THE EFFECT OF COMPOUNDS ON THE BIOCHEMISTRY
OF TRANSFORMING LYMPHOCYTES

by

JOHN JAMES GARDNER

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In vitro cultures of guinea-pig lymph node cells were stimulated to transform with phytchaemagglutinin (or antigen) and the incorporation of $[^3\text{H}]$-glucosamine, $[^{14}\text{C}]$-leucine, $[^3\text{H}]$-uridine and $[^3\text{H}]$-thymidine measured. The inhibitory effect of established anti-inflammatory drugs and novel candidate compounds on some of these parameters and on cell viability was determined.

FPL52806 affected the following measured parameters of stimulated cells, in decreasing order of sensitivity:

- $[^3\text{H}]$-glucosamine incorporation at 40h.
- $[^3\text{H}]$-thymidine incorporation at 40h.
- $[^{14}\text{C}]$-leucine incorporation at 40h.
- Reduction in number of transformed cells.
- Reduction in number of viable cells.
- $[^3\text{H}]$-uridine incorporation at 20h.

The effect of FPL52806 on $[^{3}\text{H}]$-thymidine and $[^{14}\text{C}]$-leucine incorporation were not due to interference with the binding of phytchaemagglutinin to the cells and were related to the length of time the cells had been exposed to the compound.

FPL52806-treated cells were studied by scanning electron microscopy.

The potencies of 6,8-di-alkyl chromones as inhibitors of $[^3\text{H}]$-thymidine and $[^{14}\text{C}]$-leucine incorporations were shown to be mathematically related to the lipophilicities of the compounds. The extent to which these compounds bound to bovine serum was also related to the lipophilicity of the compounds.

The order of decreasing potency of established anti-inflammatory drugs as inhibitors of $[^3\text{H}]$-thymidine incorporation was chloroquine, prednisolone, flufenamic acid, phenylbutazone, indomethacin, ibuprofen and sodium salicylate. This potency order is discussed with respect to the therapeutic potencies of the drugs in rheumatoid arthritis and to the activities of the drugs in other in vitro and in vivo test systems which may detect anti-inflammatory properties.
The advantages of the stimulated lymph node cell system as anti-inflammatory screen are described.

The biology of the lymphocyte and the evidence for the involvement of this cell in rheumatoid arthritis is described.
To Jennie, Louise and David
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'I like work; it fascinates me. I can sit and look at it for hours. I love to keep it by me; the idea of getting rid of it nearly breaks my heart.'

JEROME K. JEROME 'Three Men in a Boat'
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CHAPTER ONE

GENERAL INTRODUCTION
In the 1960s, drugs used in the treatment of chronic inflammatory diseases, such as rheumatoid arthritis, were widely studied for their effect on the metabolic activity of lymphocytes in vitro. Smith and Forbes (1967) found that salicylate, phenylbutazone, oxyphenbutazone, indomethacin, chloroquine and hydrocortisone, at concentrations considered to be clinically attainable in man, inhibited protein synthesis in short-term cultures of human blood lymphocytes. The drugs caused similar degrees of inhibition whether or not the lymphocytes were stimulated with phytohaemagglutinin (PHA). Whitehouse (1967) similarly showed inhibition of RNA, DNA and protein synthesis, in very short term cultures (30 minutes) of sheep thoracic-duct lymphocytes, by phenylbutazone, indomethacin, flufenamic acid and steroids; however, chloroquine was without effect in Whitehouse's system. Previously, Gantner and Zuckner (1965) had shown that blood lymphocytes taken from rheumatoid arthritis patients, during treatment with salicylates, responded to stimulation with PHA to a lesser extent than lymphocytes taken before drug treatment. The inhibitory effect of salicylates, administered in vitro and in vivo, on the PHA-stimulated transformation of human blood lymphocytes has been recently confirmed (Opelz and Terasaki, 1973; Crout et al, 1975).

These studies suggest that studies of the effects of drugs on lymphocytes might provide a suitable screening system for anti-inflammatory compounds (Forbes and Smith, 1967). Furthermore, they imply that the therapeutic effect of drugs, such as salicylate, in rheumatoid arthritis is, at least partially, due to their inhibitory effect on lymphocyte function. The latter conclusion is supported by the finding that acetylsalicylate, phenylbutazone and indomethacin (Winchutch et al, 1974) inhibit the cytotoxic activity of
lymphocytes, presumably due to lymphokine release (Dumonde et al., 1969). The release of lymphokines by lymphocytes has been suggested to be important in early inflammatory events (Mackler, 1971). The discovery that drugs used in the treatment of rheumatoid arthritis affect lymphocytes, supports the belief that immune mechanisms are important in rheumatoid arthritis (Winchurch et al., 1974).

The purpose of the present study was to determine the effect of established anti-inflammatory drugs and novel compounds on the synthetic activities of guinea-pig lymph node lymphocytes stimulated with PHA and to relate these effects to activities of the established drugs and candidate compounds in other anti-inflammatory screens.
The functional and anatomical division of lymphocytes into T (thymic-dependent) lymphocytes and B (bone marrow-derived, or bursal-equivalent) lymphocytes is well established (Roitt et al., 1969; Raff 1971; Good 1972; Winkelstein and Rabin, 1975). B lymphocytes are responsible for immune responses involving the synthesis of specific immunoglobulins (humoral immunity) which are the immune responses Coombs and Gell (1963) classified as types 1 to 3. T lymphocytes are responsible for cell-mediated immunity, or delayed hypersensitivity reactions (Coombs and Gell's type 4).

The foetal liver and later in life the bone marrow, are the sources of a multi-potential lymphoid stem cell capable of ultimately maturing into either T or B lymphocytes (McGregor, 1968; Good, 1972). T lymphocytes complete their differentiation in the thymus (Miller and Osoba, 1967), or under the influence of a hormone-like substance secreted by the thymus (Goldstein, 1972; Bach and Dardenne, 1973; Miller 1974). Avian B lymphocytes complete their differentiation into competent cells in the bursa of Fabricus, a gut associated organ. In mammals no comparable gut associated lymphoid tissue has been found, although the appendix and Peyer's patch were considered to be possibly such organs. It appears that maturation of B lymphocytes occurs directly within the bone marrow (Unanue et al., 1971; Abdou and Abdou, 1972).

When mature competent T lymphocytes are released some circulate continuously through the blood and lymph, passing out of the blood through post-capillary venules in the lymph nodes and Peyer's patches, travel through the lymphoid tissue and enter the efferent lymph, returning to the blood stream via the thoracic duct or other lymphatic vascular anastomoses (Gowans and Knight, 1964). Other T lymphocytes migrate into
specific areas of lymphoid tissue, the thymic dependent areas, which are in the case of lymph nodes the paracortex (Parrott and De Sousa, 1971), and remain as small inter-mitotic cells for an indeterminate time, but retaining the capacity to re-enter the circulation. T lymphocytes are in general long-lived (Howell, 1965; Buckton et al., 1967). In contrast most B lymphocytes are believed to be short-lived cells with a rapid rate of renewal (Craassdock et al., 1971; Parrott and De Sousa, 1971). The majority of B lymphocytes do not circulate but remain relatively fixed at the site of production. A minor population of B lymphocytes do circulate, and pass through different areas of the lymphoid tissue and with a slower transit time than T cells (Howard et al., 1972). The circulating B lymphocytes have a longer life span than non-circulating B lymphocytes (Howard, 1972; Sprent and Basten, 1973). In the peripheral lymphoid tissue B lymphocytes are found in areas which are different from the thymic-dependent areas occupied by T lymphocytes and, in the case of lymph nodes there are the germinal centres, subcapsular zones and medulla (Parrott and De Sousa, 1971).

T and B lymphocytes may be distinguished by criteria other than different origins, circulatory behaviour and life spans. T lymphocytes are denser, and less adherent or sticky (Bianco et al., 1970), slightly larger (Howard et al., 1972) and more negatively charged (Wioland, 1972) than B lymphocytes. The charge difference is related to the number of sialic acid residues present on T compared with B lymphocytes (Nordling et al., 1972). T and B lymphocytes have different appearances under the scanning electron microscope, B lymphocytes having many more and longer external processes than T lymphocytes (Polliack et al., 1973). Other surface membrane characteristics may be used to distinguish B and T lymphocytes. B lymphocytes have immunoglobulin present on their surface (Robellino et al., 1971; Shevach et al., 1973), receptors for the C3 component of complement (Ross et al., 1973; Shevach et al., 1973), and also
receptors for the Fc portion of IgG (Dickler and Kunkel, 1972). These are believed to be absent from T lymphocytes.

T lymphocytes in mice carry a specific membrane antigen, the theta antigen (Raff, 1971) which is absent from B lymphocytes. A similar antigen appears to be present on human T lymphocytes (Smith et al., 1973). T lymphocytes may also be identified by their unique capacity to form rosettes with native unsensitized sheep red cells (Bach and Dardenne, 1972; Wybran and Fudenberg, 1973).

T and B lymphocytes which are sensitized to antigen, respond in different ways when exposed to the antigen. B lymphocytes divide and differentiate into blast cells with abundant rough endoplasmic reticulum and some become plasma cells which secrete antibody to the stimulating antigen. This antibody is responsible for the immune responses which Coombs and Gell classified as types 1 to 3. B lymphocytes are functionally responsible for the synthesis of antibody involved in humoral immunity.

T cells proliferate and differentiate to blast cells which do not, in contrast, contain abundant rough endoplasmic reticulum and do not become antibody-secreting cells. They secrete a variety of non-antibody biological activities, or factors, ('lymphokines', Dumonde et al., 1989) such as migration inhibition factor, cytotoxic factor, mitogenic factor, inflammatory factor and various factors which are chemotactic for polymorphonuclear leucocytes, eosinaphils and macrophages. The biological activities of these factors have been reviewed by David (1971) and Pick and Turk (1972). Some of these factors at least, are involved in cell-mediated immune responses, for which T cells are considered to be primarily responsible (Bloom, 1971). It has recently been established that stimulated guinea-pig B lymphocytes can also produce macrophage-inhibiting factor (Yoshida et al., 1973), cytotoxic factor (Wahl, 1974), and macrophage-activating factor (Wilton et al., 1975). B lymphocytes from other species, for example
man, (Mackler et al, 1974) and mice (Parker and Metcalf, 1974), can also produce lymphokines. These results suggest that B lymphocytes may be involved in cell-mediated immunity in addition to their role of synthesizing antibody for humoral immunity, and that cell-mediated immunity is not exclusively a T lymphocyte function (Wilton et al, 1975). It is not yet known whether or not the same B lymphocytes responsible for antibody synthesis also produce lymphokines.

T cells in addition to their role in cellular immunity assist B cells in the synthesis of certain antibodies (Miller and Mitchell, 1968). The T cells do not synthesize the antibody but in some way, possibly involving local concentration of antigen, induce the B cells to synthesize antibody in response to the antigen. Such antigens, known as thymus-dependent antigens, usually lack the repeating identical determinants which are found in thymus-independent antigens, such as pneumococcal polysaccharide.
1.3 LYMPHOCYTE TRANSFORMATION

The response of specifically-sensitized T or B lymphocytes to antigen, that is cell proliferation and blast-cell formation followed by cell division, is known as lymphocyte transformation, stimulation, or activation. Certain substances, known as mitogens, mimic the effect of specific antigen on sensitized cells and cause lymphocyte transformation.

In contrast to antigenic stimulation where only a small number of cells respond, mitogens cause a large number of cells to transform; mitogens are polyclonal stimulants (Andersson et al., 1972b; Greaves and Janossy, 1972). The most commonly used mitogen is phytohaemagglutinin (PHA) which is isolated from the red kidney bean, Phaseolus vulgaris, (Nowell, 1960). PHA stimulates the transformation of only T cells in rats, mice and chickens (Janossy and Greaves, 1971). In man there is evidence for stimulation of B cells by PHA (Phillips and Roitt, 1973), but this could be an indirect result of stimulation of T cells by PHA (Phillips and Weisrose, 1974). Greaves et al. (1974) considered PHA to be predominantly, if not exclusively, a T cell stimulant in man.

Similar plant-derived mitogens (phytomitogens) have been prepared from Canavalia ensiformis (Weckster et al., 1968), Lens culinaris (Young et al., 1971) and Wisteria floribunda (Toyashima et al., 1971). Concanavalin A (from Canavalia ensiformis) is a T cell mitogen. These phytomitogens bind to carbohydrates, and this may be important in the mechanism of their lymphocyte-stimulating action, since addition of N-acetyl-D-galactosamine inhibits the stimulation of transformation by PHA (Fisher and Mueller, 1969a) and transformation caused by concanavalin A is inhibited by methyl-D-mannopyranoside (Novogrodsky and Katchalski, 1971a).
Simple inorganic compounds such as mercuric chloride (Schopf et al., 1967; Caron et al., 1970), and sodium periodate (Novogrodsky and Katchalski, 1971b; Parker et al., 1972) have also been shown to cause lymphocyte transformation. Sodium periodate is a T cell stimulant (Novogrodsky, 1974; Thurman et al., 1974). The oxidative activity of sodium periodate is believed to be responsible for the compound's mitogenic activity, oxidation of sugars on the lymphocyte's surface with formation of aldehydes being important (Novogrodsky and Katchalski, 1972; Zatz et al., 1972). Macrophages are also involved in the mechanism of mitogenesis induced by sodium periodate (Biniaminov et al., 1975). Treatment of lymphocytes with neuraminidase, to reveal galactose residues, and with galactose oxidase, to oxidize these to form aldehyde groups will also induce transformation (Novogrodsky and Katchalski, 1973; Biniaminov et al., 1975).

Specific B-lymphocyte mitogens include lipopolysaccharide (LPS), (Andersson et al., 1972a), pneumococcal polysaccharide SIII, (Coutinho and Moller, 1973), N-palmitoyl-D-glucosamine (Rosenstreich et al., 1974), dextran sulphate (Dorries et al., 1974), proteases such as trypsin and pronase (Kaplan and Bona, 1974) and an extract of Nocardia opaca, (Bona et al., 1975). The pokeweed mitogen, extracted from Phyto
tolacca americana will stimulate both T- and B- lymphocytes to transform (Janossey et al., 1972; Weber 1973). PHA and concanavalin A are both T-lymphocyte mitogens, as described above, but when the mitogens are covalently bound their specificities change. PHA bound to sepharose beads is mitogenic for both B- and T-lymphocytes (Greaves and Bauminger, 1972) but concanavalin A bound to the surface of plastic petri dishes is mitogenic for only B-lymphocytes (Andersson et al., 1972b).

Cyclic guanyl monophosphate (cyclic-GMP) stimulates both B- and T-lymphocytes (Diamantstein and Ulmer, 1975a). A calcium ionophore, A23187, has been shown to possess mitogenic activity (Luchasen et al., 1974, and Maino et al., 1974).
mitogenic activities of cyclic-GMP and the calcium ionophore have relevance to the understanding of the early events that occur during lymphocyte transformation.

Lymphocyte transformation may also be induced by immune complexes (Moller, 1969); the culture together of lymphocytes from genetically dissimilar individuals, mixed lymphocyte reaction, (Elves, 1968) and by anti-lymphocyte serum (Grasbeck et al, 1964) or by antibody directed against immunoglobulin present on the lymphocyte surface (Alm and Peterson, 1969). Mitogenic Factor, present in the 'lymphokines' produced by antigen-stimulated (Dumonde et al, 1969; Wolstencroft and Dumonde, 1970) or mitogen stimulated (Mackler et al, 1972; Smith and Barker, 1972) T-lymphocytes will stimulate other lymphocytes to transform.

Lymphocyte transformation is the central event in all immune responses, both those involving B-lymphocytes (Types 1 to 3 of Coombs and Gell's classification) and T-lymphocytes (Type 4). The transformation can be stimulated specifically by antigen if the lymphocyte donor has been exposed to the antigen, non-specifically by mitogens, and other ways which have immune significance, for example the mixed lymphocyte reaction.

1.3.1 The Morphology of Lymphocyte Transformation

As seen in the light microscope, mitogens, such as PHA, transform small lymphocytes, 7-8 in diameter, and containing little cytoplasm, into large pyronine-staining 'blast' cells 12-20 in diameter, which later divide (Robbins, 1964). Allison and Mallucci (1964) observed an increase in the number of granules which stain with neutral red in the cytoplasm of PHA-stimulated blast cells, which indicates an increase in the number of lysosomes. Transforming cells also tend to
aggregate in clumps (Eurenius et al., 1969). An increase in nuclear volume has been measured for guinea-pig lymph node lymphocytes (Eurenius and McIntyre, 1970), and also for human blood lymphocytes (Muniz et al., 1970).

Under the electron microscope, cell size, proportion of cytoplasm, and number of lysosomes, mitochondria, ribosomes and polysomes is increased in PHA-stimulated cells (Biberfield, 1971b). The ribosomes are not significantly organised on the rough endoplasmic reticulum, as is found in stimulated B lymphocytes (Janossy et al., 1973; Shand et al., 1973). An increase in the number of nuclear pores has been reported (Maul et al., 1971) in PHA-stimulated human blood lymphocytes; the increase is biphasic and the first increase seems to be related to an increase in protein synthesis and the second to the onset of DNA synthesis (Maul et al., 1972).

1.3.2 The Biochemistry Of Lymphocyte Transformation.

The small lymphocyte is metabolically quiescent. When the cell is induced to transform, for example with PHA, a complex series of metabolic changes take place involving increased energy production and synthesis of macromolecules which finally result in mitosis. These changes were reviewed by Lucas, 1971.

The events which occur early in lymphocyte transformation have been intensively studied, to increase the understanding of lymphocyte transformation and of the initial events occurring after gene derepression.

The early events include increased synthesis of cyclic-GMP (Hadden et al., 1972), and acceleration of phospholipid turnover, especially that of phosphatidyl inositol (Fisher and Mueller, 1969b). An enhanced uptake of ions occurs, for example, K+ (Questel and Kaplan, 1970; Kay 1972), Ca2+ (Asherson et al., 1970; Whitney and Sutherland, 1972) and Fe3+ (Cross and Ord, 1971).
The uptake of metabolic precursors, for example uridine (Peters and Hausen, 1971a), carbohydrate (Peters and Hausen, 1971b), and amino acids (Mendelsohn et al., 1971; Van der Berg and Betel, 1973 and 1974) is also increased. These early changes occur within one hour of stimulation of the lymphocyte and are related to changes in cell membrane function, (Ladoulis et al., 1975). Endocytosis, a membrane function, is also increased in stimulated lymphocytes (Smith and Hollers, 1970; Biberfield, 1971a). Further evidence for changes in cell membrane function during lymphocyte transformation is the increased membrane fluidity (Burnett et al., 1974). Burnett et al. (1974) found close correspondence between the temporal changes in membrane fluidity and intracellular cyclic-GMP levels.

Early events which are intracellular include increased phosphorylation of nuclear histones (Kleinsmith et al., 1966; Cross and Ord, 1970) and synthesis of non-histone chromatin protein (Levy et al., 1973; Johnson et al., 1974). A redistribution of lysosomal enzymes into the cytoplasm occurs soon after stimulation of lymphocytes with PHA (Hirschhorn et al., 1968). This redistribution may be important in the remodelling processes that occur in stimulated lymphocytes (Hirschhorn et al., 1969; Weissmann and Dukor, 1970).

Subsequent to the early membrane-related changes and early nuclear and cytoplasmic events, synthesis of RNA, protein, phospholipid and DNA occurs prior to mitosis (Lucas, 1971). Changes related to RNA synthesis include increased cytidine and uridine kinase (Lucas, 1967) and decreased intracellular pools of uridine nucleotides (Lucas, 1971). The activity of nucleolar and nucleoplasmic DNA-dependent RNA polymerases are increased at an early stage (Pogo, 1972a).

DNA polymerase activity is increased at about 24 hours after stimulation of the lymphocyte (Agarwal et al., 1970) and DNA synthesis is maximal from about 40 hours, depending on the lymphocyte source and stimulant used. Thymidine kinase
activity parallels the timing of DNA synthesis, with maximal increase in activity occurring with the peak of DNA synthesis (Rabinowitz et al., 1970). Enzyme activities which are associated with the synthesis of nucleic acid precursors and which are increased during lymphocyte transformation include carbamyl phosphate synthetase and aspartate transcarbamylase (Ito and Uchino, 1973); these enzymes are concerned in the synthesis of pyrimidines. Increased ornithine decarboxylases has been found (Kay and Lindsay, 1973); this enzyme is involved in histone synthesis.

At 24 hours after stimulation of lymphocytes, protein synthesis is accelerated (Mueller and LeMahieu, 1966). Ahern and Kay (1973) found an increased number of ribosomes actively engaged in protein synthesis. The activity of the proline biosynthesizing enzyme (Δ'-pyrroline-5-carboxylate reductase) is increased during lymphocyte transformation, but the proline degrading enzyme, proline oxidase, is not affected (Valle et al., 1975). Valle et al. also found that the reductase stimulated in lymphocytes was sensitive to inhibition by proline, indicating a negative feed-back mechanism to control the synthesis of proline at a rate just sufficient for protein synthesis.

The synthesis of glycoproteins is also increased (Hayden et al., 1970). Hayden et al. found that the majority of the 14C-glucosamine, used to measure glycoprotein synthesis, was associated with membranes, mainly the plasma membrane. The synthesis of phospholipid is also increased (Fisher and Mueller, 1969b) indicating new membrane synthesis. The activity of galactosyl transferase on the lymphocytes' plasma surface is also increased during transformation (Lamont et al., 1974). This effect was interpreted to indicate fresh plasma membrane synthesis on the golgi apparatus, where galactosyl transferase is found normally at higher levels than on the plasma membrane.

The above synthetic processes require energy and this is provided by the oxidation of glucose. Hedeskov (1968) found
that the rate of glucose utilization was increased twofold in PHA-stimulated lymphocytes and that pyruvate formation and the pentose cycle activity were increased. MacHaffie and Wang (1967) had previously shown that the pentose cycle was enhanced in stimulated lymphocytes and interpreted this to reflect an additional need for biosynthetic intermediates and NADPH to accommodate the mitotic activity induced by PHA. Roos and Loos (1970) found that PHA caused early changes in the levels of glycolytic intermediates and ATP and ADP, and concluded that PHA stimulates processes consuming energy, the demand for which is met initially by glycolysis and subsequently by the Krebs Cycle. Sagone et al (1974) have confirmed that increased pentose cycle activity and glycolysis occur during lymphocyte transformation. Glycogen particles accumulate after 24h in PHA stimulated cells (Quaglino and Hayhoe, 1965) indicating that not all of the glucose taken up by the cell is used immediately for energy production.

Lymphocyte transformation is biochemically a very complex process, of which 'our current understanding remains descriptive', (Lucas, 1971). A similar view was expressed by Maino et al (1974), when discussing the initial biochemical changes, 'the sequence, relative importance and control of these events are not yet defined'. This lack of definition of the importance of the various biochemical changes that occur in lymphocyte transformation may be illustrated by the assertion of Maino et al that 'lymphocyte transformation is the result of a direct affect of mitogen (PHA) on the permeability of the plasma membrane to Ca$^{2+}$', (Maino et al, 1974) and Diamantstein and Ulmer's suggestion that 'Ca$^{2+}$ is required for a step preceding DNA synthesis, but not for the early initial phase of transformation' (Diamantstein and Ulmer, 1975b).
A large number of compounds have been assessed for activity against lymphocyte transformation. The degree of inhibition of lymphocyte transformation has been usually measured by study of the effect of compound on the incorporation of $^{3}H$-thymidine in mitogen (generally PHA)-stimulated cultures.

The literature prior to 1967 has been reviewed by Ling (1968), who names chloroquine, corticosteroids, nitrogen mustard, salicylate, mercaptoethanol, actinomycin, puromycin, cycloheximide and 5-fluoro-2-deoxyuridine as potent inhibitors of lymphocyte transformation. Other compounds, such as indomethacin, flufenamic acid, also inhibit lymphocyte transformation but only at concentrations considered to be pharmacologically unrealistic (Ling, 1968).

Subsequent to Ling's review many other drugs, antibiotics and antimetabolites, have been found to inhibit lymphocyte transformation (Table 1).

Various extracts, or factors from biological material have also been shown to inhibit lymphocyte transformation (Table 2).

The relevance of these in vitro effects to the in vivo situation is not known for many of the agents, but administration of salicylates to patients has been shown to decrease the in vitro responsiveness of the lymphocytes to mitogen-stimulation (Gantner et al, 1966; Crout et al, 1975). Similarly in vitro inhibition of the stimulation of patients' lymphocytes has been reported for cis-platinous diamminodichloride (Khan and Hill, 1973) when the compounds were administered to patients.

The mechanism of action of the agents and natural
substances (Tables 1 and 2) which inhibit lymphocyte transformation are mostly uncertain. The antimetabolites, such as 6-mercaptopurine, and antibiotics, such as adriamycin, probably act by interference with nucleic acid synthesis. Rifampicin inhibits RNA polymerase in PHA-stimulated lymphocytes (Pogo, 1972b) but 3-deoxyadenosine did not affect this enzyme, although it inhibited RNA synthesis (Pogo, 1974).

Some inhibitors of lymphocyte transformation, such as prostaglandins (Webb et al., 1974) and the enterotoxin from Vibrio Cholerae (Hart and Finkelstein, 1975) have been suggested to act via the cyclic-AMP system. Cyclic-AMP and cyclic-GMP have antagonistic effects in many biological systems (Estensen et al., 1973; Goldberg et al., 1973). Cyclic-GMP is known to increase in PHA-stimulated lymphocytes (Hadden et al., 1972) and extracellular cyclic-AMP will inhibit lymphocyte transformation (Diamantstein and Ulmer, 1975a). It seems likely that compounds which raise the intracellular cyclic-AMP level by, for example, the inhibition of phosphodiesterase, or by the stimulation of adenyl cyclase, will inhibit lymphocyte transformation. Hart and Finkelstein (1975) suggested that the enterotoxin of Vibrio Cholerae stimulated adenyl cyclase.

