BIOCHEMICAL STUDIES ON CELLS INVOLVED IN IMMUNE RESPONSES

BY

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C O N T E N T S

GENERAL INTRODUCTION

One of the first observations on the phenomenon of immunity was recorded in 500 B.C., when Thucydides noted that only people who recovered from a previous epidemic were able to tend the afflicted without fear of re-infection during subsequent epidemics. Through succeeding centuries this observation was repeated with different plagues until the end of the eighteenth century when Jenner (1798) showed that individuals infected with cowpox were not susceptible to infection on subsequent exposure to smallpox. Nearly a hundred years were to elapse before the next significant advance was made, when Pasteur (1880a) injected fowl with an old culture of Pasteurella ovisoptica. The fowl failed to manifest symptoms of chicken cholera. When he followed this by injection of a fresh culture, the fowl still failed to become ill. Pasteur then extended this to make the observation that aged cultures, which had lost their virulence, retained their capacity to induce immunity.

There were different ways of explaining these observations. Pasteur (1880b) himself thought that the immunising organism consumed some nutrient factor which would be necessary if subsequent doses were to prove infective. On the other hand, Chauveau (1880) preferred to think that the micro-organism left behind something which prevented further infection of the animal by the same organism. Pasteur's work with bacteria was extended by Von Behring and Kitasato (1890) who showed that bacterial filtrates containing toxin could be used to cause immunity. Von Behring and Kitasato concluded that immune sera exerted its protective action by combination of specific components with the toxin. This observation seemed to explain why drawn blood appeared to be more resistant to putrefaction than other organic substances. Buchner (1894) proposed that toxins were modified and incorporated into antitoxins. However, when it was shown that antitoxins greatly exceeded toxins in the plasma, this theory was rejected. At the same time, Metchnikoff (1901) thought that toxins were phagocytosed by white blood cells.

In 1900, Ehrlich forwarded his "side-chain" theory which considered cells to be fitted with side-chains by which food molecules could be ingested. These could also function as agents for attack on toxins introduced into circulation. When combination occurred, the regeneration of these cells, which were responsible for these particular side-chains, took place, and an overproduction ensued, leading to a release of these side-chains into plasma, thus giving rise to humoral antitoxin. This theory held for thirty years and explained many of the features of the immune response. However, in 1930, Landsteiner demonstrated antibody formation to artificial antigens (combination between carrier proteins and small chemical groups known as haptens). Ehrlich's side-chain theory fell into discredit as it became obvious that the number of possible antibodies was too great to be considered as a side-chain on a cell.

About this time it was also noted that antibody activity was associated with the gamma-globulin fraction of serum. This observation provided the background to theories which considered that antibody synthesis was nothing more than a

modification of normal gamma-globulin synthesis. Breinl and Haurowitz (1930) and, independently, Alexander (1931) and Mudd (1932) proposed that the antigenic portions of foreign molecules in some way interfered with the incorporation of amino-acids into normal gamma-globulins, such that the "modified" gamma-globulin could now be specific for the particular antigen. In the view of these authors, the polar side groups of the antigen would give a particular orientation of amino-acids in their vicinity and maintain them in this position throughout protein synthesis.

Pauling (1940), while still in favour of the idea that antigen fashioned somehow the gamma-globulin molecule to become specific antibody, rejected the idea of interference by antigen with gamma-globulin amino-acid sequence but rather he proposed that the normal gamma-globulin molecule was able to form a wide variety of intramolecular bondings and foldings. Only when the antigen was present, was a stable conformation of the molecule adopted which would be complementary to that particular antigen. This proposal, unlike those of the early 1930s, inferred that difference in antibody activity did not

reside in variation in amino-acid sequence of the gamma-globulins.

Pauling's theory became untenable when it was shown by Koshland and Engleberger (1963) that two antibodies of different amino-acid sequence were formed in a rabbit after a single injection of antigen and also by Buckley et al, (1963) that denatured anti-BSA from rabbit serum regains its antibody activity after the removal of the denaturing guanidine salt by dialysis and in the absence of the antigen.

The above are described as Template Theories, where antigen acts as a template for gamma-globulin synthesis, but they possess a number of inherent disadvantages. Firstly, it is implied by these theories that antigen must be present while antibody is being synthesised but more recently (Nossal, 1967) it has been noted that antibody production continues in the absence of detectable antigen. Moreover, these theories fail to explain the differences between the primary and secondary response nor can they account for the phenomenon of tolerance. In 1955, Jerne proposed a theory which relied for its inspiration on Ehrlich's "side-chain theory" of more than half a century

previously. He thought that a wide variety of "natural antibody" was normally present in trace amounts in normal serum. When antigen was introduced, it then selected and combined with its specific antibody. This complex would then be taken up by macrophages which then led to production of more antibody of the same specificity.

Two years previously, in 1953, Billingham et al. described the concept of "tolerance" and in particular with regard to the neonate. The fact that the gamma-globulin fraction appears not to contain antibodies against structural components of the body leads to the concept of the particular gamma-globulin or the cell responsible for its production being eliminated at birth. These "responsible" cells were termed clones by Burnet (1957) who proposed his "Clonal Selection Theory". He postulated a population of cells called clones, capable of producing antibody to a very wide range of antigen. Incoming antigen would then select a particular clone and initiate specific antibody production. Moreover, clones which contained the potential to react with "self" could be destroyed in embryonic life, so resulting in tolerance. Neonatal

tolerance could be explained in the same way, i.e. immature clones presented with antigen to which they had the capacity to react would also be destroyed. At the beginning of the 1960s, therefore, there were broadly speaking two ways of considering the riddle of specific antibody formation. Firstly, there were the instructive theories of Haurowitz, Mudd and Alexander, and secondly, the selective theories of Jerne and Burnet. Irrespective of which theory is propounded, there are many experimental observations which must be taken into consideration.

It has been know for some time that most proteins are capable of eliciting a specific antibody response, i.e. they are antigenic, if they are injected into a foreign species. This capability is not confined to proteins, as some polysaccharides, and occasional nucleic acids, can elicit antibody production. What distinguishes the antigenic from the non-antigenic is not clear. Some pointers were provided by the work of Landsteiner (1946), who replaced hydrogen atoms on the aromatic and imidazole rings of tyrosine and histidine residues respectively, of proteins with aromatic diazonium

radicles to give substituted residues on proteins of the formula x . Benz . N = N . y, where y is the tyrosine of protein and x an anionic residue, i.e. $-SO_3H$, -COOH. Using these compounds as antigens, he found that antibodies can distinguish between ortho-, meta- and para-substituents of the ring, and also between cis- and trans-isomers, or between d- and l-stereoisomers. Following from this, it was found that the groups which acted as determinant groups in proteins possessed certain common properties :

1. A rigidity of chemical structure, and
2. The presence of groups which could interact with the combining groups of the antibody by means of hydrogen bonds or other intermolecular forces.

Thus, those structural elements of the determinant group that project distally from the central mass of the immunising antigen seem to play a dominant role in determining the antibodies' specificity. Further studies conducted on the antigenicity of fatty acids, or polyamino-acids bound to gelatin, showed that a certain rigidity and polarity is a prerequisite for antigenicity. It thus seems that the antigenicity of natural proteins might be

due to the presence of certain amino acid sequences in the polypeptide chains. Another point to be taken into consideration is whether the substance is soluble or not. In general, the more soluble a substance, the less antigenic it is. This is explained by the theory that foreign material must first be ingested by macrophages before it becomes antigenic - the more particulate the substance, the more readily ingestible by macrophages. The fact that soluble proteins are antigenic at all is thought to be due to the presence of a small amount of aggregated protein, which can gain access to the macrophages more readily.

Much work has been carried out to determine the actual structure of antibodies and some results are relevant to a study of the mechanism of the immune response.

Antibodies belong to a class of proteins known as immunoglobulins which contain heavy and light chains. The light chains contain 214 amino acids and have a molecular weight of 20,000, while the heavy chains contain approximately twice as many amino acid residues and have a molecular weight of between 50,000 and 70,000. Five classes of immunoglobulins have been differentiated on the basis of their heavy

chains, all of which possess different antigenic determinants and display different structures. Two types of light chain, common to all classes, have been described (κ and λ) which have the same molecular weight but differing amino acid sequences (see Table 1).

Porter (1962) proposed that all antibody molecules are made up of a basic unit of four chains, e.g. in the case of IgG, there are four chains - two light and two heavy. The latter constitute the core of the molecule, and are linked by - disulphide bonds, while the light chains are on the flanks, one attached to each heavy chain (see Fig. 1) by a disulphide bond. It is suggested that IgM may be formed from five IgG units joined together by disulphide bonds, while IgA might be formed from two such units. Electron microscopy has revealed that IgG may be a $\curlyvee$ - shaped structure, as depicted in Fig. 1.

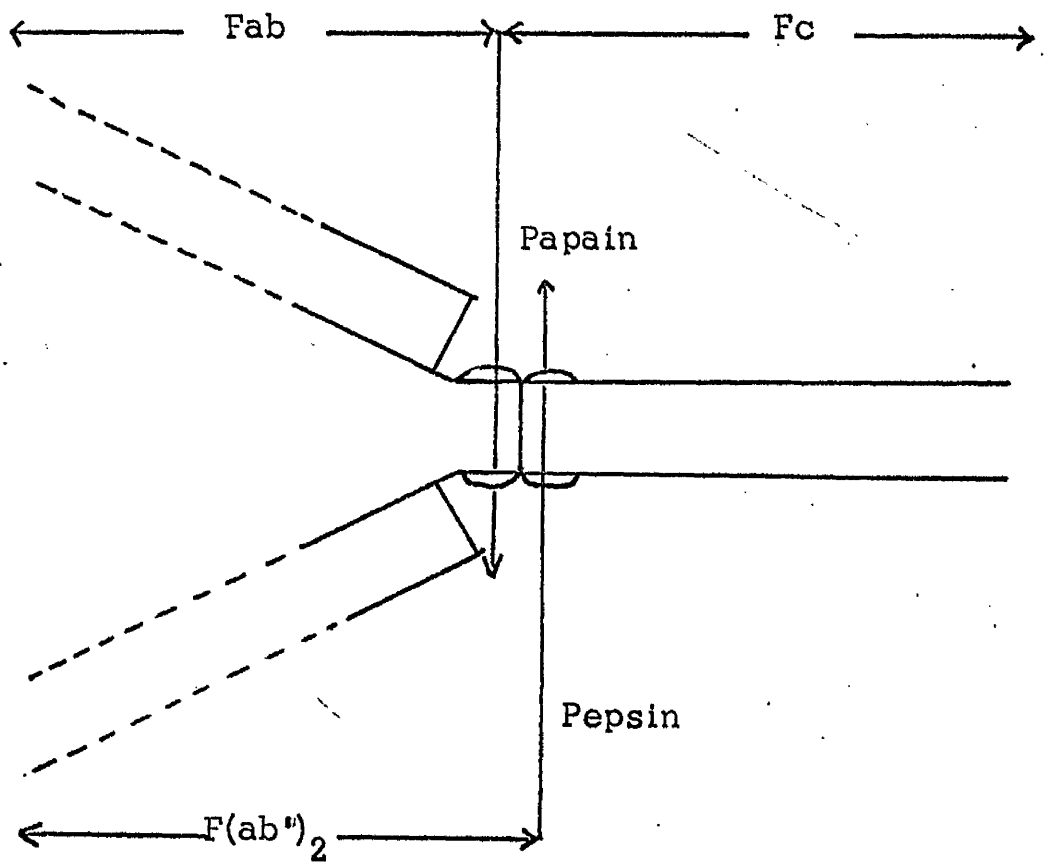
TABLE 1 : SOME PROPERTIES OF HUMAN GLOBULINS

Class	Sedimentation Coefficient	M.W.	CHO%	Heavy Chains	Light Chains
IgG	7	150,000	3	γ	κ and λ
IgM	19	900,000	12	μ	κ and λ
IgA		400,000	10	α	κ and λ
IgD				δ	κ and λ

An additional class (IgE) has been postulated to account for the reaginic antibodies which mediate various hypersensitivity responses in man.

Fig. 1 : DIAGRAMATIC REPRESENTATION OF THE 4-CHAIN UNIT STRUCTURE OF IMMUNOGLOBULINS

Chains are linked by disulphide bridges. Broken lines indicate the variable portions of the heavy and light chains. The undulating portion of the heavy chain represents the area susceptible to proteolytic digestion; papain and pepsin give rise to the fragments indicated. Light chains have a molecular weight of 20,000 and heavy chains a molecular weight of 50,000.



As Haber (1964) has indicated, purified antibodies against a single hapten antigen are a diverse population of molecules. Therefore it has proved impossible to determine the amino acid sequence of a pure antibody. However, protein chemists have been provided with relatively large quantities of Bence Jones proteins, which are recoverable from the urine of men and mice suffering from plasma cell tumours. Although no antibody activity has been attributed to these proteins, the work of Edelman and Gally (1962) has shown that their electrophoretic and antigenic behaviour is similar to that of light chains. On the basis of various peptide maps of such Bence Jones proteins, Putnam, Easley and Helling (1963) proposed that all Bence Jones proteins of the same antigenic type consist of a portion of fixed sequence and a portion of variable sequence. Subsequent work showed this to be a valid proposition.

Cohen and Milstein (1967) described the light chain as consisting of 214 amino acid residues, although variations in size as a result of insertions or deletions have been observed. Amino acids 1-107 (numbered from the amino end

of the chain) constitute what is described as the variable portion, while amino acids 108-214 constitute the constant portion. This suggested strongly that the antigen combining portion of the light chain resides in the variable half of the chain. Putnam, Titani and Whitley (1966), describing type K Bence Jones proteins, point out that not all the residues in the variant end of the chain are, in fact, variable. They describe proteins of chain length 214 amino acid residues and variation only occurred in the first 107 residues. Of these, 107 residues, making up the variable half of the chain, variation between chains is noted only in 40 residues. These variations appear to occur largely in clusters and it is probable that, in the tertiary structure of the gamma-globulin molecule, the variable residues of both heavy and light chains will be closely arranged in space. As there is an intrachain disulphide bond between amino acids 23 and 88, the residues situated near these half cystines will be near in space. In fact, considerable variation has been detected around both these half cystines.

Hydrolysis of IgG with papain yields a crystallisable fragment Fc and two identical non-crystallisable fragments Fab, which were found to contain the antigen combining site. The Fab fraction consists of amino terminal component of the heavy chain, as well as the light chain component. The former, designated Fd, is capable of binding to antigen *in vivo*, whereas the light chain does not exhibit this property. Nevertheless, the light chain appears to play some part in antibody antigen reactions as the binding capacity of heavy chain to antigen is increased in the presence of specific light chain. It is now generally thought that antibody specificity is caused by amino acid variability in the Fab fragment, while the Fc portion appears to be concerned with such physiological roles as complement fixation and transport across membranes, e.g. placenta.

Isotypic variants are described as being types of a light chain, within a given class of immunoglobulin, which differ antigenically from one another, e.g. k and λ chains. Allotypic variants occur between individuals of the same species, where light or heavy chains within the same class are antigenically different. Idiotypic variants arise where

different antibodies within an individual are antigenically different.

Some problems were presented by the actual biosynthesis of antibody by the protein synthesising machinery of the cells. Williamson and Askonas (1967), and Shapiro, Scharff, Maizel and Uhr (1966) showed that light chains were synthesised on 180 S polyribosomes, while heavy chains were synthesised on 270-300 S polyribosomes. In order that a balanced synthesis of light and heavy chain occur, it was suggested that light chains were released autonomously from the polyribosomes and these light chains controlled the rate of release of the heavy chains from the polyribosomes.

The actual site of antibody production has been investigated and it appears that most circulating antibody is synthesised by cells in the spleen and lymph nodes. Fagraeus (1948), using the fluorescent antibody technique, showed that the plasma cells of the spleen were responsible for production of antibody by this organ. Using basically the same technique, Leduc, Coons and Connolly (1955) showed that the lymph node was also involved. Depending on the route of antigen injection,

Askonas and Humphrey (1958) noted that tissues such as bone marrow, lung and liver could also produce antibody. Appendix and thymus cells have not yet been shown to produce antibody, but work over the last decade suggests a different role for the lymphoid cells in these organs. Although the thymus does not hypertrophy in response to antigen injection, as does tissue such as lymph node, it has become clear that this tissue bears a significant relation to other lymphoid tissues.

Miller (1961) has shown that thymectomy in the neonate mouse caused a reduction of the lymphocyte population and impaired immune responses in the adult animal. This suggests that the thymus is necessary for production of cells to populate other tissues. Osoba and Miller (1964) thymectomised neonates and placed the thymus in a millipore chamber in the peritoneal cavities of the animals. Their experiments showed normal development of lymphoid tissue. The fact that the capsule filter was of sufficiently small pore size to exclude the passage of cells suggested that the thymus could be responsible for the secretion of a hormone necessary for the normal development of lymphoid tissue. Thymectomy in adult

life does not lead to such a profound deficiency of competent cells but it has been shown to impair seriously the return to normal responsiveness of heavily X-irradiated animals (Cross, Leuchars and Miller, 1964), and of tolerant animals (Taylor, 1964).

More recent work has thrown fresh light on the role of the thymus in the development of immune capacity. Claman, Chaperon and Triplett (1966) injected irradiated mice with normal thymus or bone marrow cells. These animals were subsequently given antigen. The mice receiving both thymus and marrow cells displayed more areas of haemolysin activity in their spleens than mice receiving either cell type alone. It had been previously shown by Harris and Ford (1964) that some cells from the bone marrow reach lymphoid tissue after a period of residence in the thymus. Miller and Mitchell (1967) were thus able to deduce that the bone marrow precursor cell was dependent on thymus cells for its development to antigen reactive cells. In 1969, Miller and Mitchell showed that injection of viable thymus lymphocyte was essential to obtain a normal 19 S haemolysin cell response in the spleens of thymectomised neonates when challenged with sheep RBC.

Only antisera directed against host cells could significantly reduce the haemolysin forming cells present in the spleen. They suggested that the bone marrow cells of the host were the antibody forming cells. In a subsequent paper, Mitchell and Miller (1969) conducted chromosome analysis to confirm this suggestion.

Good and Cain (1970), commenting on the relationship between thymus dependent cells and humoral immunity, describe an antigen sensitive cell from the thymus which recognises the antigen, and an antigen insensitive cell from bone marrow which is capable of synthesising antibody. They propose that antibody on the surface of the antigen sensitive cells could act mechanically to enhance the antigenicity of the antigen for the insensitive antibody producing cell.

As reported above, Fagraeus (1948) found that the plasma cells of spleen were capable of producing antibody. Some authors, including Attardi et al. (1964), thought that the lymphocyte might also synthesise antibody but Fagraeus found that nearly all the cells of the spleen of immunised animals that formed antibody were plasma cells. Keuning and Van der

Silkke (1960) noted that larger immature plasma cells were far more active in synthesis of antibody than the mature plasma cell. This was also the conclusion of Thorbecke and Benacerraf (1962) who suggested that the plasma cell arose from the small lymphocyte which developed through the following steps :

Small lymphocyte —— large lymphocyte —— plasma cell.

Nossal and Makela (1962), on the basis of pulse labelling experiments using ^{3}H thymidine, found that all the antibody forming plasma cells were the result of recent mitotic division, and, on the basis of this observation, they postulated that, when small lymphocytes enlarged under antigenic stimulation, they divide unevenly to yield plasma cells and small lymphocytes. In 1962, Gowans et al. showed that in a graft versus host reaction, small lymphocytes enlarge into pyroninophilic cells which divide. Yoffey (1964), noting that some of the lymphocytes had a very long half life, considered them to be committed as they would already have received a primary stimulation. They would have arisen by asymetrical division of large pyroninophilic cells, as

postulated by Nossal and Makela (1962) and would remain static until aroused by secondary stimulation. Further support for this scheme came from the work of McGregor, McCullogh and Gowans (1967), who showed that, when rats were irradiated to block the haemolysin response to sheep red blood cells, the blockage could be overcome by the administration of small lymphocytes. Thus, small lymphocytes are found to be capable of responding to antigen.

Proof that the small lymphocyte is the carrier of immunological memory comes from the work of Gowans and Uhr (1966). They injected rats subcutaneously with 10^{8} PFU $\emptyset$ x 174 and a low primary response ensued. The thoracic ducts of a number of such primary rats were cannulated ten weeks after immunisation and the larger dividing lymphocytes removed. These were cultured for 24 hours at 37^{o}C. and the resulting small lymphocytes were injected into syngenic recipients 24 hours after they had received 500 Rads. of whole body irradiation. These recipients were then challenged with a single intravenous dose of 10^{10}PFU of $\emptyset$x174. A substantial secondary response resulted. Thus, the small

lymphocyte is the carrier of immunological memory.

The question of how many antigens a single lymphocyte is capable of responding to is still an open one and one which will be dealt with later. However, Attardi et al. (1964) has claimed that 20% of the antibody forming cells from a double immunised rabbit formed antibodies against more than one non-cross reacting antigen. On the other side, Dutton and Mishell (1967), producing a primary response to sheep and Burro erythrocytes in vitro, noted that different cell populations are involved in the response to two non-cross reacting antigens. Nossal et al. (1963) have reported that single cells formed antibody of more than one class - first IgM and then IgG, both with the same antigenic specificity.

The cell which many think to be the first to meet injected antigen is the macrophage - a large phagocytic cell. This cell occurs free in the circulation and also in the lymphoid organs. The fate of a particular antigen depends on its nature and on the method of administration. Benacerraf (1957) reported that liver macrophages seemed to play a large part in clearing large particulate matter. Thorbecke, Maurer and

Benacerraf (1960) reported that aggregated proteins are more readily phagocytosed by macrophages of the reticulo-endothelial system than native proteins.

Perkins and Leonard (1963) reported that macrophages are selective in their ability to recognise and phagocytose antigen. On the other hand, Rhodes and Lind (1960), treating preparations of macrophages to two non-cross reactive antigens, found that less than 1% of the cells contained only one antigen. In vitro experiments have indicated an important role for the macrophage in the immune response. Harris (1965) noted that the uptake of ^{14}C thymidine by spleen cell suspensions in culture from rabbits previously immunised to HSA is specifically stimulated by addition of spleen and peritoneal cells containing the same antigen. The macrophages containing antigen were more effective in this stimulation than other cells. He calculated that 5×10^6 macrophages incubated with 1000 µg HSA for 24 hours contained 0.13 µg. of this protein, yet 1×10^6 cells could stimulate to a significant degree and, if the same amount of antigen were added directly to spleen cells, there was little effect. It was thought that

the macrophage may select out a fraction of HSA which was more antigenic. Subsequently, Harris and Cramp (1968) pointed out the aggregated fraction of the soluble antigen which, more rapidly ingested by macrophages, would enhance the initiation of an immune response.

Some doubt exists about the radiosensitivity of these cells. Gallily and Feldman (1967) injected macrophages, incubated with Shigella, into mice exposed to 550 r, and noticed how this triggered the formation of agglutinating antibody in animals which did not respond to the injection of antigen alone. Unanue and Askonas (1968) on the other hand found that the secondary response, which was elicited by injection of macrophages containing antigen into primed mice, was not impaired by X-irradiation of macrophage donors. Harris (1967) was able to show that macrophages from animals tolerant to BGG incubated with this antigen took up the antigen and stimulated DNA synthesis in primed spleen cells, indicating that the macrophage performance was not affected in tolerant animals.

Further structural evidence that the macrophage might be involved in immune processes was provided by Schoenberg, Mumaw, Moore and Weisberger (1964). These workers, studying sections of spleen and lymph node from immunised and non-immunised rabbits, noticed that "islets of macrophages were always surrounded by either plasma cells or lymphocytes, though in greater numbers in immunised animals". Using the electron microscope, they showed communication between the cytoplasms of the macrophage and lymphocyte and particles the size of ribosomes were seen in the corridors connecting the two cell types. Nossal et al. (1965) and Buyukozer (1965) could not detect anatomical bridges between medullary macrophages and plasma cells or lymphocytes in lymph nodes and concluded that in the medulla of the lymph node micro-anatomical structure was not particularly conducive to the transfer of inductive factors from macrophage to antigen reactive lymphocytes. Buyukozer reported that macrophages, when activated with antigen, could turn into antibody forming cells. This view has been rejected by Nossal, among others.

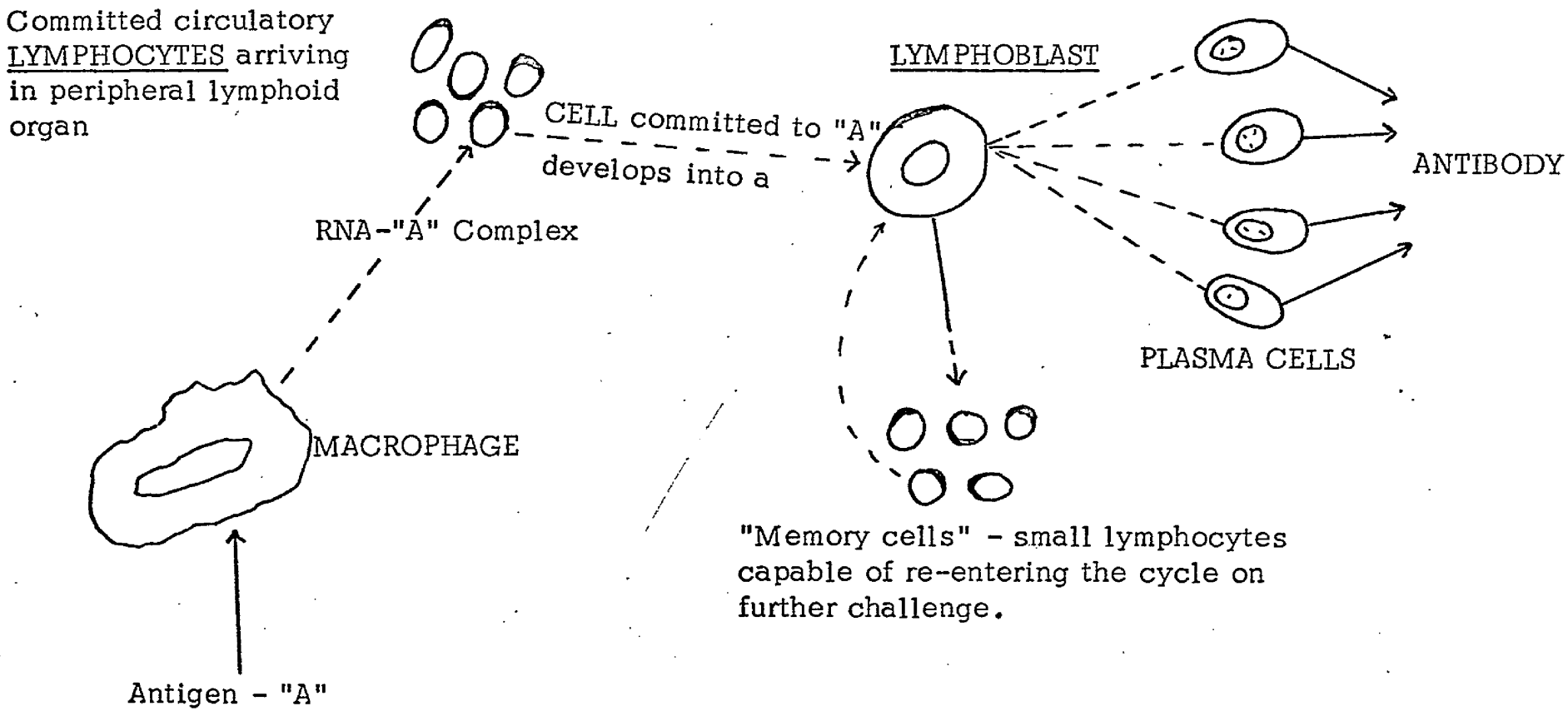
It had been noticed that antigenic flagella ^{131}I labelled persists for four weeks in the dendritic macrophages of lymph nodes, and Garvey and Campbell (1957) have reported that the antigen which persists in liver is complexed with nucleic acids.

Some doubt exists about the precise role of macrophages in immune responses and a scheme is presented in Fig. 2 which attempts to summarise their interaction with other cells involved in immune responses.

An important phenomenon which had been demonstrated is that of the production of tolerance. Any theory of antibody production must be capable of explaining the development of the state of tolerance.

Hanan and Oyana (1954) noticed that mature rabbits, which had been injected with a particular antigen shortly after birth, did not form specific antibodies if the same antigen was re-injected at a time when they were able to produce antibodies to other antigens. This phenomenon has been described as "actively acquired tolerance". A similar phenomenon, termed "immunological paralysis" can be obtained

Fig. 2 : Possible interrelationships among cells involved in the immune responses.



in adult animals by the injection of very large doses of antigen.

Smith and Bridges (1958) have shown that tolerance and paralysis are both finite and demonstrated that a state of paralysis could be maintained by further injection of antigen at a time when an animal would otherwise recover reactivity. Humphrey (1964) has described paralysis which persists long after clearance of protein antigen. Dresser (1962) reported that tolerance ensued in adult animals if they were injected with the supernatant fraction of a soluble protein solution which had been subjected to ultracentrifugation to remove aggregated material.

Burnett (1954) stated that recognition of self is something that needs to be learnt by the animal and is not an inherent genetic quality of the organism. In this respect he proposed that self recognition and tolerance are similar phenomena and that tolerance and self recognition will persist as long as the paralysing antigen or host protein is present. The analogy between tolerance and self recognition gains authority when one considers Owen's observation in 1945 of double blood groups in twin cattle which arose from separately fertilised

ova. Because there is a fusion of the placenta, the cells and antigens of both calves mix freely in each other's circulation and therefore the cows are born with two different types of red cells and will be tolerant to both. If placental fusion had not occurred, both animals would have been able to produce haemolysins and agglutins to the other's cells, should mixing have occurred in adulthood. In seeking explanations for this phenomenon, Dresser and Mitcheson (1968) have considered the possibility of peripheral failure in paralysed animals as distinct from central failure. Peripheral failure denotes interference with the access of antigen to reactive cells or interference with the antibody produced, while central failure refers to inhibition of cells involved in immune response such that no antibody will be produced. More recently, evidence has accumulated that the lymphocyte is the cell involved in paralysis. Weigle and Dixon (1959) showed that a primary and secondary response to BCG could be performed by lymph nodes transferred to paralysed out-bred rabbits, while Dietrich and Weigle (1964) transferred cells from paralysed donors to irradiated mice and

found that they could not respond to stimulation, indicating that a "central inhibition" was probably the most likely. Moreover, a suspension of circulating cells containing more than 90% lymphocytes was shown by Mitchenson (1967) to be capable of restoring the responsiveness to an animal paralysed to BSA. This indicates that the lymphocytes may be the cell involved in paralysis. Harris (1967) took peritoneal macrophages from rabbits tolerant to BGG and incubated them in medium with this antigen. The cells took up the BGG and stimulated the rate of DNA synthesis in spleen cell suspensions from a rabbit previously immunised to BGG, which suggested that macrophage function is unaffected by tolerance. Current thought on the phenomenon of tolerance is that, if the antigen acts directly on its receptive lymphocyte, then paralysis ensues, whereas if the antigen is processed by macrophages first, and then transferred to the lymphocytes, an immune reaction will then follow. This model which was first forwarded by Frei et al.(1966), explains how solutions which have been spun to remove the particles most easily phagocytosable by macrophages are poorly immunogenic or

even capable of inducing paralysis. Also, pneumococcal polysaccharides which suffer poor intracellular digestion by macrophages are highly potent as inducers of paralysis.

A further aspect of the relationship between the macrophage and immune processes was first revealed by the work of Fishman (1961) in which he produced a primary response to T_2 phage by rat lymph node cells in culture with the aid of macrophages. He found that this response could be reproduced if a cell free filtrate of macrophages, which had previously been incubated with T_2 phage, was added to lymph node cells in culture. The neutralising activity to T_2, which appeared in the lymph node cultures after addition of macrophage extract, was specific for T_2 phage and moreover the T_2 neutralising activity did not appear if T_2 phage was added directly to the lymph node culture. If the extract from macrophages incubated with T_2 was subjected either to heat treatment at 80 —— 100°C. for 15 minutes or to the action of ribonuclease, then no T_2 neutralising activity was observed in the lymph node cultures on the addition of the treated extract.

