 10 | 10 | 33 | 1.21 |
| 7 | 13 | - | 32 | 11 | 10 | 9 | 7 | 25 | 1.24 |
| 8 | 17 | 5 | 34 | 10 | 8 | 7 | 6 | 24 | 1.23 |
| 9 | 14 | 4 | 29 | 13 | 11 | 10 | 8 | 23 | 2.28 |
| 10 | 13 | 3 | 31 | 16 | 13 | 9 | 5 | 18 | 2.05 |
| 11 | 11 | 4 | 27 | 9 | 9 | 9 | 8 | 35 | 2.07 |
| Mean ± † S.D. | 14.5 ± 2.3 | 4.2 ± 0.8 | 31.8 ± 3.6 | 13.3 ± 3.6 | 10.5 ± 1.4 | 8.7 ± 1.3 | 7.1 ± 1.7 | 20.8 ± 9.2 | 1.57 ± 0.5 |

* Since not every pattern showed a distinct "n=1½" peak, the dimer peak was always taken to include this.

** Since not every pattern showed a distinct "n=6" peak, the heavy polyribosomes were considered as "n=>5".

† S.D. = Standard Deviation

TABLE 3 : 3b : DISTRIBUTION OF RIBOSOMAL AGGREGATES IN POLYRIBOSOME PELLETS EXTRACTED FROM SPLEENS OF 9 RATS KILLED 3 DAYS AFTER ANTIGEN INJECTION

| | Percent of Total Polyribosomes Present as | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Rat | n=1 | n=1½ | n=2+1½* | n=3 | n=4 | n=5 | n=6 | n=>5* | Spleen Weight (g.) |
| 1 | 11 | - | 25 | 12 | 10 | 9 | 9 | 33 | 2.43 |
| 2 | 17 | 5 | 38 | 16 | 11 | 8 | - | 10 | 1.60 |
| 3 | 14 | 6 | 33 | 15 | 13 | 9 | 6 | 16 | 1.22 |
| 4 | 11 | - | 26 | 13 | 12 | 11 | - | 27 | 0.78 |
| 5 | 11 | - | 28 | 10 | 10 | 9 | 9 | 32 | 1.29 |
| 6 | 12 | 5 | 35 | 12 | 10 | 8 | 6 | 23 | 0.77 |
| 7 | 14 | 4 | 34 | 15 | 11 | 9 | 4 | 17 | 1.34 |
| 8 | 16 | 3 | 34 | 17 | 12 | 7 | - | 14 | 2.55 |
| 9 | 17 | 4 | 33 | 12 | 11 | 9 | 5 | 18 | 1.50 |
| Mean ± S.D.* | 13.7 ± 2.6 | 4.5 ± 1.1 | 31.8 ± 4.4 | 13.6 ± 2.3 | 11.1 ± 1.1 | 8.8 ± 1.1 | 6.5 ± 2.1 | 21.1 ± 8.1 | 1.51 ± 0.6 |

* See Table 3 : 3a

TABLE 3 : 3c : DISTRIBUTION OF RIBOSOMAL AGGREGATES IN POLYRIBOSOME PELLETS EXTRACTED FROM SPLEENS OF 15 RATS KILLED 6 DAYS AFTER ANTIGEN INJECTION

| | Percent of Total Polyribosomes Present as | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Rat | n=1 | n=1½ | n=2+1½* | n=3 | n=4 | n=5 | n=6 | n>5* | Spleen Weight (g.) |
| 1 | 18 | 5 | 36 | 9 | 7 | 7 | 6 | 23 | 0.80 |
| 2 | 17 | 5 | 35 | 16 | 13 | 8 | - | 11 | 0.99 |
| 3 | 14 | 5 | 30 | 13 | 12 | 10 | 6 | 21 | 1.12 |
| 4 | 12 | 4 | 28 | 12 | 11 | 9 | 6 | 28 | 2.26 |
| 5 | 18 | 8 | 37 | 10 | 9 | 7 | 5 | 18 | 1.71 |
| 6 | 14 | 4 | 32 | 14 | 13 | 8 | - | 18 | 0.89 |
| 7 | 13 | 4 | 27 | 12 | 11 | 10 | 8 | 27 | 2.43 |
| 8 | 15 | - | 35 | 15 | 13 | 9 | 6 | 12 | 0.76 |
| 9 | 14 | 5 | 30 | 9 | 9 | 9 | 8 | 29 | 1.59 |
| 10 | 14 | - | 30 | 14 | 13 | 10 | 6 | 19 | 1.55 |
| 11 | 18 | - | 31 | 18 | 11 | 6 | - | 16 | 1.55 |
| 12 | 14 | 5 | 34 | 12 | 11 | 9 | 6 | 21 | 1.33 |
| 13 | 15 | 6 | 35 | 10 | 9 | 8 | 7 | 23 | 2.20 |
| 14 | 16 | 5 | 38 | 15 | 8 | 6 | - | 17 | 1.29 |
| 15 | 13 | 4 | 32 | 13 | 10 | 8 | 7 | 24 | 2.08 |
| Mean ± S.D.* | 15.0 ± 2.0 | 5.0 ± 1.1 | 32.7 ± 3.3 | 12.8 ± 2.6 | 10.7 ± 2.0 | 8.3 ± 1.3 | 6.5 ± 0.9 | 20.5 ± 5.4 | 1.50 ± 0.5 |