A further possible mode of action for substances that inhibit mitogen-stimulated lymphocyte transformation is the inhibition of the binding of mitogen to the lymphocyte. N-acetyl-D-galaclcosamine is believed to inhibit PHA-stimulated lymphocyte transformation in this way (Fisher and Mueller, 1969a). Yachrin (1975) showed that fetuin, a glycoprotein, inhibited PHA-stimulated lymphocyte transformation by a similar mechanism.
TABLE 1. Agents That Inhibit Lymphocyte Transformation In Vitro.

<table>
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<th>Substance</th>
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<tr>
<td>Amantadine</td>
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<td>Diphenylhydantooin</td>
<td>Mackinney and Vyas (1972).</td>
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<tr>
<td>Halothane</td>
<td>Bruce (1972); Cullen et al (1972).</td>
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<tr>
<td>Adriamycin</td>
<td>Gale and Carnes (1971).</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>Khan and Hill (1971).</td>
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<tr>
<td>Cis-platinous diamminodi-chloride</td>
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<tr>
<td>3-Deoxy adenosine</td>
<td>Pogo (1974).</td>
</tr>
<tr>
<td>Transition metal ions (e.g. Cd²⁺, Co²⁺, Mn²⁺ and Ni²⁺)</td>
<td>Berger and Skinner (1974).</td>
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<tr>
<td>Beryllium compounds</td>
<td>Jones and Amos (1975).</td>
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TABLE 2. Natural Substances and Factors From Biological Material That Inhibit Lymphocyte Transformation In Vitro.

<table>
<thead>
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<th>Substance or source</th>
<th>Reference</th>
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<tr>
<td>Factor from cultured human lymphoma cells</td>
<td>Hersh and Drewinko (1974).</td>
</tr>
<tr>
<td>Seminal fluid</td>
<td>Stites and Erickson (1975).</td>
</tr>
<tr>
<td>Fetusin</td>
<td>Yachrin (1975).</td>
</tr>
<tr>
<td>Streptolysin 0 (haemolytically-active)</td>
<td>Anderson and Cone (1974).</td>
</tr>
<tr>
<td>Enterotoxin from Vibrio Cholerae</td>
<td>Hart and Finkelstein (1975).</td>
</tr>
<tr>
<td>Factor from Streptococci, group A</td>
<td>Malakian and Kaloustian (1975).</td>
</tr>
<tr>
<td>Unsaturated fatty acids, e.g. linoleic and arachidonic acids</td>
<td>Mertin and Hughes (1975).</td>
</tr>
</tbody>
</table>
Rheumatoid arthritis is an inflammatory disease of the synovium that leads to destructive changes in the joints and is often associated with the presence of abnormal antibodies (Rheumatoid Factor) in the blood. It is a major cause of suffering and economic loss from illness (Rheumatism and Arthritis in Britain, 1973). The elderly, especially female, are most frequently affected.

The underlying cause of rheumatoid arthritis is unknown. Initially the disease was believed to be due to bacterial infection of the joints. Subsequently it was suggested that auto-immune mechanisms were important, however the antigen responsible has not been identified. The pathology of the affected joints in rheumatoid arthritis is consistent with a chronic immune response involving local antibody production. Evidence for the presence of mycoplasma (Williams et al, 1970) and viruses, especially rubella, (Ogra et al, 1975) in rheumatoid synovial tissue has been interpreted to suggest that infection of the joints with these agents may be responsible for initiating the disease. Dumonde (1971) suggested that rheumatoid arthritis is due to a central defect in cell mediated immunity, especially the surveillance function permitting the persistence of antigen in the joints, resulting in immune complex formation. The cause of the defect could be viral infection. Glynn (1972) suggested that exogenous antigen, presumably a micro-organism present in the joints could cause a short-lived inflammation in the joints due to the immune response to the antigen and that auto-antigen release could occur during this initial inflammation, resulting in the establishment of a self-perpetuating immunologically specific inflammatory reaction.

Whatever the initiating process(es?) are in rheumatoid arthritis it is believed that lysosomal enzymes are responsible
for many of the changes that occur in rheumatoid joints (Chayen and Bitensky, 1971; Weissmann, 1972). Immune complexes, possibly between rheumatoid factor and altered IgG activate complement (Zwaifler, 1973), and cause polymorphonuclear leukocytes to infiltrate the joints. These leukocytes and also the lining cells of the synovial membrane phagocytose immune complexes and release lysosomal enzymes (Weissmann, 1972).

Lysosomal enzymes are capable of hydrolysing macromolecules (Weissmann and Dukor, 1970) and of causing tissue destruction, for example, that of cartilage matrix by cathepsin D (Ali, 1964). Lysosomal enzymes may also be responsible for the hypertrophy and hyperplasia of synovial lining cells found in rheumatoid joints (Weissmann, 1972). The absence of rheumatoid factor from the serum in about 20% of rheumatoid arthritics and its presence in non-rheumatoid patients (Glynn, 1968) and the occurrence of rheumatoid lesions in patients with agammaglobulinaemia (Gitlin et al, 1959) suggest that immune complexes other than those involving rheumatoid factor may be responsible for the release of lysosomal enzymes.

A further feature of the joints of rheumatoid arthritis is infiltration of lymphocytes into the synovia, often forming follicular clusters around which plasma cells are found (Zwaifler, 1973). In the synovial fluid of rheumatoid joints the proportion of B lymphocytes is about half that found in the blood of the patients and the proportion of T lymphocytes in the synovial fluid is correspondingly increased (Froland et al, 1973; Winchester et al, 1973; Sheldon et al, 1974). The T-lymphocytes in rheumatoid synovial fluid respond poorly to PHA compared with blood T lymphocytes, but have a higher spontaneous uptake of $[^3H]$-thymidine indicating prior stimulation by antigen in the synovium (Sheldon et al, 1974). Transformation of autologous lymphocytes by rheumatoid synovial fluid, but not by non-rheumatoid synovial fluid has been described, indicating cellular immunity to antigens in the rheumatoid fluid (Kinsella, 1973). These observations suggest that cell-mediated immunity.
may be important in rheumatoid arthritis. Similarly humoral immunity is considered to be important as manifested by rheumatoid factor and the presence of immune complexes in the joints. The role of cell-mediated immunity in rheumatoid arthritis is supported by the finding of cellular immunity to autologous IgG by blood lymphocytes of rheumatoid patients (Weisbart et al, 1975) and the detection of lymphokines in rheumatoid synovial fluid (Stastny, 1973; Ziff, 1973; Stastny et al, 1975). Proliferating T lymphocytes in the rheumatoid joints may co-operate with B lymphocytes in the local production of antibody with resulting formation of immune complexes (Ziff, 1973). The presence of macrophage-lymphocyte rosettes in rheumatoid synovial fluid cell cultures, involving mainly T-lymphocytes (Hepburn et al, 1974) suggests that antigen is present in the synovial fluid and that an immune response to the antigen occurs.

The relative roles of B and T lymphocytes in the pathogenesis of rheumatoid arthritis are not yet known (Messner, 1974), but there is sufficient evidence for the belief that B and T lymphocytes provide the link between the unknown initiating course of rheumatoid arthritis and the final lysosome-mediated joint destruction.
The number of drugs used in the treatment of rheumatoid arthritis is large and this may give some indication of the effectiveness of these drugs. Many of the drugs used traditionally to treat rheumatoid arthritis are acidic non-steroidal anti-inflammatory drugs, examples are salicylates (especially acetylsalicylic acid), phenylbutazone, ibuprofen, indomethacin and naproxen. Glucocorticosteroids, such as prednisone and prednisolone are also used; hydrocortisone acetate may be injected into the rheumatoid joints. The antimalarial compound chloroquine and various gold preparations, such as sodium aurothiomalate, are employed in the treatment of rheumatoid arthritis. Recently other drug treatments, such as the use of the immunosuppressive agents cyclophosphamide and azathioprine (Currey et al., 1974), and penicillamine (Gordon, 1974) have been introduced.

The majority of these drugs only affect the symptoms of rheumatoid arthritis. Duthrie (1971) considered that only gold preparations and chloroquine have true anti-rheumatic activity. The study of Currey et al. (1974) comparing the effectiveness of immunosuppressives and gold suggests that immunosuppressives may also have true anti-rheumatic activity.

The drugs which are believed to possess true anti-rheumatic activity, namely, chloroquine, gold and immunosuppressives, have appreciable side effects and hence are generally reserved for severely affected patients. The non-steroidal anti-inflammatory drugs, which also have adverse side effects especially gastro-intestinal bleeding, are used in patients with an early stage of the disease. Duthrie (1971) considered that acetylsalicylic acid was the drug of choice for these early stages, doses of 5g or more per day being required for anti-inflammatory effect (Boardman and Hart, 1967). Indomethacin and phenylbutazone are both
effective anti-inflammatory drugs, but both can cause undesirable side effects (Brooks, 1975). Ibuprofen, although claimed to be free of side effects is not superior to acetylsalicylic acid (Davies and Avery, 1971).

Steroids are used in cases of rheumatoid arthritis more severe than those treated with non-steroidal anti-inflammatory drugs (Constable et al, 1975).

1.6.1 Mechanism Of Action Of Drugs Used In The Treatment Of Rheumatoid Arthritis.

Drugs effective in the treatment of rheumatoid arthritis, especially drugs of the acidic non-steroidal anti-inflammatory group, have been the subject of extensive investigation in the last decade in an attempt to identify their mechanism(s) of action, (Adams and Cobb, 1967; Hichens, 1974).

Much of this work was initiated to select screening techniques which would identify superior compounds for the treatment of rheumatoid arthritis and other chronic inflammatory conditions.

Acidic non-steroidal anti-inflammatory drugs are active in many test systems related to the processes which are believed to occur in inflammation, such as those in the joints of rheumatoid arthritis. The acidic non-steroidal drugs are polyvalent; their effectiveness in treating the symptoms of rheumatoid arthritis cannot be ascribed to inhibition of one particular stage of the inflammatory processes.

The inhibition of prostaglandin synthetase by acidic non-steroidal anti-inflammatory drugs has received much attention (Vane, 1971; Flower and Vane, 1974; Flower, 1974). The potency of non-steroidal anti-inflammatory drugs as inhibitors of prostaglandin synthetase correlates well with their therapeutic effects (Flower et al, 1972). Prostaglandins
are found in inflammatory sites, for example the exudate induced in animals by carrageenin (Willis, 1969) and have been shown to induce inflammatory effects when injected into dog knee joints (DiRosa and Willoughby, 1971).

Other effects of non-steroidal anti-inflammatory drugs which may be important in their therapeutic action include stabilization of lysosomal membranes (Tanaka and Iizuka, 1968; Ignarro, 1971 and 1972), inhibition of connective tissue activation (Castor, 1972), inhibition of the effects of various mediators such as bradykinin and 'slow-reacting substances' (Shen, 1972), and inhibition of proteolytic enzymes (Skidmore and Whitehouse, 1967). Non-steroidal anti-inflammatory drugs stabilize serum protein (Mizushima, 1964) and accelerate sulphydryl-disulphide interchange (Gerber et al, 1967). The latter effect may be relevant to the formation of aggregated IgG in the joints of rheumatoid arthritis. The aggregation of IgG is accelerated by hyaluronic acid (Gerber 1975) which is present in synovial fluid at a high concentration. Further effects of non-steroidal anti-inflammatory drugs are inhibition of platelet function (O'Brien, 1968) and inhibition of the migration of mononuclear cells (Di Rosa et al, 1972). The relative importance of the various actions of non-steroidal anti-inflammatory drugs mentioned above in their therapeutic effect in man is not known, but the effect on prostaglandin synthetase is probably of considerable significance.

The mode of action of other drugs used in the treatment of rheumatoid arthritis such as chloroquine, gold salts, steroids and penicillamine is as uncertain as that of the acidic non-steroidal anti-inflammatory drugs. Chloroquine accumulates in lysosomes (Allison and Young, 1964) and its anti-rheumatic activity may be due either to inhibition of the lysosomal enzymes (Cowey and Whitehouse, 1966) or to stabilization of the lysosomal membrane, preventing enzyme release (Weissmann, 1964 and 1968). Chloroquine also accumulates in lymphocytes (Fedorko, 1967). Chloroquine
inhibits nucleic acid synthesis (Domenjoz, 1971), and will therefore affect tissue proliferation, such as that found in rheumatoid joints. Chloroquine also blocks sulphydryl-disulphide interchange (Gerber, 1964).

Gold salts accumulate in lysosomes and inhibit lysosomal enzymes (Persellin and Ziff, 1966; Ennis et al, 1967; Weissmann, 1972). Gottlieb et al (1972) found that gold salts administered intramuscularly accumulated in inflammatory rheumatoid joints at sufficiently high concentrations to inhibit lysosomal enzymes. Gold salts bind to macromolecules, such as collagen (Adam et al, 1964), nucleic acids (Eberl, 1971), and immunoglobulins (Lorber et al, 1972) and this may provide a mechanism for the uptake of gold into phagocytic cells (Lorber et al, 1972). Gold salts do not stabilize lysosomal membranes (Persellin and Ziff, 1966; Ignarro, 1971).

Steroids such as prednisolone affect many of the processes which occur in inflammation (Cleman, 1975) such as lysosomal enzyme release (Weissmann, 1972), chemotaxis of polymorphonuclear leukocytes (Ward, 1966), and the mobility and rate of cell division of macrophages (Fauve and Pierce-Chase, 1967). Steroids have profound effects on cell-mediated immunity, but little or no effect on antibody synthesis in man (Tuchinda et al, 1972; Butler and Rossen, 1973). Steroids decrease the number of circulating lymphocytes (Borella and Green, 1972) but the effect is short-lived in man (Chai and Gilbert, 1973). Man is a steroid-resistant species compared with, for example, the rat, and the decrease of lymphocytes reflects a redistribution from the circulating pool rather than lymphocyte death (Borella and Green, 1972). Lewis and Piper (1975) reported that steroids inhibit the release, but not the synthesis of prostaglandins, in contrast to non-steroidal anti-inflammatory drugs which inhibit their synthesis. Recently inhibition of prostaglandin synthesis by steroids has been reported (Kantrowitz et al, 1975; Tashjian et al, 1975). Steroids are polyvalent compounds and the relative importance of these actions in rheumatoid
Arthritis is unknown.

Penicillamine is a sulphhydril compound and reacts with the disulphide bonds, such as those found in Rheumatoid Factor, resulting in depolymerization of the Factor (Pavelka et al., 1971). Clinically however the resulting decline in the amount of rheumatoid factor present in the serum of penicillamine treated rheumatoid arthritics does not correlate with clinical improvement (Huskisson and Berry, 1974).

The importance of penicillamine's bivalent metal-chelating properties, especially that of copper, as an explanation of the compound's clinical efficacy is undecided, though the elevated serum copper level in rheumatoid arthritis suggests this property may be important (Pavelka et al., 1971).
CONCLUSION

The cause of rheumatoid arthritis and the processes responsible for joint damage are uncertain, but it is likely that immune mechanisms, both those involving B lymphocytes (humoral immunity) and T lymphocytes (cell-mediated immunity), are important (Messner, 1974). Anti-inflammatory drugs possess many properties which may explain their therapeutic activity. The effect of these drugs on lymphocyte transformation (Forbes and Smith, 1967; Opelz and Terasaki, 1973; Crout et al., 1975), and on the cytotoxic activity of such lymphocytes (Winchurch et al., 1974) may be properties relevant to the therapeutic activity of drugs in rheumatoid arthritis. The effectiveness of immunosuppressive drugs, such as cyclophosphamide, in the treatment of rheumatoid arthritis (Hurd, 1972; Currey et al., 1974) supports the belief that lymphocytes are important in the disease. However, there is controversy over whether cyclophosphamide affects both B and T lymphocytes (Clements et al., 1974; Hurd and Giutiano, 1975) or solely B lymphocytes (Horwitz, 1974).

In the subsequent chapters of this thesis the effect of anti-inflammatory drugs and novel compounds on the synthetic activity of PHA-stimulated guinea-pig lymph node cells (a T lymphocyte function) is described. The role of T lymphocytes in rheumatoid arthritis is controversial. If the function of T lymphocytes, such as regulation of immunoglobulin synthesis, is depressed or disordered in some way, as has been suggested by Dumonde (1971), any inhibitory effect of drugs on T lymphocyte function in rheumatoid arthritis might be beneficial but 'normalizing' the immune response might alternatively exacerbate the disease. Interpretation of the relevance of the effect of drugs on T lymphocytes must await greater knowledge of the function of T lymphocytes in the disease process of rheumatoid arthritis.
CHAPTER TWO

MATERIALS AND METHODS
2.1 MATERIALS

2.1.1 Animals

Out-bred male Hartley-strain guinea-pigs, of 350g to 450g were obtained from Hillcrest Research Station, Belton, Leicestershire, and bedded on sterile hay in wire-bottomed cages. They received Oxoid food pellets and water containing ascorbic acid (1g per 200ml) ad libitum, and fresh cabbage daily.

2.1.2 Tissue Culture Materials

Eagle's Minimal Essential Medium (MEM) batches, M3011 and M2144 was supplied by Wellcome Reagents Limited, Beckenham, Kent, as was Eagle's Minimal Essential Medium which did not contain the amino acid leucine, batches M2042, M3734 and M692. Colostrum-deprived calf serum was obtained from Wellcome Reagents Limited (batches K2465, K2812, K3138) and Flow Laboratories, Irvine, Ayr, (batch 40377). It was found that other batches from these suppliers obtained exclusively during the winter months were not satisfactory for the culture of PHA-stimulated guinea-pig lymph node cells, due, possibly, to presence of inhibitory substances. Purified phytohaemagglutinin (PHA) was obtained from Wellcome Reagents Limited, batches K1360, K2402 and K4402. The optimum concentration of PHA which stimulated the incorporation of $^{3}H$-thymidine by $2 \times 10^6$ guinea-pig lymph node cells in 10% serum at 40 hours was determined for each batch. It was in the range 1 to $2 \mu g/ml$.

2.1.3 Chemicals

Hydroxy-ethyl-piperazine-ethyl-sulphonic acid, HEPES, was obtained from Sigma London Chemical Company as was bovine serum albumin (fraction V), batch 113C-3430. Diphenyl oxazole,
1,4-di[2-(5-phenyl-oxazolyl)]-benzene and 1M-Hydroxide of Hyamine 10-X in methanol were obtained from Packard Ltd., Caversham, Berks. Freund's Complete Adjuvant was obtained from Difco Laboratories. Ovalbumin was supplied by B.D.H. Chemicals Ltd., Poole, Dorset.

Other chemicals were of analytical grade and were obtained from B.D.H. Chemicals Ltd., Poole, Dorset, or Fisons Scientific Equipment Ltd., Loughborough, Leicestershire.

2.1.4 Anti-Inflammatory Drugs And Novel Compounds

Anti-inflammatory drugs were obtained through Mr. H. Radzivonik from Merck, Sharpe, and Dohme, (indomethacin), Geigy (phenylbutazone), Boots Pure Drug Company Ltd., (ibuprofen), May and Baker Ltd., (chloroquine), Glaxo Laboratories (prednisolone) and Parke-Davis (flufenamic acid). Sodium salicylate and p-hydroxy-benzoic acid were products of British Drug Houses Ltd. The purity of these substances was not determined. The Fisons Ltd., Pharmaceutical Division (FPL) compounds supplied by the Department of Medicinal Chemistry were pure on the criteria of one spot in 20 different thin layer chromatographic systems, and were used as the soluble sodium salts, when available.

2.1.5 Radiochemicals

Radio-active metabolic precursors were obtained from the Radiochemical Centre, Amersham, in sterile multidose vials, and after dilution with normal sterile saline stored at the temperature recommended by the supplier. 

\[ ^{3}H \text{thymidine, batches 88, 96 and 101 (5.0 Ci/m mole and } > 98\% \text{ pure), } \left[ U^{14}C \right] \text{L-leucine, batches 79 and 85 (approximately 340mCi/m mole and >97\% pure), } \left[ 5^{3}H \right] \text{uridine, batches 59 and 63 (approximately 5.0 Ci/m mole and > 98\% pure) and } \left[ 1^{3}H \right] \text{D-glucosamine, batch 7 (1.1Ci/m mole and > 96\% pure) } \]
were diluted so that stock solutions contained 100, 25, 100 and 25$^\text{Ci}$/ml respectively.

Before use these solutions were diluted with Eagle's minimal essential medium (1:9, v/v) so that the final solution contained in 0.1ml, 1, 0.25, 1 and 0.25$^\text{Ci}$ respectively. $[^3\text{H}]$-6,8-di-t-butyl chromone, $[^3\text{H}]$-FPL52806, (3.1m Ci/m mole) was a gift from Dr. C.J.P. Adderley, Fisons Ltd. The compound was generally labelled; the majority of the $[^3\text{H}]$ was in the t-butyl groups.
2.2 GENERAL METHODS

2.2.1 Cleaning Of Glassware

General glassware was soaked in 2% v/v Decon 75 detergent rinsed once with tap water and with distilled water. Stainless steel gauzes were placed in 5M-NaOH overnight to digest adhering material, and rinsed in tap water, 0.1M-HCl and distilled water. Stainless steel Millipore filters were soaked in 1% v/v Hederol detergent and rinsed in distilled water. Pipettes were soaked in Pyroneg detergent solution followed by 1M-HCl and rinsed in tap water and distilled water (six rinses).

2.2.2 Sterilization Of Glassware And Other Materials

General glassware, wrapped in aluminium foil, was sterilized in a hot air oven at 160° for 2 h. The necks of pipettes were plugged with non-absorbent cotton wool and treated similarly. 'Millipore' filters, wrapped in aluminium foil, were autoclaved at 15 lbs. per square inch pressure for 20 mins. in a vacuum autoclave. Deionized water for use in preparation of culture media was sterilized for 15 mins. at 15 lb. per square inch steam pressure. Drug solutions in Eagle's minimal essential medium were sterilized by 'Millipore' membrane filtration (0.22\(\mu\)m pore).

2.2.3 Treatment Of Guinea-Pigs To Induce Enlarged Lymph Nodes

The method of Oppenheim et al (1967), described by Wolstencroft and Dumonde (1970) was used. An emulsion (0.05ml) of Freund's Complete Adjuvant in sterile normal saline (1:1, v/v), was injected into each of the guinea-pig's paws. The enlarged draining lymph nodes were removed.
twelve to twenty days afterwards, generally at 14 ± 1 days.

2.2.4 Sensitization Of Guinea Pigs To Ovalbumin

Ovalbumin in sterile saline (1mg/ml) was emulsified with Freund's Complete Adjuvant (1:1 v/v) and injected into the paws of the animal, so that each guinea-pig received 100μg of ovalbumin in 0.2ml of the emulsion. The enlarged lymph nodes were removed at 14 ± 1 days. Skin testing at this time, by injection of 5μg of ovalbumin in 0.1ml of saline intradermally, revealed that the animals exhibited a state of delayed hypersensitivity to ovalbumin, and that antibody production had also occurred, since reddening and swelling of the ovalbumin injected sites occurred at one hour.

2.2.5 Preparation Of Culture Medium

Eagle's Minimal Essential Medium was prepared in 500ml batches which contained 25ml of 560mM HEPES 2M-NaOH buffer, pH 7.67 and also 2 x 10⁵ IU of penicillin and 100mg streptomycin. The final pH of the Eagle's medium was 7.45 ± 0.05 at 37°. Colostrum-deprived calf serum was decomplemented by heating at 56° for 30 minutes.

2.2.6 Preparation Of Lymph Node Cells

The method described by Wolstencroft and Dumonde (1970) was employed. Guinea-pigs were killed by cervical dislocation and bled by severing the neck vessels. The enlarged lymph nodes were removed aseptically, after moistening the animal's skin with ethanol. The nodes were placed in 5ml of Eagle's medium containing 5% v/v decomplemented calf serum and the adhering adipose and connective tissue removed. After transfer to fresh medium - 5% serum the nodes were teased
apart using two sterile needles. The Eagle's medium plus cells was passed through a stainless steel gauze, (80 mesh), and the torn nodes further disintegrated on the gauze using the piston of a 5ml disposable syringe. A further 25ml of Eagle's medium - 5% serum was used to wash the cells through the gauze. The cell suspension was centrifuged at 500g for ten minutes and after successive resuspension washed three times with Eagle's medium - 5% serum. The cells were finally suspended in 10ml of Eagle's medium - 5% serum. The above procedures were performed in a cabinet which had been sterilized by overnight use of an u.v. lamp.

2.2.7 Measurement Of Cell Yield And Viability

The viability of the lymph node cells was measured by an eosin-exclusion method, (Wolstencroft and Dumonde, 1970). The cell suspension (0.5ml) was diluted with 1.5ml of saline and 2.0ml of eosin Y (0.8% w/v) in saline was added. A sample of the stained cell suspension was placed in an improved Neubauer haemocytometer. After about three minutes to allow the cells to settle in the haemocytometer chamber the number of viable unstained lymph node cells was counted in an area of 1mm² using phase contrast, and a Wild microscope. The number of non-viable (stained) cells, viable large granular cells which were considered to be macrophages, and red blood cells, which were readily identified by their pale staining and bright appearance under phase contrast, were also counted. The mean yield of viable lymph node cells was $3.4 \pm 0.9 \times 10^8$ (range 2.0 to $5.1 \times 10^8$) in twenty typical experiments. The corresponding number of macrophages was $0.2 \pm 0.2 \times 10^7$ (range 0 to $0.4 \times 10^7$) and red blood cells $1.8 \pm 1.0 \times 10^7$ (range $0.8$ to $4.8 \times 10^7$).