This latter observation tempted Fishman and Adler (1963b) to isolate the RNA fraction of the extract and to test it for immunogenic activity. This they did by inserting into the peritoneal cavities of rats diffusion chambers containing various permutations of the following :

1. RNA (M + T_2) — RNA from macrophages previously incubated with T_2
2. RNA (M) — RNA from untreated macrophages
3. LN — Lymph node cells from untreated rats
4. RNase — Ribonuclease
5. T_2 — T_2 phage

Two sets of host animals were used. One set was irradiated and the other was non-irradiated. The experiment and its results are shown in Table 2.

The results confirm the idea that the immunogenic activity of the extract of T_2 treated macrophages resided in a ribonuclease sensitive RNA species. In experiments designed to investigate the incubation time necessary for the macrophage to process the T_2 and produce an active immunogenic RNA,

Fishman and Adler (1963a) found that the immunogenic activity of the RNA harvested from T_2 treated macrophages *in vitro* increased sharply for the first 30 minutes to maximal activity. It was also shown that a 10-minute incubation period with T_2 was fully sufficient to ensure an active extract if disruption of the macrophage was delayed for an additional 20 minutes.

TABLE 2 : From Fishman and Adler (1963b)

Group Material in Chamber**	Antibody Response in Rats*	
	X-irradiated	Non-irradiated
1. RNA (M + T_2) + LN	0/12	10/12
2. RNA (M + T_2)	0/12	7/9
3. RNA (M + T_2) + LN + RNase	0/11	0/11
4. RNA (M) + LN + T_2	0/11	0/11

* Numerator = Number of rats producing antibody
Denominator = Number of rats tested

** See text for details of abbreviations

Askonas and Rhodes (1965), using ^{131}I labelled haemocyanin as antigen, found that antigenic material was associated with the RNA phenol extracted from mouse peritoneal cells which had been in contact with haemocyanin in vivo. This preparation was immunogenic in previously primed mice. If the RNA was extracted from the macrophage immediately after addition of haemocyanin, the preparation was found to be immunogenic, although it was necessary to add 20-30 times the amount of haemocyanin to obtain similar amounts of macromolecular ^{131}I material in the aqueous phase. They reported that the minimum amount of ^{131}I haemocyanin necessary to elicit increases in serum antigen binding capacity in primed recipient mice was 0.001 µg. which is 20 times the amount reckoned to be present in immunogenic RNA preparations. These results seemed to refute the idea gaining currency in some quarters that the role of the macrophage may be to synthesise a new species of informational RNA but rather they point to an antigen trapping mechanism or an adjuvant role for RNA facilitating antigen entry to cells.

In their very interesting paper, Adler, Fishman and Dray (1966) provide some support for the idea that the "immunogenic RNA" preparations may contain informational RNA. Using T_2 phage as antigen and rabbits as donors of macrophages and lymph nodes, they showed first that the work of Fishman and Adler (1963) with rats could be repeated with rabbit cells. It was then shown that antibody production came in two waves with peaks on days 4 -5 and 10-13 of culture of lymph node cubes respectively. The antibody present from day 4 to day 5 was found to have a sedimentation coefficient of 19 S while that present on days 10 to 13 belonged to the 7 S globulins. When the peritoneal cells and lymph node cells came from animals of different allotypes, the 19 S antibody, which was the first formed, was shown to have the allotypic characteristics of the donor of the macrophages, while the 7 S antibody had allotypic characteristics of the lymph node donors. On the basis of these results, the authors postulated two species of peritoneal cell RNA. One of these RNAs, which is susceptible to degradation by RNase, is responsible for the appearance of IgM antibody and is formed in peritoneal cells on encountering

antigen. The other species of RNA is responsible for the IgG antibody, is resistant to RNase, and is already present in macrophage before antigen addition.

Bishop, Pisciotta and Abramoff (1967) investigated the synthesis of RNA in macrophages. They extended Fishman's observations by showing that rat peritoneal macrophages, following the phagocytosis of Sheep Red Blood Cells, synthesised a species of RNA which would promote specific haemagglutinating antibody when added to normal rat spleen cells. On fractionation of this RNA, it was found that most of the immunogenic activity resided in the 6-10 S fraction. Pulse labelling studies indicated that, during phagocytosis of Sheep Red Blood Cells, synthesis of RNA in the 6-10 S region is complete 30 minutes following the pulse in peritoneal exudate cells, while 60 minutes completed synthesis in cells not exposed to antigen.

Cohen (1967) turned his attention to the possibility of transferring specific immunity from spleen cells immunised to Sheep Red Blood Cells to non-immunised spleen cells by extraction of RNA from the immunised cells. This proved to be

feasible and, using sucrose density gradients, Cohen demonstrated that the species of RNA responsible sedimented at 8-12 S. This RNA was resistant to ribonuclease. To test the postulate that recipient spleen cells had specific recognition sites for RNA or for an RNA-antigen complex, Cohen designed the following experiment. He incubated non-immunised spleen cells with minimally digested 8-12 S RNA from spleen cells previously immunised to Sheep Red Blood Cells in an attempt to block the proposed acceptor sites. When non-digested 8-12 S RNA from cells immunised to Sheep Red Blood Cells was subsequently added, the immunogenic activity was found to be inhibited and the reaction was found to be specific because, if normal cells were previously incubated with digested or non-digested RNA from spleen cells incubated with E. Coli and then afterwards treated with RNA from spleen cells immunised to Sheep Red Blood Cells, then no inhibition was detected. He concluded that, if this immunogenic RNA originated in spleen macrophages, then the degradative enzyme might cause damage to its site of recognition on the cell, while, if it originated in spleen

lymphocytes, the blocking effect might represent the inhibition of transfer of a messenger from lymphocyte to lymphocyte. Raska and Cohen (1968) then introduced the technique of hybridisation into their study of macrophage RNA. This technique relied on two principles : the ability of two species of RNA - one labelled, the other unlabelled - to compete with one another for hybridisation sites with DNA, this capacity for competition between two RNAs being indicative of the number of molecular species they possess in common. From their work with Chick Red Blood Cells and with Sheep Red Blood Cells, they conclude that some similar and some distinct species of RNA appeared after peritoneal exudate cells had been exposed to different antigens. They then separated the peritoneal cell population on the basis of their relative tendency to adhere to glass into non-adhering cells (mainly lymphocytes) and adhering cells (mainly macrophages). Hybridisation studies were conducted on both these cell types which revealed that, while the macrophage synthesised new RNA molecules on antigen stimulation, these were not distinct for each antigen. The lymphocyte on the other hand seemed to be synthesising an

RNA which was distinct for each antigen. This finding is supported by a modification of the above hybridisation technique as described by Cohen (1969). DNA is embedded in nitrocellulose columns and RNA from cells grown in the presence of Sheep Red Blood Cells and tritiated uridine is passed down the columns. The RNA which failed to collect on the columns was assayed and found to be of lower specific activity than RNA of spleen cells untreated with antigen. This suggested that RNA from spleens of immunised animals found more hybridisation sites than spleen RNA of non-immune animals. Conversely, he found that, if cells of the peritoneal cavity were treated identically, the results showed that the RNA from immunised cells which failed to collect on the columns was of higher specific activity than the non-immune cells, indicating that large amounts of radioactive material was being formed which could not form hybrid because of the shortage of available sites. This radioactive RNA was found to lie between 6 - 10 S when analysed on a 5 - 20% linear sucrose gradient and was also found to be capable of inducing a small proportion of peritoneal cells to produce sheep cell

haemolysis. Gottlieb, Glisin and Doty (1967) devised a set of experiments which assigned RNA to an adjuvant role of informational carrier. Firstly they showed that, if mouse peritoneal cells are incubated with T_2 and 250 uc ^3H uridine, the radioactivity is found in the 4 S fraction while the immunogenic activity was found in the 28 S fraction. They then argued that the immunogenic RNA was present in any cell irrespective of whether it had encountered antigen or not. They found that RNA from T_2 infected cells competed normally for hybridisation sites on macrophage DNA with RNA from R17 infected cells and also with RNA from non-infected mice. This RNA was resistant to the activity of RNase-A. They finally showed that the RNA was associated with protein which was susceptible to pronase. This RNA-protein complex could be separated from the gross RNA using a Cs_2SO_4 gradient and it was also shown to have most of the immunogenic activity. This prompted these authors to conclude that such activity is the result of the combination of a certain RNA species non-specifically with antigen such as to make it more antigenic. Moreover, their data also indicated that a unique

RNA is not made by peritoneal macrophages in response to antigen and therefore they would argue more for an adjuvant role for RNA rather than an informational one.

On the other hand, evidence against an adjuvant role is proposed by Pinchuck et al. (1968) who report that RNA extracted from peritoneal macrophages exposed to a linear random synthetic polypeptide (A) initiated an immune response in mice which normally responds only poorly to this antigen. If 10 to 150 ug. of such RNA containing 0.02% of the antigen, which could be obtained from mice, rabbit or rat peritoneal cells incubated with the antigen, was injected intraperitoneally into recipient mice, specific antibody could be detected by passive haemagglutination 3 to 4 weeks later. The immunogenic quality of the RNA preparation was destroyed by ribonuclease. If a second synthetic polypeptide (B) was used, then an immunologically active RNA could be extracted from peritoneal cells with which the polypeptide (B) was incubated. If RNA from peritoneal cells which had been incubated with polypeptide (B) was innoculated into mice along with polypeptide (A), a subsequent anti-polypeptide (B) response followed but no

anti-A response. The authors concluded that this argued strongly against the RNA acting as an adjuvant. A very interesting point was the fact that RNA from rabbit or rat peritoneal cells incubated with antigen could then act as an immunogenic agent in a third species, the mouse.

A picture has been emerging from the various reports in the literature of an RNA-antigen complex which might be more important than either RNA or antigen alone. This RNA-protein complex was reported by Gottlieb (1968) to be present in all macrophages regardless of their exposure to antigen in the laboratory, and it is likely that these RNA-protein complexes are representative of past antigenic encounter by the macrophages. These complexes are found to be 21 - 28% protein. It was not found to be possible to convert RNA from unexposed macrophages to RNA-protein complex by incubation of the RNA with T_2 *in vitro*, nor was it possible to render immunogenic the RNA from non-infected cells by treating this RNA with T_2 *in vitro*. It therefore seems that the macrophage is necessary for complexing fragments of antigen into antigen-RNA complex. The same author notes that the macrophage,

however, may not have a general role to play in the immune response as the "Fishman effect" has not been demonstrated for soluble antigens. Roelants and Goodman (1968) used poly γ - D glutamic acid which has not been found to be immunogenic of itself but can elicit a response when complexed with methylated albumin. These authors found that in either form the tritiated polypeptide will be taken up by the peritoneal cells in vitro and in vivo, and the labelled material will be found exclusively with 4 - 5 S RNA fraction of the gradient. If labelled T_2 were used, then the labelled material was found again in the 4 - 5 S fraction. This was the only type of RNA which was significantly synthesised, as indicated by the uptake of ^{14}C uridine. They also found that, if RNA was mixed with protein in vitro, no association occurred whereas, if cell free extracts were used, there was association comparable to whole cells. These authors investigated the nature of the bonding between RNA and protein in such complexes and concluded it to be covalent. These results are at variance with those reported by Gottlieb (1969) who finds that soluble antigen can be dissociated from the RNP

complex by solutions of high ionic strength. He also concluded from a consideration of ribonucleo-protein complexes of molecular weight of 12,000 and containing 28% protein that the protein moiety must be of small molecular weight. Using polyacrylamide gels and sucrose density gradients respectively, Gottlieb also found that his RNP complex migrated ahead of the t-RNA peak in contrast to Goodman and Roelants whose labelled antigen turned up in the 4 - 5 S complex. In a subsequent paper, Gottlieb and Strauss (1969) showed the RNP complex to have an S_{20W} of 1.3 whereas, if the complex was treated with pronase, the S_{20W} value dropped to 1.3. These authors also computed that RNA component of the RNP complex had a chain length of 25 which would correspond to a molecular weight of 8,250.

Before closing this introduction, a review of current attempts to correlate the above observations with present thinking in molecular biology will be undertaken.

Although until quite recently there appeared to be two opposing schools of thought, (i) selective and (ii) instructive, claiming to explain the known facets of the immune response,

recent developments indicate that the selective theories may prove in the long run to be more tenable. The instructive theories as championed by Haurowitz (1965) considered that the antigen interfered with some stage of gamma-globulin biosynthesis in such a way as to produce a specific antibody to that antigen. More specifically, he proposed that the antigenic determinant complexed with the messenger RNA or the ribosomes. Immunoglobulin synthesis would take place as before for most of the amino acid sequence except at certain amino acid residues where the interference of the determinant would lead to the replacement of a normal amino acid by one which would contribute to altering the site in such a way that it would constitute part of the antibody forming site specific for that antigen. This immunoglobulin would thus be altered in sequence, shape and affinity and would now be specific for the interfering antigen. To explain the continued formation of antibody by an animal long after antigen can be detected in the bloodstream, Haurowitz cites Garvey and Campbell (1957) who found antigen complexed in tissue months after its administration. He also disposed of

objections about the qualitative difference between primary and secondary antibody by adopting the explanation of Najjar and Fisher (1956) who point out that primary antibody is formed against antigen whereas secondary antigen is formed against antibody-antigen complexes. Another objection was that antibody production to soluble antigens in the secondary response exhibits an exponential increase, quite distinct from a primary response. However, the particulate antigen T_2 has a secondary response similar to its primary response. Haurowitz dispensed with the questions of tolerance and self-recognition by explaining that tolerance or unresponsiveness to autologous proteins is due to their being present in excess. If concentration was dropped, then responsiveness would occur.

Quite a number of models have been presented recently by authors favouring selective models. These authors would then propose that antigen selects one clone or group of cells in the animals which may have capacity to produce antibody to only that particular antigen or that the antigen selects a cell with a capacity to react against a very wide range of

antigen but the challenging antigen selectively turns on the correct portion of the cells genome such that specific antibody only is produced. There are a number of observed facts which any such selective theory will have to be compatible with, and they are as follows :

(i) Multiplicity of amino acid replacements in the variable half and the relative invariance of sequence in the constant half of gamma-globulin chains.

(ii) The presence of a majority of amino acids interchanges consistent with single base replacement in the genetic code.

(iii) The occurrence of invariant segments within the V region.

(iv) Similarity of lengths of C and V regions.

(v) Low recombination among allotypes.

The theories which attempt to explain these phenomena can be divided into two groups :

Firstly, there is the multiple gene hypothesis which proposes that each variable region of an antibody molecule is coded for by a different gene in the germ line. Putnam

and Titani (1965), examining Bence Jones proteins, pointed out that these proteins might be the products of two separate genes. The amino terminal end would be under the control of many genes, while the carboxyl terminal would come under the control of a single gene. A linking enzyme would have to be postulated to link together the two proteins. There is no precedent for this type of condensing enzyme capable of joining what would be two basic amino acids, at positions 107 and 108. However, this theory gained some currency when it was shown that some patients with multiple myeloma excrete a whole light chain and also a half molecule virtually identical to the variable portion, but this may be a degradation product rather than a light chain precursor.

A theory not unlike the above was proposed by Dreyer and Bennett (1965) who suggested that the genetic material which codes for the variable position of the light chain is inserted into a portion of nucleic acid which contains the common gene. They propose that the genetic material comes from rings of nucleic acid within the chromosomes and the insertion mechanism is thought to be similar to that for the introduction

of a lambda virus into a bacterial chromosome. While this theory and the one considered above explain how the INV factor is independent of the subgroup of the amino terminal half, they both fail to suggest how these multiple genes may have evolved and moreover how a particular gene is selected.

The second group of theories are the Somatic Variation Hypotheses which invoke variation of replication in somatic cells. Potter, Appella and Geiser (1965) proposed that unusual triplets coding only in the variable region could bind to anticodons of transfer RNA which were lacking in complete specificity for their activating enzymes and thus introduce different amino acids by the same triplet. The fact that multiple replacements for single amino acids are noted (i.e. leucine replaced by five different amino acids at positions 21, 46, 76, 90, 92, 102 or 189) instead of the single amino acid interchange predicted by theory, reduces its ability to command credibility. Moreover, the ambiguous codons described above would have to be restricted to the region of the gene coding for the amino terminal half of the light chain. In effect, this theory requires that a single gene remains constant throughout

differentiation while a specialised translation mechanism develops in such a way as to cause changes in sequence. One final objection arises when one considers what triplets would give rise to such ambiguity. A rather ingenious hypothesis, but one somewhat difficult to prove, was put forward by Brenner and Milstein (1966) who considered that part of the nucleotide sequence for the gene specifying the sequence of amino acids in the constant region acts as a recognition site for a cleaving enzyme which cuts one strand of the double-stranded DNA, forming a 3 ' OH end on the side of the cleavage proximal to the end coding for the variant end of the chain. An exonuclease is then postulated to displace the cleavage enzyme and commence degration of DNA from the 3 ' OH end, exposing the complementary strand. A polymerase enzyme would repair the damage but in doing so would introduce mistakes. The cell would then divide, giving one daughter with wild type gene and the other with the repaired gene. Repetition of the above process could cause a large number of differing sequences with difference restricted to the amino end of the light chain. Subsequent division would

ensure that every sequence produced would be conserved. In order that these new clones remain stable, the hydrolytic enzyme must become repressed. This paper merely offers a model system for generation of diversity of antibody chain sequence but again no attempt is made to explain how these clones may be activated to produce antibody. Edelman and Gally (1967) had a different solution to offer for the riddle of the origin of antibody diversity. A number of genes are postulated to have arisen by tandem duplication. The precursor gene may have a length equal to the length of the constant portion of the light chain. It was further proposed that 50 such tandem duplicated genes would exist in parallel arrays in the maturing lymphocyte. Point mutations would arise in sequences corresponding to the variable sections of the immunoglobulin chain. A cross-over, which would be favoured by the close homology of the genes, as well as tandem duplication, would then occur. The resultant set of genes would appear to have arisen by point mutation. The objections to this theory are based on the constancy of the invariant portion within a given chain type and the question

of inheritance of allotype. A most interesting hypothesis is that of Smithies (1965), whose observations also include an explanation of tolerance. He proposed intragenic crossing over somatically between regions of homology in genes controlling antibody structure. These nucleic acids govern the synthesis of many forms of "antibody virus" containing nucleic acid which dictates the sequence of a protein coat, thought to exhibit antibody activity of similar type to 19 S globulin. These antibody viruses are being passed to cells which will be capable of primary responses to the antigen. Should encounter take place between antibody virus and antigen in these cells, then the virus will proliferate with an excess production of coat protein, i.e. 19 S antibody. The nucleic acid then transduces itself into the genome of the host cell. This cell then proliferates and retains the capacity to synthesise specific 7 S antibody. If, on the other hand, the antibody virus meets its antigen while circulating in the bloodstream, then the combination between the coat antibody and the antigen releases the nucleic acid into an environment where it is susceptible to degradation by nucleases. This

leads to elimination of antibody viruses which are responsible for the production of this specific antibody, i.e. tolerance to this particular antigen ensues. So far nothing which resembles Smithies' antibody virus has been found.

At the moment, while most immunologists would favour a selective approach to antibody diversity, no entirely satisfactory model of selection has been put forward, nor have some of the genetic implications of such theories outlined above been satisfactorily answered.

The work to be described in this thesis is concerned firstly with the response of rabbit peritoneal exudate cells with antigen. Particular attention was payed to the quality and quantity of RNA synthesised in these cells after antigen encounter. Studies were also carried out on the effect of the presence of antigen on the uptake of (i) thymidine, (ii) leucine, and (iii) inorganic phosphate into the cellular components of peritoneal exudate cells <u>in vitro</u>.

The second concern of this thesis is with the effect of antigen on the uptake of labelled thymidine by cultures of appendix cells from normal rabbits or from rabbits previously immunised to antigen. Some preliminary studies were carried out on mixed cultures of appendix cells and spleen cells.

MATERIALS AND METHODS

MATERIALS

Animals

Male Californian rabbits of varying ages were used as donors of peritoneal cells and lymph nodes.

Antigens

Crystallised Bovine Serum Albumin (BSA) and Bovine Gamma Globulin Fraction II from plasma (Armour Pharmaceuticals, Eastbourne, England).

Sheep Serum Albumin; Egg Albumin (5 x crystallised); Pig Gamma Globulin (Pentex, Kankakee, Illinois, U.S.A.) Sheep Gamma Globulin (Koch-Light Laboratories Ltd., Bucks, England).

T_2 bacteriophage in buffer (Heshey and Chase, 1962) (Miles Laboratories, Inc., Elkhart, Indiana, U.S.A.)

Paraffin (light)

Specific gravity 0.830 - 0.870 (British Drug Houses).

Media

Eagles Hela Cell Medium (EHM); Pucks; NCTC 109; TC 199 (Flow Laboratories, Irvine, Ayrshire, Scotland)

Buffers

(A) 0.01 M Acetate Buffer, pH 5.0, containing

10^{-3} M Mg^{++}

0.2 M Li Cl

0.5% Sodium dodecyl Sulphate (SDS)

(B) 0.01 M Acetate Buffer, pH 5.0, containing

10^{-3} M Mg^{++}

0.2 M Li Cl

Phosphate Buffered Saline (PBS), pH 7.2, containing

Na Cl	1.00 gram	in 1 litre of H_2O
K Cl	0.25 grams	
$Na_2 HPO_4$	144 grams	
$KH_2 PO_4$	0.25 grams	

Phenol (A.R.)

British Drug Houses. Distilled at

and made 88% W.R.T. water.

Scintillation Fluid

0.5% (w/v) Diphenol : Oxazole in toluene (A.R.)

Isotopes

^{3}H Thymidine

2 - ^{14}C - Thymidine at a specific activity of 7.10 m Ci/m M

and a concentration of 30 μ/ml.

Isotopes (continued)

Uridine T (G)	1.52 Ci/mM
^{14}C Uridine	60.7 mCi/mM
[^{3}H] Adenosine	500 mCi/mM
[^{14}C] Leucine	312 mCi/mM
[^{14}C] Iodoacetamide	52.3 mCi/mM

(The Radioactive Centre, Amersham, Bucks, England)

Normal Rabbit Serum

Uninoculated rabbits were bled from the peripheral ear vein and the sera obtained from such bleedings were pooled and spun to remove any particulate matter. If the serum was employed in the indirect haemagglutination test, it was first heated at 56°C. for 30 minutes to remove complement. The serum was then centrifuged at 80 g for 15 minutes and the supernatant fraction was incubated for 30 minutes with formalinised Sheep Red Blood Cells to remove any antibodies reacting with Sheep Red Blood Cells. The cells were removed by centrifugation and the serum was diluted appropriately with PBS (pH 7.2).

Formalinised Sheep Red Blood Cells

(Difco, Detroit, Michigan, U.S.A.)

Tannic Acid

Stock solution - 1 gram of tannic acid in 200 ml. Phosphate Buffered Saline (pH 6.4). This was diluted 1 : 25 with PBS (pH 7.2) and used in the tanning process.

Top Agar

3 grams of Difco Bacto Agar (Oxoid No. 3) was made up to 500 ml. with distilled water. This was dissolved by autoclaving and was sterilised at 15 lbs./in^2 for 15 minutes.

Bottom Agar

Difco Bacto Tryptone	10 grams
Difco Bacto Agar (Oxoid No. 3)	10 grams
Sodium Chloride	8 grams
Glucose	1 gram

The tryptone and agar were dissolved by autoclaving. Then sodium chloride and glucose were added, the mixture was made up to 1 litre with distilled water and sterilised at 15 lbs./in^2 for 15 minutes.

Bacteria

E. Coli Strain B

Penicillin/Streptomycin (Flow Laboratories, Irvine, Ayrshire)

This was added to all media used. The final concentration was 100 units Penicillin/ml. and 100 µg. Streptomycin/ml.

METHODS

Production of Peritoneal Exudate Cells

Californian rabbits of about 3 months were injected intraperitoneally with 50 ml. of sterile liquid paraffin. After 4 days, the peritoneal cavity was washed out with a balanced salt solution. The cells were then centrifuged at 80 g for 10 minutes and resuspended in EHM. A cell count was made and the cell suspension was adjusted to the required concentration.

Preparation of Cells for Estimation of Radioactivity (^{14}C and ^{3}H)

250 µg. BSA was added to the peritoneal cell suspension as carrier. 5 ml. of ice cold 10% TCA was then added and the mixture well shaken and allowed to stand at 0°C. for 15 minutes, after which it was centrifuged at 1,500 g for 10 minutes. The precipitate was then washed three times with ice cold 5% TCA. One ml. of hyamine hydroxide was added to the precipitate and incubated at 60°C. for one hour. The dissolved precipitate was washed into scintillation vials with 10 ml. of scintillation fluid.

Preparation of Cells for Estimation of Radioactivity (^{32}P)

Twenty volumes of 2 : 1 chloroform/methanol was added to cell suspension and the mixture was shaken and allowed to stand long enough to effect separation of the layers. The upper aqueous layer was then removed and 0.2 volumes of 0.9% Na Cl, which had previously been shaken with 10 volumes of 2 : 1 chloroform/methanol, was added and the mixture was shaken. The layers were again allowed to separate and the aqueous top layer was removed. The washing with 0.9% Na Cl was repeated exactly as before. When the aqueous layor was removed, an aliquot of the chloroform/methanol extract was evaporated to dryness on a planchette and counted in a gas-flow counter.

Washing of Peritoneal Exudate Cells

After the incubation of antigen with macrophages, the cells were washed free of antigen. The cells were spun at 800 g and resuspended in fresh EHM after which they were respun at 800 g. They were then resuspended in Buffer A and 0.4 ml. of 1.3% suspension of Bentonite was added.

Extraction of Ribonucleic Acid

The method used was as employed by Adler et al. (1966) which was a modification of the method of Homma and Graham (1963).

To a suspension of peritoneal cells in Buffer A was added an equal volume of 88% phenol. The mixture was shaken vigorously for 2 minutes, heated to 60°C. for 3 minutes, then cooled rapidly and spun at 800 g for 15 minutes. The aqueous layer was removed, 0.2 ml. of 1.3% Bentonite added, and washed with 2 volumes of ether. The aqueous layer was then treated with 2 volumes of ethanol at -10°C, allowed to stand at -10°C for 30 minutes and spun at 7,820 g for 15 minutes. The precipitated RNA was resuspended in Buffer B and reprecipitated with ethanol at -10°C. Finally, the RNA was resuspended in Buffer B.

Preparation of Lymph Node Cubes for Culture

The culture conditions used followed closely those of Adler, Fishman and Dray (1966). The animals were slaughtered and the popliteal lymph nodes were removed aseptically and placed in a plastic petri dish containing EHM. The node

was freed from adipose tissue and dissected into cubes of 1 cu. cm. Six such lymph node cubes were placed in a gauze pad which rested on a bed of glass beads in a sterile plastic petri dish. Each plate received 5 ml. of EHM and instead of using serum, hydrocortisone semisuccinate in a concentration of 0.1 ug./ml. was used. The plates were incubated in a moist atmosphere containing 5% CO_2/95% O_2

Harvesting of Tissue Culture Fluid and Preparation of Gamma Globulin Fraction for Assay of Antibody Activity

The culture fluid from 4 plates was pooled 4 days after initiation of culture. Normal rabbit serum, previously heated to 56°C and free of detectable T_2 neutralising activity, was added as carrier to a concentration of 1%. An equal volume of saturated ammonium sulphate was added and the gamma globulins were allowed to precipitate overnight at 0°C. Following centrifugation at 1,000 g for 10 minutes, the gamma globulins were resuspended in 2 ml. of phosphate buffered saline, pH 7.2, and dialised overnight at 0°C against phosphate buffered saline. The gamma globulin preparation, then concentrated tenfold, was stored at -10°C.

Analysis of Tissue Culture Gamma Globulin Preparations for Antibody Activity

(i) Anti-BSA Activity

Principle : Sheep Red Blood Cells were treated with tannic acid to make them more adhesive. These tanned cells were then incubated with BSA which was adsorbed on to the cell surface. These cells were then incubated with sequential dilutions of a test sera and the presence or absence of detectable anti-BSA in each dilution noted by observing the extent of agglutination of the cells.

Tanning and Coating Procedure : This was as described by Herbert (1967), with the modification that a 1 in 5000 dilution of tannic acid was used. A tanned control was also used which was not coated with BSA but rather incubated with phosphate buffered saline. Both BSA treated tanned cells and PBS treated tanned cells were mixed on a rotary mixer for 45 minutes.

The concentration of Normal Rabbit Serum in phosphate buffered saline used for resuspending coated and control cells prior to usage in the test was found to be critical and the 1% used by Herbert was found to be inadequate to

stabilise the cells. A 2% solution of NRS in PBS was found to be more satisfactory.

Titrations : Serial dilutions of test sera were performed in 0.9% saline with NRS to 0.5%. The dilutions used were 1 : 10, 1 : 20, 1 : 40, 1 : 80, 1 : 160, 1 : 1600, 1 : 3200.

Scoring : Scoring of the patterns obtained was according to Stavitsky (1954).

(ii) Anti-T_2 Activity

Principle : Tissue culture fluids to be tested were incubated with 3 dilutions of T_2 bacteriophage. Controls were set up where PBS was incubated with 3 dilutions of the phage. Samples of equal volume from these tubes were subsequently plated on agar with E. Coli Strain B and the plates were incubated overnight. The plaque formation in these plates was noted and the degree of reduction in the test plates compared to control plates was indicative of the extent of antibody present.

Assay of Anti-T_2 Activity in Culture Fluid

From a stock T_2 phage suspension, 3 dilutions were made to contain 1000, 100 and 10 PFU T_2 bacteriophage

respectively/0.6 ml. Each of these dilutions was incubated at 37°C for 30 minutes with an equal volume of gamma globulin preparation to be tested. Control tubes were set up in which the phage dilutions are incubated under similar conditions with equal volumes of phosphate buffered saline. Meanwhile, tubes are set up containing 4 ml. of top agar maintained in liquid state in a water bath at 47.5°C. Just before termination of the incubation of the test and control preparations with phage, 0.5 ml. aliquots of E. Coli Strain B were added to the top agar. On completion of 30 minutes of incubation, two 0.5 ml. aliquots were withdrawn from each of the test and control incubations and were added to separate tubes of top agar. Immediately after the addition, the agar, now containing E. Coli and an aliquot of test or control T_2 preparation, was poured into a petri dish containing bottom agar. The dish was rotated gently to ensure complete coverage of the bottom agar surface. The plates were allowed to stand for 15 minutes to allow solidification after which they were placed at 37°C in a humid incubator overnight. On the

following day, the number of plaques in the test and control plates were noted and the degree if inhibition of plaque formation was indicative of the amount of antibody present.

X-Irradiation of Cell Suspensions

3 ml. aliquots of cell suspension were drawn off when clumps were allowed to settle. They received 600 Rads in one minute. The cell concentration was adjusted with EHM to 8 million/ml and serum concentration was 5%

Separation of Particulate Matter from a Solution of Soluble Antigen

A solution of antigen was made at a concentration of 1% This solution was spun at 205,000 g for 2 hours at 2°C. The precipitate was resuspended in EHM to the original volume, and the supernatant fraction was removed.

Immunisation Procedures

Three-month-old Californian rabbits were given a single intravenous injection of antigen (30 mg./Kg. body weight) and at least 4 weeks were allowed to elapse before the animals were used. The rabbits were generally used between four and six weeks after immunisation.

Preparation of Cell Suspensions

(a) Appendix : Rabbits were killed by cervical dislocation, the appendix removed and the lumen freed of contents with phosphate buffered saline (PBS), pH 7.2. The layer of lymphoid cells was scraped clear of the sub-mucosal area using a spatula and was placed on a stainless steel grid on a filter funnel and teased gently with a glass rod to disaggregate the cells. The cells were then washed through the grid and filter funnel to a collecting vessel below. The cell clumps were allowed to settle out of this suspension. A portion of the supernatant was drawn off and cell concentration determined. This suspension was adjusted to the required concentration by the addition of EHM.

(b) Spleen : The spleen was immersed in 15 ml. of EHM in a petri dish and teased apart with forceps and scalpel. The disaggregated tissue was washed into a bottle with EHM. The tissue clumps were allowed to sediment by leaving the vessel to stand for 5 minutes at 37°C. A sample of the supernatant was removed and cell concentration determined. This suspension was adjusted to the appropriate concentration by addition of EHM.

