# LYMPHOCYTE METABOLISM

by

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John L. Smith

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## SUMMARY

Lymphocytes were separated from human venous blood by cotton wool filtration. $^{14}$C-leucine was used to measure protein synthesis by the cells in plasma-free supplemented Eagle's medium prepared without $^{12}$C-leucine. Synthesis of protein continued for 12 hours in this medium, while synthesis continued for 24 hours if this medium was supplemented with 25% autologous plasma.

$^{3}$H-Uridine was used to measure RNA synthesis by lymphocytes. Lymphocytes synthesised RNA for as long as 48 hours in plasma-free medium. $^{3}$H-Thymidine was used to measure DNA synthesis. Unstimulated lymphocytes did not incorporate this precursor during incubation in plasma-medium for 72 hours.

PHA stimulated both RNA and protein synthesis by lymphocytes. PHA-stimulated lymphocytes began to incorporate $^{3}$H-thymidine after incubation with this substance for 24 hours. Lymphocyte cultures incubated with $^{3}$H-leucine and examined by autoradiography of the dried cell smears showed that PHA both increased the amount of protein synthesised by unstimulated cells and increased the proportion of cells making protein.

Protein synthesis was blocked by puromycin at a concentration of 40 μg/ml in the medium. Actinomycin D inhibited most of the protein synthesis at concentrations of 2 μg/ml in the medium, but a small amount of synthesis continued for 6 hours. Actinomycin D inhibited RNA synthesis. It was suggested that the protein synthesis not inhibited by actinomycin D was dependent on the presence of a long-lived mRNA in lymphocytes. Actinomycin D inhibited the initial stimulation and the established response of lymphocyte protein synthesis to PHA.

Lymphocyte culture fluids were analysed by immunoelectrophoresis and autoradiography. Culture fluids from lymphocytes incubated with $^{14}$C-leucine labelled IgA, IgM and IgG and a number of arcs in the $\alpha$ and $\beta$ globulin regions of IEP patterns. A diffuse distribution of labelled material was always present around the origin and towards the anode of IEP patterns. Evidence was presented for the specificity of immunoglobulin synthesis but the results cast doubt on the specificity of the labelling of the other proteins. Evidence was also presented that lymphocytes from a patient with Hashimoto's disease made antibody to human thyroglobulin.

Labelled immunoglobulins were the only newly-synthesised

proteins detected in the cell supernatants of unstimulated cultures incubated for 24 hours. Other labelled proteins were released after freezing and thawing the cells.

PHA did not stimulate the production of any new proteins by lymphocytes in sufficient quantities for detection by the methods used here. The proteins synthesised by the cells in the presence of actinomycin D were the same as those made by untreated cultures. PHA stimulated the production of IgG less than it stimulated the production of the total proteins when included in the medium of lymphocytes for 24 hours.

Many drugs, which had anti-inflammatory and immunosuppressive activity *in vivo* inhibited lymphocyte protein synthesis *in vitro*. The activity of analogues and metabolites in lymphocyte culture suspensions paralleled their activity *in vivo*.

Three *in vivo* studies suggested that the drugs azathioprine and cortisone inhibited lymphocyte protein synthesis. Adrenocorticotrophic hormone *in vivo* strongly inhibited lymphocyte protein synthesis, probably via stimulation of corticosteroid secretion.

The drugs azathioprine, 6-mercaptopurine and cortisone differed in their effects on HeLa, Hep2 and LLmck2 cells from

their effect on peripheral lymphocytes. The effects of selected drugs on lymphoid cells from lymph and thymus glands were similar to their effect on peripheral lymphocytes.

Thymus cells cultured under conditions similar to those used with peripheral lymphocytes did not make IgA or IgM. IgG synthesis was demonstrated with one preparation of cells. PHA stimulated thymocytes to make immunoglobulins. Lymph node cells *in vitro* made immunoglobulins and a range of proteins similar to those made by peripheral lymphocytes.

The data presented in this thesis support the hypothesis that inhibition of lymphocyte protein synthesis may be one of the mechanisms by which drugs with anti-inflammatory and immunosuppressive activity produce their effect in the body.

CONTENTS

# CHAPTER 1

# LITERATURE REVIEW

## 1.1. Introduction

It seems likely that lymphocytes are involved in all immunological phenomena. Lymphoid cells have been implicated in the primary and secondary responses to antigen, in homograft rejection and in autoimmunity where antibodies are directed against host tissue. Cells of the lymphocyte series accumulate, possibly as a result of neoplastic proliferation, in a number of diseases, including chronic lymphatic leukaemia, lymphosarcoma and Waldenström's macroglobulinaemia. Lymphocyte migration into the tissues in acute inflammation is part of a well ordered response. In chronic inflammation, they are found in large numbers at the site of the lesions.

This thesis is concerned with a study of human lymphocyte metabolism. These cells can be easily obtained in sufficient numbers from blood, free of other leukocytes. Lymphocytes can be cultured in defined medium for short periods and thus present a source of morphologically homogeneous human cells for study.

The products of lymphocytes are important for the expression of their function in immunological phenomena. The literature pertaining to the metabolism of lymphocytes will be reviewed in this chapter, with special reference to protein synthesis. In subsequent chapters of this thesis studies of protein and nucleic acid syntheses in lymphocytes have been reported. In these studies radioactive precursors have been used to follow the synthesis of these products by lymphocytes cultured *in vitro*.

Immunosuppressive and anti-inflammatory drugs vary widely in their structure and action on cell metabolism. Some are known to interfere with deoxyribonucleic and ribonucleic acid synthesis, while others uncouple oxidative phosphorylation. The mechanisms by which these drugs effect their immunosuppressive and anti-inflammatory activity in the body are not fully understood. The literature relating to the effect of a number of these drugs on lymphocyte metabolism will be reviewed. The hypothesis that these drugs exert their influence, at least in part, by their effect on lymphocyte protein synthesis has been investigated in the chapters to follow.

## 1.2. Lymphocyte Function

### 1.2.1. Morphology distribution and circulation

Lymphocytes examined in stained, air-dried smears are small, round cells with a large nucleus and little cytoplasm. The nucleus is dense with a coarse lumpy non-reticular structure. The cells range in size from 8 to 20 μ and are arbitrarily graded into small, medium and large (Wiseman, 1931). Lympoid tissue in man represents about 1% of the total body weight (Braunsteiner and Zucker - Franklin, 1962). Lymphocytes are actively motile (Lewis, 1931; 1933) and are readily identifiable in tissue, blood and lymph by their characteristic biphasic movement (Ebert, et al., 1940).

Collections of lymphocytes occur in the thymus, spleen, lymph nodes and bone marrow. Lymph nodes are encapsulated lymphoid aggregates, supplied and drained by afferent and efferent lymph ducts. Non-encapsulated lymphoid aggregates such as the pharyngeal tonsils and Peyer's patches of the small intestine, receive fluids directly and are drained by efferent ducts. Lymphocytes also comprise about 20% to 30% of the nucleated bone marrow cells (Loutit, 1963) and roughly 20 times as many lymphocytes are present in bone marrow as in

peripheral blood at one time (Braunsteiner and Zucker-Franklin, 1962).

Two populations of peripheral lymphocytes have been reported to exist in man (Ottesen, 1954; Hamilton, 1959). Ottesen (1954) estimated that a minority have a lifetime of 3 days, while the majority have a mean age of 100 to 200 days. Other estimates of lymphocyte lifetimes have been reported to be as long as 540 $\pm$ 64 days (Norman, et al., 1965) and even up to 10 years (Buckton and Pike, 1964) in man. Fitzgerald (1964) suggests that these long-lived small lymphocytes are immunologically 'committed' and that they are an important basis of long term immunity. The existence of such cells lessens the need for a genetic basis for stored antibody information which can be carried adequately by a form of limited inheritance.

The central lymphoid organs are those which are thought to be responsible for the normal development and maturation of lymphocytes, which ultimately migrate to the peripheral lymphoid organs. The central lymphoid tissues in birds are the thymus and the bursa of Fabricus. The bursa of Fabricus is peculiar to birds and its equivalent

has not been found in other higher vertebrates; in these the only central lymphoid organ is the thymus. Immunological competence in the peripheral lymphoid organs, including the lymph node, spleen and lymphoid tissue in the gut is thought to require the presence of central lymphoid tissue, for supplying both cells and humoral factors (Peterson et al., 1965).

The thymus is important in this discussion not only because it may be the site of the origin or maturation of lymphocytes, but also because thymocytes have been compared with small lymphocytes from peripheral blood and lymph nodes in studies of protein synthesis reported in this thesis.

Morphological and cytological observations suggest that lymphocytes originate in the developing thymus of the mouse (Auerbach, 1961). Evidence has been presented that the thymuses of young adult rats lymphocytes arise in the epithelial tissue after an inductive stimulus by the mesenchyme (Sainte-Marie and Leblond, 1964). However, cells with the morphological characteristics of small lymphocytes occur in the bone marrow (Harris and Burke, 1957; Yoffey, 1960) and it has been suggested that they may be pluripotential stem cells (Cudkowicz et al., 1964). Bone marrow cells injected

into lethally irradiated mice colonized the thymus. Lymphoid tissue appeared later in the lymph nodes (Ford and Micklem, 1963). By contrast thoracic duct lymphocytes were not pluripotential (Anderson and Whitelaw, 1960).

Thymectomy in adult rats resulted in a fall in the number of thoracic duct small lymphocytes (Schooley and Kelly, 1961). A similar fall occurred in guinea pigs (Reinhardt and Yoffey, 1956). The development of the thymus in the animal has been found to precede the appearance of small lymphocytes. Neonatal thymectomy in a number of species has been shown to stop the normal growth and maturation of the spleen and lymph nodes (Miller, 1962; Good et al., 1962; Sherman and Dameshek, 1963; Waksman et al., 1962).

Thymocytes have been shown to be in circulation with other lymph tissue of the body and to be able to lodge in these tissues. In the guinea pig the thymus contains a network of lymphatic vessels carrying thymocytes to the general circulation (Kotani et al., 1966). Weissman (1967) has demonstrated by intrathymic radioactive labelling of thymic lymphocytes, the migration of thymocytes to peripheral lymphoid sites in the rat. Radioactive thymocytes have been identified as small lymphocytes in the spleen, bone marrow

and lymph glands, after injection into the blood of syngeneic rats (Everett et al., 1964). In mice, grafts of neonatal thymus released cells which divided in the spleen and lymph nodes; however, the grafts were eventually replaced by host cells (Harris and Ford, 1964; Miller, 1963). In the adult mouse or rat cells from the blood (Harris et al., 1964), spleen (Galton et al., 1964), thoracic duct (Gowans and Knight, 1964) and lymph node (Galton and Reed, 1966) enter the thymus.

In summary these findings emphasize the importance of the thymus for the normal development of the lymphoid tissue. The evidence suggests several roles of thymus function. The thymus in the young may be the sole progenitor of immunologically competent cells. Alternatively, it may be able to attract and provide an environment for the maturation of lymphoid precursors before 'seeding' other tissue (Harris and Ford, 1964; Miller, 1963). This second hypothesis which is based on the assumption that the thymus requires a constant replenishment of stem cells from an external source (Harris et al., 1964; Metcalf and Wakonig-Vaartaja, 1964), explains the apparently pluripotential nature of bone marrow cells. Metcalf (1956, 1958) has

demonstrated production *in vivo* and *in vitro* by thymocytes of a humoral substance termed 'lymphocytosis stimulating factor (LSF)' which bears all the characteristics of a hormone. His evidence suggests hormonal control of peripheral lymphoid tissues by the thymus.

In the body, large numbers of lymphocytes enter the blood via the thoracic and right lymphatic ducts (Yoffey and Courtice, 1956). Little was known of the fate of these cells until it was shown that lymphocytes recirculate between the blood and lymph. Lymphocyte recirculation has been demonstrated in the rat (Gowans, 1959; Gowans and Knight, 1964; Caffrey *et al.*, 1962; Everett *et al.*, 1964), calf (Cronkite *et al.*, 1964), sheep (Hall and Morris, 1964) and man (Perry *et al.*, 1967).

In the rat, the main route of recirculation appears to be within the lymph nodes and Peyer's patches, where small lymphocytes enter by transversing the endothelial cytoplasm of the post-capilliary venules (Gowans and Knight, 1964).

1.2.2. Immunological activity

The small lymphocyte has been implicated in many functions related to the immunological response. Lymphocytes from the thoracic duct (Wesslen, 1952), lymph and spleen

(Najarian and Feldman, 1961) are able to transfer tuberculin hypersensitivity. Small lymphocytes are able to transfer transplantation immunity (Najarian and Feldman, 1962), autoimmune nephritis (Hess et al., 1962) and contact sensitivity to chemicals (Najarian and Feldman, 1963).

Gowans (1962) was able to initiate a fatal wasting disease in $F_1$ hybrid rats by infusion of thoracic duct lymphocytes from parental strain rats. He observed in the course of the immune reaction the transformation of these cells into large pyroninophilic cells. Other workers (Hildemann et al., 1962; Hildemann, 1964) observed that small lymphocytes from adult mice were capable of producing transplanation disease and runting when injected into newborn hosts of a different strain. Their observations led them to conclude that small lymphocytes from adult mouse blood were immunologically reactive, although the majority of the cells after injection into the host were eliminated and did not appear to proliferate. These observations suggest that small lymphocytes are primary cells involved in homograft rejection. They are able to attack tissue directly or form large cells which are probably involved in antibody responses to the host tissue. (The role of

these large cells in antibody responses will be discussed.) It has been demonstrated in lymphocyte-depleted rats that homografts survived permanently when the antigenic differences between the donor and recipient were small (McGregor and Gowans, 1964) indicating the importance of the small lymphocyte in host-versus-graft reactions.

The precise role of the small lymphocyte in antibody responses is not known. Recent evidence suggests that the involvement of the cell in the primary and secondary response differs. Primary responses in rats were reduced by drainage of lymphocytes from the thoracic duct. The response was restored by intravenous infusion of syngeneic small lymphocytes from normal rats but not from those made tolerant to the antigen (McGregor and Gowans, 1963). Small lymphocytes appear to be necessary for the normal primary response and have been implicated as carriers of immune responses within the body. Hall and co-workers (1967) have observed in the lymph nodes of sheep stimulated with antigen, the transformation of small lymphocytes to large basophilic cells, which were able to transfer the immune response to other nodes and between chimeric twins.

Other cell types seem to be necessary for the

initiation of a primary immunological response. Ribonucleic acid (RNA) extracts from macrophages exposed to antigen, were able to induce antibody production in lymph node cultures. The response could not be elicited by antigen alone (Fischman, 1961; Fischman and Adler, 1963). The immune response can be transferred from immunized to non-immunized spleens in mice by RNA extracts (Cohen, 1967); however, the stimulus may not be due solely to RNA but to an RNA-antigen complex (Askonas and Rhodes, 1965; Friedman et al., 1965). Phagocytic cells in lymphoid tissue retain antigenic proteins for long periods, in which time they could be complexed with RNA (Garvey and Campbell, 1957; Bartfield and Juliar, 1964).

In summary, the present evidence suggests that in the primary responses, the antigen enters the macrophage, becomes complexed with RNA, or incites the formation of intracellular RNA, which can activate small lymphocytes to transform to large RNA rich cells. These cells divide rapidly and are found in the efferent lymph as lymphoblasts. It has been reported that lymphoblasts are able to transfer the immune response to remote tissue (Hall et al., 1967) and that they are able to differentiate into plasma cells (Birbeck and Hall, 1967), so that the end result in either situation is

to increase antibody production.

While lymphocyte depletion in rats had led to impaired primary responses, the secondary response was unaffected (McGregor and Gowans, 1963). This suggests that secondary responses are mediated by fixed cells in the lymphoid tissue. Nossal and Mäkelä (1962) have presented some evidence to support the contention that the fixed precursors of antibody producing cells are dividing large lymphocytes. They transferred almost pure small lymphocyte suspensions from the thoracic duct of primarily immunized rats to normal X-irradiated recipients and observed typical secondary responses in these animals after antigen challenge. It appears that the role of the small lymphocyte in the secondary response is as a memory cell. The evidence that small lymphocytes contain antibody (Attardi et al., 1964) supports this.

Collections of lymphocytes, especially small lymphocytes, are a characteristic feature of long standing inflammatory infiltrates. Small lymphocytes are constantly seen at the periphery of tuberculous and granulomatous lesions (Spector and Willoughby, 1963). Lymphocyte immigration is a part of the acute inflammatory response. In rabbits active macrophages were first seen at the site of subcutaneous injections

of egg albumin. Neutrophils began to migrate into the area after two hours. At four hours, lymphocytes began to appear and were present in greatest numbers after eight hours. Mononuclear cells replaced lymphocytes and became predominant after twelve hours (Page and Good, 1958; Kolouch, 1939). A similar cycle of events has been observed in man (Rebuck and Crowley, 1955).

The time lapse before lymphocyte migration in acute inflammation suggests that substances produced at the inflammatory site induce the lymphocyte response to inflammation. Both actinomycin D and puromycin were effective in blocking the lymphocyte response (Page, 1964). However, actinomycin D given one hour after the initiation of inflammation was ineffective. This suggests that protein synthesis by the lymphocyte is a necessary prerequisite for migration and that this synthesis appears to occur very early in the inflammatory response. The stimulus for this onset of protein synthesis has been attributed to the neutrophil (Page, 1964).

## 1.3. Protein Synthesis

### 1.3.1. Biosynthesis in mammalian tissue

It is the purpose of this summary to outline some of the

theories of protein synthesis in mammalian cells, as supported by recent experimental findings, before proceeding to a discussion of protein synthesis in lymphocytes and the effects of various agents on lymphocyte protein synthesis *in vivo* and *in vitro*. The formation of protein within a mammalian cell involves the joining together of twenty or more amino acids by α-peptide linkages into an enormous variety of genetically determined sequences. The nucleic acids are intimately involved in this process. The roles of deoxyribonucleic acid (DNA) and RNA will be discussed.

1.3.1.1. Amino acid activation

The initial step is concerned with the activation of the amino acid to form an enzyme bound amino acyl adenylate. The activation reaction becomes the first step in which the 20 unit language (amino acids) of protein synthesis is translated into the four unit language (adenine, uracil or thymine, guanine and cytosine) of nucleic acids. It is now generally accepted that bacterial and mammalian cells possess amino acid activating enzymes for each of the common amino acids. The specifity of the amino acid activating enzymes is high, but is not absolute. However, control mechanisms have been shown to exist which reduce the possibility of errors to an extremely low frequency (Baldwin and Berg, 1966; Novelli,

1967). The evidence for the existence of a single amino acid activating enzyme for each amino acid is strong and it does not appear that several types of enzymes, which activate one amino acid, exist (Novelli, 1967).

The mechanism of activation involves the carboxyl group of the amino acid, which is activated by adenosine triphosphate with the elimination of pyrophosphate (Hoagland et al., 1956; Berg, 1961). The same enzyme responsible for the activation of the amino acid transfers the amino acid to transfer ribonucleic acid (t-RNA). In doing this the enzyme must recognise not only its amino acid but also the amino acid's t-RNA (Berg, 1961).

1.3.1.2. Transfer of the amino acid to the template

After activation of the amino acid and the formation of the amino acyl RNA complex, the amino acid is transferred to the template. Transfer RNA fulfills the role of an adaptor molecule carrying the amino acid to the template (Crick, 1958) and in so doing it must possess some recognition device (Chapeville, et al., 1962; Weisblum, et al., 1962). Each transfer RNA has a strict specificity for its amino acid and amino acid activating enzyme (Novelli, 1967).

A number of multiple t-RNA's for the same amino acid in

a given species has been reported. For example E. coli has been shown to have five leucine specific t-RNA's (Apgar and Holley, 1964; Kelmers, et al., 1965), and possibly three serine specific t-RNA's, two tyrosine t-RNA's, two phenylalanine t-RNA's and three valine t-RNA's (Goldstein, et al., 1964).

The heterogeneity of amino acid specific t-RNA's has been ascribed to the degeneracy of the genetic code and in some cases these multiple forms of a given t-RNA have been shown to respond to different coding triplets (Norelli, 1967).

The acceptor site on the t-RNA molecule is the terminal adenosine group and this will not function unless the two nucleotides adjacent to it are cytosine (Hecht, et al., 1959; Zauchau, et al., 1958). Recent studies (Fresco, et al., 1966) have shown that active forms of various t-RNA's have unique tertiary structures exhibiting varying degrees of stability with respect to amino acid specificity. This finding suggests that the strict specificity of t-RNA for its amino acid and amino acid activating enzyme complex may depend on a unique tertiary conformation.

During transfer of the amino acid to the ribosome, the whole of the t-RNA molecule enters the ribosome and reappears

unchanged after transfer of its amino acid (Chapeville, et al., 1962; Weisblum, et al., 1962; Nathans and Lipman, 1961; Hoagland and Comley, 1960).

1.3.1.3. Peptide formation

Transfer ribonucleic acid is responsible for the transfer of the amino acid to the template. The template consists of aggregates of ribosomes, which are joined by messenger ribonucleic acid (m-RNA), termed polysomes (Warner, et al., 1962), or ergosomes (Wettstein, et al., 1962). Polysomes are widely distributed in bacterial and mammalian cells and are thought to be active in protein synthesis although Munro et al., (1963) suggested that single ribosomes may be active if m-RNA is attached. The association and dissociation of the ribosomal aggregates appear to be dependent on the magnesium ion concentration, the pH and ionic strength of the cell sap (Chao, 1957; Petermann and Hamilton, 1961). The ribosomes are made up of 60-65% RNA and 35-40% protein. Each contains dissociable units rougly two-thirds and one-third by weight (Schweet and Heintz, 1966).

m-RNA which is attached to the aggregates of ribosomes is synthesized as a complimentary molecule to DNA and is thought to carry a specific code from DNA. Both strands of

DNA are copied. At the surface of the ribosomes, m-RNA reacts with a complimentary code on the t-RNA molecule, after which transfer of the amino acid takes place and ordered peptide synthesis begins. The peptide formation appears to be stepwise from the N-terminal to the C terminal of the chain (Korner, 1964).

The code letters in RNA are read in triplets or codons, each of which specifies one, or sometimes more, of the twenty amino acids that form protein molecules. Six codons have been demonstrated for the amino acids leucine and arginine. Multiple codons have been demonstrated for other amino acids. There is no information available to tell us if the organism or cell is making use of all codons at any one time (Novelli, 1967).

The initiation of the peptide chain in E. coli has been found to involve a particular amino acid, N-formylmethionine. It is now thought that the initiation of the peptide chain on the template requires a special starting codon which in E. coli calls for the placement of N-formylmethionine at the N terminal of the chain (Marcker and Sanger, 1964; Webster, et al., 1966; Adams and Capecchi, 1966). The end of the peptide chain may be marked by a special terminal codon (Schweet and Heinz, 1966).

The release of protein from the ribosomal template is independent of ribosomal disintegration. Release has been demonstrated to be energy dependent, requiring precise conditions of ionic strength and potassium ion concentration (Hultin, et al., 1961; Grabowski and Munro, 1960).

1.3.2. Biosynthesis in lymphocytes

The lymphocyte is able to synthesize a variety of proteins, including the immunoglobulins. The present evidence indicates that the mechanisms of synthesis are similar to those in other mammalian cells. In this discussion the nature of the proteins made by lymphocytes, the ultrastructure of these cells and their ability to synthesize nucleic acids will be reviewed.

1.3.2.1. Nature of the proteins made

Rabbit peripheral blood lymphocytes have been reported to make antibody and $\gamma$globulin (Hulliger and Sorkin, 1963; Kearney and Halliday, 1965; Landy, et al., 1964). Human peripheral blood lymphocytes have been reported to make $\gamma$globulin (Bach and Hirschhorn, 1963; Van Furth, 1964; Parenti, et al., 1966), and a range of proteins identifiable after immunoelectrophoresis and autoradiography (Van Furth, 1964). The interpretation of these findings is subject

to criticism. A discussion of this aspect will be found in Chapters 4 and 5 of this thesis.

Phytohaemagglutin and antigen are able to stimulate the transformation of small lymphocytes into large and medium-sized cells capable of making antibody or $\gamma$ globulin (Elves, et al., 1963). It was recently shown that antigenically stimulated lymphocytes are able to transform into cells identical with plasma cells (Birbeck and Hall, 1967). Plasma cells are regarded as the principal antibody producing cells in the body (Nossal, 1959, 1962; Mellors and Korngold, 1963; Bjørneboe et al., 1947).

The synthesis of immunoglobulins like that of all proteins is under genetic control (Cohen and Milstein, 1967; Lobb, et al., 1967). Immunoglobulin proteins are composed of chains of different molecular weights. Immunoglobulins can be separated into light (L) and heavy (H) chains by mercaptoethanol (Edelman and Benacerraf, 1962; Edelman et al., 1961; Edelman and Poulik, 1961) and similar A and B chains by reduction and acidification (Porter, 1963; Fleischman, et al., 1962; Edelman, et al., 1963). The molecular weights of the B and L chains are similar and are approximately half those of the A and H chains (Edelman and

Benacerraf, 1962; Cohen, 1963). The synthesis of these two different chains is thought to occur at different ribosomal groups within the cell (Becker and Rich, 1966; Shapiro, et al., 1966).

Antibody globulins are of three main types, IgM, IgA and IgG. These three immunoglobulins differ in their types of heavy chains but have common light chains. The bulk of the antibodies in rabbits and in man is formed by the IgG globulins. Their appearance in the blood serum is frequently preceded by the formation of IgA and IgM globulins (Haurowitz, 1965a). Antibody molecules have two antigen combining sites (Porter, 1963) and these sites are identical (Mandy, et al., 1963).

1.3.2.2. Protein synthesis mechanisms in lymphocytes

When small lymphocytes are examined under the electron microscope they contain amongst other structures small amounts of endoplasmic reticulum, nucleoli and ribosomes. These structures suggest that they are capable of synthesizing deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and protein (Zucker-Franklin, 1963). When stimulated by phytohaemagglutinin these cells develop many ribosomes, the endoplasmic reticulum forms into rough vesicles and sacs and

the Golgi apparatus becomes well developed with smooth surfaced vesicles and cisternae (Elves, et al., 1964). Morphologically indistinguishable cells are found in the lymphoid tissues of animals during host vs. graft and graft vs. host reactions (Binet and Mathe, 1961; Gowans, et al., 1961). When these cells are examined under the electron microscope they have an ultrastructure which is almost identical with that of the phytohaemagllutinin stimulated lymphocyte cell (Binet and Mathe, 1961; 1962; Binet, et al., 1961), except they do not have prominent electron opague bodies (Elves, et al., 1964). These cells are thought to derive from the small lymphocyte (Porter and Cooper, 1962).

Antibody producing cells in the spleen and lymph nodes, when examined by electron microscopy, contain large numbers of ribosomes grouped as polysomes (Cunningham, et al., 1966; Becker and Rich, 1966; Shapiro, et al., 1966). These polysomes fall into two groups, one containing 7-8 ribosomes and the other 16-20 ribosomes. This biphasic distribution within the cell appears to be linked with its antibody production and has been associated with the animal's state of immunity (Becker and Rich, 1966; Shapiro, et al., 1966). The biphasic ribosome distribution is peculiar to these antibody producing

cells and differs from distributions in E. coli (Kiho and Rich, 1964), chick embryo (Kretsinger, et al., 1962) and HeLa cells (Rich, et al., 1963).

The synthesis of the light chains of immunoglobulin has been associated with the smaller polyribosomes and the synthesis of the heavy chains with the larger polysomes (Shapiro, et al., 1966; Becker and Rich, 1966). Some consider that the light and heavy chains are synthesized separately and assembled by some process (Hood, et al., 1966) while other suggest they are continuously assembled during synthesis (Becker and Rich, 1966).

Lymphocytes synthesize ribosomal and cytoplasmic RNA (Newsome, 1965; Cooper and Rubin, 1966; Winter, and Yoffey, 1966) and DNA (Hayhoc and Quaglino, 1965; Rubini, et al., 1965; Inman and Cooper, 1965). The effects of antigens and phytohaemagglutinin on RNA and DNA synthesis parallel their effect on protein synthesis. This will be discussed in the following section.

The relation of lymphocytes to antibody producing cells has been discussed. The present evidence indicates that these antibody producing cells are derivatives of antigenically stimulated small lymphocytes, which enlarge to form blast

cells. It appears reasonable to propose that the intracellular stages of protein synthesis in lymphocytes and in antibody-producing cells closely resemble those mechanisms found in other mammalian tissues. The effects of stimulators and inhibitors of protein synthesis will be discussed in relation to this general scheme.

## 1.4. Factors influencing lymphocyte metabolism

Puromycin and actinomycin D, inhibitors of protein synthesis in other mammalian cells, inhibit lymphocyte protein synthesis (Van Furth, 1964). Antigens and phytohaemagglutinin stimulate lymphocyte protein synthesis. Many drugs exhibit an anti-inflammatory and immunosuppressive activity *in vivo*. These agents will be discussed in relation to lymphocyte metabolism.

### 1.4.1. PHA and antigens

An extract of the kidney bean (*Phaseolus vulgaris*), phytohaemagglutinin (PHA) has been known for some years to be able to stimulate small lymphocytes to mitosis (Nowell, 1960; Börjeson, *et al.*, 1964; Ling and Husband, 1964: MacKinney, *et al.*, 1962).

Phytohaemagglutinin is a protein containing 6% carbohydrate. The haemagglutinating principle of the substance

can be removed by erythrocyte absorption without any detectable loss of the mitotic stimulating activity (Börjeson, et al., 1964). However, the leucoagglutinating property can not be removed from its mitogenic activity (Kolodny and Hirschhorn, 1964). The mitotic action of PHA has recently been shown to be inhibited by a specific antiserum (Byrd, et al., 1967).

PHA stimulates RNA (Cooper and Rubin, 1965), DNA (Yoffey, et al., 1965) and protein synthesis in lymphocyte (Bach and Hirschhorn, 1963) and protein synthesis in leukocyte cultures (Turner and Forbes, 1966). When PHA transformed lymphocytes are examined cytochemically, they are found to contain increased amounts of protein, RNA and glycogen compared with unstimulated cells (Gough and Elves, 1966).

Several substances other than PHA have been shown to stimulate transformation of peripheral lymphocytes into large blast like cells capable of division. These include tuberculin purified protein derivative (Schrek, 1963; Pearmain, et al., 1963), tetanus toxoid, typhoid-paratyphoid vaccine, diphtheria toxoid, smallpox vaccine, Haemophilus pertussis antigen (Elves, et al., 1963a,b; Hirschhorn, et al., 1963), leukocyte antiserum (Grasbeck, et al., 1963), pollen extract (Lycette and Pearmain, 1963) various tissue antigens (Hashem, 1963; Hashem and Barr,

1963) and pokeweed (Phytolacca americana) extract (Farnes, et al., 1964; Borjeson, et al., 1966; Chessin, et al., 1966; Chessin, et al., 1967).

A much lower percentage (0.5% - 4%) of peripheral lymphocytes were stimulated to mitosis by smallpox vaccine, PPD and E. coli filtrate than by PHA and staphlococcal culture filtrate (Ling and Husband, 1964). The stimulus by the group of antigens may be specific for a particular cell population in contrast with PHA and staphlocci filtrate which do not appear to exhibit this specificity.

When lymphocytes are stimulated by PHA or antigen, the changes, observed in the transformed cells by light and electron microscopy, are similar (Elves and Wilkinson, 1963; Elves, et al., 1963a; Pearmain and Lycette, 1963). However, the mechanisms by which these agents stimulate small lymphocytes are not known to be similar or dissimilar.

It has been shown in PHA treated cells that an increase in RNA synthesis was preceded by an early stimulation of phosphorylation and dephosphorylation of nuclear proteins, concomitant with increased activity in the nucleus (Kleinsmith, et al., 1966). In studies reported by Cooper and Rubin (1966) PHA stimulated human lymphocytes to produce RNA which did not

become associated with the ribosomes. In contrast antigen stimulated the production of RNA which became associated with the ribosomes. PHA was needed continuously for the stimulation of non-ribosomal precursor RNA otherwise the RNA synthesis reverted back to ribosomal precursor. Mach and Vassalli (1965) have reported that antigen stimulated an increase in ribosomal RNA precursor in both lymph nodes and spleen cells.

Torelli, et al. (1965) suggested that this difference between antigen and PHA-stimulated lymphocytes offers a possible explanation of the action of PHA. The postulate assumes that PHA increases the availability of t-RNA, rather than that PHA has a direct effect on m-RNA. As a consequence the ribosomal template in unstimulated cells becomes fully active. Induction of new m-RNA is followed by DNA synthesis and mitosis. This type of mechanism would in part explain the non-specificity of PHA stimulation.

The mechanism by which antigen elicits antibody formation is the subject of much controversy. Theories of antibody synthesis fall into two groups, the template and the selective. In the first, the antigen is thought to modify protein synthesis within antibody producing cells, while in the second, antigen selects those cells which are coded for the production of the

specific antibody (Haurowitz, 1965a,b).

Antigen appears in the body at the site of amino acid incorporation (Haurowitz and Crampton, 1952; Crampton, et al., 1952; Roberts and Haurowitz, 1962) and can combine with RNA fractions (Askonas and Rhodes, 1965; Friedman, et al., 1965). The molecular configuration and change of an antibody is complementary to that of the antigen (Landsteiner, 1945). These properties of the antibody depend on its amino acid sequence, which differs in antibodies to different haptens (Groff and Haurowitz, 1964; Knight, et al., 1964; Gold, et al., 1964).

This evidence favours a template role of antigen action. The template theories require the continued presence of the antigen during antibody formation and for the elicitation of the secondary response. However, the persistence in vivo of the antigen or antigen-RNA complexes is not known. The selective theories do not depend for their validity on the continued presence of the antigen, which may act selectively at a cellular or subcellular level in a population of cells (Burnett, 1964; Jerne, 1962; Szilard, 1960) for the production of antibody producing clones.

1.4.2. Drugs

In the work to be described here lymphocyte cultures were used to study the activity of a group of drugs with anti-inflammatory and immunosuppressive properties. The drugs included hydrocortisone, thiopurines, salicylate, phenylbutazone and its metabolite oxyphenbutazone and an analogue sulphinpyrazone, chlorambucil, indomethacin, chloroquine and mefenamic acid. Several of these drugs will be discussed with respect to lymphocyte metabolism *in vitro* and *in vivo*.

Drugs known to block protein synthesis by mammalian cells also block antibody synthesis. Actinomycin C was an effective inhibitor of antibody synthesis when giving 1 to 4 days after antigen *in vivo* (Brown, 1964). Actinomycin D (Smiley, *et al.*, 1964; Uhr, 1963) and puromycin (Smiley, *et al.*, 1964) completely abolished antibody synthesis by spleen and lymph node cells. Both puromycin and actinomycin D inhibited the lymphocyte response to inflammation in rabbits (Page, 1964).

The purine antimetabolites are known to have an effect on the immunological responses both *in vivo* and *in vitro*. 6-Mercaptopurine (6MP) inhibited the primary response to antigen in rabbits and mice (Cruchaud, 1966; Butler and Coons,

1964). The drug partly suppressed the secondary response in rabbits (Cruchaud, 1966) but had no effect on the secondary response in mice (Butler and Coons, 1964). In mice, 6MP induced reversible immunological paralysis similar to that induced by radiation (Brooke, 1966). In man therapeutic oral doses of 6MP caused a sharp drop in the incorporation of labelled amino acids by leukocytes *in vitro*, isolated from patients with chronic granulocytic leukaemia. This fall preceded the decrease in the number of circulating lymphocytes by several days (Nadler, *et al.*, 1961). Scott, *et al.* (1966) found that the effects of 6MP on leukocytes *in vitro* were erratic and could not be correlated with regularity with the observed effects *in vivo*. 6MP has been shown in rabbits to inhibit the lymphocyte response to inflammation (Page, *et al.*, 1962).

Azathioprine, another purine analogue, is effective in the treatment of haemolytic anaemia, an autoimmune disease in man (Dameshek and Schwartz, 1960; Schwartz and Dameshek, 1962; Hitzig and Massimo, 1966). Both 6MP and azathioprine are active in the prevention of kidney graft rejection (Hitchings and Elion, 1963; Schwartz, 1965). The purine analogue thioguanine is highly effective in inhibiting antibody

production (Berenbaum and Brown, 1964; Berenbaum, 1966) without causing gross destruction of lymphoid tissue (Phillips, et al., 1956).

The antibiotic chloramphenicol induced immunological tolerance in rabbits and mice suppressing the primary response completely or partially. However, it had little effect on the secondary response (Cruchaud, 1966; Butler and Coons, 1964). In vitro, the drug had no effect on the established immune response in rabbit lymph node fragments but did affect the initiation of the response with antigen (Ambrose, 1966). Combined with 6MP the drug was more effective than either 6MP or itself at similar concentrations on the primary response in rabbits (Cruchaud, 1966).

Salicylate, a drug with mild anti-inflammatory activity, has been reported to inhibit the secondary response to antigen in vitro (Ambrose, 1966). The drug reduced death from anaphalaxis (Lepper, et al., 1950) and inhibited the Schwartzman reaction in rabbits (Schwartzman and Schneierson, 1953). Salicylate is partially effective for the treatment of rheumatic symptoms, while meta- and para-hydroxybenzoic acid are not (Smith, 1953).

A group of analogues with greater activity as anti-inflammatory agents than salicylate, include phenylbutazone, oxyphenbutazone and sulphinpyrazone (Randall, 1963). A comparative study of the effects of these analogues on inflammation induced in animals, has been reported by Domenjoz (1960). Oxyphenbutazone, a metabolite of phenylbutazone has anti-rheumatic and pharmacological effects similar to the parent drug, but is more toxic. Sulphinpyrazone is a less striking anti-inflammatory agent than phenylbutazone, but a much better uricosuric agent in man (Goodman and Gilman, 1965).

No common action can be ascribed to the immunosuppressive and anti-inflammatory drugs. Salicylates and phenylbutazone analogues are uncouplers of oxidative phosphorylation (Whitehouse, 1963) while others like the purine analogues interfere with purine nucleotide and nucleic acid synthesis (Elion, 1967). Chlorambucil, an alkalylating agent, is thought to cross link DNA and inhibit rapidly-proliferating cells (Goldacre, et al., 1949; Timmis, 1958). The effect of chlorambucil on DNA replication exceeds its ability to impair RNA production (Wheeler, 1962). Some agents inhibiting immune responses

cause necrosis of lymphoid tissue, for example chlorambucil (Elson, et al., 1958), cyclophosphamide (Wheeler, 1962), corticosteroids (Dougherty and White, 1945) and radiation (Bloom, 1948). However, many substances such as methotrexate, 6-mercaptopurine and thioguanine in dose levels that inhibit immune responses as efficiently as the lymphotoxic agents, have little effect on lymphoid tissue (Berenbaum, 1964).

It is possible that immunologically competent cells differ in their sensitivity to immunosuppressive agents at various stages of their differentiation, for example radiation and busulfan damage cells at early stages of differentiation whereas mustards and ethyleneimines damage them at a later stage (Berenbaum, 1964).

While the drugs discussed may differ widely in their site and mechanism of action within the body, it is proposed that they in part exert their activity via the lymphocyte. This hypothesis based on the central role of the lymphocyte in inflammatory and immune responses, will be examined in this thesis with particular reference to drug effects on protein synthesis. In the studies to follow the relevance of the findings with various drugs will be discussed with respect to this postulate.

# CHAPTER 2

# THE DETERMINATION OF CONDITIONS FOR LYMPHOCYTE CULTURE

## 2.1. Introduction

In Chapter 1, the central role of the lymphocyte in immunological phenomena has been reviewed. These cells are easily isolated from the blood, providing a morphologically homogeneous cell suspension available for investigation. *In vitro* studies of lymphocyte metabolism not only assist in defining its function but also make available a biological system for the study of agents which influence its function. For example, leukocyte and lymphocyte protein synthesis (Hirschhorn, *et al.*, 1963; Van Furth, 1964; Forbes and Turner, 1965; Turner and Forbes, 1966), RNA synthesis (Newsome, 1965; Winter and Yoffey, 1966; Cooper and Rubin, 1966) and DNA synthesis (Hayhoc and Quaglino, 1965; Chapman and Dutton, 1965; Rubini, *et al.*, 1965) have been studied *in vitro*. In many of these studies the effects of PHA have been examined. Many of these investigators have employed mixed leukocyte cultures and culture medium containing human plasma or calf serum as supplement. Cooper and Rubin cultured lymphocytes in Eagle's medium (1955) while Van Furth employed a defined medium supplemented with 0.5% ovalbumin.

In this chapter investigations of *in vitro* conditions suitable for lymphocyte protein synthesis have been reported. In these investigations $^{14}$C-leucine has been used as radioactive precursor. The studies reported here are related to the following aims:

1. To obtain cell suspensions containing only lymphocytes and erythrocytes.
2. To investigate a plasma-free medium for culture purposes.
3. To determine that erythrocytes which contaminated the final cell suspensions did not contribute to the incorporation of $^{14}$C-amino acid into protein. The effect of other leukocytes in the suspensions was also investigated.
4. To determine that the incorporation of $^{14}$C-amino acid represented true protein synthesis.

## 2.2. Materials and Methods

### 2.2.1. Isolation and culture of lymphocytes

All the glassware employed in these techniques except blood bank bottles was siliconized (Siliclad, Clay Adams Inc. N.Y.). All glassware was subjected to a routine washing procedure (Appendix i).

Lymphocytes were prepared from five hundred millilitres

of venous blood collected from healthy donors into bottles containing heparin (0.5% final concentration, Commonwealth Serum Laboratories, Parkville, Victoria) and dextran (5% final concentration of a 6% dextran solution in saline, Glaxo, Greenford, England).

The erythrocytes were allowed to settle for one hour at 37°C. The supernatant was collected and centrifuged at 150 x g for 4 minutes to sediment most of the polymorphs, monocytes and erythrocytes. The lymphcoyte-rich suspension from this first centrifugation was drawn off and concentrated at 800 x g for 15 minutes and resuspended in 50 ml of plasma. This suspension was added to a glass column (2 x 30 cm) loosely packed with surgical cotton wool and plugged at both ends. (Fichtelius, 1950). The column was then incubated at 37°C for 45 minutes.

At the end of the incubation period, the cells were eluted with 50 ml of warm fresh autologous plasma. The cells were then washed twice with Hanks balanced salt solution (HBSS, Commonwealth Serum Laboratories, Parkville, Melbourne, Victoria) before final suspension in Eagle's culture medium (Eagle, 1955) made up without leucine and supplemented with "non-essential" amino acids as described by Ambrose (1964).

The composition of the medium and the nature of the amino acids are given in the Appendix (ii). When the cells were cultured in Eagle's medium containing leucine, stock medium from the Commonwealth Serum Laboratories (Parkville, Victoria) was used.

The final cell suspension was dispensed in 1 ml portions containing 1-4 x $10^6$ lymphocytes, into 20 ml universal containers for culture. Determinations were made in triplicate.

Polymorphs and monocytes, which were used in several experiments, were obtained from whole blood prepared in the manner described with the omission of the low speed centrifugation (150 x g). Columns of glass beads were substituted for cotton wool. After incubation the cells were eluted from the columns with buffered solution at 37°C (Rabinowitz, 1964). The compositions of the eluting solutions are given in the Appendix (iii). The polymorphs and monocytes were washed once with HBSS and resuspended in the same medium as that which was used for lymphocyte culture.

$^{14}$C-Leucine (L-leucine $^{14}$C(U), specific activity of 150 mc/mM, Radiochemical Centre, Amersham, England) was added to cultures in concentrations of 0.25 to 10.0 µc/ml. Cultures were incubated at 37°C, in an atmosphere of 95% $O_2$ and 5% $CO_2$ and were terminated by freezing (-15°C) at 24 hours,

or at other times as indicated.

The drugs, actinomycin D (Merck, Sharp and Dohme, Rahway, N.J.), puromycin (Lederle, Pearl River, N.Y.) and phytohaemagglutinin (PHA, Burroughs Wellcome, England) were added to the cultures as indicated in the text.

2.2.2. Determination of the incorporation of $^{14}$C-leucine into protein

At the termination of culture the cultures were frozen (-15°C) and thawed. This was repeated twice. The culture fluids were then decanted into plastic tubes and the culture bottles were rinsed once with 2 ml of 0.9% saline and the rinsings added to the tubes. The tubes were centrifuged to remove cell debris at $12.10^3$ x g for 10 minutes. The supernatants were decanted and 0.25 ml of a standard pooled serum was added to each. No carrier protein was added to the supernatants from cultures containing plasma. The protein was precipitated with cold trichloroacetic acid (TCA) in a final concentration of 5%. The precipitates were washed three times with 5% TCA and finally taken up in dilute $NH_4OH$ for plating onto 2 $cm^2$ planchettes at infinite thickness. Samples were counted in a gas flow Geiger Muller counter (Philips, Model PW 4032).

Infinite thickness was determined to be 5 mgm of protein/2 $cm^2$ (Appendix iv). Serum from a standard pool was dispensed into portions and stored at -15°C between use. The amount added to the culture supernatant was well in excess of the amount for infinite thickness. This amount was chosen so that comparison between the incorporation of isotope into protein by lymphocytes cultured in plasma free medium and by those cultured in medium containing 25% plasma, could be made easily.

. Five per cent TCA was sufficient to precipitate all acid insoluble radioactive protein. Subsequent addition of 10% TCA to such supernatants did not precipitate any more labelled protein. Two or three washes of the precipitated protein with 5% TCA by centrifugation at 1500xg, were sufficient to remove all traces of free $^{14}$C-leucine. Three washes were adopted as standard procedure.

Turner and Forbes (1966) have shown that the <u>in vitro</u> incorporation of $^{14}$C-leucine and $^{14}$C-glutamic acid by leukocytes into protein resisted treatment with hot TCA and lipid solvents. Acid hydrolysis of the labelled proteins showed that the radioactivity was present only in the amino acids used as substrate. Since hot TCA failed to remove a

significant proportion of the radioactivity from protein precipitates in their experiments it was decided to use TCA at room temperature.

The extent of binding of $^{14}$C-leucine to protein in the absence of metabolic activity of the lymphcoytes was determined by adding puromycin to the culture medium. In addition, cells were frozen (-15°C) before incubation. The incorporation of radioactivity into the TCA precipitable protein was not significantly above background in these cultures. The background level of the counter was of the order of 15-20 counts per minute. The counting efficiency of the machine was 25% for $^{14}$C-isotope.

2.2.3. Cell viability and morphology

Viability was determined by cell counting and trypan blue staining on triplicate cultures after incubation for 0, 24, 48 and 72 hours. Cell counts were determined with a Coulter Counter (Model D) at each time. Cell samples from these cultures were also incubated in medium containing 0.05% trypan blue (George T. Gurr Ltd., London, England) for 15 minutes at 37°C, and the proportion of cells containing dye was counted. The viable cells excluded dye. From this information the number of viable cells could be determined

at any time and could be expressed as a percentage of the number viable at the commencement of incubation.

The technique was not applicable to cultures of lymphocytes stimulated with PHA. The leucoagglutination caused by this substance prevented accurate determinations of viability during culture.

Air-dried smears were stained with Leishman's stain and examined by light microscopy. The stain was prepared by dissolving 0.3% Leishman stain (Townson and Mercer, Australia) in 97% methanol (Industrial grade); the stain was allowed to stand 6 months and was filtered before use. Cell suspensions were also examined by phase microscopy.

## 2.3. Results

### 2.3.1. Composition of the cell suspensions

In initial experiments loosely packed siliconized glass wool (Rabinowitz, 1964) was used to pack the columns used to isolate the lymphocyte preparations. The separation of lymphocytes from other leukocytes by glass wool filtration did not give consistent results and at times the preparations contained up to 10% polymorphs. Lymphocyte preparations by cotton wool filtration consistently gave suspensions of 99% purity. Lymphocyte suspensions in the work to be described were always obtained by cotton wool filtration unless otherwise

stated. Ten - fifteen percent of the lymphocytes in the blood were recovered in the final preparations.

Low speed centrifugation (150 x g) of the cell suspensions, after sedimentation of the bulk of the erythrocytes by dextran (Turner and Forbes, 1966), halved the yield of lymphocytes in the final preparations but reduced the number of erythrocytes by a factor of ten.

More than 99% of the nucleated cells in the final suspensions, prepared and washed as described in the methods, were lymphocytes. Granulocytes and monocytes were usually eliminated altogether. Platelets contaminating the suspension after elution from cotton wool were almost completely removed by washing the cell suspension with HBSS. Erythrocytes were always present in the suspensions in numbers five to ten times the lymphocyte count. The Leishman stained smear of the final cell suspensions showed 2 to 10% of large and medium-sized lymphocytes, the rest being typical small lymphocytes.

2.3.2. Viability and morphology of lymphocytes in medium with and without plasma

Freshly isolated lymphocytes examined by light microscopy of Leishman stained smears, and by phase contrast microscopy

of the suspensions, appeared normal. After 24 hours in plasma-free medium the lymphocytes exhibited degenerative changes. The nuclei were frequently bilobed and more homogeneous and appeared to be losing their characteristic structure. These changes had intensified by 48 hours, and by 72 hours many cells had disintegrated, while the rest were grossly degenerate. These changes were accelerated in plasma free cultures containing PHA.

By the criterion of exclusion of trypan blue, 97-99% of the cells were viable when initially suspended in culture medium. After 24 hours in plasma free medium 83-94% of the lymphocytes remained viable as determined by counting and viability testing. In medium containing autologous plasma 97-99% of the cells were alive at this time as judged by the same criteria. Death of the cells in plasma free medium continued steadily, so that after 72 hours only 20-36% of the cells remained viable.

2.3.3. Cells cultured in plasma free medium

Two washings with HBSS were sufficient to remove most of the serum protein from the lymphocyte suspensions prior to final suspension in culture medium (Appendix V)

In earlier studies (Turner and Forbes, 1960) leukocytes

had been cultured in complete Eagle's medium which was prepared with a supplement of autologous plasma. In this study plasma had been excluded in order to eliminate variable factors which may influence protein synthesis, to provide a constant defined medium and to prevent the interaction of drugs and other test substances with plasma.

Preliminary experiments were made to investigate the suitability of Eagle's medium, with and without supplement of amino acids (Ambrose, 1964) for culture purposes. The most satisfactory was Eagle's medium with the amino acid supplement (Appendix VI).

In protein synthesis studies $^{14}$C-leucine was used as a radioactive precursor. For economy it was necessary to use suboptimal amounts of precursor. As a consequence the suitability for lymphocyte culture of an amino acid supplemented Eagle's medium which was prepared without $^{12}$C-leucine was investigated. The effect of $^{12}$C-leucine in the medium on the incorporation of $^{14}$C-leucine into protein, is shown in Fig. 2.1. The concentration of $^{14}$C-leucine in this experiment was $6.4 \times 10^{-3}$ mM.

The incorporation of radioactive precursor by the cells increased markedly when no $^{12}$C-leucine was present in the

Fig. 2.1. <u>The effect of $^{12}$C-leucine in the culture medium on the incorporation of $^{14}$C-leucine by lymphocytes</u>

The lymphocytes were prepared from the blood of a normal donor, by glass wool filtration. The final suspensions contained more than 99% lymphocytes. The cells were finally suspended in supplemented Eagle's medium prepared without leucine and dispensed in 0.5 ml aliquots into culture bottles. The suspensions were brought to 1 ml volumes by the addition of supplemented Eagle's medium prepared with $^{12}$C-leucine at various concentrations. The concentration of $^{14}$C-leucine in the cultures was 1 µc/ml.

This graph shows two relationships,

(1) The relationship between the incorporation of $^{14}$C-leucine (expressed as counts per minute per $10^6$ cells per µc of $^{14}$C-leucine) by lymphocytes into protein and the concentration of $^{12}$C-leucine in the medium is shown as ● in the figure.

(2) The relationship between the uptake of leucine from the medium (expressed as $10^{-5}$ µmoles leucine incorporated from the medium by $10^6$ cells) by lymphocytes and the concentration of $^{12}$C-leucine in the medium is shown as □ in the figure.

This experiment was designed by myself and was performed with assistance from Dr. J.W.M. Lawton.

Counts /10^6 cells / µc / min
400
300
200
100
0
0·1
0·2
10
5
10^-5 µmol Incorporated /10^6 cells
Concentration of L-Leucine (mM)

medium. The incorporation by the cells of leucine, from the medium into protein fell to a minimum when the concentration of $^{12}$C-leucine in the medium was less than 0.1 mM and approached zero.

The incorporation of $^{14}$C-leucine into protein by lymphocytes was linear with increasing cell concentration and with increasing amounts of radioactivity in the medium (Appendix VII).

The effects of other cells in the lymphocyte suspensions were investigated. Since polymorphs and monocytes were removed from the suspensions, these were not of immediate interest. Platelets contaminating the suspensions obtained by cotton wool filtration, were largely removed by the washing procedures. Erythrocytes were always present in the final suspensions in numbers 5-10 times that of the lymphocytes. It was important then to determine the effect of erythrocytes on lymphocyte protein synthesis.

Erythrocytes in the final suspensions did not contribute to the synthesis of protein by lymphocytes, nor did they influence their activity (Table 2.1). Although the number of erythrocytes in culture suspensions could be reduced by lysis to a lymphocyte: erythrocyte ratio of 1:0.5, from a

Table 2.1. Influence of erythrocytes on protein synthesis by lymphocytes

Expt. 1. In this experiment erythrocytes were lysed by exposing the cell suspensions to a hypotonic solution. 1.5 Ml of water was added to 1.0 ml of cells suspended in culture medium for one minute at 37°. The cultures were restored to isotonicity by adding 1.5 ml of 2N saline. The cells were centrifuged at 800 x g and resuspended in culture medium containing $^{14}$C-leucine for incubation.

The cells were cultured in $^{12}$C-leucine-free medium with $^{14}$C-leucine at a concentration of 0.25 µc/ml. Determinations were made in triplicate.

Expt. II. Erythrocytes were lysed by adding 10 ml of water to the lymphocyte suspension in 5 ml of autologous plasma. The cells were left for 5 minutes, centrifuged at 800xg for 10 mins. and then washed with Hanks balanced saline solution in the normal way. A control culture was treated with saline.

In this experiment the cells were prepared by glass wool filtration and cultured in Eagle's medium containing $^{12}$C-leucine with $^{14}$C-leucine in the medium at a concentration of 1 µc/ml. Determinations were made in duplicate.

TABLE 2.1

| Expt. No. | Treatment | Lymphocyte count x $10^6$ | Erythrocyte count x $10^6$ | Protein Synthesis CPM | CPM/$10^6$ lympho-cytes |
|---|---|---|---|---|---|
| I | Before lysis | 2.75 | 13.7 | 307 | 112 |
| | After lysis | 1.85 | 0.9 | 202 | 109 |
| II | Before lysis | 1.8 | 10.0 | 124 | 80 |
| | After lysis | 1.3 | 0.2 | 106 | 84 |

In both experiments the lymphocyte and erythrocyte counts were determined using a Coulter counter. CPM represents the incorporation of $^{14}$C-leucine as counts per minute into protein. In the final column the incorporation of radioactive precursor into protein has been expressed as counts per minute per $10^6$ lympho-cytes cultured.

former ratio of 1:5, the protein synthesis per lymphocyte remained the same.

Platelets were removed from the final lymphocyte suspensions by the washing procedure so that there were very few or none in the final suspensions. Enriched platelet suspensions prepared on glass bead columns (Rabinowitz, 1964) did not significantly incorporate $^{14}$C-leucine into protein. In these experiments it was difficult to obtain suspensions completely free of lymphocytes.

Prior work in this laboratory (Turner and Forbes, 1966) with lymphocyte suspensions containing polymorphs prompted preliminary investigations into the effect of polymorphs on lymphocyte protein synthesis. Suspensions of polymorphs and monocytes were prepared as described by Rabinowitz (1964). Polymorph rich suspensions incorporated $^{14}$C-leucine into protein. Low percentages of polymorphs in the lymphocyte suspensions stimulated the incorporation of $^{14}$C-leucine into protein (Fig. 2.2). In one of the experiments reported in Fig. 2.2, monocyte-rich cultures were cultured with $^{14}$C-leucine. These cells incorporated precursor. Also on this occasion both the polymorph rich and monocyte rich suspensions were cultured with PHA, but

Fig. 2.2. The effect of polymorphs in lymphocyte cultures

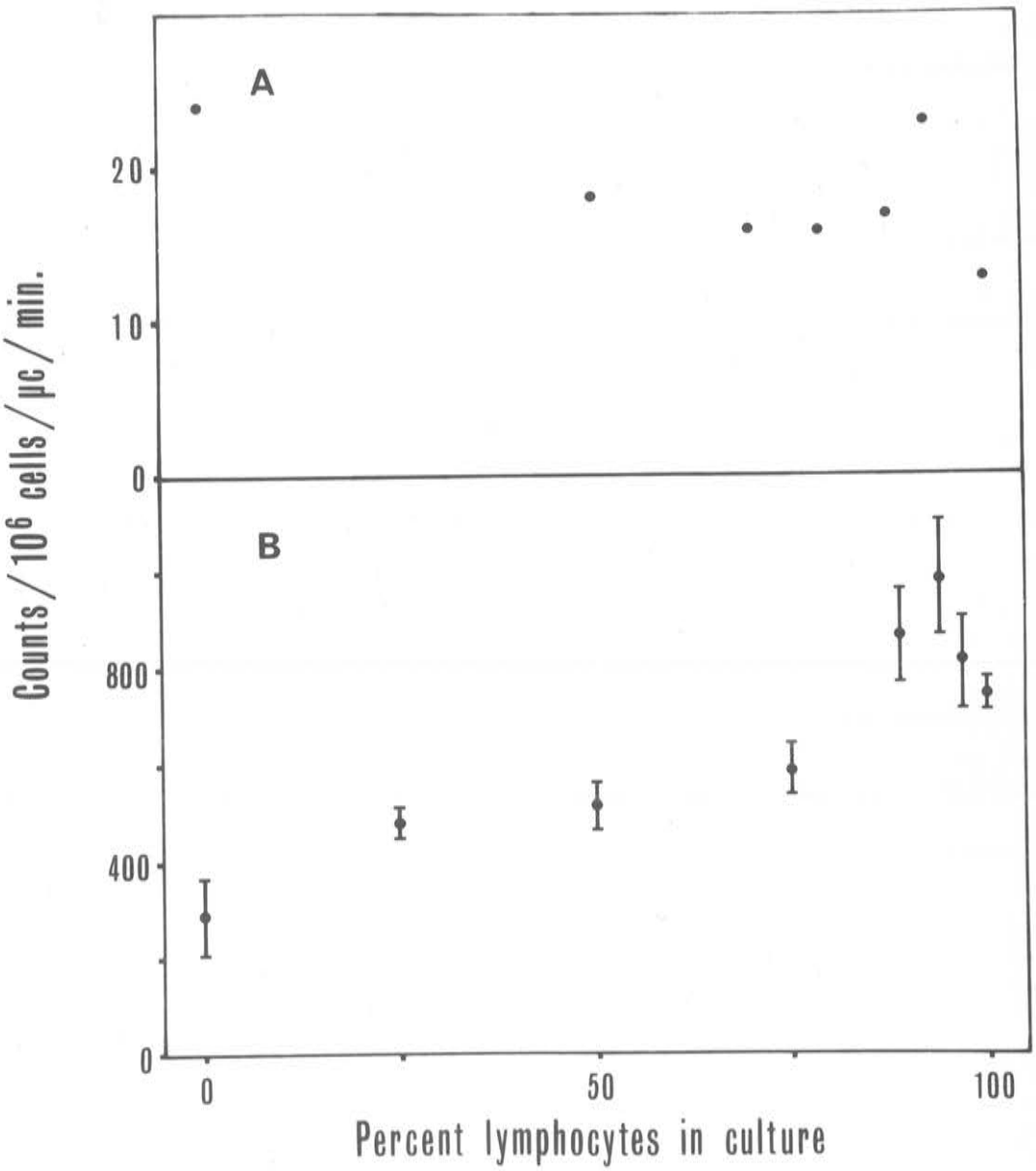
Graph A - Expt. 1. The lymphocytes in this experiment were obtained by filtration of blood through glass wool. The final suspensions contained 99% lymphocytes. The polymorph rich suspension contained 85% polymorphs and 15% monocytes. The total culture volume was 1 ml and the concentration of $^{14}$C-leucine was 1 µc/ml. The cells were cultured in commercial Eagle's medium. Determinations were made in duplicate. The cultures corresponding to 100% lymphocytes contained 6.6 x $10^6$ cells while those corresponding to 100% polymorphs contained 1.6 x $10^6$ cells of the polymorph rich preparations.