The mean percentage viability of the lymph node cells, that is:

$$\frac{\text{number of viable lymph node cells}}{\text{total number of dead and viable lymph node cells}} \times 100$$

was $49\% \pm 4\%$ (range $28\%$ to $57\%$).
Lymph node cells were cultured in vertical round-bottomed glass tubes 13mm x 65mm in external dimension stoppered with silicone rubber bungs. The volume of the cultures was 2.0ml and their depth was 2.7 cms. Each culture of 2.0ml contained Eagle's medium enriched with 10% (v/v) decomplemented calf serum and 2 x 10^6 viable lymph node cell initially. PHA-stimulated cultures generally contained 1/μg/ml of the mitogen. Drugs were added in a proportion of the Eagle's medium. Replicate cultures were prepared from bulk reactions, frequently of 15mls which provided generally five individual replicates of 2.0ml volume. The cultures were incubated in a water bath at 37° without shaking and with air as the gas phase for various times depending on the biochemical parameter measured.
2.3 DNA SYNTHESIS IN CULTURES OF GUINEA-PIG
LYMPH NODE CELLS

Lymphocyte stimulants such as PHA induce the cell to transform and eventually divide. Prior to mitosis during the 'S' phase the cell doubles its chromosome number and DNA content and will incorporate exogenously added precursors such as thymidine into DNA. Measurement of the incorporation of \(^{[5}\text{methyl-}^{3}\text{H}]\) thymidine is the most frequently determined parameter for assessing the degree of lymphocyte transformation. It has been assumed that all the radioactivity present in acid-insoluble material is due to \(^{[3}\text{H}]\)-thymidine incorporated into DNA. Goldspink and Goldberg (1973) found that slices of liver, brain and kidney incorporated the labelled material into lipid and protein after degradation and that non-specific binding could occur to protein and RNA. These effects are less important in actively growing tissue (Goldspink and Goldberg, 1973). The specificity of \(^{[3}\text{H}]\)-thymidine for DNA synthesis therefore is likely to be greater in stimulated lymph node cell cultures than in their non-stimulated controls. Evidence for the breakdown of \(^{[3}\text{H}]\)-thymidine in cultures of human blood lymphocytes to thymine and dihydrothymine when using long incubations has been reported (Cooper and Milton, 1964). In order to minimise these effects cultures were pulsed with \(^{[3}\text{H}]\)-thymidine for short periods in the present work.

2.3.1 Measurement of \(^{[3}\text{H}]\)-Thymidine Incorporation

The method of Oppenheim et al as modified by Wolstencroft and Dumonde (1970) was employed. At various times, usually 40h, \(1\mu\text{Ci} \text{ of }^{[3}\text{H}]\)-thymidine (5Ci/m mole) was added in 0.1ml of sterile saline to each 2.0ml culture. The tubes were incubated at 37° in a vertical position, for 4h. At this time the cells were deposited by centrifugation at 4°C, and washed twice at 4° with 3.0mls of saline - which was buffered to pH 7.4 - with 10 mM NaH\(_2\)PO\(_4\)-NaOH, and contained 10mg/litre
phenol red. The supernatant was poured off and the inverted tube drained on Kleenex tissue, and the cell pellet resuspended by vortex mixing in the drop of fluid remaining. The cell pellet was washed twice with 2.0ml of 5% w/v trichloroacetic acid solution at 4° to prepare the acid-insoluble material which was washed once with 3.0ml of methanol.

Initially during this work a Sorvall RC-2B refrigerated centrifuge was used. This was equipped with an GSA angle-head containing purpose-made perspex adaptors, which allowed fifty four cultures to be processed at one time. The samples of cells in culture media and in saline were centrifuged for five minutes at 5,000 rpm (= 1020g) and the samples of cells in trichloroacetic acid and methanol were centrifuged for 10 minutes at 10,000 rpm (= 4080g). Later an MSE Multex centrifuge, placed in a 4° cold room, equipped with swing-out 'Multi-tube carrier' (MSE), which allowed seventy two cultures to be processed, was employed. With this equipment the initial centrifugations were performed for 5 mins. at 2,500 rpm (= 1540g) and then subsequently for ten minutes at the same speed. The centrifuges were changed due to the greater experimental convenience of the Multex centrifuge, and the possibility that some trichloroacetic acid-precipitated material was lost when fluid was poured off samples centrifuged in the angle-head Sorvall RC-2B centrifuge. This possibility was confirmed when replicate samples were processed using both centrifuges (Table 3).

The methanol-dried trichloroacetic acid precipitated material was dissolved in 0.3M Hyamine hydroxide in methanol (0.5ml per culture) by heating at 65° for 30 mins. with occasional agitation. The resulting clear solution was transferred, using 3 x 5ml volumes of toluene-based scintillator, to a counting vial. Samples were counted either for 10 mins. or to a pre-set count of 10,000 (=1% Poisson error), if this occurred within 10 mins. Extraction blanks, that is a sample containing 1μCi of $^{3}H$-thymidine and 2.0ml of Eagle's medium but no cells, and incubated for the
<table>
<thead>
<tr>
<th>PHA concn. (μg/ml)</th>
<th>Mean Radioactivity per Culture (d.p.m. x 10³)</th>
<th>MSE Multex</th>
<th>Sorvall RC2B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>7 ± 1</td>
<td>6 ± 1</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>216 ± 10</td>
<td>157 ± 46</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>145 ± 11</td>
<td>96 ± 7</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>67 ± 12</td>
<td>51 ± 10</td>
<td></td>
</tr>
</tbody>
</table>

Figures are the means of 4 replicates ± SD
same length of time as the cultures containing cells, were exposed to the labelled precursor, and carried through the washing procedure, gave counts less than twice those of a scintillator blank. The extraction blank was deducted from the counts obtained from the cultures containing cells.

2.3.2 Investigation Of Culture Conditions To Obtain Maximum Incorporation Of $^{3}H$-Thymidine.

The number of variables which are known to affect the intensity of the response in stimulated lymphocyte cultures is large and some of these are inter-reacting, for example the concentration of stimulant and the serum concentration (Forsdyke, 1967). The conditions for maximum incorporation of $^{3}H$-thymidine by mixed lymphocyte reaction-stimulated human blood lymphocytes have been investigated by Hughes and Caspary (1970). Hersh et al (1969) described the effect of variation of the surface area on which the cells could settle; the greater the area on which cells could settle the smaller was the uptake of $^{3}H$-thymidine at low cell densities. The pH of the culture medium (Hughes and Caspary, 1970) and the nature and amount of supplementing serum (Alford, 1970a) are important. The presence of metals ions such as Ca$^{2+}$, Fe$^{2+}$ and Zn$^{2+}$ (Alford, 1970b) is necessary. The presence of cell types other than lymphocytes can modify the response. Levis and Robbins (1970) found that removal of glass adherent cells (macrophages?) decreased the PHA-stimulated response at all PHA concentrations. The number of red blood cells present modifies the amount of $^{3}H$-thymidine incorporated, (Tarnvik, 1970). The length of the pulse period, the amount of $^{3}H$-thymidine added and its specific activity also affect the incorporation (Sample and Chretien, 1971).

In the present work, certain of these parameters such as culture volume, number of cells, serum concentration, shape of culture tube, amount and specific activity of the $^{3}H$-thymidine to be added were standardized at the start of the work. These were selected on the basis of experimental
convenience and satisfactory results in the hands of other workers with cultured guinea-pig lymph node cells (R.A. Wolstencroft personal communication). The influence of other parameters, such as the concentration of PHA, and time of maximum DNA synthesis were determined experimentally, and using these results certain of the initially assumed parameters were examined.

**Determination of time of maximum $[^{3}\text{H}]$-thymidine incorporation.**

The incorporation of $[^{3}\text{H}]$-thymidine at various times was measured in cultures stimulated with $1\mu\text{g/ml}$ PHA and non-stimulated cultures. Maximum incorporation of $[^{3}\text{H}]$-thymidine by PHA-stimulated cultures occurred on the second day at 45h., Table 4. The stimulation index, that is the ratio between the $[^{3}\text{H}]$-thymidine incorporated by the stimulated cultures and by the non-stimulated was also maximal on the second day. In subsequent experiments $[^{3}\text{H}]$-thymidine incorporation was measured at a slightly earlier time, namely 40h., since this was experimentally convenient.

The time of maximum $[^{3}\text{H}]$-thymidine incorporation is in agreement with other authors (Oppenheim et al., 1967).

**Determination of PHA dose response curve for $[^{3}\text{H}]$-thymidine incorporation.**

Cultures were incubated for 40 and 64h. in the presence of various concentrations of PHA (0.25 - 20$\mu\text{g/ml}$). At these times $1\mu\text{Ci}$ of $[^{3}\text{H}]$-thymidine was added for a 4h. pulse period. Figure 1 shows that a PHA concentration of about $1\mu\text{g/ml}$ caused maximum incorporation of $[^{3}\text{H}]$-thymidine at both times.

**Investigation of the inter-dependence of serum and PHA concentrations on $[^{3}\text{H}]$-thymidine incorporation.**

Cultures containing various concentrations of heat-decomplemented calf serum and PHA were incubated for 40h. and $1\mu\text{Ci}$ of $[^{3}\text{H}]$-thymidine per culture was added for a 4h.
TABLE 4. **Incorporation of $^3$H-Thymidine at Various Times by PHA-Stimulated and Non-Stimulated Guinea-Pig Lymph Node Cells**

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>PHA, 1/γ/ml</th>
<th>No PHA</th>
<th>Stimulation Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>26</td>
<td>47.2 ± 8.0</td>
<td>3.0 ± 0.1</td>
<td>16</td>
</tr>
<tr>
<td>45</td>
<td>162.1 ± 37.4</td>
<td>1.6 ± 0.2</td>
<td>99</td>
</tr>
<tr>
<td>69</td>
<td>38.4 ± 12.0</td>
<td>0.9 ± 0.2</td>
<td>43</td>
</tr>
</tbody>
</table>

Figures are the means of 4 replicates ± SD.
Figure 1. Incorporation of $[^3H]$-Thymidine at 40 and 64 Hours by Guinea-Pig Lymph Node Cells Stimulated with Various Concentrations of PHA.
pulse period. Serum concentration of 0.25, 3.25, 10.25, 30.25 and 50.25\% v/v were included and PHA concentrations of 0, 1, 2 and 4\mu g/ml were used. Figure 2 shows that the serum concentration had a marked effect on the form of the PHA dose response curve. For each serum concentration there appeared to be a corresponding concentration of PHA which resulted in optimal incorporation of \( [^3H] \)-thymidine, so that the optimal PHA concentrations for 30.25 and 50.25 v/v\% serum were above 4\mu g/ml. This family of PHA dose response curves might reflect the increased binding of PHA to serum at high serum concentrations, reducing the 'free' PHA available for cell stimulation, or the toxic effects which have been reported to occur with high PHA concentrations (McIntyre and Cole, 1969).

At high serum concentrations the volume of Eagle's medium in the 2.0ml cultures was reduced. The decline in the amount of \( [^3H] \)-thymidine incorporated at high serum concentrations might be related to the resulting reduction in the concentration in the cultures of substances present in the Eagle's medium which are required for transformation. Other substances present in the serum are believed to augment the transformation response, which might explain the increased \( [^3H] \)-thymidine incorporation when the serum concentration is increased from 0.25\%v/v. In order to resolve these possibilities further experiments would be required, but the data does allow the conclusion that 10\% serum provides good incorporation over a wider range of PHA concentrations than do the other serum concentrations employed.

Effect of varying the length of the 'pulse' period on the incorporation of \( [^3H] \)-thymidine.

The length of the 'pulse' period was fixed at 4h., as one of the arbitrarily selected parameters at the start of this work. A 4h. pulse is experimentally convenient, and is short relative to the time of in-vitro culture of the lymph node cells, so that a close approximation to the rate
Figure 2. Effect of Culture in Various Concentrations of Serum on $[^3]H$-Thymidine Incorporation at 40 Hours by Guinea-Pig Lymph Node Cells Stimulated with Several Concentrations of PHA.
of DNA synthesis at a time in the middle of the pulse period is measured. Other authors have used longer pulse periods (for example overnight) which have the advantage of giving increased incorporation, but the disadvantage is that the rate is likely to have changed over such a long period. A short pulse period is especially necessary when the rate of DNA synthesis is rapidly changing, for example at the onset of DNA synthesis in time course studies. The criterion used to assess the suitability of the 4h pulse period was linearity of incorporation of $[^3\text{H}]$-thymidine with time. This criterion has been used by Schellekens and Eijsvogel (1968) and Bain (1970) to confirm optimal labelling conditions, but Sample and Chretien (1971) stated that this was not a reliable criterion when pulse periods longer than 4h were used. With pulse periods up to 4h, linearity was a reliable criteria.

Cultures containing optimal serum concentrations (10%v/v) and with or without 2/8/ml of PHA were incubated for 47h. At various times from 40h, 1/Cl of $[^3\text{H}]$-thymidine was added to six replicate cultures so that at 47h the labelled precursor had been present for 0.42 to 7h. Figures 3 and 4 show the incorporation in PHA-stimulated and non-stimulated cultures respectively. The incorporation in the PHA-stimulated cultures was linear with time in the 7 hour period investigated. The correlation coefficient for the mean incorporations against time was 0.998. Incorporation into non-stimulated cultures was apparently linear up to 5.5h. Thus a 4h pulse satisfied the criterion of linearity of incorporation and was therefore used routinely.

Comparison of $[^3\text{H}]$-thymidine incorporation in cultures containing complete Eagle's minimal essential medium and medium from which leucine was omitted.

In some experiments both $[^3\text{H}]$-thymidine and $[^1\text{C}]$-leucine incorporations were determined simultaneously in the same cultures as measures of DNA and protein synthesis respectively. Since leucine is a constituent of Eagle's
Figure 3. Effect of Variation of Length of Pulse Period on the Incorporation of $[3^H]$-Thymidine by PHA-Stimulated Guinea-Pig Lymph Node Cells.
Figure 4. Effect of Variation of Length of Pulse Period on the Incorporation of $^{3}H$ - Thymidine by Non-Stimulated Guinea-Pig Lymph Node Cells.
medium (thymidine is absent) and its presence resulted in low incorporations of $[^{14}C]$-leucine it was necessary to use leucine-free medium when protein synthesis was measured. The effect of absence of leucine from the Eagle's medium on $[^{3}H]$-thymidine incorporation was therefore investigated.

$[^{3}H]$-thymidine incorporation was measured in cultures containing 10% v/v serum with or without 1/20/ml PHA and Eagle's medium with or without leucine.

The omission of leucine from the medium did not reduce the incorporation of $[^{3}H]$-thymidine, (Table 5). Simultaneous $[^{14}C]$-leucine and $[^{3}H]$-thymidine incorporation could thus be measured satisfactorily.

**Statistical validity of the effect of compounds on $[^{3}H]$-thymidine incorporation.**

In the subsequent chapters of this thesis the effect of established anti-inflammatory drugs and certain drug candidates from Fisons Limited Pharmaceutical Division (FPL) on metabolic activities, such as incorporation of $[^{3}H]$-thymidine, occurring in PHA-stimulated guinea-pig lymph node cells are described. Most of these compounds had totally different chemical structures, but some of the FPL compounds were members of an homologous chemical series. The activities of the members of this homologous series on $[^{3}H]$-thymidine incorporation were compared; that is inhibition of incorporation of $[^{3}H]$-thymidine was used as a bio-assay. Bio-assay methods are divided into two types, parallel line assays and slope ratio assays (Finney, 1964).

The inhibition of $[^{3}H]$-thymidine incorporation plotted against the logarithm of the concentration of the compound under test resulted in parallel lines, for the members of the homologous series. The statistical validity of such an assay may be confirmed by dilution assay, that is several fixed volumes of the two dilutions of the compound are assayed. If the assay is statistically valid the resulting dose response
**TABLE 5.** Incorporation of $[^3H]-$Thymidine at 4Oh. by PHA-Stimulated and Non-Stimulated Guinea-Pig Lymph Node Cells Cultured in Leucine-Free and Leucine-Containing Eagle's Minimal Essential Medium.

Mean Radioactivity per Culture
(d.p.m. x $10^2$)

<table>
<thead>
<tr>
<th></th>
<th>No PHA</th>
<th>PHA, 1/μg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium containing leucine</td>
<td>5.5 ± 0.5</td>
<td>624.8 ± 132.0</td>
</tr>
<tr>
<td>Leucine-free medium</td>
<td>6.6 ± 1.0</td>
<td>775.5 ± 104.0</td>
</tr>
</tbody>
</table>

Figures are the means of 4 replicates ± SD
curves will be parallel and the volumes of the drug dilutions causing a fixed inhibition (for example 30% or 50%) will be in the inverse ratio of the drug concentrations in the two drug dilutions (Finney, 1961).

A 1mM solution of 6,8-di-t-butyl chromone (FPL52806) a member of the homologous series of substituted chromones in Eagle's medium was prepared and a portion diluted to 0.67mM. Hence the dilution ratio was 0.67. Various fixed volumes of the two 6,8-di-t-butyl chromone solutions were added to bulk lymphocyte cultures of final volume 12ml. Five replicate 2ml cultures were dispensed containing 10%v/v calf serum, 1μg per ml PHA and 1 x 10^6 viable cells per ml. After 40h. incubation the incorporation of [^3H]-thymidine was measured.

Figure 5 shows the percentage inhibition of [^3H]-thymidine incorporation as a probit transformation plotted against the volume of the 6,8-di-t-butyl chromone dilutions added to the 12ml bulk reaction. The two lines are parallel when examined by eye, and statistical analysis resulted in a relative potency of 0.62 with 95% fiducial limits of 0.55 to 0.69 compared with the dilution ratio of 0.67. This shows that measurement of the relative potencies of 6,8-di-t-butyl chromone homologues by this method is a statistically valid bio-assay. The inhibition of [^3H]-thymidine incorporation by other drugs, such as the anti-inflammatory steroid prednisolone, resulted in dose response curves of different slope to that of FPL52806. Comparison of the drug concentration causing a fixed degree of inhibition, for example, 50%, is not a statistically valid bio-assay for the relative potencies of FPL52806 and prednisolone.

2.3.3[^3H]-Thymidine Incorporation By Cultures Of Specifically-Sensitized Guinea-Pig Lymph Node Cells Stimulated With Antigen

Cultures containing 10%v/v heat-decomplemented calf serum,
Figure 5. Inhibition of $[^3]$H-Thymidine Incorporation of PHA-Stimulated Guinea-Pig Lymph Node Cells by Various Volumes of 1mM and 0.67mM FPL 52806 Added to 12ml Bulk Cultures: A Dilution Assay.
2 x 10^6 viable lymph node cells isolated from a guinea-pig sensitized to ovalbumin, and 1000, 100, 10 /µg/ml ovalbumin and no ovalbumin were prepared. At 40, 64, 88, 112 and 136h, 1/6i of [3H]-thymidine was added for a 4h. pulse period and the trichloroacetic acid-insoluble material isolated.

Table 6 shows the incorporation of [3H]-thymidine at the various times by control and ovalbumin-stimulated guinea-pig lymph node cells. The increase in the incorporation of [3H]-thymidine due to presence of the antigen was small compared to the large increase found in PHA-stimulated cultures (Page 48). The incorporation of [3H]-thymidine declined with time in both the antigen and non-stimulated cultures. The highest ovalbumin concentration tested (1000 /µg/ml) caused the greatest stimulation of [3H]-thymidine incorporation, except at 40h. when that caused by 100 /µg/ml was slightly greater.

The small incorporation of [3H]-thymidine found in antigen-stimulated cultures and the decrease with time indicates that when the effect of drug is studied the time of maximum incorporation of [3H]-thymidine (40h.) should be used. An ovalbumin concentration of 100 /µg/ml will be used, since this gave similar incorporation at 40h. to the 100 /µg/ml ovalbumin concentration and higher incorporation at other times.
### TABLE 6. Incorporation of $[^3H]$-Thymidine by Ovalbumin-Sensitized Guinea-Pig Lymph Node Cells in the Presence of Various Ovalbumin Concentrations at Various Times.

Mean Radioactivity per Culture
(d.p.m. x 10^2)

<table>
<thead>
<tr>
<th>Time, (h)</th>
<th>0</th>
<th>10</th>
<th>100</th>
<th>1000</th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td>22.1 ± 5.6</td>
<td>43.4 ± 3.7</td>
<td>51.5 ± 2.6</td>
<td>49.6 ± 6.4</td>
</tr>
<tr>
<td>64</td>
<td>18.3 ± 3.7</td>
<td>25.1 ± 7.6</td>
<td>26.7 ± 3.2</td>
<td>36.7 ± 6.7</td>
</tr>
<tr>
<td>88</td>
<td>7.1 ± 0.9</td>
<td>14.1 ± 3.6</td>
<td>14.2 ± 2.6</td>
<td>19.8 ± 0.1</td>
</tr>
<tr>
<td>112</td>
<td>4.4 ± 1.2</td>
<td>6.9 ± 1.8</td>
<td>6.5 ± 1.6</td>
<td>11.4 ± 1.4</td>
</tr>
<tr>
<td>136</td>
<td>3.0 ± 1.0</td>
<td>5.3 ± 2.6</td>
<td>4.0 ± 1.1</td>
<td>5.2 ± 2.6</td>
</tr>
</tbody>
</table>

Figures are the means of 4 replicates ± SD
The incorporation of $[^3\text{H}]$-uridine was measured as an index of the rate at which RNA was synthesized by cultured guinea-pig lymph node cells. The incorporation of $[^3\text{H}]$-uridine by PHA-stimulated cells is greatly increased compared with the rate in non-stimulated cells (Cooper and Rubins, 1965; Kay, 1968). The acceleration of RNA precursor incorporation by stimulated cells occurs earlier than the increase in $[^3\text{H}]$-thymidine uptake (Mueller and Le Mahieu, 1966). Cooper (1972) has described the complexity of RNA synthesis in lymphocytes and the unreliability of measurement of gross $[^3\text{H}]$-uridine uptake. The complexity is due to the number of RNA types synthesized, (ribosomal, heterogeneous nuclear, messenger and transfer RNA), and their different rates of synthesis and turnover. Most of the added radioactive RNA precursor is incorporated into ribosomal RNA, which is slowly synthesized and broken down but which comprises a major proportion of total cell RNA, and heterogeneous nuclear RNA which is very rapidly synthesized and broken down and comprises about 15% of total cell RNA. The different rates of synthesis of the two above RNA types result in mainly heterogeneous nuclear RNA synthesis, a minor cell RNA constituent, being measured when very short pulses of $[^3\text{H}]$-uridine are used. The use of $[^3\text{H}]$-uridine to measure RNA synthesis in PHA-stimulated cells is further complicated by the effect of PHA on the rate of entry of added radioactive uridine into the intracellular pool of uridine triphosphate (UTP), and the size of the UTP pool and the rate of flow of UTP from the pool, which are all altered by PHA (Hausen and Stein, 1968; Peters and Hausen, 1971). Gross $[^3\text{H}]$-uridine incorporation is increased in transforming lymphocytes, and easily measured, but the interpretation is complex (Cooper, 1972).
2.4.1 Measurement of $^{3}$H-Uridine Incorporation

Cultures of 2.0ml contained 10% v/v heat decomplemented calf serum, 2 x $10^{6}$ viable lymph node cells, and 1μg/ml of PHA. The PHA concentration of 1μg/ml was chosen to correspond to that in cultures in which $^{3}$H-thymidine incorporation was measured. The cultures were incubated at 37° for various times when 1Ci of $^{3}$H-uridine (5 Ci/mMole) in 0.1ml of saline was added. After 4h, the trichloroacetic acid-insoluble material was isolated in an identical manner to that described when $^{3}$H-thymidine incorporation was measured, and the radioactivity present determined.

2.4.2 Determination of Time of Maximum $^{3}$H-Uridine Incorporation

Cultures containing various concentrations of PHA (0, 0.2, 1.0, 5.0μg/ml) were incubated at 37°. At various times the incorporation of $^{3}$H-uridine was measured. Both stimulated and non-stimulated cultures incorporated large amounts of $^{3}$H-uridine immediately after initiation of the cultures (Figure 6), which declined in the non-stimulated cultures rapidly up to 20 hours and more slowly afterwards. Mueller and Le Mahieu (1966) reported a similar effect, but of smaller magnitude, with cultured human leucocytes using $^{3}$H-cytidine as RNA precursor. They suggested that the decline in the incorporation by the non-stimulated cells could either reflect cell death or repression of RNA synthesis. A mechanism for the repression was not suggested.

Cultures stimulated with 0.2 and 5.0μg/ml of PHA showed a slower decline of $^{3}$H-uridine incorporation with time than the unstimulated cultures and cultures stimulated with 1μg/ml of PHA showed a peak of $^{3}$H-uridine incorporation at about 20h. followed by a decline. Table 7 shows the stimulation index, that is, the ratio of the amount of $^{3}$H-uridine incorporated by stimulated cultures to that incorporated by the controls at various PHA concentrations.
Figure 6. Incorporation of $[^3H]$-Uridine at Various Times of Non-Stimulated and PHA-Stimulated Guinea-Pig Lymph Node Cells.
TABLE 7. Stimulation Index for $[^{3}H]$-Uridine Incorporation by PHA-Stimulated Guinea-Pig Lymph Node Cells at Various Times

<table>
<thead>
<tr>
<th>Time, h</th>
<th>Stimulation Index</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Concn. of PHA (μg/ml)</td>
</tr>
<tr>
<td>0.5</td>
<td>1.0</td>
</tr>
<tr>
<td>19.0</td>
<td>2.6</td>
</tr>
<tr>
<td>23</td>
<td>2.4</td>
</tr>
<tr>
<td>43</td>
<td>2.2</td>
</tr>
<tr>
<td>67.5</td>
<td>1.9</td>
</tr>
</tbody>
</table>

Figures are the means of 5 replicates
Maximum stimulation occurred at about 20h., but a high level of stimulation was maintained up to 67.5h. \(^{3}H\)-uridine incorporation and the effect of drugs on this was measured at 1 and 20h. in subsequent experiments.