* See Table 3 : 3a

DISCUSSION

The patterns obtained on analysis of rat liver polyribosomes compared closely with those obtained by other workers in this department (Fleck, Shepherd and Munro, 1965; Wunner, Bell and Munro, 1966). The resolution of monomer to hexamer material was slightly enhanced. This may be due to the fact that conditions for the density gradient step varied (30 mins., 1°, 200,000 g used here as against 60 min., 1°, 118,000 g) or to variation in the methods employed in analysis of the gradient. The flow-cell used in these investigations involved minimal inversion of the gradient whereas in the conventional flow-cell the gradient is completely inverted and has to pass through a U-turn before being monitored.

With the rabbit spleen tissue, the preponderance of monomers and dimers (Fig. 3 : 4) may indicate more extensive ribonuclease activity than occurred in rat spleen. One possible reason for this is that there was a longer time interval between the death of the rabbit and removal of the spleen than was required with the rat. Wettstein *et al.* (1963) point out that temperature control of the tissue involved in these procedures is critical. Alternatively, it is possible that these rats, which were reared in

communal cages until experimentation began were actively responding to infection of one kind or another and are not true "normal" controls - that is, that the pattern obtained from rabbit spleen is more normal for non-antibody forming lymphoid tissue. This possibility is further considered in the Section dealing with the effects of antigen injection on the polyribosome patterns.

Resolution of rabbit spleen polyribosomes compares with that obtained by Voss et al.(1965) using "immune" rabbit lymph node tissue, by Talal (1966) using normal rabbit spleen and by Bagdasarian, Bagdasarian and Borecka (1966) using spleen cells from hyperimmune rabbits. So far as is known, there are no reports in the literature showing significantly better resolution of rabbit spleen polyribosomes or a greater proportion of heavy polyribosomes in this tissue. Askonas and Williamson (1966) and Williamson and Askonas (1967) have concluded that the light and heavy chains of IgG antibody would require polyribosomes containing around 7 and 15 ribosomes respectively (on the assumption that 5 ribosomes can be accommodated on a mRNA of 450 nucleotides as was found in the case of haemoglobin synthesis - Warner, Knopf and Rich, 1963). This also assumes that the chains are synthesized complete and not in fragments as is postulated by La Via et al. (1966).

Thus the situation in rabbit spleen clearly requires further investigation since aggregates of 7-15 ribosomes have not been resolved in this tissue. Improved resolution would greatly facilitate studies on the ribosomal aggregates involved in antibody production.

The resolution of rat spleen polyribosomes and the recovery of heavy polyribosomes is considerably better than that reported by other authors (Talal, 1966; La Via et al., 1966) although La Via, Hammond, Vatter and Northup (1967) have some evidence that aggregates containing up to 12 ribosomes are present in rat spleens. Without further studies, either by electron microscopy or by determination of sedimentation coefficients it would be impossible to estimate the maximal polyribosomal size obtained in the investigations reported here.

Under controlled conditions, ribonuclease disaggregates polyribosomes to monomers by attacking the linking mRNA molecule but does not degrade the ribosomes themselves to any great extent. The conditions used here were similar to those employed by Talal (1966) and the results confirm that the first major peak in the polyribosome patterns is monomer material (Fig. 3 : 6). The labelling of the other peaks as dimer, trimer and so on is based on the

observations of Wettstein et al. (1963) and would also require independent confirmation for this system. The ribonuclease sensitivity of the polyribosomes is in contrast to the findings of Manner and Gould (1965). These authors, however, later suggested that only a proportion of the polyribosomes is ribonuclease resistant (Manner, Gould and Slayter, 1965).

The haemolysin response in rats has been well characterized by a number of authors. Haemolysin titres in normal rats begin to rise 1-3 days after the intravenous injection of SRBCs and is maximal 5-6 days after injection (Šljivić, Simić and Petković, 1960; Fitch, Wissler, La Via and Barker, 1956). In the spleen, which produces most of the circulating antibody (Rowley, 1950), mitotic activity is maximal round about the third day of the primary response (Wissler et al., 1960).