Graph B - Expt. 2. The lymphocytes in this experiment were obtained by filtration of blood through cotton wool. The polymorph rich suspensions contained 87% polymorphs and 13% monocytes. All the cultures contained 2 x $10^6$ cells in a total volume of 1 ml. $^{14}$C-Leucine was included in the medium (supplemented Eagle's medium prepared without leucine) at a concentration of 0.25 µc/ml. Determinations were made in triplicate. The 100% polymorph culture in the graph refers to the polymorph-rich preparations.

Monocyte rich suspensions also prepared on this occasion, incorporated precursor into protein. These suspensions containing 17% polymorphs and 83% monocytes were cultured in leucine free supplemented Eagle's medium. The culture volume was 1 ml. and 0.25 µc of $^{14}$C-leucine was included per ml. The incorporation into the TCA precipitable protein was 476 counts per min per $10^6$ cells per µc. When the polymorph and monocyte rich preparations were cultured with PHA there was no significant stimulation of protein synthesis compared with a six fold increase in the incorporation of precursor by cultures of stimulated lymphocytes.

The incorporation of $^{14}$C-leucine into protein has been corrected and expressed in graph A and B as counts/$10^6$ cells cultured/µc of $^{14}$C-leucine/minute.

Both of these experiments were designed and performed in co-operation with Dr. J.W.M. Lawton.


A
B
20
10
0
800
400
0
0
50
100
Counts / $10^6$ cells / µc / min.
Percent lymphocytes in culture

no significant stimulation of protein synthesis was observed (see legend to Fig. 2.2).

2.3.4. The effect of inhibitors

The effects on protein synthesis of the inhibitors puromycin and actinomycin D were investigated. Fig. 2.3, shows the effect of increasing puromycin concentrations in plasma free medium on the incorporation of $^{14}$C-leucine into protein by unstimulated lymphocytes. Puromycin at a concentration of 40 µg/ml completely inhibited protein synthesis by unstimulated cells. PHA-stimulated cells were inhibited at the same concentration.

Incorporation of $^{14}$C-leucine into protein by unstimulated cells was substantially but not completely inhibited by actinomycin D in the medium (Fig. 2.4). The full effect was exerted at a concentration of 1.5 µg/ml. A small amount of protein was synthesized at this concentration.

Experiments were designed to estimate thetime course of this actinomycin D resistant protein synthesis. When actinomycin D and $^{14}$C-leucine were added simultaneously to the cell suspension before incubation, synthesis continued for 6 hours (Fig. 2.5). Preincubation of the lymphocytes with actinomycin D for 30 minutes before the addition of

Fig. 2.3. Effect of puromycin on the incorporation of $^{14}$C-leucine into protein

Puromycin was included in the culture medium at concentrations up to 160 µg/ml. The lymphocytes were unstimulated and were cultured in $^{12}$C-leucine-free, supplemented Eagle's medium.

The counts/$10^6$ cells/µc/min., represents the counts measured in the TCA precipitable protein per $10^6$ cells cultured, corrected to a concentration of 1 µc of $^{14}$C-leucine in the medium. C.P.M. represents the actual counts per minute measured in the precipitate under the experimental conditions.

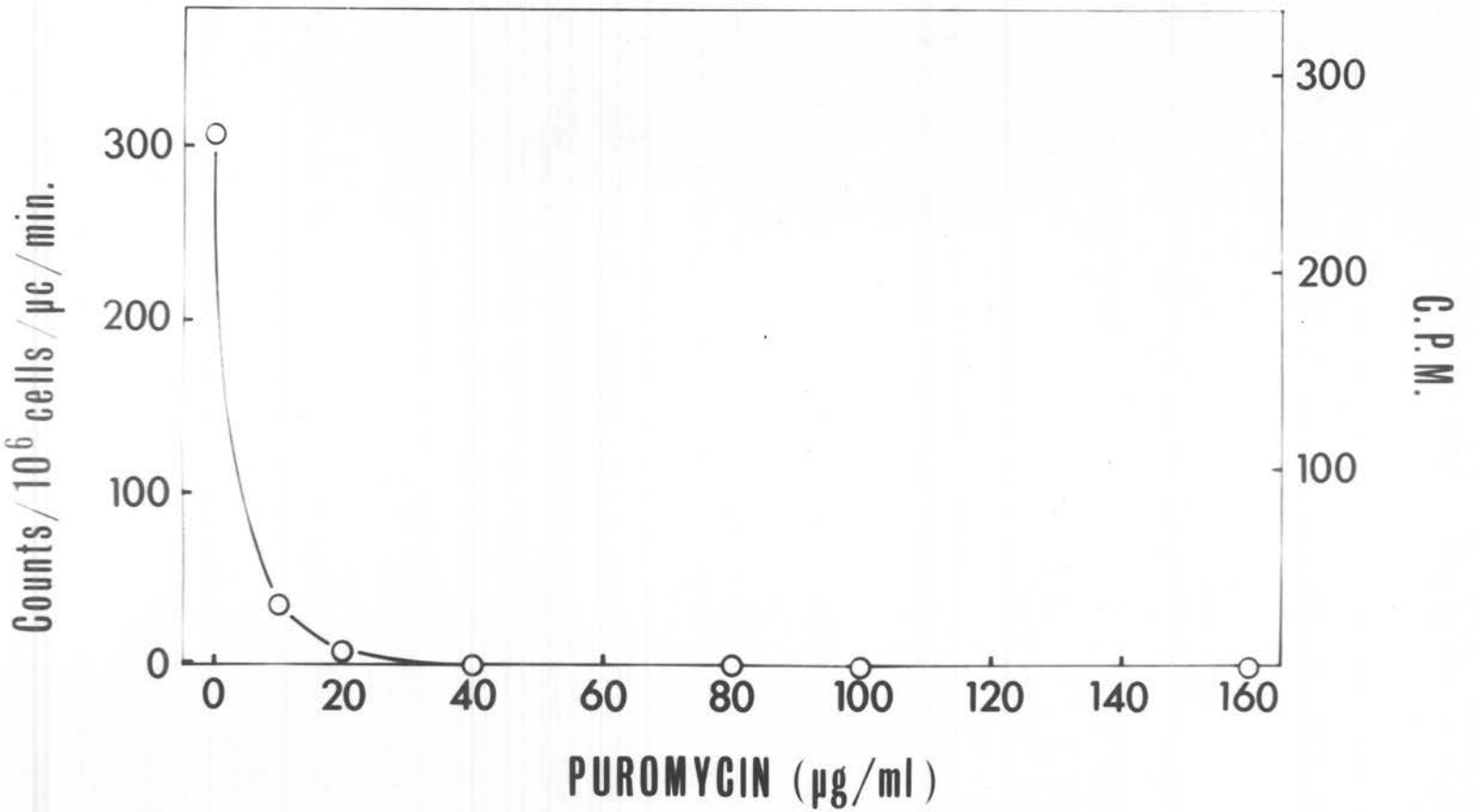

Counts/10^6 cells/μc/min.
300
200
100
0
0
20
40
60
80
100
120
140
160
PUROMYCIN (μg/ml)
300
200
100
C.P.M.

Fig. 2.4. <u>The effect of actinomycin-D on the incorporation of $^{14}$C-leucine into protein</u>

Actinomycin D was included in the culture medium at concentrations up to 3.3.µg/ml. The lymphocytes were unstimulated and cultured in $^{12}$C-leucine-free, supplemented Eagle's medium.

The counts/$10^6$ cells/µc/min., represent the counts measured in the TCA precipitable protein per $10^6$ cells cultured corrected to a concentration of 1 µc of $^{14}$C-leucine in the medium. C.P.M. represent the actual counts per minute measured in the precipitate under the experimental conditions.

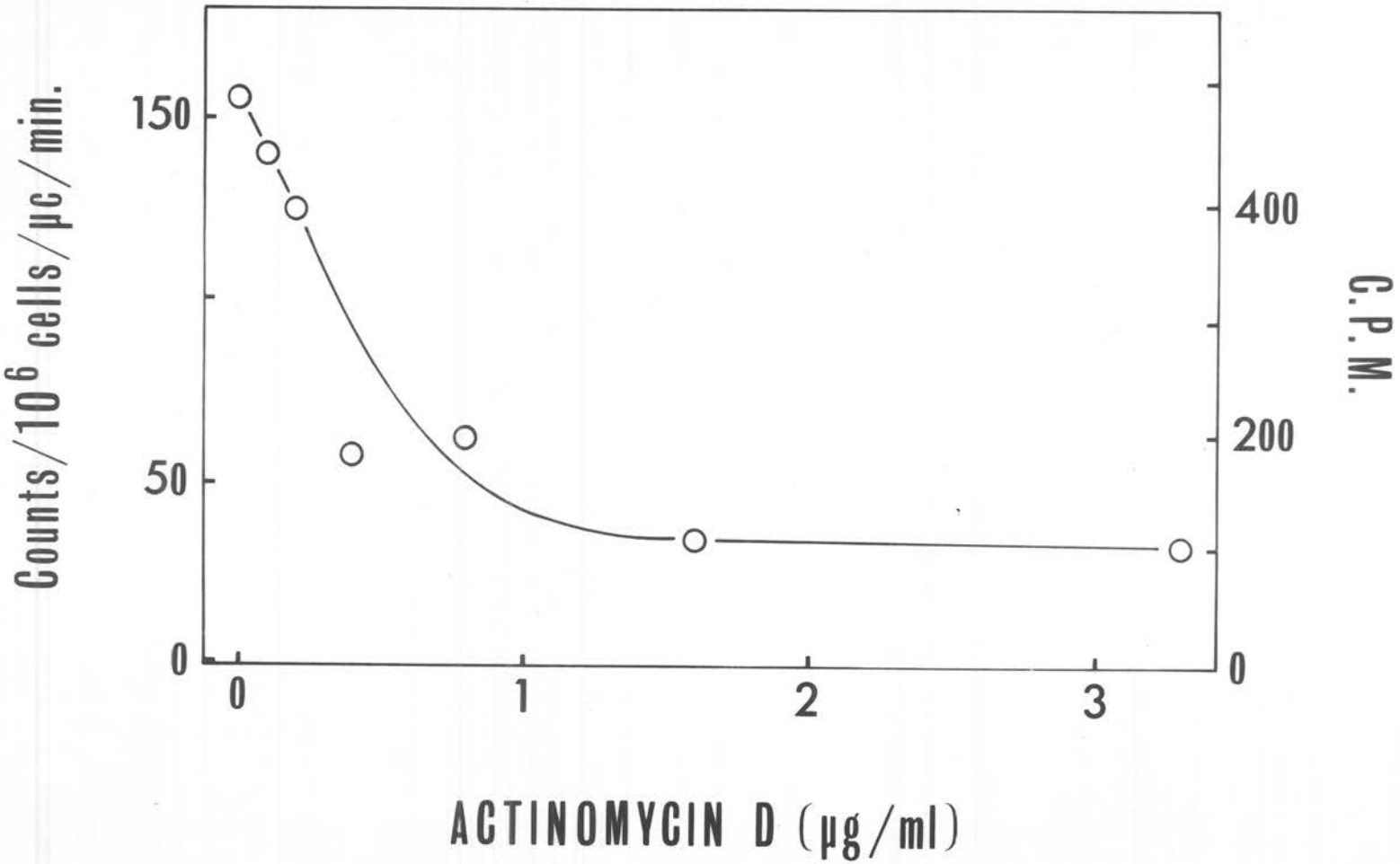

Counts/$10^6$ cells/μc/min.
150
50
0
0
1
2
3
400
200
0
C.P.M.
ACTINOMYCIN D (μg/ml)

Fig. 2.5. <u>The time course of actinomycin D resistant protein synthesis</u>

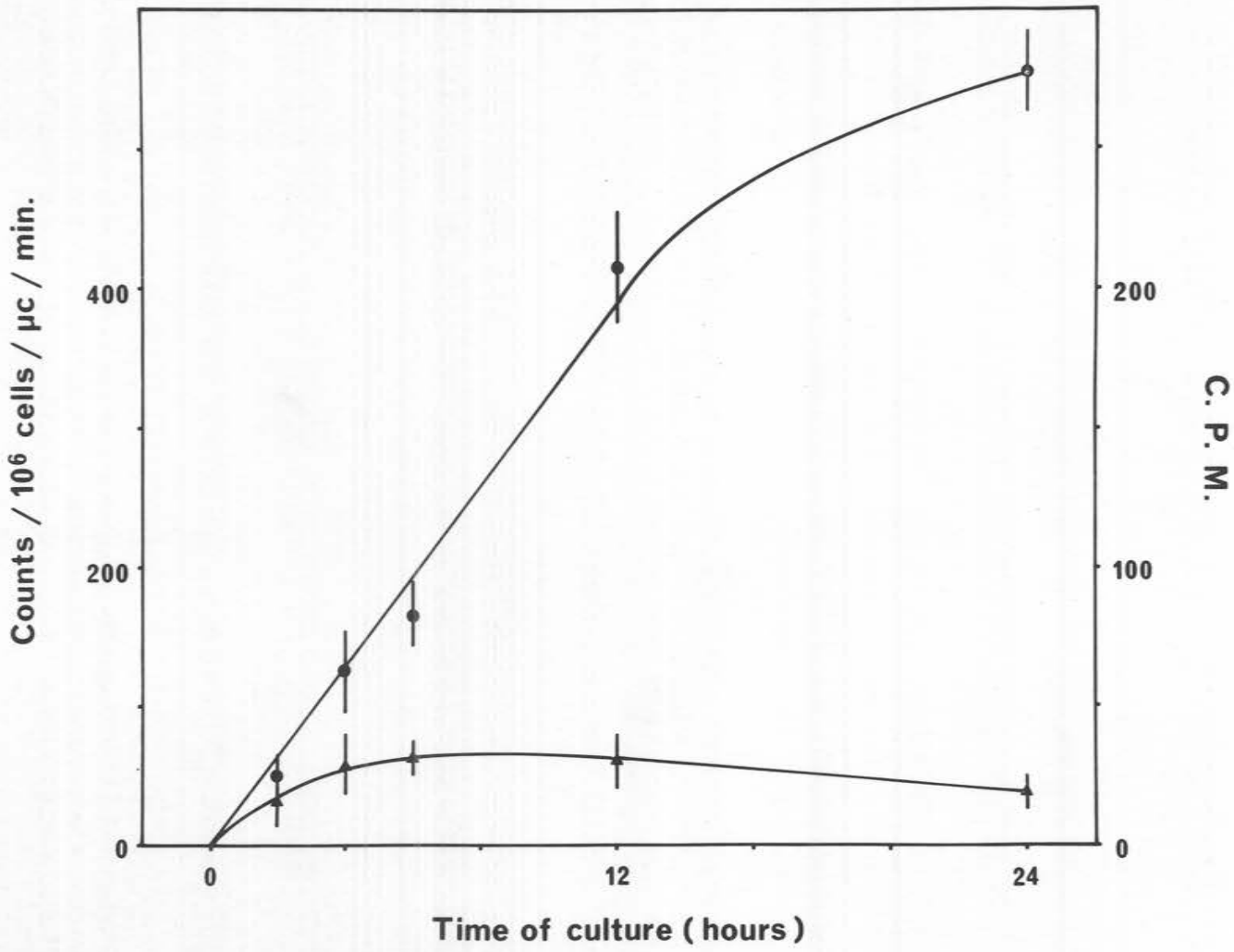
Actinomycin D was included in the medium at 3.3 μg/ml. The lymphocytes were unstimulated and cultured in $^{12}$C-leucine-free, supplemented Eagle's medium. Control cultures were incubated in the same medium.

Triplicate cultures were sampled at the times indicated and protein was precipitated with TCA. The standard deviations of the results have been included in the figure.

The counts/$10^6$ cells/μc/min represent the counts measured in the TCA precipitable protein per $10^6$ cells cultured, corrected to a concentration of 1 μc of $^{14}$C-leucine in the medium. C.P.M. represents the actual counts per minute in the protein precipitates.

● Control cultures

▲ Actinomycin D treated cultures


Counts / 10⁶ cells / µc / min.
400
200
0
200
100
0
C. P. M.
0
12
24
Time of culture ( hours )

$^{14}$C-leucine did not reduce this synthesis (Table 2.2). Cells killed by freezing at -15°C were incubated with $^{14}$C-leucine in plasma-free medium and were used as controls to these experiments. These killed cells did not incorporate $^{14}$C-leucine into protein.

When actinomycin D and PHA were added together at the beginning of incubation to the culture medium, the PHA-response was inhibited. When actinomycin D was added to the cells after the PHA-response had been initiated, the response was blocked (Table 2.2).

## 2.4. Discussion

In the development of suitable conditions for lymphocyte culture, cell preparations were prepared from blood by cotton wool filtration, which had been modified from the method of Fichtelius (1950). This method was superior to filtration of blood through columns of siliconized glass wool, which gave preparations contaminated with other cell types. Platelets and plasma were eliminated from the final culture suspensions by washing the cells with balanced salt solution. Erythrocytes contaminating the final cell suspensions, in numbers varying from 5-10 times the lymphocyte count, did not affect the incorporation of radioactive

Table 2.2. Effect of actinomycin D on protein synthesis by lymphocytes

Three experiments are summarized in this table. Cells were incubated with and without PHA and actinomycin D, which were added at the beginning of the incubation period, with one exception in experiment (c). In experiment (a) it can be seen that actinomycin D prevented the initiation of the response to PHA. In experiment (b) cells were pre-incubated with actinomycin D before addition of $^{14}$C-leucine. There was no greater inhibition of protein synthesis when actinomycin had been acting for an hour before addition of tracer. This suggests that the most important effect of actinomycin D on lymphocytes is to inhibit the synthesis of short-lived messenger RNA. In experiment (c) actinomycin D was added after the PHA response had been under way for 6 hours. Actinomycin D blocked the established response.

CPM represents the actual counts per minute in the TCA protein precipitates prepared from the cultures.

TABLE 2.2.

| Culture | PHA | Actinomycin D | Incubation (hours) | CPM |
|---|---|---|---|---|
| a | - | - | 24 | 420 |
| | + | - | 24 | 1284 |
| | + | + | 24 | 17 |
| | - | + | 24 | 17 |
| b | - | - | 24 | 360 |
| | - | added at 0 hours | 24 | 31 |
| | - | added at -0.5 " | 24 | 38 |
| | - | added at -1 " | 24 | 31 |
| c | - | - | 24 | 587 |
| | + | - | 24 | 1516 |
| | + | - | 6 | 392 |
| | + | added at 6 hours | 24 | 299 |

cursor into protein by lymphocytes. This was shown by reducing the numbers of erythrocytes by 90%, by lysis of the suspensions in hypotonic solution, without affecting the rate of incorporation of $^{14}$C-leucine into protein per lymphocyte.

Other leukocytes in the blood caused incorporation of $^{14}$C-leucine into protein. Small percentages of polymorph rich preparations when introduced into the lymphocyte cultures caused increased incorporation of precursor, beyond that expected from cultures of lymphocytes and granulocytes alone. The stimulus by small numbers of polymorphs may have been due to the release of nutrient from these cells after lysis, which at a particular concentration was stimulatory to lymphocyte protein synthesis. It would be interesting to investigate this further with reference to the work of Page (1964), who postulated that the stimulus for lymphocyte protein synthesis and migration in acute inflammation was caused by the neutrophil. This evidence emphasized the need to exclude other leukocytes from the final lymphocyte suspension.

Protein synthesis by unstimulated and PHA-stimulated lymphocytes was completely blocked by puromycin at a

concentration of 40 μg/ml and was substantially blocked by this drug at lower concentrations in the culture medium. Puromycin is a specific inhibitor of protein synthesis by mimicking transfer ribonucleic acid at the ribosome surface within the cell (Nathans, 1964). This finding provided evidence that the incorporation of $^{14}$C-leucine into protein by the cells represented protein synthesis activity. Further evidence was afforded by the work of Turner and Forbes (1966), who reported that radioactive supernatant proteins from leukocyte cultures resisted treatment with hot TCA and lipid solvents. When they hydrolysed these products, radioactivity was found only in the amino acids, leucine or glutamic acid, which were used as substrates.

$^{14}$C-Leucine aminoacyl RNA complexes not removed from the protein precipitates by cold TCA would suggest incorporation of precursor into protein in the presence of puromycin. The fact that puromycin completely inhibited the incorporation of $^{14}$C-leucine into the protein precipitates coupled with the evidence of Turner and Forbes (1966) suggest that aminoacyl RNA complexes do not contribute to the radioactivity in the precipitates. As a result of these findings protein precipitates were not

routinely washed with hot TCA.

The incorporation of $^{14}$C-leucine into protein by unstimulated and PHA-stimulated lymphocytes was not completely inhibited by actinomycin D, an inhibitor of messenger RNA synthesis (Kirk, 1960), at concentrations causing maximum inhibition. The amount of synthesis in the presence of actinomycin D was the same in cultures of unstimulated or PHA-stimulated lymphocytes. Pre-incubation of the cells with actinomycin D did not significantly reduce this synthesis. This did not suggest that the synthesis of protein in the presence of actinomycin was related to the permeability of the cell to this substance. The data provide evidence for the presence of a long-lived RNA in lymphocytes. This RNA was able to direct protein synthesis for 6 hours after the inclusion of the drug in the culture medium. This protein synthesis comprised a small part of the total synthesis, the majority of which was dependent on a continuous synthesis of messenger RNA. When actinomycin D was added to cultures of lymphocytes which were previously incubated for 6 hours with PHA, further protein synthesis was inhibited. The initiation of protein synthesis stimulation and the continued stimulus by PHA was dependent on actinomycin

D sensitive RNA synthesis.

Hirschhorn, et al., (1963) reported that the synthesis of protein continued for 2 hours in the presence of actinomycin D. He also reported that lymphocytes, which were cultured in the presence of actinomycin D and PHA, incorporated an amount of radioactive precursor into supernatant protein, which at the end of 24 hours was the same as that amount incorporated into the supernatant proteins of control cultures without these agents. The results reported in this chapter support the evidence for the presence of a long-lived ribonucleic acid in lymphocytes. The second finding of Hirschhorn is at variance with the results reported here. Hirschhorn measured the incorporation into the supernatant proteins released from the cells during culture and not the amount incorporated into protein released from the cells after freezing and thawing. This could be an explanation for the observed differences. He also stated that all of the protein liberated from the cells in control and actinomycin D treated cultures was IgG globulin. The nature of the proteins which are made by lymphocytes will be reported and discussed in Chapters 4 and 5.

Eagle's medium supplemented with amino acids (Ambrose,

1964) supported protein synthesis by lymphocytes. Some of the advantages of this medium will be discussed later in this thesis. The most immediate advantages are that the medium can be completely defined and that variable plasma factors which affect synthesis (Kamrin, 1966) are excluded.

The protein synthesis activity of lymphocytes was much reduced in medium prepared without leucine. Optimal conditions for the incorporation of leucine into protein by the cells did not occur until the leucine concentration in the medium was 0.1mM. The amount of protein synthesized was optimal at this concentration and above, under the conditions of culture. This concentration of amino acid, which limited optimal synthesis, was similar to that found by Eagle (1955) to be limiting for the growth of HeLa cells in vitro.

The incorporation of radioactive precursor into protein by lymphocytes showed a linear relationship with cell concentration up to 7-8 x $10^6$ cells. For most purposes cell cultures contained 2-3 x $10^6$ cells. The relationship between this incorporation and the concentration of $^{14}$C-leucine in the medium was also linear. The expression of the results as activity per $10^6$ lymphocytes per μc of $^{14}$C-leucine in the medium, provided a suitable means of comparing data from different experiments.

Eighty to ninety percent of the lymphocytes which were cultured in plasma-free medium, were viable at 24 hours by the criterion of trypan blue staining. Whether all of these cells are able to synthesize protein is one of the subjects of investigation to be described in the next chapter.

2.5. Summary

1. Purified lymphocyte suspensions were prepared by cotton wool filtration. In the final suspensions more than 99% of the nucleated cells were lymphocytes. The suspensions were contaminated only by erythrocytes.
2. Lymphocytes survived well in plasma free medium during culture for 24 hours. At 72 hours few of the cells were viable in this medium. The viability of the cell increased when autologous plasma was included in the medium.
3. The synthesis of protein by lymphocytes was demonstrated in plasma free medium by the inclusion of $^{14}$C-leucine in the medium. The effect of increasing concentrations of $^{12}$C-leucine in the medium on the incorporation of radioactive precursor by the cells was investigated. Plasma free Eagle's

medium supplemented with various amino acids and prepared without $^{12}C$-leucine was adopted as standard culture medium.

4. Phytohaemagglutinin (PHA), a bean extract, when included in the medium stimulated the cells to make increased amounts of protein.

5. Actinomycin D and puromycin, inhibitors of protein synthesis in other mammalian cells, inhibited protein synthesis by lymphocytes and also inhibited the lymphocyte protein response to phytohaemagglutinin. Puromycin completely inhibited protein synthesis. In the presence of actinomycin D, a small amount of synthesis continued for 6 hours.

6. Small concentrations of polymorphs in lymphocyte cultures stimulated protein synthesis.

7. Erythrocytes present in the final suspensions did not contribute to, nor influence the incorporation of $^{14}C$-leucine into protein by lymphocytes.

# CHAPTER 3

# TIME-COURSE STUDIES OF LYMPHOCYTE PROTEIN AND NUCLEIC ACID SYNTHESES

## 3.1. Introduction

In this chapter are reported investigations of lymphocyte protein and nucleic acid syntheses at various times during the incubation of the cells *in vitro*.

The final lymphocyte preparations contained cells which were morphologically uniform by the criteria of phase contrast microscopy and light microscopy after Leishman's staining. Nevertheless, this did not mean that they were metabolically similar. Experiments with $^{3}$H-leucine as radioactive precursor were designed to investigate the number of cells which were engaged in protein synthesis in unstimulated cultures and to determine the number of cells in these cultures stimulated by PHA to produce protein. Cells which had incorporated precursor were detected by the technique of autoradiography. Torelli, *et al.*, (1964) has used this technique successfully to study the metabolism of leukocytes *in vitro*.

The experiments in this chapter were designed:

1. To study the time-course of protein synthesis by lympho-

cytes cultured in medium with and without an autologous plasma supplement. The effects of PHA on the time-course of protein synthesis were also studied.

2. To investigate the time-course of nucleic acid synthesis by lymphocytes cultured in plasma-free medium, with and without PHA.

3. To detect by the technique of autoradiography the proportion of cells active in protein synthesis.

## 3.2. Materials and Methods

### 3.2.1. Isolation and culture of lymphocytes

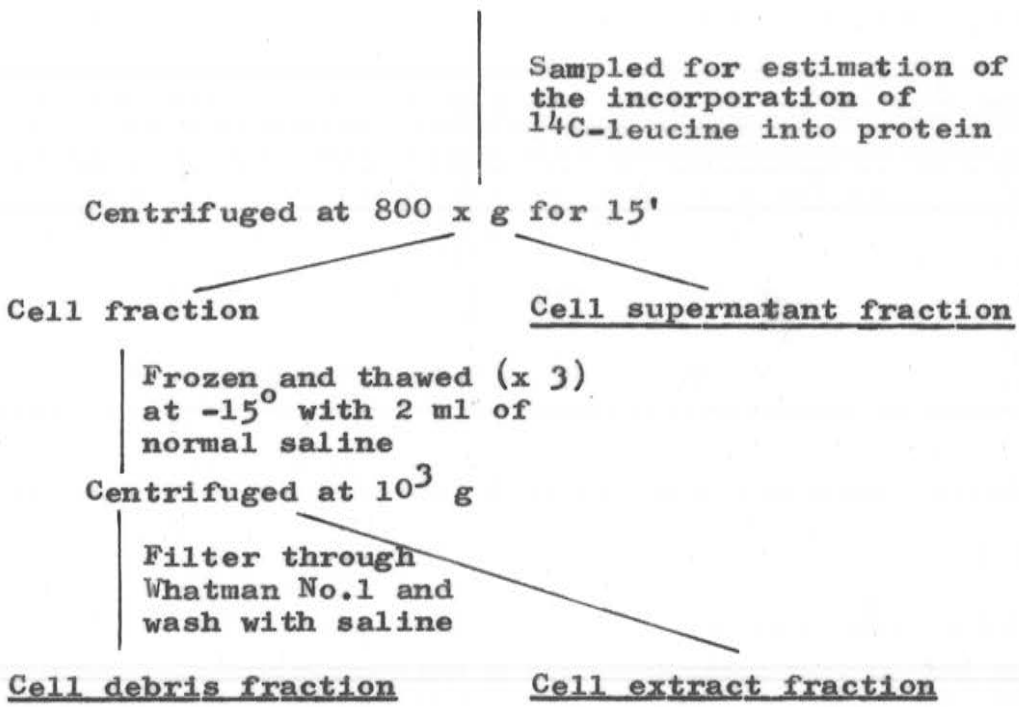
The methods used to prepare lymphocyte cultures and to determine the incorporation of $^{14}$C-leucine into protein were described in Chapter 2, section 2.2.1. The cells which sedimented at 150 x g during lymphocyte preparation were used when cultures of mixed leukocytes were prepared.

### 3.2.2. Time-course of protein synthesis

The time-course of the incorporation of $^{14}$C-leucine into protein by lymphocytes was determined in two situations: (i) culturing lymphocytes in a leucine-free supplemented Eagle's medium (ii) culturing lymphocytes in the same medium supplemented with 25% autologous plasma. Triplicate cultures were terminated at intervals up to 72 hours.

The supernatants were separated from the cells immediately at sampling by centrifugation at 800 x g. "Soluble" protein was extracted from the cells by freezing and thawing them with 2 ml. of normal saline. This cell extract fraction was removed by centrifugation at $10^3$ x g and the remaining cell debris fraction was collected and washed on filter paper (Whatman No. 1, Balston Ltd., England).

The following diagram summarized the sampling technique used in these experiments.




In the work to be described reference will be made to

the fractions which are underlined in the above diagram. The proteins in the cell supernatant and cell extract fractions were precipitated with 5% TCA. These fractions were counted on planchets at infinite thickness. The cell debris fractions were counted on the filter papers.

3.2.3. Autoradiography of cells

Cultures of lymphocytes and leukocytes were pulse-labelled at various times with $^{3}$H-leucine (L-leucine, 4,5, T, specific activity of 960 mc/mM, Radiochemical Centre, Amersham) at a concentration of 1 μc/ml in the medium. After the cells had been incubated with radioactive precursor for 15 minutes, excess medium was added to the cultures and the cell suspensions were immediately centrifuged at 800 x g for 10 minutes. After the supernatant had been discarded the cell button was resuspended in 0.2 ml of plasma and the cells were smeared on clean glass slides. The cell smears were fixed in acetic acid and methanol fixative (mixed in a ratio of 1:4) overnight at 4°C and were washed in several changes of saline and water for 48 hours.

After the smears had dried the slides were dipped into photographic emulsion (Nuclear Research Emulsion L4, Ilford, diluted with 2 volumes of water) and were exposed for three weeks at 4°C in light- and moisture-proof boxes.

At the end of this time the emulsion was developed with ID2 (Ilford) and the smears were stained with Leishman's stain. Grain counts were made over a minimum of 200 cells for each treatment. The background count did not exceed one grain per cell area in the slides examined.

3.2.4. Nucleic acid studies

$^{3}$H-Uridine (Uridine T(G), specific activity of 2.18 C/mM, Radiochemical Centre, Amersham) was used to follow RNA synthesis. $^{3}$H-Uridine was included in the culture medium at a concentration of 1-2 µc/ml and the cultures were sampled after 0,3,6,12,24,48 and 72 hours of incubation at 37°C.

The technique used to determine the incorporation of radioactive precursor into RNA, was adapted from that described by Cooper and Rubin (1965). The cultures were sampled after incubation with precursor by placing them in an ice bath to inhibit activity. The cultures were centrifuged at 800 x g for 10 minutes at 4°C. The supernatant was discarded and the cell button was washed once with cold HBSS. The cells were then fixed with cold ethyl alcohol-acetic acid fixative (3:1) for 10 minutes. The fixative was removed by centrifugation and the cells were stored at 4°C overnight in 70% ethyl alcohol. On the next

day, after the alcohol had been removed, the cells were extracted with 2% perchloric acid (PCA) for 50 minutes at 4°C. The extract was discarded and the cells were washed once with fresh cold 2% PCA. The cell button was then extracted with 1-5 ml of 10% PCA at room temperature for 5 hours.

1-2 ml of this acid extract was included in 14 ml of scintillator and counted in a Packard Scintillation counter (Model 3002). The scintillator was prepared from 1 litre of dioxane (AR grade, May and Baker Ltd., England), 10 mg of Naphthalene (Laboratory grade, May and Baker), 250 mg of 1:4 di (2-(5 phenyloxazolyl)) benzene (POPOP, AR grade, Univar Byproducts and Chemicals Pty. Ltd., N.S.W., Australia) and 10 g of diphenyloxazole (PPO, AR grade, Univar) and was stored in an opaque bottle away from light.

$^{3}$H-Thymidine (Thymidine 6-T(n), specific activity of 22.1 C/mM, Radiochemical Centre, Amersham) was included in the culture medium to follow DNA synthesis. Lymphocyte cultures were pulse-labelled with this precursor at a concentration of 1 μc/ml in the medium for 1 hour after 24, 48 and 72 hours of incubation at 37°C. After incubation with precursor, excess medium was added to the culture and the cells were prepared for autoradiography in the manner

described previously. In these experiments, the lymphocytes were cultured in medium supplemented with 25% autologous plasma.

## 3.3. Results

### 3.3.1. Time-course studies of protein synthesis

Figure 3.1 shows that the total synthesis of protein in the cell supernatant and cell extract fractions occurred at a steady rate in plasma-free medium for the first 12 hours and ceased after 24 hours' incubation. Very little release of protein took place from the cell into the supernatant during the first 12 hours of incubation. Most of the protein in the cell supernatant fraction was released after this time.

PHA stimulated protein synthesis as measured by the cell extract, cell supernatant and cell debris fractions. This substance increased the incorporation of $^{14}$C-leucine into all of these protein fractions. In stimulated cultures the time-course of synthesis was similar to that in unstimulated controls. There was a comparatively small amount of precursor incorporated into the cell debris fraction of unstimulated cultures compared with the amount incorporated into the debris of the PHA stimulated cells.

Fig. 3.1. Time course of protein synthesis by lymphocytes in plasma-free medium

The categories represent the following:

A - Radioactive protein released into the medium (referred to as cell supernatant fraction in the text).

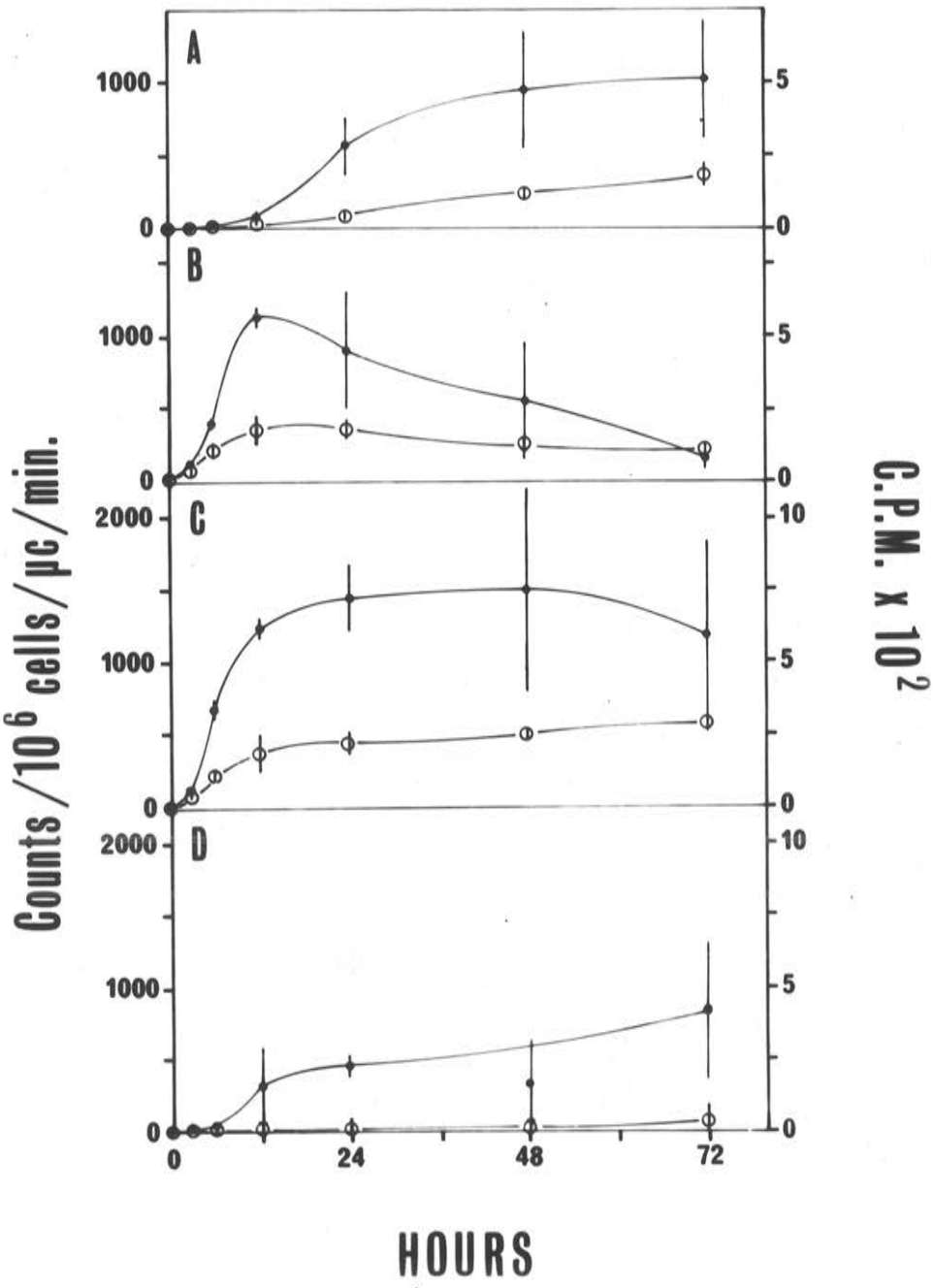
B - Radioactive protein released by freezing and thawing the cells (referred to as cell extract fraction in the text).

C - Total non cell-bound protein, which is the sum of categories A and B.

D - Radioactive protein in the cell debris which was not released by freezing and thawing (referred to as cell debris fraction in the text). The counts in this fraction have been corrected for carrier protein dilution of the other fractions. The results are then comparable. The determination of the radioactivity in this fraction was probably inaccurate because of uneven distribution of cellular debris over the filter paper.

The symbols ○ represent determinations made with unstimulated cultures and ● represent determinates made with PHA-stimulated cultures. The standard deviations of the triplicate determinations are shown in the figure.

The incorporation of radioactivity has been expressed in two ways. (1) CPM refers to the actual counts per minute in the fraction examined. (2) Counts/$10^6$ cells/μc/min., represents the number of counts expressed per $10^6$ cells cultured and corrected for a concentration of 1 μc of $^{14}$C-leucine in the medium.




This experiment was designed by myself and was performed in collaboration with Dr. J.W.M. Lawton.

Figure 3.2 shows that the time course of protein synthesis by lymphocytes in medium containing 25% plasma was similar to that by the cells in medium without plasma. The release of protein from the cells to the supernatant during incubation took place less rapidly than in plasma-free medium. The incorporation of $^{14}C$-leucine into the cell debris fraction was much greater in the cultures containing plasma. The degree of stimulation of each category of protein synthesis by PHA was similar in both types of medium.

3.3.2. The influence of phytohaemagglutinin

The stimulatory effect of PHA on lymphocyte protein synthesis in plasma free medium was dose dependent (Fig. 3.3). At high concentrations, PHA did not stimulate lymphocyte protein synthesis but had a slight inhibitory effect. The optimal concentration of PHA to stimulate protein synthesis was used in studies with this batch. When other batches of PHA were used, the optimal concentration for each was not determined. They were used at the optimal concentration determined from Fig. 3.3.

In plasma-free medium some batches of PHA did not stimulate protein synthesis, when used at this optimal concentration and in some instances synthesis was depressed.

Fig. 3.2. Time course of protein synthesis by lymphocytes in medium containing 25% plasma.

The categories represent the following:

A - Radioactive protein released into the medium (referred to as cell supernatant fraction in the text).

B - Radioactive protein released by freezing and thawing the cells (referred to as cell extract fraction in the text).

C - Total non cell-bound protein, which is the sum of categories A and B.

D - Radioactive protein in the cell debris which was not released by freezing and thawing (referred to as cell debris fraction in the text). The counts in this fraction have been corrected for carrier protein dilution of the other fractions. The results are then comparable. The determination of the radioactivity in this fraction was probably inaccurate because of uneven distribution of cellular debris over the filter paper.

The symbols ○ represent determinations made with unstimulated cultures and ● represent determinates made with PHA-stimulated cultures. The standard deviations of the triplicate determinations are shown in the figure.

The incorporation of radioactivity has been expressed in two ways. (1) CPM refers to the actual counts per minute in the fraction examined. (2) Counts/$10^6$ cells/μc/min., represents the number of counts expressed per $10^6$ cells cultured and corrected for a concentration of 1 μc of $^{14}$C-leucine in the medium.

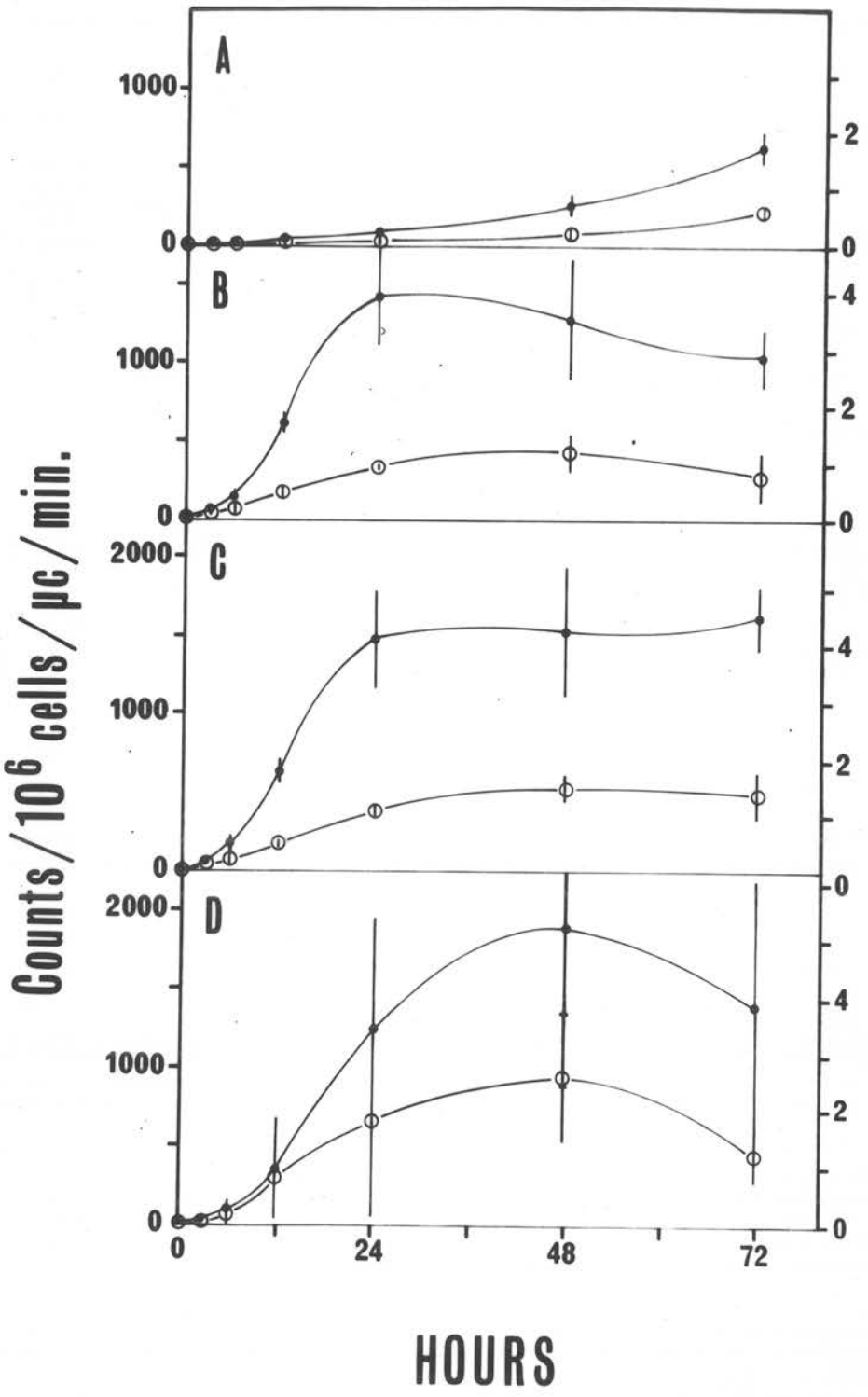

A
B
C
D
1000
0
2000
2
4
0
24
48
72
Counts/10⁶ cells/μc/min.
C.P.M. x 10³
HOURS

Fig. 3.3. Effect of PHA on the incorporation of $^{14}$C-leucine into protein by lymphocytes in plasma-free medium

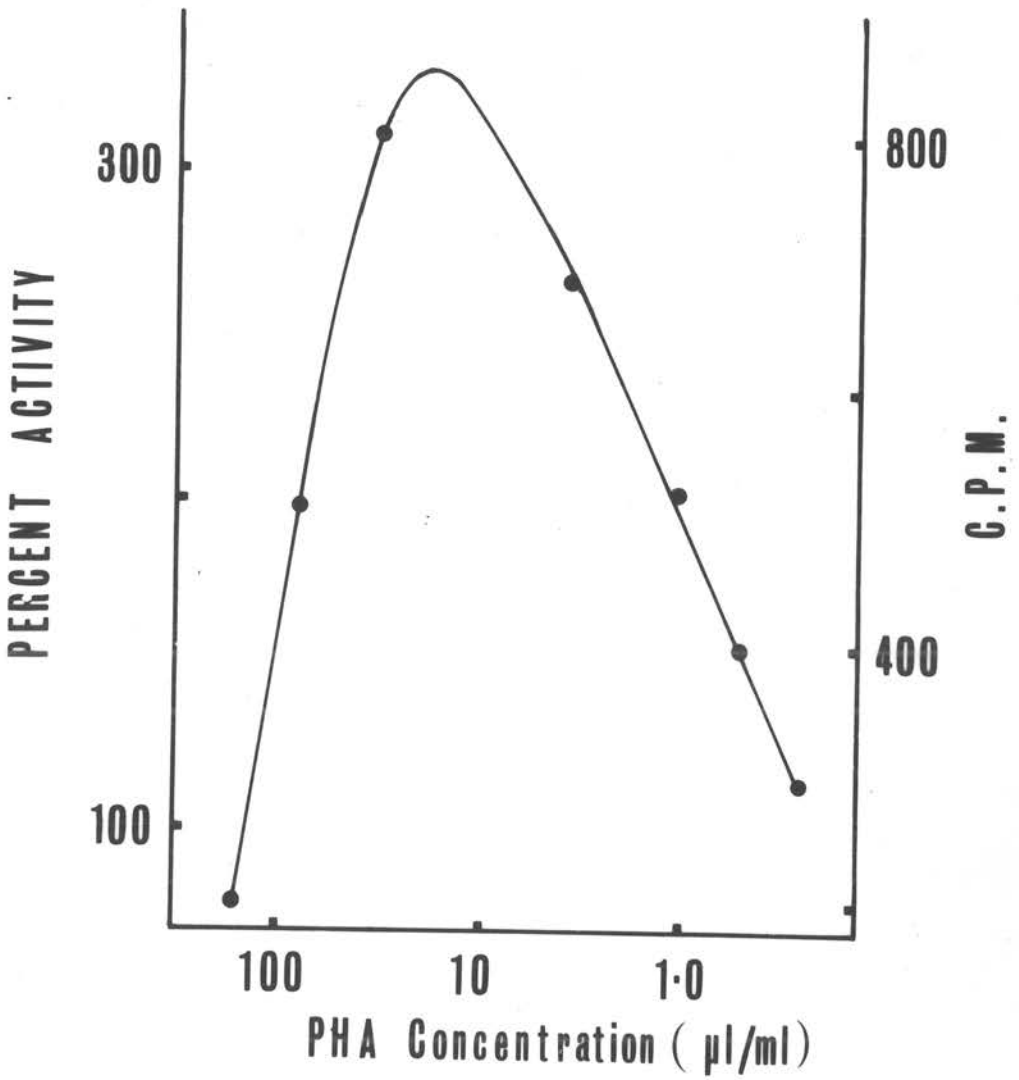
One vial of 5 ml dried extract of PHA (Batch K7346) was reconstituted with saline and added to the cultures. The concentration is expressed in terms of microlitres of reconstituted PHA per ml. The lymphocytes were cultured in supplemented Eagle's medium prepared without leucine. $^{14}$C-leucine was included in the medium at a concentration of 0.25 µc/ml. Each culture contained $2 \times 10^6$ cells.

The incorporation of $^{14}$C-leucine has been expressed as:

(1) CPM represents the actual counts per minute in the TCA precipitate.

(2) Percent Activity is defined as the

$$\frac{\text{CPM in PHA-stimulated culture}}{\text{CPM in unstimulated culture}} \times \frac{100}{1}$$


PERCENT ACTIVITY
300
100
800
400
C.P.M.
100
10
1·0
PHA Concentration ( µl/ml )

All PHA batches stimulated increased protein synthesis when the lymphocytes were cultured in medium containing plasma. In both types of medium the degree of stimulation by the same batch of PHA varied with the lymphocyte donor. Conversely the stimulation of protein synthesis by different batches of PHA, which were used in the medium at the same concentration, varied when tested with lymphocytes from the same donor. In plasma-free medium the range of stimulation by PHA varied from two to five times the control values.

### 3.3.3. Incorporation of $^{3}$H-leucine

Table 3.1 shows the percentage of cells labelled and the mean grain count per cell in PHA stimulated and unstimulated cultures, which were sampled at various times during incubation for 24 hours. A Leishman stained smear of the lymphocyte preparations prior to culture is shown in Plate 3.1(a). Cells which have incorporated $^{3}$H-leucine are shown in Plate 3.1(b).

Forty-percent of the cells;were labelled in unstimulated cultures after 2 hours' incubation. The value rose to 62-68 percent in the next 10 hours and fell to 50 percent at the end of 24 hours. The mean grain count rose sharply from 4.6 at 2 hours to 9.5 - 10.6 during the following 4 hour

Table 3.1. Incorporation of $^{3}$H-leucine by lymphocytes in plasma-free medium

Lymphocytes were cultured in leucine-free supplemented Eagle's medium. Each culture contained $1.8 \times 10^{6}$ cells. $^{3}$H-Leucine was included in the cultures at the times indicated at a concentration of 1 μc/ml. After incubation with precursor for 15 min., the cells were prepared for autoradiography. The background grain count was less than 1 grain per cell area. Each mean was obtained by counting not less than 200 cells and is given in the table with its standard deviation (SD).

TABLE 3.1

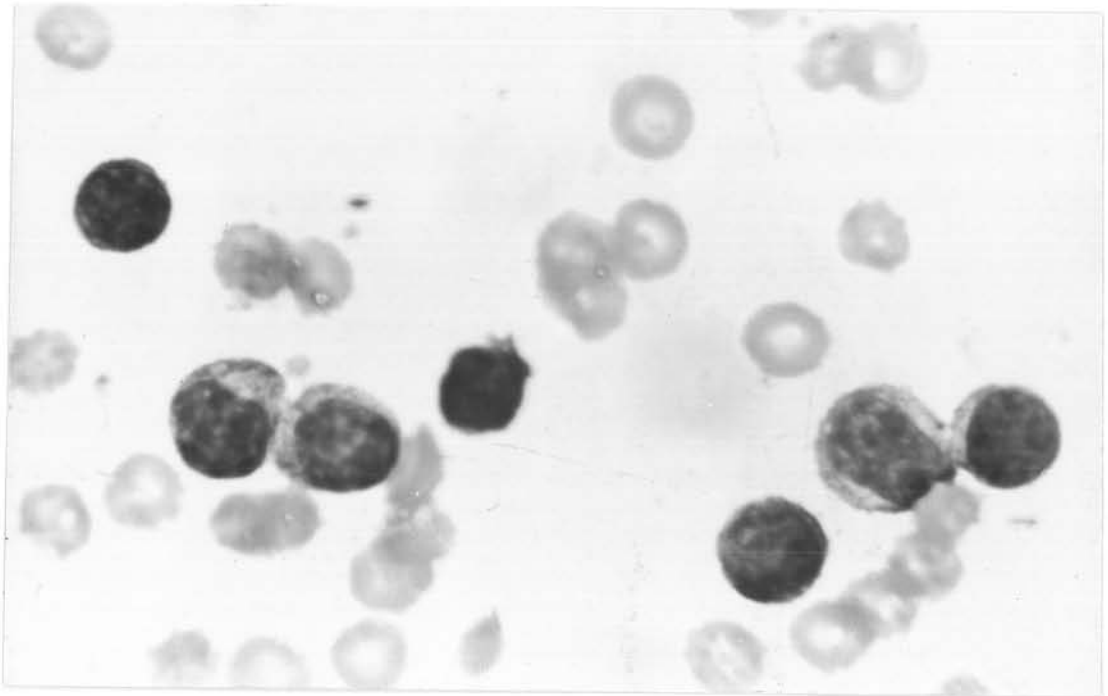
| Time (hr) | Unstimulated | | PHA-stimulated | |
|---|---|---|---|---|
| | Percent labelled | Mean grain count/cell ± SD | Percent labelled | Mean grain count/cell ± SD |
| 2 | 40 | 4.6 ± 3.2 | 90 | 11.0 ± 6.0 |
| 4 | 63 | 10.6 ± 3.2 | 84 | 13.8 ± 8.7 |
| 6 | 62 | 9.5 ± 5.5 | 88 | 14.1 ± 8.8 |
| 12 | 68 | 4.5 ± 2.2 | 84 | 10.4 ± 6.8 |
| 24 | 50 | 5.1 ± 3.0 | 60 | 10.2 ± 6.8 |

Plate 3.1. Lymphocyte preparations

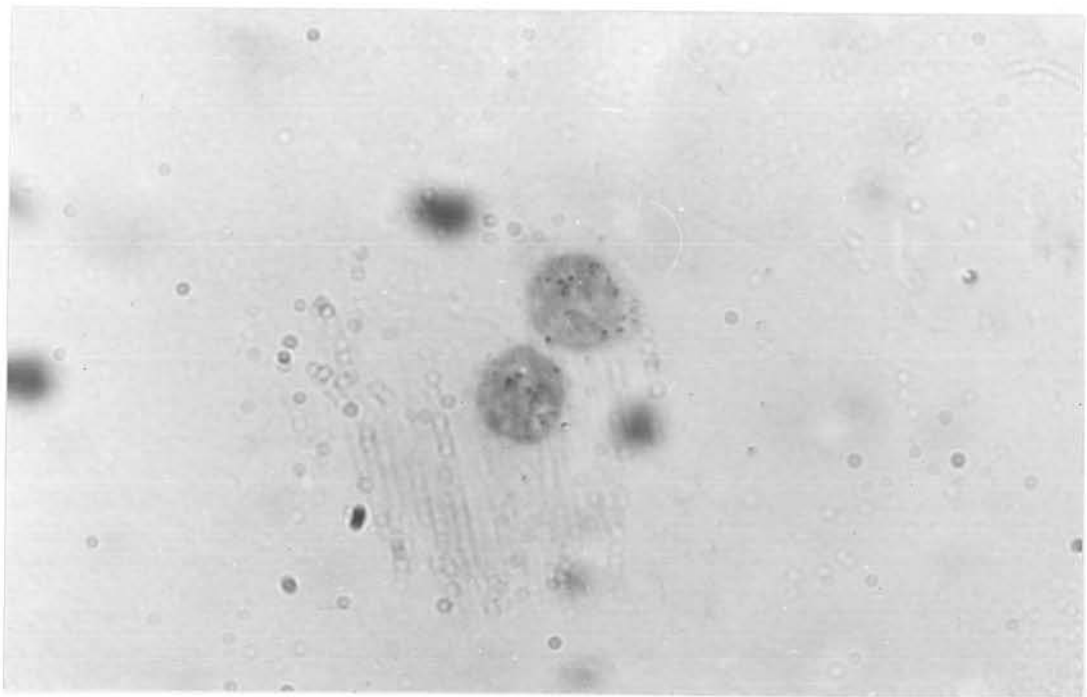
(a) Smear of a final lymphocyte preparation stained with Leishman's.

(b) Smear of unstimulated lymphocytes, incubated with $^{3}$H-leucine after 6 hours incubation. Grains (radioactivity) are evident over the cells, which have been stained with Leishman's.

a



b



incubation period. After 24 hours incubation, the mean grain count fell to 5.1. PHA in the medium increased both the mean grain count and the percentage of labelled cells in the cultures compared with those values in unstimulated controls. In stimulated cultures, the percentage of labelled cells was 84-90 during the first 12 hours of incubation. The mean grain count increased from 11.0 to 14.0 during the first 6 hours and remained at 10.4 - 10.2 for the duration of the incubation time.

Lymphocytes, polymorphs and monocytes in mixed leukocyte cultures incorporated $^{3}$H-leucine, Plate 3.2(a). No labelled erythrocytes were seen in these preparations or in the preparations of labelled lymphocytes.

3.3.4. Nucleic acid synthesis

The time-course of RNA synthesis by lymphocytes cultured in plasma-free medium is shown in Fig. 3.4. In unstimulated cultures, synthesis continued steadily for 48 hours and declined after 72 hours incubation. PHA stimulated markedly the synthesis of RNA by lymphocytes.