Harvesting of Cells

To each culture tube was added 0.1 ml. of 1% BSA and this was followed by 5 ml. of ice cold 10% TCA. The tubes, having been allowed to stand for 15 minutes, were spun at 1,500 g for 10 minutes. The supernatant was decanted and the precipitate was resuspended in 2 ml. of ice cold 5% TCA. The precipitate was similarly washed a further two times.

Autoradiography

Appendix cells were suspended at a concentration of 8 million/ml. 2 ml. of this suspension was added to a plastic petri dish containing a coverslip and the cells were incubated as before. They were pulsed with 10 uCi ^{3}H thymidine in the presence of 0.25×10^{-5} M thymidine, 12 hours before harvest. When plates were being harvested, the medium was drawn off carefully in order to avoid disturbing the cells which had settled on the coverslip. The coverslips were treated as follows :

1. A short rinse with phosphate buffered saline to remove excess medium.
2. Acetic Acid : Ethanol (3 : 1) at $0^{o}C$ for 10 minutes.

3. 10% TCA at 0°C for 10 minutes.

4. Two washes with water at 0°C for 10 minutes.

5. A short rinse with absolute ethanol at room temperature to remove water.

After drying at room temperature, the coverslips are mounted using Depex on glass slides which had previously been degreased in absolute alcohol and coated with a film of gelatine-chrome alum. The slides were covered with AR 10 stripping film. After two weeks of exposure to film, the autoradiographs were developed in D 19 b for 5 minutes, rinsed in water, fixed with Amfix for 4 minutes, washed in running water for 5 minutes and dried in air. They were then stained with Giemsa (freshly diluted 1 : 20 v/v in water) for 30 seconds, washed in water and dried in air.

Labelling of BSA with [^{14}C] Iodoacetamide

This was carried out according to the procedure of Tang and Hartley (1967).

(^{14}C) acetamide labelled BSA had a specific activity of 8980 cpm/mg.

(^{14}C) labelled T_2 bacteriophage (3.57 dpm/10^6 pfu) grown in the presence of (^{14}C) Leucine was kindly provided by Dr. J. D. Pitts.

STUDIES ON PERITONEAL EXUDATE CELLS

R E S U L T S

In a previous section of this thesis, the current lines of thought on macrophage involvement in immune responses is outlined. This is an area of considerable controversy. Many authors have proposed that ingestion of the antigen by macrophages leads to an enhancement of the immunogenicity of the antigen. Argyris (1967) reported that peritoneal cells containing Sheep Red Blood Cells caused antibody production in normal syngenic mice but not in irradiated mice. This would suggest the release of an immunogenic factor by peritoneal cells containing antigen rather than the transfer of immuno-competent cells. The work of Harris (1965) with BGG and of Askonas and Rhodes (1965) with haemocyanin suggests that the macrophage may select out an immunogenic portion of the soluble antigen and pass this to antigen sensitive cells.

On the other hand, Perkins and Makinodan (1965) reported that mice injected intraperitoneally with glycogen to increase macrophage production exhibited an impaired antibody response to Sheep Red Blood Cells. Parkhouse and Dutton (1966)

argued that the addition of macrophages to BSA stimulated spleen cell suspensions, normally low in macrophage cells, should result in an increase in DNA synthesis in these spleen cells. However, an inhibition of DNA synthesis in antigen stimulated and control cells was noted. These authors were able to show that the inhibition was not due to competition between cell types for the antigen.

The possible role of macrophage RNA in the immune response has also been reviewed in the introduction to this thesis. The fact that authors, including Adler et al. (1966) and Gottlieb et al. (1967), could produce an apparently immunogenic RNA preparation from peritoneal cells incubated with antigen for 30 minutes suggests that there is either a rapid synthesis of a new species of RNA, a conclusion supported by Cohen (1969), or that the RNA was present in macrophages prior to encounter with antigen. In this section, experiments designed to investigate the interaction between antigen and macrophages, with particular reference to RNA metabolism, are reported.

Initially, studies were carried out on leucine uptake into protein, thymidine uptake into DNA, and phosphate uptake into phospholipid, all in peritoneal cells under control and stimulated conditions. These studies were prompted by reports by Cohn et al. (1966) that mouse peritoneal macrophages in vitro undergo changes involving increase in protein synthesis, including cell growth and formation of large numbers of lysosomes. Also, it was reported by Volkman (1966) that 1% of normal mouse and rat peritoneal cells are capable of incorporating tritiated thymidine at any given time. Somewhat higher is the incorporation rate of 3% to 4% reported by Aronson and Elberg (1962) for oil induced rabbit peritoneal exudate cells. A dramatic increase in mitosis was observed by Forbes and Mackaness (1963) in peritoneal exudate cells when mice were re-injected with BSA.

It is intended in this section, therefore, to characterise the effect on the metabolism of peritoneal cells incubated in the presence of antigen and to observe what effect the presence of antigen would have on the synthesis of macromolecules of these cells over and above the changes reported to take place when the cells are merely cultured in vitro.

EXPERIMENT 1 : Time course of the uptake of ^{14}C leucine into non-stimulated and T_2 phage stimulated peritoneal exudate cells.

Peritoneal exudate cells were suspended in 8 ml. of EHM and 5 μCi of [^{14}C] leucine (312 M Ci/mM) were added. The cell concentration was 10^5 million/ml. The population was divided into two equal portions. To one was added 0.4 ml. T_2 phage (10^8 PFU/ml.) and to the other 0.4 ml. of PBS. 1 ml. samples of both control and stimulated cells were incubated at 37°C in 5% CO_2/95% O_2 and the cells were harvested at 30 minutes and 90 minutes respectively after setting up the culture.

EXPERIMENT 2 : The effect of changing the concentration of stimulatory T_2 phage incubated with peritoneal exudate cells on the uptake of ^{14}C leucine by these cells.

Peritoneal exudate cells were resuspended in 8 ml. of EHM and 6 μCi of [^{14}C] leucine (312 M Ci/mM) were added. The cell concentration was 10^5 million/ml. This population was divided into 1 ml. portions and the following additions were made to duplicate tubes :

Fig. 3 : Time course of uptake of Leucine by peritoneal exudate cells in the presence and absence of antigen.

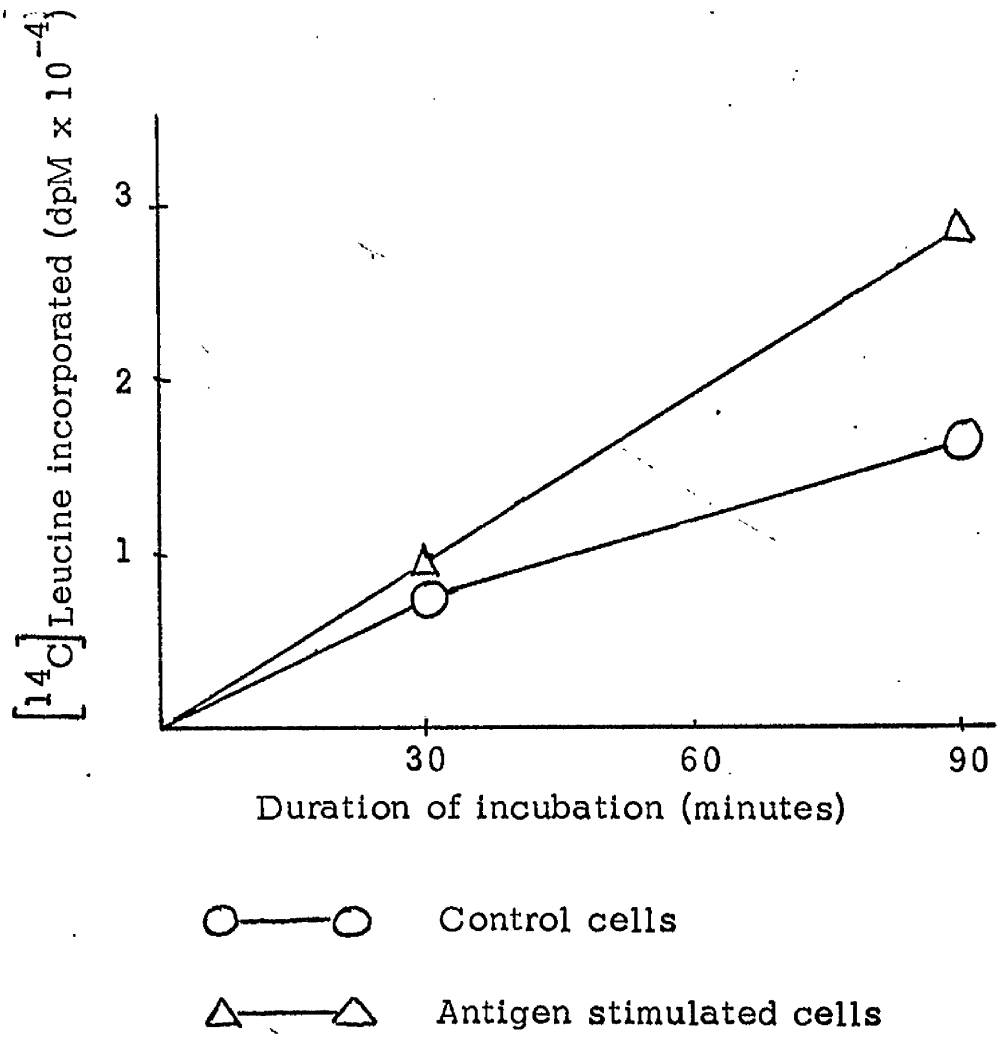
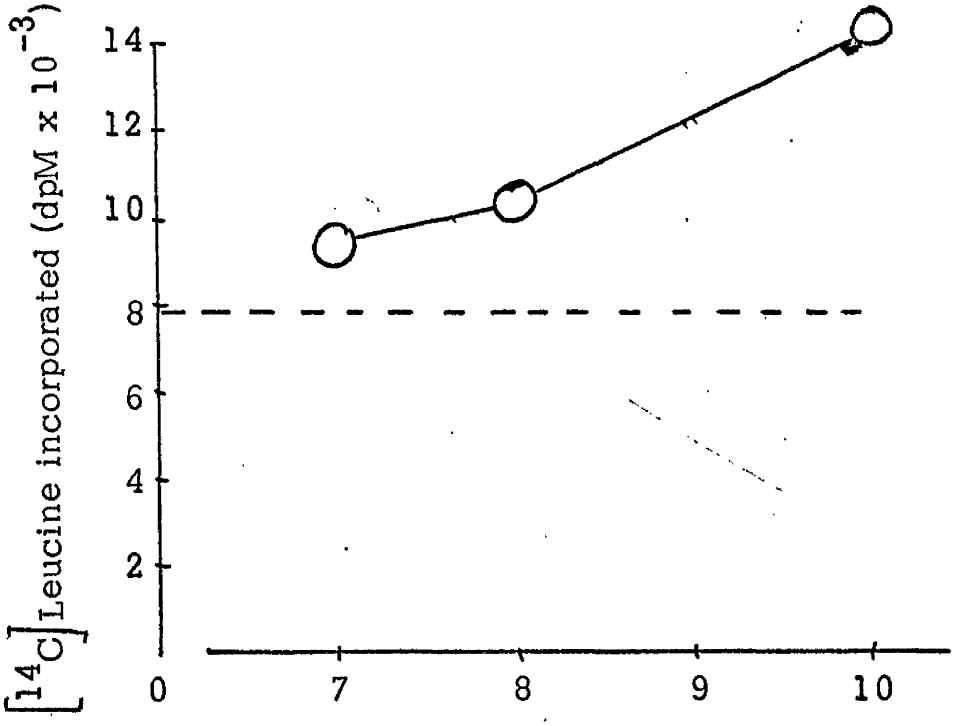


Fig. 4 : The effect on $[^{14}C]$ Leucine uptake of increasing the concentration of T_2 phage incubated for 30 minutes with peritoneal cells



Dotted line indicates Leucine uptake in absence of antigen.

(i) 0.1 ml. of T_2 phage suspension (10^{11} PFU/ml.)

(ii) 0.1 ml. of T_2 phage suspension (10^{9} PFU/ml.)

(iii) 0.1 ml. of T_2 phage suspension (10^{8} PFU/ml.)

(iv) 0.1 ml. of EHM

Control and test samples were incubated as in Experiment 1 and were harvested at 30 minutes after setting up of incubation.

Fig. 3 shows that one effect of antigenic stimulus on the peritoneal exudate cell was to increase the uptake of leucine into the cell with respect to time. This effect appears to be concentration dependent as can be seen from Fig. 4 where an increase in the ratio of T_2 phage to peritoneal exudate cells was accompanied by an increase in leucine uptake.

EXPERIMENT 3 : Time course of uptake of ^{14}C thymidine into non-stimulated and T_2 phage stimulated peritoneal exudate cells.

Peritoneal cells were resuspended in 8 ml. of EHM and the cell concentration was 10^{5} M/ml. 4 μCi of [^{14}C] thymidine 7.18 M Ci/mM was added to these cells. The cell population was divided equally and to one suspension was added 0.4 ml. of T_2 suspension (10^{8} PFU/ml.) while to the other was added

0.4 ml. of PBS.

One ml. samples were incubated at 37°C and control and test samples were harvested at 30 minutes and 90 minutes respectively after setting up of the culture.

EXPERIMENT 4 : The effect of changing the concentration of T_2 phage incubated with peritoneal exudate cells on [^{14}C]thymidine uptake by those cells.

Peritoneal exudate cells were resuspended in 0 ml. of EHM and the cell concentration was 10^5 million/ml. 4 µCi of [^{14}C]thymidine 7.18 M Ci/mM were added to the suspension. The cell suspension was then divided into 1 ml. aliquots and the following additions were made to duplicate samples :

(i) 0.1 ml. of T_2 suspension (10^{11} PFU/ml.)

(ii) 0.1 ml. of T_2 suspension (10^9 PFU/ml.)

(iii) 0.1 ml. of T_2 suspension (10^8 PFU/ml.)

(iv) 0.1 ml. of EHM

All tubes were incubated at 37°C and harvested at 30 minutes after setting up of the culture.

When T_2 phage was incubated with peritoneal exudate cells, it was found (Fig. 5) that the uptake of thymidine into acid precipitable material of the cell was depressed relative to

Fig. 5 : Time course of uptake of [2 - ^{14}C] thymidine by peritoneal exudate cells in the presence and absence of antigen.

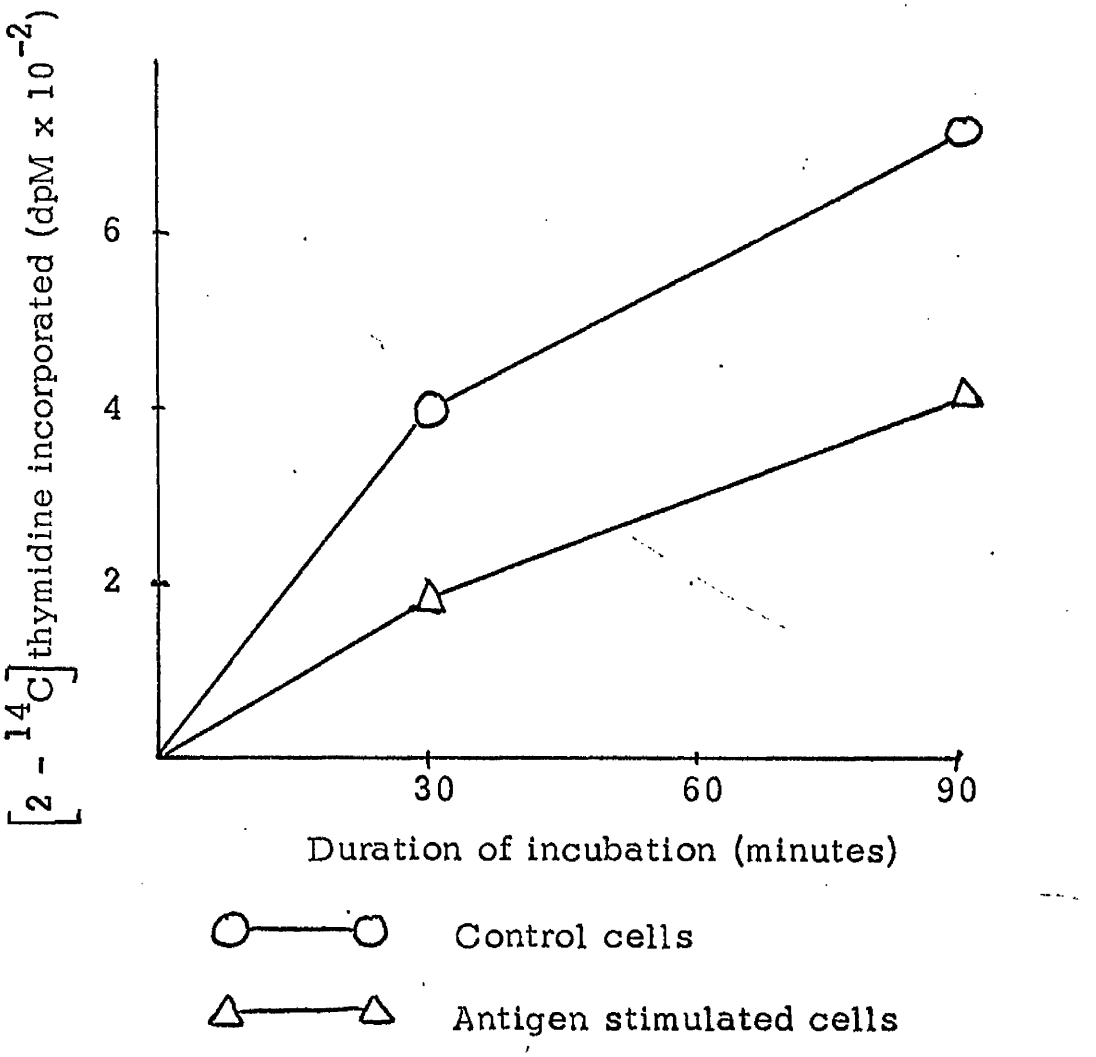
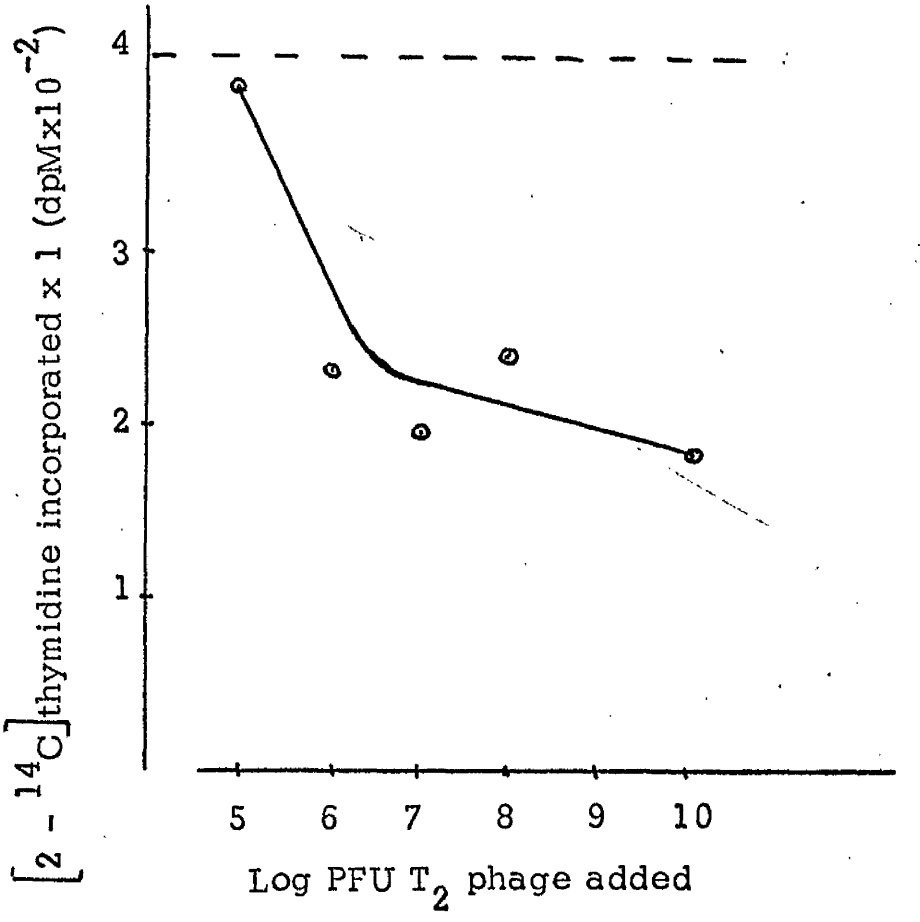


Fig. 6 : The effect of increasing the concentration of T_2 phage incubated for 30 minutes with peritoneal cells on [2 - ^{14}C] thymidine uptake.



Dotted line indicates thymidine uptake in absence of antigen.

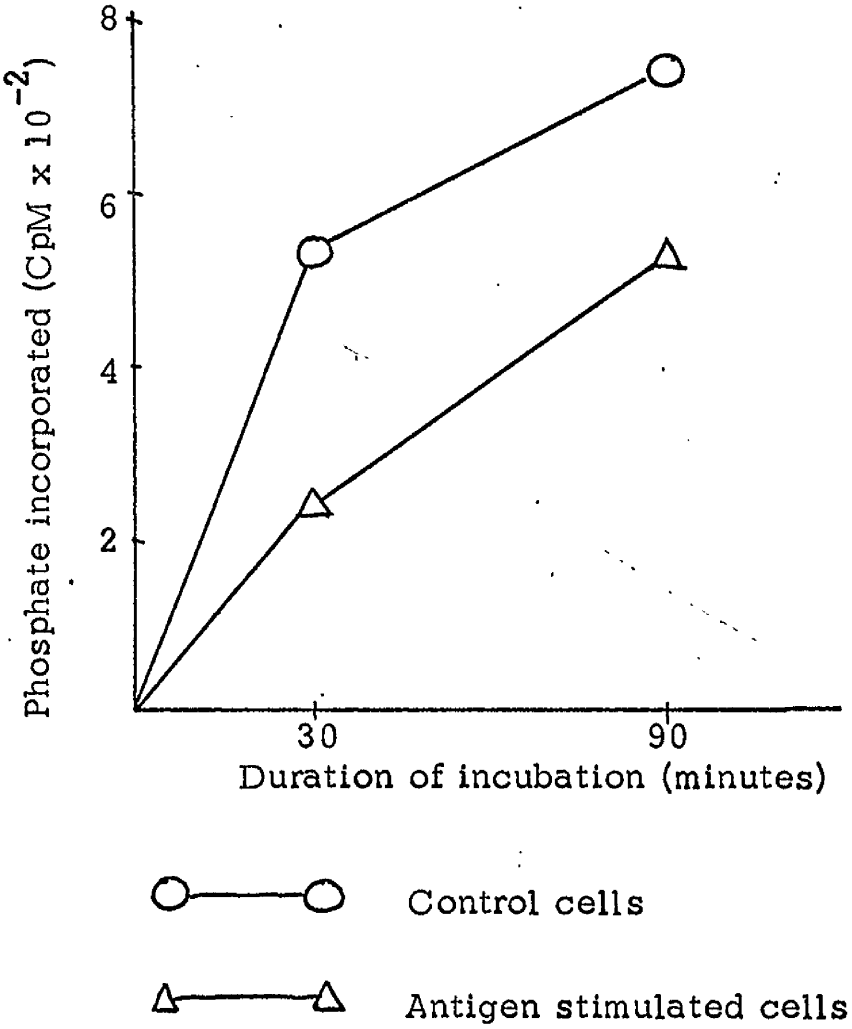
control values over a period of 3 hours. This depression bore an inverse relationship to antigen concentration as can be seen from Fig. 6.

EXPERIMENT 5 : Time course of the uptake of $[^{32}P]$ phosphate into phospholipid of non-stimulated and T_2 phage stimulated peritoneal exudate cells *in vitro*.

Peritoneal exudate cells were suspended in 8 ml. of EHM and the cell concentration was 10^5 million/ml. To the cell suspension was added 100 µCi of $[^{32}P]$ phosphate (42 Ci/mM) and the suspension was then divided into two equal portions. To one was added 0.4 ml. of T_2 suspension (10^9 PFU/ml.) and to the other 0.4 ml. of PBS. One ml. samples of both test and control suspensions were incubated as in Experiment 1 and harvested at 30 minutes and 90 minutes respectively after setting up of culture.

As can be seen from Fig. 7, the incubation of T_2 phage with peritoneal cells depressed the incorporation of inorganic phosphate into phosphate. In the next experiment, the effect of changing the ratio of T_2 phage to peritoneal cells was examined during the course of a 30-minute pulse of inorganic phosphate.

Fig. 7 : Time course of uptake of phosphate into phospholipid of peritoneal cells in the presence and absence of antigen.



EXPERIMENT 6 : The effect of changing the concentration of T_2 phage incubated with peritoneal exudate cells on the uptake of $[^{32}P]$ phosphate into phospholipid of those cells.

Peritoneal cells were suspended in 8 ml. of EHM. The cell concentration was 10^5 million/ml. To the cell suspension was added 100 μCi of $[^{32}P]$ phosphate (30 Ci/mM) and this suspension was then divided into 1 ml. aliquots. To duplicate 1 ml. samples, the following additions were made :

(i) 0.1 ml. of T_2 suspension (10^{11} PFU/ml.)

(ii) 0.1 ml. of T_2 suspension (10^{10} PFU/ml.)

(iii) 0.1 ml. of T_2 suspension (10^8 PFU/ml.)

(iv) 0.1 ml. of T_2 suspension (10^7 PFU/ml.)

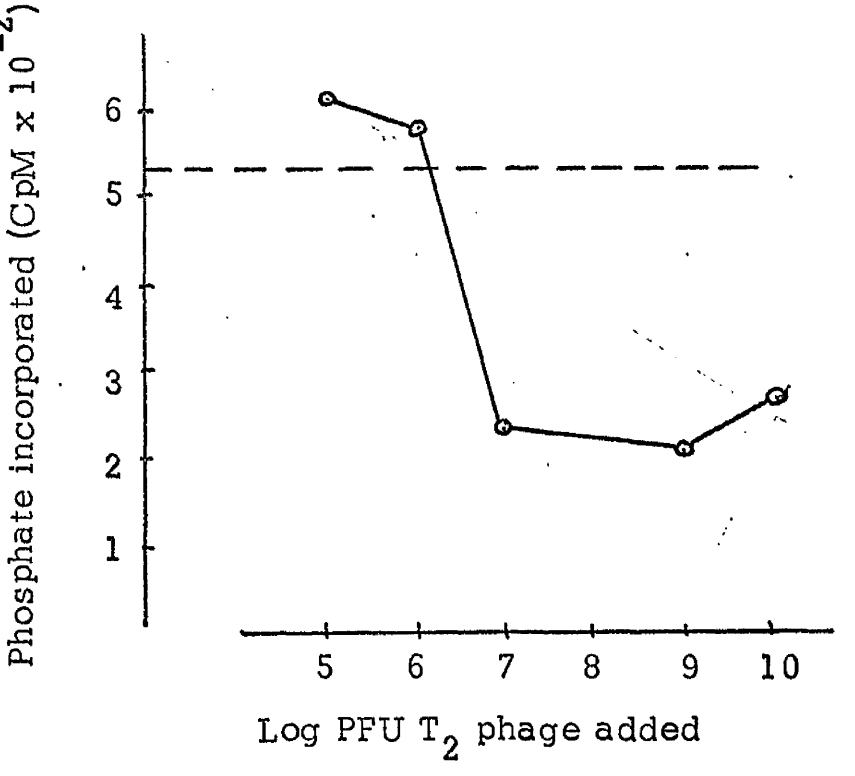
(v) 0.1 ml. of T_2 suspension (10^6 PFU/ml.)

(vi) 0.1 ml. of EHM

Samples were incubated as in Experiment 1 and these samples were harvested at 30 minutes following the setting up of incubation.

Fig. 8 shows that, when the ratio of T_2 phage to peritoneal cells was 10 to 1 or less, there was a negligible increase in

Fig. 8 : The effect of increasing the concentration of T_2 phage incubated for 30 minutes with peritoneal cells on the uptake of phosphate into phospholipid.



Dotted line indicates phosphate uptake in the absence of antigen

uptake of inorganic phosphate into phospholipid. At ratios of 100 : 1 or greater, there was a reduction of uptake of phosphate to about 50% of control values.

Initially the increase in uridine uptake as a function of time was investigated when peritoneal exudate cells were cultured in vitro with T_2 phage. Accordingly, the uptake of [^{14}C] uridine in control and test tubes was studied over the intervals 30, 60 and 120 minutes respectively after inception of culture.

As a control the incorporation of [^{3}H] adenosine into control and T_2 phage stimulated peritoneal cells was studied over the same time intervals.

EXPERIMENT 7 : Time course of the uptake of [^{14}C] uridine and [^{3}H] adenosine into RNA of both non-stimulated and T_2 stimulated peritoneal exudate cells.

Peritoneal cells were suspended in 24 ml. of EHM, giving a concentration of 2×10^6 million cells/ml. The cell population was divided into two equal suspensions and to one was added 100 μCi [^{3}H] adenosine (500 M Ci/mM) and to the other was added 10 μCi ^{14}C uridine (60.7 M Ci/mM).

Each of these cell populations was divided equally in two and to one of each group was added 0.12 ml. T_2 10^{10} PFU/ml. while the controls in each group received 0.12 ml. PBS.

One ml. samples of both control and stimulated samples were incubated at 37°C in an atmosphere of 5% CO_2/95% and were harvested at 30, 60 and 120 minutes respectively after the setting up of culture.

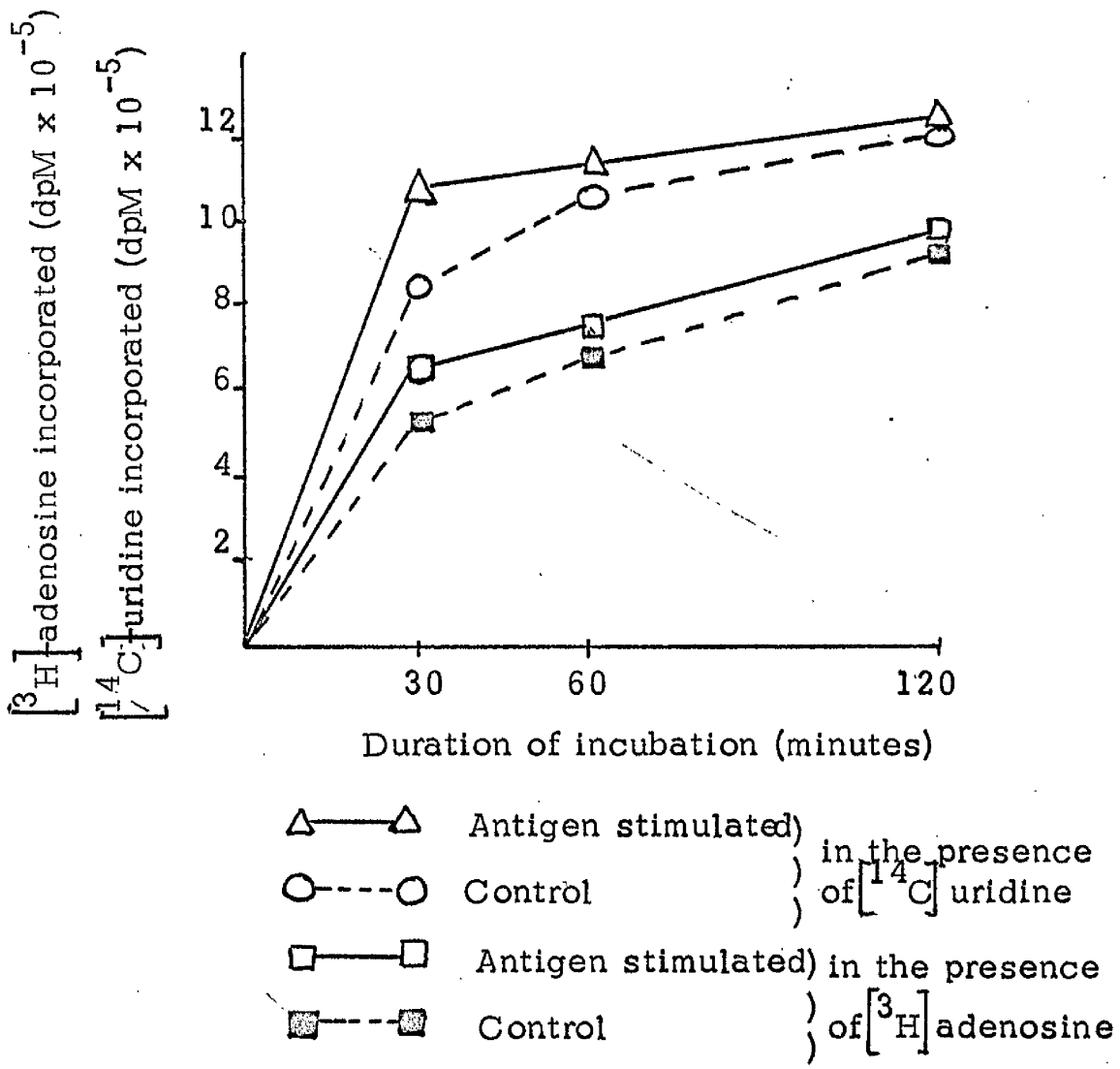
The reason for using uridine as an RNA precursor was the fact that the uridine pool in the cells is small, there is rapid equilibration between internal pool and added isotope, and most of the uridine pool is used in RNA synthesis unless there is any significant degree of glycogen synthesis. However, there are certain disadvantages associated with usage of uridine to detect increase in RNA synthesis. In other cell systems involving uridine uptake, it has been suggested that the effect of stimulus might be to alter the de novo synthesis of uridine or to alter the rate of uptake of exogenous labelled uridine into the internal uridine pool, the result being different specific activities in the uridine pools of control and stimulated cells. Thus, subsequent incorporation into

RNA in stimulated cells will appear to be artificial relative to the control value. At the same time, the incorporation of [^{3}H] adenosine into stimulated and non-stimulated cells was also studied. While [^{3}H] adenosine is a precursor in many other pathways, notably ATP biosynthesis, there is a relatively large and stable internal adenosine pool.