With the rats used here, spleen polyribosomes could readily be extracted, in contrast to the observations of La Via et al. (1966). However, the distribution of ribosomal aggregates in the spleen polyribosome populations was not significantly different using normal rats or rats killed 3 or 6 days after antigen injection. In view of the cellular response in the spleen and the increased

production of circulating gamma globulin provoked by antigen injection, absence of any effect on polyribosome size was surprising. It has been shown by electron microscopy that in the differentiation of antibody-forming cells from precursor cells there is a considerable increase in the quantity of heavy polyribosomes (De Petris and Karlsbad, 1965).

One possible reason why this was not detected here is that the control rats may not have been true controls. Two observations suggest this to be the case:

(i) The average spleen weight (1.57 g. - Table 3 : 3a) was much greater than the value reported for normal rat spleens by other authors (0.81 g. for rats weighing 400 g. - Jandl et al., 1965; 0.71 g. for rats weighing 530 g. - Peters, 1967).

(ii) The increase in spleen weight which is characteristic of the primary haemolysin response (Wissler et al., 1960) was absent here.

It is suggested that these rats, which had all been raised in communal cages, were infected to one degree or another. This would account for the variation in spleen weight and polyribosome patterns in the control animals, and would also mask any response occurring in these

parameters in animals injected with antigen. Again this problem could only be completely overcome by using animals reared in germ-free conditions.

From this work it was concluded that polyribosomes were present in considerable quantity in the rat spleens used and in less quantity in the rabbit spleens. Extraction of these could be carried out readily, and the proportion of heavy polyribosomes recovered from rat spleens varied according to the extent of dilution of the tissue during extraction, but was of the order of 20% of the polyribosome population of the tissue. Antigen injection appeared to result in no alteration in the proportion of heavy polyribosomes but for reasons discussed it was considered that this may have been an artifact.

# G E N E R A L  D I S C U S S I O N

Since the results of the 3 Sections of this thesis have already been discussed in some detail, it will be the purpose of this Discussion to relate the observations to each other and to some other relevant aspects of antibody formation. Discussion will concern events following antigen injection into normal animals (Primary Response), previously immunized animals (Secondary Response) and tolerant animals (Tolerance).

PRIMARY RESPONSE

It has been postulated (Section 2) that 12 hours after a primary intravenous injection of an antigen into an animal proliferation of macrophages is occurring in the spleen. Since such proliferation occurs in animals injected with heated autologous erythrocytes (Jandl *et al.*, 1965), it may be quite unrelated to the immune response. Nossal (1967) points out that the anatomical relationship between macrophages and lymphocytes in lymphoid tissue precludes easy transfer of material from one cell type to the other and he does not favour the view that antigen ingestion by macrophages is a necessary preliminary to the immune response.

However, since macrophages are specialized cells involved in the ingestion of foreign material and of dying cells, it seems probable that they have some specific surface sites which recognize "foreignness." That this is the case is suggested by the observations of Nossal and Ada (1964) that macrophages from immune and tolerant animals have a greater ability to recognize and ingest the specific antigen than do macrophages from normal animals.

Thus the interpretation placed on the observation that 12 hours after antigen injection into normal animals there is an increase in splenic DNA polymerase activity (Table 2:3) is that proliferation of macrophages is occurring resulting in a population of macrophages able to ingest and catabolize the antigen.

An alternative explanation is that the increase in polymerase activity at this time reflects DNA synthesis in cells whose progeny will synthesize IgM antibody. There is evidence that these cells are distinct from the cells involved in production of IgG antibody (see for example Vazquez and Makinodan, 1966). Since IgM antibody is not formed in tolerant animals nor to any great extent in the secondary response, then this would account for the absence of the 12-hour increase in DNA polymerase in these cases (Table 2 : 3).

However, 2 observations made at 24 hours support the first explanation given above. Firstly, the enzyme activity had returned to normal (Fig. 2 : 1) which would be unlikely if continued proliferation of cells involved in synthesis of IgM antibody were the explanation. Secondly, as indicated in Table 1 : 1, elimination of antigen over the first 24 hours of the primary response was lower than over the first 24 hours of the secondary response, but the elimination rate was greater thereafter.