2.4.3 Determination Of PHA Dose Response Curve For \(^{3}H\)-Uridine Incorporation

Cultures containing a range of PHA concentrations (0.1 - 25 \(\mu\)g/ml) were incubated for 20h. when \(^{3}H\)-uridine incorporation was measured. A PHA concentration of 1 to 2 \(\mu\)g/ml caused maximum incorporation of \(^{3}H\)-uridine, (Figure 7). Since 1 \(\mu\)g/ml PHA was used when the effect of drug on \(^{3}H\)-thymidine incorporation at 40h. was measured this concentration was maintained during study of the effect of drugs on \(^{3}H\)-uridine incorporation at 20h.
Figure 7. Incorporation of $[^3H]$-Uridine at 24 Hours by Guinea-Pig Lymph Node Cells Stimulated with Various Concentrations of PHA.

![Graph showing incorporation of $[^3H]$-Uridine at 24 Hours by Guinea-Pig Lymph Node Cells Stimulated with Various Concentrations of PHA.](image)
Incorporation of $^{14}\text{C}$-leucine by cultured guinea-pig lymph node cells was measured as an index of protein synthesis, (Mueller and Le Mahieu, 1966; Skoog, 1972). Mitogens cause T cells to synthesize lymphokines, (Meckler et al, 1972), which are secreted into the culture medium, and are important in cell mediated immune reactions. No attempt was made to measure the synthesis of these factors as only the trichloroacetic acid-insoluble material of washed cells was isolated. In view of the large increase in cell size most of this was probably incorporated into general cell protein.

### 2.5.1 Measurement Of $^{14}\text{C}$-Leucine Incorporation

Incorporation of $^{14}\text{C}$-Leucine was measured in 2.0ml cultures containing Eagle's medium free of unlabelled leucine. Cultures contained $10^6$ v/v heat decomplemented calf serum, $2 \times 10^6$ viable cells initially and usually $1\mu\text{g/ml}$ of PHA. The cultures were pulse labelled with $0.25\mu\text{Ci}$ of $^{14}\text{C}$-leucine usually for 4h., although 2h. pulses were used in the initial experiments. The $^{14}\text{C}$-leucine incorporated into trichloroacetic acid-insoluble material was isolated in an identical manner to that described for $^3\text{H}$-thymidine incorporation.

### 2.5.2 Determination Of Time Of Maximum $^{14}\text{C}$-Leucine Incorporation

Cultures were prepared with or without $1\mu\text{g/ml}$ of PHA and incubated at $37^\circ$ for various times up to 68.5h.; $^{14}\text{C}$-leucine incorporation was measured using 2h. pulses. In identical replicate culture the incorporation of $1\mu\text{Ci}$ of $^3\text{H}$-thymidine was measured using 2h. pulse for comparison. Maximum incorporation of $^{14}\text{C}$-leucine in the culture stimulated with $1\mu\text{g/ml}$ of PHA occurred at 45h. (Table 8). The incorporation
TABLE 8. Incorporation of $^{14}$C-Leucine and $^{3}$H-Thymidine by PHA-Stimulated and Non-Stimulated Guinea-Pig Lymph Node Cells at Various Times Using a 2h. Pulse

Mean Radioactivity per Culture (d.p.m. x $10^3$)

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>$[^{14}\text{C}]$-Leucine</th>
<th>$[^{3}\text{H}]$-Thymidine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No PHA (1/$\mu$g/ml)</td>
<td>PHA (1/$\mu$g/ml)</td>
</tr>
<tr>
<td>2</td>
<td>1.9 ± 0.4</td>
<td>2.0 ± 0.3</td>
</tr>
<tr>
<td>21</td>
<td>1.4 ± 0.1</td>
<td>2.6 ± 0.4</td>
</tr>
<tr>
<td>26</td>
<td>1.1 ± 0.3</td>
<td>3.0 ± 1.0</td>
</tr>
<tr>
<td>45</td>
<td>0.8 ± 0.1</td>
<td>4.8 ± 0.2</td>
</tr>
<tr>
<td>69</td>
<td>0.7 ± 0.1</td>
<td>1.7 ± 0.2</td>
</tr>
</tbody>
</table>

Figures are the means of 5 replicates ± SD
in the non-stimulated cultures declined with time. The incorporation of $[^{3}\text{H}]$-thymidine followed a similar time course to $[^{14}\text{C}]$-leucine in both stimulated and non-stimulated cultures.

Since the two radioactive precursors were labelled with different isotopes, $[^{3}\text{H}]$ and $[^{14}\text{C}]$, incorporation of both $[^{3}\text{H}]$-thymidine and $[^{14}\text{C}]$-leucine could be measured in the same cultures. The $[^{3}\text{H}]$ and $[^{14}\text{C}]$ incorporated may be differentiated using a dual-channel liquid scintillation spectrometer. Subsequent experiment showed that satisfactory results could be obtained using a 4h. pulse period. The effect of unlabelled-leucine-free Eagle's medium on $[^{3}\text{H}]$-thymidine incorporation has been described previously, (Section 2.3 Page 52).

2.5.3 Determination Of PHA Dose Response Curves For $[^{14}\text{C}]$-Leucine And $[^{3}\text{H}]$-Thymidine Incorporation In The Same Cultures At 40 Hours

Incorporation at 40h. was measured since this time had been previously employed for measurement of $[^{3}\text{H}]$-thymidine incorporation. Cultures of 2.0ml containing 10% v/v heat decomplemented calf serum, $2 \times 10^6$ viable lymph node cell initially, and 0.1 to 10/µg/ml of PHA in Eagle's medium free of unlabelled leucine were prepared. $[^{14}\text{C}]$-leucine (0.25/µCi) and 1/µCi of $[^{3}\text{H}]$-thymidine were added together in 0.1ml of saline for a 4h. pulse period. Figures 8 a and b show respectively the PHA dose response curves for $[^{14}\text{C}]$-leucine and $[^{3}\text{H}]$-thymidine incorporation. The optimal PHA concentration for both curves was 2/µg/ml in this experiment, but the incorporation with 1/µg/ml PHA was only slightly smaller. In view of this small difference and the previous use of 1/µg/ml of PHA for study of the effect of drug on $[^{3}\text{H}]$-thymidine incorporation, it was decided to retain this PHA concentration when the effect of drugs was investigated on $[^{14}\text{C}]$-leucine and $[^{3}\text{H}]$-thymidine incorporation in the same cultures.
Figure 6. Effect of PHA Concentration on $[^3]H$-Thymidine and $[^{14}C]$-Leucine Incorporation of Guinea-Pig Lymph Node Cells Measured Simultaneously in the Same Cultures.

a) $[^3]H$-Thymidine

b) $[^{14}C]$-Leucine
2.5.4 Statistical Validity Of The Effect Of Compounds On $^{14}$C-Leucine Incorporation

An identical approach and method to that described on Page 55 was used. Figure 9 shows that the percentage inhibition of $^{14}$C-leucine incorporation plotted as a probit against the amount of the two 6,8-di-t-butyl chromone (FPL52806) solutions added resulted in parallel lines. The relative potencies of the two FPL52806 solutions was 0.64 (with 95% fiducial limits of 0.57 to 0.70) which compared favourably with the theoretical value of 0.67. Measurement of the effect of drugs on $^{14}$C-leucine incorporation therefore is a statistically valid bio-assay.
Figure 9. Inhibition of $^{14}$C-leucine Incorporation of PHA-Stimulated Guinea-Pig Lymph Node Cells by Various Volumes of 1mM and 0.67mM FPL 52806 Added to 12ml Bulk Cultures: A Dilution Assay.
2.6 [\(^{3}\text{H}\)]-GLUCOSAMINE INCORPORATION BY CULTURES OF GUINEA-PIG LYMPH NODE CELLS

Stimulated lymphocytes secrete proteins which contain carbohydrate; B lymphocytes produce immunoglobulin and T lymphocytes lymphokines. The incorporation of [\(^{3}\text{H}\)]-glucosamine by PHA-stimulated and non-stimulated cultured guinea-pig lymph node cells into acid-insoluble material, presumably glycoprotein, (Hayden et al, 1970), was therefore investigated.

2.6.1 Measurement Of [\(^{3}\text{H}\)]-Glucosamine Incorporation

At various times 0.25\(^{\prime}\)Ci of [\(^{3}\text{H}\)]-glucosamine (1.1 Ci/mMole) was added to each 2ml culture for a 4h. period and the trichloroacetic acid-insoluble material was isolated in an identical manner to that described for [\(^{3}\text{H}\)]-thymidine incorporation. No attempt was made to eliminate the glucose present in Eagle's medium, which would be converted into glucosamine by the cells and reduce the effective specific activity of the added [\(^{3}\text{H}\)]-glucosamine, due to the absolute requirement for glucose as energy source for lymphocyte transformation.

2.6.2 Determination Of Time Of Maximum [\(^{3}\text{H}\)]-Glucosamine Incorporation

The incorporation of [\(^{3}\text{H}\)]-glucosamine into non-stimulated cultures and cultures stimulated with 1\(^{\prime}\)g/ml of PHA was measured at various times. The greatest incorporation in the PHA-stimulated cultures occurred at 42h. (Table 9). At this time the stimulation index was also greatest (Table 9). In subsequent experiments when the effect of the drug on the incorporation of [\(^{3}\text{H}\)]-glucosamine was determined the incorporation at this time was therefore used.
TABLE 9. Incorporation of $[3^3H]$-Glucosamine by PHA-Stimulated and Non-Stimulated Guinea-Pig Lymph Node Cells at Various Times

Mean Radioactivity per Culture (d.p.m.)

<table>
<thead>
<tr>
<th>Time, (h)</th>
<th>PHA (l/µg/ml)</th>
<th>No PHA</th>
<th>Stimulation Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>18</td>
<td>591 ± 123</td>
<td>82 ± 16</td>
<td>7.2</td>
</tr>
<tr>
<td>42</td>
<td>951 ± 263</td>
<td>25 ± 10</td>
<td>38.2</td>
</tr>
<tr>
<td>66</td>
<td>546 ± 59</td>
<td>28 ± 14</td>
<td>19.5</td>
</tr>
</tbody>
</table>

Figures are the means of 5 replicates ± SD
Many drugs such as phenylbutazone (Burns et al, 1953), dicumarol (O'Reilly et al, 1962), and carbenoxolone (Parke and Lindup, 1973), are known to bind reversibly to serum proteins, especially to albumin. This is important both to the efficacy and safety of the drug. When the free drug concentration is high, toxicity may be seen, for example with phenylbutazone, and this can occur unintentionally when two drugs which compete for the same protein binding site are administered simultaneously.

Protein binding is a common feature of acidic non-steroidal anti-inflammatory drugs (Hichens, 1974). A drug which is protein bound is believed to be pharmacologically inactive. When the intrinsic relative potencies of certain substituted chromones were compared as inhibitors of biochemical processes in lymph node cell cultures it was necessary to determine the degree of protein binding of these compounds to the serum present in the cultures so that the proportion of protein bound inactive drug could be subtracted from the added drug concentration. The degree of protein binding to serum varied from compound to compound, and was measured under conditions similar to those present in the lymph node cell cultures.

2.7.1 Measurement of Protein Binding By Equilibrium Dialysis

Visking tubing dialysis bags were washed in distilled water for several hours and filled with 2.5ml of 10% v/v heat decomplemented calf serum in 0.155M NaCl solution, and were dialysed for 15 hours against 9.0ml of 140mM NaCl - 28mM HEPES - NaOH, pH 7.45, at 22°. Various substituted chromones (6,8-di-alkylchromones) were present outside the dialysis bags, in amounts which if uniformly distributed
between the dialysis bag and surrounding fluid would have resulted in concentrations of 15.7, 52.2, 157 and 522/μM. The equilibrium concentration of the substituted chromones outside the dialysis bags was determined spectrophotometrically by their u.v. absorption.

2.7.2 Measurement Of Protein Binding By Fluorescence Quenching

Bovine serum albumin is the protein in calf serum to which 6,8-di-alkyl chromones bound when their binding to 10% v/v calf serum was determined by equilibrium dialysis. In this section the binding of the compounds to isolated bovine serum albumin is investigated using a spectroscopic method, quenching of the native fluorescence of the albumin.

The native fluorescence of 3.0ml of 1mg/ml bovine serum albumin in 10mM tris-HCl, pH 7.4, was measured in a Hitachi-Perkin Elmer MPF3 spectrofluorimeter (excitation 290nm, emission 340nm). Small incremental volumes, up to a total of 0.25ml, of 1mg/ml solutions of 6,8-di-alkyl chromones were added using an Agla micrometer syringe and the decline in the native fluorescence of the bovine serum albumin determined after each addition. After correction of the fluorescence values for bulk absorption and correction for the dilution of the albumin solution by addition of chromone solution the quench curve data was analysed by an iterative computer program to give values of n, the number of primary binding sites for the chromones on the bovine albumin, and K, the association constant of binding to these primary binding sites. Estimated values for n by this method are not very reliable since relatively large changes in n cause little change in the shape of the quench curve. Greater significance is attached to the values of K where relatively small changes alter the shape of the curve markedly.
The partition coefficient of a substance is the ratio of the concentrations of the substance in two immiscible solvents (for example an aqueous and organic solvent) which are in equilibrium with one another. It is related to the lipophilicity of the substance, the more lipophilic the substance is, the greater will be the concentration in an organic solvent in equilibrium with a given concentration in the aqueous phase.

The degree of lipophilicity of a drug, measured by its partition coefficient, is important, since the therapeutic potency of the drug may be a function of the lipophilicity of the substance (Hansch and Fujita, 1964). The lipophilicity of the drug determines the extent to which the drug passes through cell membranes to its molecular site of action and also contributes to the extent to which the drug is protein bound. There is often a limiting degree of lipophilicity for a given drug series to achieve maximum activity. Above a certain degree of lipophilicity the potential drug is soluble in lipophilic phases that effective drug concentrations are not achieved in the aqueous phases, preventing movement of the drug to its site of action. In order to relate the relative activities of members of a homologous series of substituted chromones to their lipophilicities their partition coefficients were determined.

2.8.1 Measurement Of Apparent Partition Coefficients

The apparent partition coefficients of the homologous series of 6,8-di-alkyl chromones were determined between octanol:ether (1:3, v/v) and an aqueous phase of 28-mM HEPES adjusted to pH 7.45 with M-NaOH. The pH of the aqueous phase was identical to that of lymph node cell cultures since
pH has an effect on the apparent partition coefficients of ionizable molecules (Fujita et al., 1964). The organic and aqueous phases were not saturated with the opposing phase. The substituted chromones were added at a concentration of 100 μM to the aqueous phase and stoppered tubes containing equal volumes of the two phases were shaken for 20 mins. at 22°. The phases were separated by centrifugation. The concentrations of the substituted chromone remaining in the aqueous phases were measured spectrophotometrically by their u.v. absorption, and reference to 100 μM solution. The concentrations of the substituted chromones present in the organic phase were calculated by difference and the ratios of the concentrations in the organic and aqueous phases, the partition coefficients, calculated. No correction was made for the degree of ionization of the chromone compounds. The values of the pKa of the chromones are all about 2, hence the degree of ionization at pH 7.4 approached unity (>99.99%). Strictly only relative partition coefficients were determined, the true partition coefficients being about 100,000 times larger.
Certain anti-inflammatory drugs, chloroquine (Allison and Young, 1964) and gold salts (Gottlieb et al., 1972), are known to accumulate in cells. This may be relevant to their mode of action. The 6,8-di-t-butyl chromone, FPL52806, whose effect on synthetic processes occurring in cultured guinea-pig lymph node cells has been determined, and which is believed to have anti-inflammatory activity, was available in a radioactively labelled form. This tritiated material was used to determine whether or not cultured cells accumulated the drug.

**2.9.1 Measurement Of Association Of $[^3\text{H}]$-6,8-Di-t-Butyl Chromone To Guinea-Pig Lymph Node Cells**

Cultures containing $2 \times 10^6$ viable lymph node cells initially, various amounts of decomplemented calf serum (0.2, 1.0 and 10% v/v), with or without 1/2g/ml PHA, 25 and 75/N $[^3\text{H}]$-6,8-di-t-butyl chromone and Eagle’s medium to 2.0ml were prepared. The cultures were incubated for 48h. at 37° when they were rapidly centrifuged, (2600xg for 2 mins.), the supernatant decanted and the cells resuspended in normal saline containing 125/M unlabelled 6,8-di-t-butyl chromone. The centrifugation and resuspension was repeated twice to wash away the $[^3\text{H}]$-6,8-di-t-butyl chromone. In one experiment a further wash with methanol containing 125 M 6,8-di-t-butyl chromone was included. The cell pellets were dissolved in hyamine hydroxide and the radioactivity present determined. The solution used for washing the cells contained 125/M 6,8-di-t-butyl chromone to reduce leakage of the $[^3\text{H}]$-6,8-di-t-butyl chromone from the cells.
2.10 VIABILITY OF COMPOUND-TREATED GUINEA-PIG LYMPH NODE CELLS

When the effect of drugs on the biochemical activity of cultured cells is determined it is desirable to assess the toxicity of the drug (Stewart and Ingram, 1967). The viability of drug-treated cultures was measured using a dye-exclusion method when the biochemical activities of cells such as $[^{3}H]$-thymidine incorporation was determined at 40h.

2.10.1 Measurement of Viability of Compound-Treated Guinea-Pig Lymph Node Cells

A replicate culture of those used for biochemical measurements was centrifuged at 1500 r.p.m. (1500xg) for 3 min. and the cell-free supernatant decanted. The cell pellet was gently suspended in 0.2ml of Eagle's medium to which was added 0.2ml of 0.8% w/v eosin Y in saline. A sample of the stained cell suspension was placed in an improved Mieubaeur haemocytometer and the number of unstained viable cells, both blast-cells and non-stimulated cells, counted in an area of 0.25mm$^2$. 
Compounds which inhibit the synthetic activities of PHA-stimulated guinea-pig lymph node cells also affect the viability of cells measured by dye-exclusion. Loss of cell viability is a gross morphological change. In order to investigate whether or not compounds also caused subtle morphological changes compound-treated cells were examined by scanning electron microscopy.

Scanning electron microscopy is a technique for examination of the surface architecture of materials at magnifications which are not attainable with the light microscope, due to loss of resolution at magnifications greater than about 1500 times under the light microscope. The resolution is a function of the wavelength of the light photons. High resolutions are achieved in electron microscopes due to the high energies of the electrons used which possess wave properties of very low wavelength.

The surface features of whole materials after coating with electron dense material, such as gold, are revealed under the scanning electron microscope in contrast to the transmission electron microscope in which the density of electron dense material, such as OsO₄, 'staining' ultra-thin sections of the material are studied.

Polliack et al (1973) reported that T and B lymphocytes could be identified under the scanning electron microscope, indicating the potential of the technique for detecting subtle morphological changes not apparent by light microscopy.

2.11 Preparation Of Material For Scanning Electron Microscopy

The culture medium from 48h. guinea-pig lymph node cell
cultures, containing either 1μg/ml PHA or none, and also containing various compound concentrations, was gently decanted. The cells were suspended in the few drops of medium remaining and placed on coverslips which were incubated at 37° for 10 mins. in a humid atmosphere to allow cell attachment. The serum present was removed by three washes with serum-free-Eagle's medium and the coverslips were immersed in 1.5% glutaraldehyde in Millonig's solution (Millonig, 1961) for 30 mins. at 4°. After three washes with Millonig's solution containing 7.5% sucrose the coverslips were placed in 1% OsO₄ in Millonig's solution for 30 mins. at 4°. The fixed cells were dehydrated with aqueous ethanol (40%, 55%, 70%, 95% and 100%, v/v) and air dried. The cells after vacuum-coating with gold were examined using a Cambridge S4 Stereoscan Scanning Electron Microscope.
CHAPTER THREE

EFFECT OF COMPOUNDS ON SYNTHETIC PROCESSES IN

STIMULATED GUINEA-PIG LYMPH NODE CELLS
The effect of established anti-inflammatory drugs, such as sodium salicylate, phenylbutazone, prednisolone and indomethacin, and candidate compounds on PHA-stimulated transformation of guinea-pig lymph node cells is described in this chapter. The effect of one candidate compound on antigen-stimulated transformation is also investigated. The inhibitory effect of compounds on lymphocyte transformation may either be assessed by morphological criteria such as increase in cell size or in the number of mitotic figures (Panayi et al., 1973) or by measurement of one of the many biochemical parameters which are increased in transforming lymphocytes. In this chapter the biochemical parameters of incorporation of $[^3H]$-thymidine, $[^14C]$-leucine, $[^3H]$-uridine and $[^3H]$-glucosamine was studied.
3.2 EFFECT OF COMPOUNDS ON DNA SYNTHESIS IN PHA-STIMULATED GUINEA-PIG LYMPH NODE CELLS

The effect on DNA synthesis of known anti-inflammatory drugs and candidate compounds was determined by exposing cultured guinea-pig lymph node cells to the compound for various lengths of time. All compounds were tested when they were present throughout the total culture period including the four hour pulse period with \( ^3 \text{H} \)-thymidine. In addition, certain compounds were added immediately before the 4h. pulse period with \( ^3 \text{H} \)-thymidine, so that the compound was not present during the culture period. One compound was also added at various times throughout the culture period.

3.2.1 Effect On DNA Synthesis Of Compounds Present Throughout The Total Culture And Pulse Periods

Cultures of guinea-pig lymph node cells stimulated with PHA were prepared as described previously. Compounds were added to give final concentrations of \( 1/\mu \text{M} \) to \( 2500/\mu \text{M} \), though often \( 10/\mu \text{M} \) to \( 1000/\mu \text{M} \) was employed. After 40h. culture \( ^3 \text{H} \)-thymidine was added for a 4h. pulse. At 44h. the radioactivity incorporated into acid-insoluble material was measured and the effect of the drug on DNA synthesis calculated by reference to the radioactivity incorporated in a drug-free control. The percentage inhibition of the rate of \( ^3 \text{H} \)-thymidine incorporation, or the probit transformation of this percentage (Gale and Carnes, 1971), was plotted graphically against the logarithm of the drug concentration causing the inhibition. From these graphs the drug concentrations which inhibited \( ^3 \text{H} \)-thymidine incorporation by 50% (ID\(_{50}\)) were determined.
Table 10 shows the effects of several anti-inflammatory drugs and sodium p-hydroxybenzoate acid on $[3^H]$-thymidine incorporation as measured by the ID$_{50\%}$. The effect of the majority of these drugs was determined only once. Chloroquine and prednisolone were more active inhibitors than the acidic anti-inflammatory drugs, having ID$_{50\%}$ that were 30 to 100 fold lower. Sodium salicylate and ibuprofen were of similar activity, and less active than indomethacin, phenylbutazone and flufenamic acid. The non anti-inflammatory structural isomer of salicylate, p-hydroxybenzoate did inhibit $[3^H]$-thymidine incorporation, but only at high concentrations.

The dose response curves for certain of these drugs are plotted, using the probit transformation of the percentage inhibition caused by the drug, in Figure 10. The anti-inflammatory steroid prednisolone gave a dose response curve which was appreciably different in slope to those of the acidic anti-inflammatory drugs and chloroquine.

The candidate compounds tested, with their structures, and their effects on $[3^H]$-thymidine incorporation, measured by the ID$_{50\%}$, are presented in Table 11. The dose response curves of the inhibition of $[3^H]$-thymidine incorporation by members of an homologous series of compounds, the 6,8-di-alkyl substituted chromones, are shown in Figure 11, and the effects of other substituted chromones and non-chromones in Figure 12. The ID$_{50\%}$ of the candidate compounds ranged from 15$\mu$M for FPL55522, which is not a chromone, to 900$\mu$M for FPL5067C (disodium cromoglycate). The un-substituted chromone, FPL52839, inhibited $[3^H]$-thymidine incorporation by less than 10% when tested at concentrations up to 2500$\mu$M.