It can be seen in Fig. 9 that the increase in uridine uptake by peritoneal cells, in the presence and absence of T_2 phage, rose sharply over the first 30 minutes of incubation, with uptake being greater in the presence of T_2 phage than in its absence. Thereafter, the uptake in both control and stimulated cultures fell off. However, at 60 minutes, the uptake of uridine in stimulated cells was still greater than in control cells. After 120 minutes, the uptake in control and test samples was similar. This would indicate a short increase in RNA synthesis in stimulated cells. In both test and control samples the [^{3}H] adenosine uptake followed a similar pattern.

The fact that uptake of both [^{14}C] uridine and [^{3}H] adenosine followed similar patterns into both control and stimulated cells indicated that (i) <u>de novo</u> synthesis of uridine or (ii) uptake of

Fig. 9 : Time course of uptake of uridine and adenosine into peritoneal cells in the presence and absence of antigen.



labelled uridine into peritoneal cells was not being altered by incubation with antigen. This work provides only an indication that uridine pools in control and stimulated cells have comparable specific activities. To be more certain of this, the specific activities of the internal uridine pools in stimulated and control cells would have to be measured. This, however, was not attempted at this time.

Fishman, Van Rood and Adler (1964) had noted different immunogenic capacity associated with RNA extracted from peritoneal cells incubated with different concentrations of T_2 phage. Experiment 8 was undertaken to note the effect of changing the ratio of T_2 phage to peritoneal cells on uridine uptake into the RNA of the peritoneal cells.

EXPERIMENT 8 : The effect of changing the concentration of stimulatory T_2 phage incubated with peritoneal exudate cells on the uptake of uridine by those cells.

Peritoneal cells were resuspended in 8 ml. of EHM, giving a concentration of 10^5 million/ml. To the cell suspension was added 150 µCi of $\left[^3H\right]$uridine. This suspension was then divided into 8 equal 1 ml. populations. Then 0.1 ml. of the

following suspensions was added to duplicate tubes :

(i) T_2 phage suspension (10^8 PFU/ml.)

(ii) T_2 phage suspension (10^9 PFU/ml.)

(iii) T_2 phage suspension (10^{11} PFU/ml.)

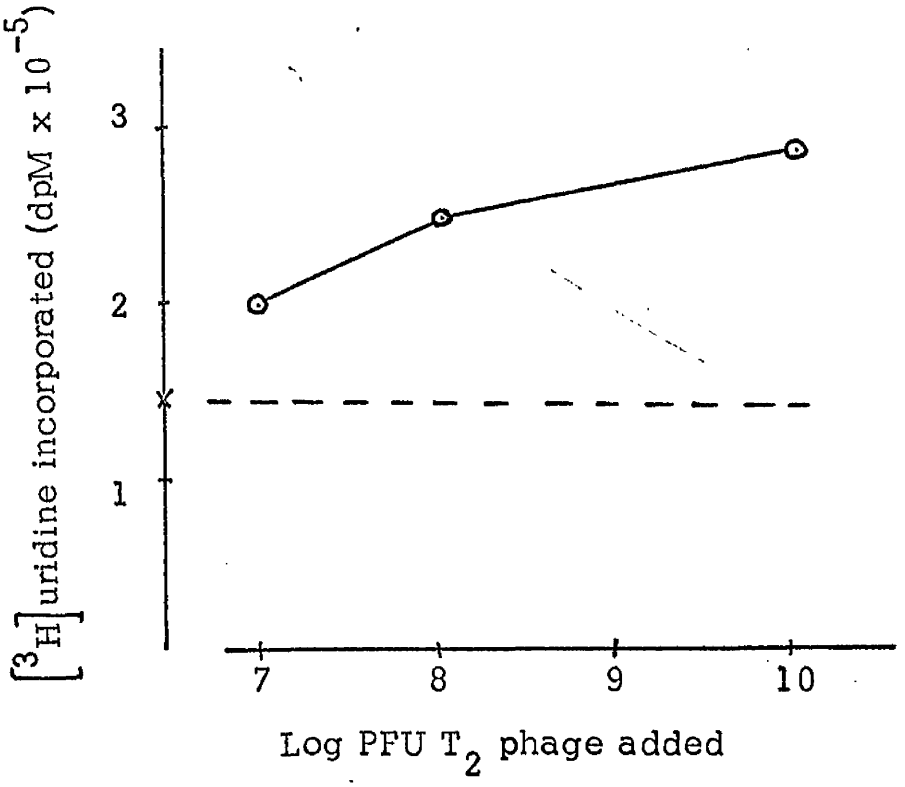
(iv) EHM

One ml. samples of the control cells and the various stimulated cells were incubated as in Experiment 1 and were harvested at 30 minutes after the setting up of the incubation.

The results of this experiment can be seen in Fig. 10 where it is observed that the ratio of T_2 phage to peritoneal macrophages was increased during a one-hour culture period then the amount of ^3H uridine incorporated into acid precipitable material correspondingly increased.

The results of Experiments 7 and 8 indicate a stimulus of RNA synthesis in the peritoneal cells stimulated by T_2 phage over control cells. This stimulus appears to be dependent on the ratio of T_2 phage to peritoneal cells - the higher the ratio, the greater the increase. A more detailed study of the newly formed RNA would be of interest since it is not clear whether the stimulus to RNA synthesis is a general one

Fig. 10 : The effect on uridine uptake of increasing the concentration of T_2 phage incubated for 30 minutes with peritoneal cells



Dotted line indicates uridine uptake in the absence of antigen.

or whether it is confined to a particular species of RNA. In order to elucidate this aspect of the problem, fractionation of RNA after a 30-minute pulse was carried out on a sucrose density gradient.

EXPERIMENT 9 : Sucrose Density Gradient analysis of RNA from control and T_2 phage stimulated peritoneal cells following a 30-minute pulse of labelled uridine.

Peritoneal cells were suspended in 10 ml. of EHM at a concentration of 2×10^7 million cells/ml. This population was divided in two. To one portion was added 125 µCi $\left[^3H\right]$ uridine (1.52 Ci/mM) along with 0.5 ml. T_2 phage 0.2×10^{11} PFU/ml., while the other portion received 5 µCi $\left[^{14}C\right]$ uridine (60.7 M Ci/mM) and 0.5 ml. PBS.

After 30 minutes of incubation, the cells were washed free of isotope and then control and stimulated cells were combined in suspension with Buffer A and RNA was extracted as described above. The RNA was resuspended in 1 ml. of Buffer B to a concentration of 470.4 ug/ml.

117 ug of RNA in 0.25 ml. of Buffer B were layered on to a 5 - 20% sucrose gradient and centrifuged for 2 hours at

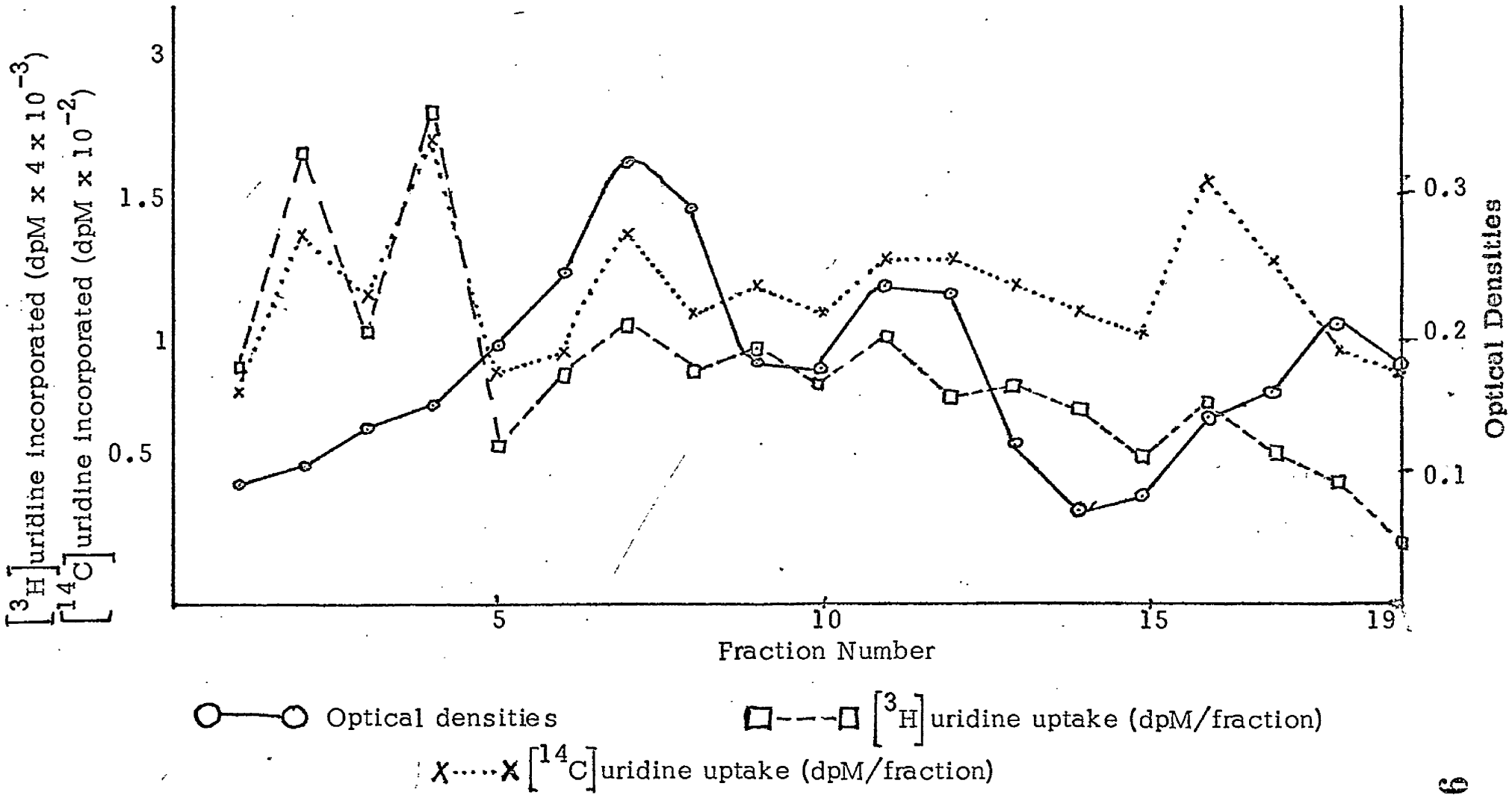
205,000 g at 2°C. The gradient was harvested by collecting drops through a hole pierced in the bottom of the tube. Absorbance at 260 mu was noted for each fraction which was then precipitated with 5% TCA on to millipore filters and radioactivity estimated in toluene with PPO using a liquid scintillation counter.

Fig. 11 shows the sucrose density gradient pattern of RNA extracted from both control and T_2 phage stimulated peritoneal exudate cells grown in the presence of radioactive uridine for 30 minutes. The control cells had been incubated with 0.002 μ moles of $\left[^{14}C\right]$ uridine and the stimulated cells with 0.002 μ moles of $\left[^{3}H\right]$ uridine. While both populations received equimolar amounts of radioactive uridine, the specific activities of the stimulated cells was 25 times greater than that given to control cells. Thus, if no stimulus of uridine uptake into RNA occurs in the peritoneal exudate cells incubated with T_2 phage, then the ratio of disintegrations per minute associated with the RNA from stimulated cells to the disintegrations per minute associated with RNA from control cells will be constant at 25 along the

gradient. Fig. 11 shows, however, that this ratio is above 25 at the heavy end of the gradient and below 25 at the light end of the gradient. This does suggest that an increase in RNA synthesis by peritoneal exudate cells occurs preferentially in the RNA which sediments at the heavy end of the gradient when peritoneal cells are presented with T_2 phage for a 30-minute culture period. Much of this increase appears to be associated with species of RNA whose sedimentation coefficients are greater than 28 S.

As much of the radioactivity in Fig. 11 is associated with species of sedimentation coefficient greater than 28 S, it is not possible to say whether it represents precursor to ribosomal RNA or whether it is heterodisperse nuclear RNA. By following the 30-minute pulse of [^{3}H] uridine with non-radioactive uridine, the further metabolism of these species of RNA may throw some light on their nature, i.e. ribosomal or nuclear RNA. Such an experiment would also give some idea of the stability of the RNA synthesised during the 30-minute incubation period.

Fig. 11 : Sucrose density gradient of RNA extracted from a suspension containing 2 populations of peritoneal cells. One population was incubated for 30 minutes with T_2 phage and $[^3H]$uridine while the other was incubated with $[^{14}C]$uridine alone.



EXPERIMENT 10 : To investigate the further metabolism of RNA species synthesised by peritoneal cells during a 30-minute pulse of [^{3}H] uridine and in the presence of T_2 phage.

Peritoneal exudate cells were resuspended in 10 ml. of EHM at a concentration of 2×10^6 million cells/ml. 0.1 ml. of T_2 phage suspension (2×10^{10} PFU/ml.) was added along with 150 µCi uridine and the suspension was divided in two. Both tubes wee incubated as in Experiment 1 for 30 minutes. One tube was removed and RNA was extracted from these cells as before. To the other tube was added 0.1 ml. cold uridine (.2 g/ml.) which was approximately 1,000 times greater than the amount of labelled uridine previously added. Incubation of this tube continued for $2\frac{1}{2}$ hours after which RNA was extracted from the chased cells.

Both RNA samples were resuspended in 1 ml. each of Buffer B. The concentration of the 30 minute incubation RNA was 320.8 ug. RNA/ml. while that of the 3-hour RNA was 270.1 ug. RNA/ml. 80 ug. of the 30 minute pulsed RNA in 0.25 ml. of buffer was layered on to a 5 - 20% sucrose

gradient. 70 ug. of the 3-hour RNA in 0.25 ml. of buffer was layered on to a 5 - 20% sucrose gradient. Both RNA specimens were separated on the gradient, collected and counted as described previously.

This experiment attempts to examine the stability of the RNA species synthesised by peritoneal cells in the presence of T_2. The results of this experiment are seen in Figs. 12 and 13 which show a comparison of radioactive profiles of RNA extracted from T_2 phage stimulated cells which were pulsed for 30 minutes with [^{3}H]uridine and RNA extracted from T_2 phage stimulated cells which were subjected to a 30-minute pulse with ^{3}H uridine followed by an incubation for 2½ hours with non-radioactive uridine. It will be seen that most of the radioactivity associated with species of RNA, of sedimentation values greater than 28 S in Fig. 12 became associated with ribosomal RNA species 28 S and 18 S and with the 4 S transfer RNA peak in Fig. 13. This indicates that a large amount of fast sedimenting RNA synthesised during the initial 30-minute pulse is probably of the pre-ribosomal RNA species. This opinion is reinforced by the

Fig. 12 : Sucrose density gradient of RNA extracted from peritoneal cells incubated with T_2 phage and [^{3}H] uridine for 30 minutes.

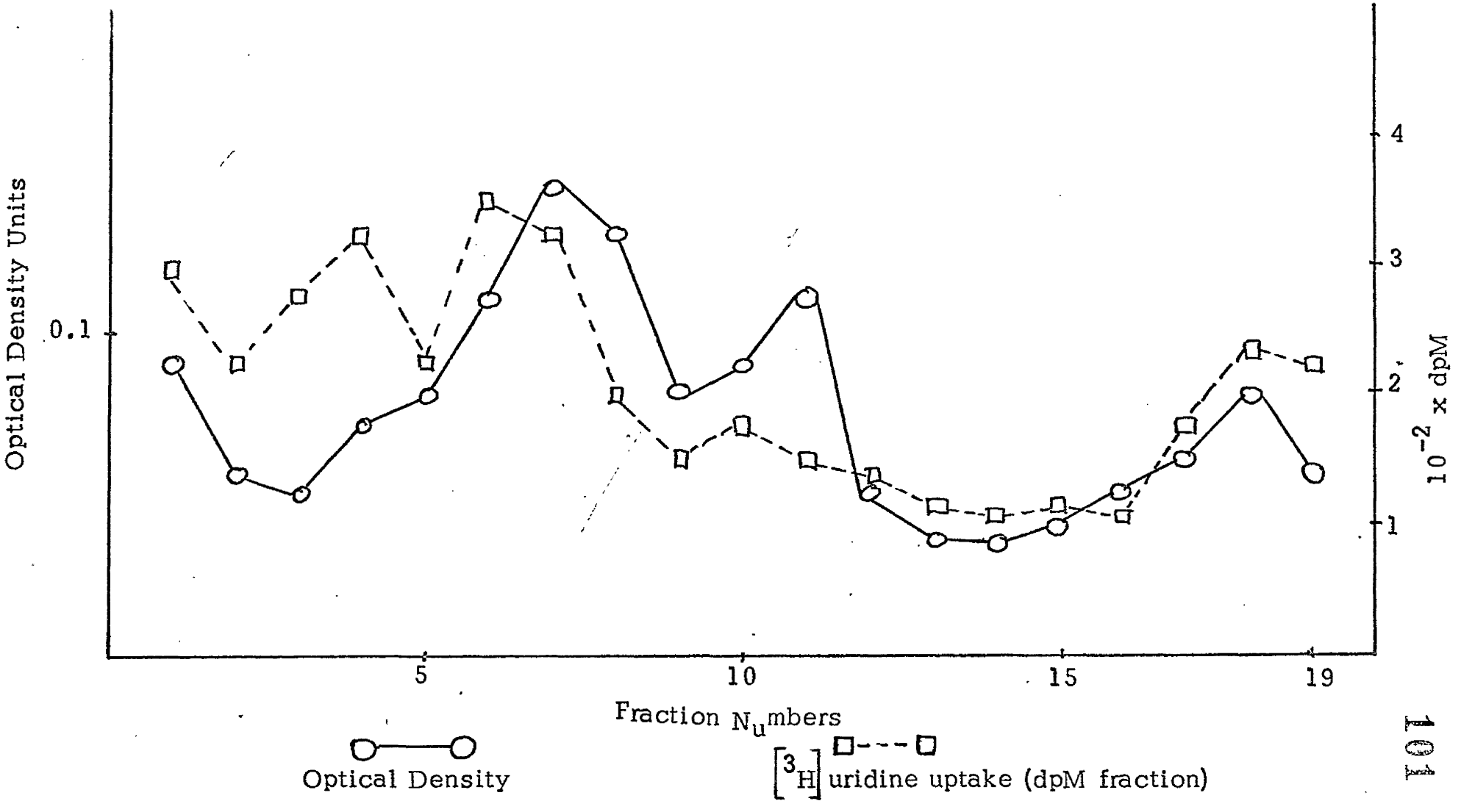
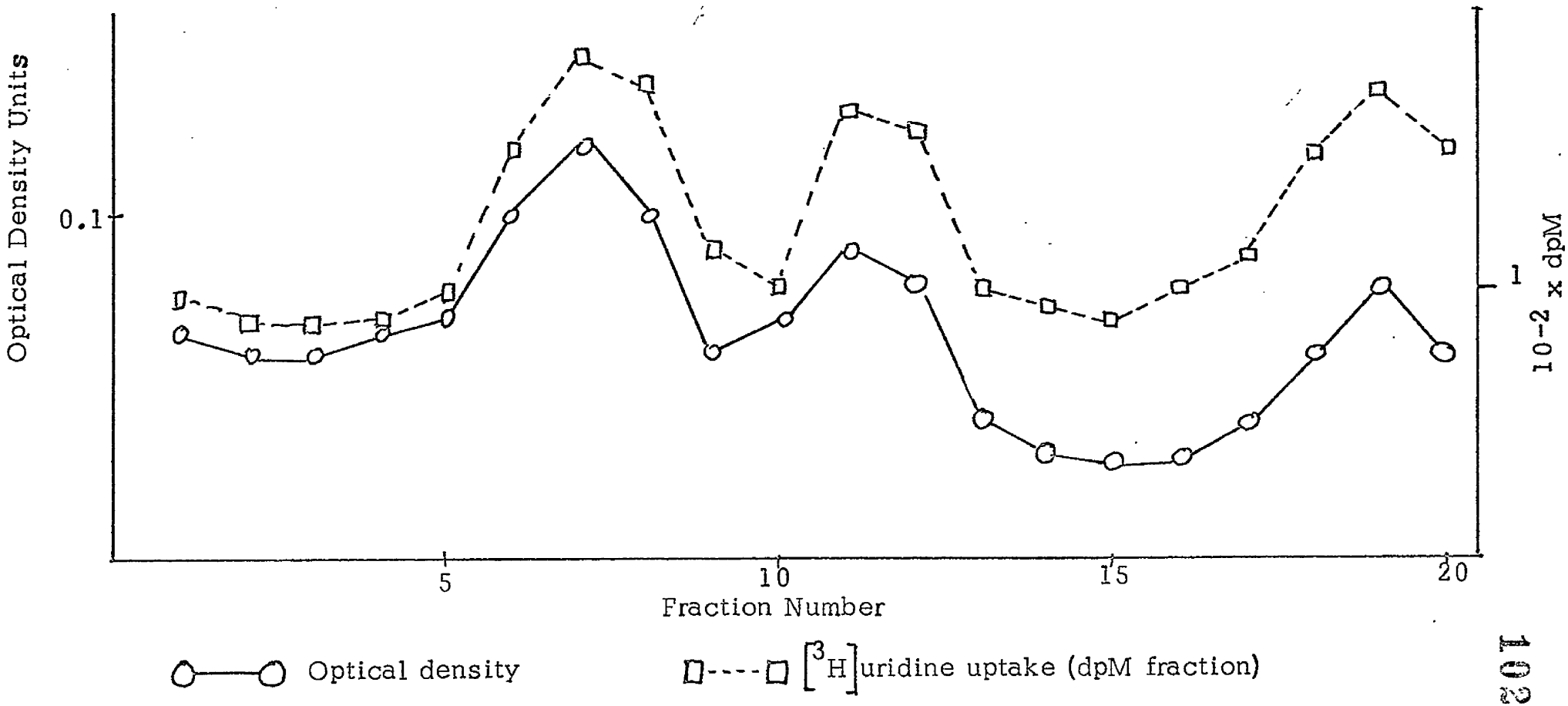


Fig. 13 : Sucrose density gradient of RNA extracted from peritoneal cells incubated with T_2 phage. [^{3}H]uridine was present for the first 30 minutes of incubation and this was followed by 1000 times the amount of unlabelled uridine for the remaining 150 minutes of incubation.



fact that there are two peaks of radioactivity in the region of the graph to the left of the 28 S optical density peak.

Askonas and Rhodes (1965) reported that antigenic fragments were associated with RNA extracted from peritoneal cells which had been incubated with antigen. In view of this, it was decided to examine RNA preparations for antigenic association. Both soluble BSA and particulate T_2 were used and an attempt was made to characterise the sedimentation coefficient of antigen associated RNA.

EXPERIMENT 11 : To scan the Sucrose Density Gradient profile of RNA from peritoneal exudate cells incubated *in vitro* with labelled T_2 phage.

Peritoneal cells were suspended in 10 ml. of EHM, giving a concentration of 5×10^6 million/ml. 150 µCi of $[^3H]$ uridine was added and the population was divided into two equal portions. To one suspension 0.25 ml. of $[^{14}C]$ labelled T_2 phage (10^{10} PFU/ml.) was added and both suspensions were incubated at 37°C for 30 minutes. After the 30-minute incubation, the second suspension was treated with a similar

volume of [^{14}C] labelled T_2 phage. RNA was then extracted separately from both suspensions. 100 µg. of both RNAs was separated on a 5 - 20% sucrose gradient.

Fig. 14 shows the radioactive profile of RNA extracted from peritoneal cells exposed for 30 minutes to [^{3}H] uridine and [^{14}C] labelled T_2 phage applied to a sucrose density gradient. The gradient conditions were 5 - 20% sucrose, centrifugation being for 2 hours at 105,000 g at 2°C. Under these conditions T_2 phage which has a particle weight of 2.2×10^8 will sediment to the bottom of the tube. It can be seen that there is a ^{14}carbon peak to the left of the 4S transfer RNA optical density peak. This cannot be undegraded T_2 phage for the reason outlined above. The band is well defined and therefore it is unlikely that it represents partially degraded T_2 phage proteins. Moreover, the band lies below the 4S peak. Degraded T_2 phage proteins would band more widely and more would be found in the lightest portion of the gradient. A more likely explanation is that small portions of degraded phage proteins are complexed with a larger RNA species of constant molecular weight. This would account for the definitive

Fig. 14 : Sucrose density gradient of RNA extracted from peritoneal exudate cells incubated for 30 minutes with [^{14}C] labelled T_2 phage.

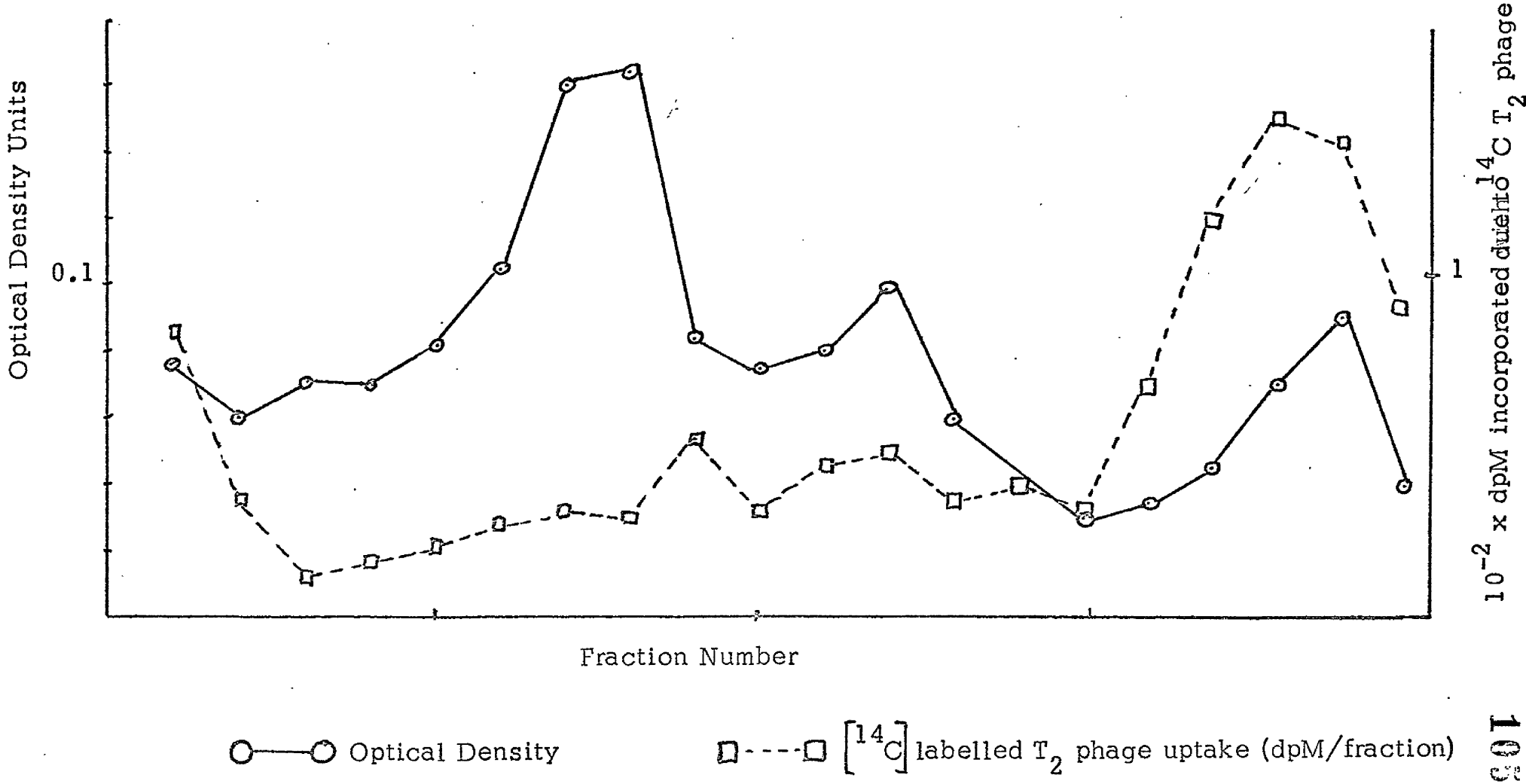
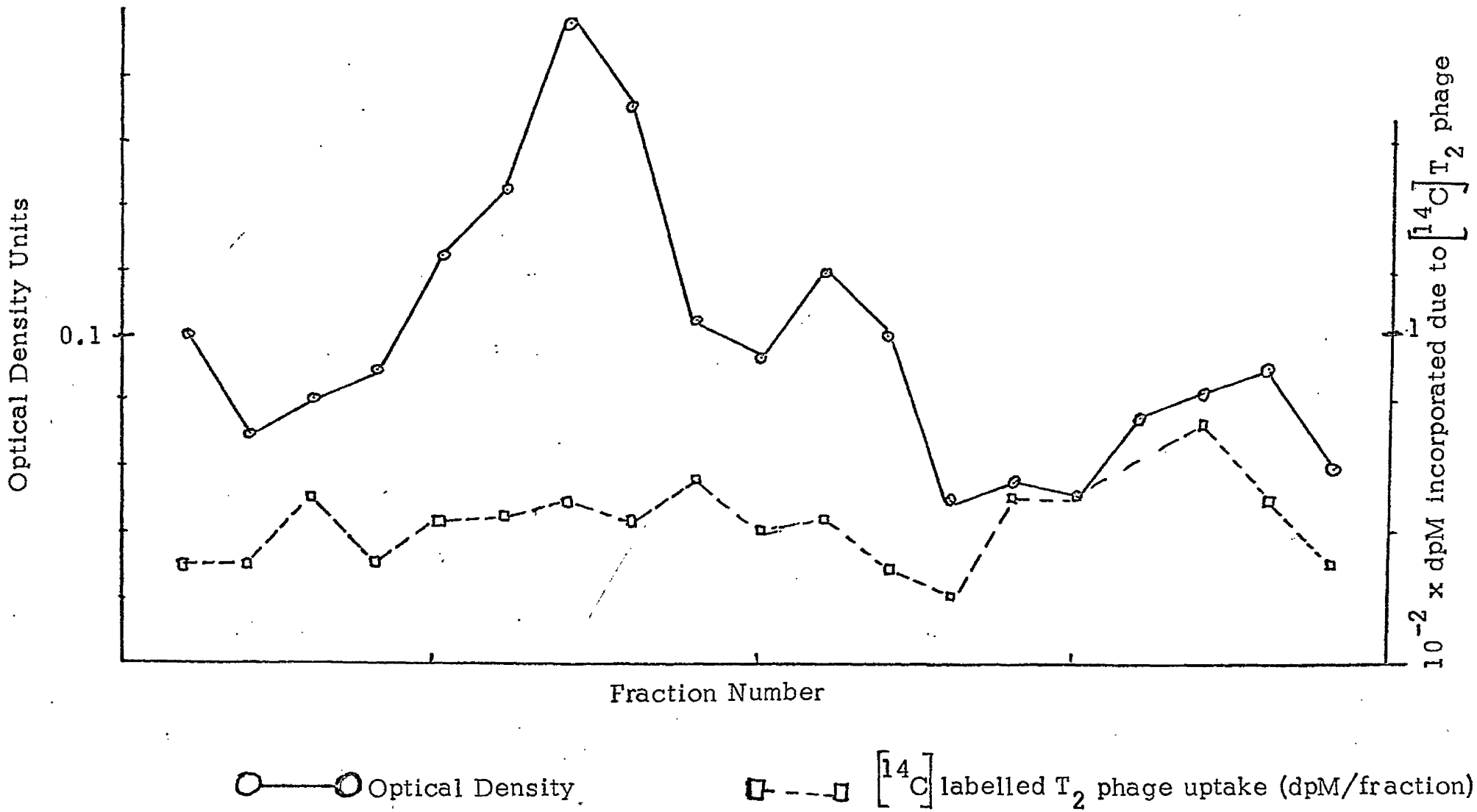


Fig. 15 : Sucrose density gradient of RNA extracted from peritoneal exudate cells to which [^{14}C] labelled T_2 phage was added just prior to RNA extraction.



banding . Fig. 15 shows that, where T_2 was added after 30 minutes of incubation, association between labelled T_2 phage fragment and an RNA species still took place and this RNA protein complex banded at the same position as the RNA protein complex in Fig. 14.