Nossal and Mäkelä (1962b) have presented evidence that the cells which produce antibody during the primary response were engaged in DNA synthesis prior to administration of antigen. It has also been suggested (Ford and Gowans, 1967) that lymphocytes which were present in the circulation at the time of antigen administration, are the cells which become involved in mediating the immune response in the spleen. Thus the possible decrease in DNA polymerase activity in the appendix 12 hours after antigen injection (Table 2 : 3) was interpreted to mean that by this time cells relatively rich in the enzyme had left the appendix and had localized in the spleen.

Consistent with the findings that 12 hours after primary antigen administration, DNA polymerase activity had

increased in the spleen but not in the appendix (Table 2 : 3) are the observations of Knight and Ling (1968). These workers reported that antigenic stimulation of spleen cells in vitro resulted in a marked increase in DNA synthesis during the first 24 hours of culture. Using cells from other sources, including appendix, this early increase was not usually observed.

The absence of any detectable increase in splenic DNA polymerase activity 1 and 2 days after primary antigen injection (Fig. 2 : 1) is of interest. The lag period before lymphoid cell proliferation begins may reflect the time taken for trapping and "processing" of the antigen in macrophages (or reticular cells) and then entry of antigen into the specific committed lymphocytes. (Evidence has been presented that antigen is present in some antibody-forming cells - see Nossal, 1967). In lymphocytes, time would be required for initiating synthesis of nucleoside triphosphates and other precursors necessary for the cellular response to take place. In this connection it is possible that RNA-antigen complexes extracted from macrophages are "immunogenic" when transferred to lymphoid cells only by virtue of the fact that the antigen has already been degraded in the macrophages. The RNA may

serve simply to give bulk to the antigenic determinant permitting its phagocytosis. That the macrophage RNA has a purely adjuvant function in these systems is suggested by the experiments of Askonas and Rhodes (1965) and Gottlieb, Glišen and Doty (1967). These latter workers found that while most of the macrophage RNA had incorporated tritiated uridine during a 30 min. incubation of macrophages with antigen, the "immunogenic" RNA contained very little label. Further, in hybridization studies, "non-immune" RNA and "immune" RNA extracted from macrophages infected with $T_2$ bacteriophage or an unrelated antigen, all competed for the DNA of the $T_2$ infected macrophages. Thus it appeared that the "immunogenic" RNA was not formed in response to antigenic stimulus and was not informational.

By the third day of the primary response, the cellular reaction in the spleen is considerable (see, for example, Langevoort, 1963) and between then and the eighth day increased DNA polymerase activity was found (Fig. 2 : 1). The timing of this increase was consistent with reports of DNA synthesis and cell division by other authors (see Section 2). The order of the increase was greater both here and in the secondary response than would have been predicted on the basis of the 2-fold reduction in the mean

generation time of the cells stimulated by antigen, which was noted by Urso and Makinodan (1963). For this reason it is considered that the increase in polymerase activity may be interpreted to mean that episomal replication of a gene coding for antibody structure occurs in the stimulated cells. As already noted, Smithies (1965) has pointed out that a process of this nature may be necessary to explain the rapidity of the primary response and he has involved this concept as part of a theory of antibody formation. The observations reported here may constitute some evidence for this concept of episomal replication.

By the seventh to eighth day of the primary response in rabbits, antibody was released into the serum as indicated by the antigen elimination tests (Fig. 1 : 2). The lag period between initiation of the cellular response and the release of antibody is a measure of the time required for the transformation of the stimulated cells to immunoblasts and their subsequent proliferation and differentiation to immature and mature plasma cells. As de Petris and Karlsbad (1965) have shown, this process involves considerable morphological changes in the cells involved including the development of endoplasmic reticulum, Golgi complex and the formation of polyribosomes.

Presumably also many enzymes (for example, amino acid activating enzymes) must be formed in increased amounts before antibody synthesis occurs.

While the polyribosome studies on rabbit and rat spleens were inconclusive, it was nevertheless shown that ribosomal aggregates could be extracted from lymphoid tissue (Section 3). Understanding of the role of polyribosomes in gamma globulin production has been considerably furthered by the studies of Shapiro, Scharff, Maizel and Uhr (1966) and independently by Williamson and Askonas (1967). These authors have presented evidence that complete light and heavy chains are synthesized respectively on 180S polyribosomes (aggregates of around 5-6 ribosomes) and on 270-300S polyribosomes (aggregates of around 12-18 ribosomes). They further suggest that free light chains are autonomously released from the ribosomes and that these may assist and control heavy chain release. This would result in balanced production of gamma globulin.

Eight weeks after primary administration of antigen, serum antibody could still be detected in the rabbits (Tables 1 : 2 and 1 : 3). Nossal (1967) considers that antibody production continues in the absence of antigen, which argues against a direct template role for antigen in antibody formation, but this is disputed by Haurowitz (1965)

who considers that antigenic determinants may remain in the antibody forming cells in minute amounts.