FPL52816, the 5,7-di-t-butyl substituted chromone had a similar ID$_{50\%}$ to FPL52806, the 6,8-di-t-butyl substituted chromone. The activity of the 6,8-di-alkyl substituted chromones (Figure 11) was related to the size of the substituent. Increase in the size of the substituent decreased the ID$_{50\%}$. For example the di-t-pentyl substituted chromone, FPL56220, was more active than the di-t-butyl
TABLE 10. Concentration of Compounds Present Throughout the Culture and Pulse Periods that Inhibited the Rate of $[^3H] \text{-Thymidine Incorporation by 50\% (ID}_{50\%\text{)}}$.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>$\text{ID}_{50%\text{}}$, $\mu \text{M}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium p-hydroxy-benzoate</td>
<td><img src="image" alt="Structure" /></td>
<td>1500</td>
</tr>
<tr>
<td>Sodium salicylate</td>
<td><img src="image" alt="Structure" /></td>
<td>300</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td><img src="image" alt="Structure" /></td>
<td>220</td>
</tr>
<tr>
<td>Indomethacin</td>
<td><img src="image" alt="Structure" /></td>
<td>150</td>
</tr>
<tr>
<td>Phenylbutazone</td>
<td><img src="image" alt="Structure" /></td>
<td>130</td>
</tr>
<tr>
<td>Flufenamic acid</td>
<td><img src="image" alt="Structure" /></td>
<td>100</td>
</tr>
<tr>
<td>Prednisolone</td>
<td><img src="image" alt="Structure" /></td>
<td>4</td>
</tr>
<tr>
<td>Chloroquine</td>
<td><img src="image" alt="Structure" /></td>
<td>1.4</td>
</tr>
</tbody>
</table>
Figure 10. Inhibition of L-[H]-Thymidine Incorporation of PHA-Stimulated Guinea-Pig Lymph Node Cells by Anti-
TABLE 11.  Concentrations of Candidate Compounds Present Throughout the Culture and Pulse Periods which Inhibited the Rate of \( ^{3}H \)-Thymidine Incorporation by 50% (\( ID_{50\%} \)).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>( ID_{50%} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>FPL 50670</td>
<td><img src="image1.png" alt="Structure" /></td>
<td>900</td>
</tr>
<tr>
<td>FPL 56112</td>
<td><img src="image2.png" alt="Structure" /></td>
<td>280</td>
</tr>
<tr>
<td>FPL 55618</td>
<td><img src="image3.png" alt="Structure" /></td>
<td>180</td>
</tr>
<tr>
<td>FPL 56294</td>
<td><img src="image4.png" alt="Structure" /></td>
<td>80</td>
</tr>
<tr>
<td>FPL 55990</td>
<td><img src="image5.png" alt="Structure" /></td>
<td>80</td>
</tr>
<tr>
<td>FPL 52816</td>
<td><img src="image6.png" alt="Structure" /></td>
<td>56</td>
</tr>
<tr>
<td>FPL 55522</td>
<td><img src="image7.png" alt="Structure" /></td>
<td>15</td>
</tr>
</tbody>
</table>

Continued.....
<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>(ID_{50}) ((\mu)M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FPL 52839</td>
<td><img src="image1" alt="FPL 52839" /></td>
<td>2500</td>
</tr>
<tr>
<td>FPL 55585</td>
<td><img src="image2" alt="FPL 55585" /></td>
<td>700</td>
</tr>
<tr>
<td>FPL 55689</td>
<td><img src="image3" alt="FPL 55689" /></td>
<td>220</td>
</tr>
<tr>
<td>FPL 55731</td>
<td><img src="image4" alt="FPL 55731" /></td>
<td>77</td>
</tr>
<tr>
<td>FPL 52806</td>
<td><img src="image5" alt="FPL 52806" /></td>
<td>69</td>
</tr>
<tr>
<td>FPL 56220</td>
<td><img src="image6" alt="FPL 56220" /></td>
<td>51</td>
</tr>
</tbody>
</table>
Figure 11. Inhibition of $[^3\text{H}]$-Thymidine Incorporation of PHA-Stimulated Guinea-Pig Lymph Node Cells by 6,8-Di-Alkyl Chromones.
Figure 12. Inhibition of $[^3\text{H}]-\text{Thymidine}$ Incorporation of PHA-Stimulated Guinea-Pig Lymph Node Cells by Miscellaneous Chromones and Non-Chromones.
Reproducibility of the determination of compound potency by measurement of the effect on $[^3\text{H}]$-thymidine incorporation of compound present throughout the culture and pulse periods.

The effect of one of the candidate compounds, FPL52806, on $[^3\text{H}]$-thymidine incorporation was determined repeatedly with the compound present throughout the culture and pulse periods. These assays were performed over many months and the dose response curves obtained are shown in Figure 13, together with the date of the assay and the ID$_{50}$. The dose response curves, and the ID$_{50}$ values obtained from those, agreed well with one another. The mean ID$_{50}$ was 69/μM with an SD of 5/μM (n=5). The determination of drug potency by this method was highly reproducible.

Effect of FPL52806 on the PHA dose response curve.

When the effect of drugs on $[^3\text{H}]$-thymidine incorporation was measured, the experimentally determined optimal concentration of PHA was used to stimulate cell transformation. It is well established that anti-inflammatory drugs can affect membranes (Hichens, 1974) and have the ability to bind to proteins and displace previously bound material (McArthur et al, 1971).

These properties of anti-inflammatory compounds may have rendered the previously determined optimal concentration of PHA (1μg/ml) sub-optimal. This might have been responsible for the inhibitions of $[^3\text{H}]$-thymidine incorporation seen with the compounds. This possibility was investigated by determining the PHA dose response curve with and without FPL52806. Cultures containing a range of PHA concentrations (0.1 - 10μg/ml), both with and without 75/μM FPL52806, were prepared. This concentration of FPL52806 had been shown previously to inhibit $[^3\text{H}]$-thymidine incorporation at 40h., using 1μg/ml of PHA as mitogen, by about 60% (Figure 11). At 40 and 64h, the incorporation of $[^3\text{H}]$-thymidine was measured.
Figure 13. Reproducibility of the Inhibition of $[^3\text{H}]$-Thymidine Incorporation of PHA-Stimulated Guinea-Pig Lymph Node Cells by FPL 52806.
There was no evidence of a displacement of the PHA dose response curve in the presence of FPL52806 relative to that in its absence, at both 40 and 64h. (Figure 14a and b). The percentage inhibition of $[^3H]$-thymidine incorporation were similar at 40 and 64h. at all PHA concentrations.

3.2.2  **Effect On DNA Synthesis Of Compound Added With The $[^3H]$-Thymidine At 40 Hours.**

The effects of anti-inflammatory drugs and candidate compounds on $[^3H]$-thymidine incorporation by PHA-stimulated guinea-pig lymph node cells with the drug present throughout the entire culture period was described in the previous sections of this chapter. To determine whether or not the inhibitions of $[^3H]$-thymidine incorporation seen were a direct and specific effect on some stage of the incorporation of $[^3H]$-thymidine into acid-insoluble material (DNA synthesis) or due to a specific or general inhibition of other processes in the cells, the effect of the presence of drugs, only during the pulse period with $[^3H]$-thymidine was determined. Compounds were added in 0.1ml of Eagle's medium to 40h. cultures immediately before the addition of $[^3H]$-thymidine for a 4h. pulse. The final drug concentrations were those which had been found to inhibit $[^3H]$-thymidine incorporation by 50% or more when the drugs were present throughout the culture and pulse periods.

The compounds had little or no effect on $[^3H]$-thymidine incorporation when present only during the 4h. pulse period, in contrast to their appreciable effect when present throughout the culture and pulse periods (Table 12). Phenylbutazone at a final concentration of 200/μM inhibited $[^3H]$-thymidine incorporation by 5% when added for the 4h. pulse period and 65% when present throughout the culture and pulse periods. Similarly sodium salicylate (300/μM) and FPL52806 (100/μM) both caused 2% inhibition when added at the time of pulsing, in contrast to 47% and 70% inhibition respectively when present throughout the total culture and pulse periods.
Figure 14. Effect of 75μM FPL 52806 on the Incorporation of [3H]-Thymidine - PHA Dose Response Curves at 40 and 64 Hours.

(a) 40 Hours

(b) 64 Hours

D.P.M. per culture

No FPL 52806

75μM FPL 52806

PHA concentration (μg/ml)
<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration((M))</th>
<th>Present Only During Pulse Period</th>
<th>Present Throughout Culture and Pulse Periods</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium Salicylate</td>
<td>300</td>
<td>2</td>
<td>50</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>500</td>
<td>-3</td>
<td>84</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>200</td>
<td>-11</td>
<td>60</td>
</tr>
<tr>
<td>Phenylbutazone</td>
<td>200</td>
<td>5</td>
<td>67</td>
</tr>
<tr>
<td>Flufenamic acid</td>
<td>100</td>
<td>0</td>
<td>50</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>10</td>
<td>9</td>
<td>92</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>12.5</td>
<td>-10</td>
<td>59</td>
</tr>
<tr>
<td>FPL 55618</td>
<td>200</td>
<td>16</td>
<td>55</td>
</tr>
<tr>
<td>FPL 52806</td>
<td>100</td>
<td>2</td>
<td>70</td>
</tr>
<tr>
<td>FPL 55522</td>
<td>50</td>
<td>12</td>
<td>70</td>
</tr>
</tbody>
</table>

- means stimulation

Effect of Compound Added at the Time of Pulsing with \( ^3H \)-Thymidine and that Present Throughout the Culture and Pulse Periods on \( ^3H \)-Thymidine Incorporation
In a subsequent experiment high concentrations of FPL52806, 6,8-di-t-butyl substituted chromone, (250 - 500μM), were added at the time of pulsing with $^3$H-thymidine, and appreciable inhibition of $^3$H-thymidine incorporation was found (Figure 15). The inhibitory action on $^3$H-thymidine incorporation of FPL52806 when present throughout the culture and pulse periods are also repeated in Figure 15 for comparison.

The dose response curve for FPL52806 added at the time of pulsing was similar in slope to that of the compound present throughout the culture and pulse periods. The ID$_{50}^{\%}$ was increased from 70μM to 350μM.

3.2.3 Effect On DNA Synthesis Of FPL52806 Added At Times Intermediate Between Initiation Of The Cultures And Addition Of $^3$H-Thymidine.

The effects of drugs on $^3$H-thymidine incorporation have been described when the drugs were present for either: a) 48h. culture period plus 4h. period, or b) the 4h. pulse period only. The effect on the rate of $^3$H-thymidine incorporation of FPL52806 added at intermediate times is now considered.

FPL52806, at final concentrations of 49 and 95μM, was added to cultures of PHA-stimulated lymph node cells at 0, 2, 4, 7, 18, 26 and 42h. after initiation of the cultures. $^3$H-thymidine was added to the cultures at 43h. for a 4h. pulse period. When the acid-insoluble material was isolated at 47h. the various cultures had been exposed to FPL52806 for 47, 45, 43, 40, 29, 21 and 5h. respectively.

The effect of FPL52806 on $^3$H-thymidine incorporation is shown in Figure 16. A progressively lower inhibition of $^3$H-thymidine incorporation with decreasing time of exposure of the lymph node cells to the drug was observed. FPL52806 (95 and 49μM) present throughout the total culture and pulse periods of 47h., caused 64 and 29% inhibition respectively.
Figure 15. Inhibition of $[^3H]$-Thymidine Incorporation of PHA-Stimulated Guinea-Pig Lymph Node Cells by FPL 52806 Present Only During the Pulse Period and FPL 52806 Present Throughout the Culture and Pulse Periods.

Inhibition of $[^3H]$-thymidine incorporation, %

FPL 52806 concentration, (µM).

- FPL 52806: present only during the pulse period
- FPL 52806: present throughout the culture and pulse periods
Figure 16. Decrease in the Inhibition of $[^3H]$-Thymidine Incorporation at 43 Hours of PHA-Stimulated Guinea-Pig Lymph Node Cells by FPL 52806 (49 and 95μM) added at Various Times After Initiation of the Culture.

- 95 μM FPL 52806
- 49 μM FPL 5206
and that present for 43h., caused 56 and 27% inhibition respectively. When present for 29h. it inhibited by 48 and 24%, for 26h. it inhibited by 38 and 19%, and for 5h. by 15 and 10%. The decrease in inhibition with reducing time of exposure to the compound was greater for 95/μM FPL52806 (64% to 15%) than for the lower concentration, 49/μM (29% to 10%).

3.2.4 Effect Of FPL52806 On DNA Synthesis In Antigen-Stimulated Guinea-Pig Lymph Node Cells.

In the previous sections of this chapter inhibition, by established anti-inflammatory drugs and candidate compounds, of the rate of incorporation of [3H]-thymidine by PHA-stimulated guinea-pig lymph node cells has been described. Cells have been stimulated with PHA since this system is reproducible and the magnitude of the increase in [3H]-thymidine incorporation caused by PHA is large, and easily and accurately measured. In this section the effect of a candidate compound 6,8-di-alkyl chromone (FPL52806) is determined on [3H]-thymidine incorporation by antigen-stimulated guinea-pig lymph node cells.

 Cultures of lymph node cells from a guinea-pig sensitized to ovalbumin were prepared as described previously. The cultures contained 1000 μg/ml of ovalbumin and FPL52806 at concentrations found to inhibit [3H]-thymidine incorporation in PHA-stimulated cells. At 4Ch. [3H]-thymidine (1/Ci) was added to the cultures for a 4h. pulse, when the trichloroacetic acid-insoluble material was isolated.

Table 13 shows the effect of FPL52806 on the incorporation of [3H]-thymidine by antigen-stimulated guinea-pig lymph node cells. The concentration of FPL52806 which inhibited [3H]-thymidine incorporation by 50% was about 45/μM which is not greatly different from the concentration of FPL52806 (69/μM) which inhibited [3H]-thymidine incorporation by 50% in PHA-stimulated cells. Results obtained using PHA-stimulated cells may therefore be applicable to antigen-stimulated cells.
TABLE 13.  Effect of FPL52806 on the Incorporation of \[^3^H\text{-Thymidine}\] by Antigen (Ovalbumin)-Stimulated Guinea-Pig Lymph Node Cells

<table>
<thead>
<tr>
<th>Concentration of FPL52806, (µM)</th>
<th>Percentage Inhibition of the Rate of Incorporation of [^3^H\text{-Thymidine}]</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>40 ± 12</td>
</tr>
<tr>
<td>50</td>
<td>53 ± 7</td>
</tr>
<tr>
<td>75</td>
<td>66 ± 7</td>
</tr>
<tr>
<td>100</td>
<td>72 ± 3</td>
</tr>
<tr>
<td>150</td>
<td>75 ± 7</td>
</tr>
</tbody>
</table>
3.3 EFFECT OF COMPOUNDS ON PROTEIN SYNTHESIS IN PHA-STIMULATED GUINEA-PIG LYMPH NODE CELLS

The effect of anti-inflammatory drugs and candidate compounds on the rate of incorporation of \(^{3}\text{H}\)-thymidine by PHA-stimulated guinea-pig lymph nodes has been described. To determine whether or not these compounds inhibited other synthetic activities of PHA-stimulated cells their effect on the rate of \(^{14}\text{C}\)-leucine incorporation was investigated. \(^{14}\text{C}\)-Leucine incorporation into acid-insoluble material was measured as an index of protein synthesis.

The compounds were tested when present throughout the culture and pulse periods. One compound, FPL52806, was tested when added at various times through the culture period.

3.3.1 Effect On Protein Synthesis Of Compounds Present Throughout The Culture And Pulse Periods.

\(^{14}\text{C}\)-Leucine incorporation by cultures of guinea-pig lymph node cells was measured at 40h. using a 4h. pulse period. The compounds were present throughout the culture and pulse periods at concentrations which had been found suitable for demonstrating the inhibition of the rate of \(^{3}\text{H}\)-thymidine incorporation. The simultaneous incorporation of both \(^{14}\text{C}\)-leucine and \(^{3}\text{H}\)-thymidine was measured in most experiments. The percentage inhibition of the rate of \(^{14}\text{C}\)-leucine incorporation due to the compound was calculated by comparison with the rate of the incorporation of \(^{14}\text{C}\)-leucine by drug-free cultures. The concentration of compound that caused 50% inhibition (ID\text{50%}) was estimated from a probit plot of the percentage inhibition of the rate of \(^{14}\text{C}\)-leucine incorporation against compound concentration.

The effect of certain candidate compounds, mainly members
of a series of 6,8-di-alkyl substituted chromones, and chloroquine on the rate of \[^{14}C\]-leucine incorporation in terms of the ID\(_{50}\%\) are shown in Table 14. The ID\(_{50}\%\) for inhibition of the rate of \[^{3}H\]-thymidine incorporation are shown also. The probit plots of the percentage inhibition are presented in Figure 17.

The ID\(_{50}\%\) for inhibition of the rate of \[^{14}C\]-leucine incorporation ranged from 1600/\(\mu\)M for FPL55585, the 6,8-di-methyl substituted chromone, to 74/\(\mu\)M for FPL56220, the 6,8-di-t-pentyl substituted chromone. Chloroquine was more active than any of the chromones tested. The values of ID\(_{50}\%\) for inhibition of the rate of \[^{14}C\]-leucine incorporation were appreciably larger than the ID\(_{50}\%\) values for inhibition of the rate of \[^{3}H\]-thymidine incorporation; i.e. \[^{14}C\]-leucine incorporation was less sensitive than \[^{3}H\]-thymidine incorporation to inhibition by the compounds tested.

The probit plots, Figure 17, of the percentage inhibition of the rate of \[^{14}C\]-leucine incorporation, against FPL compound concentration gave dose response curves of similar shape. The chloroquine dose response curve was not as steep as those of the chromones.

**Effect of FPL52806 on the PHA dose response curve.**

The effect of FPL52806 on the PHA dose response curve when \[^{3}H\]-thymidine incorporation was measured has been previously determined. It was found that FPL52806 did not displace the PHA dose response curve. Similarly the effect of FPL52806 on \[^{14}C\]-leucine incorporation using a range of PHA concentrations was measured to determine whether or not the presence of FPL52806, throughout the culture and pulse periods, affected the PHA dose response curve.

Cultures containing a range of PHA concentrations (0.1 - 10/\(\mu\)g/ml), with and without FPL52806 (75/\(\mu\)M), were prepared. This concentration of FPL52806 had previously
TABLE 14. Concentrations of Candidate Compounds and Chloroquine Present Throughout the Culture and Pulse Periods which Inhibited the Rate of $[^{14}C]$-Leucine and $[^{3}H]$-Thymidine Incorporation by 50% (ID$_{50%}$).

<table>
<thead>
<tr>
<th>Compound</th>
<th>$[^{14}C]$-Leucine</th>
<th>$[^{3}H]$-Thymidine</th>
</tr>
</thead>
<tbody>
<tr>
<td>FPL 52839</td>
<td>2500</td>
<td>2500</td>
</tr>
<tr>
<td>FPL 55585</td>
<td>1600</td>
<td>700</td>
</tr>
<tr>
<td>FPL 55689</td>
<td>400</td>
<td>220</td>
</tr>
<tr>
<td>FPL 55731</td>
<td>140</td>
<td>77</td>
</tr>
<tr>
<td>FPL 52806</td>
<td>92</td>
<td>69</td>
</tr>
<tr>
<td>FPL 56294</td>
<td>90</td>
<td>80</td>
</tr>
<tr>
<td>FPL 56220</td>
<td>74</td>
<td>51</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>2.8</td>
<td>1.4</td>
</tr>
</tbody>
</table>
Figure 17: Inhibition of 14C-Leucine Incorporation of Phytohemagglutin-Stimulated Lymph Node Cells by Chloroquine and Novel Compounds.
been found to inhibit the rate of $[^{14}\text{C}]$-leucine incorporation, using $1\mu\text{g/ml}$ of PHA, by 40\% (Figure 17). At 40 and 64h, the incorporation of $[^{14}\text{C}]$-leucine was measured. There was no evidence of a displacement of the PHA dose response curve in the presence of FPL52806 at both 40 and 64h. (Figure 18a and b). The percentage inhibition of $[^{14}\text{C}]$-leucine incorporation was similar at 40 and 64h, at all PHA concentrations.

3.3.2 Effect On Protein Synthesis Of FPL52806 Added At Times Intermediate Between Initiation Of The Cultures And Addition Of The $[^{14}\text{C}]$-Leucine Pulse.

The effect of compounds on $[^{14}\text{C}]$-leucine incorporation have been described when the compound was present throughout the total 40h. culture period and the 4h. pulse period. Here the effect of FPL52806 added at various times through the culture period, on the rate of $[^{14}\text{C}]$-leucine incorporation is described.

FPL52806, at final concentrations of 49 and 95$\mu$/M was added to cultures of PHA-stimulated lymph node cells at 0, 2, 4, 7, 18, 26 and 42h. $[^{14}\text{C}]$-leucine was added to the cultures at 43h. for a 4h. pulse period, so that when the acid-insoluble material was isolated at 47h. the various cultures had been exposed to the compound for 47, 45, 43, 40, 29, 21 and 5h. respectively.

The effect of the presence of FPL52806 (49 and 95$\mu$/M) for these various times are shown in Figure 19. There was a progressively smaller inhibition of the rate of $[^{14}\text{C}]$-leucine incorporation with decreased time of exposure of the cells to FPL52806. When the compound was present for 47h, 49 and 95$\mu$/M FPL52806 caused 29 and 64\% inhibition respectively, and when present for 29h, the corresponding inhibitions were 24 and 48\%, while drug present for 5h, caused 9 and 15\% inhibition respectively. The progressive reduction in
Figure 18. Effect of 75μM FPL 52806 on the Incorporation of [14C]-Leucine - PHA Dose Response Curves at 40 and 64 Hours.

a) 40 Hours

D.P.M. per culture

- No FPL 52806
- 75μM FPL 52806

b) 64 Hours

D.P.M. per culture

- No FPL 52806
- 75μM FPL 52806
Figure 19. Decrease in the Inhibition of $[^{14}C]$-Leucine Incorporation at 43 Hours of PHA-Stimulated Guinea-Pig Lymph Node Cells by FPL 52806 (49 and 95μM) added at Various Times After Initiation of the Cultures.
inhibition, with decreased length of time of exposure of the cells to the compound, was greater with the higher concentration of FPL52806 (95\text{\mu}M) than the lower (45\text{\mu}M).
The effect of inhibitory compounds on two bio-synthetic processes, namely, DNA and protein synthesis, of PHA-stimulated lymph node cells have been described previously. The effect of FPL52806, and chloroquine, on the rate of a further synthetic process, the incorporation of $^{3}$H-uridine is reported here. The incorporation of $^{3}$H-uridine was measured as an index of RNA synthesis.

The effect of compounds on the rate of $^{3}$H-uridine incorporation was determined, using 4h. pulses with $^{3}$H-uridine, at two times, 1h. and 20h. It had been found (Figure 6) that both non-stimulated and PHA-stimulated cultures incorporated large amounts of $^{3}$H-uridine immediately after preparing the cultures, and that the maximum difference in $^{3}$H-uridine incorporation between stimulated and non-stimulated cultures occurred at 20h. The compounds, FPL52806 and chloroquine, were present throughout the culture period, of 1 or 20h., and the 4h. pulse period.

### 3.4.1 Effect of FPL52806 on RNA Synthesis at 1 and 20 Hours

Cultures containing FPL52806, 6,8-di-t-butyl chromone, at final concentrations of 0, 50, 75, 100, 150 and 200/$\mu$M, with and without 1/$\mu$g/ml PHA, were prepared. After 1h. and 20h. of incubation the incorporation of $^{3}$H-uridine was measured. The inhibitions of the rates of $^{3}$H-uridine incorporation found at 1 and 20h. in the presence and absence of PHA are shown in Table 15 and Figure 20.

The incorporation of $^{3}$H-uridine at 20h. was more sensitive to the inhibitory action of FPL52806 than the incorporation at 1h. Though the ID$_{50}$ values obtained from the probit plots of the percentage inhibition of the rates of
### TABLE 15. Effect of FPL52806 on the Incorporation of $[^3\text{H}]$-Uridine at 1 and 20h. by PHA-Stimulated and Non-Stimulated Guinea-Pig Lymph Node Cells

<table>
<thead>
<tr>
<th>Concn. of FPL52806, (µM)</th>
<th>Percentage Inhibition of the Rate of Incorporation of $[^3\text{H}]$-Uridine</th>
<th>Pulse at 1h.</th>
<th>Pulse at 20h.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PHA (l/g/ml)</td>
<td>No PHA (l/g/ml)</td>
</tr>
<tr>
<td>50</td>
<td></td>
<td>7 ± 3</td>
<td>7 ± 1</td>
</tr>
<tr>
<td>75</td>
<td></td>
<td>12 ± 3</td>
<td>13 ± 5</td>
</tr>
<tr>
<td>100</td>
<td></td>
<td>20 ± 4</td>
<td>18 ± 2</td>
</tr>
<tr>
<td>150</td>
<td></td>
<td>36 ± 2</td>
<td>27 ± 3</td>
</tr>
<tr>
<td>200</td>
<td></td>
<td>52 ± 2</td>
<td>40 ± 3</td>
</tr>
<tr>
<td>ID$_{50%}$</td>
<td></td>
<td>200</td>
<td>300</td>
</tr>
</tbody>
</table>

Figures are the means of 5 replicate cultures ± SD.
Figure 20. Inhibition of $[^3H]$-Uridine Incorporation at 1 and 20 Hours of PHA-Stimulated Guinea-Pig Lymph Node Cells by FPL 52806.

Inhibition of $[^3H]$-uridine incorporation, %

FPL 52806 concentration, (µM)
$[^3H]$-uridine incorporation in the presence and absence of PHA were similar at a given time, for example 1h., they were not identical. The presence of PHA caused real differences in the effect of FPL52806 at any given time of pulsing with $[^3H]$-uridine.

3.4.2 Effect Of Chloroquine On RNA Synthesis At 1 and 20 Hours.

Chloroquine was tested on $[^3H]$-uridine incorporation at 1 and 20h. using a 4h. pulse period. The effect of chloroquine only on the incorporation by PHA-stimulated cells was determined, in contrast to the study of the effect of FPL52806. The effect of the presence of chloroquine (1-100/$\mu$M), throughout the culture and pulse periods, on the rates of $[^3H]$-uridine incorporation at 1 and 20h. is shown in Table 16 and the dose response curves in Figure 21, in the form of the probit transformation of the percentage inhibition of incorporation.