The next experiment attempts to ask whether the soluble antigen BSA is treated in similar fashion by the peritoneal exudate cells.

EXPERIMENT 12 : To determine the nature of association between labelled BSA and the RNA of peritoneal exudate cells with which it is incubated.

Peritoneal cells were resuspended in 10 ml. of EHM, giving a cell concentration of 8×10^5 million/ml. The cell population was divided into two equal suspensions. To one was added 0.5 ml. of a solution of $[^{14}C]$ labelled BSA (1 mg./ml.). This contained 44,900 counts. To the control suspension was added 0.5 ml. of EHM. Both tubes were incubated as before. After 30 minutes' incubation, the previously untreated tube received 0.5 ml. of labelled BSA and RNA was extracted

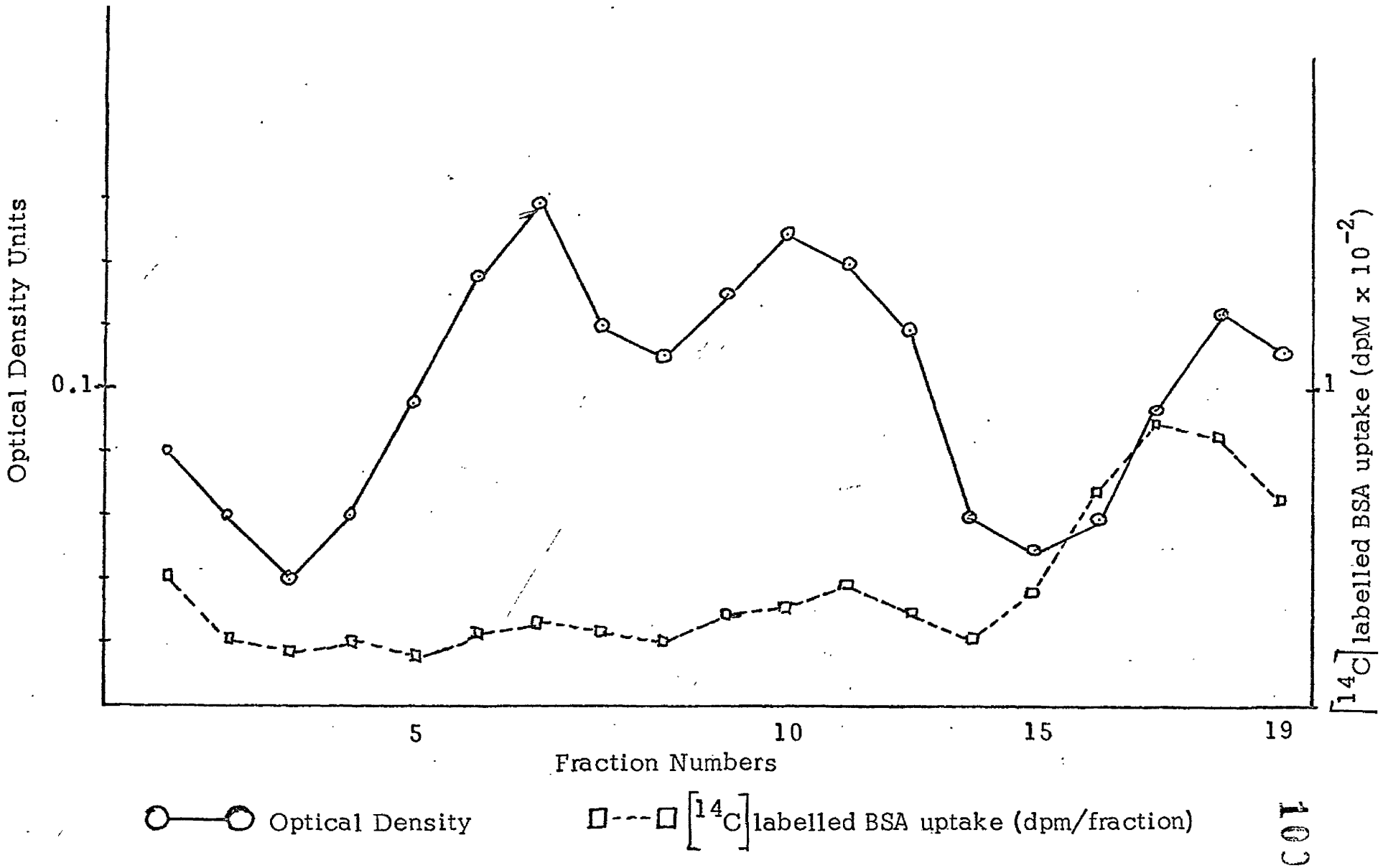
separately from each suspension. 50 µg. RNA from each suspension was separated on 5 - 20% sucrose density gradient.

As the labelled BSA was seen from Fig. 16 to band with low molecular weight RNA, and this is where undegraded BSA is expected to band, it was decided to separate the unincorporated BSA from BSA associated with RNA on a buoyant density cesium chloride gradient.

The density of the gradient was 1.500 and the tubes were centrifuged for 40 hours at 130,000 g. Under these conditions the protein associated with RNA will pellet while unassociated material will float. The pellet was then resuspended and precipitated on to millipore filters and counted as before.

Fig. 16 shows that, when Experiment II is repeated with the modification that the antigen used is soluble [^{14}C] labelled BSA instead of the particulate T_2 phage, then a similar pattern of antigen distribution on the gradient is noted. However, an additional difficulty is encountered here as undegraded, unassociated BSA also sediments at the same position on the gradient. Using buoyant density centrifugation, the unassociated BSA is separated from the BSA associated with RNA.

Fig. 16 : Sucrose density gradient of RNA extracted from peritoneal cells incubated for 30 minutes with [^{14}C] labelled BSA



The precipitated RNA with associated BSA was assayed for radioactivity and Table 3 shows the counts due to BSA associated with RNA.

TABLE 3

RNA from -	CPM due to labelled BSA fragments associated with RNA
(i) Peritoneal cells incubated with labelled BSA for 30 minutes	128
(ii) Peritoneal cells to which labelled BSA was added prior to extraction	35

Using hybridisation studies, Gottlieb, Glisin and Doty (1967) reported that the RNA species, which was complexed to fragments of the antigen to which the peritoneal cell had been exposed, was indistinguishable from RNA species present in unexposed peritoneal cells. This suggests that the RNA species which would be involved in forming an RNA antigen complex would be present in the peritoneal cell prior to antigen exposure. The effect of incubation of the cells with Actinomycin D was investigated to see if the formation

of the RNA antigen was impaired.

Gallily and Feldman (1967) used peritoneal macrophages which had been incubated with shigella and then injected this into mice exposed to 550 Rads. They noted that these cells triggered the formation of agglutinating antibody in animals which did not respond to the injection of antigen alone. It had previously been shown by Wish et al.(1952) that phagocytosis of carbon particles or colloidal gold by cells of the reticulo-endothelial system is unaffected by X-irradiation. Thus, Gallily and Feldman concluded that the irradiation of macrophages leading to immunological suppression was at least due to the impaired capacity of the macrophages to "process" the antigen. It was therefore decided to investigate the effect of X-irradiation on the capacity of the macrophage to form an antigen RNA complex.

EXPERIMENT 13 : To determine the effect of (i) Actinomycin D and (ii) X-irradiation on the ability of the peritoneal exudate cell to complex labelled BSA to its RNA.

Peritoneal cells were resuspended in 10 ml. of EHM at a cell concentration of 1.5×10^6 million/ml. The suspension was divided into 4 equal parts. Suspension 1 received 0.5 ml. of labelled BSA (1 mg./ml.) - 44, 900 counts/min. Suspension 2 received similar treatment after 30 minutes' incubation and just prior to RNA extraction. Suspension 3 received 15 µg. Actinomycin D, giving a concentration of 5 µg./ml., followed by 0.5 ml. of labelled BSA. Suspension 4 received 600 Rads. of X-irradiation followed by 0.5 ml. of labelled BSA.

All tubes were incubated as before and labelled BSA having been added to Suspension 2, RNA from each suspension was extracted and resuspended in Buffer B.

As in Experiment 12, unassociated BSA was separated from BSA complexed to RNA by buoyant density cesium chloride gradients.

The effect of X-irradiation and Actinomycin D on the ability of the peritoneal macrophage to complex antigenic fragments to its RNA is reported in Table 4. It was observed that this ability was seriously impaired by both treatments.

TABLE 4

Suspension No.	CPM above background due to presence of antigenic fragments associated with RNA
1	86.5
2	37.4
3	15.9
4	17.0

It is difficult to put an exact interpretation on these results as probably more than one function of the peritoneal cells may be adversely affected by either treatment. Further consideration of this topic will be offered in the Discussion. However, it is possible to say at this juncture that RNA synthesis is necessary for the formation of an RNA antigen complex.

The previous experiments described the formation of an antigen-RNA complex in peritoneal exudate cells incubated with antigen. Previous investigations showed that RNA from peritoneal cells exposed to antigen was capable of stimulating specific antibody formation when added to lymph node cells previously unexposed to the antigen. The immunogenic fraction of the RNA is now thought to be the antigen-RNA complex. However, in Experiments 14 and 15, gross RNA extracted from peritoneal cells exposed to (i) soluble BSA and (ii) particulate T_2 phage was tested for its ability to induce anti-BSA and anti-T_2 formation respectively in lymphoid tissue in culture.

EXPERIMENT 14

3.5×10^7 peritoneal cells in 5 ml. of EHM were incubated with 250 mg. BSA in 0.25 ml. of PBS. As control, BSA was left out of the incubation. After 30 minutes' incubation at $37^{\circ}C$, the macrophages were washed free of BSA and the RNA was extracted as before from both populations. These RNA preparations were suspended in Buffer B and their concentrations determined spectrophotometrically.

100 µg. of RNA in 1 ml. EHM from test macrophages was added to a plastic petri dish containing lymph node cubes in culture medium. Similarly 100 µg. of RNA in 1 ml. of EHM from the control macrophages was added to a petri dish containing lymph node cubes in culture medium. In all, 6 test and 6 control plates were prepared. After 4-day incubation, the gamma globulins in test and control culture fluids were isolated as described above.

To check the system, serum from a rabbit, immunised to BSA, was tested for anti-BSA activity using the indirect haemagglutination test. It was found that the end point of anti-BSA activity was outside the range of dilutions tested. In spite of this, no antibody activity could be detected in the gamma globulin preparations. Thus, in these studies it was not possible to induce antibody formation in lymph node cells in culture in the presence of RNA extracted from peritoneal cells previously incubated with soluble BSA.

In the next experiment the same procedure as that employed in Experiment 14 is followed, except that the particulate antigen T_2 phage is used in place of BSA. The assay system used in this experiment has the advantage over the indirect haemagglutination test of several orders of sensitivity in detection of specific antibody.

EXPERIMENT 15

Peritoneal exudate cells were harvested from rabbits injected 4 days previously with paraffin. They were pooled in EHM and cell count taken showing a total cell population of 7.8×10^7. This was divided into two 5 ml. sub-populations, one of which received 4×10^9 PFU T_2 phage in 0.4 ml. phosphate buffered PBS saline and the other received 0.4 ml. phosphate buffered saline. After 30 minutes' incubation at 37°C, the macrophages were washed free of antigen and RNA extracted from both populations as described above.

The RNA preparations were suspended in Buffer B and their concentrations determined spectroscopically.

100 µg. of test RNA in 1 ml. of medium was added to plates while 100 µg. of control RNA in 1 ml. of medium was added to further plates.

After the 4-day incubation period, the gamma globulins were prepared for control and test culture fluids as described in Materials and Methods and assayed for anti-T_2 activity.

TABLE 5 : ASSAY OF ANTI-T_2 ACTIVITY

Incubation	Average Plaque Count in 2 Plates*
3×10^2 PFU + PBS	302
3×10^1 PFU + PBS	28
3×10^0 PFU + PBS	5
3×10^2 PFU + gamma globulin preparation	343
3×10^1 PFU + gamma globulin preparation	24
3×10^0 PFU + gamma globulin preparation	4

* After 30 minutes' incubation the T_2 phage and PBS or gamma globulin preparation was mixed with E. Coli Strain B and poured on to agar plates. After suitable incubation, a plaque count can be taken.

The results presented in Table 5 are comparable to those in the previous experiment where a different antigen was used. Thus, RNA extracted from T_2 phage infected macrophages was unable to confer immunogenicity on a population of normal lymph node cells in culture. Test culture fluids were unable to reduce the number of plaques which were formed in control plates.

STUDIES ON PERITONEAL EXUDATE CELLS

DISCUSSION

Research over the last decade has highlighted the possibility that macrophage RNA may have an important role to play in immune responses. In this study, peritoneal exudate cells, which contain ~90% macrophages were used. The uptake of labelled uridine into control peritoneal cells and into peritoneal cells stimulated by T_2 was examined. It was found that uridine uptake was accelerated in the presence of antigen. Moreover, the greater the concentration of antigen incubated with peritoneal cells, the greater was found to be the uptake of uridine by these cells. When RNA, extracted from peritoneal exudate cells which had been incubated with labelled uridine and T_2 phage, was separated on a 5 - 20% sucrose density gradient, it was seen that a large amount of radioactivity was associated with species of RNA which had greater sedimentation coefficients than the 28 S optical density peak. The fact that there are two peaks in this area, one just to the left of 28 S, suggests strongly

that these were ribosomal precursor peaks at 45 S and 32 S respectively. This suggestion is reinforced by a comparison of Figs. 12 and 13, where the 30-minute pulse with ^{3}H uridine was followed by a 150-minute incubation with unlabelled uridine. The radioactivity in the 45 S and 32 S peaks (largely) disappears and emerges in the 28 S and 18 S peaks. This is similar to the result obtained by Girard, Penman and Darnell (1964) with hela cells and Actinomycin D.

When rat peritoneal cells were incubated with BSA in vitro, Halac, Rife and Rinaldi (1964) had reported changes in the base composition of ribonucleic acid relative to control RNA. This change involved a slight increase in G + C content content and, as ribosomal RNA and pre-ribosomal RNA have a much higher G + C content than lower molecular weight RNA species, the results may be taken to indicate an increase in RNA synthesis in the ribosomal area of the gradient. Yet there was also the possibility that the radioactivity in the rapidly sedimenting part of the gradient was not all associated with pre-ribosomal species. The (apparent) comparative increase in pre-ribosomal RNA in peritoneal cells incubated

with T_2 phage could possibly have been due in part to an increase in heterodisperse nuclear RNA. This RNA, which bands between 20 S and 100 S is of uncertain significance but it is a possible precursor of messenger RNA. Soeiro, Birnboim and Darnell (1966) having shown that heterodisperse nuclear RNA has a base composition similar to DNA. As this species of RNA has no characteristic optical density profile, it is difficult to detect its presence and therefore no attempt was made to do so.

However, while one may consider the possible contribution of heterodisperse RNA to the radioactivity appearing at the heavy end of the gradient, it is clear that much of the RNA synthesised by peritoneal exudate cells incubated with T_2 during a 30-minute pulse with labelled uridine was pre-ribosomal and ribosomal in character. This result is not in accord with the observation of Gottlieb, Glisin and Doty (1967) using rat peritoneal exudate cells. They found that these cells, exposed to a 30-minute pulse of [^{3}H] uridine which was added with T_2 phage, synthesised RNA which banded predominantly at the 4 - 5 S part of the gradient. These authors had used

T_2 phage and peritoneal cells in the ratio 20 : 1 whereas, in our studies, the ratio used was 100 : 1. The effect of increasing the concentration of antigen incubated with peritoneal cells was found from Fig. 10 to be to increase the synthesis of RNA. It may thus be that the stimulus to increased ribosomal RNA synthesis does not occur until the ratio of T_2 phage to peritoneal cells is above 20. The fact that control cells in Fig. 11 had a radioactivity profile more akin to T_2 stimulated profile in Fig. 11 rather than the radioactivity profile reported by Gottlieb et al. tends to argue against this notion. When Roelants and Goodman (1968) incubated poly γ-D-glutamic acid and ^{14}C uridine with rabbit peritoneal cells for 30 minutes, they found that 4 - 5 S RNA was the only type of RNA labelled. Other authors have claimed that 4 - 5 S RNA is the type predominantly synthesised by peritoneal macrophages in contact with antigen. Bishop, Piscotta and Abramoff (1967) incubated peritoneal cells with Sheep Red Blood Cells and followed this 15 minutes later with a 30-minute pulse of radioactive phosphate. When RNA was extracted from these peritoneal cells and separated on a sucrose density gradient,

the 4 - 5 S species were the only RNA species to be significantly labelled. Moreover, 4 - 5 S RNA from stimulated cells exhibited an increase in uptake of label over 4 - 5 S RNA from unstimulated cells. However, if a 3-hour pulse with radioactive phosphate was conducted in the absence of antigen, 28 S, 18 S and 4 - 5 S RNA species were equally labelled. An objection to the use of phosphate as a precursor of RNA species is that it would reflect the very rapid turnover of the terminal residues of the transfer RNA species. The terminal residues p C p C p A at the 3'OH end of the transfer RNA species turnover more rapidly than the transfer RNA itself. Thus, studies employing radioactive phosphate uptake into RNA would result in a relatively large uptake of phosphate in the 4 - 5 S region.

Hammond and La Via (1966) found that most of the RNA, newly synthesized by peritoneal macrophages in the presence of phage R17 and [^{3}H]uridine, sediments on a sucrose density gradient in the 4 - 10 S region. By sequential extraction of the cells at 20°C, 45°C and 65°C and by salt precipitation, they prepared messenger RNA which contained most of the

radioactive uridine. They then concluded that most of the newly synthesised RNA was messenger RNA.

These disparate results have been obtained with peritoneal exudate cells from various species and using different antigens and isotopes. In spite of these differences in experimental conditions, it is difficult to rationalise the results.

On some occasions in experiments similar to that described in Fig. 11, most of the radioactivity was found in the 4 - 5 S fraction. This was attributed to degradation of the RNA by contact with contaminated reagents or glassware. If the reagents were made up afresh and sterilized, and all glassware was thoroughly flamed before use, then results akin to those reported in Fig. 11 could consistently be achieved.

The profile of radioactivity displayed in Fig. 11 is similar to those obtained by Mach and Vassalli (1965) when immunised rat spleens and lymph nodes were pulsed with uridine for 20 minutes and also those obtained by Church, Storb, McCarthy and Weiser (1968) when immunised rat spleens were pulsed with ^{3}H uridine for 20 minutes. Now, in both spleen and

lymph node the predominant cell type is the lymphocyte. This prompts one to think that the ribosomal RNA synthesis reported in Fig. 11 may be due to contamination of the peritoneal exudate cell population by lymphocytes which may have had prior encounter with T_2 phage. Yet, when smears of peritoneal exudates were made, the macrophage content was about 90% which was similar to the 90 - 92% reported by Gottlieb, Glisin and Doty (1967). Nevertheless, it should be remembered that the peritoneal exudate contains macrophages in company with a smaller population of lymphocytes and there will be contributions probably very different by the RNAs of both types of cells to the radioactive profiles taken from unfractioned exudates. Raska and Cohen (1968) separated peritoneal exudate cells into (I) cells which adhered to glass - mostly macrophages - and (II) cells which failed to adhere to glass - largely lymphocytes, and used reciprocal competition experiments between RNA species from cells which had been exposed to different antigen. In these experiments, labelled RNA from peritoneal cells exposed to E. Coli was hybridised with DNA. The ability of unlabelled RNA from peritoneal cells

exposed to S RBC to compete with the labelled RNA was taken as an indication of the number of species the two preparations had in common. Using this technique, Raska and Cohen found that both cell populations synthesised new RNA on exposure to antigen. However, the cells which adhered to glass (macrophages) synthesised no different RNA species when exposed to different antigens. When non-adhering cells (most lymphocytes) were similarly investigated, different RNA species were synthesised when these cells were exposed to different antigens. Thus it could be concluded that peritoneal cells stimulated by antigen increase their RNA synthesis in the manner described in Fig. 11. This increase involves increase in ribosomal RNA synthesis by macrophages of the peritoneal exudate. Messenger RNA would be synthesised and would probably be specific for (i) ribosomal proteins, (ii) structural proteins associated with such organelles as lysosomes, and (iii) degradative enzymes. The lymphocytes would contribute an increase in ribosomal RNA necessary for protein synthesis. Messenger RNA would undoubtedly be synthesised. Recently, Lazda, Starr and Rachmeller (1968) investigating the synthesis

of RNA in immunised rat spleen cells concluded that two major species of messenger RNA were synthesised : a labile species which may code for enzymes and structural proteins, while a relatively stable species may be messenger for antibody synthesis.

It has been know since the work of Askonas and Rhodes (1965) that RNA, phenol extracted from peritoneal cells which had been exposed to antigen, contained traces of antigen. Using mice peritoneal cells and [^{131}I] labelled haemocyanin, they showed that a far greater amount of labelled antigen was associated with the RNA when cells had been allowed to phagocytose the antigen in vivo rather than when addition of antigen occurred just prior to phenol extraction. This indicated an active trapping mechanism. This antigen associated RNA was found to be highly immunogenic. The antigenic fragments appeared to be associated with ribosomal RNA, both 18 and 28 S types.

At about the same time, Friedman, Stavitsky and Solomon (1965) reported that they were able to detect head, tail and internal proteins of T_2 phage associated with RNA extracted

from rat peritoneal cells which had been incubated *in vitro* with T_2 phage. Consequently, opinion swung away from the idea that a specific messenger was being transferred from macrophages to lymph node cells and attention has now focussed on the RNA-antigen complex. Fig. 14 indicates that labelled T_2 fragments are associated with an RNA species to give a complex sedimenting on a sucrose density gradient to the heavier side of the 4 S peak.

Roelants and Goodman (1960) investigated the nature of interaction between peritoneal cell RNA and poly-γ-D-glutamic acid and noted that, when the interaction occurred *in vivo*, the labelled polypeptide was associated with 4 - 5 S RNA. If the interaction occurs *in vitro*, then the radioactivity of the polypeptide is displaced slightly towards the top of the gradient. Why there should be a difference between *in vivo* and *in vitro* antigenic encounter is difficult to imagine. It might be tempting to attribute the difference between the nature of association of T_2 particles to RNA and of poly-γ-D-glutamic acid to RNA to the fact that one antigen is particulate and the other is soluble. However, the association between BSA and RNA does appear to be of similar nature to that of T_2 and RNA

(see Figs. 14 and 16). Some caution must be exercised in interpreting Fig. 16 to show the presence of an RNA-antigen complex at approximately 6 S, as this is where unassociated BSA would be expected to band. However, it was seen that labelled BSA which was associated with RNA could be separated from unassociated RNA on a buoyant density gradient.

Fig. 16 and Table 3 show that, when labelled T_2 or labelled BSA were added to peritoneal cells just prior to RNA extraction, some labelled portions of the antigen were associated with the RNA. After the addition of antigen to the peritoneal cell suspension, the cells were washed with EHM and resuspended in buffer containing sodium dedecyl sulphate. It generally took 2 minutes to effect suspension of the cell precipitate prior to addition of phenol. Now, Roelants and Goodman (1968) noted that, while addition of antigen to RNA in vitro did not result in formation of an RNA complex, if the labelled antigen was added to a cell free suspension, then an RNA-antigen complex could be formed. Thus it is possible that the cell suspension would be active

enough over a two-minute interval to degrade any remaining antigen and complex the antigenic fragments to RNA.

Controversy exists about the precise nature of the interference of Actinomycin D with the formation of an immunogenic RNA. Cruchaud, Despont, Girard and Mach (1970) noted that, while there was a relationship between the amount of Actinomycin D added to a suspension of peritoneal cells with BCG and the synthesis of RNA by these cells, there was no relation betwen Actinomycine D and inhibition of the immune response if these cells were then injected to normal recipients. The inhibition they noted was to the late response occurring around day 11 rather than the early response at day 4. In a slightly different system, Fishman, Van Rood and Adler (1965) incubating T_2 with peritoneal macrophages in the presence of Actinomycin D found that the capacity of the RNA extracted from these cells was impaired in its ability to elicit an early response but not a late response when added to lymph node cubes in culture. Actinomycin D at 5 µg./ml. will certainly inhibit RNA synthesis, and in view of the reported importance of RNA-antigen complexes in the transfer of

Immunity (Gottlieb, 1968). It was considered desirable to know whether such complexes failed to form in the presence of Actinomycin D. Table 4 showed that the presence of Actinomycin D inhibited the formation of RNA-BSA complexes. It is very difficult to draw conclusions from this result as to the possible contribution of RNA antigen complexes to immune reactions until the varying reports above are resolved.

Whether the Actinomycin D exerts its inhibitory reaction by inhibiting synthesis of the particular RNA species participating in the RNA-antigen complexes or whether the RNA necessary for synthesis of linking enzymes or degradative enzymes is inhibited, it is not possible to say at present. Gottlieb et al. (1967), using hybridisation studies, concluded that the RNA participating in RNA-antigen complexes was present in peritoneal cells at the time of addition of the antigen. It would seem therefore that at the time of encounter the macrophage needs to produce enzymes capable of degrading the antigen or enzymes to link the RNA to antigenic fragment. Both situations may well prevail.

From Table 4 it can be seen that X-irradiation of the peritoneal cells prior to inhibition of these cells with BSA caused an inhibition of formation of RNA-BSA complexes. Previous reports had indicated that the capacity of the peritoneal cell to process particulate antigens may be radiosensitive (Gallily and Feldman, 1967). On the other hand, the processing of soluble haemocyamin by peritoneal exudate cells was radioinsensitive (Unanue and Askonas, 1968).

It was not found possible to induce anti-BSA synthesis in cultured lymph node cubes (from normal rabbits) when they were incubated with RNA extracted from peritoneal cells which had been incubated with BSA. To date, no in vitro transfer of immunity for soluble proteins comparable to that described for particulate matter has been reported. Transfer of immunity in a secondary response for soluble haemocyanin has been reported by Askonas and Rhodes (1965). They incubated normal mouse spleen cells for 1 hour with haemocyanin or with RNA extracted from peritoneal cells which had been treated with haemocyanin. These spleen cells were transferred to mice which had been previously injected with

haemocyanin. A normal secondary response resulted. These results and those of Harris (1965) indicate that in the case of soluble proteins the macrophage either selects out an antigen fragment which is more antigenic or that a small portion of antigen is complexed to RNA such that the capacity of such a portion to initiate a secondary response is increased. The question of how important this phenomenon is *in situ* is still uncertain. While most of the peritoneal exudate cell-ingested antigens are broken down, it appears from the work of Kölsch and Mitchison (1968) that a small portion is undegraded. Whether this portion is the immunologically active fraction or whether the antigen which is important to the role of the macrophage in the immune response is on the surface of the macrophage is an open question.

A number of reports indicate that, when RNA from peritoneal exudate cells which have been incubated with T_2 phage is added to normal lymph node cubes or spleen cells, it causes synthesis of specific anti-T_2 activity. As the results in Table 5 indicate, it has not been possible in this study to

substantiate these published findings. In assessing these reports, certain observations must be kept in mind. Michael and Kuwatch (1969) have pointed out that T_2 phage suspensions contain soluble cellular substances from the E. Coli host on which they were grown. These substances include bacterial somatic antigens which have been known to have adjuvant properties and also have been shown by Michael, Whitby and Landy (1961) to cause release of preformed antibodies from antibody forming cells. Gottlieb (1969b) has pointed out that one cannot be sure that the animal has not encountered T_2 phage previously. If the animals used in the experiments of Fishman and his colleagues had encountered T_2 phage, then his results would be pertinent to a secondary response rather than a primary response. Also, the somatic antigens contaminating RNA preparations from peritoneal cells incubated with T_2 phage would non-specifically promote the release of antibody from lymph node cells. Michael and Kuwatch (1969) found that normal mice possess anti-T_2 activity in their sera and they observed that, if a preparation of somatic antigen or endotoxin was injected into normal mice, then the T_2 activity was increased. Moreover, RNA

preparations from phenol extracted T_2 treated peritoneal cells were found to be contaminated with endotoxin. The difference between the animals used by Fishman and other workers and those used to provide the results presented in Table 5 may have been the difference between secondary responses and primary response, with its consequent reflection on the contrary results obtained. As the sera of the animals used were not tested for anti-T_2 activity, it is impossible to offer this as a concrete explanation. The RNA extracted from T_2 treated peritoneal cells and used for addition to lymph nodes was largely undegraded as adjudged by optical density profiles and radioactivity profiles on a sucrose density gradient. The culture of lymph nodes closely followed the method of Adler, Fishman and Dray (1966). An explanation of the negative result is difficult to provide. Fig. 10 has shown that there was an increase in RNA synthesis by peritoneal exudate cells when the concentration of antigen with which they were incubated was also increased. Fig. 11 has shown that the increase appears to be centred around the ribosomal and pre-ribosomal RNA species. Therefore, it

seems reasonable to expect that increasing the concentration of T_2 incubated with peritoneal exudate cells will cause a parallel increase in ribosomal protein synthesis.

Cohn et al. (1966) cultured mouse peritoneal exudate cells in vitro and made a study of new protein synthesis in these cells by means of electron microscopy autoradiography of cells exposed to a pulse of [^{3}H] leucine. Initially most of the label was associated with rough endoplasmic reticulum. Thereafter, the label was associated with the Golgi vesicles from whence it became localised in the dense granules or lysosomes. Thus, much of the new protein synthesised in peritoneal cells incubated with antigen might be expected to be (i) structural protein involved in lysosomal membranes and (ii) lysosomal enzymes. Increasing the concentration of antigen incubated with peritoneal cells would therefore cause increase in ribosomal RNA biosynthesis, leading to concomitant ribosomal protein synthesis. This increased synthesis of ribosomes would favour the increased synthesis of lysosomal enzymes and structural proteins. Therefore, in the presence of antigen, increased uptake of [^{14}C] leucine into acid precipitable

material of these cells should occur. Figs. 3 and 4 have shown that this is the case.