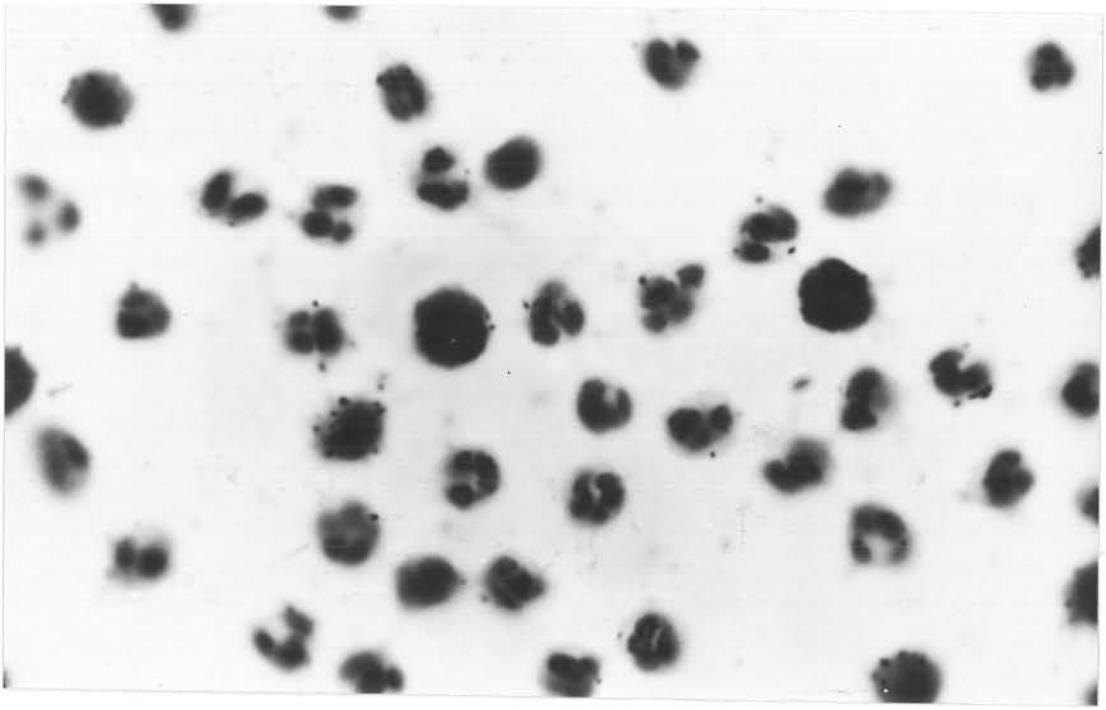
Cell death, which occurred at a steady rate when the cells were cultured in plasma-free medium (Chapter 2, section 2.3.2.), was a factor influencing the incorporation

Plate 3.2. Leukocyte and Lymphocyte preparations

(a) Smear of a mixed leukocyte preparation, stained with Leishman's, showing incorporation of $^{3}$H-leucine.

(b) Smear of a lymphocyte preparation, which had been incubated with $^{3}$H-thymidine after incubation in 25% autologous plasma medium with PHA for 24 hours, showing incorporation of precursor. The cells have been stained with Leishman's.

a



b

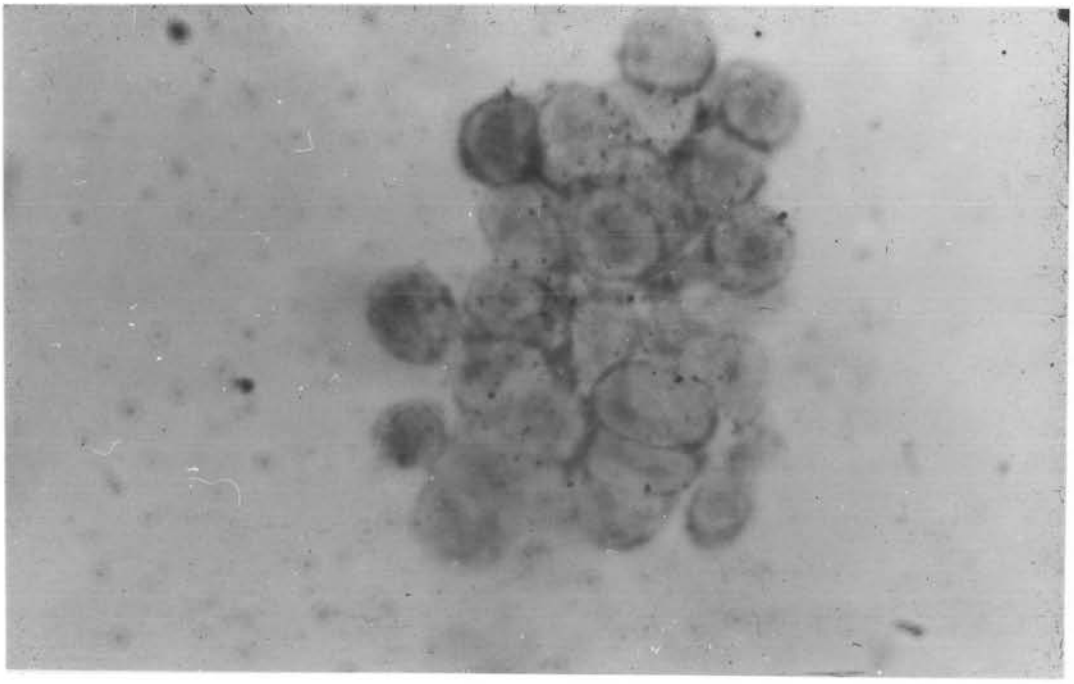


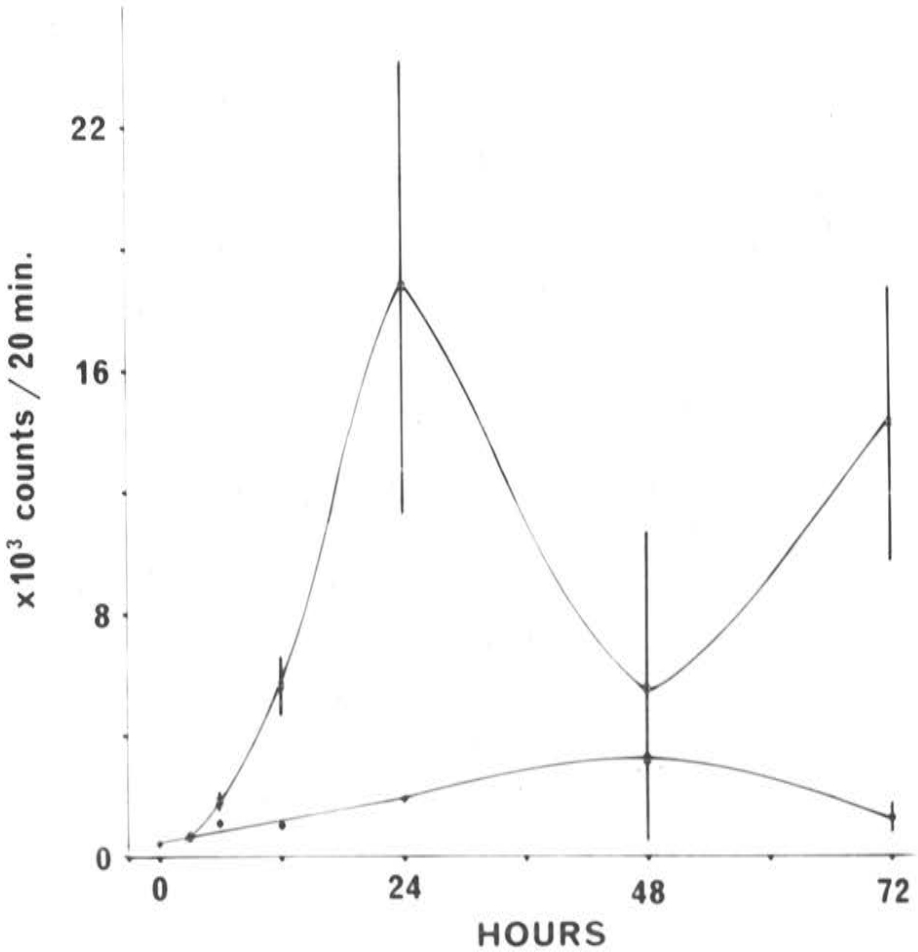
Fig. 3.4. The time course of RNA synthesis by lymphocytes cultured in plasma-free medium

The cells were cultured with $^{3}$H-uridine at a concentration of 2 μc/ml. Total culture volumes were 1 ml and RNA was determined at various times as described in the methods. The cultures contained $4.5 \times 10^{6}$ cells.

□ represents determinationsmade in PHA-stimulated cultures

● represents determinations made in control cultures

The determinations were made in triplicate. The standard deviations of the values have been indicated. The incorporation of $^{3}$H-uridine has been expressed as the actual counts per 20 minutes in 1 ml of the 10% PCA extract (total volume, 5 ml) of the samples. The extract was counted in 14 ml of scintillator. The results have not been corrected for the efficiency of the counter, or the amount of quenching by the sample carrier.


22
16
8
0
x10³ counts / 20 min.
0
24
48
72
HOURS

of radioactive precursor into RNA by lymphocytes when they were cultured for long periods of time.

Incorporation of $^{3}$H-thymidine into PHA-stimulated cells was observed as early as 24 hours (Plate 3.2(b)). At this time 26% of the cells incorporated precursor. After incubation for 48 and 72 hours, many of the stimulated lymphocytes disintegrated during preparation for autoradiography, despite the fact that the cells were cultured in medium containing plasma. No cells in the unstimulated cultures incorporated $^{3}$H-thymidine at any time during incubation for 72 hours. The percentage of cells labelled in PHA-stimulated cultures was not recorded after 48 and 72 hours incubation.

## 3.4. Discussion

Lymphocytes synthesise soluble protein released from the cells during incubation and by freezing and thawing. They also synthesise insoluble protein not released from the cells. The time courses of protein synthesis by lymphocytes were similar in medium with or without plasma. The synthesis of soluble protein continued steadily for 24 hours in the former and for 12 hours in the latter. Protein was released earlier into the supernatant by the cells cultured

in plasma-free medium. This probably was a reflection of the greater rate of cell death and lysis of the cells in this medium.

The bulk of protein synthesis by lymphocytes had been completed by 24 hours in both types of media. Moreover, lymphocytes survived well in media for periods of culture up to 24 hours. At this time 83% to 94% of the cells were viable in plasma-free medium while 97% to 99% were viable in plasma medium (Chapter 2). The morphology of the cells surviving in plasma-free medium at this time suggested that many were active (Chapter 2) and the uptake of $^{3}$H-leucine by 50% of the cells confirmed this. These data suggest that 24 hours would be a suitable period of culture for studies of protein synthesis.

Three components of protein synthesis were analysed in the time course studies. These include:

(i) supernatant protein released into the medium by the cells during incubation,

(ii) protein released from the cells after incubation by freezing and thawing,

(iii) cell protein not released by freezing and thawing.

These studies would not demonstrate the synthesis of an acid soluble nucleoprotein, which has been reported to be synthesized

by lymphocytes (Weissman, et al., 1966).

PHA stimulated the synthesis of all of these categories of protein. The burst of protein synthesis in stimulated cells preceded the onset of mitotic activity in these cells as indicated by the uptake of $^{3}$H-thymidine. The relationship between the two phenomena is not known. It is tempting to postulate that the increase in protein within the cell is responsible for the onset of mitosis, but more evidence is needed to clarify this point. In both unstimulated and stimulated cultures there was incorporation of precursor into the cell debris fraction. This fraction may represent protein not released by freezing and thawing but a fraction released from cells by other procedures. In stimulated cultures an increase in this protein fraction was probably associated with preparation of the cells for mitosis.

The stimulatory effect of PHA on theincorporation of $^{14}$C-leucine by lymphocytes was dose-dependent. The effect of PHA on lymphocyte mitosis has also been demonstrated to be dose dependent (Mackinney, 1964). All batches of PHA stimulated increased protein synthesis in cultures containing plasma but they did not always stimulate lymphocyte protein synthesis in plasma-free medium.

Each batch of PHA is tested against an arbitrary standard to ensure uniformity in agglutinating and mitogenic activity but it is not tested for its ability to stimulate lymphocyte protein synthesis (Burrough Wellcome Information Pamphlet). Kolodny and Hirschhorn (1964) have reported that the capacities of PHA to agglutinate red cells and to stimulate mitotic activity in white cells reside in two distinct separable fractions. It is possible that the capacity of PHA to stimulate protein synthesis resides in another fraction of the PHA preparation. As a consequence the optimal concentrations of PHA batches to stimulate protein synthesis may differ.

When one batch of PHA was tested at a range of concentrations for its ability to stimulate lymphocyte protein synthesis, it was inhibitory at concentrations ten times that which stimulated optimal protein synthesis. Batches of PHA, which did not stimulate lymphocyte protein synthesis at the concentrations tested, may be stimulatory at lower concentrations. Plasma may modify the action of PHA by reducing its concentration in the medium. This would occur if PHA was bound to plasma proteins.

The possibility must not be excluded that various batches of PHA contained cell toxins, which were active in plasma-

free medium but were inactivated by plasma in the medium. Lymphocyte protein synthesis when followed by autoradiographic techniques showed maximum activity after 6 hours incubation. The increase in the rate of protein synthesis as reflected by an increase in the mean grain count during the early period of incubation may reflect the recovery period by the cells from culture preparation procedures. In PHA-stimulated cultures this effect would be masked by the stimulatory action of this substance on protein synthesis.

The protein synthesis activity of lymphocytes followed by autoradiographic techniques during incubation correlated well with the quantitative time-course study of protein synthesis in plasma-free medium (section 3.3.1). PHA-stimulated cultures contained a greater number of cells which incorporated $^{3}$H-leucine. These cells also had a greater mean grain count than the controls. Active cells in both unstimulated and PHA stimulated cultures may be heterogeneous with respect to the type of protein they synthesise. For example, the fluorescent antibody technique has shown that a minority of the cells in lymphocyte cultures contain detectable amounts of the immunoglobulins (Van Furth, 1964).

PHA markedly stimulated the incorporation of $^{3}$H-uridine by lymphocytes into RNA. Actinomycin D blocked the incorporation of precursor into PHA-stimulated and unstimulated cells (Chapter 6, section 6.3.4). These data suggest,

(i) that lymphocyte protein synthesis is dependent on the continued synthesis of m-RNA, (ii) that PHA is able to stimulate an increase in the production of m-RNA.

While PHA increased protein synthesis not more than five times it increased lymphocyte RNA synthesis by 10 times, when it was included in plasma free medium. In this medium lymphocyte RNA synthesis continued for a much longer period than protein synthesis. This occurred both in PHA-stimulated and unstimulated cultures. Several possibilities may be offered to explain these data.

(i) The RNA may fulfil some other function besides that of directing synthesis of soluble protein, for example mitosis and the production of cell bound protein in PHA-stimulated cells. The RNA may be concerned with the synthesis of acid soluble nucleoprotein, which was not investigated in these studies.

(ii) The medium may be suitable for the synthesis of RNA but not for protein synthesis by lymphocytes incubated for long periods of time. Protein synthesis by unstimulated and PHA stimulated cells cultured in

medium containing plasma, extended for a similar time to RNA synthesis by the cells cultured in plasma free medium.

(iii) In stimulated cultures uridine may be incorporated into both DNA and RNA; DNA synthesis was detected as early as 24 hours in these cultures. The method which was used for the separation of these two fractions did not preclude the possibility of DNA contaminating the RNA fractions.

(iv) The greater increase in RNA synthesis compared with lymphocyte protein synthesis caused by PHA may be associated with an overproduction of m-RNA. Cooper and Rubin (1966) observed that in lymphocytes much of the PHA-stimulated RNA did not become localized with the ribosomes after synthesis. They suggest that this synthesis represents the production of large quantities of m-RNA, probably as a result of the abrogation of normal regulatory processes by PHA.

Synthesis of DNA by lymphocytes has been reported to begin 24 hours after the addition of PHA (Robbins, 1964). In the experiments reported in this chapter PHA-stimulated cells began to incorporate $^{3}$H-thymidine after incubation for 24 hours. When these cells were sampled after 48 and 72

hours incubation many of the cells ruptured during preparation for autoradiography. For studies at these times, better techniques for the preparation of the smears on gelatine coated slides may overcome this problem.

Sasaki and Norman (1966) have reported that PHA-stimulated lymphocytes in vitro completed cell enlargment after 24-48 hours' incubation and began cell proliferation at this time, with a generation time of 22 hours. The results reported here suggest that many of the stimulated cells have finished transformation by 24 hours and are entering into the phase of cell proliferation and DNA synthesis at the end of this time. These data are in agreement with Sasaki and Norman's observations.

In stimulated cultures the sudden fall of the incorporation of $^{3}$H-uridine at 48 hours and rise at 72 hours may be associated with a fall in RNA synthesis and increase in DNA synthesis, since $^{3}$H-uridine is incorporated into both nucleic acids and the preparative methods did not exclude the possibility that the RNA fractions were contaminated with DNA. However, both cell death and the variability of the triplicate determinations which were made at these times made conclusions difficult.

The choice of a better medium to support lymphocyte

growth for longer periods is necessary for further investigations into these problems. In addition, the adoption of better preparative methods for the analysis of RNA would assist in preventing confusion with DNA synthesis.

The stimulation of protein and RNA synthesis of lymphocytes by PHA occurred concurrently (Fig. 3.1 and Fig. 3.3). A comparison of the two figures does not reveal any time lag between these two events. Torelli, et al., (1966) observed similar findings by autoradiographic examination of lymphocyte cells which had incorporated radioactive precursors. These workers have discussed this evidence with respect to a protein synthesis regulatory mechanism described by Stent (1964), in which the regulation of m-RNA function is brought about by variations in the intra-cellular availability of t-RNA's. They suggest that PHA is able to increase the availability and synthesis of t-RNA, which would lead to full activation of m-RNA in lymphocytes. According to their postulate an increase in both protein and m-RNA synthesis would follow.

The marked increase in the RNA:protein ratio in PHA-stimulated cells does suggest the overproduction of m-RNA, but part of this synthesis may also represent an increase

in the production of t-RNA. The absence of an observed time lapse between increased RNA and protein synthesis in PHA-stimulated cells is not sufficient evidence that it does not exist. Such a time lapse may be very small and escape detection in the studies reported in this chapter. Until more is known about the synthesis and availability of t-RNA in mammalian cells, little can be done to resolve these problems.

## 3.5. Summary

(1) Supplemented Eagle's medium prepared without leucine and plasma supported both protein and RNA syntheses by lymphocytes *in vitro*.

(2) Protein synthesis by lymphocytes continued for 12 hours in plasma-free medium but continued for 24 hours in plasma medium. The data suggest that 24 hours is a suitable incubation time for protein synthesis studies.

(3) PHA stimulated both RNA and protein synthesis by lymphocytes but increased the former more than the latter. Lymphocyte RNA synthesis in plasma-free medium continued for a longer period than protein synthesis. These observations have been discussed.

(4) The effect of PHA in plasma-free medium on lymphocyte protein synthesis was dose dependent, but PHA in high concentrations did not stimulate protein synthesis.

(5) Some batches of PHA did not stimulate protein synthesis and in some cases were inhibitory, when lymphocytes were cultured in plasma-free medium. All batches of PHA stimulated lymphocyte protein synthesis in plasma medium.

(6) PHA-stimulated lymphocytes began to incorporate $^{3}$H-thymidine after incubation for 24 hours in plasma medium. Unstimulated cells did not incorporate $^{3}$H-thymidine during incubation for 72 hours in plasma medium.

(7) Lymphocytes, polymorphs and monocytes incorporated $^{3}$H-leucine as detected by the technique of autoradiography. Erythrocytes did not incorporate $^{3}$H-leucine. PHA included in lymphocyte cultures increased both the percentage of cells incorporating $^{3}$H-leucine and the amount incorporated.

# CHAPTER 4

# QUALITATIVE STUDIES OF LYMPHOCYTE PROTEIN SYNTHESIS

## 4.1. Introduction

It has been shown that lymphocytes synthesized protein which was released and protein which was not released from the cells by freezing and thawing. Measurement of these fractions has been reported in Chapter 3. In this chapter experiments are described in which the soluble proteins (i.e. those released from the cells) have been investigated by the techniques of immuno-electrophoresis (IEP) and auto-radiography (AR).

Recent evidence suggests that circulating lymphocytes make immunoglobulins, $\alpha$ and $\beta$ globulins *in vitro* (Van Furth, 1964). Furthermore, the formation 'de novo' of antibody by rabbit lymphocytes *in vitro* was demonstrated by Hulliger and Sorkin (1963) and Landy, *et al.*, (1964).

The aims of the studies described in this chapter were:

(1) To examine the nature of the proteins synthesized by human lymphocytes *in vitro*. PHA-stimulated lymphocyte products were examined qualitatively at various times during culture.

(2) To investigate the possibility that lymphocytes make antibody.

## 4.2. Materials and Methods

### 4.2.1. Immunoelectrophoresis (IEP) and autoradiography (AR) of culture fluids

Cultures which were prepared for these analyses usually contained 5-10 million cells and 2-5 μc/ml of $^{14}$C-leucine. The lymphocyte proteins studied by these techniques include the following fractions:

Fraction I: This fraction included the proteins released into the supernatant by the cells after freezing and thawing the cultures three times. All the soluble proteins released by the cells during culture and those proteins not released except by freezing and thawing were measured in this fraction. This fraction is referred to as the culture supernatant or culture fluid throughout this thesis.

Fraction Ia: This fraction included the soluble proteins released into the supernatant by the cells during culture. This protein fraction was equivalent to the cell supernatant fraction (Chapter 3, section 3.2.2).

Fraction Ib: The proteins not released from the cells during culture but released by freezing and thawing were included in this fractinn. This fraction was the same as the cell extract referred to in Chapter 3 (section 3.2.2).

Fraction Ia(cell supernatant) and fraction Ib(cell extract) were prepared as described in Chapter 3 (section 3.2.1). These fractions were investigated in PHA-stimulated cultures sampled at various times during incubation for 24 hours. These fractions were examined in unstimulated cultures after incubation for 24 hours.

The culture fluids which were to be investigated by IEP and AR, were concentrated and dialysed in Collodion bags (Membranfiltergesellschaft, Göttingen, Germany) under vacuum, against 2 x 500 ml changes of phosphate buffered normal saline (pH 7.5) which contained 0.2% $^{12}$C-leucine at 4°C. The culture fluids were concentrated to a final volume of 0.1 ml and stored at -15°C between analyses in small sealed bottles. Autologous plasma or pooled serum were used as protein carrier in the investigations. Equal volumes of carrier and sample were loaded into a standard well (volume 0.01 ml) cut into the centre of an agar coated slide. Immunoelectrophoresis of the sample was then carried out by the micromethod of Scheidegger (1955) using sodium barbiturate buffer, $\mu$ 0.05, pH 8.6. Agar (Bacto-Agar, Difco, Michigan, U.S.A.) was made up to a final concentration of 1% in this buffer before using it to coat the slides.

The bands of precipitation were developed by cutting

side troughs in the agar at constant and equal distances from the centre well and filling them with antisera. Patterns were developed with the following antisera: horse anti-whole human serum (Hyland Laboratories, Los Angeles, California), goat anti-IgG (Hyland), goat anti-IgM (Hyland), rabbit anti-transferrin (Boehringer Gmbh, Ingelheim am Rhein) and goat anti-IgG (Hyland). Antiserum to IgG was also prepared by immunization of rabbits (see Chapter 5). After development the slides were washed for 3 days at 4°C with normal saline and finally with water, dried over $P_2O_5$ and stained with amido-black 10B (Chroma-Gesselschaft, Schmid and Co., Germany).

The dried slides were placed in contact with strips of 35 mm film (HPS, 800 ASA, Ilford, England) and stored in sealed packages for approximately four weeks before developing.

4.2.2. Characterization of antibody

Plasma from a patient with Hashimoto's disease was used as a source of antibody to human thyroglobulin. This plasma was used as carrier protein with the culture supernatants to be investigated. Purified human thyroglobulin (Derrien, et al., 1948) was prepared and supplied by Miss Aileen Thompson, Institute of Medical and Veterinary Science, Adelaide.

After electrophoresis of the sample and carrier in agar, human thyroglobulin solutions were included in the side troughs at concentrations of 2 or 4 mgm/ml to precipitate the antibody in agar.

## 4.3. Results

### 4.3.1. Characterization of the proteins

Immunoelectrophoretic patterns in agar, obtained after electrophoresis of serum and precipitated with anti-whole human serum, anti-IgG, anti-IgM, anti-IgA, anti-transferrin and anti-haptoglobulin are illustrated in Fig. 4.1. The immunoelectrophoretic patterns have been stained with amido black 10B.

Autoradiographic patterns prepared after immuno-electrophoresis of fraction I from normal lymphocyte cultures are shown in Fig. 4.2. The immunoglobulins were clearly labelled. Protein arcs in the $\alpha$ and $\beta$ globulin regions were labelled in all the patterns. The identification of these proteins was difficult because of the great number of arcs in these regions. In each autoradiograph there was a relatively large amount of labelled material in the region of the origin and towards the anodal end of the slide. This dense labelling did not correspond to a heavily stained area

Fig. 4.1. Immunoelectrophoretic patterns of human serum developed against various antisera

Human plasma was loaded into the centre well and electrophoresed as described in the methods. The various patterns were developed with:

A - whole human antiserum, note the albumin (Alb) arc, the proteins in the $\alpha$ and $\beta$ globulin region and the immunoglobulins IgA, IgM, IgG;

B - IgM antiserum;

C - IgA antiserum;

D - IgG antiserum;

E - transferrin antiserum;

F - haptoglobulin antiserum.

The proteins transferrin and haptoglobulin fall in the region of the $\alpha$ and $\beta$ globulins. IgA, IgM and IgG extend from the $\beta$ globulin area towards the cathodal end of the pattern.

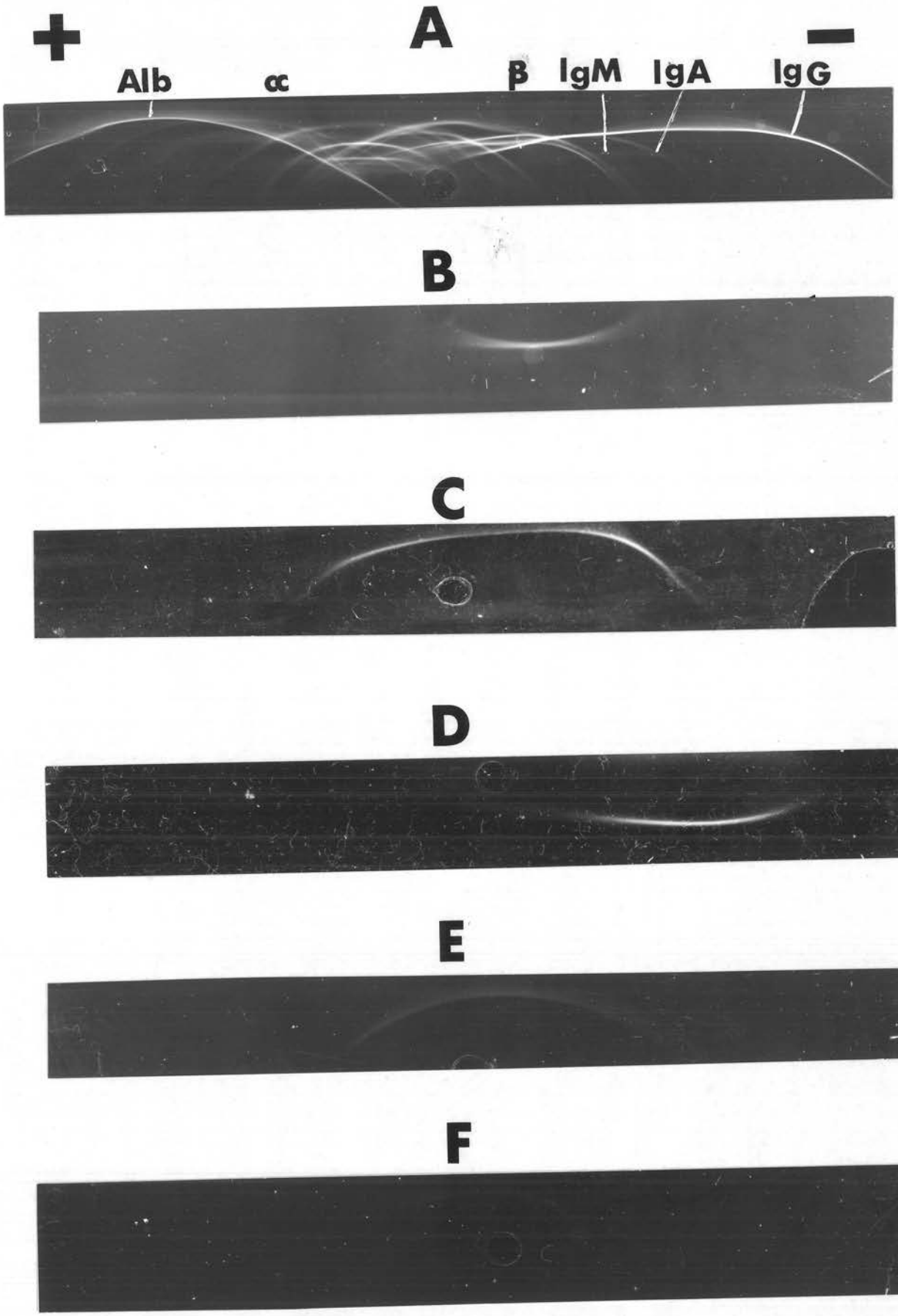

+
A
−
Alb
α
β
IgM
IgA
IgG
B
C
D
E
F

Fig. 4.2. Autoradiographs of IEP patterns prepared from fraction 1 (culture supernatants) of normal lymphocytes

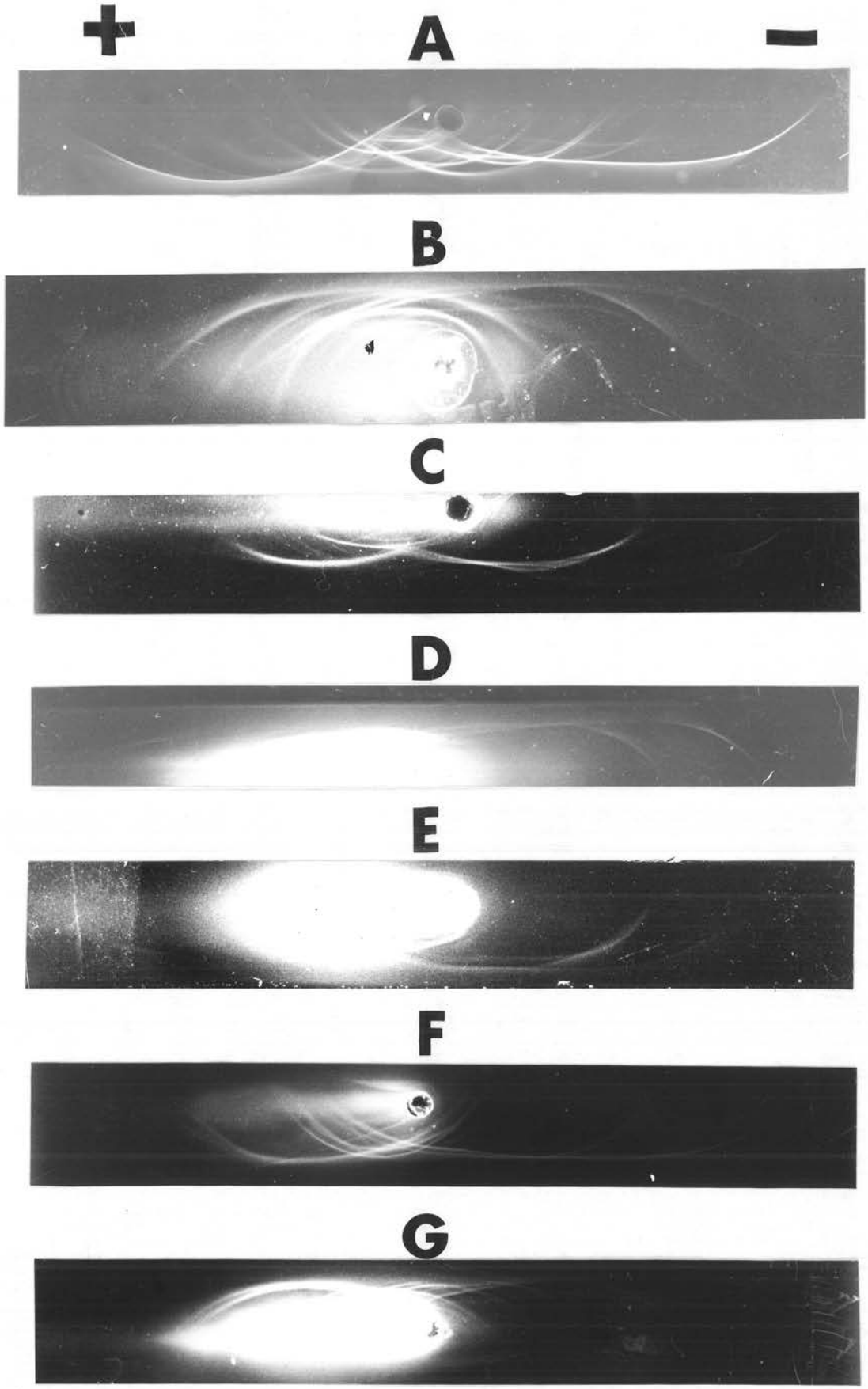
A is an IEP pattern of normal plasma developed with whole human antiserum.

The following autoradiographs were prepared from IEP patterns developed with whole human antiserum. The IEP patterns were prepared from culture supernatants of normal cultures containing:

B - $7.0 \times 10^6$ lymphocytes and 2.5 μc of $^{14}$C-leucine;
C - $7.7 \times 10^6$ lymphocytes and 5 μc of $^{14}$C-leucine;
D - $10.0 \times 10^6$ lymphocytes and 5 μc of $^{14}$C-leucine;
E - $7.0 \times 10^6$ lymphocytes and2 μc of $^{14}$C-leucine;
F - $7.9 \times 10^6$ lymphocytes and 2.5 μc of $^{14}$C-leucine;
G - same as F but the cells were stimulated with PHA.

Autoradiographs B, C, D and E were prepared from lymphocytes cultured in supplemented Eagle's medium prepared without leucine. Autoradiographs F and G were prepared from lymphocytes prepared from the same donor, but cultured in medium supplemented with 25% autologous plasma.

IgG, IgA, IgM and α and β globulins are labelled in the autoradiograph patterns. The intensity of the immunoglobulin labelling varied. PHA did not stimulate the synthesis of new proteins.


+
A
−
B
C
D
E
F
G

of protein in the immunoelectrophoretic pattern (Fig. 4.1). When the immunoelectrophoretic patterns were stained with Sudan red, a lipid stain, the only arc stained was $\beta$-lipoprotein; no other arcs or region on the slides were stained.

Autoradiographic patterns prepared after immunoelectrophoresis of fraction I from lymphocytes which had been cultured in plasma-free medium, medium containing plasma and medium containing 0.5% ovalbumin, were similar (Fig. 4.3). The intensity of immunoglobulin labelling was similar in all the patterns. There was a large amount of unidentified labelled material in the reginn of the origin in all the patterns. The amount of this material was less in the pattern prepared from lymphocytes cultured in plasma medium.

IEP patterns were formed to specific antisera to show individual proteins. Fraction I from normal lymphocyte cultures labelled the individual protein arcs IgG, IgM, IgA, transferrin and haptoglobulin in IEP patterns (Fig. 4.4).

Phytohaemagglutinin did not stimulate lymphocytes to produce new proteins, as determined by the labelling of immunoprecipitin lines in the IEP patterns (Fig. 4.2). In these patterns the intensity of the labelling of all the proteins which were labelled in the control patterns was increased. This was determined by visual observation, a more

Fig. 4.3. Autoradiographs of IEP patterns prepared from fraction 1 (culture supernatants) of unstimulated lymphocytes cultured in various media

All the cultures contained 7.9 x $10^6$ cells in a culture volume of 1 ml. Patterns B and C were prepared from cultures containing 2.5 µc of $^{14}$C-leucine. Pattern D was prepared from a culture containing 5.0 µc of $^{14}$C-leucine.

A is an IEP pattern of normal plasma developed with whole human antiserum.

The following autoradiographs were prepared from culture supernatants of lymphocytes cultured in:

B - plasma-free medium;

C - 25% autologous plasma medium;

D - plasma-free medium containing 0.5% ovalbumin.

In all the patterns the immunoglobulins, IgA, IgM and IgG are labelled. IgM labelling is the weakest of the three. The extent of the labelling of the proteins in the $\alpha$ and $\beta$ globulin region varied. The large amount of diffuse labelled material in patterns B and D obscured labelling of protein arcs in these regions. The labelling of the protein arcs in the $\alpha$ and $\beta$ globulin region of pattern C are more clearly labelled.

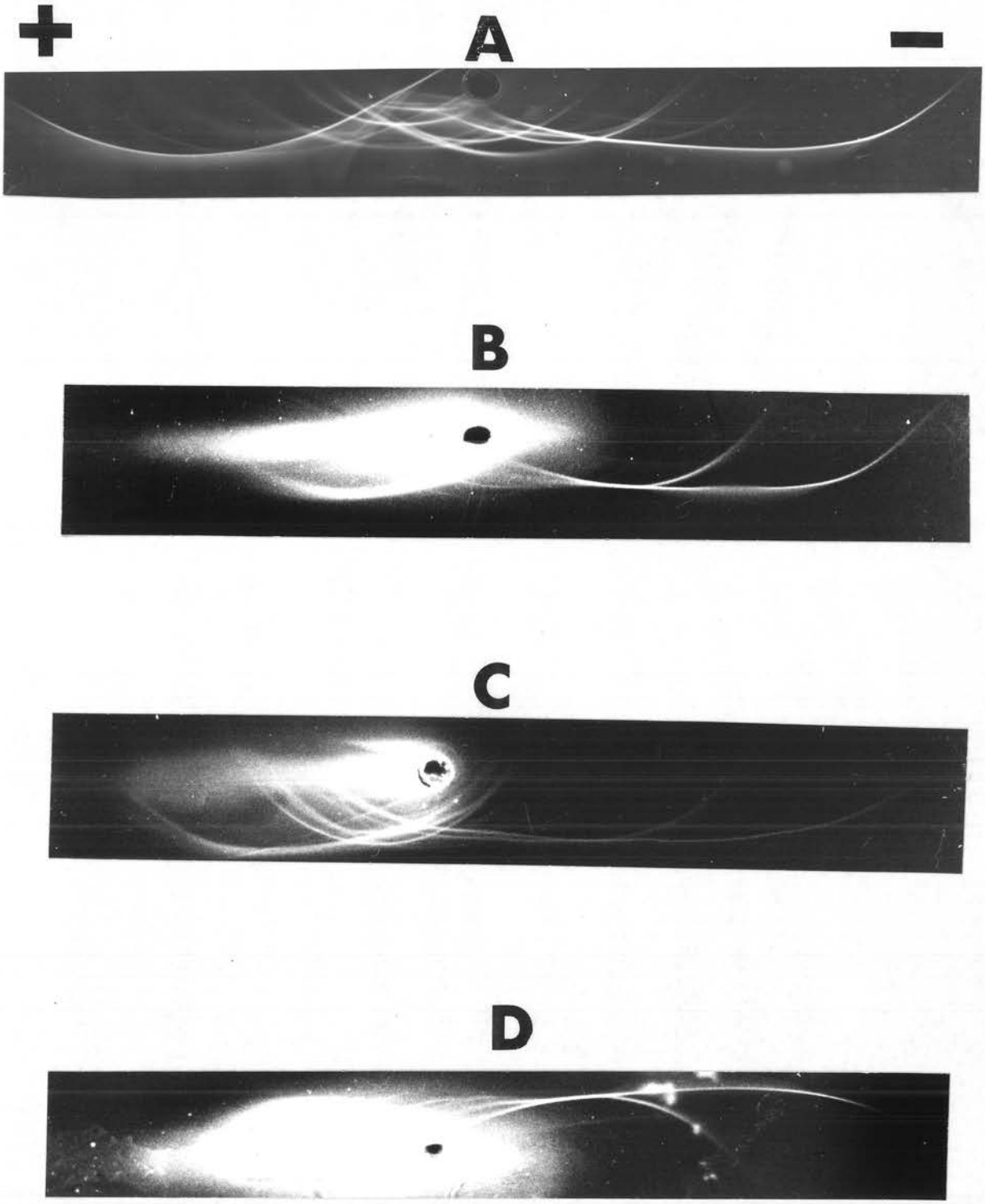

+
A
−
B
C
D

Fig. 4.4. Autoradiographs of specific IEP patterns prepared from fraction 1 (culture supernatants) of normal human lymphocytes

Pattern B was prepared from a culture containing $7.0 \times 10^6$ lymphocytes and 2.5 μc of $^{14}$C-leucine. Patterns C, D, E and F were prepared from a culture containing $7.9 \times 10^6$ cells and 2.5 μc of $^{14}$C-leucine.

A is an IEP pattern of normal plasma developed with human antiserum.

The following autoradiographs were prepared from IEP patterns developed with antisera to:

B - IgM;

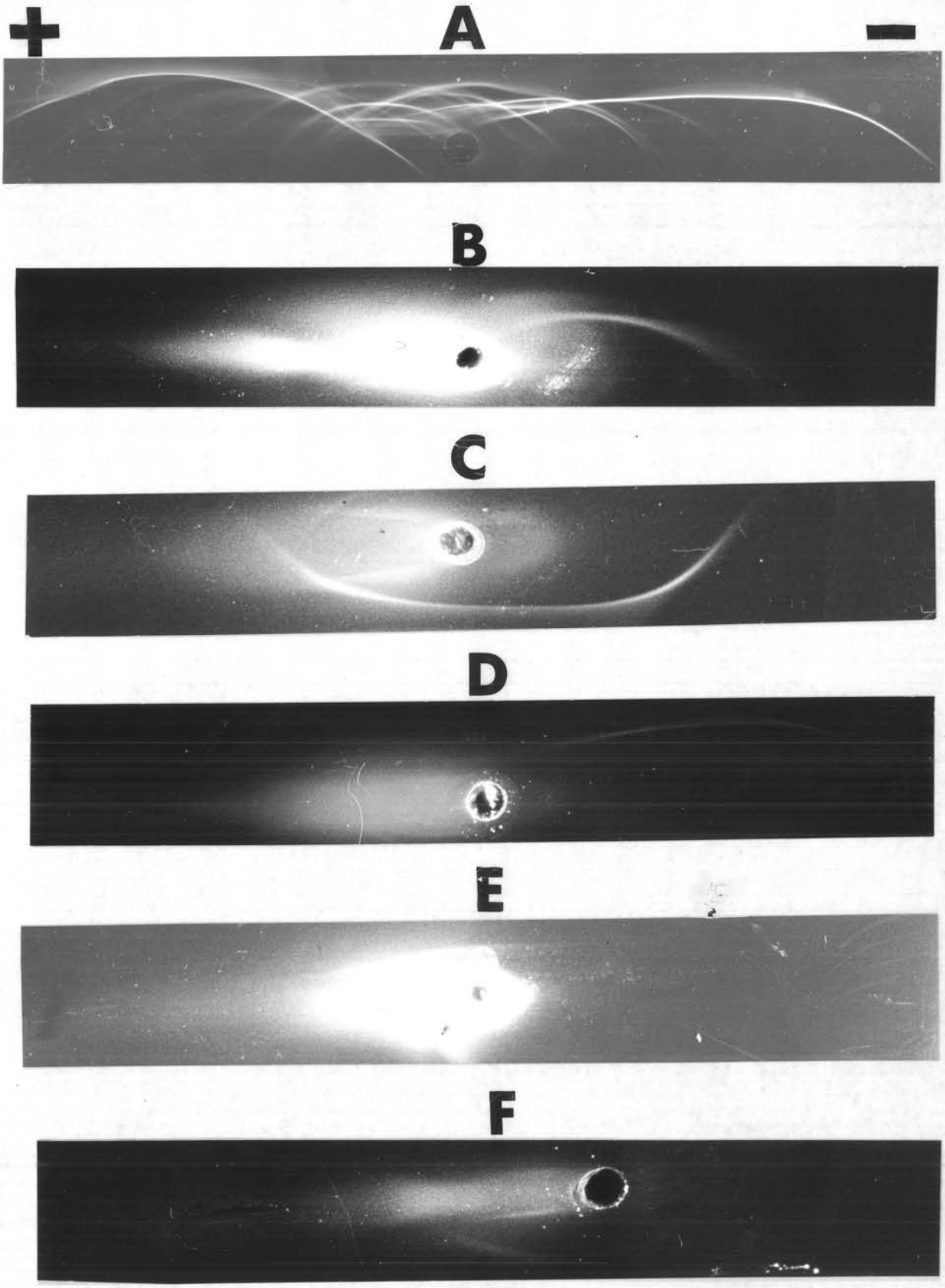
C - IgA;

D - IgG;

E - Transferrin;

F - Haptoglobulin.

The immunoglobulin arcs (IgM, IgA and IgG) are labelled. Both transferrin and haptoglobulin arcs are labelled more heavily at the anodal end. The transferrin arc is not completely labelled. In all patterns there is a significant amount of labelled diffuse material around the origin and towards the anode.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reliable method would be required for an accurate quantitative assessment of the intensity of the labelling of each protein. Actinomycin D included in the culture medium (3.3 μg/ml) reduced the labelling of all proteins found in control autoradiograph patterns (Fig. 4.5). When puromycin was included in the culture medium at a concentration of 40 μg/ml, no labelling of any part of the IEP patterns was observed.

4.3.2. Qualitative time course-studies of protein synthesis

These studies are complementary to the quantitative time-course studies reported in Chapter 3, section 3.3.1. In these investigations, both the cell supernatant and cell extract fractions were analysed by immunoelectrophoresis and autoradiography. Unstimulated cultures were sampled at 24 hours. The proteins made by PHA-stimulated lymphocytes during the incubation periods 0-12 hours, 12-24 hours and 0-24 hours were also studied this way.

In the accompanying autoradiographic patterns, reference will be made to proteins in fractions Ia, and fraction Ib of lymphocyte cultures. The proteins in each fraction were examined by electrophoresis of the sample with carrier pooled

Fig. 4.5. Autoradiographs of IEP patterns prepared from fraction 1 (culture supernatant) of normal lymphocytes incubated with actinomycin D

A is an IEP pattern of normal plasma developed with whole human antiserum.

The following autoradiographs were prepared from cultures of lymphocytes (isolated from the same donor) prepared as described:

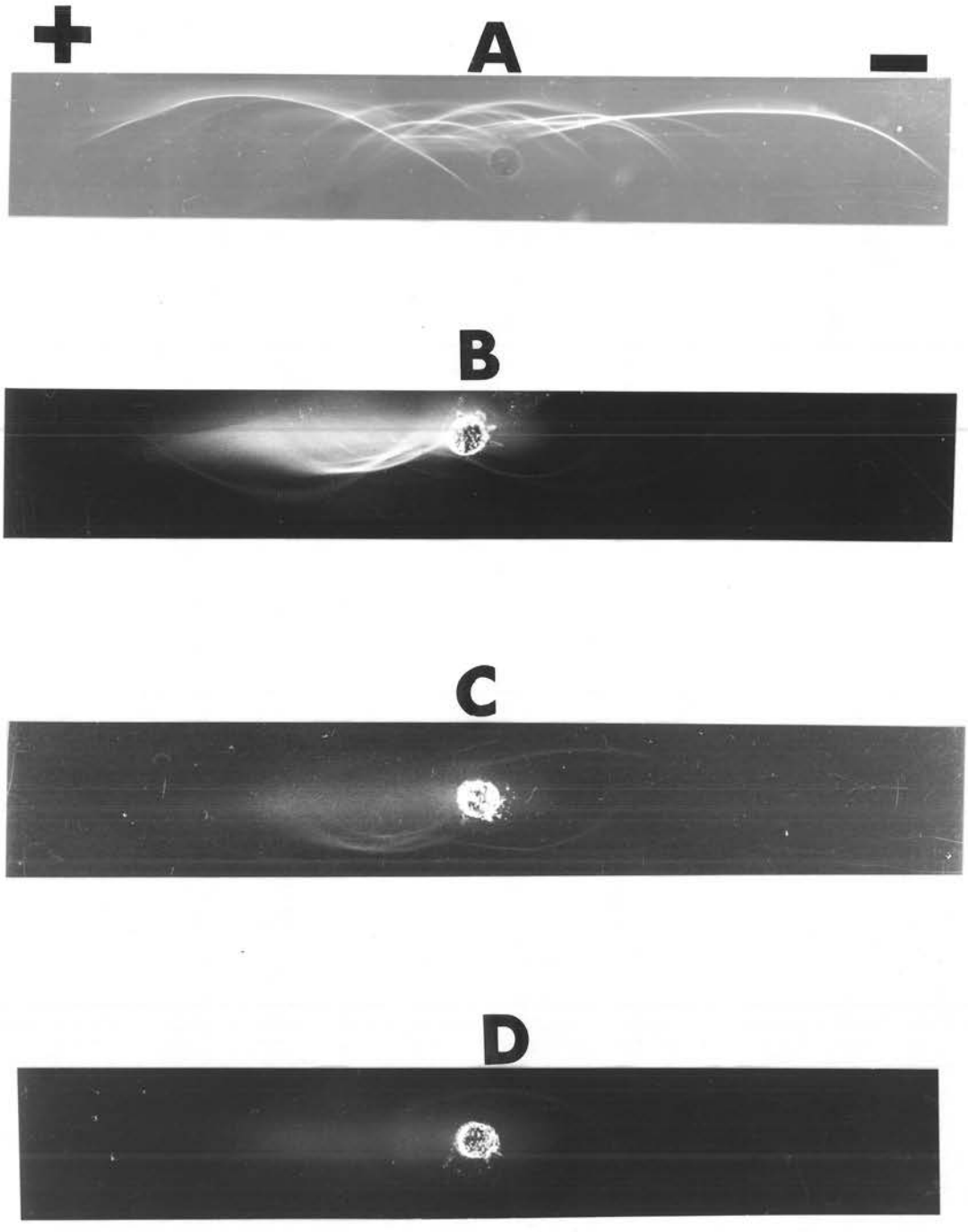
B - is an autoradiograph prepared from a culture containing 5 μc of $^{14}$C-leucine and 10 x $10^6$ cells; the IEP pattern was developed with whole human antiserum.

C - is an autoradiograph prepared from a culture containing 10 x $10^6$ cells, 10 μc of $^{14}$C-leucine and 3.3 μg of actinomycin D. The IEP pattern was developed with IgG (upper trough) and whole human antiserum (lower trough).

D - is an autoradiograph prepared from the same culture described in C. The IEP pattern was developed with IgA antiserum (upper trough) and IgM antiserum (lower trough).

The same labelled protein arcs present in B, are present in C with reduced intensity. IgG and IgM arcs are labelled by culture fluid from the actinomycin D treated culture, but the IgA arc is only labelled at the anodal end.

The diffuse labelled material present in pattern B is present in both patterns, C and D, but with reduced intensity.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D

serum and development of the immunoelectrophoretic pattern with (i) whole antiserum (ii) anti-transferrin (iii) anti-IgG (iv) anti-IgM, and (v) anti-IgA antisera. The labelled proteins in each pattern were investigated by autoradiography.

In these experiments the lymphocytes were prepared from a normal donor, by filtration of the blood through glass wool. 1 Ml portions of the final suspensions containing 99% lymphocytes were dispensed into culture bottles so that all the cultures contained $7.9 \times 10^6$ cells and 4 μc of $^{14}$C-leucine. The experimental conditions have been detailed in the texts to the figures illustrating the autoradiographs in this chapter.

In a control, unstimulated culture sampled at 24 hours, all the immunoglobulin proteins were labelled in fraction Ib. These were best identified with specific antisera. Proteins in the $\alpha$ and $\beta$ globulin region were heavily labelled (Fig. 4.6). Part of the transferrin protein arc was also labelled. The specific protein arcs were labelled heaviest at the anodal end. In fraction Ia the only protein labelled in the whole antiserum pattern was IgG globulin (Fig. 4.7). IgM, IgA and IgG precipitated in IEP patterns with specific antisera, were labelled in the accompanying autoradiographs. Transferrin was not labelled. The absence of labelled material in the

Fig. 4.6. Autoradiographs of IEP patterns prepared from fraction 1b (cell extract) of an unstimulated culture of normal lymphocytes incubated for 24 hours

The culture fluid analysed in this study was from a lymphocyte culture containing $7.9 \times 10^6$ cells and 4 μc of $^{14}$C-leucine. The lymphocytes were prepared by glass wool filtration and the final suspensions contained 99% lymphocytes.

A is an IEP pattern of normal plasma developed with whole human antiserum.

The following autoradiographs were prepared from IEP patterns developed with antisera to:

B - human antiserum;

C - IgG;

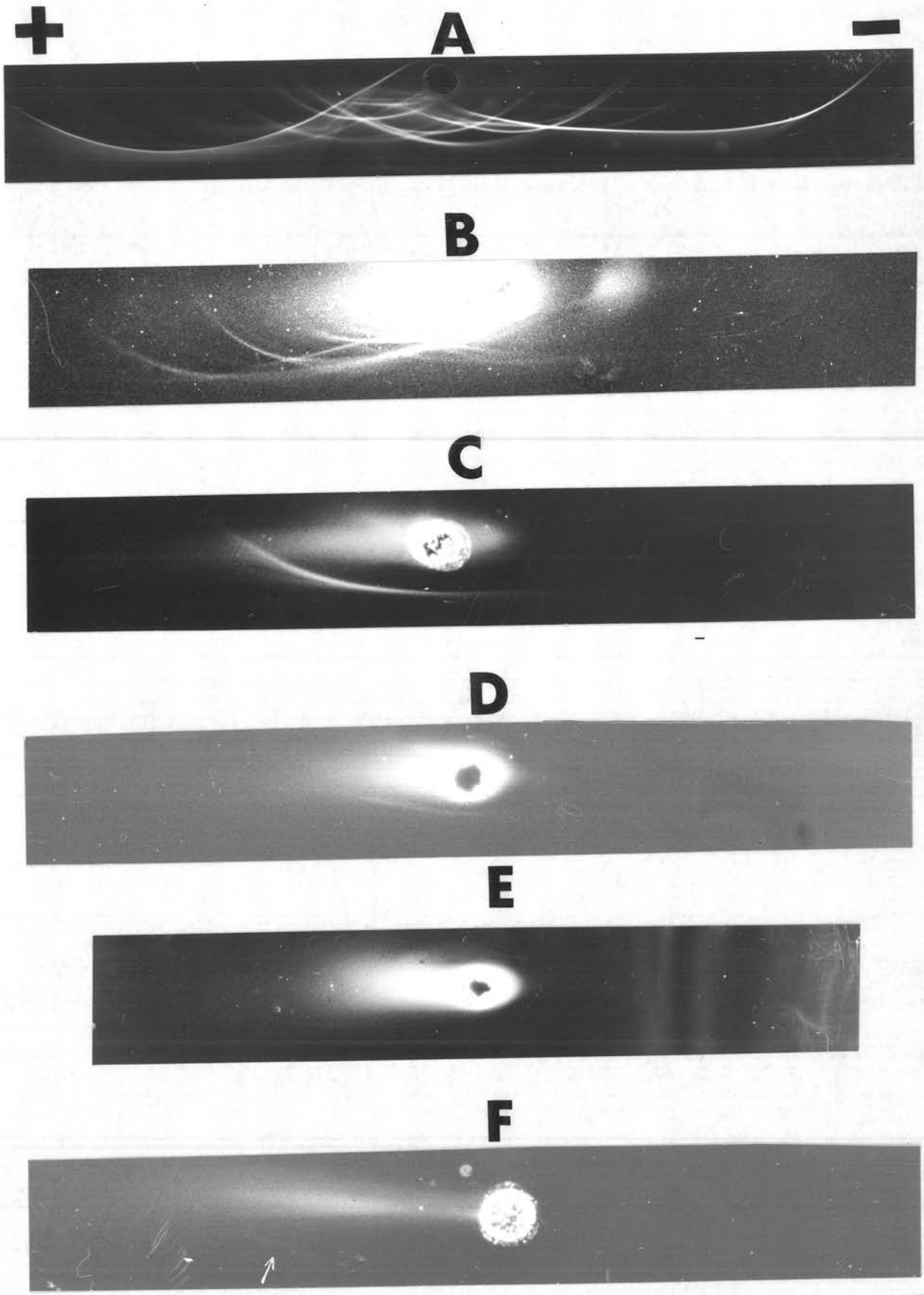
D - IgM;

E - IgA;

F - Transferrin.

In pattern B the protein arcs in the α and globulin region are labelled heaviest. In the other patterns the immunoglobulin arcs IgG and IgA are labelled, but IgM and transferrin arcs are labelled only at the anodal end.

There is diffuse labelled material around the origin in all the patterns.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F

Fig. 4.7. Autoradiographs of IEP patterns prepared from fraction 1a (cell supernatant) of an unstimulated culture of normal lymphocytes incubated for 24 hours

The culture fluid analysed in this study was from the same culture as that described in figure 4.6.

A is an IEP of normal plasma developed with whole human antiserum.

The following autoradiographs were prepared from IEP patterns developed with antisera to:

B - human antiserum;

C - IgA;

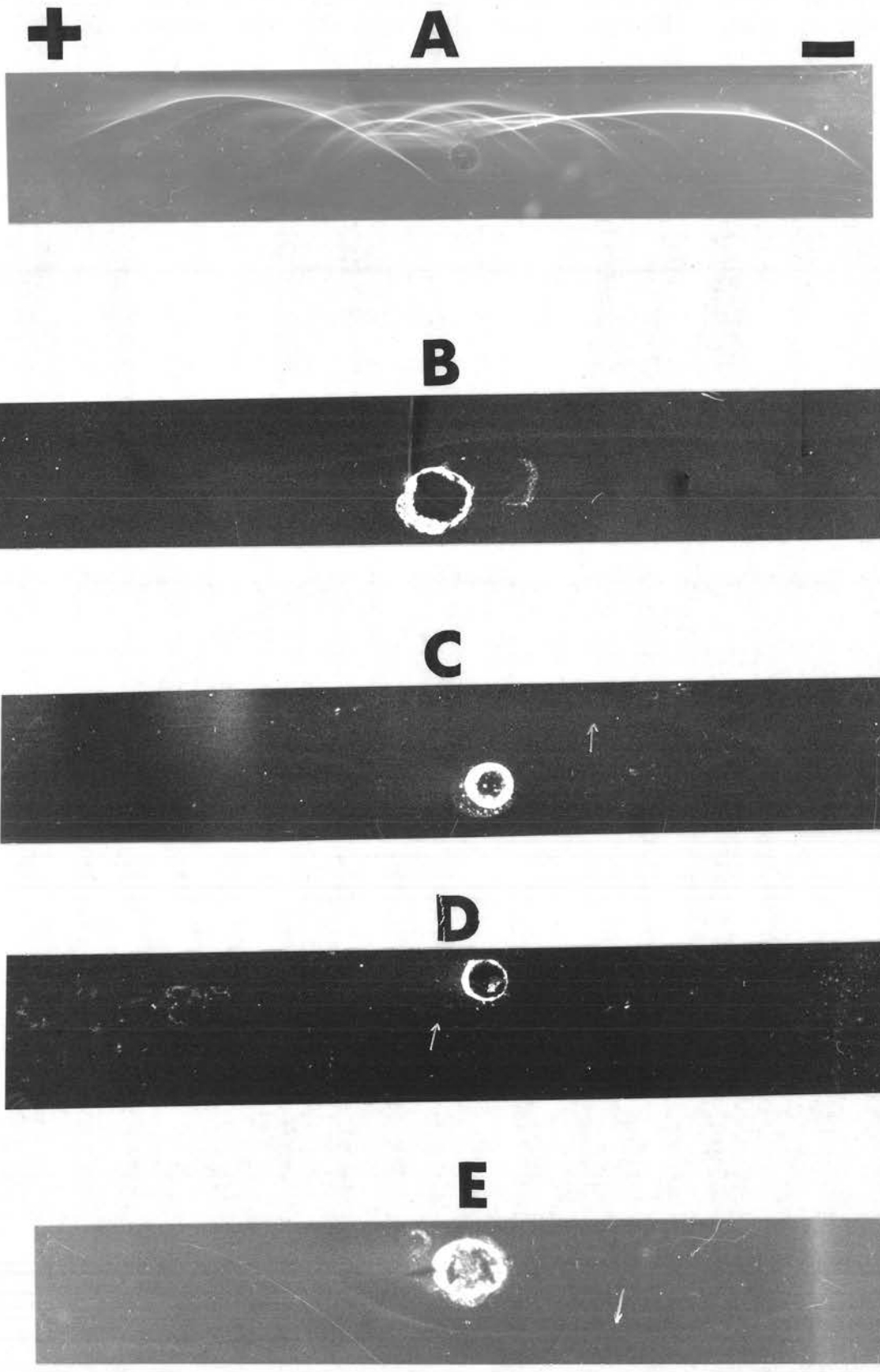
D - IgM;

E - IgG.