Chloroquine present for 20h. before measurement of $[^3H]$-uridine incorporation caused greater inhibition than when present for only 1h. before pulsing with $[^3H]$-uridine. 100/$\mu$M chloroquine inhibited $[^3H]$-uridine incorporation by 95% when incorporation was measured at the later time, but only 18% at 1h. Similarly 10/$\mu$M chloroquine caused 26% inhibition at 20h., but stimulated the incorporation of $[^3H]$-uridine at 1h. by 2%. The probit transformed dose response curve for the effect of chloroquine on $[^3H]$-uridine incorporation at 20h. was not linear, Figure 21. The drug concentration causing 50% inhibition of incorporation, ID$_{50}$%, at 20h. was 20/$\mu$M. The corresponding ID$_{50}$% for the effect of chloroquine at 1h. was estimated by extrapolation to be about 300/$\mu$M.
TABLE 16. Effect of Chloroquine on the Incorporation of $[^3\text{H}]$-Uridine at 1 and 20h. by PHA-Stimulated Guinea-Pig Lymph Node Cells

<table>
<thead>
<tr>
<th>Concentration of Chloroquine ($\mu M$)</th>
<th>Percentage Inhibition of the Rate of Incorporation of $[^3\text{H}]$-Uridine Pulse at 1h.</th>
<th>Percentage Inhibition of the Rate of Incorporation of $[^3\text{H}]$-Uridine Pulse at 20h.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$-1 \pm 2$</td>
<td>$5 \pm 3$</td>
</tr>
<tr>
<td>2</td>
<td>$-1 \pm 2$</td>
<td>$7 \pm 10$</td>
</tr>
<tr>
<td>5</td>
<td>$-3 \pm 3$</td>
<td>$17 \pm 6$</td>
</tr>
<tr>
<td>10</td>
<td>$-2 \pm 4$</td>
<td>$26 \pm 6$</td>
</tr>
<tr>
<td>20</td>
<td>$2 \pm 3$</td>
<td>$51 \pm 3$</td>
</tr>
<tr>
<td>50</td>
<td>$10 \pm 3$</td>
<td>$83 \pm 1$</td>
</tr>
<tr>
<td>100</td>
<td>$18 \pm 2$</td>
<td>$95 \pm 1$</td>
</tr>
</tbody>
</table>

- means stimulation of $[^3\text{H}]$-uridine incorporation

Figures are the means of 5 replicates ± SD.
Figure 21. Inhibition of $[^3H]$-Uridine Incorporation at 1 and 20 Hours of PHA-Stimulated Guinea-Pig Lymph Node Cells by Chloroquine.
The inhibition by compounds of three bio-synthetic processes in stimulated lymph node cells have been described in the previous sections of this chapter. Here the effect of FPL52806 on the rate of incorporation of $[^3\text{H}]$-glucosamine is described.

The incorporation of $[^3\text{H}]$-glucosamine was measured as an index of glycoprotein synthesis (Hayden et al, 1970).

Cultures containing a range of FPL52806, 6,8-di-t-butyl chromone, concentrations (33 to 300/μM) were prepared. At 40h, the rate of incorporation of $[^3\text{H}]$-glucosamine during a 4h pulse was measured and the percentage inhibition due to the presence of the compound calculated by reference to the incorporation by a drug-free control.

Table 17 shows the effect of FPL52806 on $[^3\text{H}]$-glucosamine incorporation, the inhibition ranged from 98% with 200/μM compound, to 24% with 33/μM FPL52806. The ID$_{50%}$ was estimated to be 38/μM (Figure 22). This figure is similar to the ID$_{50%}$ for $[^3\text{H}]$-thymidine incorporation at 40h (69/μM), though lower than that of $[^{14}\text{C}]$-leucine incorporation at 40h (92/μM).
TABLE 17. Effect of FPL52806 on the Incorporation of $^{3}$H-Glucosamine by PHA-Stimulated Guinea-Pig Lymph Node Cells

<table>
<thead>
<tr>
<th>Concentration of FPL52806 (nM)</th>
<th>Percentage Inhibition of the Rate of Incorporation of $^{3}$H-Glucosamine</th>
</tr>
</thead>
<tbody>
<tr>
<td>33</td>
<td>24 ± 13</td>
</tr>
<tr>
<td>50</td>
<td>40 ± 8</td>
</tr>
<tr>
<td>75</td>
<td>52 ± 4</td>
</tr>
<tr>
<td>100</td>
<td>79 ± 3</td>
</tr>
<tr>
<td>150</td>
<td>89 ± 4</td>
</tr>
<tr>
<td>200</td>
<td>98 ± 2</td>
</tr>
<tr>
<td>300</td>
<td>96 ± 3</td>
</tr>
</tbody>
</table>

Figures are the means of 5 replicates ± SD.
Figure 22. Inhibition of $[^3H]$-Glucosamine Incorporation at 40 Hours of PHA-Stimulated Guinea-Pig Lymph Node Cells by FPL 52806.
The effects of established anti-inflammatory drugs and candidate compounds on the synthetic activities of stimulated guinea-pig lymph node cells have been investigated. PHA has generally been used as the stimulant of the lymph node cells. The synthetic activities measured have included incorporation of $[^3H]$-thymidine, $[^{14}C]$-leucine, $[^3H]$-uridine and $[^3H]$-glucosamine.

Established anti-inflammatory drugs, when present throughout the entire 40h. culture and 4h. pulse period, inhibited $[^3H]$-thymidine incorporation in the following order of increasing potency, sodium salicylate, ibuprofen, indomethacin, phenylbutazone, flufenamic acid, prednisolone and chloroquine (Table 10). Candidate compounds also inhibited the incorporation of $[^3H]$-thymidine when tested under the same conditions (Table 11). However when drugs and candidate compounds were added at the same time as the $[^3H]$-thymidine at 40h. negligible inhibition was seen (Table 12), though higher concentrations of one candidate compound, 6,8-di-t-butyl chromone (FPL52806), was shown to inhibit the incorporation of $[^3H]$-thymidine when added with the $[^3H]$-thymidine (Figure 15). Addition of FPL52806 at times intermediate between initiation of the cultures and 40h. caused a declining inhibition of $[^3H]$-thymidine incorporation the later the compound was added (Figure 19). FPL52806 did not displace the dose response curve between concentration of PHA and incorporation of $[^3H]$-thymidine (Figure 18), indicating that the inhibition of $[^3H]$-thymidine incorporation by the compound was not caused by interference with the binding of PHA to the lymph node cells. FPL52806 inhibited $[^3H]$-thymidine incorporation in antigen-stimulated cells when present throughout the culture and pulse periods to a similar extent as it inhibited PHA-stimulated cells.
Chloroquine and candidate compounds inhibited $[^{14}\text{C}]$-leucine incorporation when present during the entire 40h. culture and 4h. pulse periods. The order of compound potency was identical to that found with $[^{3}\text{H}]$-thymidine incorporation, but the concentration of compound inhibiting $[^{14}\text{C}]$-leucine incorporation by 50% were higher than those found to inhibit $[^{3}\text{H}]$-thymidine incorporation (Table 14). The inhibition of $[^{14}\text{C}]$-leucine incorporation of FPL52806 declined the later the compound was added to the cultures (Figure 19). The inhibition of $[^{14}\text{C}]$-leucine incorporation by FPL52806 added at the time of initiation of cultures was not caused by interference with the binding of PHA to the lymph node cells.

Chloroquine and FPL52806 inhibited the incorporation of $[^{3}\text{H}]$-uridine at 1 and 20h. when present throughout the entire culture and pulse periods. Greater inhibition at 20h. than 1h. was seen with both compounds.

FPL52806 inhibited $[^{3}\text{H}]$-glucosamine incorporation when present throughout the 40h. culture and 4h. pulse period.
CHAPTER FOUR

THE RELATIONSHIP BETWEEN THE PHARMACOLOGY OF

SOME CHROMONES AND THEIR PHYSICAL PROPERTIES
In this chapter the physical properties of some of the compounds (6,8-di-alkyl chromones), whose effects on the synthetic activities of PHA-stimulated guinea-pig lymph node cell cultures were described in Chapter 3, are investigated. The lipophilicity of compounds is important in assessing the ease with which they cross cell membranes to their site of action. Lipophilicity can be measured by determining the partition coefficient of the compound.

The extent to which drugs bind to serum protein can modify the potencies of the drugs. Since 10% v/v serum was present in the culture medium when the potencies of the 6,8-di-alkyl chromones as inhibitors of synthetic activity was investigated in Chapter 3 the extent to which the compounds were bound to serum protein was investigated. The extent to which compounds bind to protein may relate to their lipophilicities if hydrophobic bonding is important.

The presence of serum in the cultures may have acted as a trap for much of the added compound reducing the amount of compound which associated with the lymph node cells and hence its apparent potency. This possibility was experimentally confirmed for one of the 6,8-di-alkyl chromones.
In Chapter 3 the effects on the synthetic activities of PHA-stimulated guinea-pig lymph node cells of various established anti-inflammatory drugs and candidate compounds were described. Among the candidate compounds was an homologous series of 6,8-di-alkyl substituted chromones, the alkyl substituents of which ranged in size from the t-pentyl group to the methyl group. The parent non-alkylated chromone was also tested.

It was found that the potencies of the members of 6,8-di-alkyl substituted chromone series differed considerably as inhibitors of $[^3H]$-thymidine or $[^{14}C]$-leucine incorporation. The potency, measured by the drug concentration causing 50% inhibition of the rate of incorporation of $[^3H]$-thymidine, or $[^{14}C]$-leucine, relative to that present in a drug-free control, of the di-t-pentyl substituted chromone was the greatest and the potency of the other members of the homologous series decreased with the diminishing size of the substituent. The differences in potency between adjacent members of the series were greater when the alkyl substituents were small, for example the ID$_{50\%}$ for the rate of $[^3H]$-thymidine incorporation were 52/M and 69/M for the di-t-pentyl and di-t-butyl chromones (FPL56220 and 52806), but 220/M and 700/M for the di-ethyl and di-methyl chromones (FPL55689 and 55585).

This variation in potency as inhibitors of a biological test system for members of an homologous series has frequently been reported in the literature. The biological test systems have ranged in complexity from the inhibition of the in-vitro activity of enzymes e.g. carbonic anhydrase (Kakeya et al, 1969), to whole animal systems, such as the hypnotic effect of barbiturates in the rat (Shonle et al, 1930). Various attempts have been made to relate the potencies of
members of chemical series in a given biological test system
to the electronic, steric and lipophilic properties of the
compounds (Fujita, 1972). These attempts to determine
structure-activity relationships were made to further the
understanding of the compound's mode of action at the
molecular level. For example the optimum size of an
enzyme inhibitor (steric factor) will give some indication
of the dimensions of the enzyme's active site, and the
electronic factor (σ or Hammett factor) should give
information concerning the electronic processes occurring
at the enzyme's active site. Hansch's Π factor, which
measures the lipophilicity of the compound can give
understanding of the importance of lipophilic binding at
the enzyme's active site (and hence presumably of the
natural substrate of the enzyme or process) or of the ease
with which the drug will cross cell membranes to travel
to its site of action. By measuring these factors for a
series of chemicals and correlating the potencies of the
chemicals in a given biological test system it is possible
to determine which of the factors (electronic, steric or
lipophilic) is dominant in controlling the potency and the
contribution, if any, the other factors make. Once this
is done, that is the structure-activity relationship is
expressed as a mathematical equation, it is possible to
predict the activity in the biological test system of novel
or yet unsynthesized compound if the relevant factors
(steric, σ, or Π) can be calculated. This predictive
value is an additional reason for the study of structure-
activity relationships.

Once sufficient compounds of a chemical series have
been assayed in a biological test system to allow
determination of the structure-activity relationships
equation it is, in theory, possible to predict the structure
of the compound which would be the most potent inhibitor.
This is obviously of great value in the pharmaceutical
industry if the biological test system is a true model of
a disease.
In the case of the 6,8-di-alkyl substituted chromones studied in Chapter 3 only the size of the alkyl side chains was varied. It is known that alkyl side chains have little effect on the electronic character of the molecule on which they are present (Hansch, 1972). It is therefore unlikely that the difference in the potencies of the members of this homologous series on the rate of [3H]-thymidine and [14C]-leucine incorporation by guinea-pig lymph node cells can be interpreted in a manner involving Hammett's electronic factor, \( \sigma \). It is more probable that the differing potencies of 6,8-di-alkyl substituted series may be due to changes in lipophilicity of the molecules or possibly due to changes in the volume of the molecules (steric factor).

4.2.1 Apparent Partition Coefficients Of 6,8-Di-Alkyl Chromones

The apparent partition coefficients of 6,8-di-alkyl substituted chromones between octanol:ether (1:3 v/v) and pH 7.45 buffer were determined as described previously.

The structures of the 6,8-di-alkyl substituted chromones and their partition coefficients are shown in Table 18. The experimentally determined partition coefficients ranged from 4.6 for the highly lipophilic di-t-pentyl substituted chromone (FPL56220) to less than 0.1 for the di-methyl substituted and un-substituted chromones (FPL55585 and 52839).

The logarithms of the apparent partition coefficients plotted against the number of carbon atoms in the substituents are shown in Figure 23. The relationship between the logarithm of the apparent partition coefficient and the number of carbon atoms in the substituent was not linear. The apparent partition coefficient of the di-methyl substituted chromone (FPL55585) was estimated by extrapolation to be about 0.05.
<table>
<thead>
<tr>
<th>Compound</th>
<th>Substituents</th>
<th>Partition Coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>FPL 52839</td>
<td>hydrogen</td>
<td>0.1</td>
</tr>
<tr>
<td>FPL 55585</td>
<td>methyl-</td>
<td>0.1</td>
</tr>
<tr>
<td>FPL 55689</td>
<td>ethyl-</td>
<td>0.09</td>
</tr>
<tr>
<td>FPL 55731</td>
<td>n-propyl-</td>
<td>0.23</td>
</tr>
<tr>
<td>FPL 52806</td>
<td>t-butyl-</td>
<td>0.70</td>
</tr>
<tr>
<td>FPL 56220</td>
<td>t-pentyl-</td>
<td>4.61</td>
</tr>
</tbody>
</table>
Figure 23. Relationship between the Apparent Partition Coefficient of 6,8-Di-Alkyl Chromones and the Number of Carbon Atoms in the Alkyl Side Chain.

![Graph showing the relationship between apparent partition coefficient and number of carbon atoms in alkyl side chain.](image-url)

- Apparent partition coefficient
- Number of carbon atoms in alkyl side chain
The true partition coefficient can be calculated from the apparent partition coefficients by correction for the proportion of the compound which is dissociated (Fujita et al., 1964). The pKa of the chromones are about 2. At pH 7.45 at least 99.999% of the chromones will be dissociated and the correction factor to convert apparent partition coefficient to true partition coefficients will be very large (> $10^5$).

4.2.2 Relationship For 6,8-Di-Alkyl Chromones Between Apparent Partition Coefficient And Potency As Inhibitors Of DNA And Protein Synthesis

Table 19 shows the partition coefficients and potencies of the 6,8-di-alkyl substituted chromones as inhibitors of $[3^H]$-thymidine and $[14C]$-leucine incorporation presented as the drug concentration causing 50% inhibition of the rate of incorporation (from Table 14). The ID$_{50}$s for both $[3^H]$-thymidine and $[14C]$-leucine incorporation were not related in a linear fashion to the partition coefficient. In Figure 24 the data of Table 19 is presented in the more usual form used for structure-activity relationships, that is the logarithms of the reciprocal of the ID$_{50}$ plotted against the logarithm of the partition coefficient (Hansch, 1972; Fujita, 1972).

The increasing potency of the 6,8-di-alkyl chromones with increasing lipophilicity should be noted, and also that the curves flatten. This indicates that optimum lipophilicity for 6,8-di-alkyl substituted chromones which inhibit $[3^H]$-thymidine and $[14C]$-leucine incorporation is approached by the di-t-pentyl substituted chromone. The extent to which compounds bind to serum protein is related to lipophilicity and it is possible that the proportion of added compound bound to the serum in the culture medium increased with increasing lipophilicity. This may be
### TABLE 19.

**Apparent Coefficients and ID<sub>50</sub><sup>°</sup> for [<sup>3</sup>H]-Thymidine and [<sup>14</sup>C]-Leucine Incorporation for 6,8-Di-Alkyl Substituted Chromones**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Substituents</th>
<th>Partition Coefficient</th>
<th>ID&lt;sub&gt;50&lt;/sub&gt; &lt;sup&gt;°&lt;/sup&gt;, (µM)</th>
<th>[&lt;sup&gt;3&lt;/sup&gt;H]-Thymidine</th>
<th>[&lt;sup&gt;14&lt;/sup&gt;C]-Leucine</th>
</tr>
</thead>
<tbody>
<tr>
<td>FPL 55585 methyl-</td>
<td></td>
<td>0.05</td>
<td>700</td>
<td>1600</td>
<td></td>
</tr>
<tr>
<td>FPL 55689 ethyl-</td>
<td></td>
<td>0.09</td>
<td>220</td>
<td>450</td>
<td></td>
</tr>
<tr>
<td>FPL 55731 n-propyl-</td>
<td></td>
<td>0.23</td>
<td>77</td>
<td>140</td>
<td></td>
</tr>
<tr>
<td>FPL 52806 t-butyl-</td>
<td></td>
<td>0.70</td>
<td>69</td>
<td>92</td>
<td></td>
</tr>
<tr>
<td>FPL 56220 t-pentyl-</td>
<td></td>
<td>4.61</td>
<td>52</td>
<td>78</td>
<td></td>
</tr>
</tbody>
</table>

* means extrapolated value.
Figure 24. Relationship between the Reciprocal of the Potencies (ID$_{50}$) of 6,8-Di-Alkyl Chromones as Inhibitors of $[^3H]$-Thymidine- and $[^{14}C]$-Leucine Incorporation and the Apparent Partition Coefficients of the Compounds.
responsible for the flattening of the curves in Figure 24. This possibility is investigated in the next section of this Chapter.
Many drugs are known to bind reversibly to serum protein. The extent of this binding varies considerably from drug to drug; examples of drugs which are highly protein bound are dicumarol (O'Reilly et al., 1962) and phenylbutazone (Burns et al., 1953), which are at least 95% bound at therapeutic levels. Carbenoxolone, an anti-gastric ulcer agent is protein-bound to greater than 99.9% at 100 times the therapeutic levels (Parke & Lindup, 1973). Other drugs, such as the salicylates, are normally bound to a smaller extent and some such as antipyrine, an analgesic, are only slightly protein-bound if at all (Smith and Rawlins, 1973).

The protein binding of drugs is important if the majority of the drug is protein-bound, and also pharmacologically inactive. The protein-bound drug will act as a reservoir for the non-protein-bound and pharmacologically active free drug, decreasing in general the rate of elimination of drugs by both metabolism and excretion.

The majority of protein-bound drugs bind mainly to serum albumin, but some drugs bind to other protein, for example prednisolone binds to transcortin, an α-globulin (Desgnez, 1966). Normally many of the sites to which drugs are bound are occupied by normal body constituents, for example fatty acids are extensively bound to serum albumin (Dole, 1956), as is bilirubin (Ostrow and Schmid, 1963). Thyroxine is normally only slightly bound to albumin, binding to pre-albumin and a specific thyroxine-binding protein being more important (Salvatore et al., 1966). Cortisol is normally 95% bound to transcortin (Sandberg et al., 1966). It is possible for drugs to displace these normal body constituents from their binding sites, if the
drug has a greater affinity for the binding site than the body constituents. Such effects may have clinical significance, for example, drugs such as sulphisoxazole may displace bilirubin from albumin in the new born premature infant with disastrous results (Silverman et al, 1956).

Competition for binding sites also occurs between drugs. A highly protein-bound drug will displace a weakly bound one and increase the free drug level of the weakly bound drug potentiating its action. This effect has been described for the highly protein-bound anticoagulant warfarin and the even more highly protein-bound chlorophenoxyisobutyric acid (Clofibrate). It was found that administration of the two drugs together potentiated the anticoagulant activity of the warfarin (Schrogie and Solomon, 1967). The importance of protein binding to the pharmacology of drugs has been reviewed by Meyer and Guttman (1968).

Several types of bonding have been suggested to be responsible for the binding of drugs to proteins. Ionic bonds may occur between ionized groups of the drug, for example – COO⁻, and oppositely charged groups on the protein, for example – NH₃⁺. Hydrogen bonding may also be involved. Weak electrostatic forces such as the Keesom, Debye and London forces opposed by the Born force may be important (Settle, 1971). An additional bonding system is the so-called hydrophobic bond.

Hydrophobic bonds result from the repulsion between lipophilic regions on a compound, or protein, and water and the resulting mutual attraction between the lipophilic groups of the drug and protein. This attraction is analogous to the partitioning of a lipophilic drug between an aqueous polar solvent and a lipophilic less polar one.

If the binding of the 6,8-di-substituted chromones
involves hydrophobic bonding the more lipophilic member of the series will be bound to a greater extent than that of the less lipophilic members of the series and the degree of protein binding will parallel their lipophilicity measured by partition coefficient.

Measurement of the extent to which 6,8-di-alkyl chromones are bound to serum protein, under the experimental conditions used to study the effect of the compounds on the synthetic processes in guinea-pig lymph node cells (Chapter 2), will allow corrections of the added drug concentration for the proportion of drug bound to the serum in the cultures, enabling the actual free and (pharmacologically active?) drug concentration to be calculated.

4.3.1 Protein Binding Of 6,8-Di-Alkyl Chromones

By Equilibrium Dialysis

The chromones were those studied in the previous section of this Chapter, and whose effects on \( ^3H \)-thymidine and \( ^{14}C \)-leucine incorporation by lymph node cells had been determined. The binding of the compounds to serum was determined under conditions similar with respect to pH and proportion of serum to those of the lymph node cell cultures when the effect of the compounds on synthetic processes was investigated.

The percentage of the various chromones binding to serum at various concentrations of these compounds is shown in Table 20.

The more lipophilic chromones, (that is with larger alkyl substituents), were bound to serum proteins to a greater extent at a given concentration than the less lipophilic. For example, 52\(^\mu\)M 6,8-di-t-pentyl chromone, FPL56220, was 93% serum-bound, whereas 52\(^\mu\)M 6,8-di-methyl chromone, FPL55585, was 60% bound. The serum binding depended on the concentration of the drug for most of the
TABLE 20. **Percentage of 6,8-Di-Alkyl Substituted Chromones Bound to 10% (v/v) Calf Serum at Various Chromone Concentrations**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Substituents</th>
<th>Chromone Concentration (μM)</th>
<th>Percentage Chromone Serum Bound</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>16</td>
<td>52</td>
</tr>
<tr>
<td>FPL 52839</td>
<td>hydrogen</td>
<td>23</td>
<td>35</td>
</tr>
<tr>
<td>FPL 55585</td>
<td>methyl-</td>
<td>58</td>
<td>60</td>
</tr>
<tr>
<td>FPL 55689</td>
<td>ethyl-</td>
<td>73</td>
<td>65</td>
</tr>
<tr>
<td>FPL 55731a-</td>
<td>propyl-</td>
<td>82</td>
<td>77</td>
</tr>
<tr>
<td>FPL 52806</td>
<td>t-butyl-</td>
<td>95</td>
<td>81</td>
</tr>
<tr>
<td>FPL 56220</td>
<td>t-pentyl-</td>
<td>99</td>
<td>93</td>
</tr>
</tbody>
</table>
compounds; the lower the added concentration of FPL compound, the greater percentage of the compound that was serum-bound. This effect was slight for the 6,8-di-methyl chromone, and absent in the case of the un-substituted chromone. The di-t-butyl substituted chromone was, for example, 95, 81, 64 and 52% serum bound at concentrations of 16, 52, 156 and 522 μM respectively.

The data in Table 17 was used to calculate the free non-protein-bound drug concentration in the lymph node cell cultures subsequently. The importance of lipophilicity in determining the proportion of the 6,8-di-alkyl chromone which was protein-bound is illustrated in Figure 25, where the percentage of added chromone (52 μM) bound to serum protein is plotted against the apparent partition coefficient of the chromone. The values for the un-substituted chromone, FPL52839, are not plotted since the value for the compound's apparent partition coefficient had not been determined. Figure 25 shows that lipophilicity, as measured by apparent partition coefficient, does contribute to the extent to which the 6,8-di-alkyl chromones bind to serum proteins, but that the size of the alkyl side chains does not entirely determine the extent to which the compounds bind to serum proteins.

4.3.2 Protein Binding Of 6,8-Di-Alkyl Chromones By Fluorescence Quenching

In this section the binding of 6,8-di-alkyl chromones to isolated bovine serum albumin is described in contrast to the previous section where the binding to 10% v/v calf serum in 0.155M NaCl was reported. The fluorescence quenching method measures association mainly with the primary binding sites to which compounds bind with high affinity.

The quenching of the native fluorescence of proteins
Figure 25. Relationship between the Percentage of 6,8-Di-Alkyl Chromone (52μM) Bound to 10% (v/v) Bovine Serum and the Apparent Partition Coefficient of the Compound.

Percentage of 52μM chromone bound to bovine serum

Apparent Partition Coefficient

- FPL 56220
- FPL 52806
- FPL 55731
- FPL 55689
- FPL 55585
by compounds as they bind is one of many spectroscopic techniques which have been used to investigate the binding of compounds to proteins (Chignell, 1971 and 1974). For a compound that binds to a protein to quench the native fluorescence of the protein it is necessary for the absorption spectrum of the compound to overlap the fluorescence emission spectrum of the protein (Chignell, 1971 and 1974). The 6,8-di-alkyl chromones fulfil the criteria with respect to bovine serum albumin.

Table 21 shows the apparent number of primary binding sites and the apparent association constants of binding for the 6,8-di-alkyl chromones derived from the quenching of the native fluorescence of bovine serum albumin by the compounds. The values for the number of primary binding sites determined by this method are known to be imprecise, since large variations in their number have little effect on the form of the fluorescence quench curves. The association constants tend to increase with the increasing size of the alkyl substituents of the 6,8-di-alkyl chromones, and hence the lipophilicity of the compounds. This trend is not regular, however. The irregularities are especially marked in the case of the di-methyl chromone, FPL55585, which has an association constant which is close to that of the 6,8-di-t-butyl chromone, rather than being intermediate between that of the un-substituted and 6,8-di-ethyl chromones (FPL52839 and 55689 respectively). It is possible that this anomaly is caused by 6,8-di-methyl chromone having a molecular size which is especially favourable for binding to bovine serum albumin.