SECONDARY RESPONSE

The decrease in the specific activity of DNA polymerase in the appendix 12 hours after a second injection of antigen (Table 2 : 3) is interpreted to mean that, as in the primary response, cells relatively rich in the enzyme leave the appendix and localize in the spleen. It is inferred from the absence of any concomitant increase in polymerase activity in the spleen at this time that no macrophage proliferation is occurring and that an increase in localization of antigen and in formation of other non-polymerase protein in the tissue masks any increase in DNA polymerase which may be present in the lymphoid cell population. Enhanced uptake of antigen is supported by the observations of Nossal and Ada (1964) and by the fact that elimination of antigen (in this case PGG) was greater at 24 hours in the secondary response than in the primary response (Table 1 : 1).

No increase in DNA polymerase activity was evident at 24 hours and it is again considered that the lag period before lymphoid cell proliferation begins may be related to the time taken for catabolism of the antigen. Apart

from the 12-hour increase in the primary response the detectable increase in splenic DNA polymerase was earlier in the secondary response (beginning at 2 days - Fig. 2 : 1) and this may reflect the involvement of a greater number of cells. It is also known from histologic evidence (see, for example, Movat and Fernando, 1965) that the cellular reaction does begin earlier in the secondary response. If it is established that "processing" of antigens in one cell type is a necessary preliminary to antibody formation, then the greater ability of macrophages to ingest antigen in the secondary response may explain the earlier initiation of the cellular reaction. If macrophages (or reticular cells) are not involved, then it seems necessary to postulate that the "memory" cells (small lymphocytes?) also have a greater capacity to recognize, ingest and catabolize the specific antigen. It is also possible that the presence of antibody in the serum would result in a more rapid immune response by complexing with the injected antigen and rendering it more readily phagocytosed.

By the third day of the secondary response, antibody has been released into the serum (Section 2 and Dixon et al., 1954) and in these studies free serum antibody was detected in 1 rabbit at this time (Table 1 : 3). In the spleen,

DNA polymerase activity has increased over control values to a level which is again consistent with the suggestion that episomal replication of a gene coding for antibody structure is occurring. Activity in the appendix is not significantly different from control values (Table 2 : 3) suggesting that any role this tissue has in the secondary response is complete by this time.

By the sixth day, free serum antibody was detected in all cases (Table 1 : 3) and DNA polymerase activity was still higher than control values in the spleen. From the studies of Bauer et al. (1963) it is known that in the secondary response only small amounts of IgM antibody are formed and that most of the antibody is of the IgG class. Uhr (1964) has shown that IgM synthesis ceases in the absence of antigen so it is possible that the kinetics and characteristics of IgM antibody formation are identical in both primary and secondary responses and that the earlier production of IgG antibody in the latter case results in the removal of antigen and the cessation of IgM production. That the IgM-synthesizing system does not develop immunological "memory" has been suggested by Vazquez and Makinodan (1966).

TOLERANCE

As determined by the absence of immune elimination of antigen (Fig. 1 : 2) and by the absence of circulating antibody, the injection of a large amount of BSA into a rabbit at birth results in paralysis of the antibody-forming mechanism in the mature animal (Section 1).

However, tolerance (or immune paralysis) can be induced in other ways:

(a) administration of very large quantities of antigen to the adult animal either over a prolonged time interval or in one injection (see Smith, 1961);

(b) administration of small quantities of antigen either filtered (Frei et al., 1965) or ultra-centrifuged (Dresser, 1962) to remove aggregated material.

Using BSA as antigen and mice as experimental animals, Mitchison (1964) has further developed this concept of 2 zones of paralysis. Mice were immunized against fluid BSA - 3 intraperitoneal injections per week for varying time intervals using a range of doses of the immunizing antigen. The ability of such mice to give an immune response to BSA in Freund's Adjuvant was then determined. Paralysis was

induced to varying degrees using low doses of antigen administered up to approximately 8 weeks (depending on actual antigen dose) and using high doses administered for more than 2 weeks (again depending on antigen dose). The pattern emerging from these studies suggested that any dose of antigen could eventually paralyse were it not for the limit set by the life-span of the mouse.