In pattern B the only protein arcs labelled are IgG and part of IgA.

In the others patterns the IgA and IgG arcs are labelled while the IgM arc is labelled at the anodal end. Transferrin was not labelled.

Note the absence of the diffuse labelled material around the origin and towards the anode in the patterns.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D
E

region of the origin was most striking, when compared with the amount present in the cell extract fraction.

Fraction Ib of a PHA-stimulated culture which was sampled at 12 hours, contained labelled α and β globulins, labelled immunoglobulins and labelled transferrin (Fig. 4.8). In all the patterns there was a considerable amount of diffuse labelled material in the region of the origin. The arcs were labelled heaviest at the anodal end. Fraction Ia of this culture contained labelled α and β proteins, labelled IgG and labelled IgA (Fig. 4.9). IgM and transferrin were not labelled. The labelled material around the origin was much reduced when compared with the amount in fraction Ib.

In a stimulated culture which was incubated with $^{14}$C-leucine during the incubation period 12-24 hours, fraction Ib contained labelled α and globulins, and labelled IgG. No other proteins were labelled (Fig. 4.10). Fraction Ia from this culture contained similar labelled proteins (Fig. 4.11), but as was observed previously there was less labelling around the origin than in the cell extract.

In the final study of this series, it was shown that fraction Ib from a PHA-stimulated culture sampled at 24 hours contained labelled α and globulin, labelled transferrin,

Fig. 4.8. Autoradiographs of IEP patterns prepared from fraction 1b (cell extract) of a PHA-stimulated culture of normal lymphocytes incubated for 12 hours

The culture fluid analysed in this study was prepared from a culture similar to that described in figure 4.6. PHA was included in the medium and the lymphocytes were incubated with $^{14}$C-leucine for 12 hours.

A is an IEP pattern of normal plasma developed with whole human antiserum.

The following autoradiographs were prepared from IEP patterns developed with antisera to:

B - human serum;

C - IgG;

D - IgM;

E - IgA;

F - Transferrin.

In pattern B the protein arcs in the $\alpha$ and $\beta$ globulin region are heavily labelled. The labelling of the immunoglobulin arcs is not distinct, but in the other patterns IgA, IgM and IgG arcs are labelled heaviest at the anodal end. The transferrin arc is labelled only at the anodal end.

Note the large amount of diffuse labelled material in the region of the origin and towards the anode of the patterns.

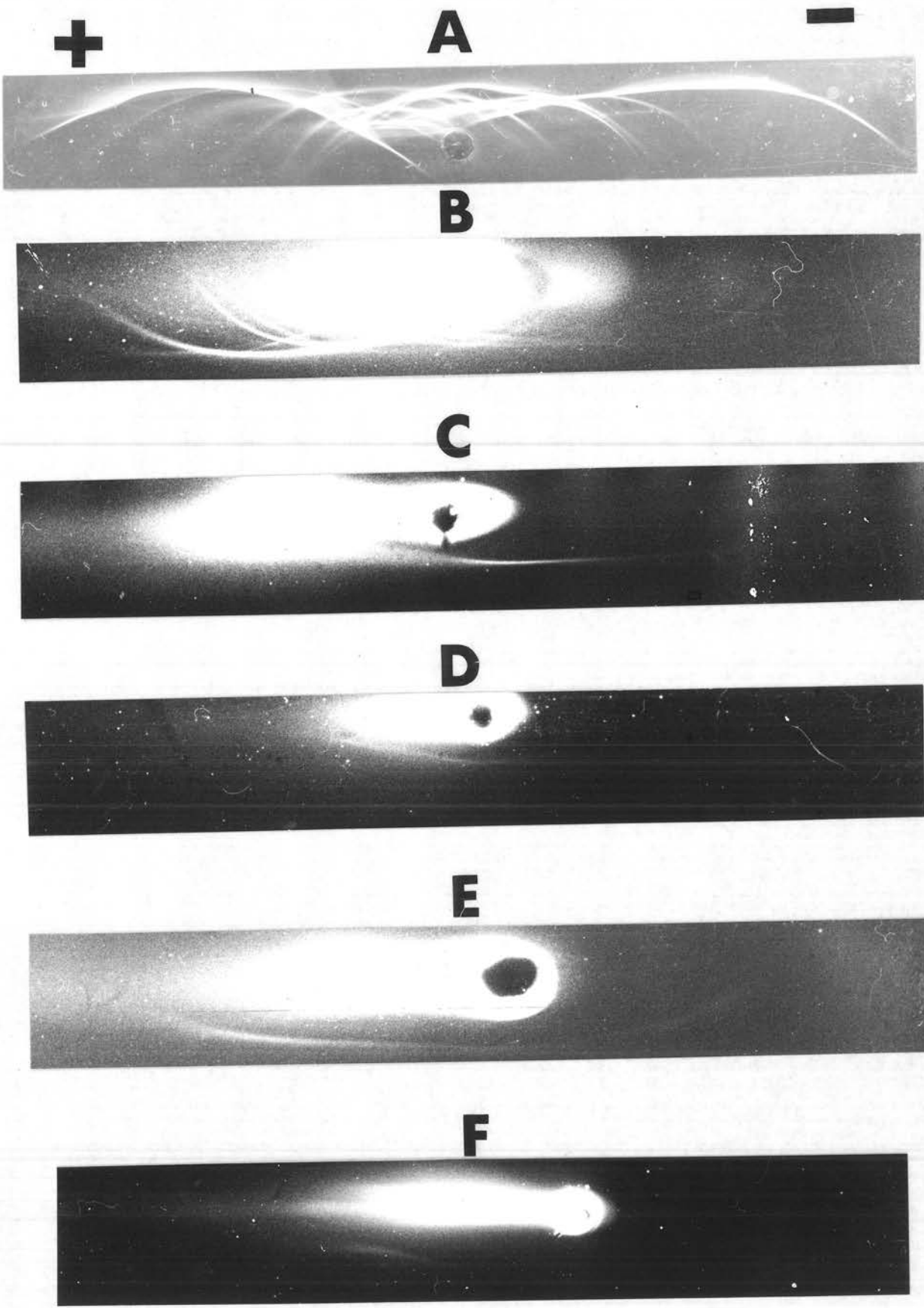

+
A
−
B
C
D
E
F

Fig. 4.9. Autoradiographs of IEP patterns prepared from fraction 1a (cell supernatant) of a PHA-stimulated culture of normal lymphocytes incubated for 12 hours

The culture fluid analysed in this study was from the same culture as that described in figure 4.8.

A is an IEP pattern of normal plasma developed with whole human antiserum.

The following autoradiographs were prepared from IEP patterns developed with antisera to:

B - human serum;

C - IgG;

D - IgA.

In pattern B none of the protein arcs are heavily labelled. In the other patterns IgG and IgA are both weakly labelled, but IgM and transferrin were not labelled.

Diffuse labelled material present in the autoradiographs shown in figure 4.8 is barely evident in pattern B. It is absent from patterns C and D.

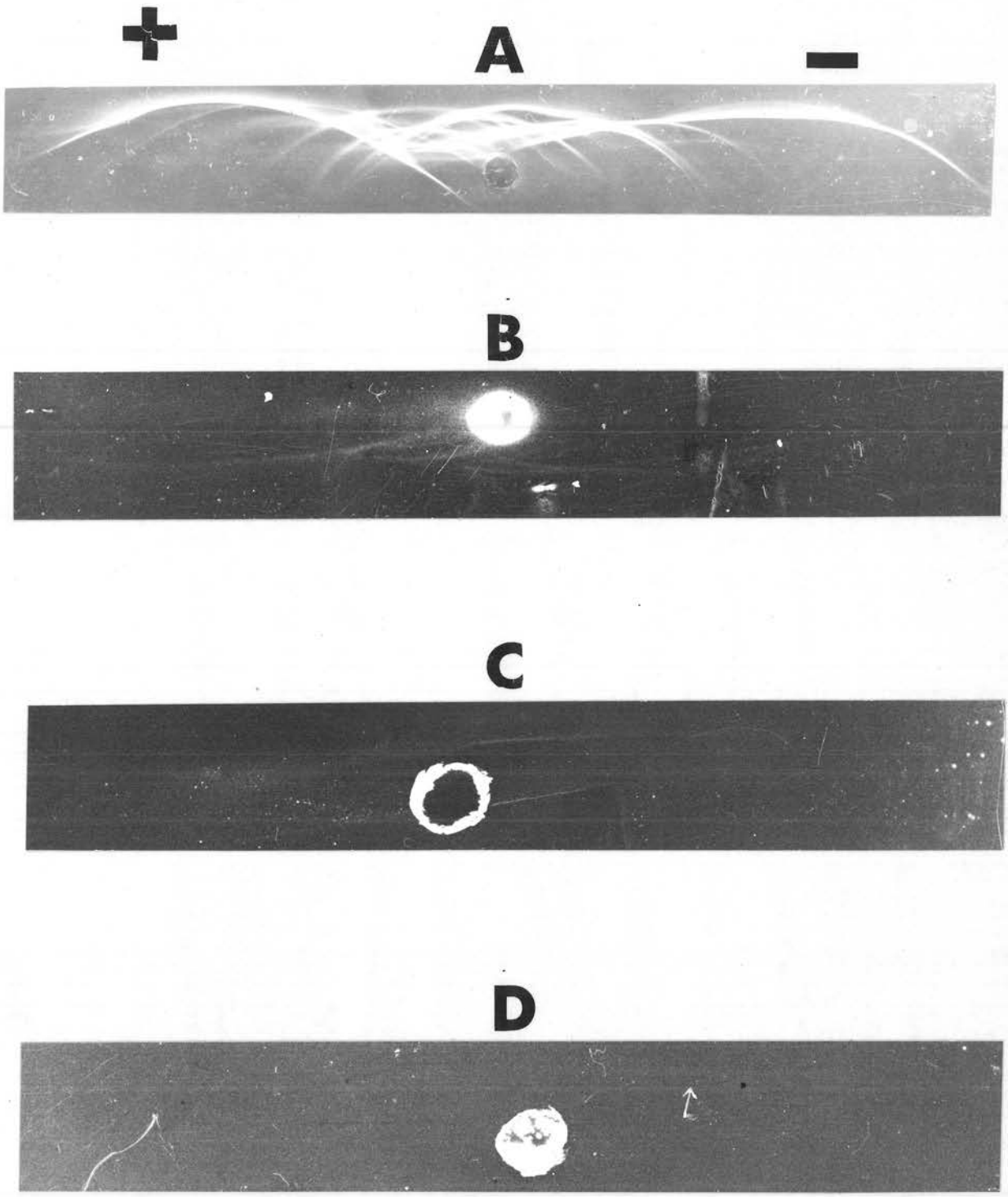

+
A
−
B
C
D

Fig. 4.10. Autoradiographs of IEP patterns prepared from fraction 1b (cell extract) of a PHA-stimulated culture of normal lymphocytes incubated for 24 hours after radioactive precursor had been added at 12 hours

The culture fluid analysed in this study was prepared from a culture similar to that described in figure 4.6. PHA was included in the medium. $^{14}$C-Leucine was included after the cells had been incubated for 12 hours.

A is an IEP pattern of normal plasma developed with whole human antiserum.

The following autoradiographs were prepared from IEP patterns developed with antisera to:

B - human serum;

C - IgG.

In B various arcs are weakly labelled. Part of the IgG arc is labelled, but IgA and IgM arcs were not labelled. In the other pattern IgG is labelled. IgA, IgM and transferrin were not labelled.

More diffuse labelled material is associated with pattern C than with pattern B.

+ A −

B

C

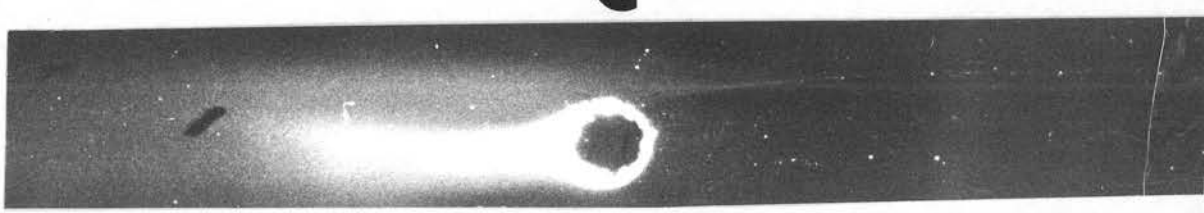


Fig. 4.11. Autoradiographs of IEP patterns prepared from fraction 1a (cell supernatant) of a PHA-stimulated culture of normal lymphocytes incubated for 24 hours after radioactive precursor had been added at 12 hours

The culture fluid analysed in this study was from the same culture as that described in figure 4.10.

A is an IEP pattern of normal plasma developed with whole human antiserum.

The following autoradiographs were prepared from IEP patterns developed with antisera to:

B - human serum;

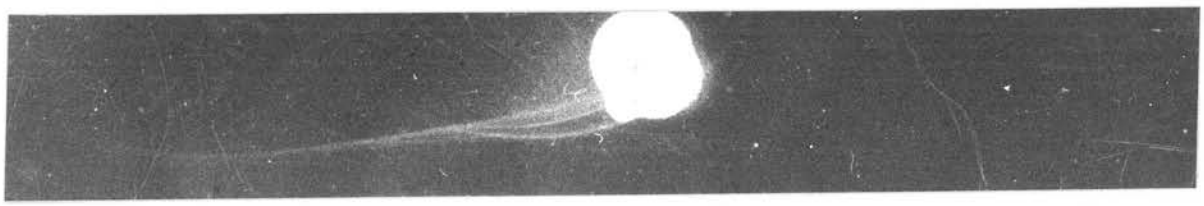
C - IgG.

In pattern B various arcs in the $\alpha$ and $\beta$ globulin region are labelled. IgG arc is labelled at the anodal end. IgM and IgA were not labelled. In the other patterns the IgG arc is labelled heaviest at the anodal end, but IgA, IgM and transferrin arcs were not labelled.

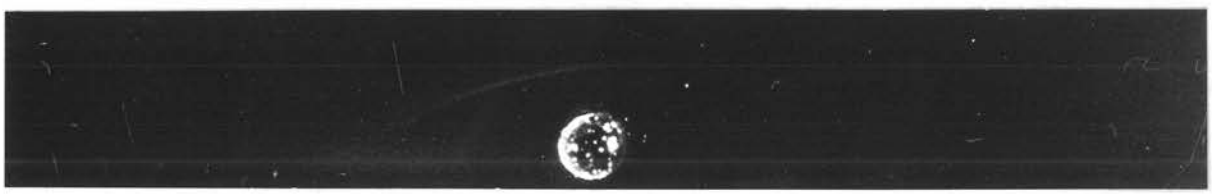
The diffuse labelled material about the origin is barely evident in both patterns.

+ A −

B



C



IgM, IgA, and IgM. The amount of labelled material around the origin and towards the anode was much greater than that seen in previous autoradiographs. All the precipitin arcs were labelled heaviest towards the anodal end (Fig. 4.12). Fraction Ia contained all the labelled proteins found in fraction Ib, except transferrin (Fig. 4.13), but the labelling around the origin was much reduced.

These findings have been tabulated (Table 4.1). For the purpose of comparison the intensity of labelling was graded arbitrarily by visual observation. The order of grading ranked from +++ (heaviest labelling) to + (faint).

4.3.3. Antibody synthesis

Lymphocyte cultures were prepared from the blood of a patient with Hashimoto's disease and terminated at 24 hours by freezing and thawing. The proteins in fraction I were electrophoresed and the immunoelectrophoretic pattern was developed against thyroglobulin and whole anti-human serum. The corresponding autoradiograph showed distinct and strong labelling of all the immune globulins and in particular the thyroglobulin antibody line (Fig. 4.14).

Fractions I from twelve normal lymphocyte cultures were analysed in this way. None contained labelled antibody.

Fig. 4.12. Autoradiographs of IEP patterns prepared from fraction 1b (cell extract) of a PHA-stimulated culture of normal lymphocytes incubated for 24 hours

The culture fluid analysed in this study was prepared from a culture similar to that described in figure 4.6. PHA was included in the medium.

A is an IEP pattern of normal plasma developed with whole human antiserum.

The following autoradiographs were prepared from IEP patterns developed with antisera to:

B - human serum;

C - IgM;

D - IgG;

E - IgA;

F - Transferrin.

In pattern B the $\alpha$ and $\beta$ globulin arcs are labelled heaviest. In the other patterns IgA, IgM and IgG arcs are labelled heaviest at the anodal end. The transferrin arc is only labelled at the anodal end. Note the large amount of diffuse labelled material around the origin and towards the anode in all the patterns.

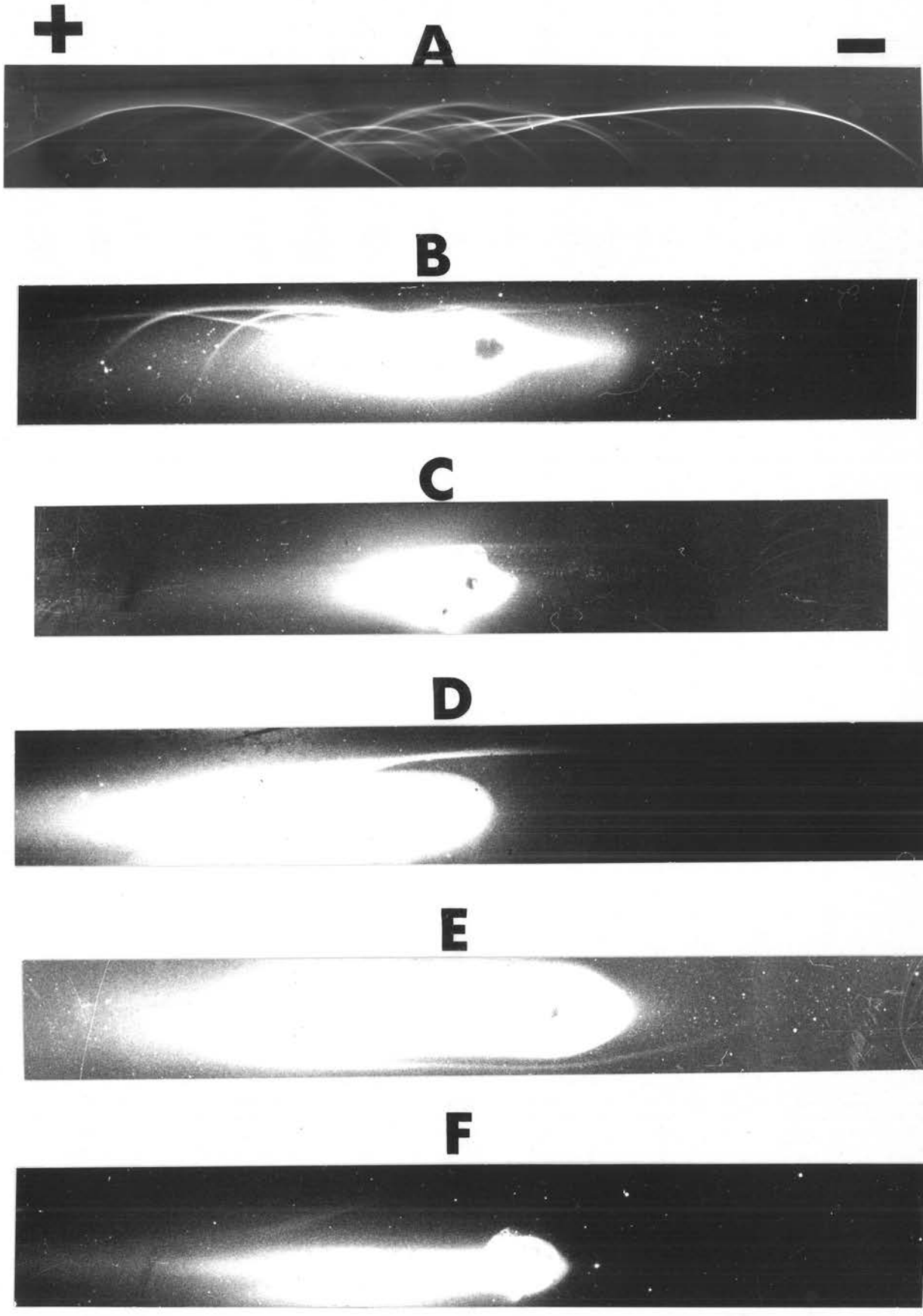

+
A
−
B
C
D
E
F

Fig. 4.13. Autoradiographs of IEP patterns prepared from fraction 1a (cell supernatant) of a PHA-stimulated culture of normal lymphocytes incubated for 24 hours

The culture fluid analysed in this study was from the same culture as that described in figure 4.12.

A is an IEP pattern of normal plasma developed with whole human antiserum. The following autoradiographs were prepared from IEP patterns developed with antisera to:

B - human serum;

C - IgG;

D - IgA;

E - IgM.

In pattern B the $\alpha$ and $\beta$ globulin arcs are labelled heaviest. In the other patterns the IgG arc is labelled, while IgM and IgA arcs are faintly labelled. The transferrin arc was not labelled.

Very little diffuse labelled material is evident in any of the patterns.

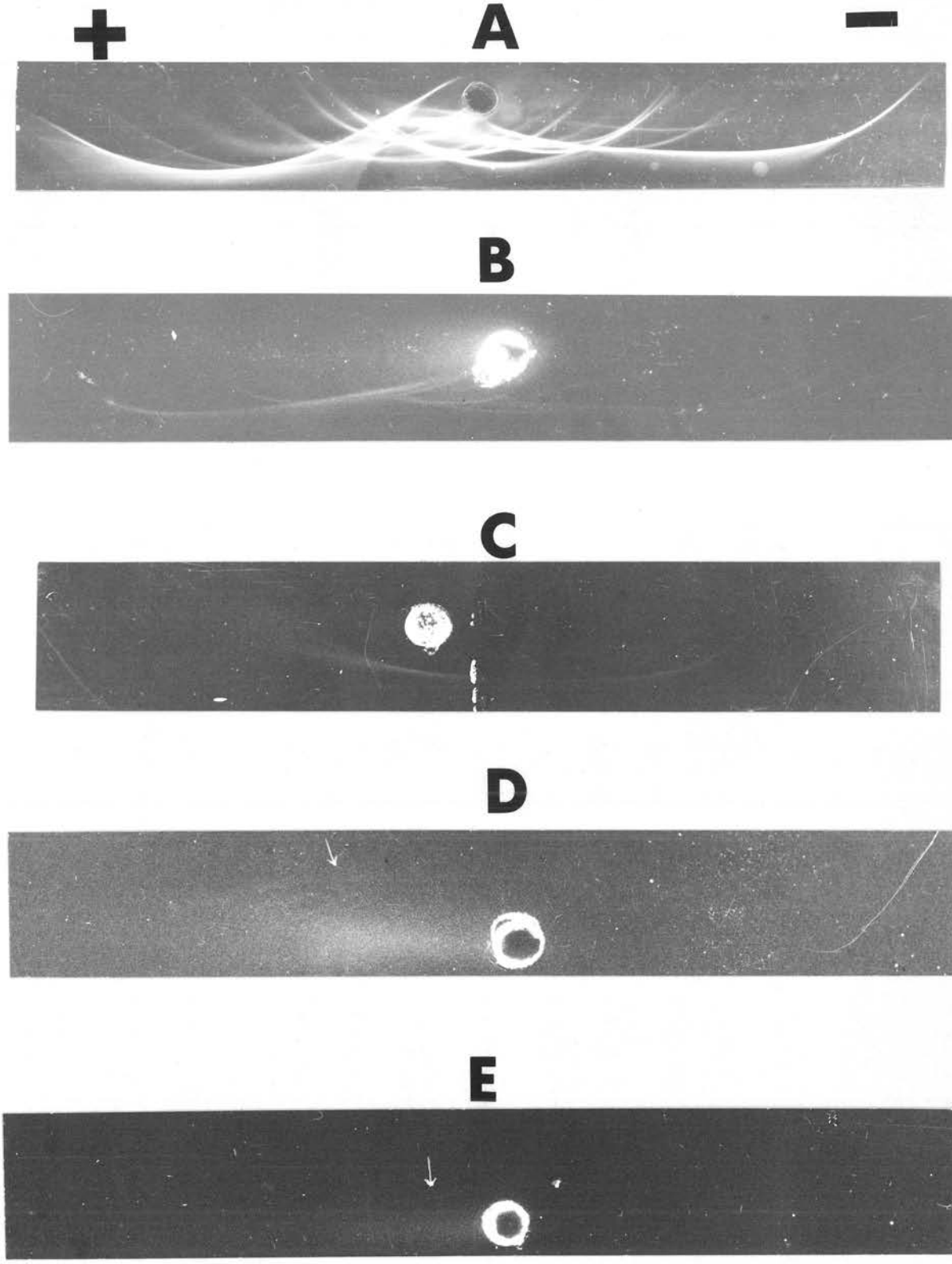

+
A
−
B
C
D
E

Table 4.1. Summary of labelled protein arcs in autoradiographs of IEP patterns of lymphocyte culture material

This table is a summary of the data presented in figures 4.6 - 4.13.

Labelling was arbitarily graded from + to +++ by visual observation of the autoradiographs. +++ represents heavy labelling, while + indicates faint labelling of the protein arcs.

The labelling of the proteins in the $\alpha$ and $\beta$ globulin region was graded by reference to the autoradiographs of IEP patterns developed with whole human antiserum. The grading of the other labelled proteins was determined by reference to the autoradiographs of IEP patterns developed with specific antisera.

TABLE 4.1

AUTORADIOGRAPHY OF IEP PATTERNS OF LYMPHOCYTE CULTURES

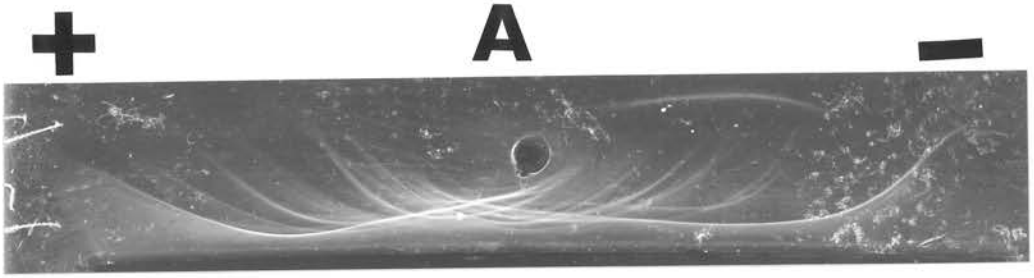
| Time (hr) | Soluble Protein Fraction | α globulins | β globulins | Transferrin | IgM | IgA | IgG | Fig. |
|---|---|---|---|---|---|---|---|---|
| 0-24 | Cell extract | +++ | ++ | + | ++ | + | ++ | 4.6 |
| | Cell supernatant | - | - | - | + | + | ++ | 4.7 |
| 0-12 | Cell extract | +++ | ++ | + | ++ | ++ | ++ | 4.8 |
| | Supernatant | + | ++ | - | - | + | + | 4.9 |
| 12-24 | Cell extract | ++ | + | - | - | - | + | 4.10 |
| | Supernatant | ++ | + | - | - | - | + | 4.11 |
| 0-24 | Cell extract | +++ | ++ | + | ++ | ++ | ++ | 4.12 |
| | Supernatant | ++ | ++ | - | + | + | ++ | 4.13 |

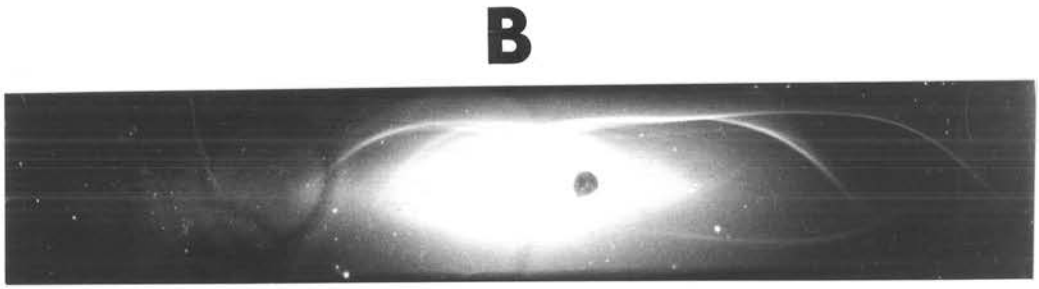
Fig. 4.14. Autoradiograph prepared from fraction 1 (culture supernatant) of a culture of lymphocytes prepared from a patient with Hashimoto's disease

Lymphocytes were prepared from a patient with Hashimoto's disease. The culture contained 10.0 x $10^6$ cells and 5 μc of $^{14}$C-leucine. The cells were cultured in supplemented Eagle's medium prepared without leucine.

A - is an IEP pattern of the patient's plasma developed with human thyroglobulin (upper trough) and whole human antiserum (lower trough).

B - is an autoradiograph of the IEP pattern A. The thyroglobulin antibody arc is labelled. Protein arcs in the α and β globulin region of the IEP pattern developed with whole human antiserum are labelled. The IgG, IgA and IgM arcs are also labelled.


+
A
−


B

## 4.4. Discussion

The immunoglobulins IgG, IgA and IgM were labelled in all the IEP patterns prepared from fractions I of normal lymphocyte cultures. The extent of the labelling of each of the protein arcs varied between individuals. The proteins in the $\alpha$ and $\beta$ globulin region of the IEP pattern were always labelled. These proteins could not be readily identified except by the use of specific antisera. The proteins identified this way were transferrin and haptoglobulin.

A diffuse amount of radioactively labelled material, which did not correspond to any identifiable protein arc, was always present towards the anodal side of the origin in IEP patterns prepared from fraction I of normal lymphocyte cultures. This material was not stained with lipid stain but was lightly stained with amido black.

No qualitative differences between the autoradiographs prepared from fractions I of unstimulated and PHA-stimulated lymphocyte cultures could be detected. PHA appeared to stimulate all the proteins. Whether PHA caused a similar increase in the amount of each individual protein produced by unstimulated cells was not tested.

Only small differences were detected between the

autoradiographic patterns prepared from culture fractions I of lymphocytes, which were cultured in medium containing plasma, medium without plasma or medium containing ovalbumin. The IEP pattern prepared from lymphocytes cultured in plasma medium contained a greater number of labelled arcs in the $\alpha$ and $\beta$ globulin region and contained less of the diffuse labelled material around the origin compared with the other patterns. This material in all the IEP patterns masked the labelled arcs in the $\alpha$ and $\beta$ globulin region. The significance of the labelling of the protein arcs in these regions of the IEP patterns will be discussed in Chapter 5. The lymphocytes appeared to synthesise all the immunoglobulins when cultured in the three types of medium.

These experiments further demonstrated the suitability of a plasma-free medium for culture purposes. The inclusion of ovalbumin in the medium (Van Furth, 1964) did not change the types of proteins made by lymphocytes cultured for 24 hours.

The proteins synthesised by lymphocytes in the presence of actinomycin D were the same as those found in control cultures without drug. All the proteins showed reduced labelling in the autoradiograph patterns compared with

autoradiographs prepared from untreated cultures. It appeared that the actinomycin D-resistant protein synthesis was associated with all the proteins made by lymphocytes.

Fraction I of a lymphocyte culture, which was prepared from a patient with Hashimoto's disease, labelled the thyroglobulin antibody line in an IEP pattern. Fraction I of lymphocyte cultures, which were prepared from normal donors, did not label this arc. These data provided evidence that:

(i) the labelling of this thyroglobulin antibody line represented specific labelling and

(ii) lymphocytes from this patient with Hashimoto's disease synthesised antibody.

The observation that lymphocytes are able to make antibody has been reported by others (Hulliger and Sorkin, 1963; Landy, et al., 1964). Whether thyroglobulin and PHA included in the medium were able to stimulate the production of antibody will be discussed in Chapter 5.

In an unstimulated culture, which was sampled at 24 hours, only labelled immunoglobulins could be detected in fraction Ia. Hirschhorn, et al., (1963) has reported that IgG was the only detectable labelled protein released by lymphocytes cultured in vitro. However, the technique they used, paper electro-

phoresis, was inferior to the techniques used here for the separation and analysis of protein. The reasons for this will be discussed in Chapter 5. The results reported here suggest that unstimulated lymphocytes released only labelled immunoglobulins during culture, while the other newly synthesised protein remained within the cells.

In the PHA-stimulated culture sampled after incubation for 24 hours fraction Ia contained all the labelled proteins found in fraction Ib except transferrin. The release of other labelled proteins besides the immunoglobulins in PHA-stimulated cultures during incubation may represent cell death and lysis rather than cellular release. Stimulated cells did not survive as well in plasma-free medium as unstimulated cells.

A PHA-stimulated culture incubated for 12 hours, contained all the labelled proteins found in stimulated cultures incubated for 24 hours. Fraction Ia of this culture contained all those labelled proteins found in fraction Ib except IgM and transferrin.

No labelled IgM, IgA or transferrin were found in either fraction Ia or Ib of a PHA-stimulated culture incubated with $^{14}$C-leucine during the 12-24 hour period of culture. Labelled $\alpha$ and $\beta$ globulin proteins and labelled IgG were detected in

both of these fractions. The intensity of the labelling of these protein arcs in the autoradiographs was not as great as the intensity of labelling of the proteins made in the first 12 hours by stimulated lymphocytes. These data suggested that most of the synthesis had been completed in the first 12 hours of incubation, a result which was in agreement with the time course studies.

Labelled transferrin was found in fraction Ib of all the cultures examined except that prepared from the PHA-stimulated culture incubated with $^{14}$C-leucine during the 12-24 hour period. This labelled protein was not found in fraction Ia of any of these cultures. This would suggest that newly synthesised transferrin did not appear to be released from the cells during incubation in contrast to the other proteins made by the lymphocytes.

The presence of a large amount of unidentified labelled material in the region of the origin suggested that radio-activity in some arcs may represent non-specific labelling. The absence of this material in fraction Ia compared with that in fraction Ib of an unstimulated lymphocyte culture suggested that this material was not released during the culture period. This material was present in both fractions Ia and Ib of PHA-stimulated cultures, but in much reduced

intensity in fraction Ia. In these cultures the presence of this material in fraction Ia may not be the result of PHA stimulating its release but rather cell degeneration, which was accelerated by this substance in plasma-free medium. The identity of this material remains to be determined; this problem will be discussed in Chapter 5.

Non-specific labelling of the arcs in IEP patterns have been reported by other workers. Phillips and Thorbecke (1965) observed non-specific labelling of $\alpha_2$-macroglobulin, using spleen and liver cultures from rat into mouse chimeras. They reported that $\alpha_2$-macroglobulin is labelled in culture fluids from many tissues and suggested that the protein may be combined with other labelled tissue products such as enzymes. The finding that esterase activity is associated with transferrin (Uriel, 1961) supports this idea. In light of this evidence it is evident that the artifacts of the technique are not fully understood. In Chapter 5 investigations of the specificity of the labelling of the arcs in the IEP patterns have been reported.

Because of the possibility that several arcs in the IEP patterns may be labelled non-specifically, a discussion of the types of proteins made by lymphocytes will be left to Chapter 5. The evidence suggests that lymphocytes are able

to make all the immunoglobulins and a wide range of $\alpha$ and $\beta$ globulin proteins.

4.5. Summary

(1) Fluids from radioactive plasma-free lymphocyte cultures labelled the immunoglobulin (IgA, IgM and IgG) arcs and a range of protein arcs including transferrin and haptoglobin located in the $\alpha$ and $\beta$ globulin region after immunoelectrophoresis with plasma.

(2) There were no qualitative differences between the autoradiographs of IEP patterns prepared from PHA-stimulated and unstimulated lymphocyte culture fluids.

(3) There were no qualitative differences in the autoradiographs of IEP patterns prepared from fluids of lymphocytes cultured in plasma medium, plasma-free medium and medium containing ovalbumin.

(4) Actinomycin D in the culture medium reduced the labelling of all the proteins found in the culture fluids of control cultures.

(5) The only labelled components released by unstimulated cells incubated for 24 hours were IgA, IgM and IgG.

(6) PHA-stimulated cells released all of those labelled proteins found in the cells, except transferrin, during incubation. IgA, IgM and transferrin labelled during the first 12 hours of culture, were not labelled by the cells in the latter half of the incubation period.

(7) A large amount of unidentified radioactive material was detected around the origin and towards the anodal end of most IEP patterns prepared from lymphocyte culture fluids.

(8) A thyroglobulin-anti-thyroglobulin precipitin arc was labelled after immunoelectrophoresis only by radioactive protein from lymphocytes of a patient, who produced antibodies to thyroglobulin. These data provide evidence for the synthesis of antibody by lymphocytes.

# CHAPTER 5

# THE SPECIFICITY OF IMMUNOGLOBULIN RADIOACTIVE LABELLING OF (IEP) PATTERNS.

## 5.1. Introduction

Antibody antigen complexes are formed by molecules which combine specifically with each other. When antigen-antibody complexes involving constituents of the medium are formed in lymphocyte culture fluids, radioactive material coprecipitates non-specifically. A high proportion of this material can be removed by pre-treatment with an unrelated antibody-antigen precipitate (Thorbecke, 1950; Parenti, et al., 1966). For example, if lymphoid cells synthesize radioactive antibody to A, other radioactive products coprecipitate with an A-anti A complex. These other radioactive products also precipitate with a B-anti B complex, which is not made by the lymphoid cells.

Until the extent of the co-precipitation of these substances with antibody-antigen complexes is determined, the findings in Chapter 4 cannot be interpreted with full confidence. The presence of radioactive material which appears as a smudge in autoradiographs of immuno-electrophoretic patterns prepared from labelled lymphocyte culture fluids, suggests that the labelling of some immuno-arcs in these patterns may not represent synthesis.

Experiments in this chapter were designed to demonstrate

(1) That antibody-antigen precipitates, which are not related to human proteins contain radioactivity when formed in radioactive human lymphocyte culture fluid.

(2) That radioactive labelling of the immunoglobulins in IEP patterns represents synthesis. This was investigated by,

   (a) forming antibody-antigen precipitates not related to human proteins, in agar after electrophoresis with radioactive lymphocyte culture supernatants,

   (b) elution of IgG globulin from the agar after electrophoresis and precipitation of this protein in the eluate with antibody to IgG.

## 5.2. Materials and Methods

### 5.2.1. Antibody and antigens

Formation of antibodies to egg albumin and human IgG globulin was induced in rabbits by injection of 5 mg of purified protein in 0.5 ml, mixed with an equal volume of Freund's complete adjuvant (Difco, Michigan, U.S.A.) into both fore and hind limbs. This was repeated at one week's interval. At further one week intervals, 10 mg of protein in 1 ml volumes were given intravenously, via the lateral ear vein.

After three weeks of intravenous antigen administration, the rabbits were bled from the lateral ear vein and the serum was tested for antibody. The course of antigen administration was continued with antibody producing rabbits. These rabbits were also bled at weekly intervals (20 - 30 ml of blood).

Egg albumin (3x crystalline, British Drug Houses (Australia), N.S.W., Australia) was made up to a stock solution at 10 mg/ml and portions were stored at -15°C before use. Pure IgG globulin was prepared from normal pooled sera by precipitation with sodium sulphate to a final concentration of 14%. [The preparation method has been described in full detail by Strauss, et al., (1964).] The precipitate was taken up in 0.9% saline (buffered at pH 7.5 with 0.01M phosphate) and twice precipitated with sodium sulphate at 14%. The final precipitate was redissolved in buffered saline and dialysed for six days at 4°C against repeated changes of buffer at pH 8.6 (0.04M Tris [Tris (hydroxylmethyl) aminomethnae, Sigma Chemical Co., St. Louis, Mo., U.S.A.]; 0.005M phosphate). The dialysed material was loaded onto a column (6 x 60 cm), packed with washed DEAE-cellulose (Whatman, Balston Ltd.,

England), which had previously been equilibrated with buffer (0.04M Tris, 0.005M phosphate, pH 8.6). The column was eluted with this buffer at a flow rate which was not in excess of 3 ml/min. The eluate fractions were collected at 10°C.

Optical density (OD) of individual tubes was measured at 280 mμ. Protein in tubes showing a progressive increase in OD up to 2.0 units and all those readings > 2.0 units were pooled and concentrated in dialysis tubing against Carbowax 20M (G.T. Gurr Ltd., London). The eluate was concentrated to 10 mg of protein/ml and equilibrated by dialysis for 24 hours at 4°C with buffered saline. Portions were stored at -15°C between use.

Human thyroglobulin was prepared by the method of Derrien, et al. (1948) and supplied by Miss Aileen Thompson, Institute of Medical and Veterinary Science, Adelaide. This was used as antigen in studies with thyroglobulin antibody. Mouse serum and sheep anti-mouse serum antibody were gifts from Dr. Keven Turner, Department of Microbiology, University of Adelaide.

Immune precipitates were formed at equivalence point

in the culture supernatant to be studied. Precipitation techniques were performed aseptically by filtering, at 4°C, the culture supernatants, antiserum and antigen through Hemming's filters fitted with bacterial filter papers (Standard grade, Oxoid, Courtaulds, Ltd., England). After addition of antibody and antigen, the solutions were left to stand at 37°C for 30 minutes and were kept at 4°C for two to three days. The precipitates were washed carefully twice with buffered saline at 4°C, taken up in dilute ammonium hydroxide and plated out on planchets to be counted.

5.2.2. IEP and electrophoresis in Agar

Immunoelectrophoresis of the culture supernatants in agar and autoradiography were performed as described previously (Chapter 4, Section 4.2.1).

In several experiments the protein arcs were not developed with antisera after the culture supernatants had been electrophoresed. In these experiments samples were loaded into slots (1 x 30 mm) cut across the centre of large plates (10 x 7.5 cm) coated with agar to a depth of 2-3 mm. The sample was electrophoresed in the manner described previously except that electrophoresis was extended from

1½ to 2 hours. After electrophoresis a transverse cut, 1 mm behind the slots towards the cathodal end was made in the agar. Agar from the cathodal end was scraped and washed with buffer into a small beaker. The agar was frozen at -15°C and thawed on glass sinters fitted into Buchner flasks. The agar was washed with buffered saline and the eluate was collected into the flasks under vacuum.

Immune precipitates with antibodies to egg elbumin and human IgG were formed in aliquots of the eluate. The precipitations were not formed aseptically but sodium azide (1 part in 1000) and merthiolate (1 part in 1000) were included in the eluates to inhibit microbial growth. The immune precipitates were washed carefully twice with 20 ml lots of buffered saline at 4°C before being taken up in dilute $NH_4OH$ to be plated out on planchets and counted .

5.2.3. Paper electrophoresis

Portions (0.01 to 0.02 ml) of concentrated dialysed culture supernatants were electrophoresed on paper strips (13 cm x 2 cm) with barbitone buffer. Electrophoresis was continued for 16½ hours at a constant amperage of 2 amps for each strip. At the completion of electrophoresis, the strips were dried in an oven at 100°C for 20 min, stained with

Bromophenol blue, washed twice with 5% acetic acid and once with 5% acetic acid and sodium acetate. The strips were dried again at 100°C and passed over concentrated ammonia fumes.

Buffer: 30.9 gm sodium barbitone (LR grade, British Drug Houses) and 5.52 gm barbituric acid in 2 litres of distilled water ($\mu$ = 0.075, pH 8.6).

Stain: 1 gm of bromophenol blue (British Drug Houses) in 1 litre of ethyl alcohol saturated with 300 gm of mercuric chloride (LR grade, May and Baker Ltd., England).

Washing Solution (i) 5% acetic acid (commercial)

(ii) 5% acetic acid with 3 gm of sodium acetate (AR grade, British Drug Houses) per litre added.

After the strips had been stained and washed they were cut into 5 mm sections, taped on planchets and counted under the end window of a Geiger Mueller counter (Phillips Model PW 4032).

## 5.3. Results

### 5.3.1. Quantitation of antibody and formation of immune precipitates

The equivalence point of the rabbit antiserum to human IgG globulin was determined with pure IgG globulin and whole human serum. The antiserum gave only one line in the IEP pattern when developed against whole human serum (Appendix VIII). The equivalence points of the anti-human thyroglobulin and anti-egg albumin antibodies against antigen were also determined. These data with the corresponding IEP patterns, appear in the Appendices IX and X.

When antibody-antigen precipitates were formed in culture fluids of lymphocytes, they contained radioactivity irrespective of their specificity (Table 5.1). The radioactivity in the thyroglobulin antibody precipitates was of the same order when they were formed in the supernatants from cultures of lymphocytes from patients with Hashimoto's disease as when they were formed in the supernatants from cultures of normal lymphocytes. The radioactivity of egg albumin-antibody precipitates was of the same order as that associated with human IgG globulin-antibody precipitates.

Table 5.1. Radioactivity in immune precipitates formed in culture fluids from lymphocytes

Antigen-antibody precipitates were formed in fluids of lymphocytes cultured for 24 hours in plasma-free, supplemented Eagle's medium prepared without leucine. The cultures were terminated by freezing and thawing. Immune precipitates were formed in aliquots of the culture fluid. Proteins in another aliquot were precipitated with TCA.

The radioactivity in the precipitates has been expressed as counts per $10^6$ cells per µc of $^{14}$C-leucine in the medium. The radioactivity has not been corrected for the amount of protein in the precipitates, but the ratio of the counts in the immune precipitate to the total counts in the TCA precipitate has been expressed as a percentage.

The cultures were stimulated with PHA or unstimulated as indicated.

I - Thyroglobulin-antibody (TgAb) precipitates wereformed in culture fluids of lymphocytes from patients with Hashimoto's disease and from normal donors. The protein in these precipitates was 16.5 mgm (determined by O.D. measurement).

II - Egg albumin-antibody (EaAb) precipitates were formed in culture fluids of normal lymphocytes. The protein in these precipitates was 5.5 mgm.

III - Human IgG - antibody (IgAb) precipiates were formed in culture fluids of normal lymphocytes. The protein in these precipitates was 7.32 mgm.

TABLE 5.1

| | Lymphocyte Donor | PHA stimulated | Counts/$10^6$ cells/ µc | | Percent (Immune ppt / TCA) |
|---|---|---|---|---|---|
| | | | Immune pp't TgAb | TCA pp't | |
| | Hashimoto A | - | 50 | 656 | 7.4 |
| | | + | 218 | 2082 | 10.4 |
| | | - | 23 | 324 | 6.9 |
| | | + | 243 | 1603 | 15.2 |
| I | Hashimoto B | - | 66 | 384 | 17.1 |
| | | + | 40 | 1178 | 3.4 |
| | Normal A | - | 48 | 640 | 7.5 |
| | | + | 78 | 1660 | 4.7 |
| | B | - | 28 | 248 | 11.0 |
| | | + | 30 | 992 | 3.1 |
| | C | - | 80 | 406 | 19.7 |
| | | + | 54 | 838 | 6.5 |
| | D | - | 87 | 849 | 10.2 |
| | | + | 54 | 855 | 6.3 |
| | E | - | 96 | 508 | 18.8 |
| | | + | 279 | 1561 | 17.8 |
| | F | - | 29 | 642 | 4.6 |
| | | + | 47 | 863 | 5.5 |
| | | | EaAb | | |
| II | Normal G | - | 50 | 217 | 23.0 |
| | | + | 210 | 1127 | 18.6 |
| | | | IgAb | | |
| III | Normal H | - | 43 | 508 | 8.5 |
| | | + | 279 | 1561 | 17.8 |

Immune precipitates formed in the culture fluids of lymphocytes, which had been incubated with puromycin or had been killed by freezing at -15°C before culture, contained no radioactivity.

Immune precipitates not related to human proteins contained radioactivity when they were formed in culture fluids which had been dialysed and heated at 57°C for 30 minutes (Table 5.2a). These precipitates when formed in dialysed concentrated lymphocyte culture fluids prepared for IEP analysis, contained radioactivity (Table 5.2b).

5.3.2. Specificity of labelling of IEP patterns

The protein arcs labelled in normal IEP patterns have been discussed in Chapter 4. In the studies reported in this chapter culture fluids were electrophoresed with mouse serum as carrier protein and the IEP pattern was developed with rabbit anti-mouse serum. Several arcs in the region of the origin and towards the anodal end of the slide were labelled in the accompanying autoradiograph (Fig. 5.1). No arcs were labelled towards the cathodal end of the IEP pattern.

When rabbit serum containing antibodies to egg albumin was used as carrier during electrophoresis of the culture supernatant, the precipitation arc developed with egg albumin

Table 5.2. (a) Egg albumin antigen-antibody (EaAb) precipitates formed in heat treated and dialysed culture fluids of normal lymphocytes

Lymphocytes were cultured in supplemented Eagle's medium prepared without leucine and with $^{14}$C-leucine. The cultures were unstimulated or stimulated with PHA as indicated. The culture fluids were dialysed against 3 x 500 ml of phosphate buffered saline for 2 days. Aliquots were kept as controls while the others were heated for 30' at 56°C.

(b) Egg albumin antigen-antibody precipitates (EaAb) formed in a normal lymphocyte culture fluid prepared for immunoelectrophoresis

Normal lymphocytes (7.3 x $10^6$ cells) cultured in supplemented Eagle's medium prepared without leucine and containing 2.5 µc of $^{14}$C-leucine. The culture fluids were dialysed and concentrated as described in the methods for immunoelectrophoresis (section 5.2.1).

After dialysis and concentration, the fluids contained radioactive material which coprecipitated with egg albumin-antibody precipitates.

In both table 5(a) and (b) the incorporation of radioactivity into the immune precipitates has been corrected and expressed as counts/$10^6$ cells cultured/µc of $^{14}$C-leucine in the medium.

TABLE 5.2

| | Lymphocyte donor | PHA | Treatment | Counts/$10^6$ cells/μc in immune pp't (EaAb) |
|---|---|---|---|---|
| (a) | Normal | - | Dialysed | 86 |
| | | - | Dialysed and heat | 53 |
| | | + | Dialysed | 214 |
| | | + | Dialysed and heat | 365 |
| (b) | Normal | - | Dialysed and | 22 |
| | | + | Concentrated | 108 |

Fig. 5.1. Autoradiographs of IEP patterns prepared with mouse serum and human plasma as carrier proteins

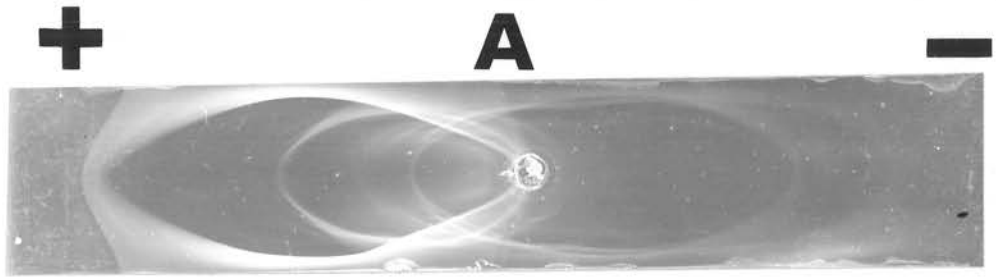
Culture fluid was prepared from a culture containing $7.9 \times 10^6$ cells and 2.5 µc $^{14}$C-leucine.

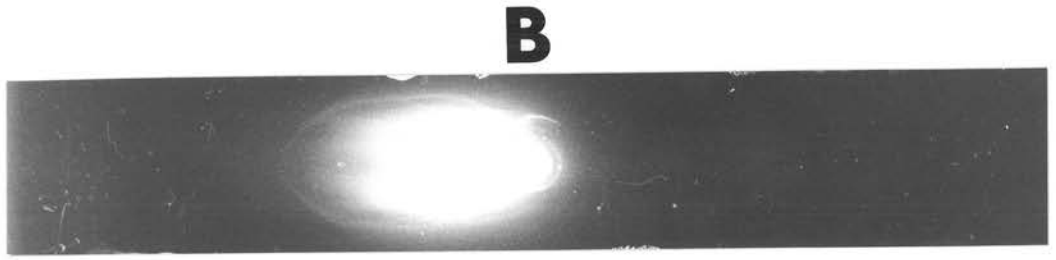
Pattern A is an IEP prepared with mouse serum and rabbit anti-mouse serum. Mouse serum was used as carrier protein during electrophoresis of culture fluid.

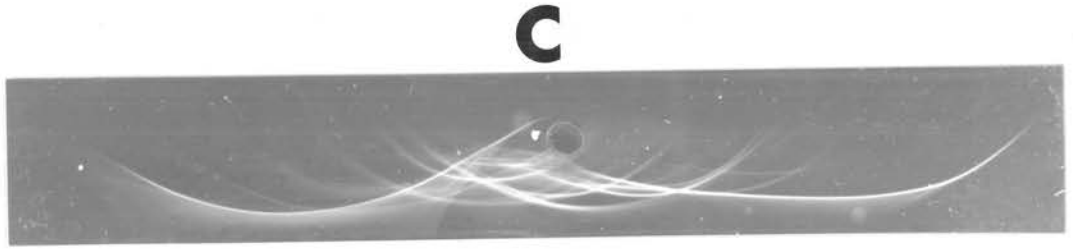
Pattern B is an autoradiograph of the IEP shown in A. No immunoglobulin arcs are labelled in the cathodal area of the IEP patterns. Arcs around the origin and towards the anodal end of the pattern are heavily labelled.

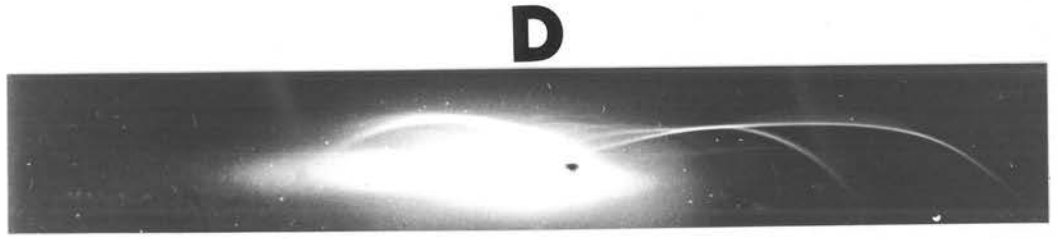
Pattern C is an IEP of human plasma developed with whole human antiserum.

Pattern D is the corresponding autoradiograph prepared from the IEP shown in C.


+
A
−


B


C


D

was not labelled (Fig. 5.2). Similarly, the thyroglobulin antibody precipitin arc, discussed in Chapter 4, was not labelled by culture fluids (Fig. 5.2).

### 5.3.3. Elution of immune globulins from agar after electrophoresis

The cathodal portion of the agar was eluted after electrophoresis of radioactive culture fluids from normal lymphocytes. Egg albumin-anti-egg albumin immune precipitates formed in the eluates always contained some radioactivity. When immune precipitates to IgG and egg albumin were formed in aliquots of the eluate, the radioactivity associated with the IgG precipitates was always significantly greater than that associated with egg albumin precipitates, (Table 5.3).

When PHA was included in plasma cultures it stimulated the production of IgG. The data indicate that this substance did not always stimulate IgG synthesis by cells cultured in plasma-free medium, although in each case it stimulated the total synthesis of protein, (Table 5.3). PHA stimulated the total production of protein by lymphocytes more than it stimulated the production of IgG globulin, as far as could be determined by measuring incorporation of $^{14}$C-leucine into immune precipitates. The incorporation of $^{14}$C-leucine into IgG globulin represented 1-2% of the total incorporation by

Fig. 5.2. IEP patterns prepared with thyroglobulin antibody and rabbit antibody to egg albumin

Pattern A is an IEP pattern prepared with plasma from a patient with Hashimoto's disease. One side of the pattern has been developed with whole-human-antiserum while the other has been developed with human thyroglobulin to precipitate antibody in the plasma.

Pattern B is an IEP pattern prepared with rabbit serum containing antibodies to egg albumin. Egg albumin was included in both side troughs. The egg albumin-antibody arcs can be identified in the cathodal region of the IEP pattern.

Pattern C is an autoradiograph prepared from normal lymphocyte culture fluid.

The thyroglobulin-antibody arc and the egg albumin-antibody arc were not labelled by culture fluids of normal lymphocytes. These arcs occur in the same region of IEP patterns as the immunoglobulins, which were labelled by normal lymphocyte culture fluids.

+ A −

B

C

Table 5.3. The radioactivity associated with IgG and egg albumin immune precipitates formed in eluates from agar after electrophoresis of culture fluids

Fluids from normal lymphocyte cultures were concentrated and dialysed. Samples of the culture fluid were electrophoresed with carrier plasma in agar. After electrophoresis the proteins were eluted from the agar as described in the methods. Immune precipitates to human γ-globulin (IgG) and egg albumin (Ea) were formed in aliquots of the eluate. Both precipitates contained 7.3 mg of protein (determined by OD).

Fluids from the following normal lymphocyte cultures were analysed in this study. All the cells were cultured in supplemented Eagle's medium prepared without leucine and plasma unless otherwise stated. The sample volume electrophoresed and the total culture fluid volume are given in parenthesis.

The cultures contained:

I - $7.2 \times 10^6$ cells and 5 μc of $^{14}$C-leucine (sample vol. 0.15 ml; total volume - );
II- $8 \times 10^6$ cells and 5 μc of $^{14}$C-leucine (sample vol. 0.15 ml; total volume - );
III-$14.5 \times 10^6$ cells and 10 μc of $^{14}$C-leucine (sample vol. 0.15 ml; total volume 0.3 ml);
IV- $10.0 \times 10^6$ cells and 10 μc of $^{14}$C-leucine (sample vol. 0.14 ml; total volume 0.3 ml).
(a) plasma free medium,
(b) 25% autologous plasma medium;
V - $8.0 \times 10^6$ cells and 7.5 μc of $^{14}$C-leucine both (i) and (ii) are duplicates (sample volume 0.14 ml; total volume 0.3 ml)
VI- $7.0 \times 10^6$ cells and 7.5 μc of $^{14}$C-leucine, both (i) and (ii) are duplicates (in this experiment the medium was supplemented with 25% autologous plasma; sample volume 0.15 ml; total volume 0.32 ml).

In experiment VII lymphocytes from a Hashimoto patient were cultured in (a) plasma-free medium and (b) 25% autologous plasma medium. The cultures contained $7.2 \times 10^6$ cells and 10 μc of $^{14}$C-leucine (sample volume 0.15 ml; total volume 0.32 ml).

In every study the counts per minute in the IgG immune precipitate were greater than the counts per minute in the egg albumin precipitate. These data provide evidence that lymphocytes synthesise IgG immunoglobulin. PHA stimulated IgG synthesis once out of three times when it was tested in plasma-free medium, although at every occasion it stimulated total protein synthesis. PHA in plasma-medium always stimulated the synthesis of IgG immunoglobulin and total proteins by lymphocytes.