Work by Sastry and Hokin (1966) showed that phagocytosis by polymorphonucleotides specifically increased their rate of incorporation of phosphate into phosphatidic acid and phosphatidyl inositol. In this respect, the results reported in Fig. 7 seem rather surprising. However, Karnovsky (1964) had demonstrated that, while the specific activity of monocytes phospholipid showed an increase of 39% during phagocytosis, no change occurred in alveolar macrophages. Oren et al. (1963) showed that, during phagocytosis, guinea pig peritoneal macrophages displayed an increase in inorganic phosphate uptake into phospholipid. This effect could not be shown for guinea pig alveolar macrophages. Thus it seems that in phosphate uptake into phospholipid is not a general property of cells which are phagocytosing. As no study was made to see if the cells were phagocytosing the T_2 phage, it is difficult to know whether the inhibition was due to phagocytosis or not.

Fig. 5 has shown that over a 90-minute interval uptake of thymidine into peritoneal cells incubated with T_2 phage is lower than uptake into cells incubated without T_2 phage. This result is surprising in view of the report by Forbes and Mackaness (1963) that about 50% of mouse peritoneal cells from an immunised rabbit incorporated tritiated thymidine 20 to 30 hours after re-injection of the same antigen. It is possible that (i) the difference in time intervals studied and (ii) the difference between primary and secondary responses may account for the difference in results.

The macrophage is known to possess a very low rate of mitosis (Spiers et al., 1961). The work of Spector and Coote (1965) indicates that it is derived from the blood monocyte, a cell which Whitelaw (1966) has shown to have a half-life of 3 days. The observations of Forbes and Mackaness cited above may be explained by proposing that an increase in macrophage population was required to effectively combat reinfection of the animal by an antigen. On the other hand, the observations in Fig. 5 may indicate that macrophages ingesting antigen may suspend mitotic activity. An interesting point in Fig. 6 is

that the inhibition of thymidine uptake by peritoneal cells incubated with increasing concentrations of T_2 phage does not manifest itself until the T_2 phage : peritoneal cell ratio is in excess of 10 : 1.

STUDIES ON APPENDIX CELL SUSPENSIONS

R E S U L T S

INTRODUCTION

The appendix is unique among the lymphoid organs of the body in that little research work on this organ is reported. In spite of this lack, some of the reports which have been published have indicated that the lymphoid cells of the appendix may be active in immune responses. So far, appendix cells have not been shown to be capable of antibody production, yet extensive DNA synthesis in this tissue is indicated by the observations of Shepherd (1965) who reported that the activity of DNA polymerase extracted from normal rabbit appendices was eleven times greater than the value reported for DNA polymerase extracted from spleen. Current opinion favours the idea that cell division precedes antibody formation. Nossal, Mitchell and MacDonald (1963), using autoradiography and noting tritiated thymidine uptake in lymphoid tissue undergoing a primary response, concluded that the presence of antigen stimulated cell division.

Mäkelä and Nossal (1962) showed that antibody production in secondary responses was also preceded by cell division. This conclusion concurred with that of Cohen and Talmage (1965) who found that in a secondary response antibody forming cells were derived from cells which underwent cell division earlier in the response. Thus it is possible that, while antibody is not produced by lymphoid cells of the appendix, these cells may provide the precursor cells of antibody forming cells in the spleen. This thought is supported by the results of Ford and Gowans (1967) who suggested that potentially antigen responsive cells are not fixed in the tissue mediating the response but rather that they are the lymphocytes circulating in the blood at the time of antigen administration. Nossal (1967) also indicated that the antigen responsive cells in mice are not fixed but they are probably derived from the bone marrow and circulate with the morphology of small lymphocytes in the blood.

Merritt and Johnson (1965) found that, if mice were treated with 2-deoxyuridine (a specific inhibitor of DNA synthesis), the primary response to bovine gamma globulin was abolished.

If the drug was administered any time up to 14 hours after injection of antigen, the immune response was enhanced, whereas, if administration of the drug was delayed until 18 hours or more after antigen injection, then the response was abolished. Thus, in mice it appeared that not only was DNA synthesis necessary but that it did not begin until at least 16 hours after primary injection of antigen. In the secondary response, the work of Capalbo, McKinodan and Gude (1965) showed that spleen cells, transferred to X-irradiated isologous recipient mice, were capable of a two-fold reduction of their mean generation time - from about 24 hours to about 12 hours - and this response was observed on the first day of the secondary response.

A very interesting system was described by Dutton and Eady (1962) and (1964) in which they showed that the uptake of [^{14}C] thymidine by spleen cell suspensions from rabbits previously immunised to heterologous serum proteins was stimulated by the addition of the soluble antigen *in vitro*. This response was specific and dependent upon the concentration of the antigen. They suggested that this response was the

in vitro counterpart of cellular proliferation seen in whole animals following secondary injection of antigen. This system has been used by Harris and Littleton (1966) who demonstrated that secondary encounter of spleen cells *in vitro* with Sheep Red Blood Cells resulted in stimulation of [2 - ^{14}C] thymidine uptake, and also by Harris and Cramp (1968) who showed that [^{14}C] thymidine uptake by spleen cell suspensions from rabbits previously immunised to heterologous proteins, was specifically stimulated by the deposits obtained after high speed centrifugation of solutions of these antigens.

The work to be described in this section concerns an attempt to characterise the effect on DNA synthesis of primary and secondary encounter between antigen and the lymphoid cells of the rabbit appendix. The approach of Dutton and Eady (1964), described above for spleen cells, will broadly be followed.

Before embarking on this programme, it was considered desirable to attain some idea of the conditions suitable for achieving a reasonable response in terms of radioactive thymidine uptake from the cultured appendix cells. Therefore,

the uptake of [2 - ^{14}C] thymidine/million appendix cells/ml. was observed while the following parameters were varied (i) cell concentration, (ii) medium of suspension and (iii) serum concentration.

EXPERIMENT 1 : The effect of cell concentrations on thymidine uptake by appendix.

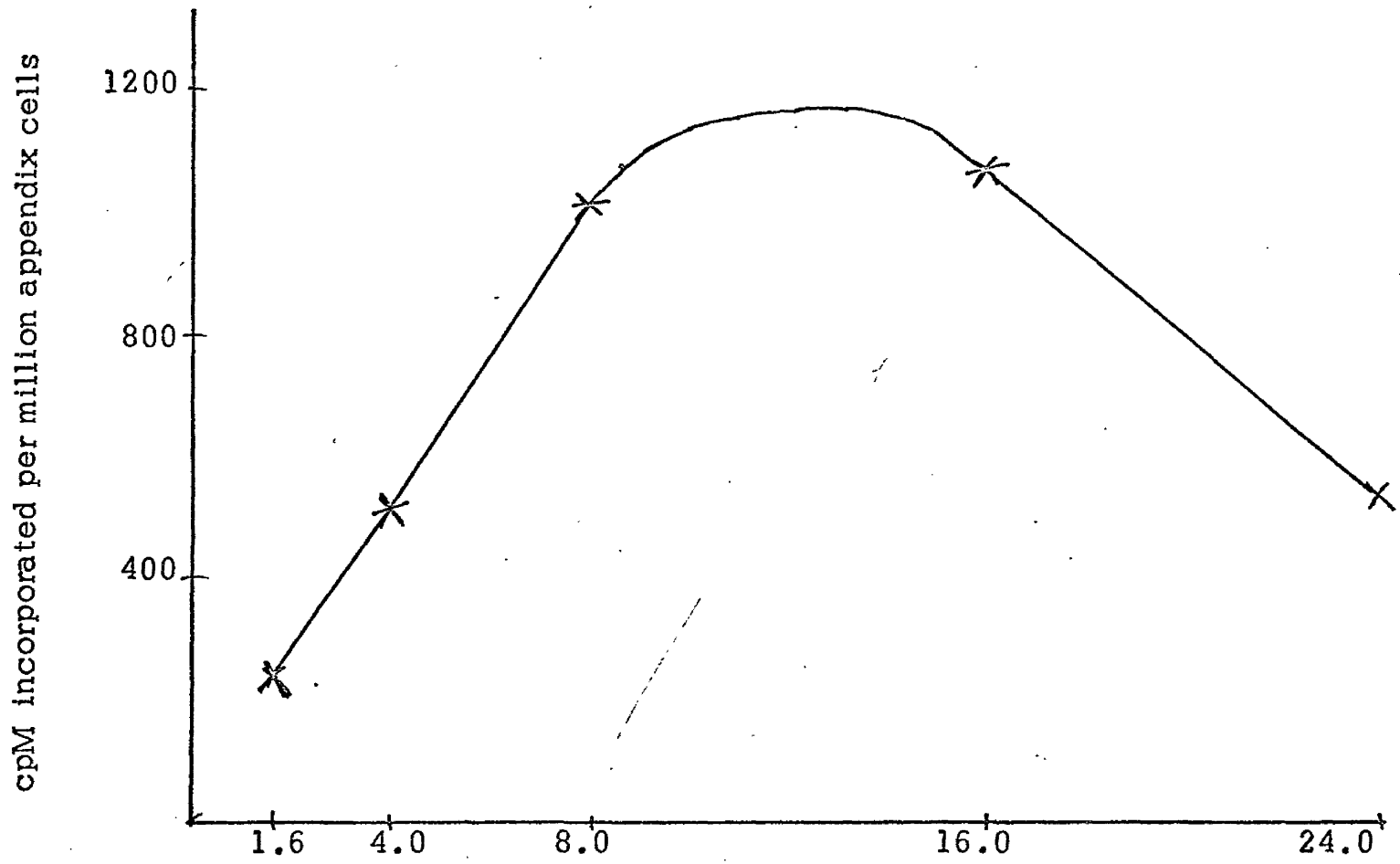
Rabbit appendix cells were suspended in EHM containing 10% serum at a concentration of 24 millions/ml. This stock suspension of appendix cells was mixed with EHM containing 10% rabbit serum to give the following concentrations :

1.6 million/ml., 4.0 million/ml., 8.0 million/ml., 16.0 million/ml. and 24.0 million/ml.

0.88 µCi [2 - ^{14}C] thymidine (7.16 mCi/mM) was added to each tube at 12 and 60 hours respectively after the inception of the culture, and the cells were harvested 12 hours later in each case.

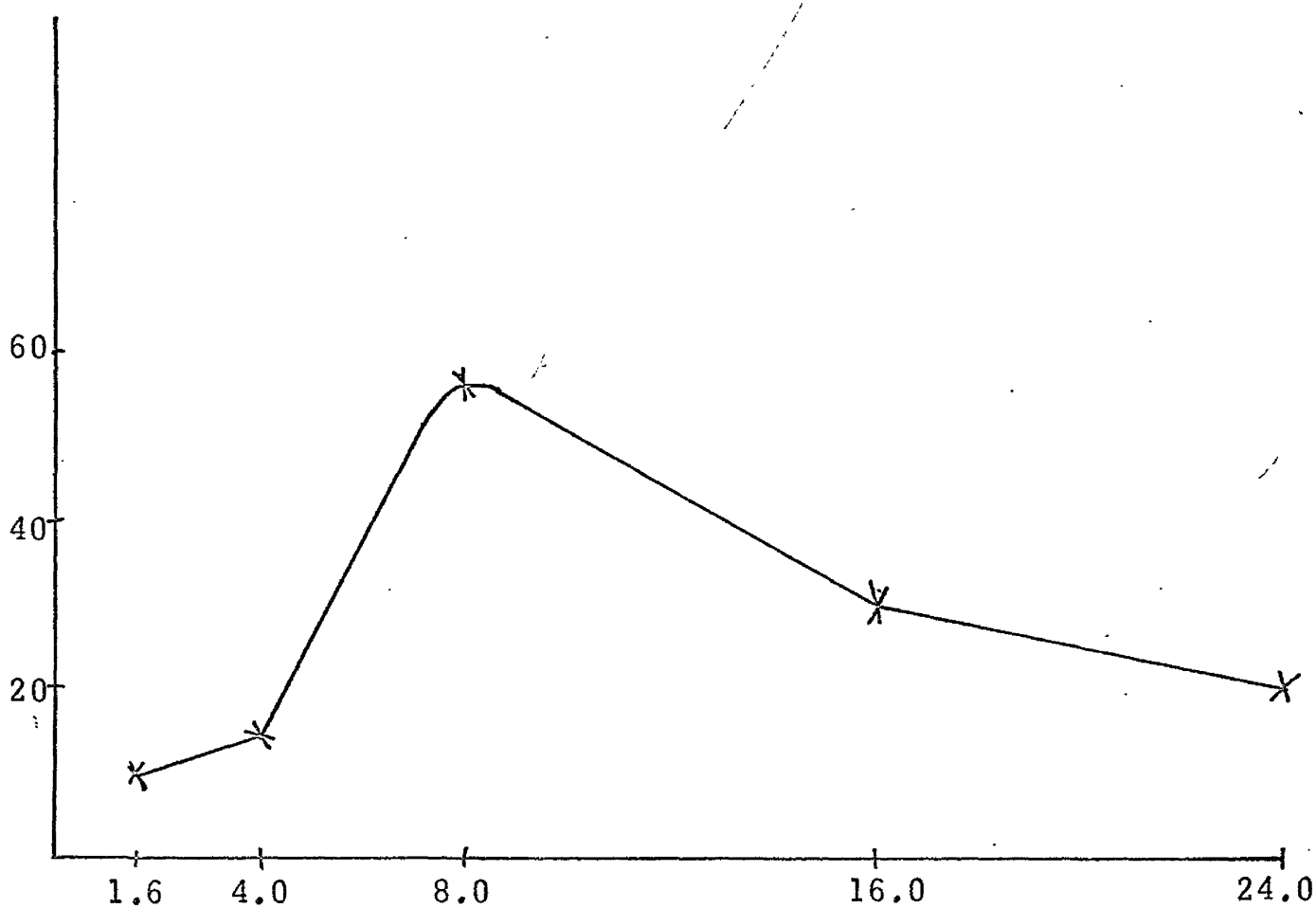
It can be seen from Fig. 17 that optimum uptake of [2 - ^{14}C] thymidine between 12 and 24 hours after the inception of culture occurred when the appendix cells were cultured at a concentration between 8 million and 16 million/ml. Fig. 18

Fig. 17 : The effect of incubation of appendix cell suspensions at different concentrations on the uptake of [2 - ^{14}C] thymidine/million cells.



Thymidine was added after 12 hours of culture and the cells were harvested 12 hours later.

Fig. 18 : The effect of incubation of appendix cell suspensions at different concentrations on the uptake of 2 - ^{14}C thymidine/million cells.



Thymidine was added after 60 hours of culture and the cells were harvested hours later.

shows that the optimum uptake of [2 - ^{14}C] thymidine between 60 and 72 hours after the inception of culture occurred when the cells were cultured at a concentration of 8 million/ml. Therefore for all further studies a concentration of 8 million/ml. was adopted for culture of appendix cells.

EXPERIMENT 2 : The effect of different suspending media on the uptake of [^{14}C] thymidine by appendix cells.

A cell suspension from appendix was made in PBS and a cell count showed a concentration of 12.8 million cells/ml. Four equal volumes were spun at 80 g for 10 minutes and the pellets obtained were each resuspended at a concentration of 8 million cells/ml. in EHM, Fucks, NCTC 109 or TC 199. These four appendix cell solutions had serum added to a final concentration of 10% and duplicate tubes containing 2 ml. of suspension were set up. The tubes were harvested 24 hours after the setting up of the culture and 0.88 µCi [^{14}C] thymidine (7.18 mCi/mM) was added to each tube 12 hours before harvesting.

TABLE 6 : A comparison of labelled thymidine uptake between 12 and 24 hours by appendix cells when suspended in four different media.

Suspending Medium	CPM incorporated between 12 and 24 hours
EHM	1798
Puck's	802
NCTC 109	206
TC 199	5

Table 6 shows that appendix cells at a concentration of 8 million cells/ml. incorporated the greatest amount of $[2\text{-}^{14}C]$ thymidine between 12 and 24 hours after inception of culture, when cultured in EHM. This medium was used in all succeeding experiments for culture of appendix cells.

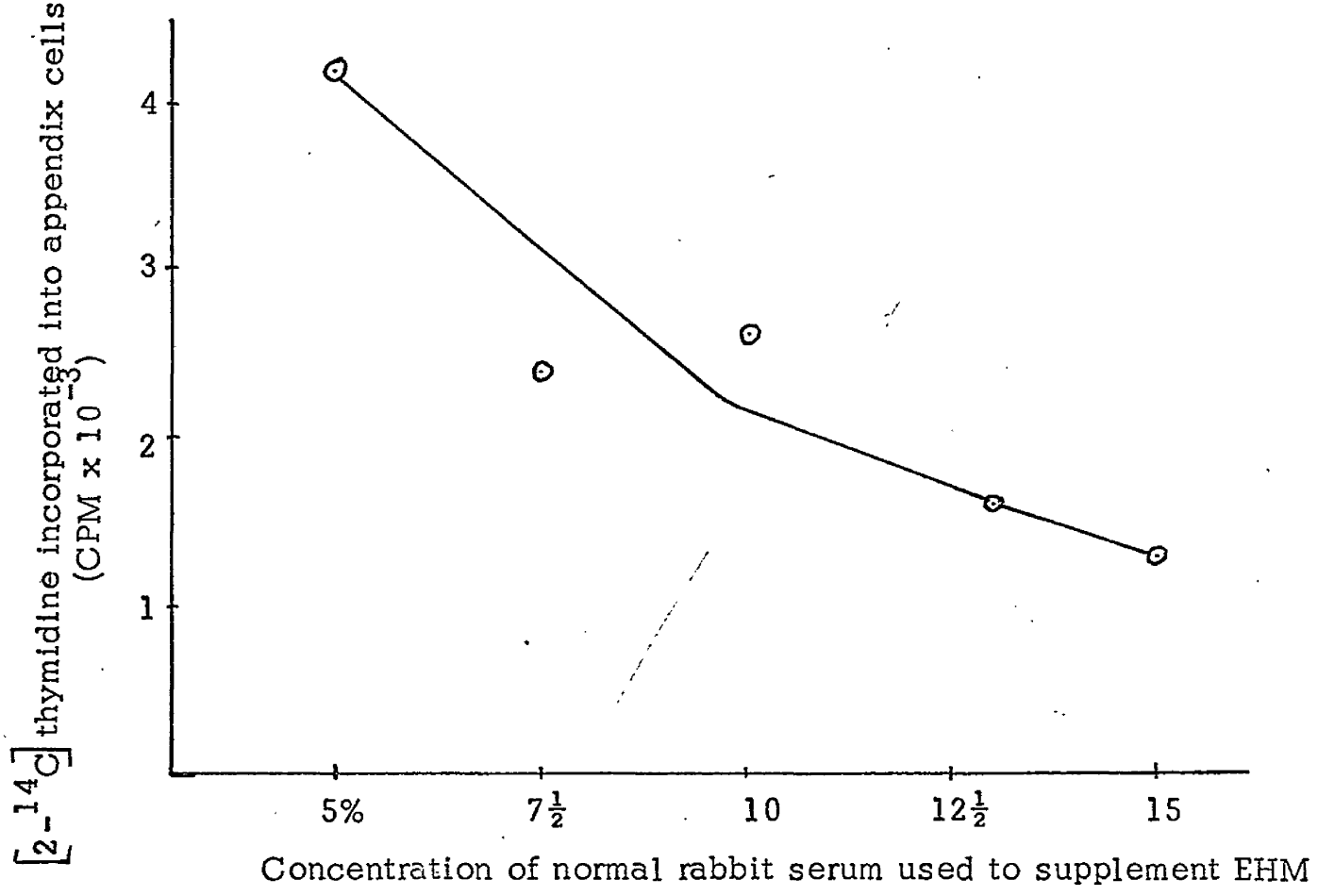
EXPERIMENT 3 : The effect of concentration of rabbit serum on the uptake of [2 - ^{14}C] thymidine into rabbit appendix cells *in vitro*.

Five suspensions of appendix cells were set up with EHM and serum concentrations to 5%, 7½%, 10%, 12½% and 15% respectively. Cell concentration was set at 8 million/ml. in all cases. Tubes were harvested at 24 hours after the setting up of the culture and 0.80 µCi [2 - ^{14}C] thymidine (7.10 mCi/mM) was added to each tube 12 hours prior to harvest.

Fig. 19 shows that, of the serum concentrations tested, 5% stimulated the greatest uptake of 2 - ^{14}C thymidine by appendix cells harvested at 24 hours after the setting up of the culture. Therefore, appendix cells were cultured in the presence of 5% Normal Rabbit Serum.

As a result of these experiments, appendix cells were cultured at a concentration of 8 million cells/ml. in EHM with Normal Rabbit Serum to 5%.

Fig. 19 : The ability of rabbit appendix cell suspensions to incorporate [2-^{14}C] thymidine in the presence of various concentrations of normal rabbit serum between 12 and 24 hours after setting up culture.



It was considered of interest to investigate the effect of antigen on thymidine uptake by appendix cells from rabbits previously immunised to this antigen. Initially the effect of different concentrations of antigen incubated with appendix cells on the uptake of thymidine by these cells was studied. The time intervals investigated were 12 to 24 hours, and 60 to 72 hours respectively after incubation of the cells with antigen.

EXPERIMENT 4 : The effect of varying the concentration of antigen incubated *in vitro* with appendix cells taken from a previously immunised animal on the uptake of $[2\text{-}^{14}C]$ thymidine by these cells.

A cell suspension at 6 million cells/ml. was prepared as before from the appendix of an immunised rabbit. Tubes containing 2 ml. each of appendix cell suspension were divided into 7 groups, a control group and groups each containing one of the following concentrations :

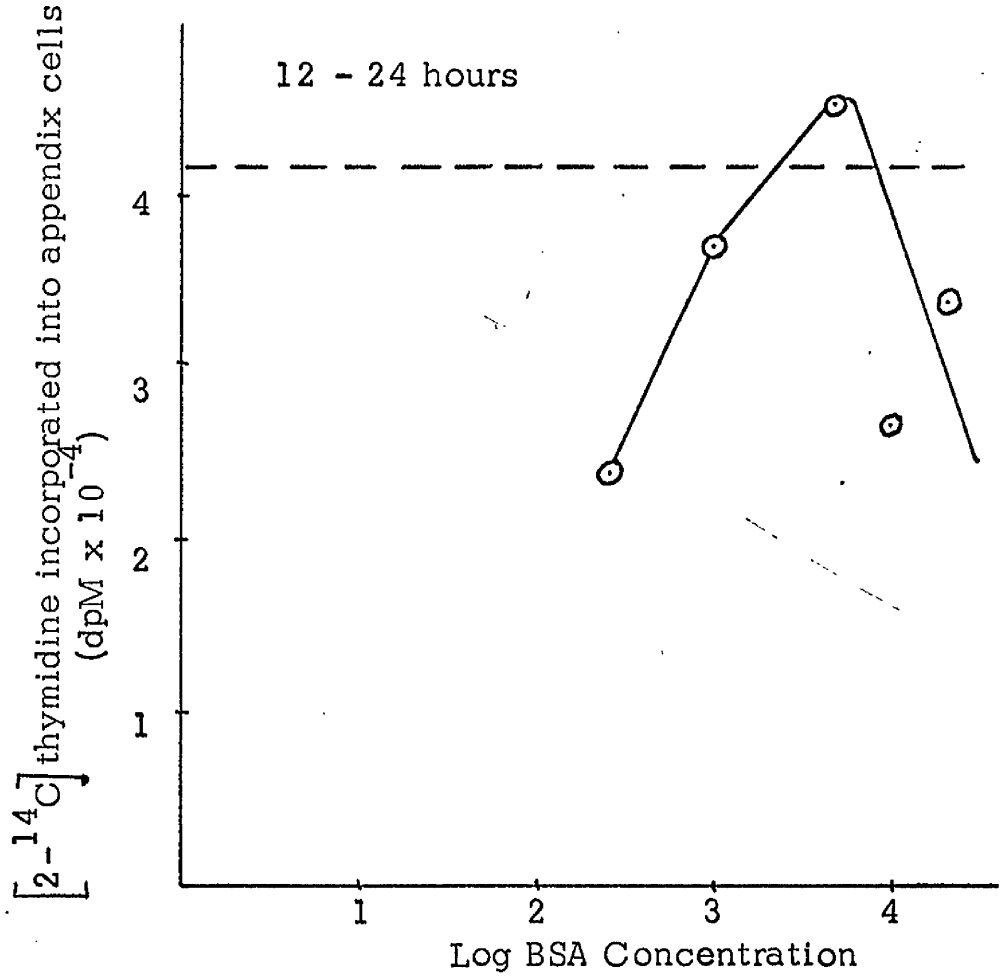
50 µg./ml., 250 µg./ml., 1,000 µg./ml., 5,000 µg./ml.

10,000 µg./ml., and 20,000 µg./ml.

The cells were incubated at 37°C in an atmosphere of 5% CO_2/95% O_2 and tubes were harvested at 24 and 72 hours after the addition of the antigen. 0.88 uCi [^{14}C] thymidine (7.18 mCi/mM) was added to each tube 12 hours prior to harvesting.

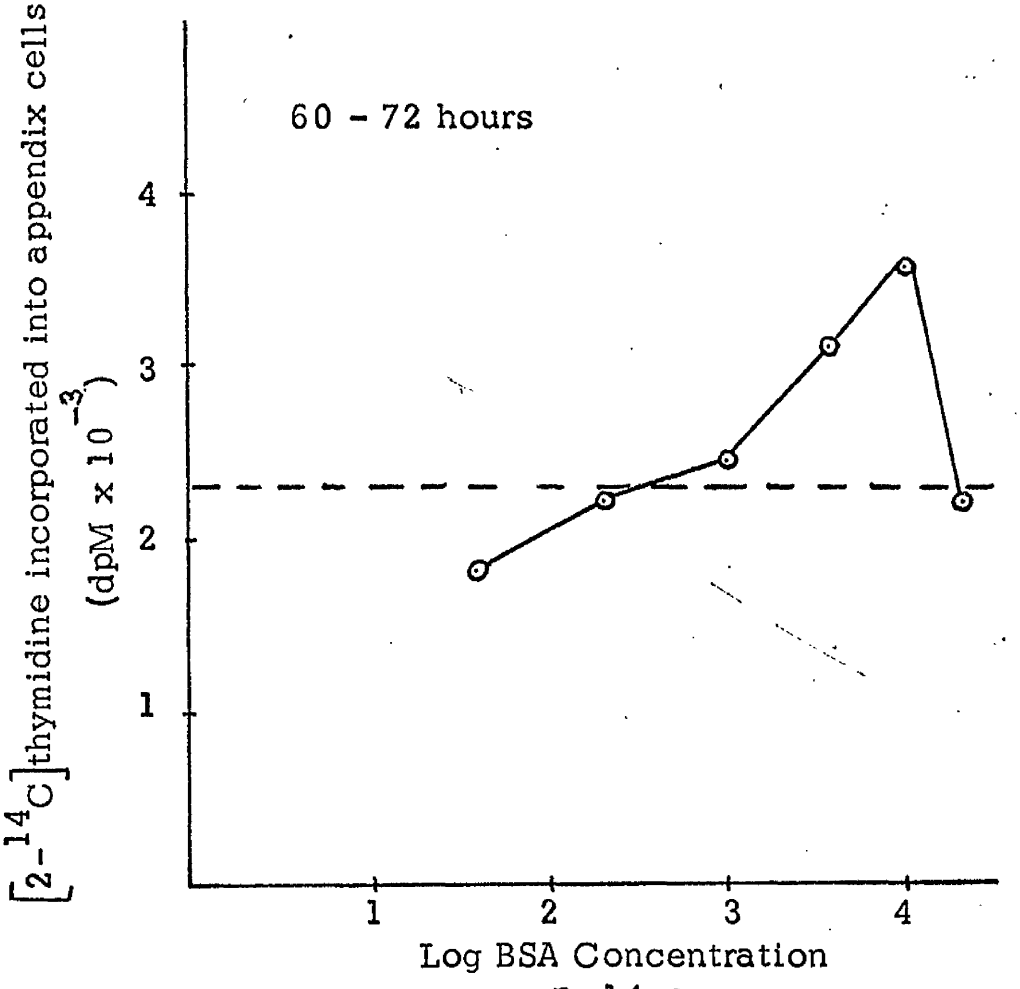
Figs. 21 and 20 show the effect on thymidine uptake by cells from immunised rabbits of changing the concentration of the immunising antigen incubated with the cells in vitro. The time intervals were between 12 and 24 hours and between 60 and 72 hours. The optimum concentration was found to be at 5,000 µg. BSA/ml. of culture fluid over the period 12 to 24 hours while over the period 60 to 72 hours the optimum was 10,000 µg. BSA/ml. However, in as much as the concentration 10,000 µg. BSA/ml. was found to inhibitory over the interval 12 to 24 hours and as 5,000 µg./ml. was stimulatory over both of the intervals studied, this latter concentration of 5,000 µg./ml. was used for further studies.

Fig. 20 : The ability of appendix cells to incorporate $[2-^{14}C]$ thymidine between 12 and 24 hours after setting up cultures in presence of various concentrations of BSA.



Dotted line indicates the value for $[2-^{14}C]$ thymidine uptake obtained when appendix cells were cultured in the absence of antigen.

Fig. 21 : The ability of appendix cells to incorporate [2-^{14}C] thymidine between 60 and 72 hours after setting up cultures in the presence of various concentrations of BSA.



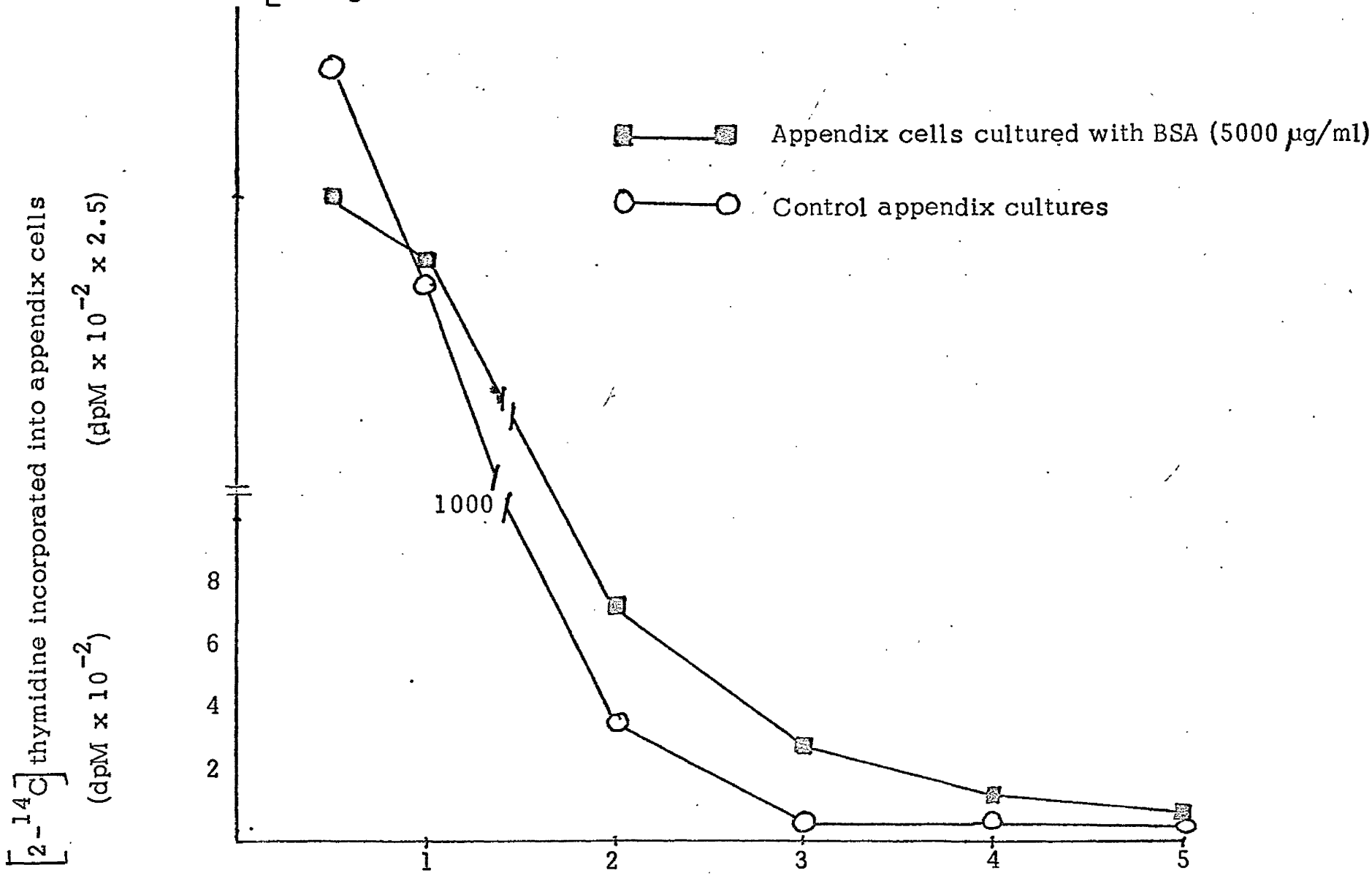
Dotted line indicates the value for [2-^{14}C] thymidine uptake obtained when appendix cells were cultured in the absence of antigen.