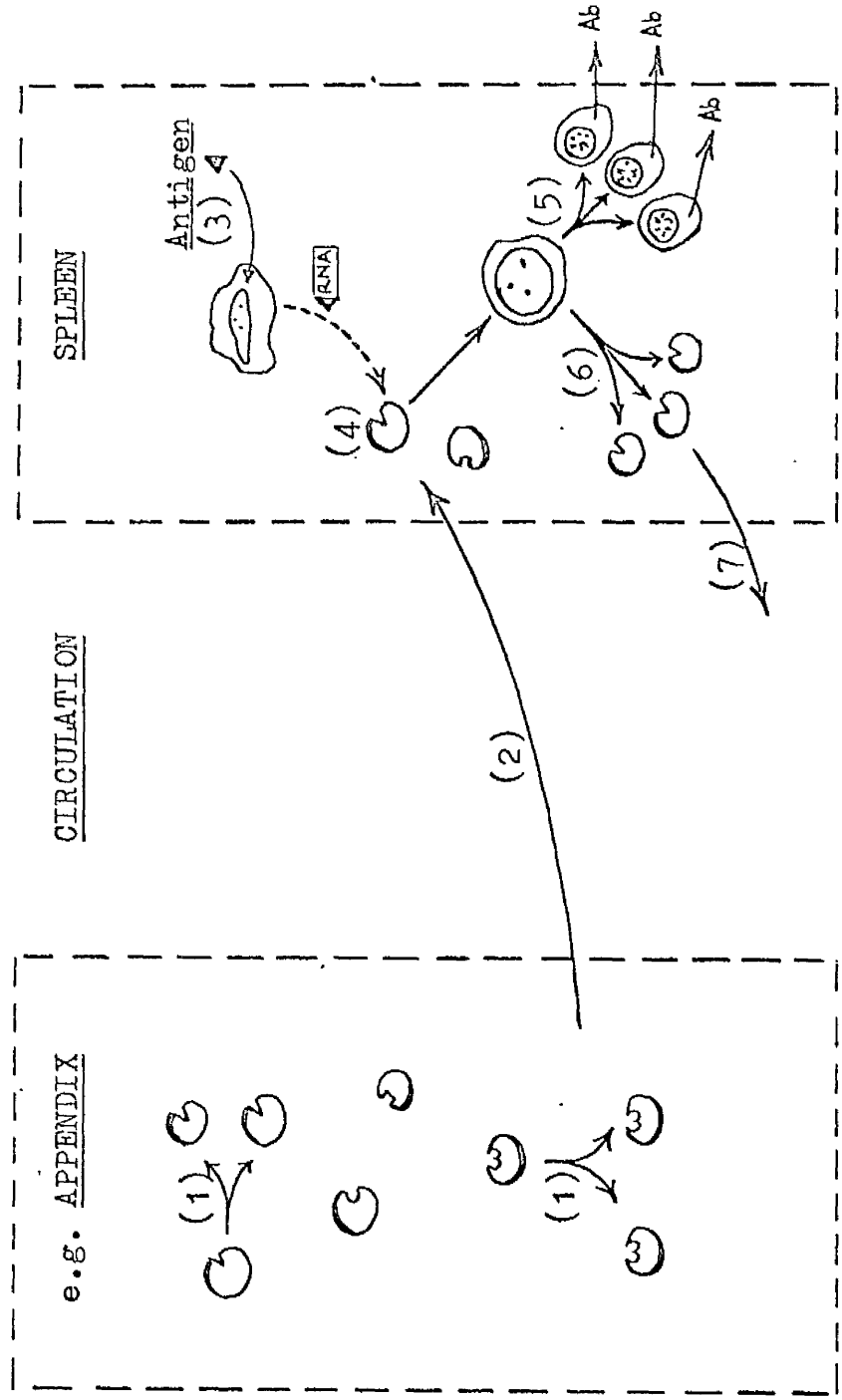
Concerning the cellular aspects of tolerance, the suggestion of Frei et al. (1965) and Mitchison (1967) that antigens reaching lymphocytes intact (without "processing" in macrophages) induces tolerance, may relate to the observations of McGregor et al. (1967) that tolerant cells do exist (i.e. that tolerance is not simply a result of absence of committed cells). Mitchison (1964) has suggested that even 1 molecule of antigen interacting with a cell may be sufficient to induce paralysis.

It is clearly necessary to gain more information on the intracellular events which follow encounter of antigen with a potential antibody-forming cell. Under some circumstances immunity results and under other circumstances there is paralysis. Thus, the presence of a sharp fall in the specific activity of splenic DNA polymerase in tolerant

animals was of considerable interest - especially since this depression occurred mainly at those times after antigen administration when splenic polymerase activity in non-tolerant animals had increased (Figs. 2 : 2a and 2b, cf. Figs. 2 : 1a and 1b). Absence of DNA polymerase from tolerant cells would make little difference to the specific activity of the enzyme extracted from the whole tissue since these committed cells represent only a small proportion of the total lymphoid cells of the spleen. Thus a decrease in enzyme activity may imply that some inhibitor of the enzyme is present in sufficient concentration as to affect the measured specific activity of the enzyme extracted from the whole tissue. Thus, it is considered that the tolerant state may result from formation of some inhibitor of DNA polymerase when antigen encounters the cell specifically committed to it. On the other hand, antigen which has first been "processed" may present a different stimulus resulting in cell proliferation and differentiation.