The incorporation of $^{14}C$-leucine into IgG immunoglobulin by unstimulated lymphocytes represents 0.6 - 2.1% of the total incorporation. This would not represent the percentage IgG immunoglobulin synthesised with respect to the synthesis of total proteins, for this would depend on the proportion of leucine in the proteins synthesised.

Table Explanation: The columns from the left represent:
Expt - Experiment number (the experimental detail is given);
Tg - human thyroglobulin was included in some cultures at a concentration of 0.5 mgm/ml;
PHA - several of the cultures were stimulated with phytohaemagglutinin;
CPM - represents the incorporation of $^{14}C$-leucine into the immune precipitates IgG and Ea (egg albumin), expressed as counts per minute;
Diff - difference in CPM of IgG precipitate and CPM of EA precipitate;
$\%\frac{PHA}{Cont}$ - the difference in CPM in the immune precipitates of PHA-stimulated cultures expressed as a percentage of the control value;
CPM total - CPM of total proteins in sample volume analysed, corrected to 7.3 mgm of protein carrier (the incorporation of $^{14}C$-leucine into the total proteins was found by plating out small amounts of dialysed, concentrated culture fluid on planchetts for counting);

TABLE 5.3

| Expt. No. | Tg | PHA | CPM IgG | CPM Ea | Diff. | % PHA Cont | CPM Total | % PHA Cont | %IgG-Ea Total |
|---|---|---|---|---|---|---|---|---|---|
| I | - | - | 16 | 3 | 13 | - | - | - | - |
| II | - | - | 82 | 13 | 69 | - | - | - | - |
| III | - | - | 267 | 42 | 225 | - | - | - | - |
| IV(a) | - | - | 139 | 57 | 82 | | 3960 | | 2.1 |
| | - | + | 131 | 89 | 42 | 50 | 11434 | 292 | 0.37 |
| (b) | - | - | 472 | 348 | 124 | | 7128 | | 1.7 |
| | - | + | 421 | 199 | 222 | 179 | 28710 | 402 | 0.78 |
| V (i) | - | - | 173 | 72 | 100 | | 5445 | | 1.8 |
| | - | + | 277 | 123 | 154 | 154 | 22374 | 411 | 0.68 |
| (ii) | - | - | 146 | 57 | 89 | | | | 1.6 |
| | - | + | 248 | 84 | 164 | 184 | | | 0.73 |
| VI(i) | - | - | 156 | 68 | 88 | | 4257 | | 2.1 |
| | - | + | 294 | 180 | 114 | 130 | 13810 | 324 | 0.82 |
| (ii) | - | - | 120 | 52 | 68 | | | | 1.6 |
| | - | + | 153 | 48 | 105 | 154 | | | 0.76 |
| VII(a) | - | - | 678 | 256 | 422 | | 21334 | | 1.9 |
| | + | - | 480 | 207 | 283 | 67 | 21334 | 100 | 1.3 |
| | - | + | 662 | 511 | 151 | 35 | 68508 | 321 | 0.22 |
| | + | + | 618 | 421 | 197 | 47 | 60390 | 283 | 0.33 |
| (b) | - | - | 236 | 139 | 97 | | 16087 | | 0.60 |
| | + | - | 276 | 166 | 110 | 113 | 13563 | 84 | 0.81 |
| | - | + | 813 | 580 | 233 | 240 | 68062 | 423 | 0.34 |
| | + | + | 497 | 321 | 176 | 181 | 48510 | 301. | 0.36 |

% PHA Cont - CPM of PHA-stimulated culture expressed as a percentage of the control value (total protein);

%IgG-Ea total - The CPM of the difference column expressed as a percentage of the CPM of the total proteins, this is a measurement of the incorporation of $^{14}C$-leucine into IgG globulin compared to the total incorporation.

unstimulated cells.

Lymphocytes from a Hashimoto patient (studies with this patient are reported in Chapter 4) were cultured in plasma-free and plasma medium for 24 hours. The lymphocytes were cultured with and without PHA and human thyroglobulin. Fluids from these cultures were electrophoresed in agar and the cathodal portions were eluted after electrophoresis. The radioactivity associated with egg albumin immune precipitates was of the same order as that associated with thyroglobulin anti-thyroglobulin precipitates formed in aliquots of the eluate. The radioactivity associated with immune precipitates to IgG globulin was greater than that associated with egg albumin precipitates (Table 5.3, Expt. VII).

PHA stimulated the production of IgG in plasma cultures but did not in plasma-free medium. Thyroglobulin included in unstimulated and PHA-stimulated plasma-free cultures and in unstimulated plasma cultures did not stimulate the production of IgG globulin. Thyroglobulin included in PHA-stimulated plasma cultures, appeared to increase the stimulation of IgG globulin production above that expected by PHA alone (Table 5.3, Expt. VII).

Labelled antibody could not be detected by the techniques of immunoelectrophoresis and autoradiography in the culture fluids from Hashimoto lymphocytes, except in that from a PHA-stimulated plasma culture. The fluid from this culture weakly labelled part of the thyroglobulin arc in the IEP pattern.

Lymphocytes from the Hashimoto patient were also cultured for 72 hours in Eagle's medium supplemented with 20% calf serum. The lymphocytes were cultured with and without PHA. Thyroglobulin was included in the medium of one unstimulated culture. After electrophoresis and elution from agar of the culture fluids, no labelled antibody could be detected in the eluates.

5.3.4. Paper electrophoretic studies

Samples, which had been prepared for IEP analysis, were electrophoresed on paper with carrier protein. The electrophoretic patterns obtained with the supernatants from cultures of unstimulated and PHA-stimulated lymphocytes are summarized in Figure 5.3. Radioactivity was spread throughout the electrophoretic patterns except the albumin region. Much of the activity resided at the origin.

Fig. 5.3. Paper electrophoresis of lymphocyte culture fluids

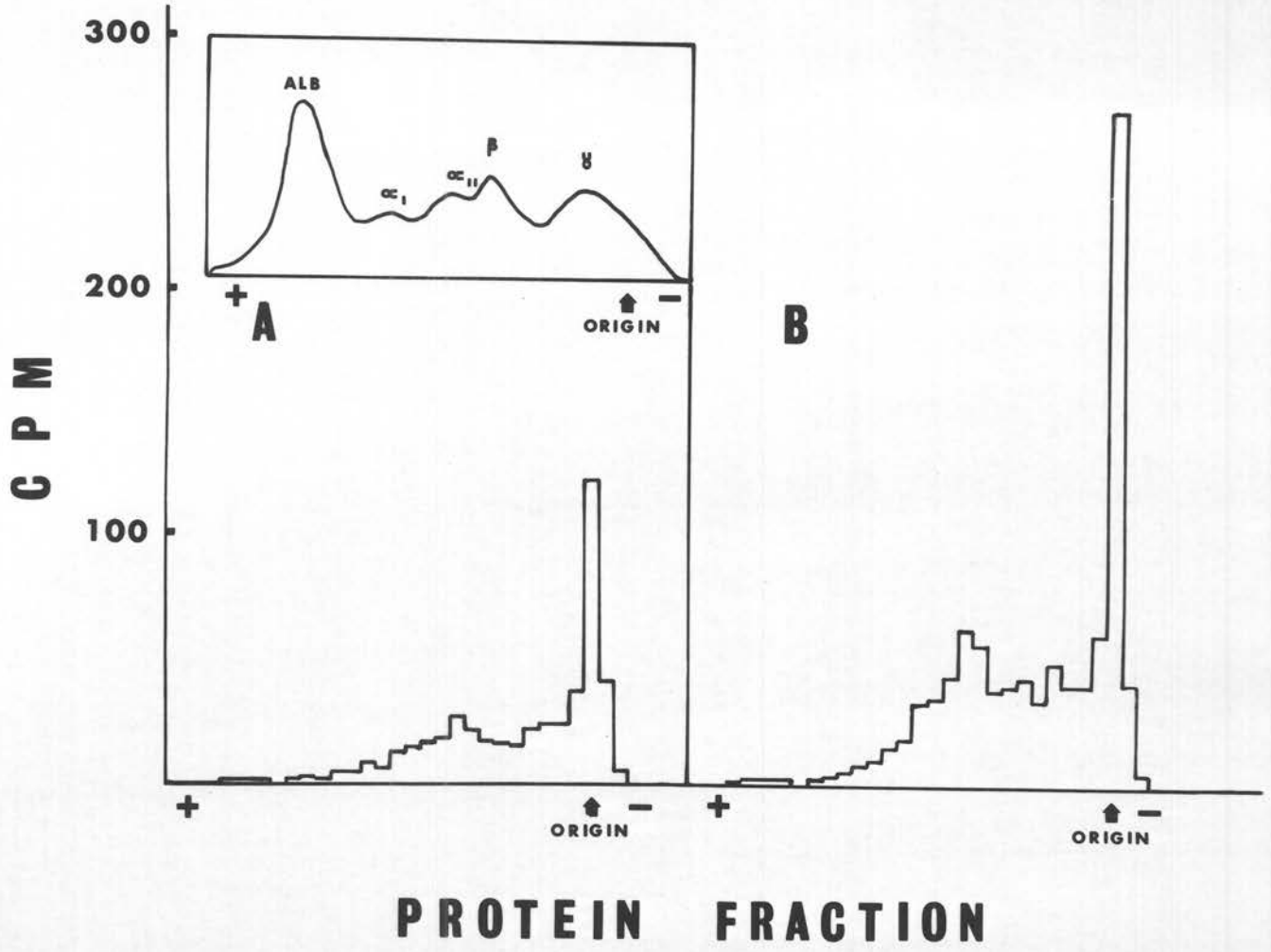
Normal lymphocytes were cultured in commercial Eagle's medium with 2.5 μc of $^{14}$C-leucine per ml. The cultures contained 7.6 x $10^6$ cells. Fluids from these cultures were concentrated and dialysed; 0.1 ml samples (total volume 0.3 ml) were electrophoresed on paper strips with 0.1 ml of carrier protein (human serum) as described in the text.

A - shows the radioactivity associated with a pattern prepared from unstimulated lymphocyte culture fluid.

B - shows the radioactivity associated with a pattern prepared from a PHA-stimulated culture fluid.

The radioactivity associated with the electrophoretic patterns has been expressed as counts per minute (CPM) measured in 5 mm strips (see methods).

A scan showing the positions of the proteins after electrophoresis is shown in the inset. PHA stimulated the incorporation of $^{14}$C-leucine into all the proteins labelled in control patterns. Very little activity is associated with the albumin peak. Most of the activity resided at the origin.


300
200
100
CPM
ALB
α I
α II
β
γ
+
A
ORIGIN
−
B
+
ORIGIN
−
+
ORIGIN
−
PROTEIN FRACTION

PHA stimulated the incorporation of radioactivity into all categories of protein.

5.4. Discussion

Radioactive proteins would be expected to have similar chemical and physical properties to non-radioactive proteins. $^{14}C$-labelled antibodies and antigens would therefore be expected to combine with antigens and antibodies to form immune complexes in the same manner as their unlabelled naturally occurring counterparts. The incorporation of radioactivity into protein and its subsequent identification by immune precipitation should prove to be a valuable technique in qualitative investigations.

Nevertheless, the technique is not without its difficulties, as other substances are known to combine with antibody antigen immune precipitates, for example complement and immunoconglutinin. Before it can be demonstrated that the $^{14}C$ labelled proteins identified by immuno-precipitation techniques are synthesized by lymphocytes, one has to exclude the possibility of other labelled substances co-precipitating with the complexes. In the studies reported in this chpter several techniques have been employed to demonstrate that the radioactivity associated with the immunoglobulin arcs

in IEP patterns which have been prepared from culture supernatants, represents synthesis of these proteins by lymphcytes.

Antigen-antibody precipites, irrespective of their relation to human proteins, contained radioactivity when they were formed in lymphocyte culture supernatants. The radioactivity associated with thyroglobulin anti-thyroglobulin immune precipitates was of the same order whether they were formed in culture fluids of lymphocytes prepared from normal donors or from people suffering from Hashimoto's disease. Antibody to thyroglobulin had been demonstrated in the culture fluids of lymphocytes prepared from Hashimoto A, by the technique of immunoelectrophoresis (Chapter 4). Lymphocytes from Hashimoto B may also be expected to be able to synthesize antibody although this could not be demonstrated by immunoelectrophoresis and autoradiography. However, Hashimoto A plasma was used as carrier protein in these studies; it is possible that the antibodies produced by Hashimoto B had different characteristics. Normal lymphocytes would not be expected to produce anti-thyroglobulin antibodies.

PHA did not always stimulate the amount of radioactivity associated with these immune precipitates. It did not stimulate the radioactivity in the precipitate more than that in the

total proteins except when it was tested with lymphocytes from Hashimoto A. PHA may have stimulated the production of antibody by these lymphocytes, so that an increase in activity in the immune precipitates was associated not only with an increase in radioactive material co-precipitating with the immune precipitate but with an increase in antibody production.

The activity associated with the egg albumin-antibody precipitates was similar to that associated with the IgG-antibody precipitates. PHA stimulated an increase in the radioactivity associated with both precipitates. However, the increase associated with the IgG antibody precipitates was greater than that associated with the total proteins. This may represent not only an increase in the radioactive substance co-precipitating with immune precipitates but an increase in the production of IgG in the presence of PHA.

Parenti, et al. (1966) demonstrated by immune precipitation the synthesis of IgG globulin by lymphocytes. They absorbed out radioactive culture supernatants with immune precipitates not specific for human proteins in order toremove co-precipitating radioactive material. The incorporation of precursor into IgG globulin was based on the difference in

radioactivity associated with the immune precipitate to IgG globulin and that associated with an immune precipitate not related to human proteins. These precipitates were performed in aliquots of the absorbed culture fluids.

Techniques similar to those used by Parenti were employed in the investigations reported in this chapter. However, the differences between the immune precipitates were not significantly greater than the variation in the background counts of the counter. These difficulties would be resolved to some extent by providing adequate $^{12}$C-leucine in the medium for lymphocyte growth and by increasing the amount of $^{14}$C-leucine to ensure significant incorporation of precursor.

The techniques employed by Parenti had been used previously by Thorbecke (1960) and Helmreich, et al., (1961) to demonstrate the synthesis of IgG globulin by rabbit lymph node cells. Hulliger and Sorkin (1963) demonstrated the synthesis of antibody by arterial blood leukocytes prepared from hyper-immune rabbits. They employed radioactive precursors, but found that the radioactivity associated with non-related antibody-antigen complexes was relatively small.

The radioactivity associated with immune precipitates not related to human proteins, was not removed by heating the lymphocyte culture fluids at 56' for 30 min, nor by dialysis. Some components of complement would be expected to be destroyed by these procedures (Kabat and Mayer, 1961).

The problem was to investigate which arcs in IEP patterns were labelled by this material and in particular if the labelling of the immunoglobulin arcs represented synthesis of these proteins. This problem was approached in a number of ways.

Egg albumin-antibody precipitin arcs formed in agar after electrophoresis of culture fluids were not labelled. Similarly, thyroglobulin-antibody precipitin arcs were not labelled after electrophoresis of normal lymphocyte culture fluids. Both of these precipitin arcs occur on the cathodal side of the origin in IEP patterns.

When mouse serum was used as carrier protein with lymphocyte culture fluids during immunoelectrophoresis the arcs in the region of the origin and towards the anodal side of the pattern were labelled. No arcs towards the cathodal end of the pattern contained activity. The labelling of the arcs corresponded to the area of the smudge of radioactivity on the anodal side of the origin in most autoradiographs

(Chapter 4).

The observations suggest that radioactive material co-precipitating with immune precipitates in the liquid phase did not label arcs in the cathodal region of IEP patterns prepared from labelled lymphocyte culture fluids. Substantial portions of the immunoglobulin arcs are found in this region of IEP patterns. Since the immunoglobulins migrate towards the cathode during electrophoresis it should be possible to elute these from the agar and recover them from the eluate.

The radioactivity in the IgG immune precipitates formed in the eluate was always significantly greater than that found in the egg-albumin precipitate formed in an aliquot of the eluate. However, the radioactivity associated with the egg-albumin immune precipitate was not reduced to zero. This suggested that a small amount of radioactive substance had migrated towards the cathode. A proportion of this material was probably sampled when the proteins were eluted from the agar on the cathodal side of the origin. The radioactive smudge evident in autoradiographs did extend back a small distance towards the cathode from the origin (Chapter 4). Nevertheless, the possibility cannot be excluded that non-specific attachment of labelled immuno-

globulin to the egg albumin immune precipitate occurred.

PHA stimulated the production of IgG by lymphocytes, each time it was included in plasma medium. This substance stimulated the increased production of IgG only once out of three times when it was included in plasma-free medium, although it stimulated the production of the total proteins each time. The degree of IgG globulin stimulation was much less than the stimulation of the total proteins in every study.

These observations suggest that the cultured cells were heterogeneous with respect to their metabolism. The reduced stimulation of IgG production compared with the stimulation of the total proteins by PHA may be explained by recalling an observation made in Chapter 3, that PHA stimulated the production of protein by cells which were not making protein in unstimulated cultures.

If inactive cells in unstimulated cultures were not stimulated by PHA to produce IgG, the increase in the total proteins would exceed that of IgG production provided that the stimulation of all the cells was proportional. Alternatively, if PHA did not stimulate all the cells equally, then it is possible that there was a greater increase in protein production by those cells not making IgG globulin. Both of

these effects may have been at work in the system.

PHA did not always stimulate IgG synthesis in plasma-free medium. This observation suggests that plasma may have been necessary for the stimulatory effect of PHA on IgG producing cells, isolated from several normal donors. Similarly, PHA did not always stimulate an increase in the total protein production by lymphocytes cultured in plasma-free medium. The stimulatory effects of one batch of PHA has been shown to be variable between normal lymphocyte donors.

Parenti, et al., (1966) reported the stimulation by PHA of IgG globulin produced by lymphocytes. The stimulation of IgG was less than the stimulation of total proteins. In their experiments autologous plasma was used to supplement the medium.

Ripps and Hirschhorn (1967) have recently reported the synthesis of IgG globulin by human peripheral lymphocytes cultured for seven days in a calf serum supplemented Eagle's medium. They found that the synthesis of total protein and IgG was a maximum on the third or fourth day of culture. PHA stimulated both the total proteins and IgG production so that the relative amounts of each were not markedly affected by PHA. The stimulation of IgG globulin was usually

5-7 times the control values, while that of total protein was more than five.

PHA in autologous plasma medium stimulated lymphocytes from a Hashimoto patient to make antibody, detectable by the techniques of immunoelectrophoresis and autoradiography. No labelled antibody could be detected in unstimulated plasma cultures or in plasma-free cultures by these techniques.

No labelled antibody could be detected in the eluates after electrophoresis and elution from agar of the culture fluids. No labelled antibody could be detected by this technique in fluids from lymphocytes cultured for 72 hours in 20% calf serum supplemented Eagle's medium.

These data suggest that the antibody producing characteristics of this patient had changed. Some twelve months separated the observations reported in Chapter 4 and the observations reported in this chapter. During this time the patient was pregnant and blood was not taken until some time after the birth of the child. The antibody titer of the patient's serum was much less at the second observation.

Lymphocytes from this patient made detectable amounts of IgG globulin. PHA stimulated its production in plasma medium but depressed its production in plasma-free medium. Thyroglobulin in plasma-free medium had a slight inhibitory

effect on the amount of labelled IgG detected, but this variation maybe within the range of experimental error. However, in plasma medium, thyroglobulin with PHA stimulated both the amount of labelled IgG detected and the total incorporation of $^{14}$C-leucine into protein beyond that expected by PHA alone. The significance of this effect needs to be substantiated by more experimental data. This observation may represent an increase in the amount of antibody, but in the absence of evidence to support this, conclusions cannot be made. Until detectable amounts of antibody are made by lymphocytes from this Hashimoto patient further investigation into this problem is limited.

The specificity of the labelling associated with the precipitin arcs in the $\alpha$ and $\beta$ globulin region of IEP patterns cannot be interpreted with full confidence. Arcs formed with mouse serum and antisera were labelled in this region after the serum had been electrophoresed with culture supernatants. The labelling of the arcs, haptoglobulin and transferrin, reported in the last chapter are subject to this criticism. Further investigation into the specificity of the radioactive labelling of these proteins using the methods described by Thorbecke (1960) and Helmreich (1961), is required.

Hirschhorn, et al., (1963) in an earlier report demonstrated the synthesis of IgG by cultures of human lymphocytes using radioactive tracers and paper electrophoresis. The results reported in this chapter indicate that this technique is unreliable, suggesting that large amounts of radioactivity remain at the origin and in the region of IgG migration. If this is so, labelling in this region cannot be attributed to IgG alone.

The nature of the material co-precipitating with immune precipitates has not been investigated. This material is stable to heat and is not dialysable, tests which suggest that it is not complement. The smudge of radioactivity associated with IEP patterns of normal culture fluids has been identified with this material but this radioactivity may also be associated with denatured labelled proteins.

The areas of the IEP patterns corresponding to the smudge of radioactivity in the autoradiographs, were lightly stained with amido black, a stain for protein. The heavy radioactive labelling associated with these areas suggests that the substance has a high specific activity. No radioactivity was detected in IEP patterns prepared from fluids of lymphocyte cultures incubated with puromycin or killed before incubation by freezing at -15°C. Immune precipitates

performed in the supernatants from such cultures were not labelled.

In summary the present evidence suggests that this material is synthesized by lymphocytes and is blocked by puromycin. It appears to be a protein containing a high amount of radioactivity. It is stable to heat (56°) and is not dialysable.

5.5. Summary

(1) Immune precipitates not related to human serum proteins were radioactively labelled when they were formed in culture fluids containing radioactive lymphocyte products.

(2) Radioactive material which co-precipitated with immune precipitates, was protein synthesized by lymphocytes as shown by its absence in culture fluids of lymphocytes killed before incubation or incubated with puromycin. This material was lightly stained with amido black in IEP patterns.

(3) After proteins, which were not related to human serum proteins, had been electrophoresed in agar with labelled culture fluids, the precipitin arcs formed with antibody were labelled in the $\alpha$ and $\beta$ globulin region but were not labelled in the cathodal

region of IEP patterns.

(5) The proteins in the cathodal region of IEP patterns were eluted from agar after electrophoresis and immune precipitates were formed in the eluates. The radioactivity in the human IgG - anti IgG precipitates was always greater than that in the control egg albumin - anti-egg albumin precipitates.

These data indicate that:

(i) some of the material co-precipitating with immune precipitates can be removed by electrophoresis from the cathodal end of IEP patterns and,

(ii) that the radioactive labelling of IgG can be demonstrated by identification in autoradiograph patterns and by precipitation by specific antibody in eluates from agar after electrophoresis.

(6) PHA stimulated the production of IgG by lymphocytes cultured in plasma medium. The stimulation of this protein by PHA was irregular in plasma-free medium. The degree of stimulation of IgG was less than the stimulation of the total proteins by PHA.

# CHAPTER 6

# THE EFFECTS OF ANTI-INFLAMMATORY AND IMMUNOSUPPRESSIVE DRUGS ON LYMPHOCYTE PROTEIN SYNTHESIS

## 6.1. Introduction

The importance of the lymphocyte in the immunological responses of the body and in inflammation has been reviewed. Lymphocytes are found in inflammatory lesions of different types. They are present in chronic inflammations of known, aetiology, for example tuberculosis, in experimental auto-immune lesions and in various non-specific inflammatory processes caused by physical and chemical agents. Lymphocytes are abundant in chronic inflammation of unknown aetiology in man, for example rheumatoid arthritis. The mode of action of drugs which modify this disease and are effective in the chemotherapy of autoimmune diseases and homograft rejection are unknown. Observations such as these led to the studies reported in this chapter.

In earlier studies it has been shown that plasma-free medium is suitable for short term lymphocyte incubation. Lymphocytes cultured in this medium were removed from substances which may affect protein synthesis (Kamrin, 1966) and in the studies to be described, the effects of the

interaction of drugs with plasma has been avoided. Conversely the interaction of drugs with plasma can be detected.

The studies reported in this chapter were designed to demonstrate:

(1) That a wide range of immunosuppressive and anti-inflammatory drugs used in clinical practice affect protein and ribonucleic acid metabolism by lymphocytes.

(2) That many drugs can be screened for their effect on protein synthesis. This was done in a number of ways; (i) by testing drugs at selected concentrations with lymphocytes from one donor, (ii) by testing each drug at a range of concentrations with one preparation of lymphocytes and, (iii) by testing drugs at selected concentrations with lymphocyte preparations from a number of donors.

## 6.2. Materials and Methods

### 6.2.1. Culture techniques

The preparation of lymphocyte cultures from whole blood has been described previously (Chapter 2). The cultures were terminated by freezing (-15°C) and the incorporation of $^{14}$C-leucine into protein was determined by precipitation with 5% trichloracetic acid.

In many of the figures which are included in this chapter, the incorporation of $^{14}C$-leucine has been expressed in one of two ways or both:

(i) CPM represents the actual counts per minute measured in the TCA precipitate under the experimental conditions.

(ii) Percent Activity is defined as the

$$\frac{\text{CPM in culture with drug}}{\text{CPM in culture without drug}} \times \frac{100}{1}$$

The following drugs were added to the culture medium (the abbreviations used in the figures in this section and throughout the thesis are given in parenthesis, together with the trade name):

(LEU) - Chlorambucil (-Leukeran, pure powder, a gift of Burroughs Wellcome and Co., London).

(ANT) - Sulphinpyrazone (-Anturan, pure powder, a gift of Geigy).

(TAN) - Oxyphenbutazone (-Tanderil, pure powder, a gift of Geigy).

(MEF) - Mefenamic acid (-Ponstan, pure powder, a gift of Parke Davis and Company, Sydney).

(IND) - Indomethacin (-Indocid, pure powder, a gift of Merck, Sharp and Dohme, Rahway, N.J., U.S.A.).

(CHL) - Chloroquine phosphate (-Chlorquine, ampoules for injection, Drug Houses of Australia).

(SAL) - Sodium salicylate (-reagent grade, British Drug Houses Ltd.).

(NIT) - Mustine N-oxide HCl (-Nitromin, Yoshitomi Pharmaceutical Industries, Ltd., Japan).

(THI) - Triethylene phosphoramide - (Thiotepa, Lederle Laboratories Division, American Cyanamid Company, New York).

(MTX) - Methotrexate (-Methotrexate Sodium Parental, Lederle Laboratories Division).

(SAN) - Actinomycin C (-Sanamycin, Bayer Pharma Pty. Ltd., Germany).

(ACT) - Adrenocorticortrophic hormone (-prepared from pig pituitary, batch 0715 containing 52.1 units/mg, Commonwealth Serum Laboratories, Melbourne, Aust.).

(ADR) - Adrenaline tartrate (1 part in 1000, Farmer Hill Pty. Ltd., N.S.W., Australia).

(COR) - Hydrocortisone sodium succinate (-Solucortef, Upjohn Kalamazoo, Mich., U.S.A.).

(END) - Cyclophosphamide (-Endoxan Asta, ampoules for injection, Mead Johnson Pty. Ltd., N.S.W., Australia).

( - ) - Chloramphenicol (-Synthomycetin, Lepetit (Pharmaceuticals) Pty. Ltd., Sydney).

(PHA) - Phytohaemagglutinin (-Burroughs Wellcome, England).

(PUR) - Puromycin (-Lederle Laboratories Division).

The drugs were made up into stock solutions with medium, sterilized by millipore filtration and kept at 4°C before use. The stock solutions were kept and used in the cultures for varying periods of this time. This period of time depended on the stability of the various drugs in solution according to information supplied by the manufacturer. Drugs which were not readily soluble in medium for example, sulphinpyrazone, indomethacin and chlorambucil, were dissolved in ethyl alcohol in clean dry beakers before use. These solutions were discarded after use.

The drugs were added to the cultures by dropwise addition from 25-2o gauge needles, which had been specially calibrated. Drugs dissolved in alcohol were added as one drop from a 20 gauge needle from solutions made up at various concentrations. Drugs in alcoholic solutions were always added to empty culture bottles, which were warmed and flamed without the caps before the addition of medium and cells. When alcohol without drug was added in this way to lymphocyte

cultures, the incorporation of precursor by these cells did not vary significantly from that by lymphocytes cultured in medium with alcohol. In experiments with drugs in alcoholic solution, control cultures containing alcohol without drug were always included.

6.2.2. Viability determinations

The viability of lymphocytes was determined by trypan blue exclusion and cell counting. These methods have been described previously (Chapter 2). Lymphocyte viability was determined in cultures with and without drugs in the medium at various times during incubation.

The viability of lymphocyte cells has been expressed as Percent Viability which is defined as,

$$\frac{\text{Number of cells viable in culture at time of sampling}}{\text{Number of cells viable in culture at the commencement of incubation}} \times \frac{100}{1}$$

6.2.3. Effects of drugs on ribonucleic acid synthesis

The synthesis of RNA was measured by including $^{3}$H-uridine in the medium. The methods of RNA extraction from the cells and estimation of the incorporation of precursor have been described in Chapter 3. Drugs were included in the medium at selected concentrations.

Fig. 6.1. <u>The effects of a wide range of drugs on lymphocyte protein synthesis</u>

Normal lymphocytes were cultured in plasma-free, supplemented Eagle's medium prepared without leucine. The cultures contained $3 \times 10^6$ cells and 0.25 µc of $^{14}$C-leucine.

The drugs:

TAN - Oxyphenbutazone

BTZ - Phenylbutazone

NIT - Mustine-N-oxide HCl

CHL - Chloroquine

END - Cyclophosphamide

THI - Triethylene phosphoramide

MTX - Methotrexate

SAN - Actinomycin C

ACT-- Adrenocorticotrophic hormone

ADR - Adrenaline

were included in the medium at the concentrations shown (these concentrations were chosen to be in the estimated pharmacological range).

CPM refers to the actual counts per minute measured under the experimental conditions.

Percent activity is defined as:

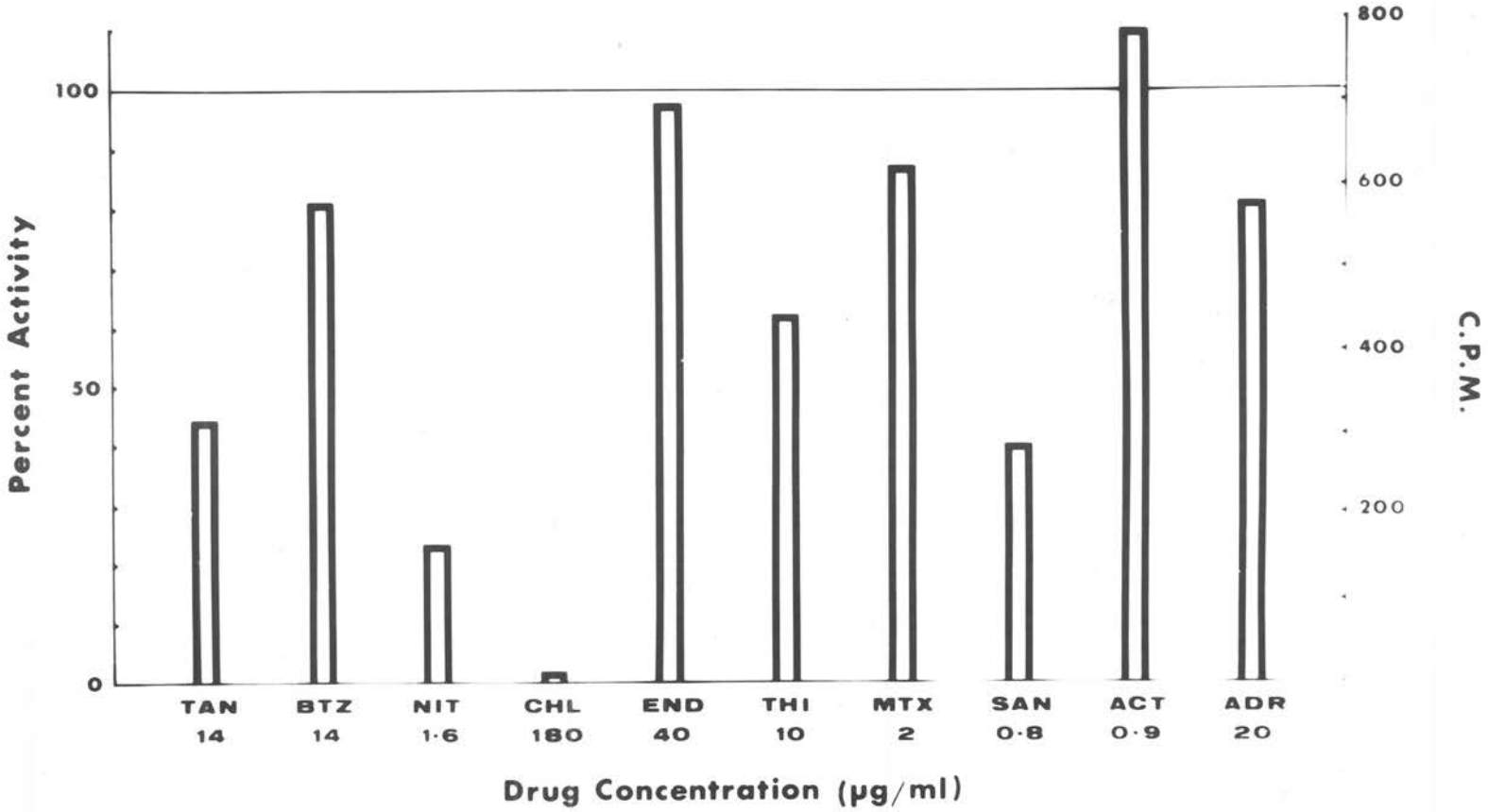
$$\frac{\text{CPM in culture with drug}}{\text{CPM in control culture}} \times \frac{100}{1}$$

## 6.3. Results

### 6.3.1. Preliminary drug trials

A wide range of drugs were tested for activity with one preparation of lymphocytes from a normal donor (Fig. 6.1). In this experiment the concentration of drugs was chosen to be within a pharmacological range, based on the clinical dose levels. This is a very approximate guide to the concentration of a drug. For example, if a drug is administered at X mg/day, then for a blood volume of 5 litres and ignoring other factors such as excretion and binding to tissues, the blood level could be arbitarily assigned to be (X/5) µg/ml.

The concentrations of the drugs used in Fig. 6.1 were most probably overestimates. However, when the drugs were tested, a pattern of activity emerged. Some of the drugs were active, for example, phenylbutazone, oxyphenbutazone and chloroquine, while some were inactive, for example, cyclophosphamide and adrenocorticotrophic hormone. Chloroquine and mjstine N-oxide HCl inhibited lymphocyte protein synthesis markedly, while oxyphenbutazone, phenylbutazone, thio-tepa methotrexate, actinomycin D and adrenalin inhibited protein synthesis to varying extents.


Percent Activity
100
50
0
800
600
400
200
C.P.M.
TAN 14
BTZ 14
NIT 1·6
CHL 180
END 40
THI 10
MTX 2
SAN 0·8
ACT 0·9
ADR 20
Drug Concentration (µg/ml)

### 6.3.2. The effects of individual drugs

The effects of various drugs, tested at a range of concentrations, have been summarized in Figures 6.2 and 6.3. The degree of maximum protein synthesis inhibition varied considerably with the individual drugs. For example hydrocortisone usually caused less than 50 percent suppression of synthesis whereas chloroquine at fully inhibitory concentrations almost abolished protein synthesis. With most of the drugs tested, a general plateau effect was observed at high concentrations. Chloroquine was the only drug which at low concentrations significantly stimulated protein synthesis. Both phenylbutazone and oxyphenbutazone were more active than sulphinpyrazone.

Mefenamic acid was highly inhibitory. Salicylate was inhibitory to protein synthesis at levels which are found in the blood (Burns, et al., 1953). Parahydroxybenzoate did not inhibit lymphocyte protein metabolism. Indomethacin, cortisone and chlorambucil were inhibitory, but not to the same degree as the other drugs tested.

Chloramphenicol did not significantly inhibit lymphocyte protein synthesis at concentrations up to 640 µg/ml in the medium. Cyclophosphamide was inactive at concentrations

Fig. 6.2. <u>The relationship of protein synthesis to concentration of drugs in the culture medium</u>

The data were compiled from several experiments. Each drug at a range of concentrations was tested with one preparation of normal lymphocytes. The lymphocytes were cultured in plasma-free, supplemented Eagle's medium prepared without leucine and containing 0.25 µc of $^{14}$C-leucine.

The drugs:

CHL - Chloroquine

ANT - Sulphinpyrazone

BTZ - Phenylbutazone

TAN - Oxyphenbutazone

MEF - Mefenamic acid

were included in the culture medium at a range of concentrations shown in the figure.

The incorporation of $^{14}$C-leucine has been expressed as percent activity.

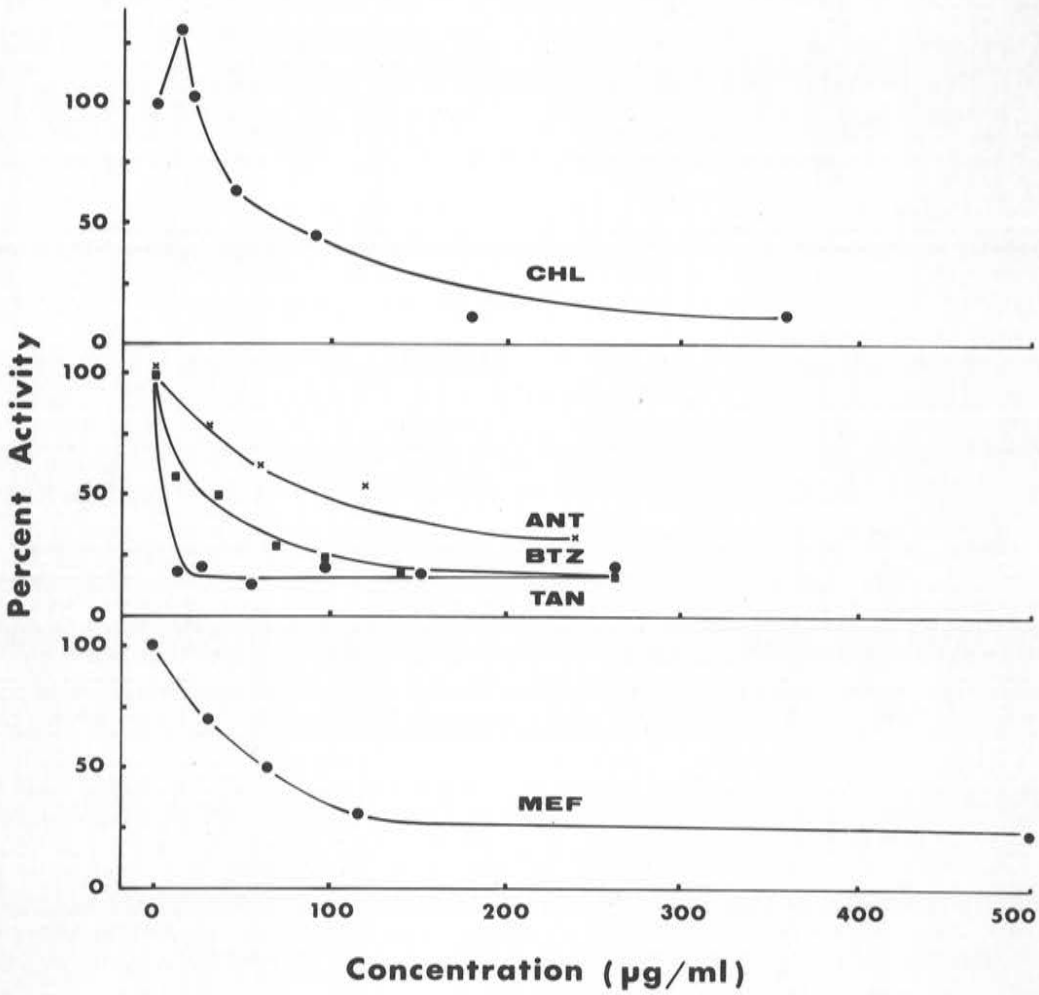

Percent Activity
100
50
0
CHL
ANT
BTZ
TAN
MEF
0
100
200
300
400
500
Concentration (µg/ml)

Fig. 6.3. <u>The relationship of protein synthesis to concentration of drugs in the culture medium</u>

The data were compiled from several experiments. Each drug at a range of concentrations was tested with one preparation of lymphocytes. The lymphocytes were cultured in plasma-free, supplemented Eagle's medium prepared without leucine and containing 0.25 μc of $^{14}$C-leucine.

The drugs:

IND - Indomethacin

p-OH-B - para-Hydroxybenzoate

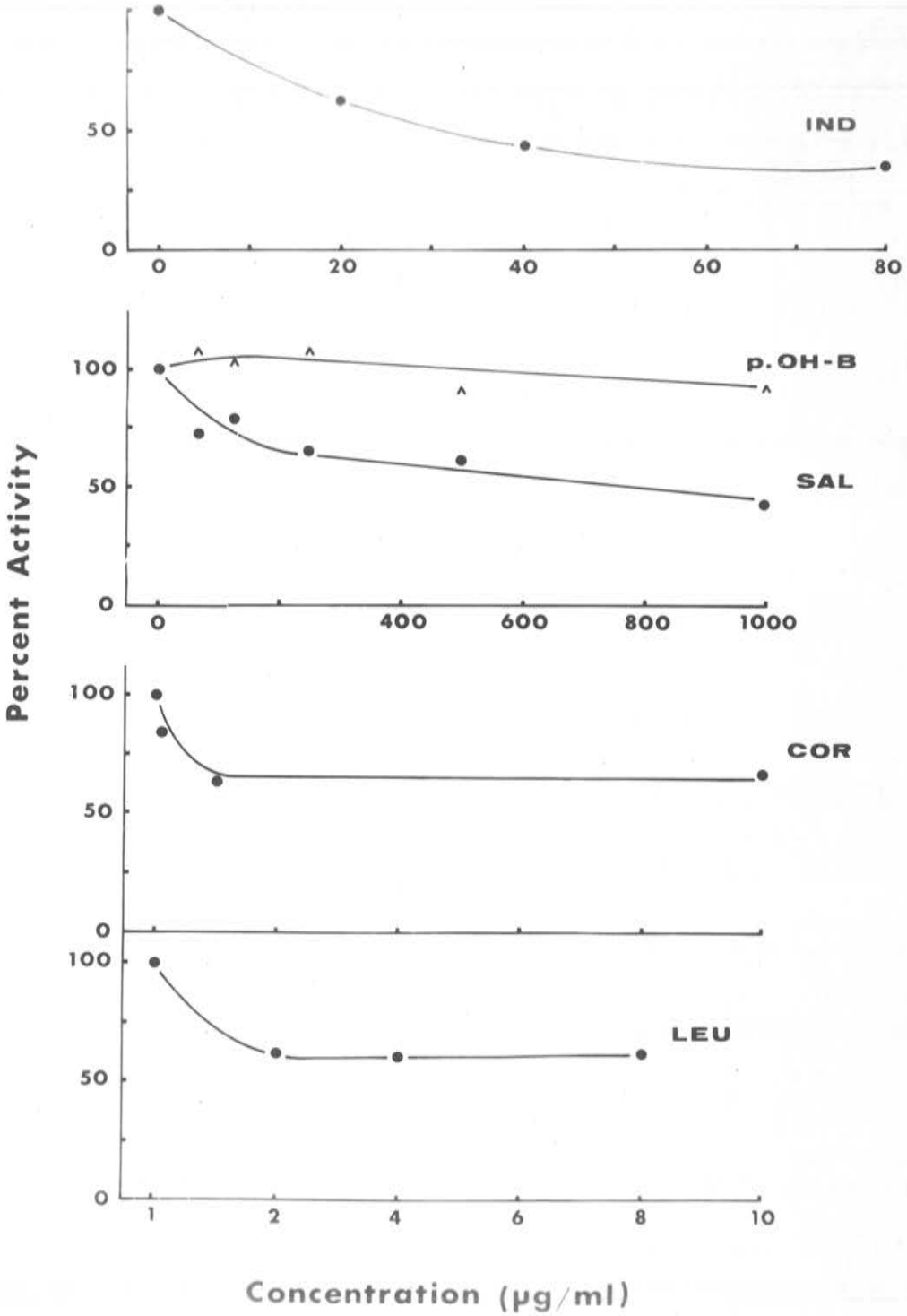
SAL - Salicylate

COR - Cortisone

LEU - Chlorambucil

were included in the culture medium at a range of concentrations shown in the figure.

The incorporation of $^{14}$C-leucine has been expressed as percent activity.


IND
p.OH-B
SAL
COR
LEU
Percent Activity
Concentration (µg/ml)
0
50
100
0
20
40
60
80
400
600
800
1000
1
2
4
6
8
10

up to 160 μg/ml in the culture medium.

The variability of drug responses between individuals was determined by testing the drugs with lymphocytes from a number of donors at selected concentrations, which had been selected from the dose response curves to cause significant inhibition and to be within the estimated pharmacological range. Each drug was tested with preparations of lymphocytes from at least three donors (Fig. 6.4). Chloroquine, indomethacin and mefenamic acid were always highly inhibitory. Salicylate in one case was not inhibitory. All the drugs varied in their inhibitory effects on lymphocytes from different individuals; the greatest variation occurred with phenylbutazone and oxyphenbutazone.

PHA stimulated protein synthesis by 2.5 to 4 fold in this system (Chapter 3). The presence of this substance in the culture medium did not markedly alter the response to various drugs (Fig. 6.5).

Many of the drugs which inhibited protein synthesis were also lethal (Fig. 6.6). Cortisone, salicylate, chlorambucil and sulphinpyrazone were not as toxic to cells as the drugs oxyphenbutazone, mefenamic acid, indomethacin and chloroquine. The increased toxicity of these drugs was paralleled by their

Fig. 6.4. The effect of drugs on protein synthesis by normal lymphocytes

The data were compiled from many experiments. In all of these the lymphocytes were cultured in plasma-free, supplemented Eagle's medium prepared without leucine and containing 0.25 µc of $^{14}$C-leucine. Each point represents a determination made in triplicate with one preparation of normal lymphocytes.

The drugs:

CHL - Chloroquine

IND - Indomethacin

LEU - Chlorambucil

MEF - Mefenamic acid

SAL - Salicylate

COR - Cortisone

ANT - Sulphinpyrazone

TAN - Oxyphenbutazone

BTZ - Phenylbutazone

were included in the medium of lymphocyte preparations at the concentrations shown in the figure.

The incorporation of $^{14}$C-leucine has been expressed as percent activity.

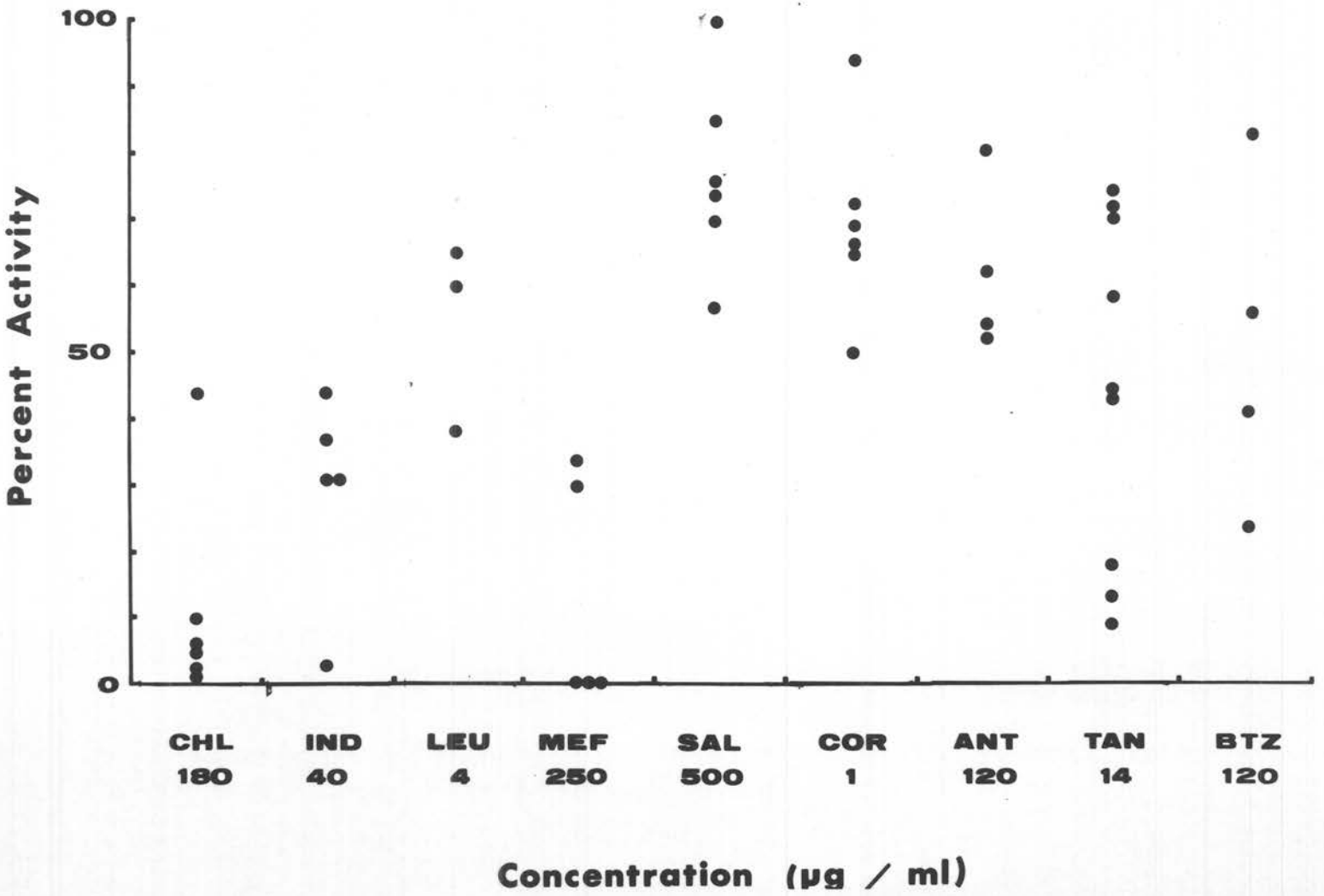

100
50
0
Percent Activity
CHL
180
IND
40
LEU
4
MEF
250
SAL
500
COR
1
ANT
120
TAN
14
BTZ
120
Concentration (µg / ml)

Fig. 6.5. <u>The effect of drugs on preparations of normal lymphocytes stimulated with phytohaemagglutinin</u>

The data were compiled from a number of experiments. In these the lymphocytes were cultured in plasma-free, supplemented Eagle's medium prepared without leucine and containing 0.25 µc of $^{14}$C-leucine/ml. PHA stimulated lymphocyte protein synthesis 3-4 times compared with that in unstimulated cultures. Each point in the figure represents a determination made in triplicate with one preparation of normal lymphocytes.

The drugs:

ANT - Sulphinpyrazone

TAN - Oxyphenbutazone

MEF - Mefenamic acid

SAL - Salicylate

IND - Indomethacin

COR - Cortisone

CHL - Chloroquine

LEU - Chlorambucil

were included in the culture medium of lymphocyte preparations at the concentrations shown in the figure.

The incorporation of $^{14}$C-leucine has been expressed as percent activity.

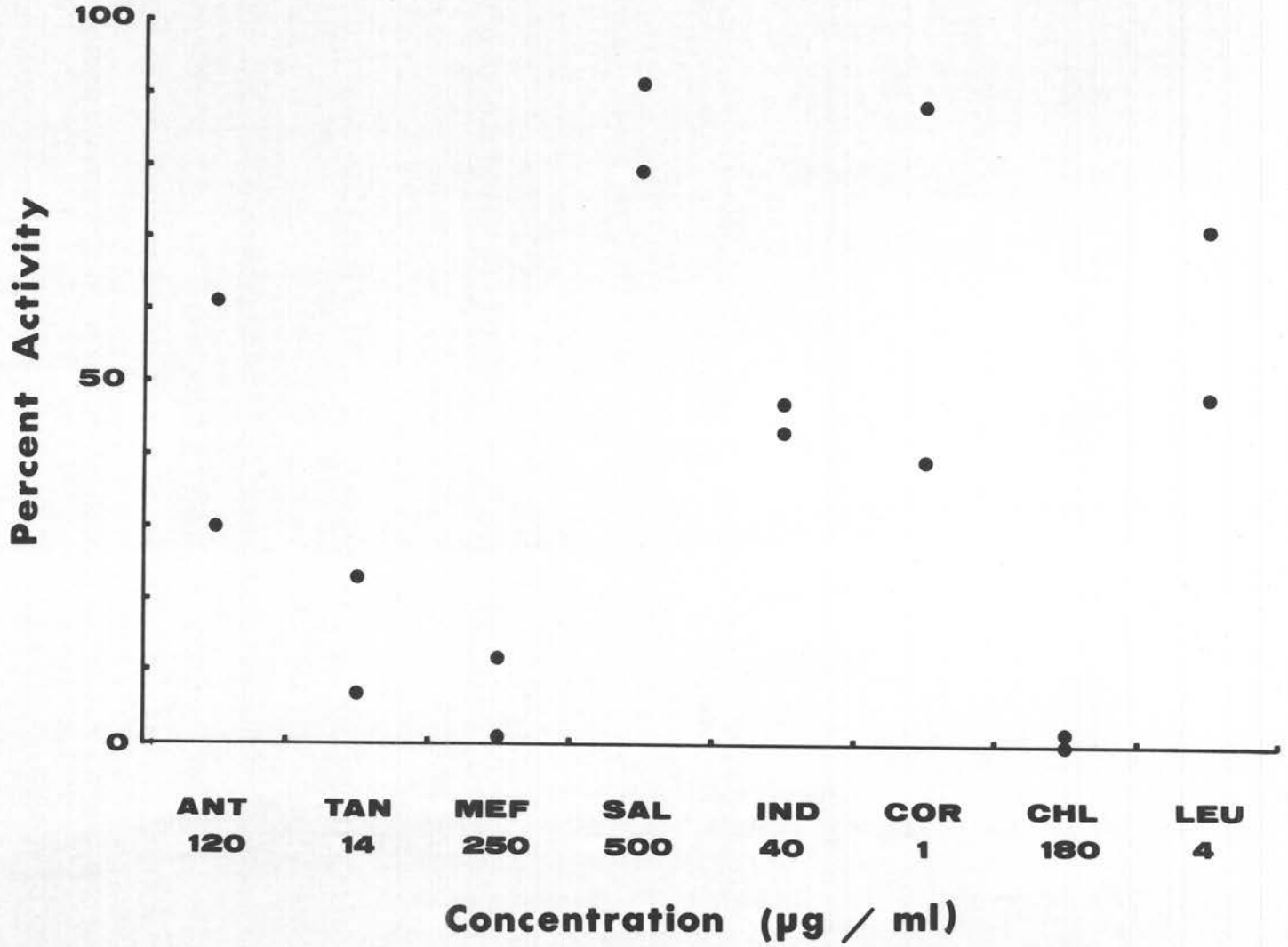

100
50
0
Percent Activity
ANT
120
TAN
14
MEF
250
SAL
500
IND
40
COR
1
CHL
180
LEU
4
Concentration (µg / ml)

Fig. 6.6. The effect of drugs on the viability of normal lymphocytes

The viability of lymphocyte was determined by methods described in the text at various times during incubation. Normal lymphocytes were cultured in plasma-free, supplemented Eagle's medium prepared without leucine. The data were compiled from several experiments and each set of points for each drug was determined in triplicate with one preparation of lymphocytes. In each experiment cultures without drug were always included as controls.

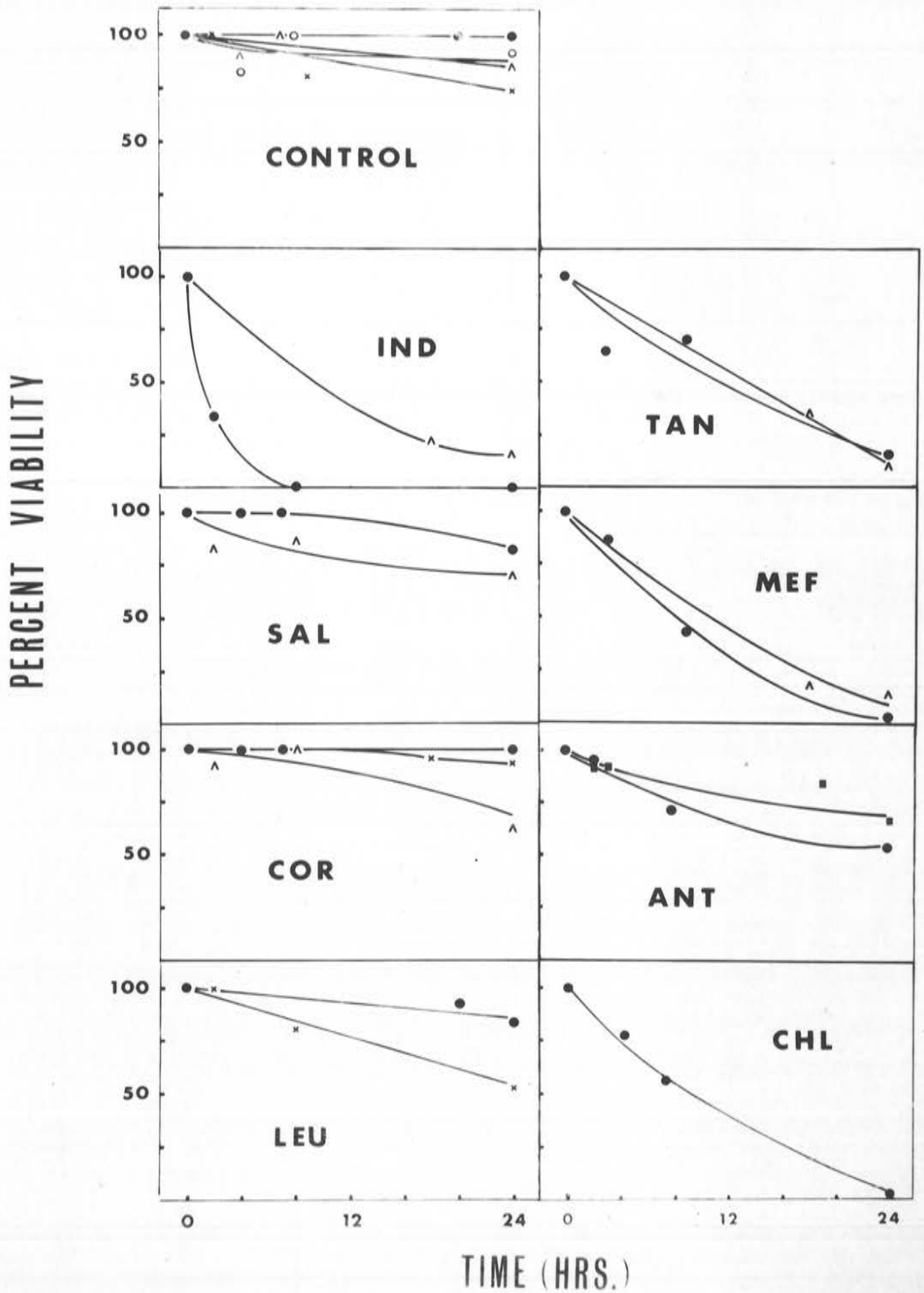
The drugs:

| | |
|---|---|
| IND - Indomethacin | (40 µg/ml) |
| SAL - Salicylate | (500 µg/ml) |
| COR - Cortisne | (1 µg/ml) |
| LEU - Chlorambucil | (4 µg/ml) |
| TAN - Oxyphenbutazone | (14 µg/ml) |
| MEF - Mefenamic acid | (250 µg/ml) |
| ANT - Sulphinpyrazone | (120 µg/ml) |
| CHL - Chloroquine | (180 µg/ml) |

were included in the culture medium at the concentrations shown above.