Study of the binding of 6,8-di-alkyl chromones by both equilibrium dialysis and fluorescence quenching show that there is a trend for the more lipophilic compound to be more highly bound to calf serum, or have a larger apparent association constant of binding to bovine serum albumin. This trend is more apparent in the equilibrium dialysis studies with calf serum than in the fluorescence
<table>
<thead>
<tr>
<th>Compound</th>
<th>Substituents</th>
<th>Apparent Number of Primary Binding Sites</th>
<th>Apparent Association Constant of Binding ($\mu$-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FPL 52839</td>
<td>hydrogen</td>
<td>0.28</td>
<td>$0.28 \times 10^5$</td>
</tr>
<tr>
<td>FPL 55585</td>
<td>methyl-</td>
<td>1.02</td>
<td>$3.51 \times 10^5$</td>
</tr>
<tr>
<td>FPL 55689</td>
<td>ethyl-</td>
<td>0.01</td>
<td>$0.87 \times 10^5$</td>
</tr>
<tr>
<td>FPL 55731</td>
<td>n-propyl-</td>
<td>0.58</td>
<td>$0.95 \times 10^5$</td>
</tr>
<tr>
<td>FPL 52806</td>
<td>t-butyl-</td>
<td>1.75</td>
<td>$4.91 \times 10^5$</td>
</tr>
<tr>
<td>FPL 56220</td>
<td>t-pentyl-</td>
<td>1.52</td>
<td>$4.74 \times 10^5$</td>
</tr>
</tbody>
</table>
quenching studies with isolated bovine serum albumin. Serum contains many substances other than albumin and it is possible that effects of these other substances, some of which will themselves be bound to serum albumin, for example fatty acids, may explain the inconsistencies found in the binding of 6,8-di-alkyl chromones to calf serum and isolated bovine serum albumin. An alternative explanation is the absence of a high salt concentration from the bovine serum albumin solutions used in the fluorescence quenching experiments, since chloride ions are known to bind to albumin and may compete with the 6,8-di-alkyl chromones for binding. Sodium chloride was present in the equilibrium dialysis experiments at a physiological concentration.
Earlier in this Chapter the importance of the binding of drugs to serum proteins was described and the experimental determination of the extent to which 6,8-di-alkyl chromones were protein-bound was reported.

It is possible that, though the free non-protein-bound drug concentration controlled the rate of drug entry into lymph node cells the eventual equilibrium concentration of drug in the cells was similar to the total drug concentration in the culture medium. This possibility requires drug binding substances to be present in the lymph node cells. The amount of \( ^3\text{H} \)-6,8-di-t-butyl chromone associated with the cells was determined therefore to investigate this possibility. It was assumed that if the amount of \( ^3\text{H} \)-6,8-di-t-butyl chromone associated with the cells gradually increased during the exposure of the cells to drug, equilibrium had been reached by 40h.

4.4.1 Association Of \( ^3\text{H} \)-6,8-Di-t-Butyl Chromone To Guinea-Pig Lymph Node Cells

The uptake of \( ^3\text{H} \)-6,8-di-t-butyl chromone by PHA-stimulated and non-stimulated lymph node cells after 40h culture in the presence of radioactively labelled material was determined. Two concentrations of \( ^3\text{H} \)-6,8-di-t-butyl chromone (25 and 75\( ^\mu\text{M} \)) and three different concentrations of calf serum (0.2, 1.0 and 10% v/v) were used.

Table 22 shows the amount of the total \( ^3\text{H} \)-6,8-di-t-butyl chromone present in the cultures associated with the cells.
TABLE 2. Percentage of 25\textsuperscript{M}M and 75\textsuperscript{M}M [\textsuperscript{3}H]-6,8-Di-t-Butyl Chromone (FPL 52806) Associated with PHA-Stimulated and Non-Stimulated Guinea-Pig Lymph Node Cells at 40h.

Percentage of [\textsuperscript{3}H]FPL52806 associated with cells (25\textsuperscript{M}M) (75\textsuperscript{M}M)

<table>
<thead>
<tr>
<th>Serum concn.</th>
<th>PHA (l\textsuperscript{g}/ml)</th>
<th>No PHA</th>
<th>PHA (l\textsuperscript{g}/ml)</th>
<th>No PHA</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2</td>
<td>6.0 ± 0.2</td>
<td>6.1 ± 0.4</td>
<td>5.2 ± 0.2</td>
<td>4.6 ± 0.1</td>
</tr>
<tr>
<td>1.0</td>
<td>3.3 ± 0.1</td>
<td>3.2 ± 0.1</td>
<td>3.4 ± 0.3</td>
<td>3.8 ± 0.3</td>
</tr>
<tr>
<td>10.0</td>
<td>1.0 ± 0.1</td>
<td>0.9 ± 0.1</td>
<td>1.0 ± 0.1</td>
<td>1.1 ± 0.1</td>
</tr>
</tbody>
</table>

Figures are the means of 4 replicates ± SD
At a given serum concentration the cells were found to bind a similar percentage of the total $[^3\text{H}]$-6,8-di-t-butyl chromone whether they had been exposed to PHA or not. A similar proportion of the total $[^3\text{H}]$-6,8-di-t-butyl chromone was associated with the cells at both 6,8-di-t-butyl chromone concentrations. The association depended on the serum concentration. The lower the serum concentration the greater the percentage of the total $[^3\text{H}]$-6,8-di-t-butyl chromone associated with the cells. For example with 0.2% (v/v) serum, 6% of total $[^3\text{H}]$-6,8-di-t-butyl chromone was bound to the cells with 25 nM 6,8-di-t-butyl chromone and stimulated cells. The corresponding percentages were 3.3% and 1.0% with 1% and 10% (v/v) serum respectively.

This indicates that drug-binding substances (proteins or possibly lipids) are present in (or on) the cells and that they compete with the serum for the binding of 6,8-di-t-butyl chromone. For this chromone, the amount associated with the cells is related to the free non-protein-bound concentration rather than the total concentration (both protein-bound and free). For other chromones of different lipophilicities, the amount of chromone associated with the cells would not be identical to the amount of 6,8-di-t-butyl chromone associated. The amount of other chromones associated with the cells would be related to the free non-protein-bound chromone concentration (protein binding is related to some extent to lipophilicity), and also related to the ease with which the non-protein-bound chromone could enter the cell. The latter is also related to lipophilicity.
SUMMARY AND CONCLUSIONS

The lipophilicity of 6,8-di-alkyl chromones, measured by their apparent partition coefficients, increase as the size of the alkyl side chains increase (Figure 23). The activity of these compounds as inhibitors of the rate of incorporation of $[^3\text{H}]$-thymidine and $[^1\text{C}]$-leucine by PHA-stimulated guinea-pig lymph node cells increase as the lipophilicity of the compounds increase (Figure 24). The rate of increase in the activity of the compounds as inhibitors of $[^3\text{H}]$-thymidine and $[^1\text{C}]$-leucine incorporation is large at low lipophilicities (6,8-di-methyl, 6,8-di-ethyl, and 6,8-di-n-propyl chromones) but slight at high lipophilicities (6,8-di-t-butyl and 6,8-di-t-pentyl chromones).

The 6,8-di-alkyl chromones bind to calf serum (Table 20) and bovine serum albumin (Table 21). The more lipophilic compounds bind to calf serum to a greater extent than less lipophilic 6,8-di-alkyl chromones, (Figure 25). The amount of 6,8-di-t-butyl chromone (FPL52806) associated with lymph node cells is related to the non-serum-bound compound (Table 22) rather than to the total compound (both free and non-serum-bound).

The activity of 6,8-di-alkyl chromones as inhibitors of the synthetic activities of PHA-stimulated guinea-pig lymph node cells is largely determined by the molecular property of lipophilicity. It is suggested that the decline in the rate of increase in the activity of the compounds at high lipophilicities may partially be caused by the increased proportion of the 6,8-di-alkyl chromones which are serum-bound. The serum-bound compound is inactive. This is supported by the experiment with FPL52806 which showed that the amount of compound associated with the cells is related to the free compound rather than to the total compound concentration.
CHAPTER FIVE

THE RELATIONSHIP BETWEEN THE PHARMACOLOGY OF SOME CHROMONES AND ANTI-INFLAMMATORY DRUGS AND THEIR EFFECT ON THE MORPHOLOGY OF GUINEA-PIG LYMPH NODE CELLS
In Chapter 3 the inhibitory effect of anti-inflammatory drug and novel compounds, the majority of which were chromones, on the synthetic activity of PHA-stimulated guinea-pig lymph node cells were described. It is important when studying the effect of compounds on the biological activities of living systems to distinguish between a true inhibition of the biological activity by the compound, and the death of the biological system due to the compound's toxic action, which will result in an apparent inhibition. Eurenius and MacIntyre (1970) stressed the importance of making this distinction when the effect of compound on lymphocyte transformation was determined. They described a technique involving the counting of nuclei of viable lymphocytes after digestion of the nuclei from compound-killed cells by cetrimide and pronase. Earlier workers, for example Tormey et al (1967) had ignored the possibility that the inhibitory effects of prednisolone on the synthetic activities of transforming lymphocytes may have been due to cytotoxicity. Other workers, for example Forbes and Smith (1967) studied the lymphocyte cytotoxic action of the compounds they investigated. They found that salicylate and hydrocortisone had little effect on the viability of lymphocytes, but that indomethacin was highly cytotoxic. Forbes and Smith used a dye exclusion method to determine the viability of their cells. A similar method, the exclusion of eosin, was used in the present work to determine cell viability after microscopic examination.

Anti-inflammatory drugs are known to stabilise lysosome membranes (Weissmann, 1972, and Ignarro, 1972) and to prevent the haemolysis of erythrocytes by hypotonic lysis (Inglot and Wolna, 1968), that is, they have membrane-active properties. In Chapter 3 it was reported that FPL52806, 6,8-di-t-butyl chromone inhibited the synthetic activities
of stimulated lymph node cells. The effect of this compound on the surface character of stimulated lymph node cells was determined by scanning electron microscopy since it was possible that the membrane-active properties of the compound might manifest themselves by affecting the cell membrane and external appearance of the cells. It was also thought possible that the cytotoxic activity of the compound would affect the appearance of the cells under the scanning electron microscope.
5.2 VIABILITY AND MORPHOLOGY OF COMPOUND-TREATED PHA-STIMULATED GUINEA-PIG LYMPH NODE CELLS

The viability of treated lymph node cell cultures after 40h. incubation with compound was measured by a eosin-exclusion method.

The microscopic examination of many of the treated cultures was brief, only the lowest concentrations of chromones and other drugs which caused complete cell death being recorded. The lowest concentrations which caused complete cell death, and the concentrations which inhibited the rate of \( ^{3}H \)-thymidine incorporation by 50% are shown in Table 23.

At the concentration which caused complete cell death there was always complete inhibition of the rate of \( ^{3}H \)-thymidine incorporation, but the concentration which caused 50% inhibition of \( ^{3}H \)-thymidine incorporation was always appreciably lower (usually at least 2 fold) than the concentration which caused complete cell death.

The toxicity of some compounds was examined in detail, and the number of viable cells in cultures treated with a series of 6,8-di-alkyl chromones over a range of doses was counted. The number of viable cells present in the treated cultures was expressed as a percentage of the number in a drug-free culture. The number of viable cells in the drug-free controls were generally 30% of the number of viable cells added initially to the cultures. The results of these experiments are shown in Figure 26. The chromone concentration which caused 50% loss of cell viability and the concentration which caused 50% inhibition of the rate of \( ^{3}H \)-thymidine incorporation are shown in Table 24. The results confirm that inhibition of \( ^{3}H \)-thymidine incorporation occurred at lower concentrations of added drug than did the corresponding percentage of cell death. For example, 77'M FPL55731, 6,8-di-n-propyl chromone, caused 50% inhibition
<table>
<thead>
<tr>
<th>Compound</th>
<th>Conc. which caused Loss of Cell Viability, relative to compound-free control, of Greater than 90% (μM)</th>
<th>Conc. which Inhibited the Rate of $^{3}$H-Thymidine Incorporation by 50%, ID$_{50}$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ibuprofen</td>
<td>1000</td>
<td>220</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>1000</td>
<td>150</td>
</tr>
<tr>
<td>Phenylbutazone</td>
<td>300</td>
<td>130</td>
</tr>
<tr>
<td>Flufenamic acid</td>
<td>200</td>
<td>100</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>30</td>
<td>1.4</td>
</tr>
<tr>
<td>FPL 52816</td>
<td>300</td>
<td>56</td>
</tr>
<tr>
<td>FPL 55618</td>
<td>300</td>
<td>180</td>
</tr>
</tbody>
</table>
Figure 26. Effect of 6,8-Di-Alkyl Chromones on the Viability of PHA-Stimulated Guinea-Pig Lymph Node Cells at 40 Hours.
TABLE 24. Effect of 6,8-Di-Alkyl Chromones on the Incorporation of $^3$H-Thymidine by PHA- Stimulated Guinea-Pig Lymph Node Cells and the Viability of PHA-Stimulated Guinea-Pig Lymph Node Cells at 40 Hours

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concn. which Inhibited the Rate of $^3$H-Thymidine Incorporation by 50%, $ID_{50}$ ($\mu$M)</th>
<th>Concn. which caused Loss of Cell Viability relative to a compound free control, of 50% ($\mu$M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FPL 55585</td>
<td>700</td>
<td>2500</td>
</tr>
<tr>
<td>FPL 55689</td>
<td>220</td>
<td>820</td>
</tr>
<tr>
<td>FPL 55731</td>
<td>77</td>
<td>230</td>
</tr>
<tr>
<td>FPL 52806</td>
<td>69</td>
<td>130</td>
</tr>
<tr>
<td>FPL 56220</td>
<td>51</td>
<td>90</td>
</tr>
</tbody>
</table>
of the rate of $[{}^3\text{H}]$-thymidine incorporation, while 50% loss of cell viability occurred at an FPL55731 concentration of 230$\mu$M.

In further experiments the effect of FPL52806, 6,8-di-t-butyl chromone, and also the number of transforming viable cells (determined by deducting the number of viable small lymphocytes from the total number of viable cells) was studied. Figure 27 shows the percentage of viable cells and the percentage of viable cells which were transforming (or transformed) relative to the number in a compound-free control at various concentrations of FPL52806.

The concentration of FPL52806 found to inhibit the rate of $[{}^3\text{H}]$-thymidine incorporation by 50% is 69$\mu$M. At this concentration the loss of cell viability was 30%, confirming that the effect of FPL52806 on $[{}^3\text{H}]$-thymidine incorporation was not solely due to its toxic action on the lymph node cells.

The values for the number of transforming (or transformed) cells in Figure 27 show that FPL52806 reduced the number of stimulated cells, to an extent greater than its effect on the total number of viable cells. This effect was greater at FPL52806 concentrations above 100$\mu$M. At 150$\mu$M FPL52806 the number of stimulated cells was 3% of the number present in the drug-free controls, while the percentage viability relative to that of the control was 37%.

The results reported in this section should be treated with caution due to the problems which are inherent in the experimental procedure such as sampling error, small number of cells counted, generally 100-200, and the difficulty of determining visually whether a cell was a small lymphocyte or was increased in size. The results do indicate that, although the compounds are toxic to lymph node cells, the effect of compounds on the rates of $[{}^3\text{H}]$-thymidine incorporation, etc., cannot solely be explained on the basis of this toxicity, and that FPL52806 reduced the number of cells which were stimulated to a greater extent than it reduced cell viability.
Figure 27. Effect of FPL 52806 Present Throughout the Culture and Pulse Periods on the Number of Transformed and Number of Viable PHA-Stimulated Guinea-Pig Lymph Node Cells at 40 Hours.

- Decrease in number, %
- Number of Transformed Cells
- Number of Viable Cells

FPL 52806 concentration, (μM)
Scanning electron microscopy reveals the surface appearance of examined material, rather than the internal structures. Polliack et al (1973) reported that by their external appearance lymphocytes could be differentiated into two groups corresponding to T or thymus-dependent and B or bursa-equivalent lymphocyte classes. The T lymphocytes were smooth surfaced in general in contrast to the B lymphocytes which had many external processes.

Since anti-inflammatory drugs have membrane active properties, FPL52806, 6,8-di-t-butyl chromone, in addition to inhibiting the synthetic activities of guinea-pig lymph node cells, might affect their external appearance in a subtle manner, as revealed by the scanning electron microscope. High concentrations of FPL52806 were shown in the previous section of this chapter to be cytotoxic by eosin-exclusion to lymph node cells. It was expected that dead cells might be differentiated from viable cells under the scanning electron microscope and that this might provide a further method for the quantitation of cytotoxicity by this compound.

PHA-stimulated guinea-pig lymph node cells incubated for 40h. with and without FPL52806, 6,8-di-t-butyl chromone, (50, 100, 150, 200, 300/μM) were prepared for scanning electron microscopic examination as described previously.
Cells which had not been treated with FPL52806 were essentially spherical and had irregular surfaces (Figures 28, 29 and 30). The cells had processes projecting on to the surface to which they were attached (Figures 28 and 29). Some cells (Figure 30) had a bulge on their surface, similar to that on a lemon. This may correspond to the uropod described by McFarland et al (1966).

In contrast, cells treated with FPL52806 (300/M) did not have any of these features; FPL52806 (300/M) was completely cytotoxic to the cells, determined by eosin-exclusion and inhibition of synthetic activity. These dead cells killed by 300/M FPL52806 (Figure 31) appeared smooth-surfaced, with much debris present. Cells treated with 200/M FPL52806 were similar (Figures 32 and 33) but some cells had the processes and irregular surface of non-compound treated cells (Figure 34). The presence of FPL52806 at concentrations of 100 and 150/M resulted in some cell death (Figures 35 and 36), but most cells had the irregular surface and processes of the drug free control cells (Figures 37 and 38). The smooth-surfaced cells and debris were absent from the 50/M FPL52806-treated samples (Figures 39 and 40), indicating little toxicity.

Cells from a non-stimulated and FPL52906-free culture are shown in Figure 41. These have the wrinkled irregular surface of stimulated cells (Figures 28-30) with processes attaching the cells to the surface of the coverslip, though the processes may be fewer in number than those of stimulated cells.

The preparation of the cells for examination under the scanning electron microscope, which entails many washes, may selectively alter the relative proportions of cell types (wrinkled-irregular, and smooth surfaced) present in the final preparation relative to that in the cell culture tube. Some degree of selectivity may also be subjectively introduced at the time of examination under the microscope and choice of cells to be photographed.
Figure 28. Scanning Electron Micrograph of PHA-Stimulated Guinea-Pig Lymph Node Cells at 40 Hours:
Magnification = 2100

Figure 29. Scanning Electron Micrograph of PHA-Stimulated Guinea-Pig Lymph Node Cells at 40 Hours:
Magnification = 19000
Figure 30. Scanning Electron Micrograph of PHA-Stimulated Guinea-Pig Lymph Node Cells at 40 Hours:
Magnification = 19000

Figure 31. Scanning Electron Micrograph of 300μM FPL 52806 - Treated PHA-Stimulated Guinea-Pig Lymph Node Cells at 40 Hours:
Magnification = 9500
Figure 32. Scanning Electron Micrograph of 200μM FPL 52806 - Treated PHA-Stimulated Guinea-Pig Lymph Node Cells at 40 Hours:
Magnification = 9500

Figure 33. Scanning Electron Micrograph of 200μM FPL 52806 - Treated PHA-Stimulated Guinea-Pig Lymph Node Cells at 40 Hours:
Magnification = 9500
Figure 35. Scanning Electron Micrograph of 150μM FPL 52806 - Treated PHA-Stimulated Guinea-Pig Lymph Node Cells at 40 Hours:
Magnification = 2050
Figure 37. Scanning Electron Micrograph of 150μM FPL 52806 - Treated PHA-Stimulated Guinea-Pig Lymph Node Cells at 40 Hours:
Magnification = 2050
Figure 39. Scanning Electron Micrograph of 50uM FPL 52806 - Treated PHA-Stimulated Guinea-Pig Lymph Node Cells at 40 Hours:
Magnification = 2050
Figure 40. Scanning Electron Micrograph of 50μM FPL 52806 - Treated PHA-Stimulated Guinea-Pig Lymph Node Cells at 40 Hours:
Magnification = 1000

Figure 41. Scanning Electron Micrograph of Non-Stimulated Guinea-Pig Lymph Node Cells at 40 Hours:
Magnification = 19000
Accepting these reservations the scanning electron micrographs indicate that, though high FPL52806 concentration changed the appearance of the majority of the cells (presumably by killing them), lower concentrations, which inhibited synthetic processes by at least 50%, changed the appearance of only a small proportion of the cells. This supports the conclusion from the eosin-exclusion experiments that the effect of the compound on the synthetic processes was not solely due to its toxic action.

Attempts were not made to quantitate the cytotoxicity caused by increasing concentration of 6,8-di-t-butyl chromone, however, when the practical difficulties and variability of the technique were appreciated. For example the preparations from the cells treated with 200 and 300\mu M compound chromone contained very few cells and little cell debris compared with preparations treated with lower chromone concentrations. The chromone not only killed the cells but also affected the number of cells and amount of cell debris which adhered to the coverslips and was carried through to final preparations.

Polliack et al (1973) used critical point drying for the preparation of their material for scanning electron microscopy. Previous workers (Boyde et al, 1972) used air drying, as was used in the present work. Polliack et al state that the air drying causes distortion of material and is unsatisfactory. It is interesting that the appearance of the cells they observed, smooth but slightly irregular cells, (and considered to be T lymphocytes), was very similar to the appearance of cells observed in the present work. Guinea-pig lymph nodes are many T lymphocytes. None of the cells in the present work was similar in appearance to the B lymphocytes described by Polliack et al; however, the absence of these may reflect the deficiencies of air drying for the preservation of delicate details, rather than the total absence of B lymphocytes from guinea-pig lymph node cell cultures after 48h. incubation.
Established anti-inflammatory drugs and candidate compounds which inhibited the synthetic activities of PHA-stimulated guinea-pig lymph node cells were cytotoxic to the cells, but the concentrations causing complete cell death were generally at least 2-fold higher than the concentrations inhibiting \( [3^\text{H}] \)-thymidine incorporation by 50% (Table 23). The concentrations of 6,8-di-alkyl chromones which caused 50% loss of cell viability were 2 to 3-fold higher than the concentrations which inhibited the incorporation of \( [3^\text{H}] \)-thymidine by 50% (Table 24). The 6,8-di-t-butyl chromone (FPL52806) reduced the number of morphologically transformed cells to a greater extent than the compound reduced total cell viability (Figure 27).

Scanning electron microscope examination of FPL52806-treated cells (which allowed viable and dead cells to be differentiated) supported the view that appreciable loss of cell viability occurred at compound concentrations appreciably higher than those found to inhibit incorporation of \( [3^\text{H}] \)-thymidine by 50%.

It would seem that, though the compounds studied were cytotoxic, the effects on the synthetic activities of PHA-stimulated lymph node cells of the compounds, particularly FPL52806, cannot be explained solely in terms of cytotoxicity.
CHAPTER SIX

THE RELATIONSHIP BETWEEN THE MOLECULAR PROPERTIES
OF COMPOUNDS AND THEIR PHARMACOLOGIC ACTIVITIES
AND DISCUSSION OF THE RELEVANCE OF THESE Activities
TO THEIR THERAPEUTIC ACTION
6.1 GENERAL INTRODUCTION

In Chapter 3 the inhibitory effects of established anti-inflammatory drugs and chromones on the incorporation of precursors of some of the major biological macromolecules by PHA-stimulated guinea-pig lymph node cells was described.

In Chapter 4 it was shown that the potency of certain chromones could be related to their lipophilicity and that the extent to which these compounds bind to serum protein was also related to their lipophilicity. The amount of one compound (6,8-di-t-butyl chromone) which was associated with the lymph node cells in the presence of serum was shown to be related to the free non-serum bound drug.

In Chapter 5 the effect of anti-inflammatory drugs and chromones on the viability, and for one compound the morphology, of lymph node cells was described. The properties of compounds have been studied at the molecular level (Chapter 4), at the biochemical pharmacology level (Chapter 3) and at the level of gross morphology (Chapter 5). The relationships between these properties will be discussed here and the thesis developed that the molecular property of lipophilicity of some of the compounds tested (i.e. the 6,8-di-alkyl chromones) controls the biochemical pharmacology and gross morphology effects of the compounds. The effect of anti-inflammatory drugs and chromones on other in-vitro tests and animal model tests (studied by colleagues at Fisons Ltd., Pharmaceutical Division) will be briefly described and the similarities and differences between the results of these tests and results obtained by myself using the lymph node cell culture systems discussed. Finally the value of the lymph node cell culture systems for detection of therapeutic activities will be considered.
The inhibitory effects of all the anti-inflammatory drugs and candidate compounds (chromones) tested on the synthetic processes of PHA-stimulated guinea-pig lymph node cells are summarized in Tables 25 and 26. The concentrations of compound at which appreciable toxicity to the cells was seen are shown, and in some cases the compound concentration causing 50% loss of cell viability.

The 6,8-di-t-butyl chromone, FPL52806, was studied in greater detail than any of the other compounds. For example, the number of cells transforming in the presence of this drug were determined. In Figure 42 the dose-response curves of the effect of FPL52806 on synthetic processes and also morphological parameters are shown. The slopes of the dose-response curves of inhibition of the rate of incorporation of \[ ^3H \]-thymidine, \[^{14}C \]-leucine and \[^3H \]-glucosamine at 4Oh., and \[^3H \]-uridine at 1 and 20h, and also the loss of cell viability and reduction in the number of transforming cells at 40h, were all similar. These similar slopes indicate that the effect of the compound on these parameters may involve a common mechanism. There is no doubt that the compound was toxic to lymph node cells (measured by the loss of cell viability), but appreciable inhibition of \[^3H \]-thymidine and \[^3H \]-glucosamine incorporation occurred at FPL52806 concentrations which caused little loss of cell viability. The effect of FPL52806 on these synthetic parameters cannot be explained solely by the toxic activity of the drug.