In conclusion, the scheme shown in Fig. 3 is presented as a hypothesis concerning some of the cellular aspects of antibody formation. This is an elaboration of the scheme shown in Fig. 1 in the Introduction.

FIG. 3. POSSIBLE INTER-RELATIONSHIPS AMONG THE CELLS INVOLVED IN THE IMMUNE RESPONSE



(1) Conditioned lymphocytes replicate in the appendix and presumably other lymphoid tissues.

(2) These cells re-circulate via the blood stream through the spleen.

(3) When antigen is given to normal animals it is ingested by macrophages (or reticular cells) and "immunogenic" RNA-antigen complexes are formed. The RNA may be informational (Adler et al., 1966) or may not be informational (Askonas and Rhodes, 1965; Gottlieb et al., 1967).

(4) Lymphocytes have specific surface recognition sites (Cohen, 1967).

(5) After ingestion of "processed" antigen, cell proliferation and episomal replication of the genes coding for antibody structure takes place - resulting in differentiated antibody-producing plasma cells.

(6) In some cases, differentiation results in cells which appear morphologically as small lymphocytes. These are "memory cells."

(7) These cells re-enter the circulation and can engage in the cellular response on further contact with antigen derived material.

(8) Antigen encountering conditioned lymphocytes without processing results in immunological tolerance (Frei et al., 1965; Mitchison, 1967) perhaps by initiating formation of an inhibitor of DNA polymerase.

The more fundamental question of the origin of diversity of antibodies is, of course, not considered in the above scheme. As information accumulates concerning the modifications in amino acid sequences in different antibody molecules then this will permit fuller understanding of the differences in the genes coding for these molecules, and the eventual formulation of a theory which satisfactorily explains the origin of antibody diversity.

# SUMMARY

The work presented in this thesis was directed towards developing a clearer understanding of some of the biochemical aspects of antibody production. The systems employed were rabbit-anti-bovine serum albumin (BSA) for studies on the specific activity of DNA polymerase, and rat-anti-sheep red blood cells (SRBCs) for polyribosome studies.

Some rabbits were rendered tolerant to BSA by the intraperitoneal injection of this protein at birth. The establishment of tolerance was confirmed by antigen elimination tests in the mature animal. Comparative studies were made on the specific activity of DNA polymerase from the spleens and appendices of normal untreated rabbits, rabbits undergoing a primary or secondary response to BSA and rabbits previously rendered tolerant to BSA.

The results obtained indicated that in the case of the splenic polymerase activity was increased by the immunization procedures. Increased activity was observed on days $\frac{1}{2}$, 3, 4, 6 and 8 of the primary response and on days 2, 3, 4, 6 and 8 of the secondary response. In the case of the animals rendered tolerant to BSA enzyme activity was

depressed 3, 4 and 6 days after one injection of this protein into the adult animal and 1, 4 and 6 days after a second injection. The significance of these results and their possible relationship to lack of macrophage proliferation in the secondary response and in tolerant animals was discussed.

Results from comparable studies in the appendix showed no consistent pattern but there appeared to be a decrease in the specific activity of the enzyme at various times after antigen injection. A possible role of the appendix in the immune response was considered on the basis of these results.

In view of this antigen stimulated cellular response in the spleen, studies were made on the polyribosome patterns of the spleens of control and immunized animals. A procedure was developed whereby polyribosomes could be extracted and analysed from rat spleens. No consistent differences between the polyribosomes of control and immunized rats were observed. Possible reasons for this inconsistency were discussed.