The viability of the cells has been expressed as percent viability, which is defined as:

$$\frac{\text{No. cells viable in culture at time of sampling}}{\text{No. cells viable in culture at commencement of incubation}} \times \frac{100}{1}$$


PERCENT VIABILITY
100
50
CONTROL
IND
TAN
SAL
MEF
COR
ANT
LEU
CHL
0
12
24
TIME (HRS.)

greater effect on protein synthesis.

In puromycin-treated cultures, 80% of the lymphocytes survived incubation for 24 hours in plasma-free medium. Viability was determined by cell counting and trypan blue staining.

When culture fluids from plasma-free cultures containing cortisone (1.0 µg), salicylate (500 µg), oxyphenbutazone (14 µg), indomethacin (40 µg), mefenamic acid (250 µg) and chlorambucil (4 µg) were examined by immunoelectrophoresis and autoradiography, they contained the same labelled proteins as found in control cultures without drug. No labelled proteins could be detected by these techniques in culture fluid from lymphocytes incubated with chloroquine (180 µg).

6.3.3. Effect of 25% autologous plasma

The inhibitory effects of all the drugs tested, except chloroquine, were reduced by plasma (Fig. 6.7). When the concentration of oxyphenbutazone was increased to levels of 560 µg/ml in medium containing plasma, the degree of inhibition of protein synthesis was similar to that caused by a concentration of 56 µg/ml in plasma-free medium (Fig. 6.8).

Fig. 6.7. <u>The effect of 25% plasma in the medium on the inhibition of protein synthesis by drugs</u>

Normal lymphocytes were cultured in supplemented Eagle's medium prepared without leucine and with or without autologous plasma. $^{14}$C-leucine was included in the medium at a concentration of 0.25 µc/ml. Lines join paired observations of the same drug with one preparation of lymphocytes.

○ - represents triplicate determinations made in 25% plasma-medium.

● - represents triplicate determinations made in plasma-free medium.

The drugs:

CHL - Chloroquine

IND - Indomethacin

LEU - Chlorambucil

MEF - Mefenamic acid

SAL - Salicylate

COR - Cortisone

ANT - Sulphinpyrazone

TAN - Oxyphenbutazone

were included in the medium at the concentrations shown in the figure.

The incorporation of $^{14}$C-leucine has been expressed as percent activity.

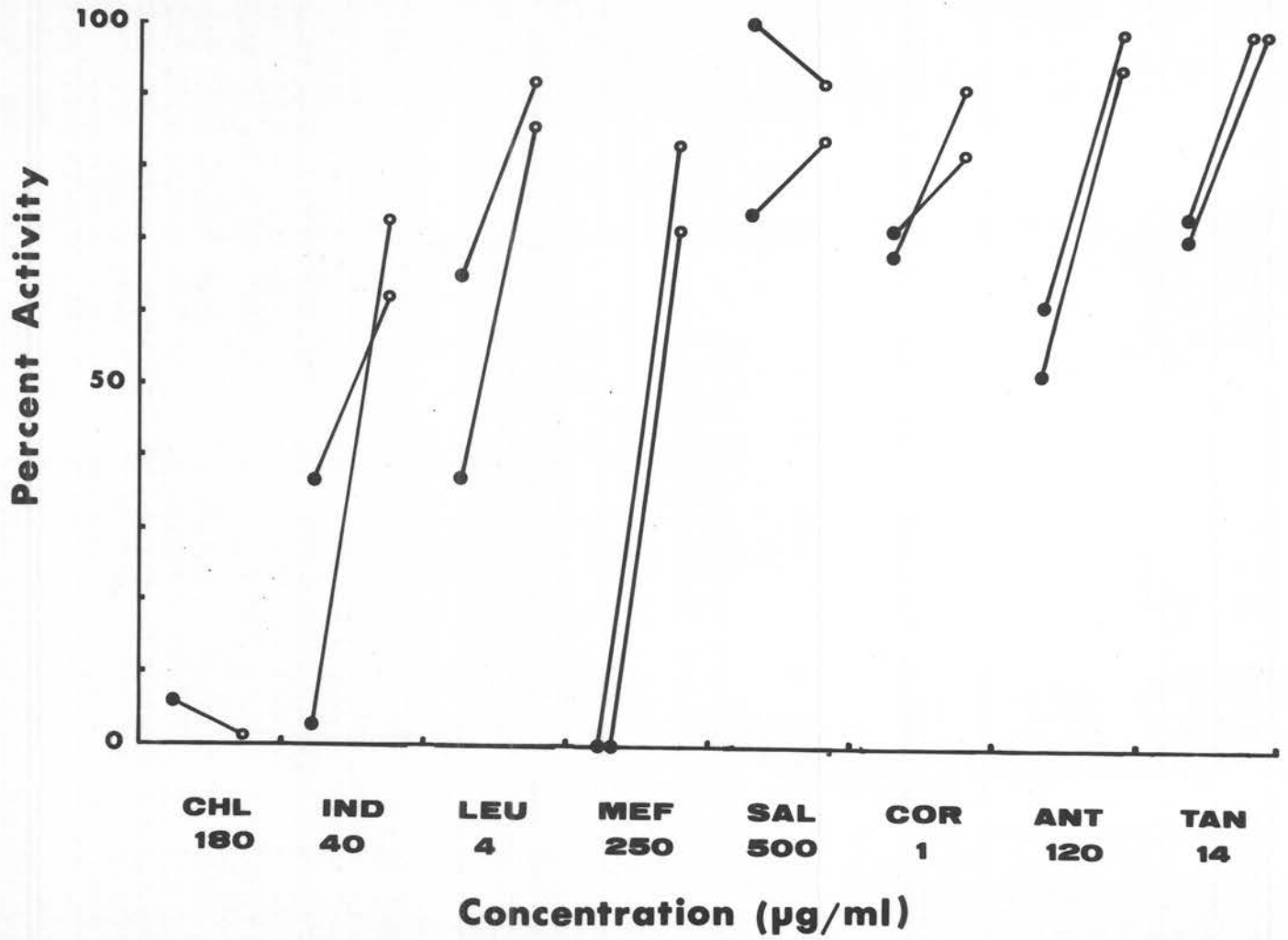

100
50
0
Percent Activity
CHL
180
IND
40
LEU
4
MEF
250
SAL
500
COR
1
ANT
120
TAN
14
Concentration (µg/ml)

Fig. 6.8. <u>The effect of plasma in the medium on the inhibition of normal lymphocyte protein synthesis by oxyphenbutazone</u>

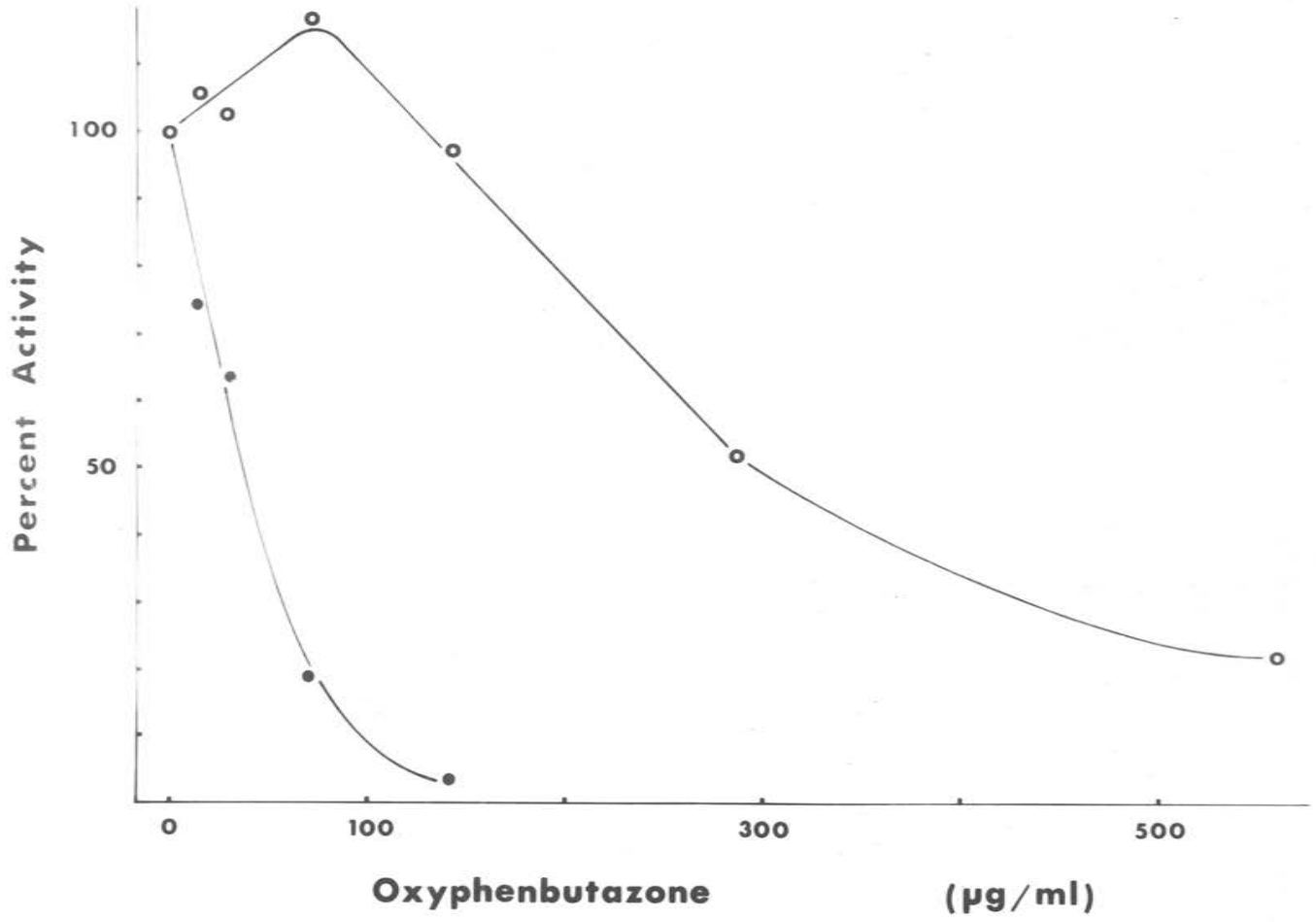
The determinations were made with one preparation of normal lymphocytes. Lymphocytes were cultured in supplemented Eagle's medium prepared without leucine and with or without autologous plasma. $^{14}$C-leucine was included in the medium at a concentration of 0.25 µc/ml. Each culture contained 2.1 x $10^6$ cells.

Oxyphenbutazone was included in the medium at a range of concentrations shown in the figure.

○ - represents triplicate determinations made with lymphocytes cultured in 25% autologous plasma medium.

● - represents triplicate determinations made with lymphocytes cultured in plasma-free medium.

The incorporation of $^{14}$C-leucine has been expressed as percent activity.


Percent Activity
100
50
0
100
300
500
Oxyphenbutazone
(µg/ml)

6.3.4. Effect on RNA

The effects of various drugs on the incorporation of $^{3}$H-uridine into RNA by lymphocytes are shown in Fig. 6.9. In the studies reported in graph B, the drugs were included in unstimulated cultures at the concentration shown. The cultures were sampled after incubation for three hours. The drugs actinomycin D, mefenamic acid and chloroquine almost completely suppressed RNA synthesis. A range of drugs, indomethacin, puromycin, chlorambucil and sulphinpyrazone partially suppressed synthesis. Cortisone had a very small effect, while oxyphenbutazone and salicylate did not significantly inhibit the incorporation of $^{3}$H-uridine into RNA. The presence of PHA in the culture medium did not significantly alter the effects of the drugs tested except puromycin (Fig. 6.9A). These PHA-stimulated cultures were sampled after incubation for 12 hours. Actinomycin D, puromycin and mefenamic acid completely inhibited synthesis, Sulphinpyrazone and indomethacin partially inhibited synthesis while cortisone and oxyphenbutazone had no significant effect.

6.3.5. In vivo study with cortisone

In one study blood was collected from a patient before and after cortisone therapy, for the preparation of lymphocyte

Fig. 6.9. <u>The effects of drugs on the incorporation of $^{3}$H-uridine into RNA by unstimulated and PHA-stimulated lymphocytes</u>

In experiments A and B, normal lymphocytes were cultured in supplemented Eagle's medium prepared without leucine. The incorporation of $^{3}$H-uridine has been expressed as counts/20 min., in 1 ml of RNA extract (total volume 5 ml). The incorporation was determined as described in Chapter 3, section 3.2.4.

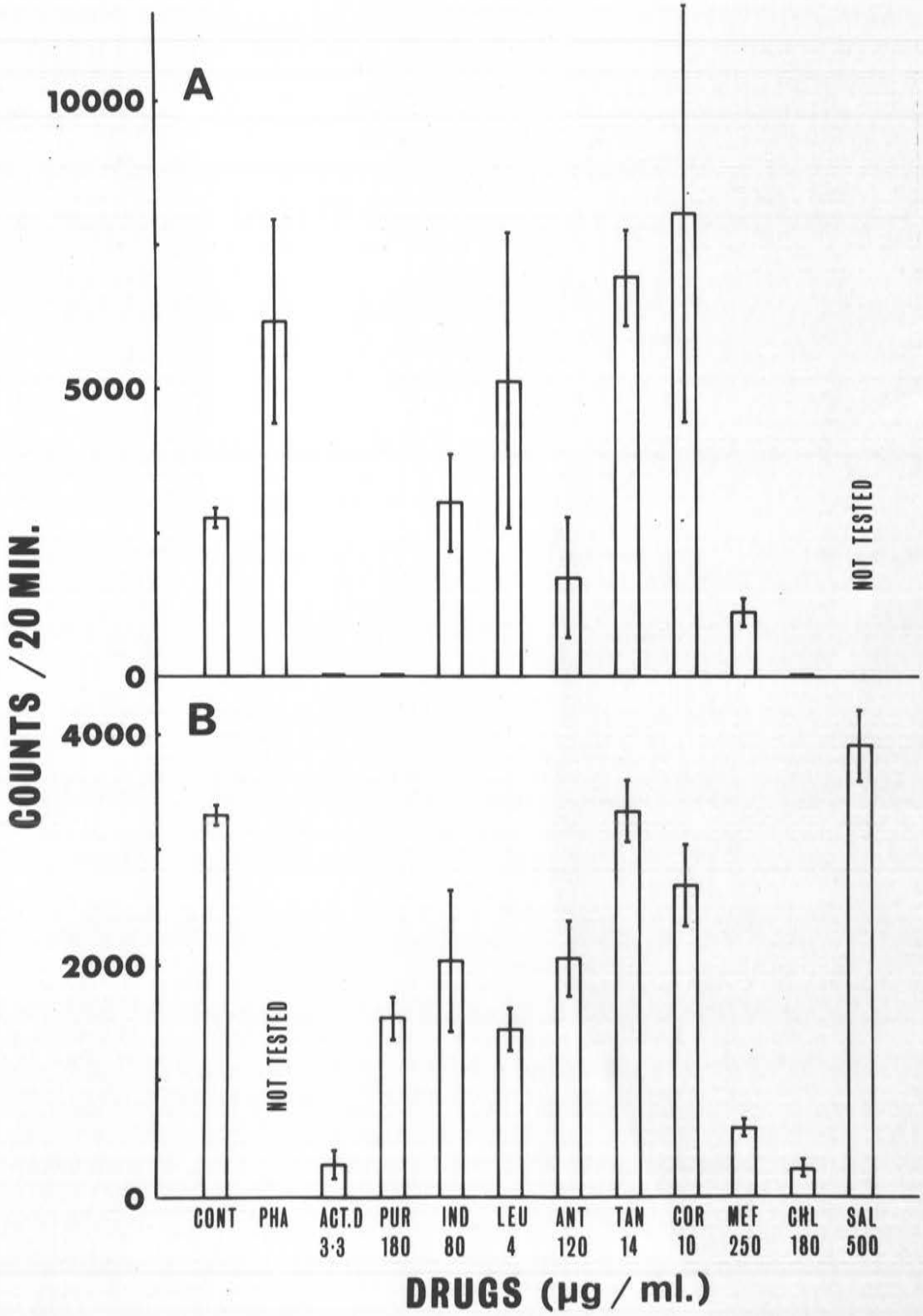
A - In this experiment drugs were includedin the medium of PHA-stimulated lymphocytes. Unstimulated lymphocytes were included as a control (CONT). The cultures contained $2.7 \times 10^{6}$ cells and 2 µc of $^{3}$H-uridine and were terminated after incubation for 12 hours.

B - In this experiment drugs were included in the culture medium of unstimulated lymphocytes. The cultures contained $3.3 \times 10^{6}$ cells and 2 µc of $^{3}$H-uridine and were terminated after incubation for 3 hours.

The drugs:

ACT D - Actinomycin D
PUR - Puromycin
IND - Indomethacin
LEU - Chlorambucil
ANT - Sulphinpyrazone
TAN - Oxyphenbutazone
COR - Cortisone
MEF - Mefenamic acid
CHL - Chloroquine
SAL - Salicylate

were included in the medium of lymphocyte preparations at the concentrations shown in the figure.


A
10000
5000
0
NOT TESTED
B
4000
2000
0
NOT TESTED
COUNTS / 20 MIN.
CONT
PHA
ACT.D 3·3
PUR 180
IND 80
LEU 4
ANT 120
TAN 14
COR 10
MEF 250
CHL 180
SAL 500
DRUGS (µg / ml.)

cultures. The patient was a 52 year-old Italian woman, who suffered from retroperitoneal fibrosis. Blood was collected on two occasions before treatment and a number of times after treatment. Lymphocytes were cultured at these times in medium with and without autologous plasma. PHA-stimulated cultures were also set up when sufficient lymphocytes were available for study. The incorporation has been expressed as counts per $10^6$ lymphocytes per 0.25 µc of $^{14}$C-leucine in the medium (Table 6.1).

Before the administration of prednisolone and for a one week period after administration there was little change in the incorporation of $^{14}$C-leucine into protein by lymphocytes cultured in plasma-free medium. On the twelfth day of drug administration (14th February), the incorporation of $^{14}$C-leucine by lymphocytes cultured in plasma-free medium, fell significantly and remained depressed throughout the course of drug therapy.

The incorporation of $^{14}$C-leucine into protein by lymphocytes cultured in plasma supplemented medium was variable. The incorporation was steady on the two days before and on the second day of drug administration. After this time the incorporation was widely variable throughout the duration of drug therapy. PHA stimulated the lymphocytes to produce

Table 6.1. A study of the effects of cortisone 'in vivo' on the ability of lymphocytes toincorporate $^{14}$C-leucine into protein 'in vitro'

The patient was a 52 year-old Italian woman with retroperitoneal fibrosis. Blood was collected on the dates shown in Table 6.1A and the lymphocytes were prepared in the usual way, except that the low speed centrifugation (150 x g) was omitted. The cultures contained 0.8 - 1.5 x $10^6$ cells and 0.25 µc of $^{14}$C-leucine. The cells were cultured in supplemented Eagle's medium prepared without leucine and with or without autologous plasma sampled at the same time with the lymphocytes.

PHA was used tostimulate the cultures at various times shown in the table. Freeze-dried aliquots of the same batch of PHA were used on every occasion at the same concentration.

The drug regimes during the collection of blood samples are given in table 6.1B. After 12 days the incorporation of $^{14}$C-leucine by lymphocytes cultured in plasma-free medium fell significantly and remained depressed. The incorporation by the cells in plasma-medium was variable.

PHA stimulated protein synthesis by lymphocytes cultured in both types of medium. The stimulation of $^{14}$C-leucine incorporation by this substance was greater in plasma-free medium than in plasma medium.

The incorporation of $^{14}$C-leucine into protein has been expressed as counts/$10^6$ cells cultured/ 1/4 µc of $^{14}$C-leucine in the medium/min.

TABLE 6.1

A. Incorporation of $^{14}$C-leucine

| Date | Drugs | Counts/$10^6$ cells/1/4 μc/min | | | | | |
|---|---|---|---|---|---|---|---|
| | | Plasma-free | | | 25% Plasma | | |
| | | No PHA | PHA | Increase % | No PHA | PHA | Increase % |
| 30 Jan | - | 460 | - | | 110 | - | |
| 1 Feb | - | 410 | 980 | 239 | 90 | - | |
| 3 Feb | + | 490 | 1205 | 246 | 100 | - | |
| 7 Feb | + | 426 | 1303 | 306 | 278 | - | |
| 9 Feb | + | 435 | 1198 | 275 | 155 | - | |
| 14 Feb | + | 248 | 697 | 281 | 52 | 104 | 200 |
| 3 March | + | 181 | 544 | 300 | 125 | 207 | 163 |
| 5 April | + | 298 | 444 | 149 | 320 | 424 | 132 |
| 12 April | + | - | - | | 69 | 131 | 189 |
| 27 April | + | 83 | 384 | 457 | - | - | |

B. Drug Regime

| Drug | Dose mgm/day | Start | Finish |
|---|---|---|---|
| Prednisolone | 10 | 2nd February | 13th April |
| Hydrocortisone | 1 x 100 | 14th April | 14th April |
| | 2 x 100 | 15th April | 15th April |
| | 2 x 50 | 16th April | 18th April |
| Prednisolone | 5 | 18th April | 8th May |

increased protein on every occasion when the substance was tested in medium with and without plasma. The stimulation by PHA was greater when the cells were cultured in plasma-free medium than when the cells were cultured in plasma medium.

## 6.4. Discussion

### 6.4.1. Comparison of the effects of drugs

The anti-inflammatory drugs which were studied, inhibited lymphocyte protein synthesis to varying extents. A wide variety of drugs were tested, including nitrogen mustards, phenylbutazone, salicylate, actinomycin, methotrexate, chloroquine and hydrocortisone. All the drugs were inhibitory at the concentrations used in the culture medium except chloroquine. This drug was inhibitory at concentrations greater than 20 μg/ml but stimulated lymphocyte protein synthesis at concentrations less than this.

The degree of inhibition by some drugs was apparently related to their effectiveness *in vivo* as anti-inflammatory agents. This was best seen with analogues differing by a small change in molecular structure. These showed significant changes in their ability to suppress lymphocyte protein synthesis and similar differences in anti-inflammatory activity. For example oxyphenbutazone, a metabolite of

phenylbutazone and more potent therapeutically at a lower dose (Goodman and Gilman, 1965), was more inhibitory than the parent drug. Phenylbutazone was slightly more inhibitory than its analogue sulphinpyrazone, which has less anti-inflammatory activity in man (Goodman and Gilman, 1965).

Salicylate, a mild anti-inflammatory agent, inhibited lymphocyte protein synthesis but its analogue, parahydroxybenzoate, was not active. Parahydroxybenzoate, is also inactive *in vivo* (Goodman and Gilman, 1965).

The relation between the ability of several alkalyating agents to inhibit lymphocyte protein synthesis reflected their activity *in vivo*. Chlorambucil, reported to be the least toxic and slowest acting of the nitrogen mustard group of drugs (Goodman and Gilman, 1965), was not as effective as mustine N-oxide hydrochloride. Triethylene phosphoramide was less active than mustine N-oxide hydrochloride when tested at the same occasion. Cyclophosphamide found by others to be inactive *in vitro* (Hampel, *et al.*, 1966) did not inhibit lymphocyte protein synthesis. This drug is activated *in vivo* to its active form (Brock and Hohorst, 1963).

Adrenocorticotrophic hormone (ACTH) is a hormone which acts to stimulate the adrenal cortex. ACTH did not inhibit

lymphocyte protein synthesis. This hormone is an anti-inflammatory agent because it stimulates the production of hydrocortisone. Hydrocortisone inhibited lymphocyte protein synthesis at concentrations of 0.1 µg/ml in the medium. By contrast adrenaline, which is released by the adrenal medulla had a small inhibitory effect at a concentration of 20 µg/ml in the medium.

Lymphocyte preparations offer unique opportunities for testing a wide range of drugs and hormones for their effect on protein synthesis. These tests may be suitable for comparing the activity of analogues and hormones in order to predict their activity in vivo. The effect of these substances on other metabolic pathways could also be observed.

### 6.4.2. Pathways by which drugs may interfere with protein synthesis

The mechanisms by which some of these drugs act in the body have been described while the mechanisms of action of others are unknown. Despite the wide range of effects these drugs have on cell metabolism they inhibited protein synthesis.

The alkalyating drugs for example, chlorambucil, are thought to act by cross linking with DNA and inhibiting cell function (Goodman and Gilman, 1965). This effect is thought

to inhibit DNA replication more than to inhibit RNA production (Wheeler, 1962).

Mefenamic acid, phenylbutazone, oxyphenbutazone and salicylate have the common property of uncoupling oxidative phosphorylation (Brody, 1956; Whitehouse, 1963). Chloroquine is thought to interact with the nucleoproteins in the cell. This drug is concentrated by the liver, spleen, kidneys, lung and leukocytes from the blood (Goodman and Gilman, 1965). Methotrexate has its greatest effect on dividing cells by interfering with folic acid metabolism (Goodman and Gilman, 1965). All of these drugs inhibited lymphocyte protein synthesis.

Actinomycin C was inhibitory. This drug acts to inhibit protein synthesis in a similar way to actinomycin D (Chapter 2).

The drugs which were used in the investigations reported in this chapter interfere with many metabolic pathways. Despite this fact all of these drugs affect lymphocyte protein synthesis either as a direct effect of drug action or as a result of the effects of the drugs on pathways associated with protein synthesis. Studies with each drug using different techniques would be necessary to investigate in detail the mechanisms, by which these drugs interfere with

lymphocyte protein synthesis.

Some of the drugs were highly toxic to cells, for example mefenamic acid, chloroquine and indomethacin. The inhibition of protein synthesis by these drugs was probably a consequence of cell death. These lethal drugs must exert an immediate effect on metabolic pathways, structures or membranes which are vital. Other drugs for example, hydrocortisone, salicylate, chlorambucil and sulphinpyrazone inhibited protein synthesis by mechanisms which did not lead to cell death.

Hydrocortisone inhibited lymphocyte protein synthesis at very small concentrations in the medium without causing cell death. Observations in other systems have shown that cortisone inhibits the early phenomena and later manifestations of the inflammatory response (Spain, 1961). Cortisone also inhibits lymphocyte function in vivo (Berenbaum, 1965). Reports have been made by Ambrose and Coons (1963a) that hydrocortisone replaced serum as a requirement for antibody synthesis by fragments of rabbit lymph nodes. This discrepancy has been resolved to some extent by subsequent studies of Ambrose (1966) showing that cortisone exerted its greatest effect during the inductive phase of the secondary immune response. This substance had little effect on the established

response. It appears that lymphocytes may vary in their sensitivity to cortisone during differentiation. The possibility also exists that cortisone was effective on cells other than lymphocytes in Ambrose's system.

Chloramphenicol, an antibiotic, has been reported to inhibit lymph node antibody synthesis (Ambrose and Coons, 1963b) and to inhibit bone marrow protein synthesis (Djordjevic and Szybalski, 1960). Chloramphenicol did not inhibit lymphocyte protein synthesis at concentrations up to 640 μg/ml in the medium during culture for 24 hours. Djordjevic and Szybalski measured protein synthesis by bone marrow cells five and six days after exposure of the cells to this drug. Ambrose (1966) has subsequently shown that chloramphenicol had its main effect on the induction of the secondary immune response and that the drug had little effect on the established response.

There are several explanations for the inactivity of chloramphenicol in lymphocyte cultures. Djordjevic and Szybalski experimented with a different cell population and incubated the cells for long periods of time with the drug. The experiments of Ambrose have shown that lymphocytes may vary in their sensitivity to the drug during differentiation

or that chloramphenicol was effective on other cell types.

6.4.3. Interaction of the drugs with PHA and plasma

The degree of inhibition by the drugs was not markedly altered when PHA was included in the medium. PHA stimulated lymphocyte protein synthesis three to four times (Chapter 3). The drugs did not inhibit the mechanism of PHA stimulation, apart from those that almost completely absolished protein synthesis (chloroquine, mefenamic acid).

Plasma in the culture medium reduced the inhibitory effects of the drugs on lymphocyte protein synthesis except in the case of chloroquine. Plasma probably bound the drugs and so reduced their effective concentration in the medium. Salicylate, phenybutazone and oxyphenbutazone have been reported to be strongly bound by plasma proteins (Randall, 1963). Inhibitory levels of oxyphenbutazone were reached in plasma medium when the concentration was increased to ten times its value in plasma-free medium. Presumably at this concentration drug had filled the plasma binding sites leaving free drug to inhibit lymphocyte protein synthesis.

The concentrations of the drugs, salicylate, hydrocortisone and phenylbutazone used in the investigations reported in this chapter were chosen to be within the range

of measured blood levels (Burns, et al., 1953) and below estimated maximum blood levels in the case of the other drugs.

During drug therapy, the binding sites of plasma proteins may be filled initially so that an accumulation of drug would occur.

If plasma binding did occur then the free blood level of the drug would be an underestimate of the combined amount of free drug and bound drug in the blood. The concentrations of the drugs at the site of inflammation and in the lymph fluid are not known. Nevertheless, the concentrations of some drugs used in the investigation reported may be never reached *in vivo* because of breakdown and excretion. Measurement of the capacity of lymphocytes to synthesise protein before and after therapy may help clarify the influence of the drugs *in vivo*.

The possibility also exists that lymphocytes *in vivo* are able to concentrate drugs from the blood, for example, chloroquine (Goodman and Gilman, 1965), so that the operative levels within the cell are higher than those in the blood.

6.4.4. *In vivo study of drug action*

In this chapter one *in vivo* study has been reported. Lymphocytes from a patient receiving prednisolone showed a reduced ability to incorporate $^{14}C$-leucine into protein after

the initiation of therapy, when these cells were cultured in plasma-free medium. By contrast the incorporation by the cells when cultured in plasma medium was variable. Factors in plasma may have masked the impaired function of the lymphocytes to incorporate $^{14}$C-leucine. Variable plasma drug concentrations too would have added to the inconsistency of the results.

PHA continued to stimulate the lymphocytes to produce more protein, although the degree of stimulation was less in plasma medium than in plasma-free medium.

The investigations reported suggest that cortisone *in vivo* had impaired lymphocyte protein synthesis, a result which had been observed with cortisone *in vitro*. The observed effects of the drug came some twelve days after the initiation of therapy. Forbes and Henderson (1966) reported a fall in serial measurements of globulin synthesis *in vitro* by lymphocytes from two patients after they had received treatment with prednisolone and ACTH respectively. These observations are compatible with the inhibitory effects of cortisone on immune responses *in vivo*.

6.4.5. RNA studies

Actinomycin D, mefenamic acid and chloroquine almost completely inhibited RNA synthesis by unstimulated lymphocytes

cultured for three hours. Chlorambucil, indomethacin, sulphinpyrazone and puromycin partially suppressed synthesis while oxyphenbutazone, cortisone and salicylate did not. Oxyphenbutazone suppressed protein synthesis more than sulphinpyrazone but the latter suppressed RNA synthesis more than the former. This suggests that a depression in RNA synthesis in lymphocytes may not be correlated with a proportional fall in protein synthesis. The toxic drugs, mefenamic acid and chloroquine may have had an indirect effect on RNA synthesis by killing the cell.

PHA, which stimulated lymphocyte protein and RNA synthesis, did not significantly alter the effects of the drugs on RNA synthesis. Actinomycin D, puromycin and mefenamic acid completely inhibited RNA synthesis by PHA-stimulated cells cultured for twelve hours. Sulphinpyrazone and indomethacin partially inhibited synthesis, while cortisone and oxyphenbutazone had no significant effect.

It is evident from these experiments that the drugs which inhibited lymphocyte protein synthesis did not all inhibit RNA synthesis.

The small amount of RNA synthesis by unstimulated lymphocytes incubated with actinomycin D for three hours was not found in two other cultures of normal cells treated

this way. This very small amount of synthesis was probably a consequence of (i) the variation of the non-specific incorporation of radioactive precursor into the RNA fraction as prepared by the methods described. (ii) The time for actinomycin D to enter the cell and block RNA synthesis. (iii) The life of the newly synthesised RNA.

RNA synthesis in PHA stimulated cells was blocked by actinomycin D, a finding confirmed by others (Cooper and Rubin, 1966). The small amount of protein synthesised in the presence of actinomycin D (Chapter 2) by unstimulated and PHA stimulated lymphocytes did not appear to depend on an actinomycin D resistant RNA synthesis.

The inhibition of RNA synthesis by puromycin was unexpected. In puromycin treated cultures 80% of the cells were viable by the criterion of trypan blue exclusion, after incubation for 24 hours. An experiment which is not reported in this chapter showed that the inhibition of protein synthesis by puromycin was reversible after three hours incubation (Appendix XI). These data suggest that the effect of puromycin on RNA synthesis was not associated with cell death caused by inhibition of protein synthesis.

Puromycin may have interfered with other metabolic pathways associated with RNA synthesis. Although puromycin has a pyrimidine ring structure the possibility cannot be excluded that it interferes with the incorporation of the purine, uridine, into RNA. These problems could be resolved by designing experiments with other protein inhibitors, which inhibit at the level of the ribosomes. Actidione, which has a different structure from puromycin but inhibits protein synthesis in a similar way (Siegel and Sisler, 1964) could be used in these investigations.

6.5. Summary

(1) Drugs with anti-inflammatory activity *in vivo* depressed lymphocyte protein synthesis *in vitro*. The activity of drug analogues and metabolites against protein synthesis reflected their activity as anti-inflammatory agents *in vivo*.

(2) Hydrocortisone *in vivo* depressed protein synthesis by lymphocytes cultured *in vitro* in plasma-free medium.

(3) Some of the drugs used were highly toxic to cells cultured in plasma-free medium. These drugs probably inhibit protein synthesis indirectly by killing the

cells. Many of the drugs were not toxic to cells. These drugs inhibited protein synthesis by mechanisms which did not lead to cell death.

(4) The inhibitory effects of many of the drugs were reduced when plasma was included in the medium. The binding of the drugs to plasma proteins was discussed as a possible mechanism for reducing their effectiveness.

(5) Studies of drug action on RNA synthesis by unstimulated and PHA-stimulated lymphocytes were reported. The unexpected depression of RNA synthesis by puromycin was discussed.

(6) PHA did not alter the degree of inhibition of protein or RNA synthesis by the drugs.

(7) It is suggested that suppression of protein synthesis by lymphocytes *in vitro* is related to the efficiency of a drug as an anti-inflammatory agent *in vivo*. It must therefore be considered likely that the anti-inflammatory effects of these drugs are due, at least in part, to their effects on cells of the lymphoid system.

Screening for capacity to inhibit protein synthesis by lymphocytes may be a useful procedure in selecting drugs for anti-inflammatory and immunosuppressive activity.

# CHAPTER 7

# THE EFFECTS OF THIOPURINES ON LYMPHOCYTE PROTEIN SYNTHESIS

## 7.1. Introduction

In Chapter 6 it has been reported that several anti-inflammatory drugs in clinical use inhibited lymphocyte protein synthesis *in vitro*. It was shown that the activity of many of the drugs *in vitro* could be correlated with their activity *in vivo*. In this chapter special attention has been given to the thiopurines. These include 6-mercaptopurine (6MP), an analogue of adenine and hypoxanthine, azathioprine (a derivative of 6-mercaptopurine that is rapidly converted into 6MP (Chalmers, *et al.*, 1967), and 6-thioguanine (6TG), an analogue of guanine.

The thiopurines are thought to interfere with purine synthesis at a number of points (Elion, 1967). The precise mechanisms by which these drugs interfere with the immune responses are not known. It has been suggested that they do this by inhibition at the level of nucleotide or nucleic acid synthesis. The studies reported in this chapter demonstrate the inhibitory effects of these drugs on lymphocyte protein synthesis.

Two pieces of evidence in the literature suggest that these drugs act by more rapid mechanisms than by inhibition of cell division and differentiation. One is the inhibition of the destructive effect of immunized lymph node cells on target cells by azathioprine (Wilson, 1965). The second is the inhibition by 6MP of lymphocyte migration in the inflammatory response, a process that is also blocked by actinomycin D (Page, 1964). These effects could be brought about by inhibition of protein synthesis by the thiopurines.

Azaguanine potently inhibits protein synthesis in Bacillus cereus (Chantrenne and Devreux, 1958; Mandel, 1958) but the thiopurines do not inhibit this synthesis (Mandel, et al., 1965). The effects of the thiopurines on mammalian cell protein synthesis has not been well documented. Several experiments were therefore designed to investigate the effects of these drugs on lymphocyte protein synthesis.

In the work reported in Chapter 6 attention was given to drug analogues; for example salicylate was active in vivo and in vitro, while para-hydroxybenzoate, which was inactive in vivo was also inactive in vitro. Since a number of thiopurine metabolites in vivo are becoming available and can be isolated, lymphocyte cultures offer unique opportunities to compare the activity of these metabolites with the parent drug. Such studies may assist in the detection of the

active form of the drugs and may lead to more effective administration. Experiments were therefore designed to investigate the activity of a number of thiopurine metabolites.

The aims of this chapter were to investigate:

(1) The effects of azathiopurine, 6MP, 6TG and a number of thiopurine metabolites on lymphocyte protein synthesis *in vitro*.

(2) The effects of azathioprine, 6MP and 6TG on the viability of lymphocytes cultured *in vitro*.

(3) The effects of azathioprine and 6MP on ribonucleic acid synthesis by lymphocytes.

(4) The metabolism of lymphocytes from patients treated with azathioprine.

## 7.2. Methods and Materials

### 7.2.1 Preparation of lymphocyte cultures

The lymphocytes were prepared and cultured from normal venous blood in the manner described previously. These methods and the determination of the incorporation of $^{14}C$-leucine into protein have been described in Chapter 2.

In the figures which are included in this chapter, the incorporation of $^{14}C$-leucine into protein in the TCA precipitate has been expressed as Percent Activity. Percent

Activity is defined as

$$\frac{\text{Counts per minute (CPM) in culture with drug}}{\text{Counts per minute (CPM) in control culture}} \times \frac{100}{1}$$

(This notation has been used in Chapter 6.)

The viability of the lymphocyte suspensions was determined by cell counting and trypan blue staining as described in Chapter 2.

The viability of the lymphocyte suspensions was expressed as Percent Viability (the same as that used in Chapter 6). Percent Viability has been defined as:

$$\frac{\text{Number viable cells in culture at time of sampling}}{\text{Number viable cells in culture at the commencement of incubation}} \times \frac{100}{1}$$

7.2.2. Nucleic acid synthesis

$^{14}$C-Guanine (Guanine-8-$^{14}$C sulphate monohydrate, specific activity 3.03 mc/mM, Radiochemical Centre, Amersham, England) and $^{3}$H-uridine were used in the culture medium at concentrations of 1-2 µc/ml to measure nucleic acid synthesis.

The incorporation of $^{3}$H-uridine into RNA was determined by methods previously described in Chapter 3. The incorporation of $^{14}$C-guanine into nucleic acid was determined by washing the cells on filter membranes (Standard Grade, Oxoid Courtaulds Ltd., Coventry, England). The cultures were kept

in an ice bath after incubation to inhibit activity. The cells were filtered on membrane filters with suction and were washed with 10 ml of normal saline followed by three washes with 15 ml volumes of 2% trichloroacetic acid (TCA). The incorporation was estimated by measuring the radioactivity of the cells on the membranes under the end window of a Geiger Meuller counter.

7.2.3. Drugs

The following drugs were added to the cultures (the abbreviations used in the figures are given in parenthesis together with the trade name):

6MP - 6-Mercaptopurine (-purinethol, pure powder, gift of Burroughs Wellcome and Co., (Australia) Ltd.).

AZA - Azathioprine (-imuran, pure powder, gift of Burroughs Wellcome).

6TG - 6-Thioguanine (Sigma Chemical Co., St. Louis, Mo., U.S.A.).

6MMPR - 6-Methyl mercaptopurine riboside (Sigma Chemical Co.).

IMI (1) - Imadazole compound isolated from the urine of patients on azathioprine therapy (gift from Mr. D. Chalmers, Department of Surgery, Adelaide,

identical with IMI(2) [Chalmers, et al., 1967]).

IMI (2) - 1-Methyl-4-nitro-5-mercapto imidazole, ammonium salt (pure powder, gift of Burroughs Wellcome).

2OH-6MP - 2-Hydroxy-6-mercaptopurine (pure powder, gift of Burroughs Wellcome).

6TU - 6-Thiouric acid (pure powder, gift of Burroughs Wellcome).

6MPN - 6-Mercaptopurine nucleotide (Atkinson, et al., 1963).

6MPR - 6-Mercapto purine riboside (powder, domestic grade, Sigma Chemical Co., Mo., U.S.A.).

## 7.3. Results

### 7.3.1. Effects on protein synthesis

The effects of 6MP, azathioprine and 6TG on lymphocyte protein synthesis are shown in Figure 7.1. 6TG and azathioprine showed similar inhibitory effects on protein synthesis while 6MP was least inhibitory of the three drugs.

Fig. 7.2 shows the effects of a number of metabolites of, and related thiopurines to, 6MP and azathioprine. The inhibitory effects of 6MP and azathioprine, which were tested simultaneously, are shown in each figure. These drugs were

Fig. 7.1. The effects of increasing concentrations of 6MP, azathioprine and 6TG in the medium or lymphocyte protein synthesis

Normal lymphocytes were cultured in supplemented Eagle's medium prepared without leucine and with $^{14}$C-leucine at a concentration of 0.25 μc/ml.

The drugs:

6MP - 6-Mercaptopurine

AZA - Azathioprine

6TG - 6-Thioguanine

were included in the medium at the concentrations shown. Each set of points represents triplicate determinations made by using the drugs with one preparation of lymphocytes.

The incorporation of $^{14}$C-leucine has been expressed as Percent Activity, which is defined as:

$$\frac{\text{Counts per minute in culture with drug}}{\text{Counts per minute in control culture}} \times \frac{100}{1}$$

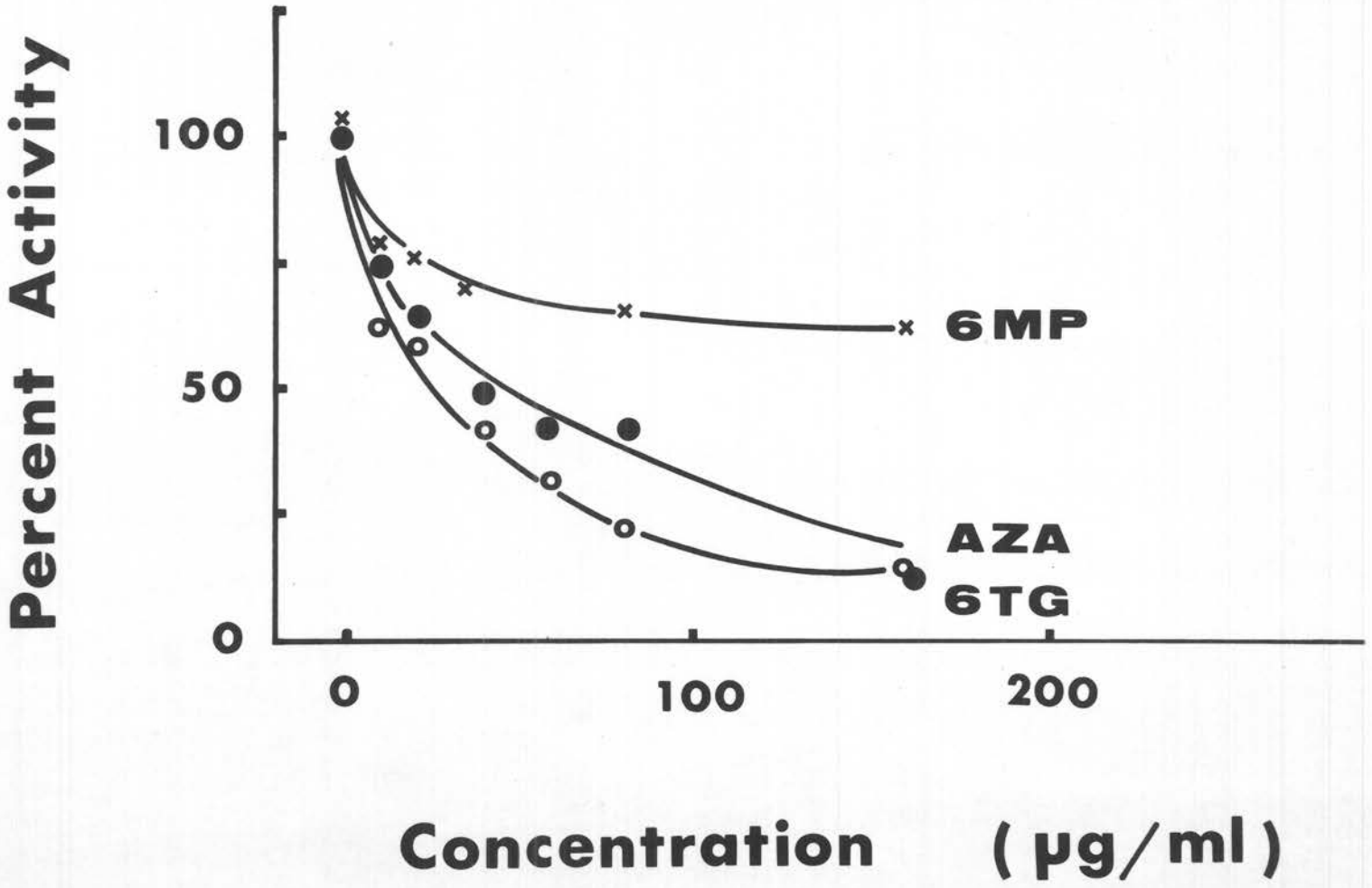

Percent Activity
100
50
0
0
100
200
6MP
AZA
6TG
Concentration (µg/ml)

Fig. 7.2. <u>The effects of a number of metabolites and analogues of 6MP and azathioprine on lymphocyte protein synthesis</u>

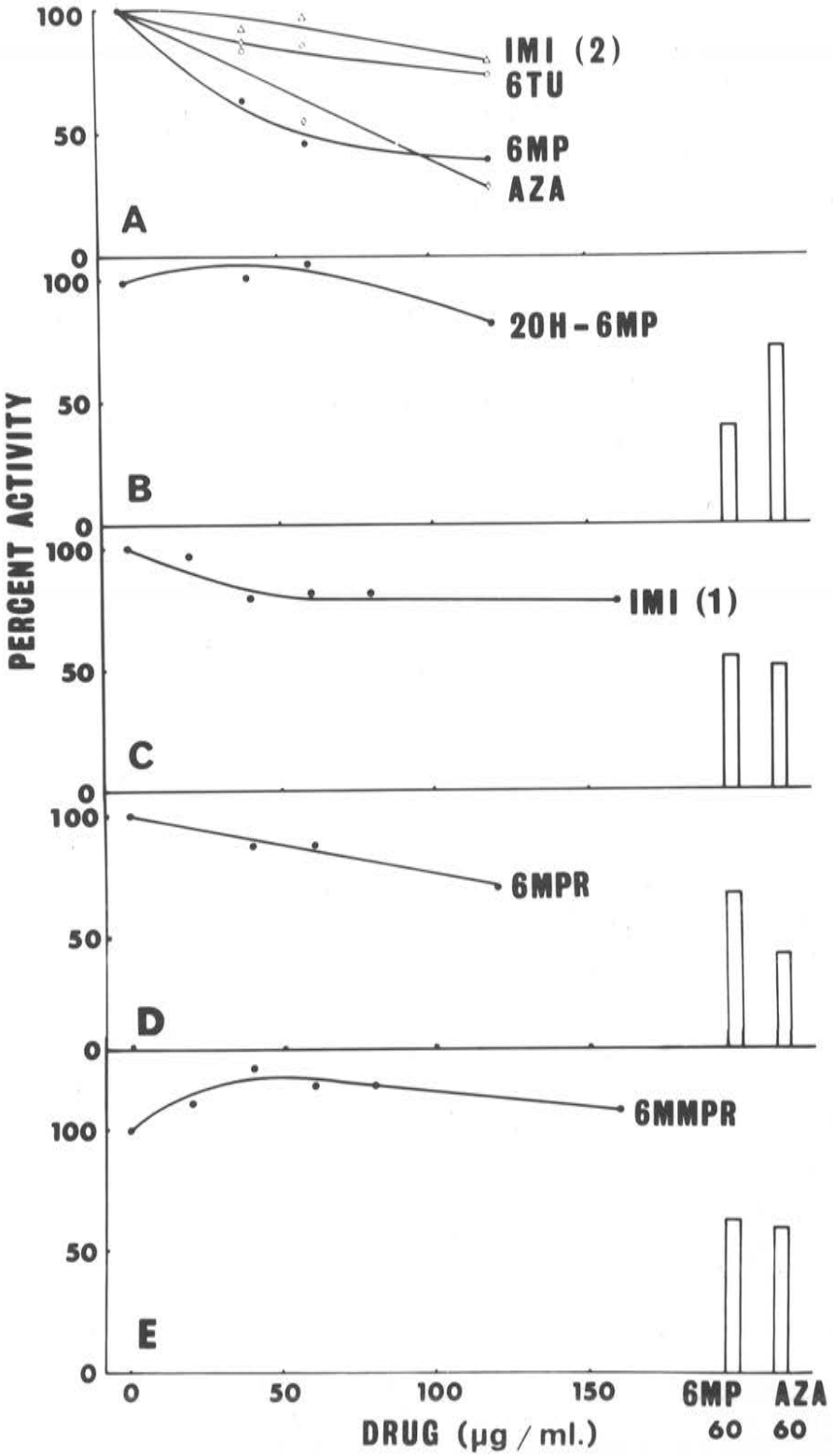
Normal lymphocytes were cultured in supplemented Eagle's medium prepared without leucine and with $^{14}$C-leucine at a concentration of 0.25 μc/ml. A, B, C, D, and E represent experiments performed with different preparations of normal lymphocytes. The drugs

IMI(2) - 1-Methyl-4-nitro-5-mercaptoimidazole
6TU - 6-Thiouric acid
6MP - 6-Mercaptopurine
AZA - Azathioprine
2OH6MP - 2-Hydroxy-6-mercaptopurine
IMI(1) - Imidazole metabolite from azathioprine
6MPR - 6-Mercaptopurine riboside
6MMPR - 6-Methyl mercaptopurine riboside

were included in the culture medium of lymphocyte preparations at the concentrations shown in the figure.

In experiment A, 6MP and AZA were included at three concentrations in the medium. In the other experiments these drugs were included at one concentration only. The inhibitions produced at these concentrations are shown as histograms.

The incorporation of $^{14}$C-leucine has been expressed as percent activity.


PERCENT ACTIVITY
100
50
0
IMI (2)
6TU
6MP
AZA
A
20H-6MP
B
IMI (1)
C
6MPR
D
6MMPR
E
0
50
100
150
6MP
60
AZA
60
DRUG (µg / ml.)

always more inhibitory than the metabolites.

The inhibitory effects of the drugs varied when they were tested with lymphocytes from different normal donors (Fig. 7.3). 6MP was the most variable of the drugs in its effect on protein synthesis and in some cases this drug was not inhibitory. By comparison, azathioprine inhibited to the same extent and often more than 6MP, when these drugs were used at the same concentrations in lymphocyte cultures prepared from the same donor. In a minority of tests, 6MP was more inhibitory than azathioprine. 6TG was inhibitory in every test. The metabolites and analogues of the thiopurines had little inhibitory effect on lymphocyte protein synthesis. The imidazole derivatives IMI (1) and IMI (2), and 6-thiouric acid had a slight inhibitory effect. 6-Methyl mercaptopurine riboside was not inhibitory, while 2-hydroxy 6 mercaptopurine was slightly inhibitory at high concentrations.

6-Mercaptopurine nucleotide, tested on one occasion, produced a small amount of inhibition, but 6MP tested on the same occasion was twice as inhibitory. 6-Mercaptopurine riboside also produced a small amount of inhibition.

Culture fluids from lymphocytes incubated with 6MP and with azathioprine at concentrations of 60 μg/ml, labelled

Fig. 7.3. <u>The effects of drugs on preparations of lymphocytes from different normal donors</u>

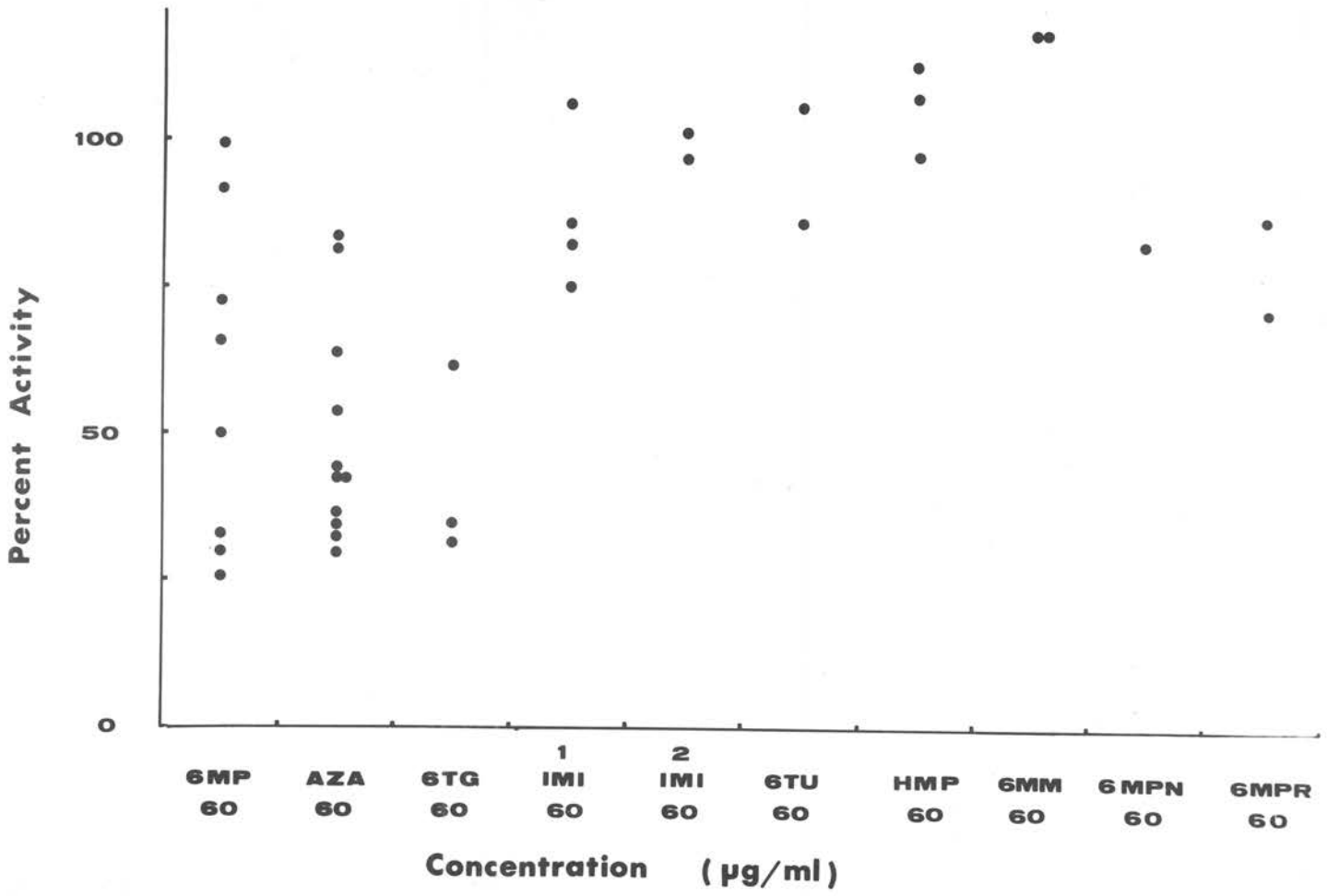
Normal lymphocytes were cultured in supplemented Eagle's medium prepared without leucine and with $^{14}$C-leucine at a concentration of 0.25 μc/ml.

The drugs:

- 6MP - 6-Mercaptopurine
- AZA - Azathioprine
- 6TG - 6-Thioguanine
- IMI(1) - Imadazole metabolite from azathioprine
- IMI(2) - 1-Methyl-4-nitro-5-mercaptoimidazole
- 6TU - 6-Thiouric acid
- HMP - 2-Hydroxy-6-mercaptopurine (2OH6MP)
- 6MM - 6-Methyl mercaptopurine riboside (6MMPR)
- 6MPN - 6-Mercaptopurine nucleotide
- 6MPR - 6-Mercaptopurine riboside

were included in the medium of lymphocyte preparations at the concentrations shown in the figure.

Each point in the figure represents a determination made in triplicate with one preparation of normal lymphocytes. The incorporation of $^{14}$C-leucine has been expressed as percent activity.


Percent Activity
100
50
0
6MP 60
AZA 60
6TG 60
1 IMI 60
2 IMI 60
6TU 60
HMP 60
6MM 60
6MPN 60
6MPR 60
Concentration (µg/ml)

the same protein arcs in IEP patterns as those found in patterns prepared from cultures of untreated lymphocytes. These drugs did not appear to selectively inhibit the production of any particular protein.

7.3.2. Interaction with plasma

The presence of 25% plasma in the medium modified the effects of azathioprine, 6MP and 6TG on protein synthesis (Fig. 7.4). The effect of plasma was not as marked with 6TG and azathioprine as it was with 6MP. On one occasion with 6MP and on two occasions with azathioprine, the inhibitory effects of these drugs were increased in the presence of plasma.

7.3.3. Interaction with PHA

The degree of inhibition of lymphocyte protein synthesis by the three drugs 6MP, azathioprine and 6TG was the same in cultures stimulated with PHA as in unstimulated cultures (Fig. 7.5).

7.3.4. Effects on lymphocyte viability

The effects of the three thiopurines, 6MP, azathioprine and 6TG on lymphocyte viability at concentrations of 60 µg/ml in the culture medium, are shown in Fig. 7.6. Azathioprine was the most cytotoxic of the three drugs. The survival curve for this drug showed an accelerated rate of cell death in the

Fig. 7.4. Effect of plasma in the medium on the inhibitory effects of 6MP, azathioprine and 6TG on protein synthesis

Normal lymphocytes were cultured in supplemented Eagle's medium prepared without leucine and with or without autologous plasma. $^{14}$C-leucine was included in the medium at a concentration of 0.25 µc/ml. Lines join paired observations of the same drug with one preparation of lymphocytes.

○ - represents triplicate determinations made in 25% plasma medium.

● - represents triplicate determinations made in plasma-free medium.

The drugs:

6MP - 6-Mercaptopurine

AZA - Azathioprine

6TG - 6-Thioguanine

were included in the culture medium at the concentrations shown in the figure.

The incorporation of $^{14}$C-leucine has been expressed as percent activity.