Using BSA at a concentration of 5,000 µg./ml. a comparison of the uptake of thymidine into appendix cells *in vitro* in the presence and absence of BSA was undertaken over a series of intervals after the inception of culture. This study would give an indication of the duration and nature of the stimulus of thymidine uptake into appendix cells in the presence of immunising antigen.

EXPERIMENT 5 : The effect of antigen on the uptake of [2 - ^{14}C] thymidine *in vitro* by appendix cells of an immunised rabbit.

A cell suspension at 8 million cells/ml. was prepared as before from the appendix of an animal immunised to BSA. Tubes containing 2 ml. of cell suspension were divided into stimulated samples containing 5,000 µg. BSA/ml. and control samples without added antigen. Duplicate tubes of both stimulated and control were harvested at 12, 24, 48, 72, 96 and 120 hours after setting up culture. In every case 0.88 µCi thymidine (7.18 mCi/mM) was added 12 hours prior to harvest.

Fig. 22 : The relative abilities of appendix cells from an immunised rabbit to incorporate [2-^{14}C] thymidine in the presence and absence of the immunising antigen.



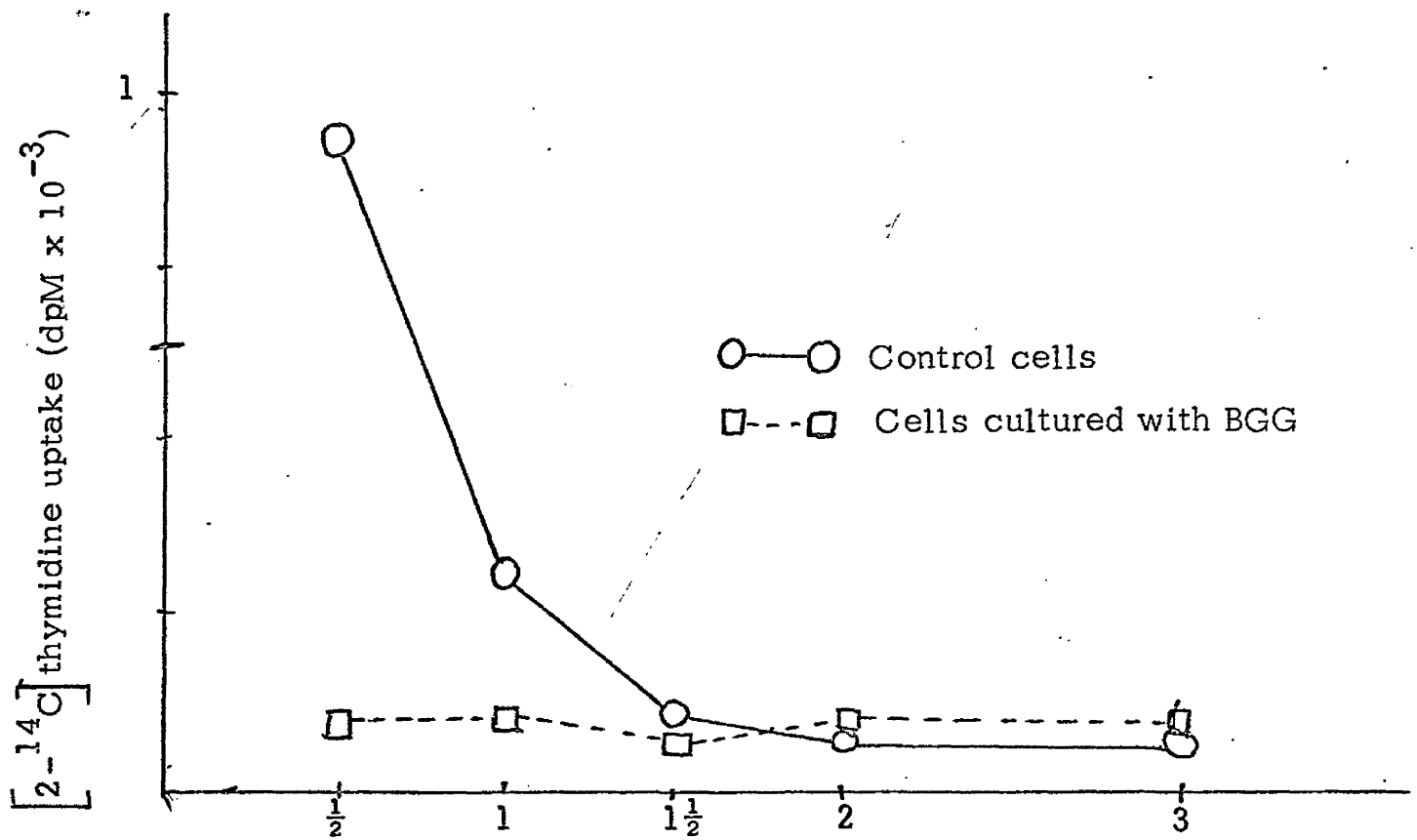
[2-^{14}C] thymidine was added to cultures 12 hours prior to harvest.

The above experiment was carried out on 10 separate occasions using BSA immunised rabbits. A typical result is indicated in Fig. 22. Such a result was obtained on 8 of the 10 occasions. It thus appears that the uptake of [2 - ^{14}C] thymidine into cells incubated *in vitro* with the immunising antigen was greater than uptake into cells which were incubated without antigen. The only interval where the incorporation of thymidine into the control was above the incorporation into stimulated cells was over the 12-hour period following the inception of the culture. This was a consistent observation. In both test and control cultures, there was a very sharp fall off in uptake of thymidine between 12 and 24 hours after which the uptake was much less.

When the response did not conform to that described in Fig. 22, in one experiment, the uptake rose after 24 hours to 72 hours and then fell away. In the other experiment, the uptake of thymidine by cells incubated with antigen was severely inhibited.

When this experiment was repeated with a BCG stimulated rabbit and BCG as the stimulating antigen *in vitro*, the results

Fig. 23 : The relative ability of appendix cells from a rabbit immunised to BGG to incorporate [2-^{14}C] thymidine in the presence and absence of BGG the immunising antigen.



[2-^{14}C] thymidine was added to cultures 12 hours prior to harvest

were very different. In 3 out of 5 experiments, total inhibition of thymidine uptake by cells incubated with BGG was observed at all times (see Fig. 23). In the remaining cases, BGG had a faintly inhibitory effect or no effect at all. Thus, in some cases, the incubation of appendix cells from a rabbit previously immunised to BGG with the same antigen *in vitro* led to a complete inhibition of uptake of thymidine into the appendix cells indicating perhaps that some mechanism for general inhibition was being activated.

Harris and Cramp (1968) had noted that the stimulation of thymidine uptake, reported when spleen cells from an immunised rabbit were incubated with the immunising antigen *in vitro*, was due to the presence of aggregated deposits obtained when the antigen solution was ultracentrifuged. Accordingly, the effect of such deposits on the uptake of thymidine into appendix cells from an immunised rabbit was noted and compared with the effect obtained when unspun antigen was incubated with appendix cells.

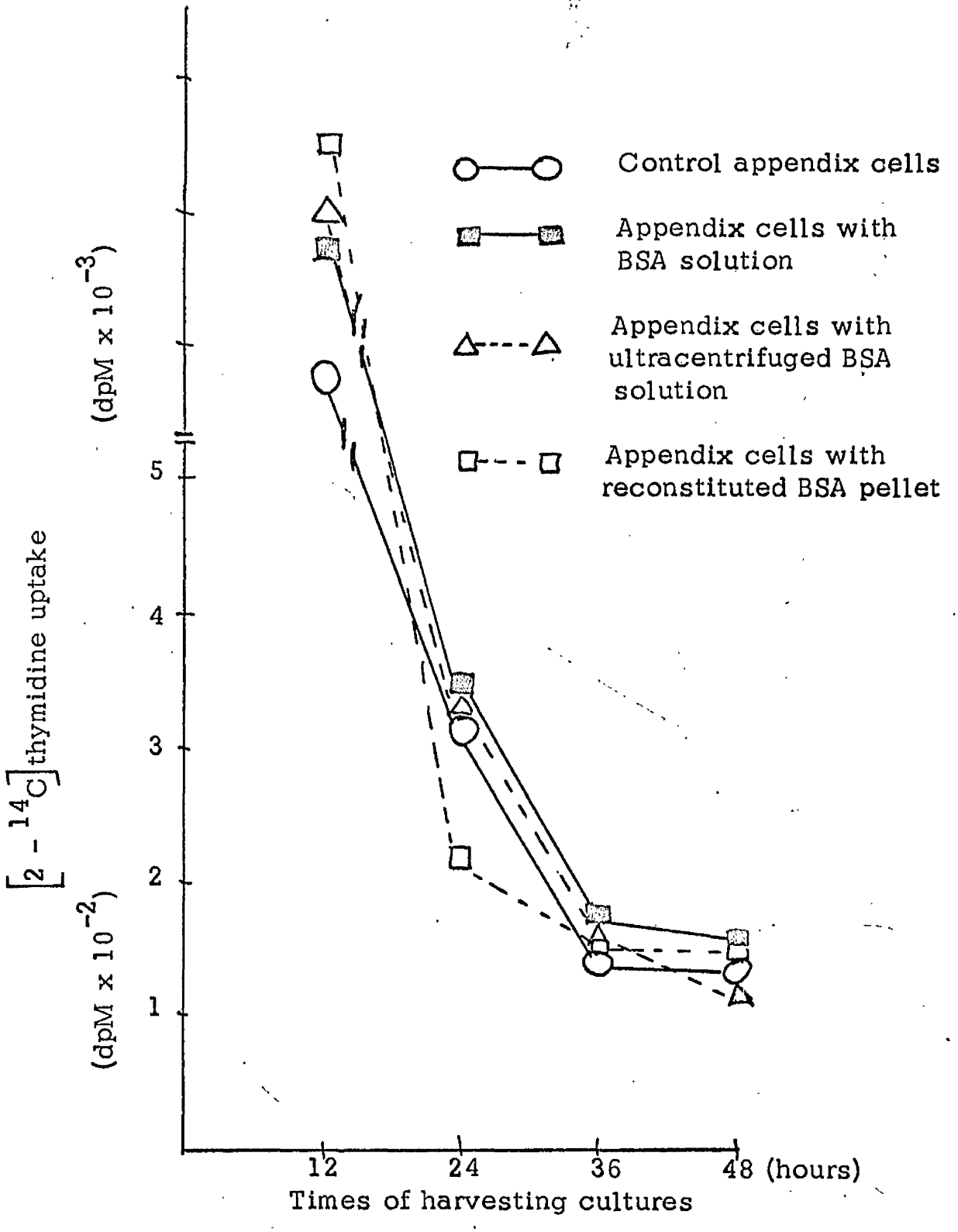
EXPERIMENT 6 : To compare the effect of deposits obtained after high speed centrifugation of BSA solutions on the rate of thymidine uptake by appendix cell suspensions taken from animals immunised to BSA.

Control and stimulated cell suspensions were prepared as in Experiment 4. In addition, 2 further cultures were set up. To the first was added 0.1 ml. of 1% BSA which had been spun free of particulate material while to the second was added 0.1 ml. of the resuspended high speed deposit.

Duplicate tubes of each type were harvested at 12, 24, 36 and 48 hours following the setting up of the culture. As before, 0.88 μCi $\left[2 - {}^{14}C\right]$ thymidine was added 12 hours before harvest.

Appendix cells from unimmunised animals were cultured *in vitro* with antigens and the effect on $\left[2 - {}^{14}C\right]$ thymidine uptake by the cells was noted. Thus, by a comparison of the results of Experiments 5 and 7, an idea of the extent to which the *in vivo* immunisation of rabbits was contributing to the results obtained in Experiment 5.

Fig. 24 : The effect on $[2-^{14}C]$ thymidine uptake of incubating (i) BSA solution, (ii) ultra centrifuged BSA solution, (iii) reconstituted BSA pellet or (iv) no BSA with appendix cells from a rabbit immunised to BSA.



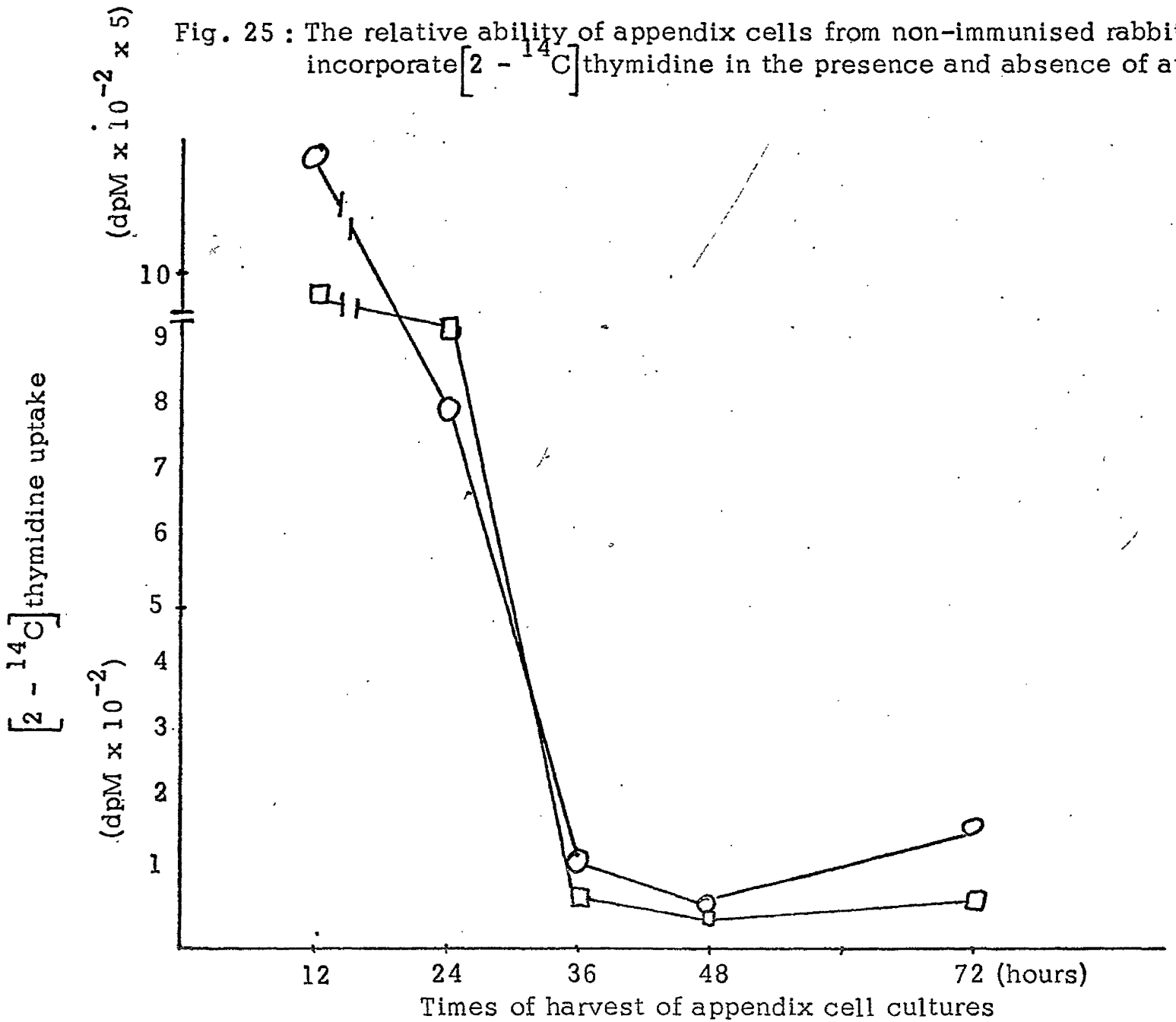
EXPERIMENT 7 : The effect of antigens on the uptake of [2 - ^{14}C] thymidine by appendix cells of non-immunised rabbits *in vitro*.

Experimental details were as described for Experiment 4, except that appendix cells were taken from an unimmunised animal and 3 different populations were tested : BSA stimulated, BCG stimulated and control appendix cell suspensions.

The cells were incubated as in Experiment 4 and were harvested at 12, 24, 36, 48 and 72 hours respectively after the addition of antigen. 0.88 μCi [2 - ^{14}C] thymidine (7.18 mCi/mM) was added 12 hours prior to harvesting.

When cells were taken from an unimmunised rabbit, no consistent difference in thymidine uptake was noted between control cells and those incubated with antigen *in vitro* (see Fig. 25). Thus it appeared that the stimulations and inhibitions reported for Experiment 4 were as a consequence of prior immunisation of the rabbits donating the appendix cells for culture.

Fig. 25 : The relative ability of appendix cells from non-immunised rabbits to incorporate [2 - ^{14}C] thymidine in the presence and absence of antigen.



[2 - ^{14}C] thymidine was added to cultures 12 hours prior to harvest.

It seemed logical at this stage to enquire if the stimulation and inhibition of thymidine uptake by appendix cells from immunised rabbits, cultured in the presence of the immunising antigen, was specific for the immunising antigen. Accordingly, appendix cells from immunised rabbits were cultured with immunising antigen and two other antigens, one of which might be expected to contain determinants similar to the immunising antigen.

EXPERIMENT 8 : To compare the effects of different protein antigens on the uptake of [2 - ^{14}C] thymidine by appendix cells of a stimulated rabbit.

As in Experiment 4, appendix cells from a BSA stimulated rabbit were used to provide a cell suspension at 8 million cells/ml. Tubes containing 2 ml. of this suspension were divided into 4 groups, a control group containing no antigen and 3 stimulating groups, each containing respectively BSA, BGG or SSA at a concentration of 5,000 µg./ml. culture fluid.

Duplicate tubes from each group were harvested at various times after the setting up of the culture. In each case

[2 - ^{14}C] thymidine was added 12 hours prior to harvest.

This experiment was repeated using an animal previously stimulated with BGG. In this case the cells were challenged in vitro with BGG, BSA and SGG.

In Table 7, the average disintegrations per minute representing thymidine uptake in each set of tubes over a particular time period is presented. It can be seen that the response, whether stimulation or inhibition, was specific for the immunising antigen. In the case of rabbit no. 1, thymidine uptake in the presence of BSA, the immunising antigen, was higher for those of either control cells or those stimulated by other antigens. In the case of rabbits no. 2 and 3, the incubation of the cells with BSA and BGG respectively, the immunising antigens, resulted in inhibition of thymidine uptake by the cells. Again the inhibition was specific for the immunising antigen. Other antigens had little effect on the rate of thymidine uptake.

TABLE 7

Rabbit No.	Antigen used in Immunisation	Rate of DNA Synthesis : 0 - 12 hours				
		None	BSA	BGG	SBA	SGG
1	BSA	96452	64808	-	-	-
2	BSA	11263	4489	11230	-	-
3	BGG	1496	1516	136	-	1440
		Rate of DNA Synthesis : 12 - 24 hours				
1	BSA	43801	44927	-	-	-
2	BSA	4933	183	4603	3375	-
3	BGG	489	-	156	-	-
		Rate of DNA Synthesis : 24 - 36 hours				
1	BSA	9800	12500	4356	9697	-
2	BSA	1901	669	1736	1374	-
3	BGG	166	242	107	-	161

In view of the suggestion by Hanaoka (1970) that, while lymphoid cells of the appendix did not produce detectable antibody in the appendix they may be involved in antibody production in spleen and lymph node, it was decided to compare the thymidine uptake by appendix cells and spleen cells cultured alone in the presence of antigen with thymidine uptake by a mixed cell culture of both spleen and appendix cells with antigen.

EXPERIMENT 9 : A comparison of the effect of antigen on the thymidine incorporating capacity of the following cell types in culture : (i) spleen cells, (ii) appendix cells and (iii) a mixed culture of spleen and appendix cells, all from immunised rabbits.

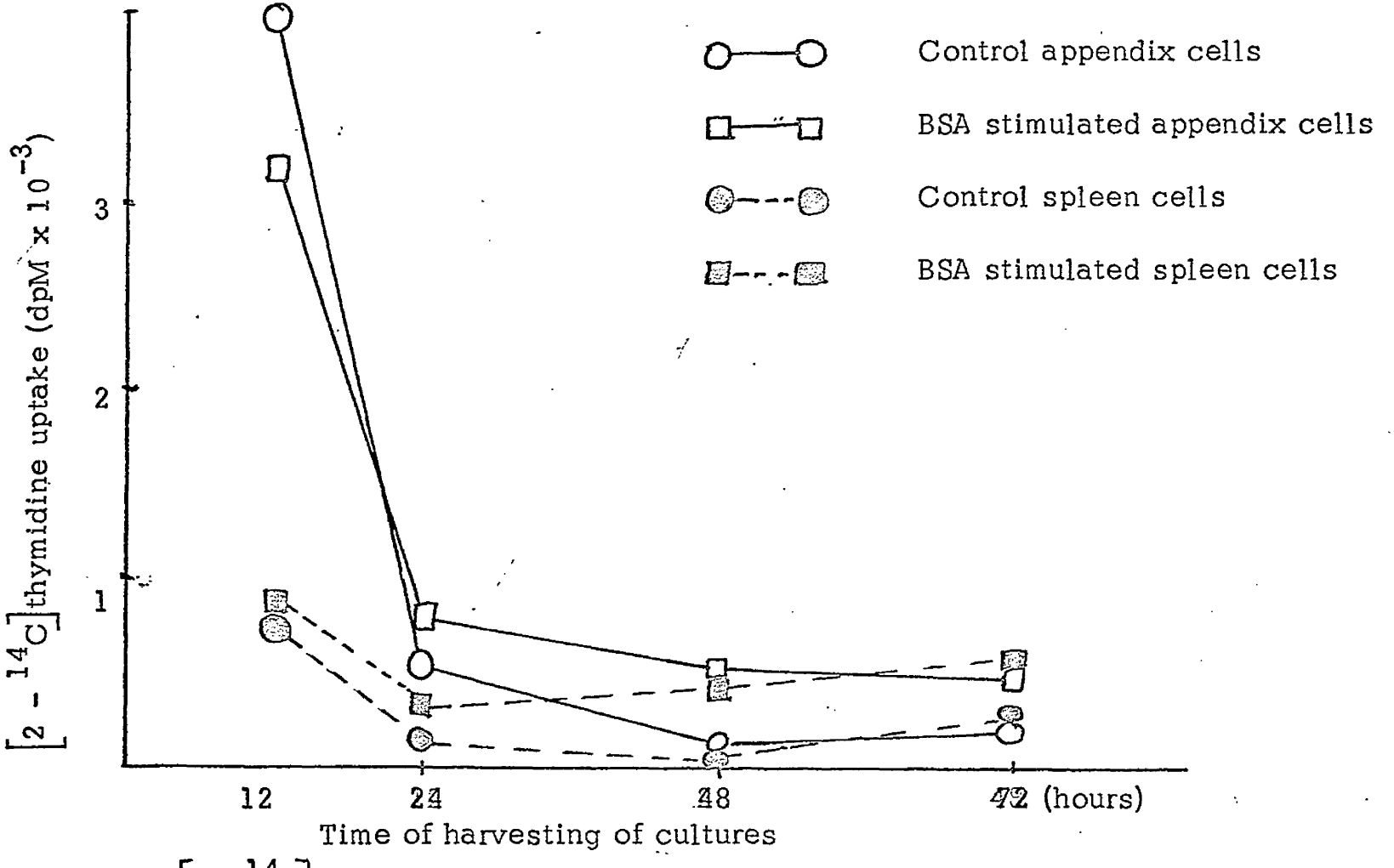
As before, a spleen cell suspension and an appendix cell suspension were made at 8 million cells/ml. 2 ml. aliquots of the spleen cell suspension were added to one set of tubes and 2 ml. aliquots of appendix cell suspension were added to a second set of tubes. In addition, a third set of tubes was set up containing 1 ml. of appendix cell suspension and 1 ml.

of spleen cell suspension. All three groups were subdivided into controls, which received no antigen, and stimulated cultures, which received antigen to a concentration of 5,000 µg./ml. The tubes were incubated as before and were harvested at 12, 24, 48 and 72 hours after the setting up of the culture. 0.88 µCi [2 - ^{14}C] thymidine was added 12 hours prior to harvesting.

The effects of the in vitro administration of an antigen to appendix and to spleen cells from a rabbit previously immunised to that antigen are seen for BSA in Fig. 26 and for BGG in Fig. 27. With spleen and appendix cells mixed, the results were as shown in Fig. 28 for BSA and in Fig. 29 for BGG. It was noticed that in the presence of BSA the uptake of thymidine by mixed appendix and spleen cell populations was higher than would have been predicted on the basis of thymidine uptake by each cell type incubated alone with the antigen (see Fig. 26).

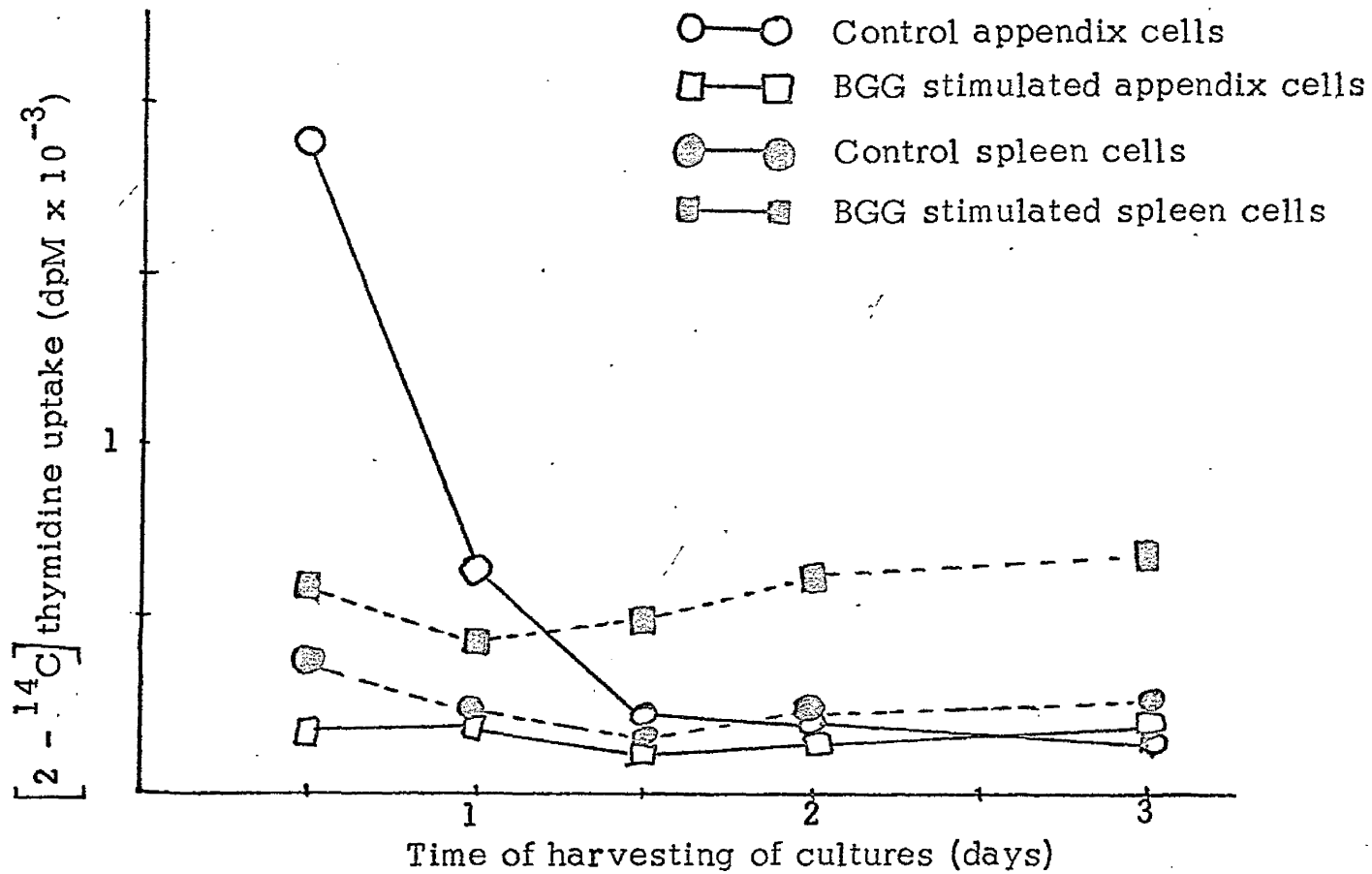
If the same experiment was conducted with BGG stimulated cells from a rabbit previously immunised to BGG, it was found (1) that the appendix cells were inhibited relative to the control

Fig. 26 : The relative abilities of separate cultures of appendix and spleen cells from an animal previously immunised to BSA to incorporate [2 - ^{14}C] thymidine in the presence and absence of BSA.



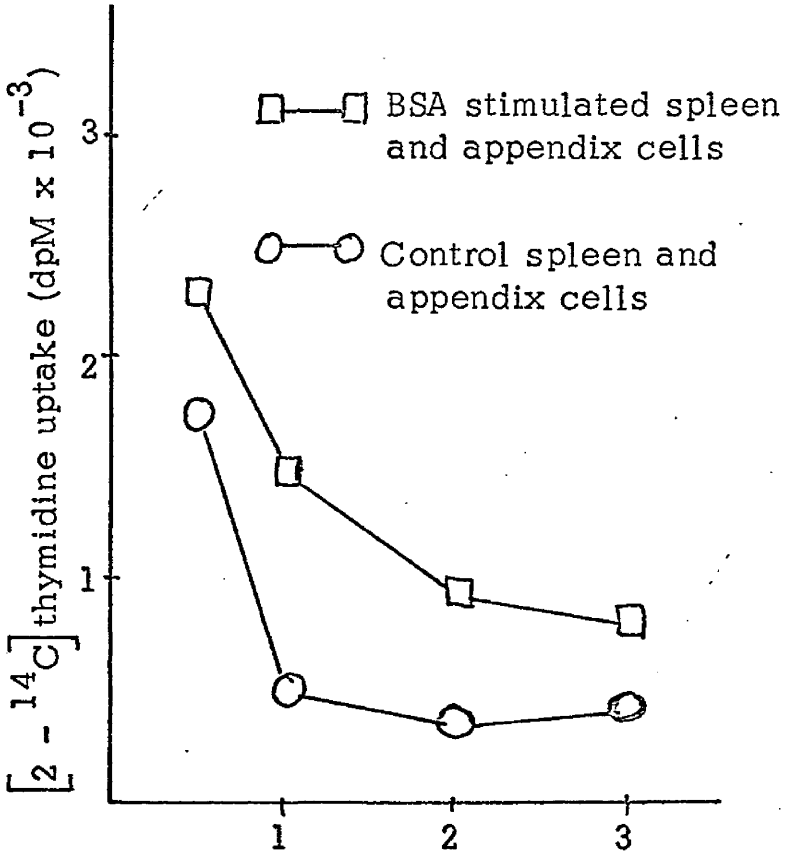
[2 - ^{14}C] thymidine was added 12 hours before harvesting.

Fig. 27 : The relative abilities of separate cultures of appendix and spleen cells from an animal previously immunised to BGG to incorporate [2 - ^{14}C] thymidine in the presence and absence of BGG.