# A P P E N D I X

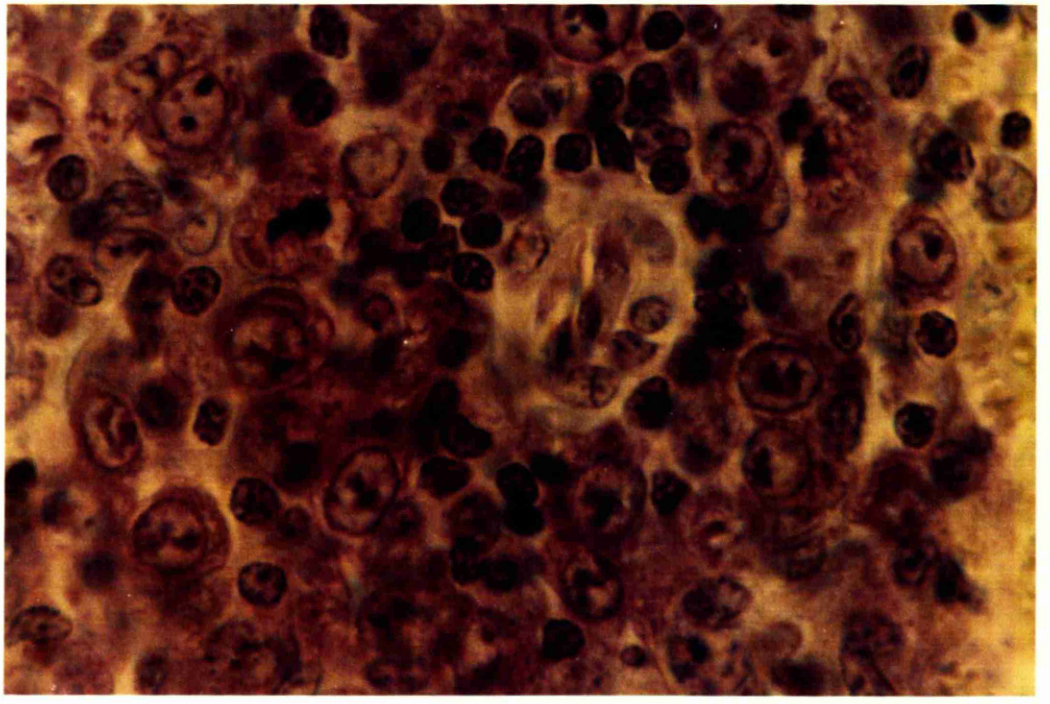


PLATE 1. Immunoblast cells in periarteriolar sheath of lymphocytes in Splenic White Pulp.
(x 500; Stain - methyl-green, thionine).

Rabbit spleen 2 days after 2nd injection of BSA.

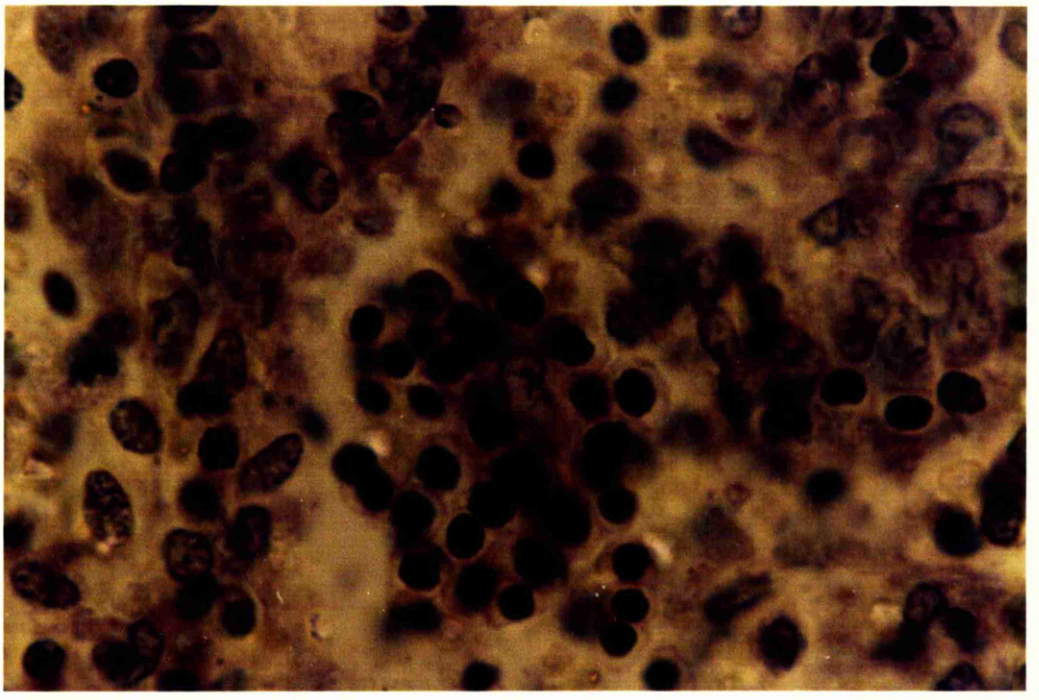


PLATE 2. Concentration of darkly staining lymphocytes surrounding larger pale staining macrophage. (x 500; Stain - methyl-green, thionine).

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