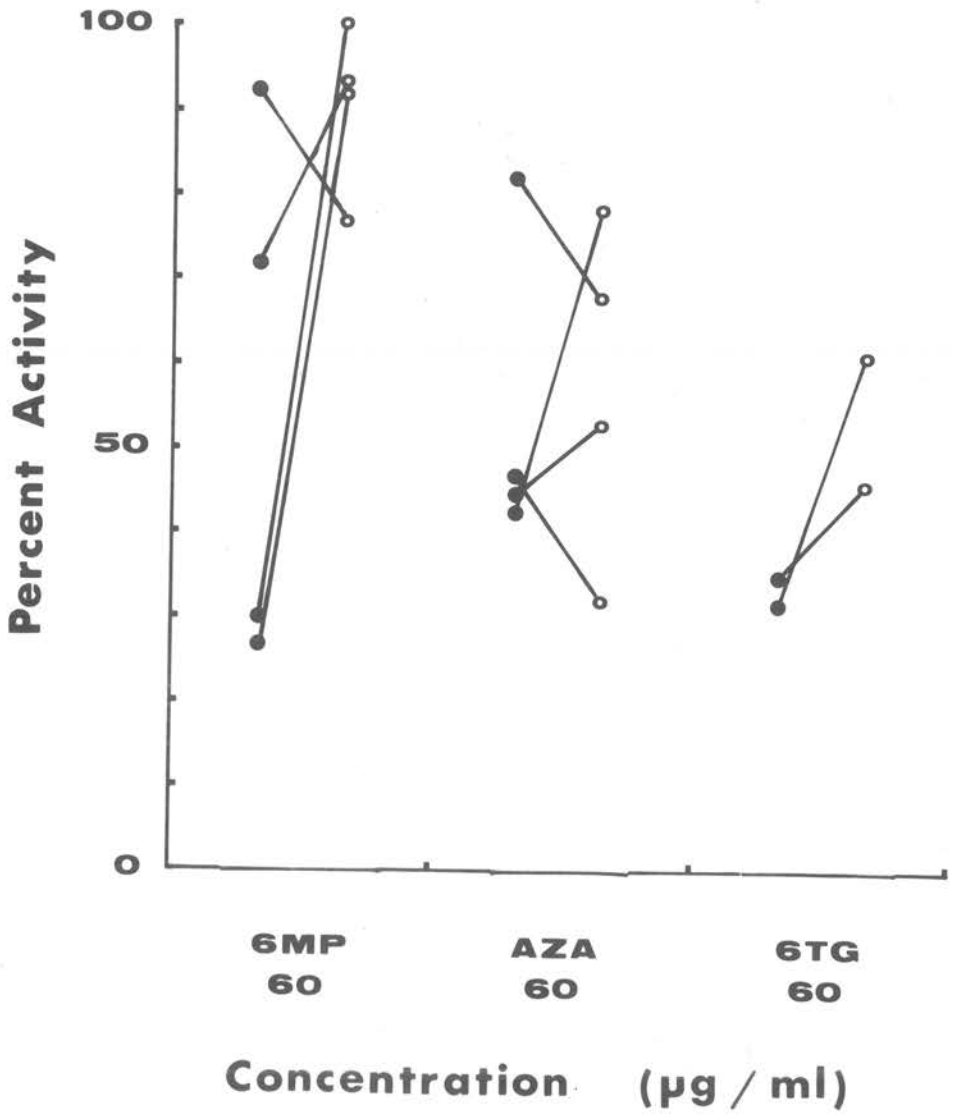

100
50
0
Percent Activity
6MP
60
AZA
60
6TG
60
Concentration (µg / ml)

Fig. 7.5. Interaction of the drugs 6MP, 6TG and azathioprine with PHA

The data were compiled from a number of experiments. Normal lymphocytes were cultured in plasma-free, supplemented Eagle's medium prepared without leucine and with $^{14}$C-leucine at a concentration of 0.25 μc/ml. PHA stimulated protein synthesis 3-4 times that in control cultures. Each point in the figure represents a determination made in triplicate with one preparation of lymphocytes.

The drugs:

6MP - 6-Mercaptopurine

6TG - 6-Thioguanine

AZA - Azathioprine

were included in the medium at the concentrations shown in the figure.

The incorporation of $^{14}$C-leucine has been expressed as percent activity.

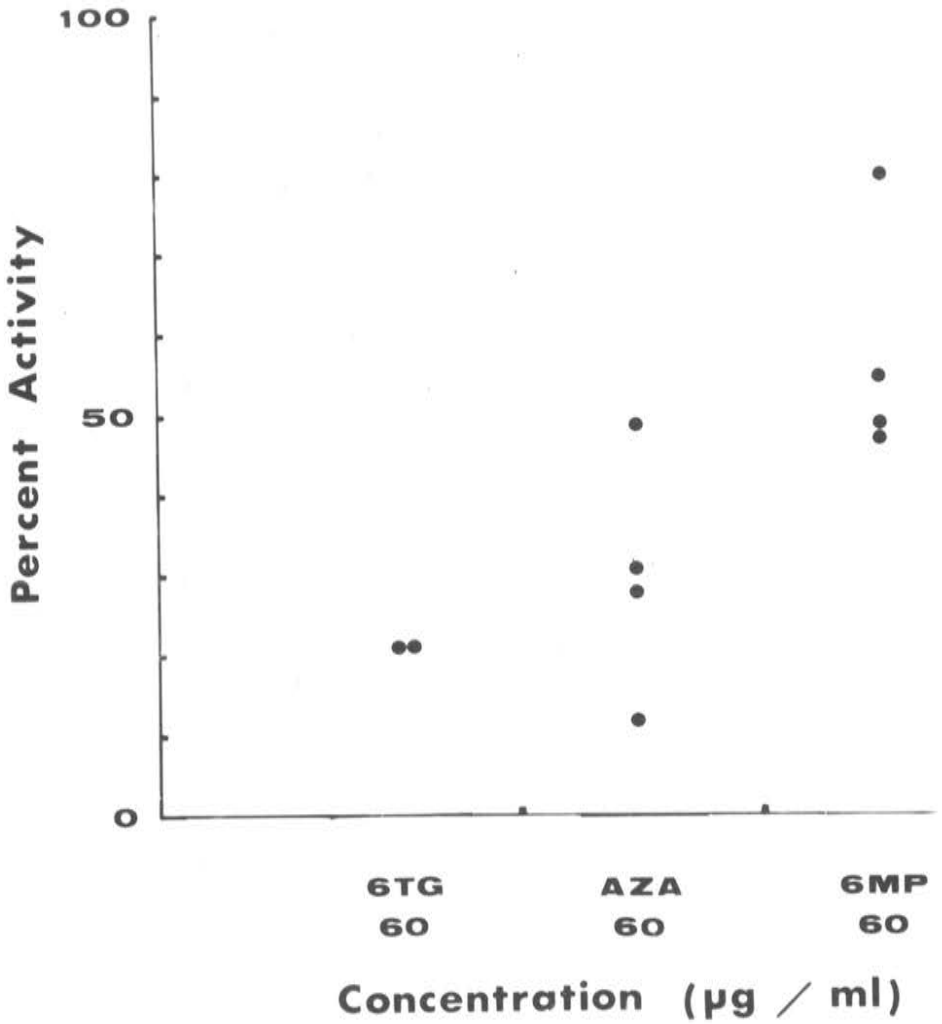

100
50
0
Percent Activity
6TG
60
AZA
60
6MP
60
Concentration (µg / ml)

Fig. 7.6. The effect of drugs on the viability of normal lymphocytes

Normal lymphocytes were cultured in plasma-free supplemented Eagle's medium prepared without leucine. Lymphocyte viability was determined as described in the text at various times during incubation. Each set of points for each drug was determined in triplicate with one preparation of lymphocytes. In each experiment cultures without drug were always included as controls.

Viability of the cells has been expressed as percent viability which is defined as:

$$\frac{\text{No. cells viable in culture at time of sampling}}{\text{No. cells viable in culture at the commencement of incubation}} \times \frac{100}{1}$$

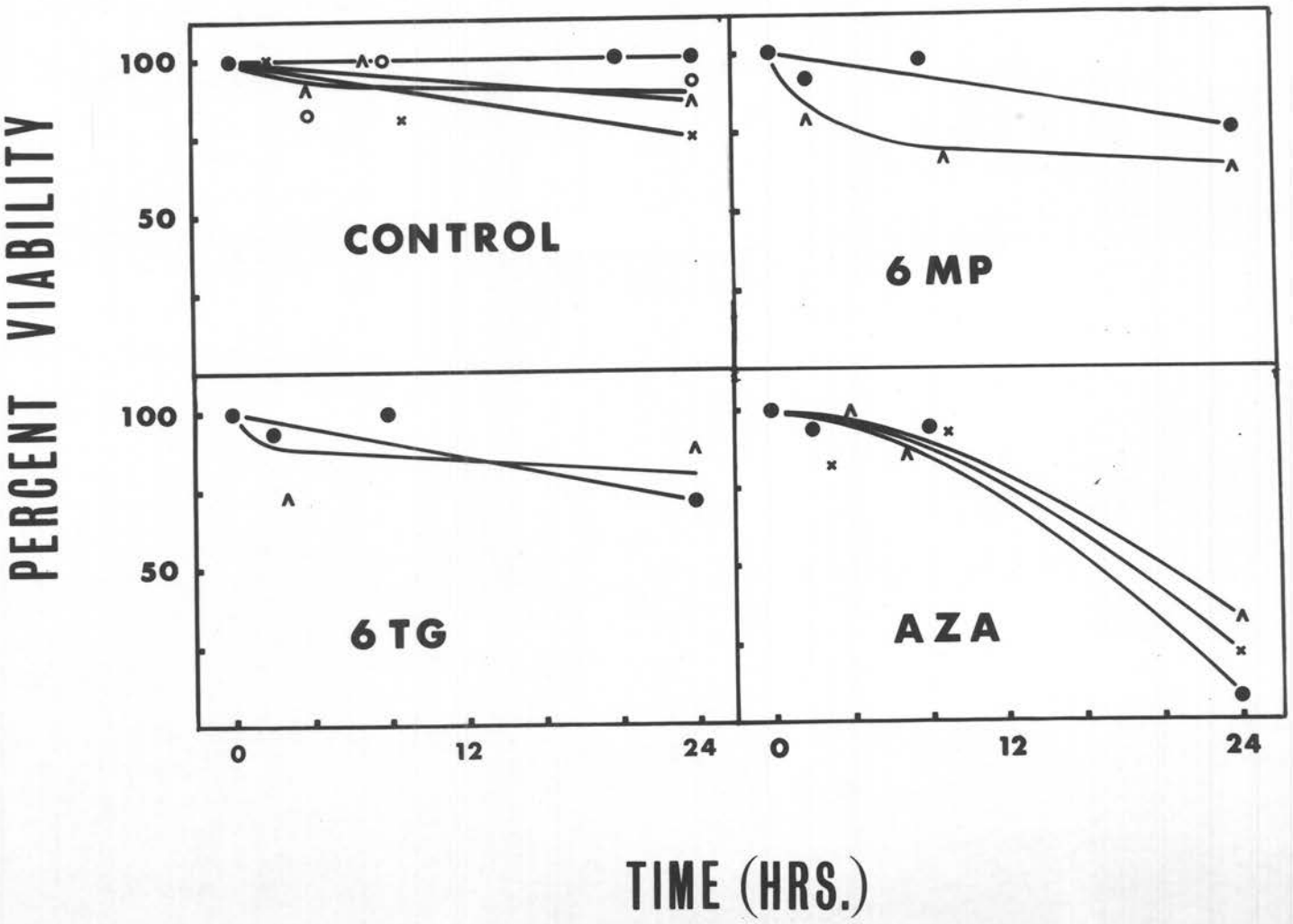
The drugs:

6MP - 6-Mercaptopurine

6TG - 6-Thioguanine

AZA - Azathioprine

were included in the medium at concentrations of 60 μg/ml.


PERCENT VIABILITY
100
50
CONTROL
6 MP
100
50
6 TG
AZA
0
12
24
0
12
24
TIME (HRS.)

second half of the incubation period. This suggested a second order effect, such as the accumulation of a toxic product. The effects of 6MP and 6TG on lymphocyte viability did not suggest that they were lethal.

7.3.5. Effects on nucleic acid synthesis

6MP had a small inhibitory effect on the incorporation of $^{3}$H-uridine into RNA by unstimulated lymphocytes, but the drug had no significant effect on the incorporation into RNA by PHA-stimulated cells. Azathioprine did not have any significant effect on the incorporation of precursor by unstimulated or stimulated cultures of lymphocytes (Fig. 7.7).

The effects of 6MP and azathioprine on the incorporation of $^{14}$C-guanine into nucleic acid are shown in Table 7.1. On three occasions 6MP inhibited uptake, while azathioprine inhibited only once. The results were variable and actinomycin D was not completely inhibitory. Killed cells did not incorporate precursor into nucleic acid.

7.3.6 "In vivo" studies

Two patients were studied. Blood was taken at intervals before and during drug therapy. The lymphocytes were prepared and cultured in plasma-free medium and medium containing autologous plasma taken at the same occasion. The cells were cultured for 24 hours and after freezing and thawing,

Fig. 7.7. The effects of 6MP and azathioprine on the incorporation of $^{3}$H-uridine into RNA

In experiments A and B, normal lymphocytes were cultured in supplemented Eagle's medium prepared without leucine. The incorporation of $^{3}$H-uridine has been expressed as counts/20 min, in 1 ml of the RNA extract (total volume 5 ml). The incorporation was determined as described in Chapter 3, section 3.2.4.

The drugs:

6MP - 6-Mercaptopurine

AZA - Azathioprine

were included in the medium of unstimulated and PHA-stimulated lymphocytes at the concentrations shown in the figure.

A. In this experiment the drugs were included in the medium of unstimulated lymphocytes. The incorporation by a culture without drug is indicated as CONT in the figure. The cultures contained 2.7 x $10^{6}$ lymphocytes and 2 μc $^{3}$H-uridine. The cultures were terminated after 3 hours' incubation.

B. In this experiment drugs were included in the medium of PHA-stimulated lymphocytes. Unstimulated lymphocytes were included as a control (CONT). The cultures contained 3.3 x $10^{6}$ cells and 2 μc $^{3}$H-uridine. The cultures were terminated after incubation for 12 hours.

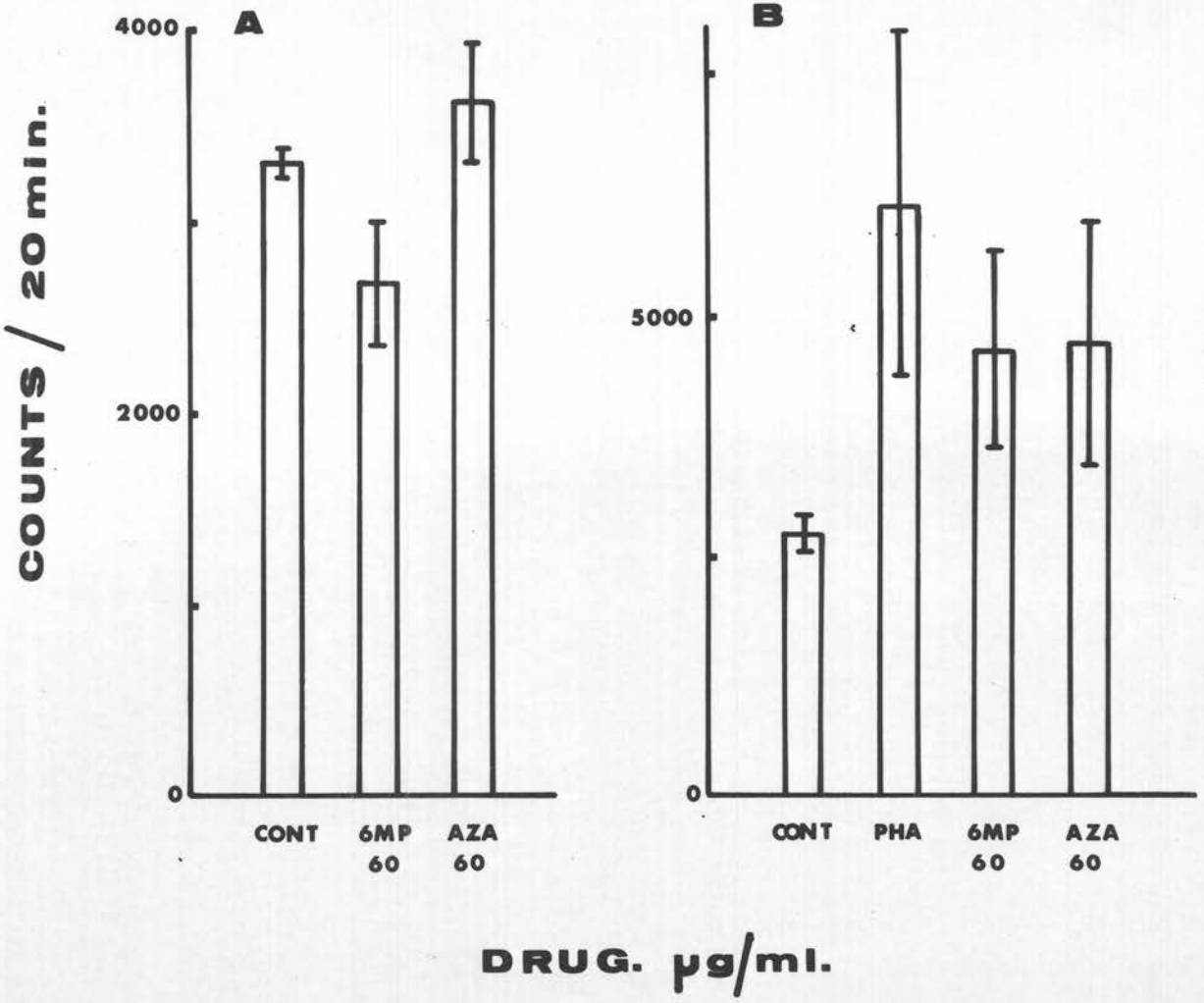

A
B
4000
2000
0
5000
0
COUNTS / 20 min.
CONT
6MP 60
AZA 60
CONT
PHA
6MP 60
AZA 60
DRUG. µg/ml.

Table 7.1. The effect of drugs on the incorporation of $^{14}$C-guanine into nucleic acid by cultures of unstimulated lymphocytes

Normal lymphocytes were cultured in supplemented Eagle's medium prepared without leucine. The other experimental conditions were:

A - these cultures contained 1.9 x $10^6$ cells and 1 µc of $^{14}$C-guanine;

B - these cultures contained 2.7 x $10^6$ cells and 1 µc of $^{14}$C-guanine;

C - these cultures contained 1.5 x $10^6$ cells and 1.5 µc of $^{14}$C-guanine.

The drugs:

Act D - Actinomycin D

AZA - Azathioprine

6MP - 6-Mercaptopurine

were included in the culture medium at the concentrations shown in the table. The incorporation of $^{14}$C-guanine was measured as described in the Methods. The incorporation has been expressed as counts per minute (CPM) in the cells after preparation for counting. These values are given with their standard deviations.

Actinomycin D did not inhibit completely the uptake of $^{14}$C-guanine. 6MP inhibited the uptake on the three occasions tested, while azathioprine inhibited only once.

TABLE 7.1

| Expt. | Drug | Dose (μg/ml) | CPM |
|---|---|---|---|
| A | - | - | 806 ± 24 |
| | Act D | 3.3 | 170 ± 43 |
| | AZA | 60 | 295 ± 18 |
| | 6MP | 60 | 378 ± 89 |
| B | - | - | 692 ± 281 |
| | Act D | 3.3 | 329 ± 111 |
| | 6MP | 60 | 421 ± 212 |
| | AZA | 60 | 685 ± 244 |
| C | - | - | 83 ± 27 |
| | Act D | 3.3 | 60 ± 21 |
| | AZA | 60 | 91 ± 18 |
| | 6MP | 60 | 50 ± 15 |

the radioactivity in proteins in the TCA precipitate was determined.

The results of these investigations are shown in Table 7.2 and 7.3. The drug regimes are shown in each table. Patient I was receiving ACTH, prednisolone and azathioprine; patient II was receiving azathioprine only.

Lymphocytes from two patients receiving azathioprine showed a change in their lymphocyte protein synthesis ability. Patient I was receiving betamethasone and ACTH prior to azathioprine therapy. ACTH was continued for a short period while betamethasone was continued as before the initiation of the patient to azathioprine. Lymphocytes from this patient synthesised little protein when cultured in plasma or plasma-free medium while ACTH treatment was continued. The synthesis by the lymphocytes rose when ACTH was discontinued and fell subsequently as the administration of azathioprine and betamethasone was continued. The fall in lymphocyte protein synthesis was most marked when the cells were cultured in plasma medium.

PHA did not stimulate protein synthesis on three occasions when included in plasma-free medium. On the other two occasions the stimulation of protein occurred when

Table 7.2. 'In vivo' study of the effects of ACTH, betamethasone and azathioprine

Lymphocytes were isolated from a male patient suffering from a skin allergy caused by an industrial accident with chromium. The lymphocytes were prepared as described in the methods except that the low speed centrifugation (150 x g) was omitted. The cultures contained 1-2 x $10^6$ cells and 0.25 µc of $^{14}$C-leucine.

In Table A, the incorporation of precursor by the cells has been expressed as counts/$10^6$ cells/min/ 1/4 µc $^{14}$C-leucine in the medium. Blood was taken from the patient and the lymphocytes were prepared at the times shown in the table. The culture volumes were 1 ml. Freeze-dried portions of the same batch of PHA were used at every occasion at the same concentration in the medium. Supplemented Eagle's medium prepared without leucine was used routinely with or without autologous plasma sampled at the same time with the lymphocytes.

The drug regimes during the collection of blood samples are given in B.

TABLE 7.2

A.

| Date | PHA | Counts No plasma | % Increase c̄ PHA | Counts 25% plasma | % Increase c̄ PHA |
|---|---|---|---|---|---|
| 18/10 | - | 26 | | 26 | |
| | + | - | - | 58 | 207 |
| 19/1o | - | 7 | | 17 | |
| | + | 2 | - | 26 | 153 |
| 20/10 | - | 109 | | 185 | |
| | + | 45 | - | 369 | 199 |
| 23/10 | - | 105 | | 79 | |
| | + | 79 | - | 220 | 279 |
| *25/10 | - | 484 | | 251 | |
| | + | - | - | - | - |
| 27/10 | - | 317 | | 354 | |
| | | 626 | 197 | 946 | 267 |
| 3/11 | - | 497 | | 265 | |
| | + | - | - | - | - |
| 10/11 | - | 60 | | 92 | |
| | + | 160 | 270 | 343 | 370 |
| 23/11 | - | 257 | | 125 | |
| | + | - | - | - | - |

B.

| Drug | Dose | Start | Finish |
|---|---|---|---|
| ACTH (long acting) | 20 i.u. | | 19/10 |
| ACTH (short acting) | 20 i.u. | 25/10 | 25/10 |
| | | 30/10 | 30/10 |
| Betamethasone | 0.75 mg t.d.s. | CONTINUOUS | |
| Azathioprine | 50 mg t.d.s. | 18/10 | |

Table 7.3. 'In vivo' study of the effects of azathioprine

Lymphocytes were isolated from a female patient suffering from kerato-conjunctivitis sicca. The lymphocytes were prepared as described in the Methods with the exception that the low speed centrifugation (150 x g) was omitted. The cultures contained 0.8 - 1.3x $10^6$ cells and 0.25 μc of $^{14}$C-leucine.

In Table A the incorporation of precursor by the cells has been expressed as counts/$10^6$ cells/min/1/4 μc of $^{14}$C-leucine in the medium. Blood was taken from the patient and the lymphocytes were prepared at the times shown in the table. The culture volumes were 1 ml. Freeze dried aliquots of the same batch of PHA were used at every occasion at the same concentration in the medium. Supplemented Eagle's medium prepared without leucine was used routinely with or without autologous plasma sampled at the same time with the lymphocytes.

The drug regime during the collection of blood samples is given in B.

TABLE 7.3

A.

| Date | PHA | Counts No plasma | % $\bar{c}$ PHA Increase | Counts Plasma | % increase $\bar{c}$ PHA |
|---|---|---|---|---|---|
| 24/10 | - | 159 | | 74 | |
| | + | 465 | 292 | 227 | 306 |
| *25/10 | - | 60 | | 94 | |
| | + | - | - | - | - |
| 27/10 | - | 51 | | 60 | |
| | + | 68 | 133 | - | - |
| 31/10 | - | 60 | | 50 | |
| | + | 150 | 250 | - | - |
| 7/11 | - | 136 | | - | |
| | + | - | - | - | - |
| 14/11 | - | 50 | | 48 | |
| | + | 73 | 146 | 126 | 262 |
| 22/11 | - | 136 | | 54 | |
| | + | - | - | - | - |

B.

| Drug | Dose | Start | Finish |
|---|---|---|---|
| Azathioprine | 50 mgm t.d.s. | 25/10 | |

* Blood taken before administration of drug.

ACTH administration was discontinued. Patient II received azathioprine only. Protein synthesis by the lymphocytes cultured in plasma-free medium did not change significantly, but the protein synthesis activity of the lymphocytes in plasma medium showed a slight fall in their ability to incorporate precursor. PHA increased protein synthesis, but on two occasions in plasma-free medium the stimulation by PHA was less than expected.

7.4. Discussion

The thiopurines, 6-thioguanine, 6-mercaptopurine and azathioprine all inhibited protein synthesis. Azathioprine (with a molecular weight approximately twice that of 6-mercaptopurine) was more effective or as effective as 6-mercaptopurine at the same concentration in the culture medium. On a molar basis azathioprine was twice as effective as 6MP. 6-Thioguanine at the same concentration showed inhibition of protein synthesis similar to that with azathioprine. The effects of 6MP were more variable than those with azathioprine when tested with lymphocytes from different patients. The differences in inhibition were most marked when PHA was included in the medium. In the presence of PHA azathioprine was always more inhibitory than 6MP. The percentage inhibitions produced by the various drugs was of the same order whether PHA

was included in the medium or not. This result suggests that the drugs did not interfere with the mechanism of PHA stimulation.

Plasma in the medium acted in most cases to reduce the inhibitory effects of the drugs except in one case with 6MP and in two cases with azathioprine.

The cells survived well in the presence of the thiopurines except azathioprine. The survival curve with azathioprine showed a decline in the latter half of the incubation period, an effect which suggested the accumulation of a toxic product, but it is unlikely that this drug inhibited protein synthesis by being lethal during the early hours of incubation. The other drugs did not appear to exert their inhibitory effects by being lethal to cells. Berenbaum (1965) has observed that thioguanine was immunosuppressive *in vivo*, without causing destruction of lymphoid tissue.

Elion (1967) has suggested a number of places at which the thiopurines could interfere with protein synthesis. In the experiments of Grünberger and Mandel (1965) with *Bacillus cereus*, 8-azaguanine did not interfere with the formation of m-RNA or with its ability to function. However, this drug is not an immunosuppressive agent. 6MP had a small but

significant effect on $^{3}$H-uridine incorporation into RNA by unstimulated cells but had no significant effect on the incorporation by PHA-stimulated cells. Azathioprine had no significant effect on the incorporation by unstimulated or PHA-stimulated cells. This incorporation was actinomycin D sensitive, suggesting that most of the RNA was m-RNA. It does not appear therefore that the drugs exert their inhibitory effects on lymphocyte protein synthesis by inhibiting m-RNA synthesis.

The thiopurines may be expected to interfere with t-RNA, but in other systems t-RNA containing 8-azaguanine continued to function normally (Levin, 1965; Weinstein and Grönberger, 1965). The drugs may interfere with the coenzyme function of guanine triphosphate (GTP) in protein synthesis, although in the experiments reported by Roy and co-workers (1961) thioinosine triphosphate and thioguanine triphosphate did not inhibit GTP coenzyme function. The thiopurines may be active at the level of ribosomal RNA but further investigation is needed to investigate this point.

The possibility cannot be excluded that the thiopurines may be incorporated into m-RNA, producing a 'nonsense'-code and malfunction of the messenger.

The greater inhibitory effects of azathioprine compared with 6-mercaptopurine may be caused by a number of factors: (i) Azathioprine itself may be more effective than 6MP. (ii) Azathioprine which is an imidazole derivative of 6MP may act as an immunosuppressive agent by the slow release of 6MP. (iii) Azathioprine derivatives may be more active than 6MP or its derivatives.

Azathioprine and 6MP are both inhibitors of the homograft reaction *in vivo* (Berenbaum, 1965). However, in the dog, azathioprine was more effective than 6MP (Calne, 1961; Calne, *et al.*, 1962) in blocking the homograft reaction.

Azathioprine was also more effective than 6MP in inhibiting the formation of haemagglutinating antibodies in mice, following the injection of sheep red cells (Nathan, *et al.*, 1961). When the mice were treated with glutathione prior to the administration of azathioprine, the responses to azathioprine and 6MP were the same.

Azathioprine is split by sulphydryl groups, which are present in glutathione, to release 6MP (Bresnick, 1959; Chalmers, *et al.*, 1967). In the presence of glutathione, azathioprine would release 6MP and be as effective as this drug, provided the imidazole derivative was inactive.

It is therefore possible that azathioprine was more effective against lymphocyte protein synthesis in the studies reported here because it acted to release 6MP throughout the incubation period. In contrast, 6MP administered initially, may have been rapidly oxidized to 6 thiouric acid by xanthine oxidase. This enzyme would reduce the effective concentration of 6MP in themedium. Alternatively it is possible that the permeability of the cells to azathioprine and 6MP differ. Lymphocytes may be more permeable to azathioprine, so that once within the cell, azathioprine acted to release 6MP and inhibit protein synthesis.

Modification of the inhibitory effects of drugs in plasma medium may have been the result of:

(i) The drugs may have been bound to plasma, reducing their effective concentration. This would have explained a reduction, but not an increase in the effectiveness of the drugs.

(ii) An increase in xanthine oxidase activity in the presence of plasma would have increased the oxidation of 6MP to 6 thiouric acid and hence would have reduced the activity of 6MP.

(iii) Sulphydryl groups in plasma proteins may be expected to increase the production of 6MP from azathioprine. An

increase in the activity of azathioprine in the presence of plasma may have been explained by the enhanced but incomplete release of 6MP which would have been effective (unless there was a permeability barrier to 6MP) throughout the incubation. Where the activity of azathioprine was reduced in the presence of plasma, there may have been sufficient sulphydryl groups to split azathioprine rapidly to 6MP, reducing the effectiveness of the drug to that of 6MP included in the medium initially.

The inhibitory effect of 6MP on one occasion was increased in plasma medium. The explanations offered do not fully explain the observed differences in the inhibitory activities of the drugs in plasma and plasma-free medium. These differences may be in some cases related to differences in the permeability of the cells in plasma and plasma-free medium. The data suggest that the inhibitory effects of azathioprine and 6MP in both types of media vary between preparations of normal lymphocytes.

In vivo 6MP is metabolized by two main pathways (Elion, 1967). One is by the direct oxidation of the drug to 6-thiouric acid. The other involves the methylation of the sulphur moiety followed by similar oxidative reactions of the methylated derivatives.

Azathioprine is metabolized in man to produce 6-thiouric acid as its principal metabolite (Elion, et al., 1961). The urine of patients also contains a yellow metabolite that has been identified as 6-mercapto- 1-methyl-4-nitroimidazole (Chalmers, et al., 1967). The same compound is formed under alkaline conditions by the action of sulphydryl groups, where the sulphur of azathioprine remains with the purine moiety (6MP) and the attacking sulphydryl compounds attaches to the nitro methylimidazole moiety (Bresnick, 1959, Elion, et al., 1961; Elion, et al., 1962).

When 6-thiouric acid and 5-mercapto-1-methyl-4-nitroimidazole were tested in vitro for their effects on protein synthesis, they had a very small inhibitory effect compared with the inhibition produced by 6MP and azathioprine tested at the same occasion. 2-Hydroxy-6MP, a hydroxy derivative of 6MP and related to the metabolites of 6MP in vivo (Elion, 1967) did produce significant inhibition of lymphocyte protein synthesis at high concentrations in the medium. This substance stimulated protein synthesis at concentrations of 60 μg/ml in the medium. Another metabolite of 6MP, 6-methyl-mercaptopurine riboside was not inhibitory on the two occasions tested, despite the fact that 6MP was

active on these occasions.

The data suggest that 6MP and azathioprine, inhibited lymphocyte protein synthesis via mechanisms which depended on the structure and activity of these drugs and not via their known metabolites *in vivo*. The activities of 6-mercaptopurine ribonucleotide and 6-mercaptopurine riboside were exceptional. 6-Mercaptopurine ribonucleotide was tested only once. The activity of this compound was half that of 6MP, but on a molar basis, the activities of these two substances were the same. It is possible that the riboside and nucleotide are broken down at the cell wall to 6MP, which enters the cell and acts to inhibit protein synthesis. Under physiological conditions, one would not expect passage of the nucleoside and nucleotide through the cell wall into the cell.

Experiments reported in this chapter suggested that 6MP inhibited the incorporation of $^{14}$C-guanine into nucleic acid; azathioprine was inhibitory once out of three occasions tested. The results of these experiments were variable and do not justify firm conclusions. The purpose of these studies was to investigate this problem in a preliminary way. Considerable difficulty was experienced in the deter-

mination of the incorporation of precursor. Two of these difficulties were, (i) to prevent the hydrolysis of guanine from nucleic acid during extraction, (ii) to remove from the extracts free guanine and guanine nucleotide not incorporated into RNA. The first of these difficulties was resolved by carrying out all extraction procedures at $4^{o}C$. The second was overcome to some extent by washing the cells with large quantities of TCA, but this difficulty would contribute to variation in the triplicate determinations.

6MP may inhibit guanine uptake into nucleic acid via its nucleotide (Elion, 1967). The difference in activity between 6MP and azathioprine suggests that this inhibitory mechanism may help to explain the effects of 6MP but not of azathioprine.

The work reported in this chapter has shown that the thiopurines inhibit lymphocyte protein synthesis. This may be one of the mechanisms by which they exert their function *in vivo* as immunosuppressive agents. The effect on protein synthesis would assist in explaining the results of Wilson (1965) who observed that azathioprine prevented sensitized lymphocytes from attacking target cells *in vitro*. It would also help to explain the effects of 6MP on lymphocyte migration in inflammation, a process which Page (1964) suggests

is dependent on protein synthesis.

These studies have shown that several metabolites of 6-mercaptopurine and azathioprine had little effect on lymphocyte protein synthesis compared with the parent drugs.

The work reported may be criticized in that the concentrations of the thiopurines used in these studies are much higher than those found *in vivo*. The concentrations of the drugs used was 60 µg/ml while it is estimated that blood levels of these drugs do not exceed 1-2 µg/ml (Elion, *et al.*, 1960; Hamilton and Elion, 1954; Smith, *et al.*, 1965). Nevertheless the concentration of free drug in the lymphocyte preparations was not measured. This may be considerably lower than that incorporated into the medium because of the oxidation of the drugs to 6 thiouric acid (Elion, 1967). *In vivo* the drugs may be accumulated by lymphocytes. If this occurred then the concentrations within the cells would be greater than the blood levels. In addition the concentrations of these anti-metabolites in the lymph and at the sites of inflammation are not known.

One study has shown that the protein synthesis ability of lymphocytes cultured in plasma medium *in vitro*, fell after the donor had been introduced to a course ofazathioprine. This fall was not consistent when the lymphocytes were

cultured in plasma-free medium. PHA on two occasions stimulated the lymphocytes less than expected when included in plasma-free medium.

The data, although incomplete because of insufficient studies before the patient was introduced to the drug, suggest that the protein synthesis ability of the lymphocytes had been impaired. This was consistently evident when the cells were cultured in plasma medium. In this medium bound and free drug may be expected to depress lymphocyte protein synthesis, a result which suggests that the continued presence of the drug may be necessary for impairment of lymphocyte function. PHA stimulated the cells on every occasion tested but conclusions about the degree of stimulation are wanting because of the few times this substance was used.

A second *in vivo* study of the effects of azathioprine on lymphocyte protein synthesis *in vitro* is compounded with the effects of ACTH and betamethasone.

In this study lymphocyte protein synthesis by the cells in plasma and plasma-free medium is strongly depressed by long acting ACTH. When the patient was given short acting ACTH the effects of this hormone were not as marked. The lymphocytes markedly recovered their ability to synthesise protein after long acting ACTH had been discontinued, but with the continued administration of betamethasone and azathioprine the protein synthesis ability of the lymphocytes fell when they were cultured in plasma medium. PHA did not stimulate the lymphocytes in plasma-free medium throughout the period of long acting ACTH administration. The degree of stimulation by PHA of the cells in plasma medium was less during the long acting ACTH administration.

The data suggest that long acting ACTH has a marked inhibitory effect on the protein synthesis ability of lymphocytes. The effects of this hormoneare probably mediated by its ability to stimulate the adrenal cortex to produce corticosteroids which are powerful anti-inflammatory agents. An <u>in vivo</u> study reported in Chapter 6, showed that the effects of hydrocortisone were most marked when the cells were cultured in plasma-free medium. The effects of ACTH mediated by corticosteroids are also most marked in plasma-free medium in the study reported in this chapter. The

ability of the cells to respond to PHA in plasma-free medium was abolished while in plasma medium it was depressed, throughout long acting ACTH administration.

When azathioprine therapy was continued the ability of lymphocytes to synthesise protein fell when the cells were cultured in plasma medium. This effect was not evident until ACTH was discontinued. The result nevertheless does suggest that the continued presence of the drug in the plasma medium of the cultures is necessary for the impairment of lymphocyte function. This is in contrast to the effects of the corticosteroids which appear to exert a residual inhibitory effect on lymphocyte cells.

In summary the data reported in this chapter suggest that 6MP, azathioprine and thioguanine inhibit lymphocyte protein synthesis *in vitro*. In two cases the data suggest that azathioprine has an inhibitory effect on lymphocyte protein synthesis *in vivo*.

A number of metabolites and related analogues (except two derivatives of 6MP) did not significantly inhibit lymphocyte protein synthesis *in vitro*, a result which suggests that the effect resides with the thiopurines, azathioprine, 6-mercaptopurine and thioguanine. The inhibitory activity of two derivatives of 6MP, 6-mercaptopurine riboside and nucleotide

suggest that 6-mercaptopurine may be released from these substances at the cell wall, enter the cell and inhibit lymphocyte protein synthesis.

7.5. Summary

(1) The thiopurines, azathioprine, 6MP and 6TG inhibited lymphocyte protein synthesis.

(2) The inhibition by these drugs varied when tested with lymphocytes from different donors.

(3) Azathioprine was as inhibitory or more inhibitory than 6MP at the same concentration (μg/ml) in lymphocyte culture medium. Azathioprine which has a molecular weight twice that of 6MP was much more effective than 6MP on a molar basis.

(4) Plasma in the medium modified the inhibitory effects of the drugs.

(5) PHA in the medium did not significantly alter the inhibitory effects of the drugs.

(6) With the exception of azathioprine the thiopurines did not have a marked effect on lymphocyte viability as determined by trypan blue exclusion and cell counts. Survival curves in the presence of azathioprine showed an increased toxicity in the second half of the incubation period, in contrast to the other drugs.

(7) 6-mercaptopurine had a small but significant effect on the incorporation of $^{3}$H-uridine into RNA. Azathioprine had no effect. Neither drug had a significant effect on the incorporation into PHA-stimulated cells. On three occasions, 6-mercaptopurine inhibited the incorporation of $^{14}$C-guanine into nucleic acid. Azathioprine inhibited the incorporation on one occasion.

(8) None of the available metabolites except 6-mercaptopurine riboside and nucleotide had a significant effect on lymphocyte protein synthesis.

(9) In vivo studies showed that lymphocytes from two patients receiving azathioprine therapy had a reduced ability to incorporate radioactive protein precursor when cultured in medium with autologous plasma sampled at the same time with the lymphocytes.

(10) The data are complementary to those reported in Chapter 6. They show that drugs which have anti-inflammatory and immunosuppressive activity in the body inhibit lymphocyte protein synthesis in vitro. The results suggest that this may be one mechanism by which these drugs are active in the body. The suitability of lymphocyte suspension for detecting

drugs with anti-inflammatory and immunosuppressive activity has been demonstrated by showing the inactivity of related analogues and metabolites.

# CHAPTER 8

# STUDIES WITH OTHER CELL SYSTEMS

## 8.1. Introduction

The work that has been previously described has been concerned with the characterization of the proteins synthesized by peripheral small lymphocytes from the blood. Further studies have demonstrated that lymphocyte preparations may be used to test the effects of drugs on protein synthesis with the purpose of screening them for activity as anti-inflammatory and immuno-suppressive agents *in vivo*.

These investigations have been extended to include studies of the proteins made by lymph node and thymus cells *in vitro* and the effects of drugs on protein synthesis by these cells. This work will now be described and discussed with reference to the differences and similarities between these lymphoid cells.

The specificity of the drugs with respect to the cells of the lymphoid system has been investigated by testing them with human and monkey kidney tissue culture cell lines.

The work described in this chapter includes studies of:

(i) The types of proteins made by lymph node and thymus cells using the techniques of immunoelectrophoresis and autoradiography.

(ii) The sensitivity of these cells to drugs which have anti-inflammatory and immunosuppressive activity *in vivo*.

(iii) The specificity of selected drugs for their effects on cells of the lymphoid system. These drugs were tested with the cell lines, HeLa and Hep2, and a monkey kidney cell line Llmck2.

## 8.2. Methods and Materials

### 8.2.1. Preparation of cell suspensions

Biopsies of thymus gland were obtained at cardiac surgery from patients 9-12½ years old. The tissues were collected into sterile capped jars containing medium, minced thoroughly and shaken vigorously. Fragments of the gland were allowed to settle and the supernatants were drawn off into sterile bottles. This washing procedure was repeated twice by adding fresh medium. The thymus cells were washed twice with Hank's balanced salt solution (HBSS) and were finally suspended in culture medium (supplemented Eagle's medium prepared without leucine).

Lymph nodes were collected, at abdominal surgery, into sterile jars containing medium. In the jars the nodes were minced with scissors and the fragments and cell suspension were filtered through sterile gauze into small sterile flasks. The suspensions were transferred into sterile capped bottles and were washed twice with HBSS, before final suspension in culture medium (supplemented Eagle's medium, prepared without leucine).

The cell lines Hep2 and HeLa cells and a monkey kidney cell line, Llmck2 were prepared and obtained from Mr. Brian Moore of the Institute of Medical and Veterinary Science, Adelaide. The cells were supplied as single cell suspensions in Eagle's medium supplemented with 25% foetal calf serum. The cells were washed once with HBSS and resuspended in supplemented Eagle's medium prepared without leucine.

The cell suspensions from thymus gland, lymph nodes and various cell lines, were prepared aseptically at room temperature.

8.2.2. Cell culture

Cells were cultured in the manner described in Chapter 2. $^{14}C$-Leucine was included in the medium at concentrations of 0.25 - 5.0 µc/ml. The culture medium, which was used for all

the studies described in this chapter, was supplemented Eagle's medium prepared without leucine. The medium did not contain autologous plasma.

The cultures were terminated after 24 hours' incubation and the incorporation of precursor was determined by precipitation with 5% TCA. Cell viability was estimated by exclusion of trypan blue and counting. These methods have been previously described in Chapter 2.

The drugs azathioprine, 6-mercaptopurine, oxyphenbutazone, phenylbutazone and hydrocortisone were added to the culture medium at a range of concentrations. (For details of the drugs see Chapter 6.)

The incorporation of radioactive precursor into protein was examined qualitatively by the techniques of immuno-electrophoresis and autoradiography. These techniques have been described in detail in Chapter 3.

## 8.3. Results

### 8.3.1. Thymocytes

Smears of thymus cell preparations were prepared and stained with Leishman stain. 75 Percent of the cells in the smears were identified as small lymphocytes while 43 percent were erythrocytes and the remainder were not classified.

The viability of the cell suspensions at the commencement

of incubation was 98-99%. After incubation for 24 hours in plasma-free medium 50% of the cells were judged viable by the criteria of trypan blue exclusion and cell counting.

Autoradiographs (AR) prepared after immunoelectrophoresis (IEP) of thymus gland cell culture supernatants are shown in Fig. 8.1. IgG, IgA and IgM were not labelled except in one pattern, where part of the IgG arc was weakly labelled. When PHA was included in the culture medium the production of IgG, IgA and IgM was stimulated (Fig. 8.1).

The drugs 6-mercaptopurine (6MP), oxyphenbutazone (TAN), phenylbutazone (BTZ) and cortisone (COR) inhibited thymus cell protein synthesis (Fig. 8.2). The inhibitory effects of the drugs varied when tested with thymus cells from three donors. PHA always stimulated the incorporation of precursor into protein (Table 8.1).

8.3.2. Lymph node cell preparations

Smears of lymph node preparations were prepared and stained with Leishman stain. 68 Percent of the cells in the smears were small lymphocytes, 6 percent were erythrocytes and the remaining cells were not classified.

The viability of the cell suspensions at the commencement of culture was 99-100% as determined by trypan blue

Fig. 8.1. Autoradiographs of IEP patterns prepared from culture fluids of unstimulated and PHA-stimulated thymocytes

Thymus cultures were prepared as described in the methods. The cultures contained $3.8 \times 10^6$ cells (total white cell count) and 2.5 μc of $^{14}$C-leucine.

Pattern A is an IEP pattern of normal plasma developed with whole human antiserum.

Patterns B,C,D,E and F are autoradiographs of IEP patterns prepared from unstimulated thymus cell cultures and developed with whole human antiserum. In patterns, B, C, D and E no labelling of the immunoglobulins is evident; proteins in the α and β globulin regions are labelled. In pattern E the α and β globulin arcs are labelled but part of the IgG arc is also labelled.

Patterns G and H are IEP patterns prepared from PHA-stimulated thymus cell cultures. Patterns E and G were prepared from culture fluids from the same gland. Similarly patterns F and H were prepared from thymocyte culture fluids from the same gland. In pattern G the IgG arc is weakly labelled but labelling of the other immunoglobulins is not evident. In pattern H the immunoglobulins IgA, IgM and IgG are labelled.

In all the autoradiograph patterns an amount of labelled material is present around the origin and towards the anode of the IEP patterns. The intensity of labelling of this material was increased when PHA was included in the medium.

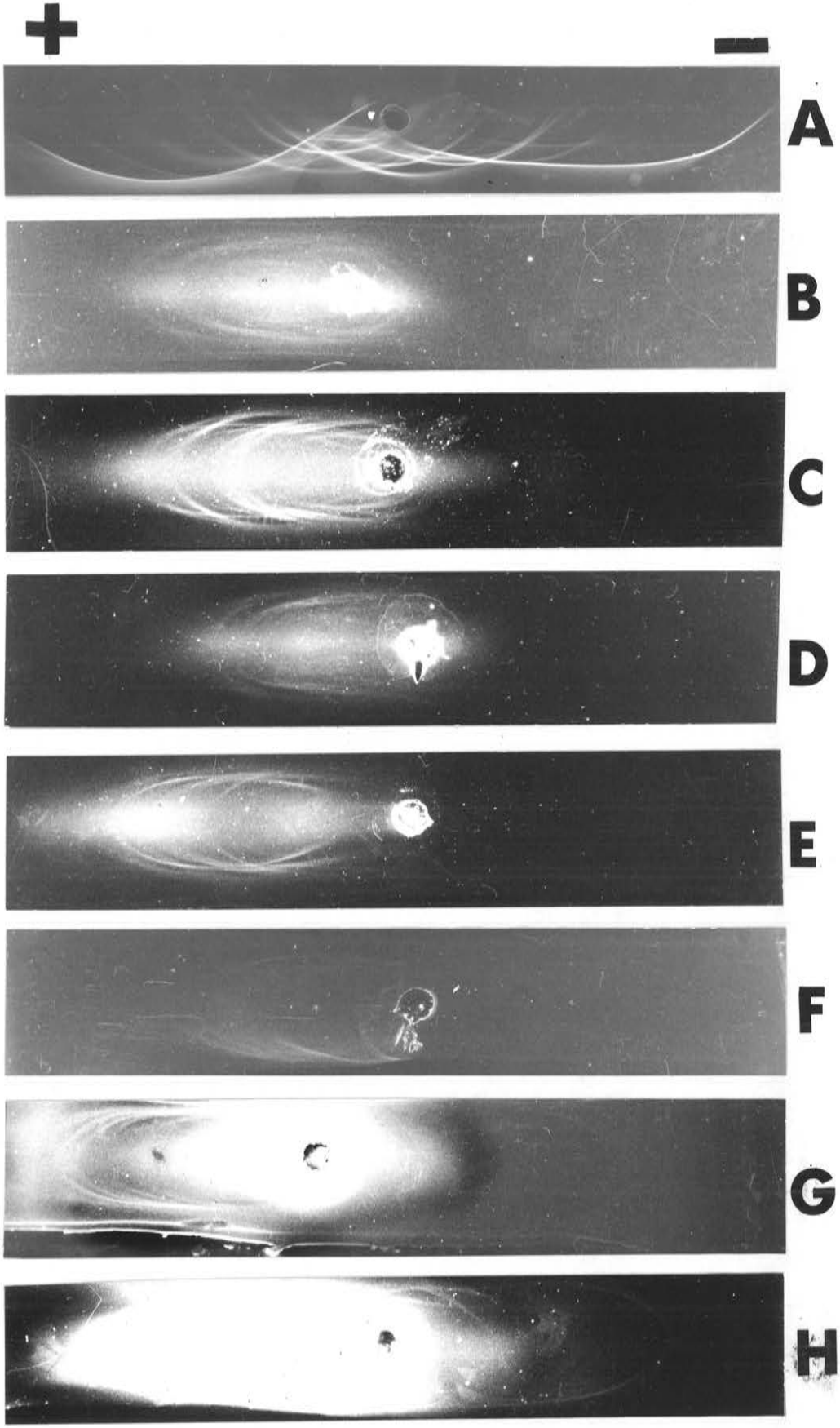

+
−
A
B
C
D
E
F
G
H

Fig. 8.2. <u>The relationship of protein synthesis to concentration of drugs in the culture medium of thymocytes</u>

Thymocytes were prepared and cultured as described in the methods. $^{14}$C-Leucine was included in the medium at a concentration of 0.25 µc/ml. The cultures contained $6.3 \times 10^6$ cells/ml (total white cell count).

The drugs:

COR - Cortisone

6MP - 6-Mercaptopurine

AZA - Azathioprine

BUT - Phenylbutazone

TAN - Oxyphenbutazone

were included in the medium at a range of concentrations shown in the figure.

The incorporation of $^{14}$C-leucine into protein has been expressed as:

(i) percent activity, which is defined as

$$\frac{\text{Counts per minute in culture with drug}}{\text{Counts per minute in control culture}} \times \frac{100}{1}$$

(ii) CPM represents the actual counts per minute measured under the experimental conditions.

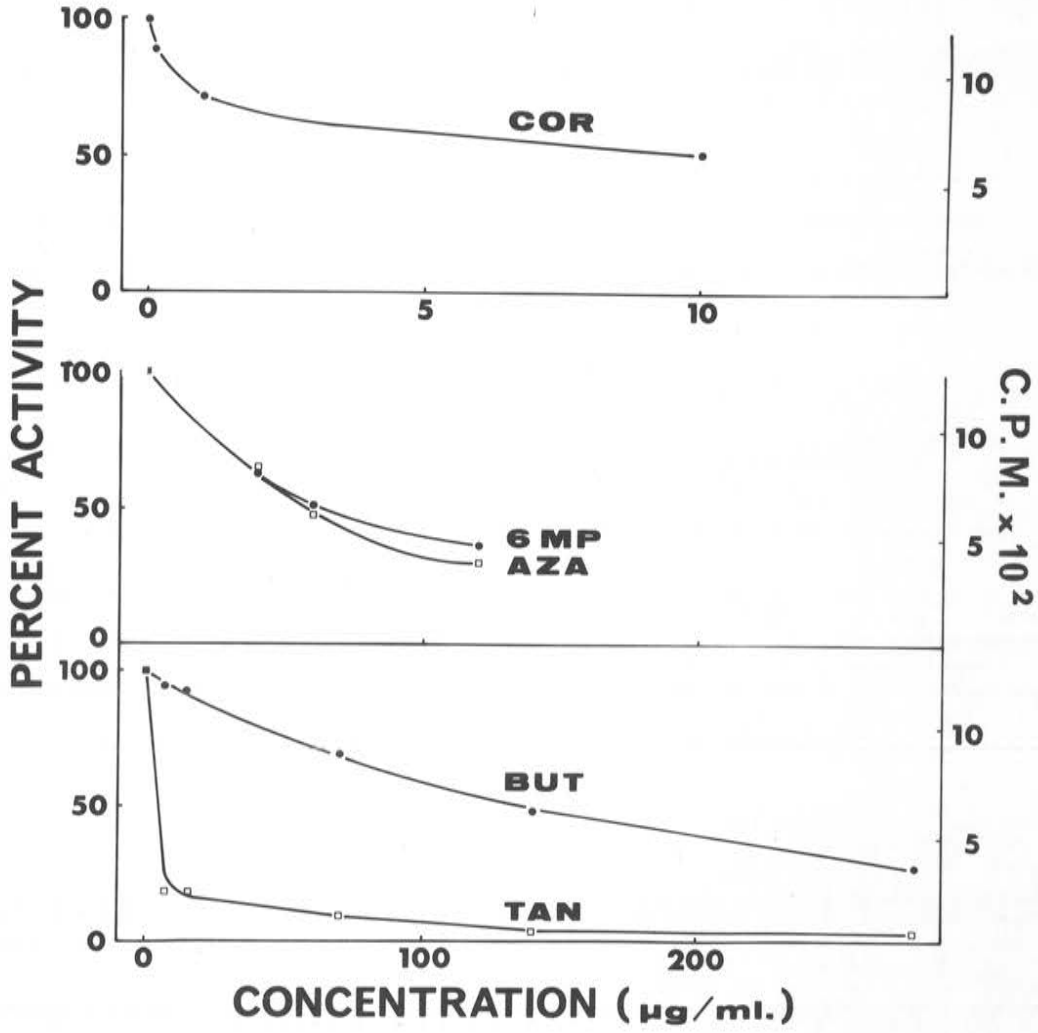

100
50
0
COR
10
5
0
5
10
100
50
0
6MP
AZA
10
5
100
50
0
BUT
TAN
10
5
0
100
200
PERCENT ACTIVITY
C.P.M. x 10 2
CONCENTRATION (µg/ml.)

Table 8.1. The effects of drugs on preparations of thymocytes from different donors

Thymocytes were prepared and cultured as described in the Methods; $^{14}$C-leucine was included in the medium at a concentration of 0.25 µc/ml. The drugs:

BTZ - Phenylbutazone

TAN - Oxyphenbutazone

AZA - Azathioprine

6MP - 6-Mercaptopurine

COR - Cortisone

were included in the medium at the concentrations shown in the table.

The incorporation of $^{14}$C-leucine has been expressed as percent activity. PHA stimulated the incorporation of precursor by the cells each time it was tested.

TABLE 8.1

| | Percent Activity | | | | | |
|---|---|---|---|---|---|---|
| Drug µg/ml | PHA | BTZ 140 | TAN 14 | AZA 60 | 6MP 60 | COR 1.0 |
| Expt. I | 161 | 51 | 88 | 76 | 97 | 57 |
| Expt. II | 241 | 49 | 19 | 47 | 51 | 72 |
| Expt. III | 381 | 84 | 22 | 74 | 32 | 70 |

exclusion. After incubation for 24 hours in plasma-free medium 56% of the cells were judged viable.

Autoradiographs prepared after immunoelectrophoresis of lymph node culture fluids are shown in Fig. 8.3. All the immunoglobulins were distinctly labelled together with proteins in the $\alpha$ and $\beta$ globulin regions of the IEP patterns. PHA did not stimulate the production of new proteins as determined by the examination of the autoradiographs of IEP patterns prepared from PHA-stimulated cultures (Fig. 8.3). In these patterns the labelling of all the protein arcs was increased as judged by visual observation.

Cortisone, oxyphenbutazone, phenylbutazone, azathioprine and 6-mercaptopurine inhibited lymph node protein synthesis (Fig. 8.4). The inhibitory effects of the drugs varied when tested with lymph node cells from different donors (Table 8.2).

8.3.3. Tissue culture cell lines

No cell smears of the final suspensions were prepared. The viabilities of the cell suspensions at the commencement and termination of the incubation were not measured. The cells were known to contain a strain of mycoplasma organisms, which had not been isolated or identified.

The effects of 6-mercaptopurine and azathioprine on

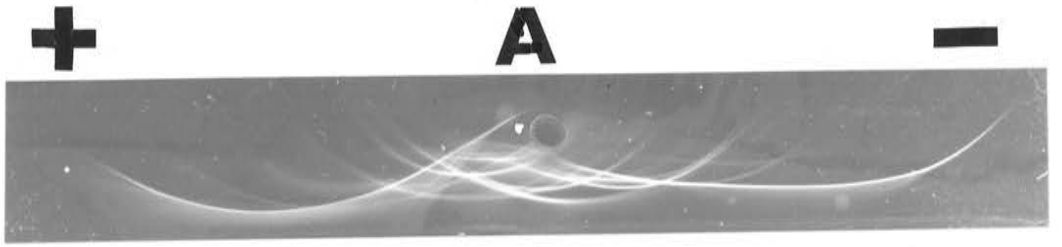
Fig. 8.3. Autoradiographs of IEP patterns prepared from culture fluids of unstimulated and PHA-stimulated lymph node cells

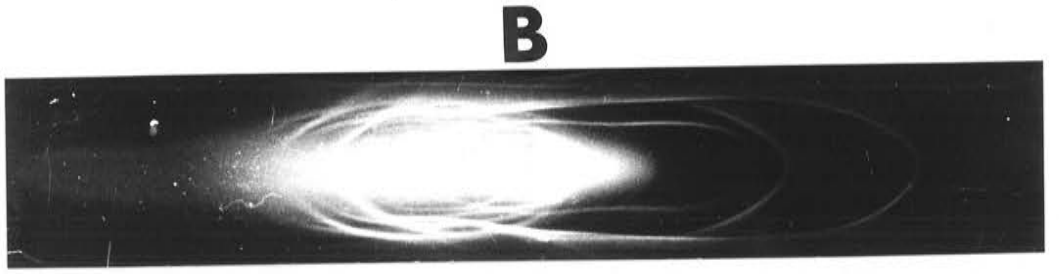
Lymph node cell cultures were prepared as described in the methods. The cultures contained $56 \times 10^6$ cells (total white cell count) and 2.5 μc of $^{14}$C-leucine and were prepared from the same node. Pattern A is an IEP pattern of normal plasma developed with whole human antiserum.

Patterns B and C are autoradiographs of IEP patterns developed with whole human serum. Pattern B was prepared from an unstimulated culture. Pattern C was prepared from a culture stimulated with PHA.

In both B and C IgA, IgM and IgG are uniformly and distinctly labelled. Protein arcs in the α and β globulin region of the IEP patterns are also labelled. In both patterns labelled material is deposited diffusely about the origin and towards the anode.

PHA increased the intensity of labelling of all arcs and the intensity of labelling of the material around the origin.


+
A
−


B

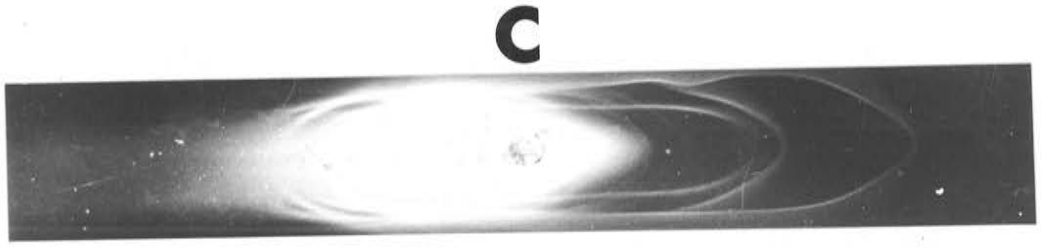

C

Fig. 8.4. The relationship of protein synthesis to concentration of drugs in the culture medium of lymph node cells

The drugs were tested with one preparation of lymph node cells prepared and cultured as described in the methods. $^{14}$C-Leucine was included in the medium at a concentration of 0.25 µc/ml. The cultures contained $1.3 \times 10^6$ cells (total white cell count).