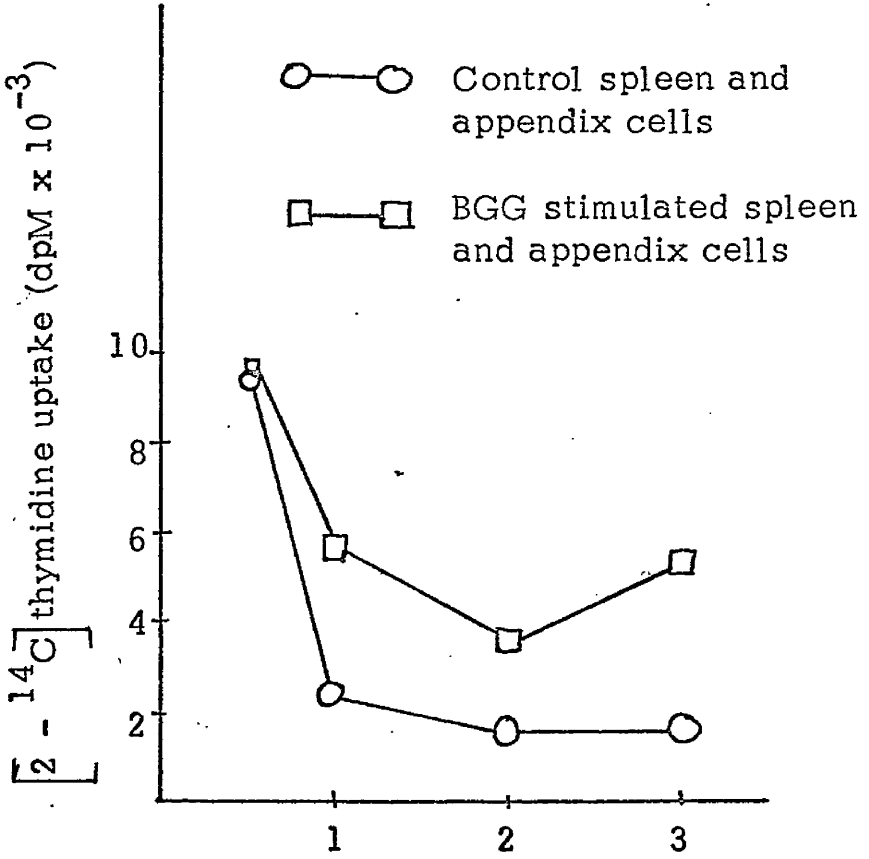
[2 - ^{14}C] thymidine was added 12 hours prior to harvesting

Fig. 28 : The ability of a combined culture of appendix and spleen cells from a rabbit previously immunised to BSA to incorporate [2-^{14}C] thymidine in the presence and absence of BSA in vitro.



[2 - ^{14}C] thymidine was added 12 hours before harvesting

Fig. 29 : The ability of a combined culture of appendix and spleen cells from a rabbit previously immunised to BGG to incorporate [2-^{14}C] thymidine in the presence and absence of BGG in vitro.



[2 - ^{14}C] thymidine was added 12 hours before harvesting

value in the presence of BCG; (2) spleen cells were stimulated in the presence of BCG and the stimulus was increasing from the 24 hours onwards; and (3) that spleen cells incubated with appendix cells in the presence of BCG caused the removal of the inhibition displayed by the latter cells when they were incubated alone with BCG.

In order to characterise the co-operative effect between spleen and appendix cells, it was decided to investigate the relative ability of these cells to co-operate in the presence of antigen when mixed in different proportions.

EXPERIMENT 10 : The effect of antigen on [2 - ^{14}C] thymidine uptake by spleen and appendix cells taken from previously immunised rabbits, mixed in different proportions.

As in Experiment 8, two stock solutions of spleen and appendix cells respectively were prepared. In all experimental tubes used below, the total volume of cell suspension used was 2 ml. and cell concentration was always 8 million cells/ml. Control tubes and stimulated tubes of appendix cells alone and of spleen cells alone were set up. In addition, stimulated

mixed appendix and spleen cell cultures were set up and these were of three types : 1½ ml. appendix cells with ½ ml. spleen cells, 1 ml. of both spleen and appendix cells, and 1 ml. of appendix cells with 1½ ml. of spleen cells. Incubation and harvesting procedures were as before. Thymidine uptake was measured over the period 36 to 48 hours following the addition of antigen.

Preliminary studies indicated that the co-operative effect between BSA stimulated appendix and spleen cells from BSA primed animals was optimal when the cells were mixed in the ratio 75% spleen cells and 25% appendix cells.

The next question to be asked was which cell type was stimulating which, in the presence of antigen, in order to give the apparent co-operation. Experiment 11 is an attempt to answer this question.

EXPERIMENT 11 : The nature of co-operation between spleen cells and appendix cells from immunised animals in their ability to take up [2 - ^{14}C] thymidine in the presence of the immunising antigen in vitro.

Spleen and appendix cells, taken from rabbits immunised to BSA, were suspended separately at concentration of 8 million cells/ml. Both populations were divided into control suspensions containing no antigen and stimulated suspensions containing 5,000 µg./ml.

Aliquots of both control spleen and control appendix cell suspensions were drawn off and subjected to 600 Rads of X-irradiation. These suspensions were adjusted to 8 million cells/ml. and stimulated by addition of EHM$_A$ (CONTAINING ANTIGEN) to 5,000 µg./ml. There were 6 cell suspensions and, as in Experiment 8, control and stimulated suspensions of mixed spleen and appendix cells were set up. Two additional mixed cell suspensions were set up as follows : firstly, 1 ml. aliquots of stimulated spleen cells were mixed with 1 ml. aliquots of stimulated irradiated appendix cells, and secondly 1 ml.

aliquots of stimulated appendix cells were mixed with 1 ml. aliquots of stimulated irradiated spleen cells. All tubes were incubated as before and were harvested at 24 hours. 0.88 μCi [2 - ^{14}C] thymidine was added at 12 hours.

TABLE

		Average dpM Incorporated*
Unstimulated appendix	A	4785
Stimulated appendix	AX	5200
Stimulated spleen	SX	550
Unstimulated spleen	S	347
Irradiated appendix (stimulated)	AXR	1823
Irradiated spleen (stimulated)	SXR	340
Mixed culture containing irradiated spleen and non-irradiated appendix	AX + SXR	3625
Mixed culture containing irradiated appendix and non-irradiated spleen	AXR + SX	1177

* Incorporation is over 12 hours between 12 and 24 hours after starting culture.

$$\frac{AX + SXR}{2} = 2770 < 3625$$

$$\frac{SX + AXR}{2} = 1186 \approx 1177$$

If the values for stimulated irradiated appendix cells were added to those for stimulated non-irradiated spleen cells, and the total divided by 2, the value obtained could be compared with that obtained from mixed stimulated culture of irradiated appendix cells and non-irradiated spleen cells. Likewise, comparison could be made between the mean of stimulated separate cultures of non-irradiated appendix cells and irradiated spleen cells and a stimulated culture containing both non-irradiated appendix and irradiated spleen cells. If it was assumed that irradiation diminishes the capacity of a population to be stimulated by another cell type, then by observing the values for the combined cultures and the mean values for the separate cultures, it was seen that the value for stimulated combined culture of non-irradiated appendix and irradiated spleen cells was greater than that obtained by taking the mean of separate stimulated cultures of non-irradiated cultures of appendix cells and irradiated spleen cells. When the value for mixed stimulated irradiated appendix and non-irradiated spleen cultures was compared with the mean value for these cell types incubated separately, no difference was noticeable. From this it was concluded that the spleen cell

was stimulating the cells of appendix origin over and above the stimulus by the presence of the immunising antigen in the medium.

Autoradiographic studies were carried out on appendix cell suspensions from rabbits immunised to BSA. These suspensions were incubated with BSA and control suspensions were set up without BSA. Tritiated thymidine was added 24 hours after start of culture and cells were harvested 12 hours later. These studies showed that the majority of the cells were lymphocytes. Little difference can be seen between the autoradiographs of stimulated (Plate 1) and Control (Plate 2) suspensions. It was found that 1.91% of the cells incubated with BSA were incorporating thymidine over the period examined, while 1.78% of the control cells were incorporating thymidine over the same period.

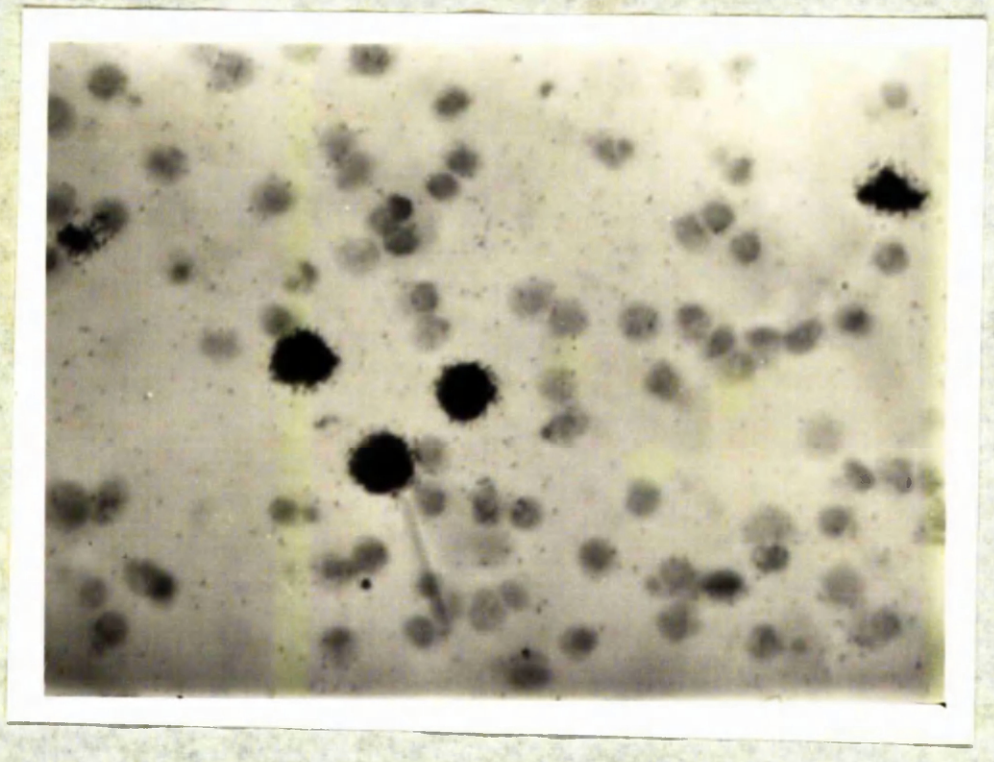


Plate 1 : Autoradiograph of appendix cells cultured in the presence of antigen. Tritiated thymidine was added 24 hours after inception of culture and cells were harvested 12 hours later.

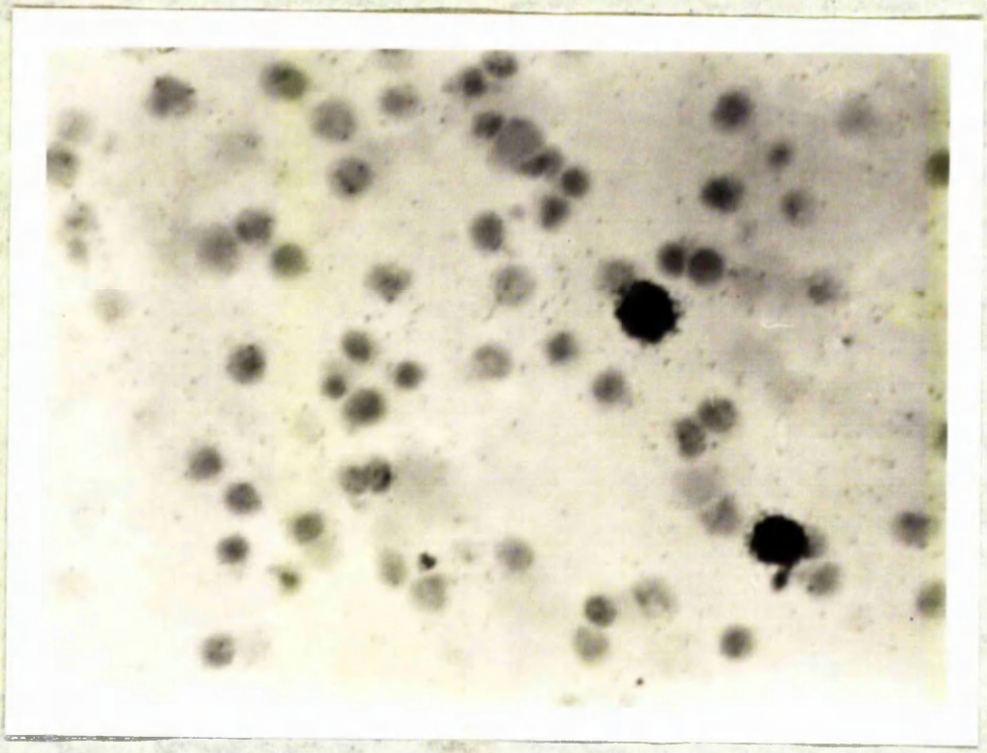


Plate 2 : Autoradiograph of appendix cells in culture. Tritiated thymidine was added 24 hours after inception of culture and the cells were harvested 12 hours later.

STUDIES ON APPENDIX CELLS IN VITRO

D I S C U S S I O N

The appendix cells were grown in suspension at a concentration of 8 million cells/ml. in Eagle's hela cell medium in the presence of 5% normal rabbit serum. This cell concentration is quite high compared with the concentration selected for culture of other cells but it is of the same order as that used by Dutton and Eady (1964) for culture of spleen cells. The concentration used by these authors on that occasion was 6.6 million cells/ml.

Four media were tested for their ability to support [2 - ^{14}C] thymidine uptake. NCTC 109 contains thymidine to a concentration of 10 mg./l. and thus dilution of added [2 - ^{14}C] thymidine would occur, resulting in decreased uptake of [2 - ^{14}C] thymidine. That this occurred can be seen from Table 6. TC 199 contains thymine to a concentration of 0.3 mg./l., and this could be converted to thymidine within the cell with consequent dilution of added radioactive thymidine. Low uptake of [2 - ^{14}C] thymidine occurred when this medium was used. Both the remaining media tested contained neither thymidine nor thymine. Eagle's hela cell medium was used

for culture of leucocytes and this medium consistently supported greater thymidine uptake than Fuck's medium. The serum concentration was 5%.

Fig. 25 has shown that there was no difference in thymidine uptake in appendix cells from a non-immunised animal cultured for 3 days in the presence and absence of BSA. On the other hand, if the appendix cells came from an animal previously immunised to BSA, then the uptake of thymidine into these cells in vitro was found in most cases to be greater in the presence of BSA than in its absence. This increase was noted after one day of culture. The appendix has not been shown to produce antibody but recent findings suggest that it does have a role to play in the immune response. Hanaoka, Nomoto and Waksman (1970) have found that the lymphoid cells of appendix have a critical role to play in 19 S antibody production. They suggest that 19 S antibody is produced by cells which are derived from the appendix.

The injected antigen may not necessarily cause an increase in cell division in the non-antibody forming appendix, as the cells may have already migrated to another locus where they

would then produce 19 S antibody. In a primary response it has been shown by Dixon et al. (1954) that 19 S antibody does not make its appearance until the eighth day after stimulus. Thus, with in vitro primary encounter, the cell division which precedes antibody production may not fall within the period of investigation.

As reported in the results section of this thesis, when BSA was used for secondary stimulus in vitro, 9 out of 10 rabbits showed an increased thymidine uptake by their appendix cells. This could be explained by the fact that, as there is a more rapid response on secondary exposure to antigen, the cell division which necessarily precedes this response is more likely to fall within the time over which the experiment was conducted. Dixon et al.(1954) have observed that in this system free serum antibody occurs 4 days after secondary challenge with antigen. Since the secondary response involves 19 S antibody initially, it is therefore likely that the appendix cells will be involved in this component of the response.

The fact that the optimum concentration of BSA for stimulating uptake of thymidine into DNA of appendix cells is as high as 5,000 µg./ml. may indicate that very few memory cells derived from *in vivo* BSA stimulation may return to the appendix. On the other hand, these memory cells may require an environment other than that provided by appendix alone *in vitro* in order to commence division.

Autoradiographic studies were carried out on thymidine uptake into appendix cell suspensions from rabbits immunised to BSA. Appendix cell suspensions were incubated in the presence and absence of BSA and no discernible difference could be found in the percentage of appendix cells incorporating tritiated thymidine between BSA stimulated cells and control cells. The period over which uptake was examined was between 24 and 36 hours after inception of culture. The increased incorporation of [2 - ^{14}C] thymidine into stimulated cells may therefore represent an acceleration of uptake of label into some of the cells already undergoing division.

In one experiment in ten, using rabbits previously innoculated with BSA, incubation of the appendix with the same

antigen *in vitro* led to complete inhibition of thymidine uptake (Table 7). A similar result was also noted with BCG primed animals in 3 of the 5 separate experiments conducted (see Fig. 23). With both the BSA primed and the BCG primed appendix cells, the inhibition of thymidine uptake was specific for the immunising antigen. If another antigen was incubated *in vitro* with these cells, the pattern of thymidine uptake closely resembled that of control cells (see Table 7). On the other hand, spleen cells from the same animals, incubated *in vitro* with the immunising antigen, showed an increase in thymidine uptake over control spleen cells (i.e. no antigen present). Thus, the inhibitory effect on thymidine uptake was not general throughout the lymphoid tissues of the body.

A number of issues are raised by these observations:

1. Why on certain occasions does the incubation of appendix cells from an immunised animal *in vitro* with the same antigen lead to inhibition of the uptake of thymidine?

It might be suggested that the rabbit did not respond to the antigen or on the other hand that tolerance had occurred. This would seem very unlikely as spleen cells from the BCG

stimulated animals showed an increase in thymidine uptake on re-encountering BGG *in vitro*, which was comparable in quality, if not in quantity, to that described by Dutton and Eady (1964) for spleen cells from immunised animals in culture with the immunising antigen. Moreover, if present theories on the nature of tolerance were considered, it would be expected that a certain inhibition of thymidine uptake by appendix cells might occur rather than total inhibition. Unfortunately, the sera of these animals was not tested for the presence of antibody. Thus, it was not possible to state whether the rabbits in question had responded to immunisation or whether they had become tolerant.

2. Is there a genuine difference in the incidence of inhibition when different antigens are used? For example, 1 out of 10 with BSA and 3 out of 5 with BGG. BSA has a molecular weight of 69,000 and BGG has components varying in molecular weight from 150,000 - 1,000,000, and differences in their effects on immunised appendix cells may be related to these differences.

Hanaoka, Nomoto and Waksman (1970) had suggested that, while the cells of the appendix were involved in 19 S antibody formation, they did not synthesise antibody while in the appendix but rather migrated to the spleen or lymph nodes where they may give rise to the antibody forming cells of these tissues. The reported co-operation between immunised spleen and appendix cells in culture in the presence of the immunising antigen in respect of the ability of both cell types to incorporate thymidine is in agreement with the above report and also with the results of Ford and Gowans (1967) who suggested that the important cells in the immune response were the cells which were entering the spleen shortly after antigen administration. Two preliminary results may help to give some idea of the nature of the interaction between appendix cells and spleen cells. It was found that co-operation between these two cell populations was greatest when the spleen cells were in excess. In fact, this is the relative proportion which one would expect to find generally in splenic tissue.

Preliminary work also suggests that the spleen cells stimulate the appendix cells to greater thymidine uptake in the presence of the immunising antigen. A number of authors, including Konda and Harris (1966), have shown that cells derived from the appendix are responsible for 19 S antibody production and that antibody production is preceded by cell division. The spleen cells may act in conjunction with injected antigen which, as Nossal (1967) points out, localises in the lymphoid follicles of the white pulp, and causes proliferation of the incoming appendix derived cells and the subsequent production of 19 S antibody.

There is some evidence that the appendix may function as a central lymphoid organ in the rabbit. Archer, Sutherland and Good (1963), examining the lymphoid organs of rabbits after neonatal thymectomy, noticed that the appendix was the only one which was not noticeably affected. However, if they removed both the thymus and the appendix in the neonate, a deficiency in immune capacity resulted which was far more severe than if the thymus alone was removed. Meuwissen et al. (1969) showed that *in vitro* DNA synthesis was greater in the

appendix than in other lymphoid organs except for the bone marrow. This is in agreement with the studies reported in this thesis where a higher rate of incorporation was observed in appendix cell cultures than in spleen cell cultures (Fig. 26). Thus, the appendix could expand the lymphoid population by rapid proliferation. In agreement with the previous observation that antibody is not produced in the appendix, Friedenstein and Goncharenko (1965) found that the appendix contains few plasma cells.

Archer, Sutherland and Good (1964) found that the appendix is not dependent on the thymus for its development. Whether this would mean that the appendix does not contain thymus dependent cells is not certain at this time. If this were the case, it would be understandable that antibody formation, which would in many cases require the presence of thymus-dependent lymphocytes, would be absent from this organ. It would also explain the co-operation between appendix and spleen reported in this thesis. The inhibition reported to occur when appendix cells from immunised animals re-encounter

antigen *in vitro* might be explained by an excess of unprocessed antigen encountering the potential antibody forming cells without the co-operation of the thymus derived cells. Thus, when such inhibited cells are incubated with spleen cell suspensions containing thymus-dependent cells, the inhibition is removed and co-operation occurs.

GENERAL DISCUSSION

Since the results obtained in the two sections of this thesis have already been discussed in some detail, it will be the purpose of this discussion to relate these findings to each other and to general aspects of antibody production.

Research over the last decade indicates that antigen introduced into an animal's circulation is quickly taken up by macrophages in the peripheral lymphoid organs. Most of this antigen is degraded but some undegraded antigen may remain on or just inside the surface of the macrophage. Craddock et al. (1967) noted apparent proliferation of macrophages in rat spleen in response to antigen. The relevance of this observation on the immune response is not certain, as Jandl et al.(1965) had noted the same response in the spleens of animals injected with heated autologous red blood cells which would presumably be non-antigenic. The results obtained in this thesis indicate that, when peritoneal macrophages are incubated in vitro with antigen, the rate of incorporation of thymidine into these cells is inhibited. The degree of inhibition bore an inverse

relationship to the concentration of antigen present.

Before comparing these varying results, a number of points should be kept in mind. In vitro studies on macrophages are generally conducted with peritoneal exudate cells. These populations have been found to consist of approximately 90% macrophages. The peritoneal exudate is raised by injection of such materials as paraffin, peptone or oyster glycogen into the peritoneal cavity. These cells therefore would have ingested a quantity of paraffin, or whatever irritant was used, and may in consequence be altered in their properties. Moreover, macrophages from different sites within the same animal have been found by Forbes and Machaness (1963) to be metabolically distinct.

With these points in mind, however, it may be possible to explain the inhibition of thymidine uptake into peritoneal cells in the presence of antigen by postulating that cells which are phagocytosing foreign material will concentrate on degradation and elimination of this material.

It is generally recognised that the macrophage does not produce antibody. There remains therefore the problem of transferring antigenic stimulus from the macrophage to the cell line responsible for antibody production. The small lymphocyte is recognised to be the precursor of the antibody forming cell.

In vitro studies suggest that either (i) undegraded antigen, (ii) messenger RNA whose synthesis was directed by the antigen, or (iii) partially degraded antigen associated with low molecular weight RNA will be important in transferring stimulus to the appropriate reactive lymphocyte. The experiments of Adler et al. (1966) indicated the possibility that messenger RNA may migrate from macrophages to lymphocytes. This view was supported by the results of Jacherts (1966) who extracted RNA from macrophages treated with antigen and added it to spleen cells. The RNA was then extracted from these spleen cells and, when added to fresh spleen cells, antibody production resulted.

On the other hand, the idea that antigen-RNA complex may be important in transfer of stimulus was first suggested by

the work of Askonas and Rhodes (1965) and Friedman et al. (1965). These workers demonstrated an RNA preparation containing traces of antigen which was capable of inducing antibody production by lymphoid cells. The results obtained in this thesis show that, when soluble or particulate antigen is incubated with peritoneal cells, then RNA extracted from these cells will have antigen associated with its lower molecular weight species. Moreover, the effect of antigen on peritoneal cells was found to be to increase the synthesis of RNA, in particular the synthesis of ribosomal and pre-ribosomal species. This increase was proportional to the concentration of antigen. As was expected, the incorporation of leucine into protein of peritoneal cells was increased in the presence of antigen and the increase was again found to be proportional to the concentration of antigen. These increases were interpreted to indicate development of the protein synthesising ability in these cells with a view to extending lysosome production to cope with the ingested foreign material. As was mentioned earlier, cell division may be temporarily halted during this period. In this study, attempts to stimulate

antibody production in lymph node cultures by the addition of RNA extracted from antigen treated peritoneal cells were unsuccessful. At this time no conclusion has been reached as to the role of RNA in the transfer of antigenic stimulus from macrophages to lymphocytes.

It has been known for some time that the plasma cell is the cell involved in active antibody production (Fagraeus, 1948). These cells are derived from the lymphocytes following appropriate stimulus.

Recent work suggests that two distinct lymphocyte populations may be necessary for antibody production. Both of these populations of lymphocytes are derived from bone marrow and are distinct on the grounds that one population requires the thymus for its development - thymus dependent lymphocytes - while the other does not - thymus independent lymphocytes. Good (1969) suggests that the latter population in rabbits may require a "certain microchemical environment", provided perhaps by appendix, for their development. These thymus independent cells are thought to be the producers of antibody. The role of the thymus

dependent lymphocyte is uncertain. It is suggested that these lymphocytes localise the antigenic determinants on their cell surface and present them to the thymus independent lymphocytes.

The results reported in the second section of this thesis confirm the existence of two types of lymphocytes as cited above. Spleen cells contain both thymus dependent lymphocytes and thymus independent lymphocytes and thus spleen cells from an immunised rabbit can exhibit increased thymidine uptake when incubated with the immunising antigen. Appendix, on the other hand, may only have thymus independent cells and thus appendix cells from an immunised rabbit would not be able to offer a very significant increase in thymidine uptake in the presence of the immunising antigen. The reported co-operation between appendix cells is also in agreement with the idea of two distinct lymphocyte populations. The occasional inhibition of uptake of thymidine into appendix cells from immunised rabbits in the presence of immunising antigen is interesting and may represent the activation of a general inhibitory mechanism when thymus independent cells alone are unable to treat the antigen.

When the thymus dependent cell presents antigen to the thymus independent cell, the latter develops into a lymphoblast. Subsequent unequal divisions give rise to (i) a ppulation of plasma cells capable of producing antigen and (ii) memory cells - small lymphocytes which will respond to further challenge with the same antigen by rapid proliferation. In this study, co-operation between spleen and appendix cells occurred. The spleen cells were shown to cause increased proliferation among the appendix cells. This preliminary finding is in agreement with the scheme presented above.

Miller's work (1969) with tolerant animals suggested that the thymus-derived lymphocyte may be the cell susceptible to tolerance, and that these cells may be unispecific. The inhibition of thymidine uptake into appendix cells from immunised animals on re-encountering the immunising antigen in vitro may well have been a manifestation of tolerance and this inhibition was specific for the immunising antigen. Frei et al. (1965) first proposed that tolerance occurred when antigen reacted directly with the lymphocyte whereas if the

antigen had only indirect access to the lymphocyte via the macrophage, then immunity was induced.

What then is the relationship between the macrophage and the immune response? It may be that the macrophage separates out the various determinants of antigenic protein. The various RNA-antigen complexes may leave the macrophages and home to the lymphocytes capable of responding to each determinant.

What then is the role of thymus-dependent lymphocytes? It is postulated that these cells possess a hypothetical immunoglobin on their surface. This immunoglobin "IgX" would concentrate antigen on its surface and present it to the thymus independent cells which would then develop into antibody forming plasma cells.

Much work remains to be done to clarify the role of the appendix in the immune response. Results obtained so far from studies on this organ indicate that it is pertinent to studies concerning distinct lymphocyte populations. Further work is also required to clarify the relation between the macrophage and antibody forming cells.

SUMMARY

The work presented in this thesis was directed towards obtaining a clearer understanding of the metabolic reactions taking place in cells involved in the immune response. Two systems were employed to do this. Peritoneal exudate cells were taken from a rabbit and cultured *in vitro*. Studies were carried out on the effect of the presence of antigen on the metabolism of these cells. The reactions studied were : the rate of uridine incorporation into RNA, the rate of thymidine incorporation into DNA, the rate of leucine uptake into protein and the rate of phosphate uptake into phospholipid.

Uridine and leucine incorporation into peritoneal cells was increased when antigen was present and the increase was related to the amount of antigen present. Thymidine and phosphate uptake into peritoneal cells was decreased in the presence of antigen and the decrease bore an inverse relationship with the amount of antigen. Sucrose density gradients were used to evaluate the character of the RNA species synthesised by peritoneal cells in the presence of antigen.

Studies were carried out on the nature of interaction between antigen and RNA of peritoneal cells.

The second system involved the *in vitro* culture of appendix and spleen cells. A comparison was made between thymidine uptake by appendix cells from immunised rabbits in the presence and absence of the immunising antigen. Thymidine uptake was found to be increased in the presence of antigen but not as dramatically as had previously been reported by other authors for spleen cells under similar conditions. This uptake of thymidine was found on some occasions to be severely inhibited. No correlation could be found between the cells and conditions used and the incidence of inhibition. Studies were carried out on mixed suspensions of spleen and appendix cells cultured in the presence of antigen. In this case uptake was higher than would have been predicted on the basis of uptake by each cell type separately. Where antigen inhibited appendix cells were incubated with non-inhibited spleen cells, the inhibition of the thymidine uptake into appendix cells was removed. Possible reasons for this result are discussed.

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SUMMARY

BIOCHEMICAL STUDIES ON CELLS INVOLVED IN IMMUNE RESPONSES

BY

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Research over the last decade has indicated a possible role in the immune response for RNA extracted from peritoneal exudate cells after exposure to antigen for short time intervals in vitro.

The synthesis of RNA was studied in antigen stimulated peritoneal exudate cells. Studies were also carried out on the capacity of a particulate antigen (T_2 phage) and a soluble antigen (BSA) to combine with RNA. The effect of antigen on the rate of uptake of precursors into protein, DNA and lipid was also investigated and an attempt made to correlate these changes with changes found in RNA metabolism.

The results obtained showed that the uptake of (^3H) uridine and (^{14}C) leucine into acid-precipitable material of rabbit peritoneal exudate cells was increased in the presence of T_2 phage. The increase was found to be proportional to the amount of T_2 phage added. At the same time incorporation of (^{14}C) thymidine and ^{32}phosphate into DNA and phospholipid of peritoneal exudate cells was decreased in the presence of phage. This decrease bore an inverse relationship to the amount of T_2 phage added. Sucrose

gradients were used to evaluate the character of RNA species synthesised by peritoneal cells in the presence of the antigen and it was found that most of the (^{3}H) uridine had been incorporated into ribosomal and pre-ribosomal species. When antigen was added to a similar culture, over the same 30 minute period, there was no increase in labelling of low molecular weight RNA as described by others, but rather there was (if anything) a small increase in labelling of ribosomal and pre-ribosomal RNA.

These results could be interpreted along with the increased leucine uptake into protein in presence of antigen to indicate increased ribosomal protein and RNA synthesis directed towards increased production of lysosomal enzymes. The depression of the uptake of thymidine and phosphate by antigen suggests an antigen induced inhibition of cell division on the part of cells reorganising themselves to degrade the ingested antigen.

A second system involving possible co-operation between cell types was selected for study. Many authors had noted that the appendix exhibited a very high rate of DNA synthesis yet this tissue has not yet been shown to produce antibody. Suggestions have been made that cells from the appendix migrate to the spleen

where they become producers of IgM antibody.

A comparison was made between thymidine uptake by appendix cells from immunised rabbits in the presence and absence of the immunising antigen. Thymidine uptake into appendix cells was found to be slightly increased in the presence of antigen. However, when bovine serum albumin was used as the immunising antigen, subsequent incubation of the appendix cells with bovine serum albumin led to inhibition of thymidine uptake in 1 of 10 animals investigated. On the other hand if bovine gamma globulin was used as the immunising antigen then secondary re-encounter of appendix cells with this antigen in vitro would lead to inhibition of thymidine uptake on 3 out of 5 occasions.

Studies were carried out on mixed suspensions of spleen and appendix cells cultured in the presence of antigen. In this case, uptake was higher than would have been predicted on the basis of uptake by each cell type separately.