The drugs:

COR - Cortisone

6MP - 6-Mercaptopurine

AZA - Azathioprine

BUT - Phenylbutazone

TAN - Oxyphenbutazone

were included in the medium at a range of concentrations shown in the figure.

The incorporation of $^{14}$C-leucine has been expressed as percent activity and counts per minute (CPM).

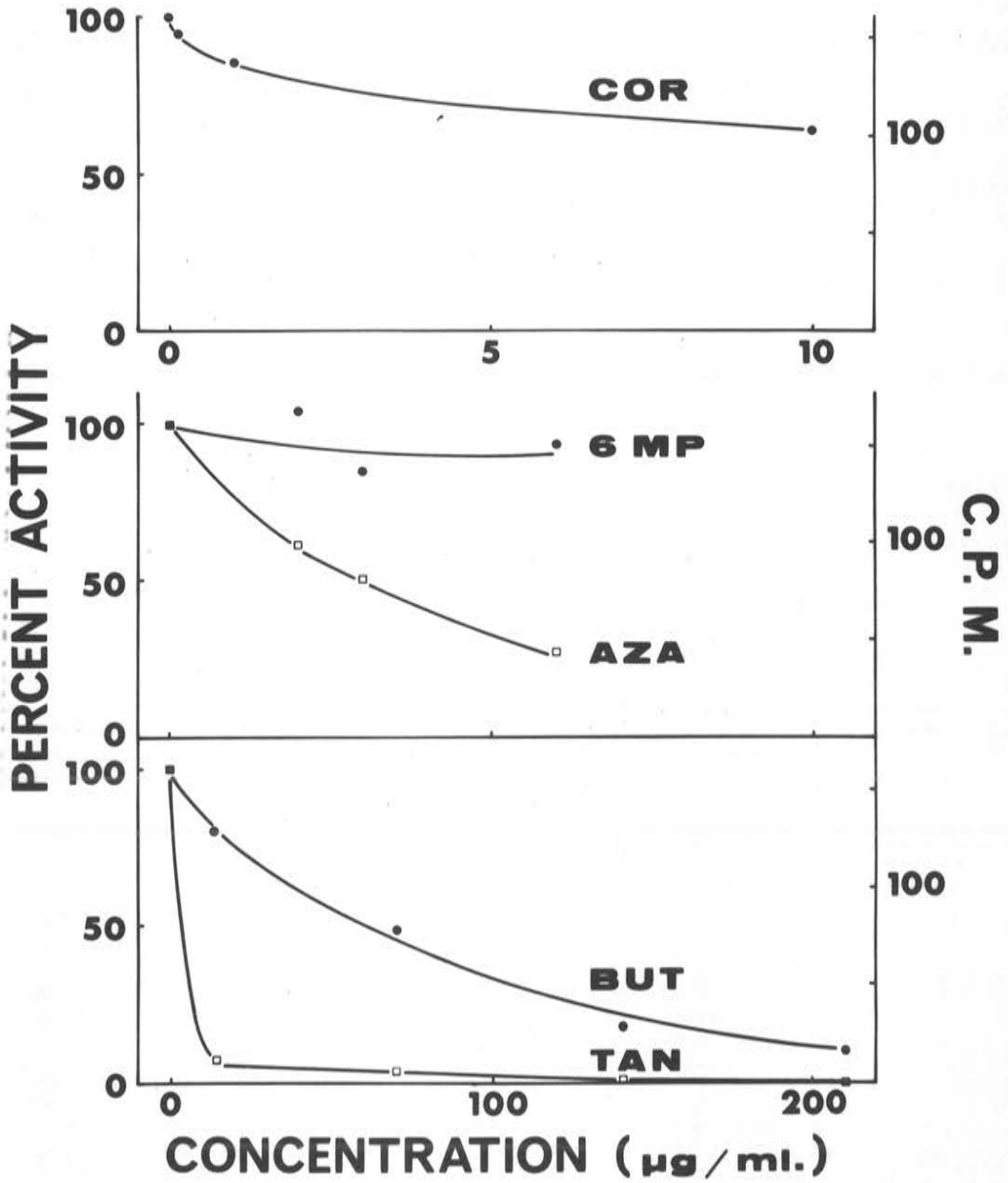

100
50
0
COR
100
0
5
10
100
50
0
6 MP
AZA
100
100
50
0
BUT
TAN
100
0
100
200
PERCENT ACTIVITY
C.P.M.
CONCENTRATION (µg/ml.)

Table 8.2. The effects of drugs on preparations of lymph node cells from different donors

Lymph node cells were prepared and cultured as described in the methods. $^{14}$C-leucine was included in the medium at a concentration of 0.25 µc/ml.

The drugs:

BTZ - Phenylbutazone

TAN - Oxyphenbutazone

AZA - Azathioprine

6MP - 6-Mercaptopurine

COR - Cortisone

were included in the medium at the concentrations shown in the Table.

The incorporation of $^{14}$C-leucine has been expressed as percent activity. PHA stimulated the incorporation of precursor by the cells.

TABLE 8.2.

| | Percent Activity | | | | | |
|---|---|---|---|---|---|---|
| Drug µg/ml | PHA | BTZ 140 | TAN 14 | AZA 60 | 6MP 60 | COR 1.0 |
| Expt. I | - | - | 9.5 | 60 | 80 | - |
| Expt. II | 248 | 18 | 7.0 | 50 | 85 | 86 |
| Expt. III | - | 7 | 9 | - | - | 71 |

protein synthesis are shown in Fig. 8.5. The drugs were tested on two occasions with the three cell-culture lines. 6-Mercaptopurine was more inhibitory than azathioprine on the synthesis of protein by Hep2, HeLa and LLmck2 cells.

Azathioprine did not significantly inhibit protein synthesis by HeLa cells. Cortisone did not inhibit protein synthesis by the three cell lines when it was included in the culture medium at concentrations up to 10 μg/ml. This drug was tested on two occasions with the three cell lines.

PHA did not stimulate protein synthesis by Hep2 cells, when it was included in the medium.

8.4. Discussion

Cells from the lymph node made the immunoglobulins IgG, IgM and IgA, together with a number ofproteins in the α and β globulin regions of IEP patterns. The protein products were similar to those of cultures of peripheral lymphocytes. PHA did not stimulate lymph node cells to make new proteins, but increased the labelling of all proteins, which were found in the autoradiographic patterns of unstimulated cells.

Unstimulated thymus gland cell cultures made a range of proteins present in the α and β globulin regions of the IEP patterns. Only in one pattern was part of the IgG arc

Fig. 8.5. <u>The effects of drugs in the medium at increasing concentrations on protein synthesis by HeLa, Hep2 and LLmck2 cells</u>

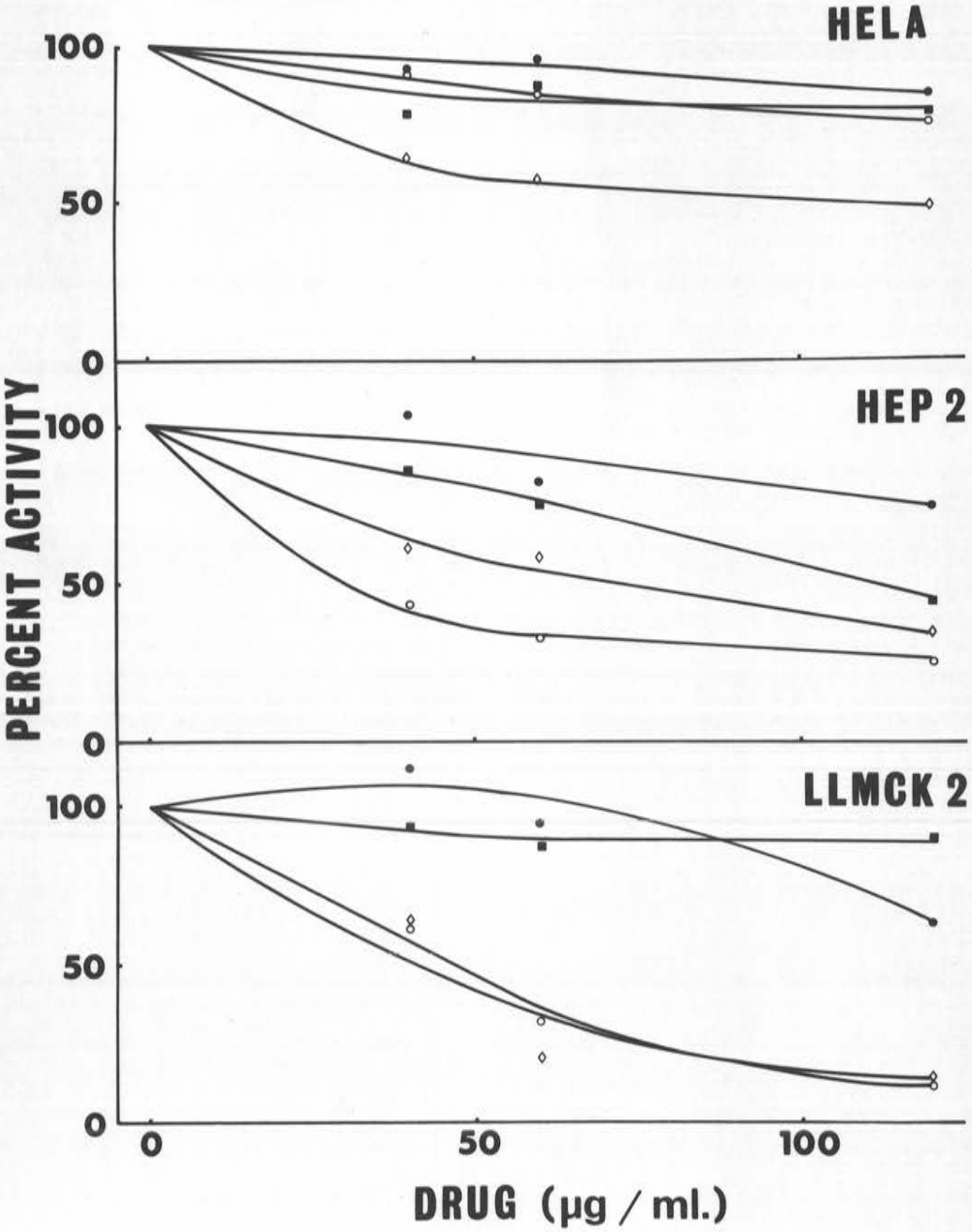
The cells were prepared and cultured as described in the methods. The cultures contained 0.3 - 0.5 x $10^6$ cells and 0.25 µc of $^{14}$C-leucine.

The drugs 6-mercaptopurine and azathioprine were included in the culture medium of HeLa, Hep2 and LLmck2 cells at the concentrations shown in the figure.

● ■ represents triplicate determinations made with azathioprine on two different occasions with the cell lines.

○ ◇ represents triplicate determinations made with 6-mercaptopurineon two different occasions with the cell lines.

The incorporation of $^{14}$C-leucine has been expressed as percent activity.


HELA
HEP 2
LLMCK 2
100
50
0
PERCENT ACTIVITY
0
50
100
DRUG (µg / ml.)

weakly labelled. PHA stimulated the synthesis of the immunoglobulins, when it was included in the medium. This substance also stimulated an increase in the labelling of the $\alpha$ and $\beta$ globulin arcs.

In IEP patterns prepared from culture fluids of thymus and lymph node cells, diffuse labelled material was always detected around the origin and towards the anode. The presence of this material in these patterns was similar to that observed in IEP patterns prepared from peripheral lymphocyte culture fluids.

The differences and similarities of thymus, lymph node and peripheral small lymphocyte protein products may be discussed with reference to the present knowledge of the function of these glands and cells in the body.

Several observations suggest that cells of the lymphoid system are constantly in flux within the body. Thymus cells in the rat and mouse have been shown to be able to colonize lymph tissue (Everett, et al., 1964, Weissman, 1967). Cells are able to leave the thymus and lodge in lymph tissue of the body while cells from the blood, spleen, and lymph nodes are able to enter this gland (Harris, et al., 1964; Galton, et al., 1964; Galton and Reed, 1966). Lymphocyte recirculation from the blood to the lymph has been demonstrated

in a number of species including man (Gowans, 1959; Gowans and Knight, 1964; Caffrey, et al., 1962; Everett, et al., 1964; Cronkite, et al., 1964; Hall and Morris, 1964; Perry, et al., 1967).

The turnover of cells is rapid in the thymus and bone marrow in contrast to the slow turnover of peripheral lymphocytes (Craddock, 1965; Ford and Micklem, 1963). Marrow cells in contrast to thoracic duct lymphocytes are able to colonize the thymus (Ford and Micklem, 1963; Anderson and Whitelaw, 1960).

The thymus differs from other lymphoid tissue significantly in function. It has been shown by several workers that neonatal thymectomy in different species stops the normal growth and maturation of the spleen and lymph nodes (Miller, 1962; Good, et al., 1962; Sherman and Dameshek, 1963; Waksman, et al., 1962).

Observations point to the fundamental dissimilarity between cells of the thymus and other lymphoid tissue. In birds the development of the lymphoid tissue is dependent on two organs, the thymus and the bursa of Fabricus. Man has a thymus, but no organ equivalent to the bursa has been detected, although the palatine tonsil has been suspected of this function (Peterson, et al., 1965). These organs are

thought to be necessary for the maturation and development of the immune system.

The differences observed in the nature of the proteins made by the thymus, lymph node cells and peripheral lymphocytes agree with reports made by several workers. Thymocytes have been shown to be inferior to peripheral lymphocytes in their ability to synthesise IgG, to transfer passive sensitization and to induce graft versus host reactions (Thorbecke and Cohen, 1964; Metcalf, 1964; Billingham and Silvers, 1964; Miller, 1965). The thymus in the animal has been found not to react to repeated injections of egg albumin, whereas the lymphoid tissue in general hypertrophies (Wiseman, 1931). This may be correlated with the fact that antibody has not been found in the thymus of hyper-immune animals (Bjørneboe, et al., 1947; Harris, et al., 1948; Fagraeus, 1948; Thorbecke and Keuning, 1956; Askonas and White, 1956).

Mowbray (1963a,b) has reported that a blood protein, $\alpha_2$-glycoprotein, inhibited immunoglobulin production when present in high concentrations in vivo. It has been suggested that this protein, which is present in the thymus in high concentrations, may act as a homeostatic control of immune function (Peterson, et al., 1965). This substance may not

have been removed during the washing procedure so that it remained with the thymocytes to depress immunoglobulin synthesis during incubation.

The results of Van Furth (1964) are at variance with those reported in this chapter. He isolated newly synthesised IgG and IgA but not IgM from cultures of thymocytes. Ovalbumin was included in the culture medium, but he stated that this substance was not antigenic. In the experiments reported in this chapter thymus cells were cultured in plasma-free medium. The labelling of the immunoglobulin arcs was detected in IEP patterns developed with whole antiserum. These arcs were not investigated with specific antisera. The investigation of the immunoglobulin arcs with specific antisera would assist in positive identification of these arcs in IEP patterns.

PHA stimulated the thymus to produce IgG. Antigen introduced directly into the thymus is able to stimulate the thymus cells to make antibody (Stoner and Hale, 1955), develop into plasma cells (Marshall and White, 1961) and reject heterologous cells (Van-Bekkum and Vos, 1957).

Some differences may be expected between the quantity of immunoglobulins synthesised by peripheral lymphocytes and lymph node cells. The lymph nodes and spleen are the principal

antibody producing organs in the body and contain the fixed cells which are responsible for the initiation of the secondary response (McGregor and Gowans, 1963). Similarity between the types of proteins made by lymph node cells and peripheral lymphocytes was observed, but no quantitative measurements were made. The IgM arc in the IEP patterns was strongly labelled in contrast to the labelling of this arc by culture fluids from peripheral lymphocytes (Chapter 4).

PHA stimulated cells of the thymus and lymph node to produce increased amounts of protein. The degree of stimulation was similar to that observed in cultures of peripheral lymphocytes. The response to PHA was not observed in cells of the culture Hep2.

The drugs oxyphenbutazone, phenylbutazone, azathioprine, 6-mercaptopurine and cortisone inhibited protein synthesis to varying extents, when they were tested with cultures of lymph node and thymus cells. The degree of inhibition produced by these drugs was similar to that observed when they were tested with cultures of peripheral lymphocytes.

The common action of these drugs on these cells emphasizes the similarity of the metabolism of the cells of the lymphoid system. These observations also support the hypothesis that

these drugs are acting in part via depression of protein synthesis by lymphoid cells.

Cortisone did not inhibit protein synthesis by the three cell lines Hep2, HeLa and LLmck2, at concentrations up to 10 μg/ml in the culture medium. 6MP was consistently more inhibitory than azathioprine when tested with the cell lines Hep2 and LLmck2. 6MP was inhibitory on one occasion when tested with cultures of HeLa cells, but on the other occasion had little effect. Azathioprine did not significantly inhibit protein synthesis by HeLa cells.

These data suggest differences in the metabolism of these cells from lymphoid cells. Although differences in permeability between tissue culture cells and lymphoid cells to the drugs, would contribute significantly to differences in the action of the drugs on these cells.

## 8.5. Summary

In this chapter investigations have been reported which show that:

(1) Lymph node cells made all the immunoglobulins and α and β globulins *in vitro*. Production of new proteins could not be detected in PHA-stimulated lymph node cultures.

(2) Thymus cells cultured *in vitro* in plasma-free medium, made α and β globulins. IgG labelling was detected in one culture. PHA stimulated the production of immunoglobulins.

(3) The drugs phenylbutazone, oxyphenbutazone, cortisone, 6-mercaptopurine and azathioprine inhibited both thymus and lymph node cell protein synthesis to varying extents. The degree of inhibition was similar to that when these drugs were included in cultures of peripheral lymphocytes. PHA stimulated the cells to make increased amounts of protein.

(4) Cortisone had no effect on the protein synthesis by HeLa, Hep2 and LLmck2 tissue culture cell lines. 6MP was more effective than azathioprine in inhibiting protein synthesis by all three cell lines. Azathioprine had no significant effect on the synthesis by HeLa cells and had little inhibitory effect on the synthesis by the other two tissue culture cells. PHA did not stimulate the production of new proteins.

# CHAPTER 9

## RECAPITULATIONS AND CONCLUSIONS

The work described in this thesis may be divided into three parts:

(1) quantitative studies of lymphocyte protein synthesis;

(2) qualitative studies of lymphocyte protein synthesis;

(3) the effects of anti-inflammatory and immunosuppressive agents on lymphocyte protein synthesis.

This discussion will follow this scheme.

### 9.1. Quantitative studies

Lymphocyte suspensions were prepared by cotton wool filtration of blood, a method adapted from techniques used by other workers (Fichtelius, 1950; Cooper and Rubin, 1965). Leukocytes adhered to the fibres and were held back while the lymphocytes filtered through, but the erythrocytes moved with the lymphocytes and were collected with this fraction. The majority of the platelets were also eluted with this faction while some were caught in the cotton fibres. Both the platelets and plasma proteins were removed from the final lymphocyte suspensions by washing them with balanced salt solutions, but the erythrocytes were not. 99% of the white cells in the final suspensions were lymphocytes, but erythro-

cytes were present in numbers 5 to 10 times the lymphocyte count.

The proteins produced by lymphocytes were studied quantitatively at various times during incubation for 72 hours in plasma and plasma-free medium. Quantitative studies of lymphocyte RNA synthesis in plasma-free medium were also reported. Various substances were tested for their ability to stimulate or inhibit protein and RNA syntheses.

Puromycin completely inhibited lymphocyte protein synthesis at concentrations of 40 μg/ml in the medium. Cells killed before incubation did not incorporate radioactive precursor into protein. These results show that the incorporation of radioactive precursor represents synthesis and that this synthesis is protein synthesis (Nathans, 1964). Furthermore, the greater part of this synthesis was inhibited by actinomycin D. A small fraction was not inhibited and appeared to be dependent on long lived m-RNA.

Protein synthesis continued for 24 hours in plasma medium and 12 hours in plasma-free medium. The proteins synthesised by lymphocytes could be divided into three fractions:

(1) Protein released into the medium during incubation;

(2) Protein not released into the medium but released by freezing and thawing the cells;

(3) Protein bound to the cell debris.

In plasma-free medium newly synthesised protein was released earlier into the medium by lymphocytes than when they were cultured in plasma medium. A protein-free environment may be expected to be unfavourable to lymphocytes but studies of cell survival showed that 6-17% of the cells were dead in plasma-free medium and 1-3% were dead in plasma-medium after incubation for 24 hours. The morphology of the cells surviving in plasma-free medium at this time suggested that the majority were metabolically active and the uptake of $^{3}$H-leucine by 50% of the cells confirmed this.

The radioactivity associated with thecell debris fraction of plasma cultures was greater than that associated with this fraction of plasma-free cultures. The total amount of protein synthesised was greater in plasma cultures where the specific activity of the $^{14}$C-leucine was less than in plasma-free cultures.

RNA synthesis by lymphocytes continued for 48 hours in plasma-free medium. RNA synthesis was inhibited by actinomycin D. The results suggest that protein synthesis by lymphocytes was dependent on RNA synthesis (Kirk, 1960). It did not appear that the small fraction of protein synthesised in the presence of actinomycin D depended on RNA synthesis which was not inhibited by actinomycin D.

PHA stimulated both protein and RNA syntheses by lymphocytes. This substance was always stimulatory in

plasma medium but was not consistently stimulatory in plasma-free medium. Protein synthesis was stimulated 2-5 times and RNA synthesis 8 times. The response to PHA varied between individuals and between cultures of the same cells in plasma and plasma-free medium. Both the initiation and the established response could be blocked by actinomycin D, a result which suggested that PHA stimulated the synthesis of short-lived m-RNA (Cooper and Rubin, 1966).

PHA stimulated protein synthesis by lymphoid cells from preparations of thymus and lymph glands. PHA did not stimulate protein synthesis by cultures of monocytes and polymorphs, nor did it stimulate the tissue culture cell line Hep2 to produce increased amounts of protein.

Cultures of unstimulated lymphocytes incubated with $^{3}$H-leucine and studied by autoradiography of cell smears showed that 60-70% of the cells were active in these preparations. In PHA-stimulated cultures the percentage of cells incorporating precursor was as high as 90%. The cells, which did not incorporate $^{3}$H-leucine, may synthesise protein in such small amounts that they escape detection by this technique. The data suggest that there is a significant

and probably quite large difference in the rate at which the cells synthesise protein. PHA stimulated 30-40% of the cells not detected in unstimulated cultures to make detectable amounts of protein.

PHA stimulated the cells to incorporate $^{3}H$-thymidine after incubation for 24 hours in plasma-medium. The relation of the initial stimulus of protein synthesis by PHA to the initiation of mitotic activity is not known.

Erythrocytes contaminating the final suspensions did not incorporate precursor or influence the incorporation of precursor by lymphocytes. Small numbers of polymorphs in lymphocyte cultures stimulated the cells to produce increased amounts of protein from that expected by cultures of polymorphs and lymphocytes alone. This observation was discussed with reference to a report made by Page (1964), who suggested that the migration of lymphocytes to the site of inflammation depended on protein synthesis which was initiated by a stimulus from neutrophils at the site.

9.2. Qualitative protein synthesis studies

The proteins made by lymphocytes have been characterized qualitatively by immunoelectrophoresis and autoradiography. The types of proteins synthesised by lymph nodes and thymus glands were also investigated.

The qualitative studies reported in this thesis confirm the findings of Parenti, *et al.*, (1966); Van Furth (1964) and Ripps and Hirschhorn (1967). Lymphocyte culture fluids examined by immunoelectrophoresis and autoradiography, labelled IgA, IgM, IgG, transferrin, haptoglobin and a number of arcs in the $\alpha$ and $\beta$ globulin region of IEP patterns. In the IEP patterns there was a considerable amount of unidentified protein of high specific activity in the region of the origin and towards the anode.

PHA did not stimulate the production of new proteins by lymphocytes but increased the labelling of all the proteins labelled in autoradiographs prepared from unstimulated cultures.

Culture fluids from lymph node cells labelled the same arcs in IEP patterns as those labelled in patterns prepared from lymphocyte culture fluids. PHA did not stimulate lymph node cells to produce new proteins but increased the labelling of those arcs labelled by unstimulated cultures.

Culture fluids from five thymus cell cultures did not label IgA, IgM and IgG arcs in IEP patterns. Culture fluid from one thymus culture labelled part of the IgG arc but IgA and IgM were not labelled. All the culture fluids labelled arcs in the $\alpha$ and $\beta$ globulin region of IEP patterns.

PHA stimulated one preparation of thymus cells to make IgA, IgM and IgG and another to make IgG. PHA also increased the intensity of labelling of the α and β globulins.

In IEP patterns prepared from culture fluids of lymph node and thymus cells, diffuse material was labelled in the region of the origin and towards the anode, corresponding to that observed in patterns prepared from peripheral lymphocyte culture fluids. The presence of this material suggested that some labelled arcs in these patterns did not represent synthesis.

It was shown that precipitin lines not related to human proteins when formed in the cathodal region of IEP patterns with lymphocyte culture fluid were not labelled. Precipitins so formed in the α and β globulin region of IEP patterns were labelled. Culture fluids of lymphocytes from a patient with Hashimoto's disease labelled the thyroglobulin-antibody arc in the cathodal region of IEP patterns. Fluids from cultures of normal lymphocytes did not label this arc. These data suggest:

(1) That the labelled immunoglobulin arcs IgA, IgM and IgG formed in the cathodal region of IEP patterns represented synthesis of these proteins;

(2) That the labelling of arcs in the $\alpha$ and $\beta$ globulin region of IEP patterns may not represent synthesis;

(3) That lymphocytes from a patient with Hashimoto's disease synthesised antibodies to thyroglobulin.

Immune precipitates to IgG and egg albumin formed in lymphocyte culture fluids contained similar amounts of radioactivity. However, the immunoglobulins could be eluted from the cathodal region after electrophoresis of culture supernatants in agar, and immune precipitates formed in portions of the eluate showed that significantly more radioactivity was associated with IgG-antibody precipitates than with egg albumin-antibody precipitates. These data support the findings that lymphocytes are able to make IgG globulin.

PHA stimulated the production of IgG to a lesser extent than the production of the total proteins. This substance did not always stimulate the production of IgG by cells in plasma-free medium even though the production of the total proteins were stimulated. PHA was always stimulatory to IgG synthesis in plasma medium. Ripps and Hirschhorn (1967) reported the stimulation of IgG synthesis and protein synthesis by PHA to similar extents when lymphocytes were cultured for

72 hours. They reported that the incorporation of $^{14}$C-leucine into IgG represented 2.2 - 5.3% of the total incorporation, while in the experiments reported in this thesis with unstimulated lymphocytes the radioactivity in IgG represented 0.6 - 2.1% of the total incorporation. The nett amounts of IgG and total protein synthesised would depend on the proportion of leucine in these proteins.

The radioactive labelled material coprecipitating with immune precipitates was stable to heat and was not dialysable, suggesting that it is neither the heat labile or dialysable components of complement (Kabat and Meyer, 1961). It appeared that this material localized itself towards the anode and around the origin after immunoelectrophoresis.

In qualitative time course studies unstimulated cultures released only labelled IgA, IgM and IgG into the supernatant during incubation for 24 hours. The other newly synthesised proteins remained stored in the cells and were not released except by freezing and thawing. The diffuse labelled material associated with IEP patterns of culture fluids was not released by unstimulated cells during incubation.

Both the cell supernatant and cell extract fluids from stimulated cultures, which were incubated for 12 or 24 hours,

labelled similar arcs after immunoelectrophoresis. However, the cell supernatant fractions from these two cultures did not label transferrin, a protein which was labelled at the anodal end by the cell extract fraction. A small amount of diffuse labelled material was associated with IEP patterns prepared from the cell supernatant fraction, but the bulk of this material was identified in the cell extract.

The data suggest that most of the protein synthesised by lymphocytes was not released during incubation. PHA stimulated the production and release of a newly-formed protein. PHA also caused an accelerated rate of cell death in plasma-free medium, which would also cause the release of cell protein. Electron microscopy coupled with autoradiography would be useful techniques to investigate and localize the storage depots of newly synthesised radioactive products within lymphocyte cells. The release of the immunoglobulins appeared to continue throughout the culture but the release of other proteins from lymphocytes may depend on stimuli or death.

The identity of the radioactive material co-precipitating with immune precipitates was not investigated. Its synthesis was blocked by puromycin andit was lightly stained with amido

black in IEP patterns, factors which suggest it was protein of high specific activity.

Investigations of lymphocyte protein products with lymphocyte antisera may reveal soluble proteins present in lymphocytes and not present in plasma. The synthesis of diffuse material in IEP patterns may represent synthesis of these proteins, which have not been washed out of IEP patterns.

It was shown by immunoelectrophoresis and autoradiography of culture fluids of lymphocytes incubated with actinomycin D that these fluids contained the same labelled proteins found in control cultures, but in reduced amounts.

9.3. Anti-inflammatory and immunosuppressive agents

Lymphocyte suspensions are easily obtained and can be used for both immunological and biochemical investigations. The hypothesis that anti-inflammatory and immunosuppressive drugs depend in part for their action on inhibition of lymphocyte protein synthesis has been investigated.

Many drugs with anti-inflammatory and immunosuppressive activity *in vivo* inhibited lymphocyte protein synthesis *in vitro*. All of a wide range of drugs tested were active against protein synthesis. Some of the drugs were lethal while others inhibited

protein metabolism by mechanisms which did not lead to cell death.

The response to various drugs varied between lymphocyte preparations from normal donors. This variability is reflected in the clinic by the variability of the patient's response to drugs. It has not however been established whether there is a correlation between sensitivity *in vitro* and *in vivo* of an individual's lymphocytes.

One of the most interesting findings was that the relationship *in vitro* between drug analogues and metabolites reflected their activity *in vivo*. For example parahydroxybenzoate, which is an isomer of salicylate and is inactive *in vivo*, was inactive *in vitro*. Salicylate which is active *in vivo*, was active *in vitro*. Oxyphenbutazone, a metabolite of phenylbutazone is more active *in vivo* at a lower dose (Goodman and Gilman, 1965). Oxyphenbutazone was more effective against lymphocyte protein synthesis *in vitro* at a lower dose than phenylbutazone. An analogue of phenylbutazone, sulphinpyrazone which has less anti-inflammatory activity *in vivo* and is a more potent uricosuric agent (Goodman and Gilman, 1965) was slightly less active than phenylbutazone *in vitro* on lymphocyte protein synthesis at the same dose levels.

The inhibitory effects of the thiopurines on lymphocyte protein synthesis may reflect their effectiveness as immunosuppressive agents *in vivo*. Azathioprine was more effective than 6-mercaptopurine in inhibiting lymphocyte protein synthesis. 6-Thioguanine was as effective as azathioprine. A number of thiopurine metabolites found *in vivo* were inactive *in vitro*, an effect which was correlated with their effects *in vivo* (Elion, 1967). The activity of two derivatives suggested that the nucleotide and riboside of 6-mercaptopurine were active against lymphocyte protein synthesis, probably via conversion of these derivatives to 6-mercaptopurine and perhaps eventually to intracellular nucleotide. 6-Mercaptopurine inhibited the uptake of $^{14}C$-guanine into nucleic acid but azathioprine did not appear to be active against lymphocyte metabolism in this way.

The activity of many drugs and drug analogues paralleled their activities in clinical use. Furthermore these observations suggest that lymphocyte cultures can be used successfully to screen drugs which have immunosuppressive and anti-inflammatory activity in the body.

Culture fluids from lymphocytes incubated with 6-mercaptopurine, azathioprine, cortisone, salicylate, oxyphenbutazone,

mefenamic acid and indomethacin labelled the same protein arcs in IEP patterns as those found in patterns prepared from untreated lymphocyte cultures. Culture fluid from lymphocytes incubated with chloroquine, which is highly lethal, did not label any arcs. None of the drugs tested appeared to inhibit the synthesis of any particular protein, but the synthesis of all proteins was affected.

Many of the drugs, except those which were highly lethal, did not interfere with the mechanism of the PHA response by lymphocyte cells. Plasma in many instances reduced the inhibitory effects of the drugs when it was included in the medium. This phenomenon was attributed to plasma binding, an effect which is known to occur with salicylate and phenylbutazone (Goodman and Gilman, 1965).

Selected drugs (cortisone, 6-mercaptopurine, azathioprine, phenylbutazone and oxyphenbutazone) had a common effect on lymphoid cells from thymus, peripheral blood and lymph node. By contrast the drugs, 6-mercaptopurine, azathioprine and cortisone differed in their effects on protein synthesis by HeLa, Hep2 and LLmck2 cells. 6-Mercaptopurine was more inhibitory than azathioprine, which had little effect on protein synthesis by these cell lines. Cortisone was not

inhibitory. The data suggest differences in the metabolism between lymphoid cells and the three cell lines.

Adrenocorticotrophic hormone (ACTH), hydrocortisone and azathioprine were studied for their effects on lymphocyte protein synthesis *in vivo*. Lymphocytes from a patient receiving ACTH showed a reduced ability to synthesise protein *in vitro*. This drug probably had its effect in the body by stimulating the production of corticosteroids. Lymphocytes from a patient receiving hydrocortisone showed a reduced ability to synthesise protein when the cells were cultured in plasma-free medium. By contrast, lymphocytes from patients receiving azathioprine showed a fall in their ability to incorporate $^{14}$C-leucine when they were cultured in medium with autologous plasma sampled at the same time. These observations suggest that the effect of hydrocortisone was residual and that factors in plasma masked the inhibitory effects of this drug. The findings with azathioprine suggest that the continued presence of this drug (in plasma) is necessary for its inhibitory effect on lymphocyte cells. More studies are needed to correlate the effects of the drugs *in vitro* with their effects *in vivo*, nevertheless the findings do suggest that the effects of azathioprine and cortisone *in vivo* parallel their effects on lymphocyte protein synthesis

in vitro.

9.4. Criticisms and Prospects

The culture medium chosen for lymphocyte studies supported the growth of these cells for short incubation periods. An incubation period of 24 hours was chosen principally because of its convenience. Most of the protein synthesis by the lymphocytes had been completed after 12 hours incubation. When plasma was included in the medium synthesis continued for 24 hours. In the studies reported in this thesis substances were investigated for their immediate effects on lymphocytes. For this reason it might have been better to limit investigations to shorter incubation times. Alternatively studies involving long term investigations such as the effect of PHA on mitosis would require the choice of a better medium.

Radioactive material coprecipitated with immune precipitates formed in lymphocyte culture fluids. Egg albumin-antibody precipitates formed in culture fluids contained an amount of radioactivity similar to that associated with IgG-antibody precipitates. This material was associated with diffuse labelled material spread around the origin and towards the anode in IEP patterns. As a result the labelling of the

arcs in the α and β globulin region of IEP patterns may not represent synthesis but non-specific labelling.

Further studies are needed both into the identity of the coprecipitating material and the specificity of the labelling of arcs in the α and β globulin regions of IEP patterns. Initial studies showed that the coprecipitating material was protein, which was stable to heat and was non-dialysable.

Studies of protein synthesis with radioactive isotopes have limitations, conclusive evidence would be provided by the demonstration of nett increase in protein production. It is quite probable that the release of the labelled immunoglobulins represented synthesis and excretion of these proteins, while the labelling of the other proteins represented metabolic turnover.

Coprecipitation of labelled material with immune precipitates limits the studies that can be made in this way. The absence of diffuse labelled material in IEP patterns prepared from cell supernatants (i.e. those proteins released from the cells during incubation) suggests that less of this coprecipitating material is present in these fractions. Analyses in the cell supernatant fraction rather than in the culture fluid released after freezing and thawing the cells,

may be more successful.

Many drugs which have anti-inflammatory and immunosuppressive activity *in vivo* inhibited protein synthesis *in vitro*, and the concentrations at which these drugs were used were in some cases within measured blood levels and below maximum expected blood levels in the case of others. Nevertheless the concentrations at which some of the drugs were tested may never be reached *in vivo*, in fact azathioprine, 6-thioguanine and 6-mercaptopurine were tested at very high concentrations.

Lymphocyte cultures have been shown to be suitable to screen drugs with anti-inflammatory and immunosuppressive activity *in vivo*. Further investigations may reveal drugs, which are useful in clinical practice or as biochemical tools.

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## APPENDIX (i)

In between use all glassware was subjected to a routine washing procedure. Immediately after use the glassware was soaked in water prior to the following steps:

(1) Glasware was washed and brushed in hot tap-water with Diwash detergent (Diversey, A'sia., Pty. Ltd.).

(2) Rinsed in tap-water then distilled water.

(3) Boiled in distilled water with 1 part of Calgon metasilicate in 100 parts of water for 20 minutes. Calgon metasilicate was made up with 40 gm of Calgon (Allbright and Wilson, Aust. (Ltd.)) and 360 gm of sodium metasilicate (Anax., Drug Houses of Australia, Ltd.) in 1 gallon distilled water.

(4) Rinsed in tap water, then distilled water.

(5) Boiled in distilled water for 20 minutes.

(6) Rinsed in distilled water and dried.

(7) Packaged for autoclaving.

## APPENDIX (ii)

Eagle's (basal) medium 10 x concentrate contains in every 100 ml:

| | |
|---|---|
| Sodium Chloride | 6.8 gm |
| Potassium Chloride | 0.4 gm |
| Monosodium Phosphate | 0.16 gm |
| Calcium Chloride | 0.2 gm |
| Magnesium Chloride | 0.08 gm |
| Glucose | 1.0 gm |
| l-Tyrosine | 18.0 mgm |
| l-Cystine | 12.0 mgm |
| l-Arginine | 21.0 mgm |
| l-Histidine | 10.5 mgm |
| l-Isoleucine | 26.2 mgm |
| l-Leucine | 26.2 mgm |
| l-Lysine | 36.5 mgm |
| l-Methionine | 7.5 mgm |
| l-Phenylalanine | 16.5 mgm |
| l-Threonine | 23.8 mgm |
| l-Valine | 23.4 mgm |
| l-Tryptophane | 4.1 mgm |
| Biotin | 1.0 mgm |
| Choline | 1.0 mgm |
| Folic Acid | 1.0 mgm |
| Nicotinamide | 1.0 mgm |
| Pantothenic Acid | 1.0 mgm |
| Pyridoxal | 1.0 mgm |
| Thiamin | 1.0 mgm |
| Riboflavin | 0.1 mgm |
| Phenol Red | 20.0 mgm |
| Penicillin Sodium | 50 mgm |
| Steptomycin Sulphate | 50 mgm |

To prepare 100 ml of Eagle's medium for use the following was added to sterile double distilled water (so that the final volume was 100 ml):

(1) 10 Ml of Eagle's medium 10 x concentrate

(2) 2 Ml of glutamine solution (contains 1.46 gm of N-glutamine per 100 ml).

The final pH of the medium was adjusted to 7.0 with sterile sodium bicarbonate solution.

Eagle's medium was prepared without leucine and the following amino acids were added to 100 ml of the 10 x concentrate:

| | |
|---|---|
| L-Glycine | 7.5 mgm |
| L-Serine | 10.5 mgm |
| L-Proline | 11.5 mgm |
| L-Asparagine $H_2O$ | 13.2 mgm |
| L-Aspartic | 13.3 mgm |
| L-Alanine | 8.9 mgm |
| L-Glutamic | 14.7 mgm |

The medium was then prepared as before. This medium was used routinely and has been referred to in the text as supplemented Eagle's medium prepared without leucine.

## APPENDIX (iii)

Leukocyte rich suspensions were loaded onto columns (2.5 x 40 cm) packed with glass beads and incubated for 3/4 hour at 37°C. The columns were then eluted with the following solutions at 37°C and various fractions were collected.

(1) 100 Ml of fresh plasma was used to elute the lymphocyte rich suspensions.

(2) 500 Ml of Hanks balanced salt solution with 20% plasma was used to elute platelets.

(3) The columns were finally washed with 500 ml of $Ca^{++}$ and $Mg^{++}$ free disodium ethylenediamine tetraacetic acid (EDTA). The reagent contained per litre: disodium EDTA 0.2 gm, NaCl 8.0 gm, KCl 0.2 gm, $Na_2HPO_4$ 1.15 gm, $KH_2PO_4$ 0.2 gm, and glucose 0.2 gm. The solution was sterilized with a Seitz filter and was buffered at pH 7.4. Polymorph rich preparations were collected at first while monocyte rich preparations were collected towards the end of eluate.

## APPENDIX (iv)

**Determination of infinite thickness.**

| Protein (mg) | CPM |
|---|---|
| 0.7 | 646 |
| 1.2 | 980 |
| 2.4 | 1310 |
| 3.6 | 1875 |
| 4.8 | 2200 |
| 6.0 | 2400 |
| 7.2 | 2400 |

Serum proteins were precipitated with 27% sodium sulphate. The protein was taken up in saline and to this solution 2.5 µc of $^{14}$C-leucine was added. Varying amounts of this protein solution were plated out on 2 sq. cm. planchets. The planchets were dried and counted.

The protein content of the stock protein solution was determined by the method of *Lowry et al., (1951). From this the amount of protein plated out could be determined. Counts per minute (CPM) of the samples are shown in the table.

*Lowry, O.H., Rosenbrough, N.J., Farr, A.L., and Randall, R.J. (1951). J. biol. Chem. 193:265.

## APPENDIX (v)

### The effect of washing on the removal of plasma proteins from lymphocytes

| No. of washings | CPM |
|---|---|
| 0 | 621 |
| 1 | 136 |
| 2 | 51 |
| 3 | 45 |
| 4 | 63 |
| 5 | 25 |

Lymphocytes were prepared by siliconized glass wool filtration. The yield was > 99% lymphocytes. These lymphocytes were incubated with $^{14}$C-leucine for 24 hours and the supernatant recovered after termination of the culture by freezing and thawing.

Lymphocytes were again prepared by glass wool filtration (, > 99% lymphocytes) and incubated with the isolated supernatant containing labelled proteins for 2 hours at 37°C. At the end of the incubation the cell suspension was divided into six aliquots. These were then washed with Hank's Balanced saline solution (HBSS) a various number of times as indicated. The cells were then plated out onto planchets and counted.

CPM represents the counts per minute of the plated lymphocytes.

## APPENDIX (vi)

**Lymphocyte protein synthesis in Eagle's medium prepared with and without an amino acid supplement**

| Media | CPM |
|---|---|
| I | 535 |
| II | 837 |
| III | 737 |
| IV | 632 |

$2.1 \times 10^6$ Lymphocytes were cultured in Eagle's medium, with and without an amino acid supplement (Appendix (ii)) and containing 1 μc of $^{14}$C-leucine. Medium I was Eagle's medium, Medium II was Eagle's medium with supplement, medium III was Eagle's medium with twice the amount of supplement and medium IV was Eagle's medium and supplement, used at twice the normal concentration. The counts per minute (CPM) in the TCA culture precipitates are shown in the table.

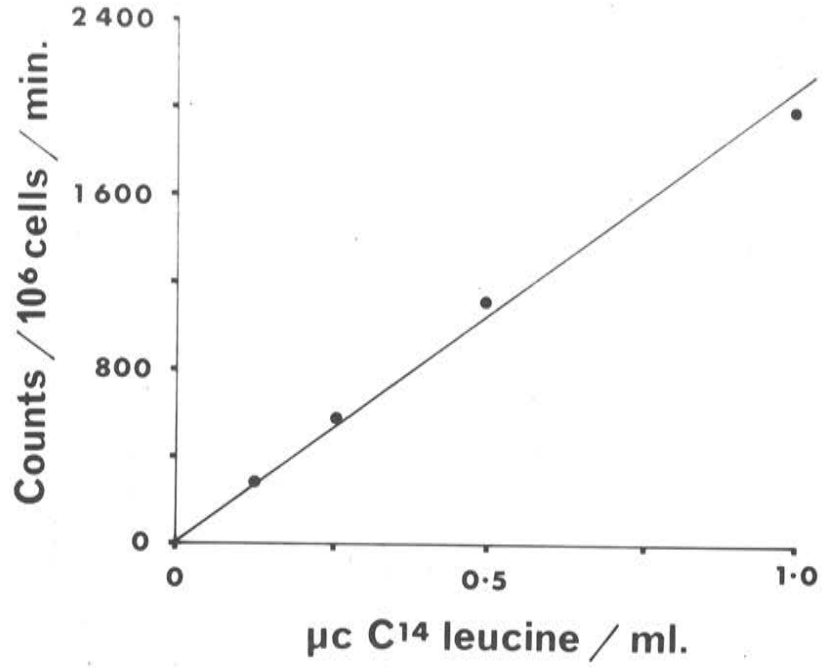
## APPENDIX (vii)

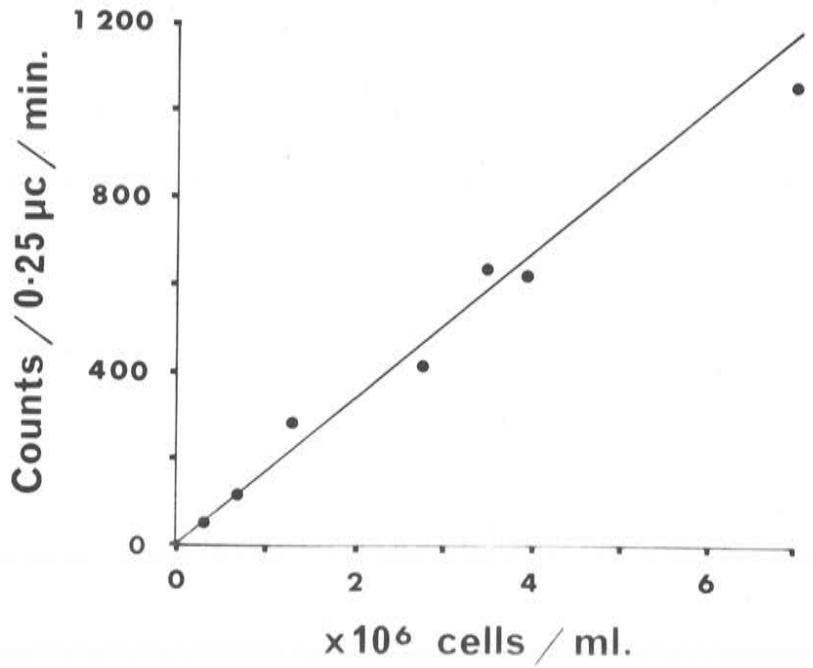
### Relation of cell numbers and μc of $^{14}$C-leucine in the medium to the incorporation of precursor into protein

Normal lymphocytes were cultured in supplemented Eagle's medium prepared without $^{12}$C-leucine. Determinations were made in triplicate.

The top graph shows the relationship between the incorporation of $^{14}$C-leucine from the medium by lymphocytes, while varying the amount of $^{14}$C-leucine in the medium. The incorporation of precursor into the TCA culture precipitates has been expressed as counts/ $10^6$ cells cultured/min.

The lower graph shows the relationship between the incorporation of $^{14}$C-leucine from the medium by lymphocytes, while varying the number of cells in the medium. The amount of $^{14}$C-leucine in the medium was constant and was 0.25 μc/ml. The incorporation has been expressed as counts/0.25 μc of $^{14}$C-leucine in the medium/ min.


2 400
1 600
800
0
Counts / 10⁶ cells / min.
0
0·5
1·0
µc C¹⁴ leucine / ml.

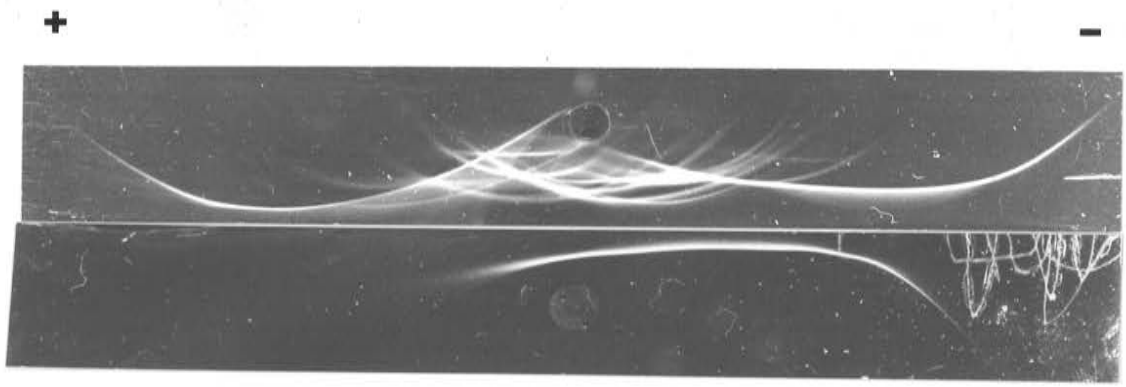

1 200
800
400
0
Counts / 0·25 µc / min.
0
2
4
6
x10⁶ cells / ml.

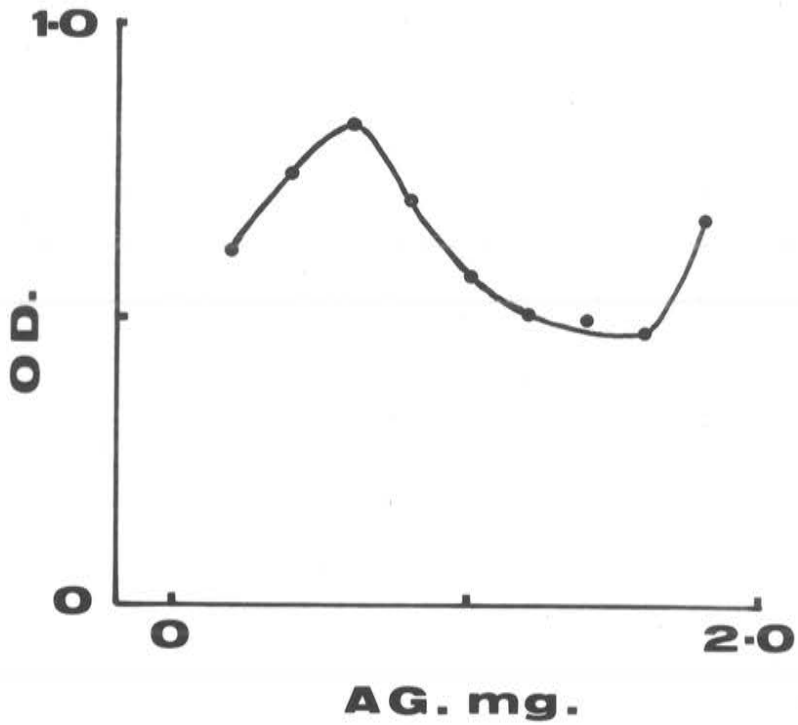
## APPENDIX (vii)

### Human IgG antiserum

Human plasma was electrophoresed in agar. The upper immunoelectrophoretic pattern (IEP) was developed with whole human antiserum. The lower pattern was developed with rabbit antiserum to human IgG.

The effect of adding increasing amounts of human IgG globulin (AG) to rabbit-anti-IgG serum is shown in the graph. Varying amounts of antigen (AG mg.) were added to 2 ml of antiserum (1:1 dilution with saline). The precipitates were washed and resuspended in 0.1M $Na_2CO_3$ (4 ml). The protein was then determined by optical density (OD) measurements at 280 mμ.


+
-

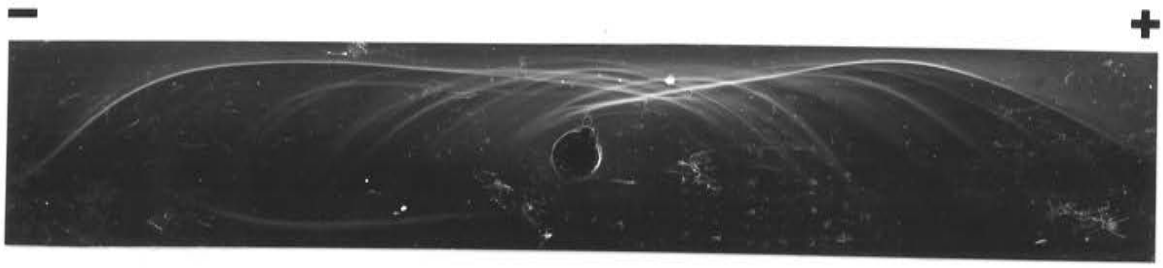

1·0
O.D.
0
0
2·0
AG. mg.

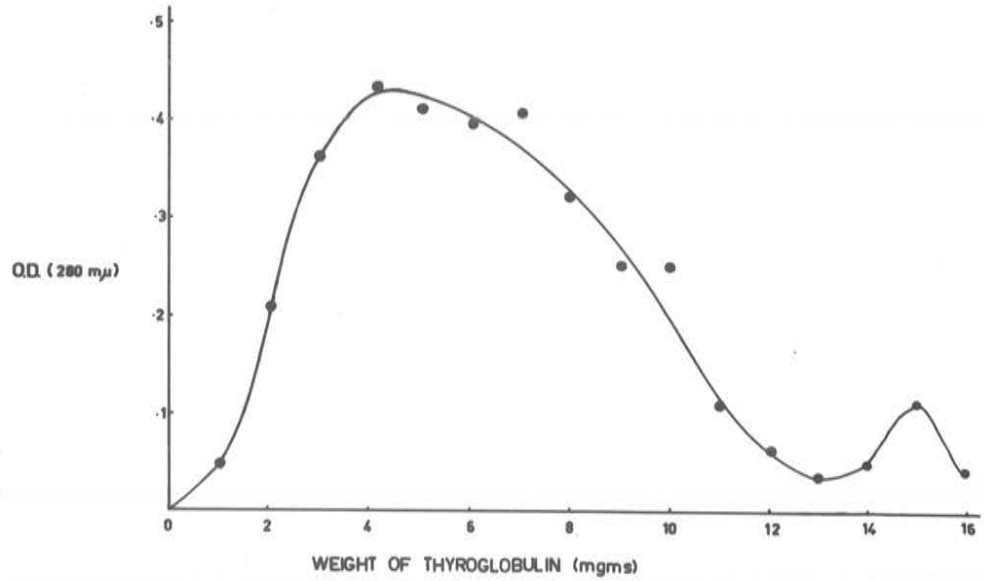
## APPENDIX (ix)

### Human thyroglobulin antibody

Plasma from a Hashimoto patient was electrophoresed in agar. The upper IEP pattern was developed with whole human antiserum. The lower IEP pattern was developed with human thyroglobulin.

The effect of adding increasing amounts of human thyroglobulin to Hashimoto plasma is shown in the graph. Varying amounts of antigen (mgms) were added to 1 ml of plasma. The precipitates were taken up in 4 ml of 0.1M $Na_2CO_3$ and the protein was determined by optical density (OD) at 280 mμ. OD was determined on 1:10 dilutions of the protein solutions.


−
+

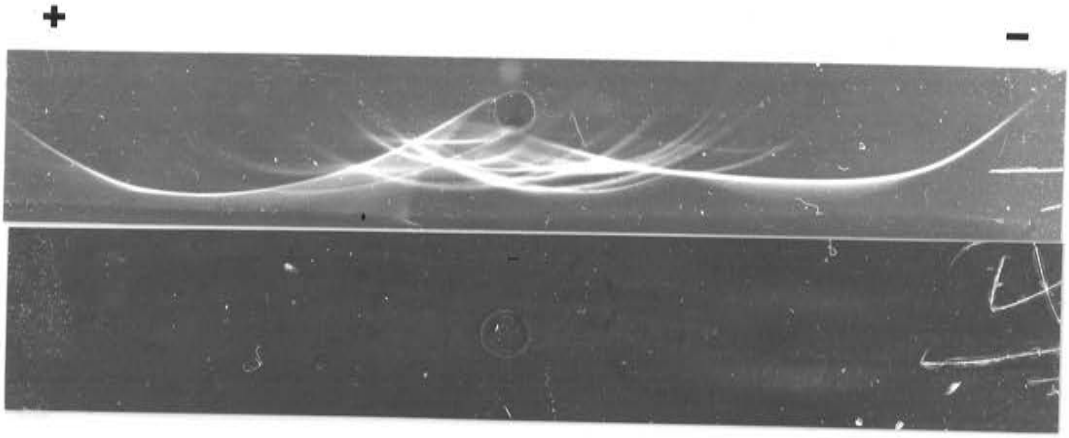

O.D. ( 280 mμ)
·5
·4
·3
·2
·1
0
2
4
6
8
10
12
14
16
WEIGHT OF THYROGLOBULIN (mgms)

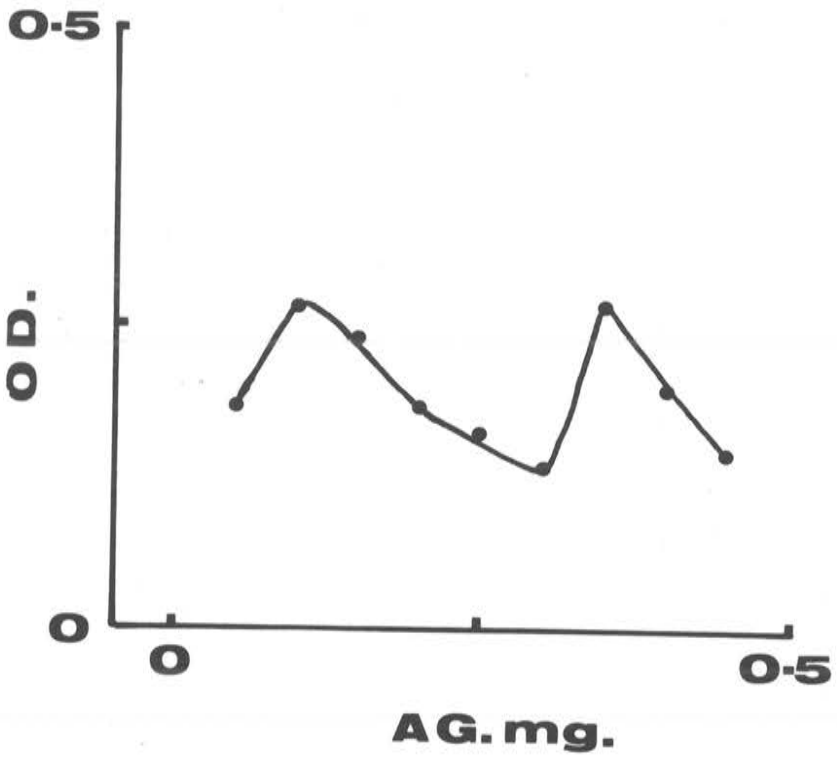
## APPENDIX (x)

Egg albumin antibody

The upper IEP pattern was developed with human plasma and whole human antiserum. The lower pattern was developed with rabbit anti-serum to egg albumin and egg albumin.

The effect of adding increasing amounts of egg albumin (AG) to rabbit-anti-egg albumin serum is shown in the graph. Varying amounts of antigen (AG mg) were added to 1 ml of antiserum (1:1 dilution with saline). The precipitates were washed and resuspended in 0.1M $Na_2CO_3$ (4 ml). The protein was then determined by optical density (OD), measurements at 280 mμ.


+
-


0·5
O.D.
0
0
0·5
AG. mg.

## APPENDIX (xi)

Reversible effect of puromycin on the incorporation of $^{14}$C-leucine by lymphocytes

| | Puromycin | Incubation time | CPM |
|---|---|---|---|
| i | - | 24 hr | 80 |
| ii | added at 0 hr. | 3 hr | 0 |
| iii | added at 0 hr washed off at 3 hr. | 24 hr | 110 |

Normal lymphocytes were cultured in supplemented Eagle's medium prepared without leucine. The cultures contained $1.2 \times 10^6$ cells and 0.25 µc $^{14}$C-leucine. Determinations were made in triplicate. Puromycin was added and removed at the times incubated. The cultures were sampled at various times and the counts per minute (CPM) of the TCA precipitates were determined.