The relative sensitivities to FPL52806 of the various parameters measured was \[^3H \]-glucosamine incorporation \( \approx \) \[^3H \]-thymidine incorporation \( > \) \[^{14}C \]-leucine incorporation \( \approx \) reduction in the number of transformed cells \( > \) reduction in cell viability \( \approx \) \[^3H \]-uridine incorporation at 20h. \( > \) \[^3H \]-uridine incorporation at 1h., in order of declining sensitivity. An identical order of sensitivity was seen
<table>
<thead>
<tr>
<th>Compound Concentration</th>
<th>Incorporation of ([^3]H)-Thymidine by Guinea-Pig Lymph Node Cells</th>
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<tr>
<td></td>
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<td></td>
</tr>
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<td>&gt; 1000.00</td>
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</tr>
</tbody>
</table>

**TABLE 25.** Summary of the Effect of Compounds on Incorporation of \([^3]H\)-Thymidine by Guinea-Pig Lymph Node Cells

*By 50%, ID50%

% viability at 48h, to greater than 90%

Compound concn, that caused loss of cell

Concn, which inhibited the rate of
<table>
<thead>
<tr>
<th>Concentration</th>
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<th>3.0</th>
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<tr>
<td>50</td>
<td>95</td>
<td>60</td>
<td>0</td>
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<td>74</td>
<td>51</td>
<td>2</td>
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<td>69</td>
<td>57</td>
<td>0.800</td>
<td>5.6806</td>
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<td>250</td>
<td>77</td>
<td>70</td>
<td>0.1600</td>
<td>5.7131</td>
</tr>
<tr>
<td>300</td>
<td>66</td>
<td>60</td>
<td>0.1500</td>
<td>5.7569</td>
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<td>350</td>
<td>70</td>
<td>60</td>
<td>0.1600</td>
<td>5.7585</td>
</tr>
<tr>
<td>400</td>
<td>66</td>
<td>60</td>
<td>0.1600</td>
<td>5.7589</td>
</tr>
<tr>
<td>450</td>
<td>70</td>
<td>60</td>
<td>0.1600</td>
<td>5.7595</td>
</tr>
<tr>
<td>500</td>
<td>66</td>
<td>60</td>
<td>0.1600</td>
<td>5.7599</td>
</tr>
</tbody>
</table>

**TABLE 26**

**Summary of the Effect of Compound on the Synthetic Activity and Morphology of PHA-Stimulated Guinea-Pig Lymph Node Cells**

- **Compound**: The compound that caused the initiation of the rate of incorporation by PHA-stimulated Guinea-pig lymph node cells
- **Concentration**: The concentration at which 50% inhibition occurred
- **Inhibition Rate**: The percentage of cell viability at the compound concentration that caused the initiation of the rate of incorporation

The table above shows the effect of various concentrations of a compound on the synthetic activity and morphological changes of PHA-stimulated Guinea-pig lymph node cells. The inhibition rate is calculated as the percentage of cell viability at the compound concentration that caused the initiation of the rate of incorporation.
Figure 42. Effect of FPL 52806 on the Synthetic Activity and Morphology of PHA-Stimulated Guinea-Pig Lymph Node Cells.
for the other compounds (Table 26). For example the ID$_{50\%}$ of $[^{1}H]^{-}$leucine incorporation was always higher than that of $[^{3}H]^{-}$thymidine incorporation, and the compound concentration causing 50% loss of cell viability was even higher (220/$\mu$M, 450/$\mu$M and 820/$\mu$M in the case of FPL55689, 6,8-di-ethyl chromone).

The differing sensitivities of $[^{3}H]^{-}$uridine incorporation to FPL52806, 6,8-di-t-butyl chromone, at 1 and 20h., may be explained by supposing that a gradual uptake of the compound by the cells takes place, which is still occurring at 1h., hence the requirement of a higher FPL52806 concentration for 50% inhibition to be achieved at 1h. then at 20h., the intra-cellular FPL52806 concentration which caused 50% inhibition being identical at both times. This explanation is supported by experiment, in which it was shown that the longer the cells had been exposed to a fixed concentration of FPL52806 the greater the inhibition of $[^{3}H]^{-}$thymidine and $[^{14}C]^{-}$leucine incorporation. This theory of a gradual uptake of compound by the cells is unlikely however to explain the different sensitivities of $[^{3}H]^{-}$thymidine and $[^{14}C]^{-}$leucine incorporation to compound at 40h., unless it is further suggested that these synthetic processes occur in different intra-cellular compartments and that the compartment in which protein synthesis occurs, the endoplasmic reticulum, contains less compound than the compartment where $[^{3}H]^{-}$-thymidine incorporation occurs, the nucleus. A simpler explanation for the difference in compound potencies as inhibitors of $[^{3}H]^{-}$thymidine and $[^{14}C]^{-}$leucine incorporation is that $[^{3}H]^{-}$-thymidine incorporation is more susceptible to inhibition than the incorporation of $[^{14}C]^{-}$leucine.

The reduction in the number of transforming (or transformed) cells occurred, in the case of FPL52806, at compound concentrations when all the synthetic processes measured at 40h. were inhibited to an appreciable degree. At higher compound concentrations the inhibition of the synthetic processes at 40h. approached 100% and the loss of cell viability increased.
It is suggested that the parameters of synthesis and morphology represent a continuum and that they possess differing susceptibilities to inhibition by compounds. The incorporation of $[^3H]$-thymidine and $[^3H]$-glucosamine are the most sensitive parameters. Higher compound concentrations progressively affect $[^1H]$-leucine and $[^3H]$-uridine incorporation. Reduction in the rates of these synthetic activities corresponds to a decrease in the number of lymph node cells which are transformed in the presence of PHA. The correspondence between the inhibition curves for $[^1H]$-leucine incorporation and the number of transformed cells by FPL52806 is close. This suggests that measurement of $[^1H]$-leucine incorporation is the best synthetic parameter for assessing lymphocyte transformation. At even higher compound concentration the number of viable cells decreases due to large inhibition of the synthetic processes. The morphological changes result from the biochemical effects of the compounds on the synthetic processes.
6.3 RELATIONSHIP BETWEEN THE LIPOPHILICITY OF 6,8-DI-ALKYL CHROMONES AND THEIR POTENCIES AS INHIBITORS OF DNA AND PROTEIN SYNTHESIS

In Chapter 4 measurement of the apparent partition coefficient of 6,8-di-alkyl substituted chromones was described. Tables of the contribution which various chemical groups make to the total lipophilicity of a molecule have been published (Hansch, 1972). It is therefore possible to calculate the contribution which the various alkyl substituents make to the total lipophilicity of the 6,8-di-alkyl substituted chromones, and knowing also the lipophilicity of the chromone nucleus, to calculate theoretical partition coefficients. These are used to plot structure-activity relationships in Figure 43. Figure 43 is Figure 24 re-plotted using calculated partition values for the respective 6,8-di-alkyl substituted chromones, instead of experimentally measured partition coefficients. The use of calculated partition data has little effect on the form of the curve, when compared with Figure 24, Page 131.

It was shown (Table 20) that much of the chromone added to the lymph node cell cultures would have been bound to serum proteins. Protein-bound compound is believed to be pharmacologically inactive. In confirmation it was shown that the $[^3\text{H}]$-6,8-di-t-butyl chromone associated with the lymph node cells (and presumably within the cells and pharmacologically active) is related to the free compound concentration rather than to the total compound concentration. The ID$_{50\%}$ for $[^3\text{H}]$-thymidine and $[^1\text{C}]$-leucine incorporation were therefore corrected for proportion of added chromone which was bound to serum protein, Table 20. The resulting potencies, ID$_{50\%}$ free compound, and the potencies of total added compound (Figure 43) are shown as the logarithm of their reciprocal plotted against the logarithm of the calculated theoretical partition coefficient in Figure 44.
Figure 43. Relationship between the Reciprocal of the Potencies (ID$_{50}$) of 6,8-Di-Alkyl Chromones as Inhibitors of $[^3\text{H}]$-Thymidine and $[^{14}\text{C}]$-Leucine Incorporation and the Calculated Partition Coefficients of the Compound.

- FPL 55585
- FPL 55689
- FPL 55731
- FPL 52806
- FPL 56220
Figure 44. Relationship between the Potencies ($\text{ID}_{50\%}$) of 6,8-Di-Alkyl Chromones, Expressed as Free Non-Protein Bound and Total Compound, as Inhibitors of $[^3\text{H}]-\text{Thymidine}$ and $[^{14}\text{C}]-\text{Leucine}$ Incorporation and the Calculated Partition Coefficients of the Compounds.

- $[^3\text{H}]-\text{Thymidine}$, free compound
- $[^{14}\text{C}]-\text{Leucine}$, free compound
- $[^3\text{H}]-\text{Thymidine}$ total compound
- $[^{14}\text{C}]-\text{Leucine}$ total compound

Calculated Partition Coefficient, $P^0$
The definite curves found when the compound potencies were plotted using the total compound concentration were converted to curves, which approximated to straight lines, over the range methyl to t-butyl substituents, when the free compound concentrations were used. This shows that the potency of the free 6,8-di-alkyl chromones was closely related to their lipophilicity.

Hansch (1969) described general equations relating the potency of compounds in a biological test system to their physical properties, such as partition coefficient. An example of such is:

\[ \log \frac{1}{ID} = k_1 \log P^0 - k_2 (\log P^0)^2 + k_3 + k_4 \]

where \(k_1, k_2, k_3\) and \(k_4\) are constants

\(ID\) is the concentration of compound causing a given response, for example, 50% inhibition.

\(P^0\) is the partition coefficient calculated theoretically and is Hammett's electronic factor.

The data for the free compound illustrated in Figure 44 was fitted to this equation by Mr. M.T. Stevens, Statistics Department, Fisons Ltd. It was assumed that the term involving Hammett's electronic factor, \(\sigma\), made no contribution, since it is known that alkyl substituents have negligible effect on \(\sigma\) (Hansch, 1972). The \(\sigma\) values for the 6,8-di-alkyl chromones were also not known.

The derived equation for inhibition of \([^{14}C]\)-leucine incorporation was:

\[ \log \frac{1}{ID_{50\% \text{ free compound}}} = 0.825 \log P^0 - 0.042 (\log P^0)^2 - 3.99 \]

\(n = 5, \ r = 0.999\)

and that for inhibition of \([^{3}H]\)-thymidine incorporation:

\[ \log \frac{1}{ID_{50\% \text{ free compound}}} = 0.834 \log P^0 - 0.049 (\log P^0)^2 - 3.62 \]

\(n = 5, \ r = 0.998\)
If Hammett's σ-factor made any contribution this is included in the final term of the equations. The correlation coefficients (0.998) indicate that Hammett's σ-factor did not make a varying contribution and that the activity of the free non-protein bound 6,8-di-alkyl chromones as inhibitors of the rates of $\left[^{14}C\right]$-leucine and $\left[^{3}H\right]$-thymidine incorporation by PHA-stimulated guinea-pig lymph node cells can be completely described in terms of lipophilicity. Since n is small (n=5) this conclusion must be treated with caution, but there can be no doubt that lipophilicity is the dominant molecular property of the 6,8-di-alkyl chromones that determines their potencies, in terms of free non-protein bound compound, in the lymph node cell systems.

The curved form of the structure-activity relationship using the potencies of the 6,8-di-alkyl substituted chromones expressed as the added chromone concentration, (Figure 43), can also be understood in terms of lipophilicity. It has been shown that the extent to which the substituted chromones are bound to serum protein is related to their lipophilicity to some extent (Figure 25). As the intrinsic activity of the chromone increases with increasing lipophilicity, the proportion of the chromone which is bound to serum proteins increases, so that the observed activity of the added chromone will not increase as rapidly as its intrinsic activity. The structure-activity data for the potencies of 6,8-di-alkyl chromones as inhibitors of $\left[^{14}C\right]$-leucine and $\left[^{3}H\right]$-thymidine incorporation in terms of the total added drug concentration was analysed therefore in a similar way to that for the free compound potencies. The following equation for inhibition of the rate of $\left[^{14}C\right]$-leucine incorporation was obtained:

$$\log \frac{1}{ID_{50\%} \text{ total compound}} = 0.974 \log P^0 - 0.097 (\log P^0)^2 - 4.31$$

n = 5, r = 0.998
and for $[^3\text{H}]$-thymidine incorporation:

\[
\log \frac{1}{\text{ID}_{50\%} \text{ total compound}} = 0.770 \log P^0 - 0.070 (\log P^0)^2 - 3.78
\]

\[n = 5, r = 0.976\]

The magnitude of the constants in the term involving $(\log P^0)^2$ is greater than those in the equations for the free compound potencies and this reflects the importance of protein binding (which is dependent to some extent on lipophilicity), when the potencies of the 6,8-di-alkyl chromones is considered in terms of the total added compound concentration. The high correlation coefficients (>0.97) indicate that the potency of the compounds expressed as the total added compound concentration is also controlled dominantly by the physical property of lipophilicity.

Similar structure-activity equations were derived involving the experimentally measured apparent partition coefficients, $P'$ (Table 18). These were for inhibition of the rate of $[^{14}\text{C}]$-leucine incorporation:

\[
\log \frac{1}{\text{ID}_{50\%} \text{ free drug}} = 0.636 \log P' - 0.536 (\log P')^2 - 1.18
\]

\[n = 5, r = 0.994\]

\[
\log \frac{1}{\text{ID}_{50\%} \text{ total drug}} = 0.244 \log P' - 0.592 (\log P')^2 - 1.82
\]

\[n = 5, r = 0.990\]

and for inhibition of $[^3\text{H}]$-thymidine incorporation:

\[
\log \frac{1}{\text{ID}_{50\%} \text{ free drug}} = 0.595 \log P' - 0.495 (\log P')^2 - 0.910
\]

\[n = 5, r = 0.982\]

\[
\log \frac{1}{\text{ID}_{50\%} \text{ total drug}} = 0.234 \log P' - 0.518 (\log P')^2 - 1.66
\]

\[n = 5, r = 0.982\]

The correlation coefficients show that the lipophilicity of the 6,8-di-alkyl chromones, determined experimentally by
the apparent partition coefficient, is dominant in determining the potencies of the compounds.

The form of the Hansch equation, which the correlation coefficients show to be a good model describing the relationships between the potency of 6,8-di-alkyl chromones as inhibitors of $^{14}$C-leucine and $^{3}$H-thymidine incorporation and their lipophilicities, dictates a bell-shaped structure-activity curve. It is possible to calculate the lipophilicity corresponding to the inflection point of this bell-shaped curve ($\log P = -k_1$) and hence to identify the compound having this lipophilicity which will be the most potent inhibitor of $^{14}$C-leucine and $^{3}$H-thymidine incorporation in the lymph node cell system. The values for lipophilicity derived from the structure-activity curves involving the experimentally measured apparent partition coefficients were 0.593 and 0.206 ($^{14}$C-leucine incorporation, free and total compound) and 0.601 and 0.226 ($^{3}$H-thymidine incorporation, free and total compound). The partition coefficients derived from these lipophilicities (lipophilicity = $\log P'$) were 3.92 ($^{14}$C-leucine, free drug), 1.61 ($^{14}$C-leucine, total drug), 3.99 ($^{3}$H-thymidine, free drug) and 1.68 ($^{3}$H-thymidine, total drug) respectively.

These partition coefficients may be compared with the experimentally measured apparent partition coefficients of the 6,8-di-alkyl chromones (Table 18). The compound which is theoretically the most potent inhibitor of the rates of $^{14}$C-leucine and $^{3}$H-thymidine incorporation in terms of the total compound would have an experimentally measured apparent partition coefficient (1.61 and 1.68 respectively) which is intermediate between that of the 6,8-di-t-butyl chromone (C.70) and that of the 6,8-di-t-pentyl chromone (4.61). The 6-t-butyl, 8-t-pentyl chromone would have a partition coefficient similar to this theoretical value and should be more potent in terms of total drug as an inhibitor of $^{14}$C-leucine and $^{3}$H-thymidine incorporation than either of the di-t-butyl or di-t-pentyl substituted chromones.
This asymmetrically substituted chromone was not available for testing so that the theoretical activity of the compound could not be confirmed.

In terms of free non-protein bound compound the apparent partition coefficient of the theoretically most potent compound is 3.92 for $[^{14}C]$-leucine incorporation and 3.99 for $[^{3}H]$-thymidine incorporation. The values are very similar to the apparent partition coefficient of the 6,8-di-t-pentyl chromone (4.61), and indicate that this compound corresponds closely to the theoretical compound which would be the most potent inhibitor of $[^{14}C]$-leucine and $[^{3}H]$-thymidine incorporation in terms of the free non-protein bound compound.

The effect of the homologous series of 6,8-di-alkyl substituted chromones on $[^{3}H]$-thymidine and $[^{14}C]$-leucine incorporation by PHA-stimulated guinea-pig lymph node cells can be explained in terms of the molecular property of lipophilicity. It cannot be assumed that the effect of other compounds, of disparate structure and differing ability to bind to serum proteins, on the synthetic activities of lymph node cells can be explained similarly. However the dose-response curves for inhibition of, for example, $[^{3}H]$-thymidine incorporation, by most of the compounds tested were, with the exception of chloroquine and prednisolone, of similar slope to those of the 6,8-di-alkyl substituted chromones, indicating a similar mechanism of action. Furthermore, most of the compounds tested (established anti-inflammatory drugs and chromone compounds) were monobasic acids, and many are known to be bound to serum proteins to some extent. These similarities suggest that the potencies of the majority of the compounds tested could be related to their lipophilicities, in a similar manner to that shown for the 6,8-di-alkyl chromones.
Forbes and Smith (1967) reported that anti-inflammatory drugs inhibited the rate of $[^{14}\text{C}]$-leucine incorporation by human lymphocytes \textit{in vitro}. Forbes and Smith suggested that the study of the effect of compounds on the rates of $[^{14}\text{C}]$-leucine incorporation by human lymphocytes might be a useful \textit{in vitro} screening system for the detection of anti-inflammatory drugs. The number of \textit{in vitro} systems in which known anti-inflammatory drugs have an effect is large (Hichens, 1974), and many of these have been suggested to be valuable as, or indeed have been used as, screening systems. Examples of such systems are heat denaturation of protein, Mizushima Test (Mizushima, 1964), the reaction between trinitrobenzaldehyde and albumin (Skidmore and Whitehouse, 1966) and sulphydryl exchange reaction (Gerber et al., 1967), hypotonic erythrocyte haemolysis (Inglot and Wolna, 1968), platelet aggregation (O'Brien, 1968) and stabilization of isolated lysosomes (Ignarro, 1972). Additional systems are the incorporation of $[^{35}\text{S}]$-sulphate by connective tissue (Brostrom et al., 1964), histidine decarboxylase (Skidmore and Whitehouse, 1966) and prostaglandin synthetase (Vane, 1971).

The rationale for many of the anti-inflammatory drugs screens listed above is mainly that the drugs at present used in the clinic for treatment of inflammation are active in the particular test. The connection between these tests and the processes occurring during inflammation, especially chronic inflammation, is extremely tenuous (for example, inhibition of histidine decarboxylase) or is non-existent (heat denaturation of protein). For other tests a stronger connection can be argued, for example soluble lysosomal enzymes are present at inflammatory sites.
(Caygill and Pitkeathly, 1966), and can cause tissue breakdown and inflammation (Weissmann et al., 1969). The lysosome membrane stabilization test can therefore be justified as a model of a process which occurs in inflammation. The inflammatory process in, for example, rheumatoid arthritis, is incompletely understood. Hence the importance of lysosome-mediated tissue damage, compared with that of other inflammatory processes is not yet known, although it is believed to be considerable. It is therefore difficult to assess the value of lysosome membrane stabilization by a drug as an indicator of the drug's potency as an inhibitor of inflammation in man. Similar doubts and reservations apply to the other in vitro screens for anti-inflammatory drugs.

The attraction to the pharmaceutical industry of in vitro systems is the ease and speed with which they can be performed. The small amount of compound required is also important, in contrast to in vivo tests, such as rat adjuvant arthritis (Newbould, 1963) which need large quantities of compounds and require much man-power, animal holding space and large numbers of animals.

Many in vivo animal models have been used in the search for anti-inflammatory drugs. These can be divided into two groups: the models of acute inflammation and those which mimic chronic inflammation. The short-lived inflammation induced in the paws of rats by injection of carrageenin (Winter et al., 1962) or kaolin (Hillebrecht, 1954) or the erythema caused on the shaved backs of guinea-pigs by ultraviolet light (Wilhelmi, 1950), are examples of the first group. The carrageenin oedema test is probably the most commonly used. These animal tests lack the chronic and progressive nature which is found in many human inflammatory conditions, for example rheumatoid arthritis.

In answer to the criticism that the chronic tests listed were not good models for persistent human inflammation, longer-term animal models were developed. Examples of these
chronic tests are rat adjuvant arthritis (Newbould, 1963) and rabbit immune synovitis (Wepsic and Hollingworth, 1968). These two animal models involve immune processes which may be important in human rheumatoid arthritis (Messner, 1974). Rat adjuvant arthritis is the most commonly used model of chronic inflammation. Evidence is available that Type 4 immune reactivity is involved in this model (Whitehouse and Whitehouse, 1968).

Differences in the metabolism of drugs in laboratory animals and man may affect the interpretation of drug activities obtained in animal models and the relevance of these results to man. It is known, for example, that phenylbutazone has an unusually long half-life in man compared to the rat ($T_1$, rat = 6h., $T_1$, man = 72h., Burns et al., 1953) and this may increase the potency of phenylbutazone in man compared to that in the rat. In vitro models of inflammation often involve considerable pain to the animals used and are unattractive to many because of this.

In summary, both in vitro and in vivo screens for anti-inflammatory activity have disadvantages. There is no single test which is completely satisfactory. It is possible that the simultaneous use of a battery of these tests and interpretation of the results together may eliminate the weaknesses of a given test and be the best method for the detection of new compounds.

The initial objectives of the work described in this thesis was to confirm that anti-inflammatory drugs do affect the synthetic activities of guinea-pig lymph node cells in vitro and to validate the system as a screen for the detection of anti-inflammatory drugs. To validate a new screening system it is necessary to show that the order of drug potency in the screen is similar to that of the therapeutic potencies of the drugs in the human disease and less importantly in other screening procedures which are accepted to be of value and are in common use.
The best validation of a screening test is the detection of a novel compound which is found later to be therapeutically active in man. The number of screens for anti-inflammatory drugs, of the many mentioned in the literature, that have been validated in this way is small.

It has been suggested by Paulus and Whitehouse (1972) that the current approach to the detection of anti-inflammatory drugs, using the established anti-inflammatory drugs (salicylate, phenylbutazone, indomethacin, etc.) as standards is fundamentally wrong; that only drugs with similar activities to the established drugs will be selected, and that since these drugs only affect the symptom rather than the basic underlying and initiating process of the inflammation, for example in rheumatoid arthritis, effort should be directed towards developing new models of inflammation. These should be more relevant to the initiating and underlying pathology. It is possible however that established drugs will not be active in these new models. Greater understanding of the pathology of inflammation, for example rheumatoid arthritis, is necessary before the new models can be developed.

In the absence of the superior (and at present hypothetical) models described by Paulus and Whitehouse it is necessary, in order to validate the lymph node cell system, to compare the effect of compounds in this system with their effects in the other screening systems which are at present in use. In the following sections the inhibitory effects of standard anti-inflammatory drugs and novel chromone compounds on the synthetic processes of PHA-stimulated guinea-pig lymph node cells are compared with the effects of the compounds on various other in vitro and in vivo test systems.
The sulphydryl-exchange reaction and hypotonic haemolysis of erythrocytes are frequently mentioned in the literature as indicators of anti-inflammatory action. It is known that the SH - SS redox system is changed in certain inflammatory states, for example rat adjuvant arthritis (Butler et al., 1969) and rheumatoid arthritis in man (Chayen and Bitensky, 1971). Sulphydryl group substances, such as cysteine, will inhibit the anti-inflammatory action of indomethacin and acetylsalicylic acid, but a direct and causal relationship between the effect of compounds on the sulphydryl-exchange reaction and their anti-inflammatory action in man cannot yet be justified. The strong correlation between effect of compounds on the sulphydryl-exchange reaction and anti-inflammatory action however, suggests that the sulphydryl-exchange reaction does have some value as a predictive screen for anti-inflammatory action (Grant et al., 1971).

The effect of compounds on hypotonic erythrocyte haemolysis may be related to stabilization of protein (Mizushima and Sakai, 1969) or to their membrane stabilizing properties, for example that of the lysosome. The role of lysosome-mediated damage in inflammation is well established (Chayen and Bitensky, 1971), so it is possible to accept that compounds which do stabilize cell membranes will possess anti-inflammatory action, when lysosome-mediated damage is involved. The membrane stabilizing effect of many compounds is biphasic. At high concentration the compound, for example a steroid, is lytic but at lower concentrations the compound stabilizes the membrane (Weissmann, 1969). This biphasic effect was seen with one of the compounds, chloroquine, tested on erythrocyte haemolysis (Table 27). The sul phydryl-exchange reaction and erythrocyte haemolysis data in Table 27 was determined by other colleagues at Fisons Ltd. I would like to thank particularly Drs. D.H. Hall and J. Mann.
Table 27 shows that there was considerable similarity between the effect of compounds on the synthetic activities of guinea-pig lymph node cells and the sulphydryl-exchange reaction. The order of potency of the acidic non-steroidal anti-inflammatory drugs, sodium salicylate, ibuprofen, phenylbutazone and indomethacin and flufenamic acid, was identical in both tests. Chloroquine was, however, inactive in the sulphydryl-exchange reaction, whereas it was extremely potent as an inhibitor of $^3\text{H}$-thymidine and $^{14}\text{C}$-leucine incorporation by guinea-pig lymph node cells. The anti-allergic compound FPL50670, disodium cromoglycate, was of low potency in both tests. The order of potency of the 6,8-di-alkyl substituted chromones FPL52839, 55585, 55689, 52806 and 56220 (hydrogen, methyl, ethyl, n-propyl, t-butyl and t-pentyl respectively) was broadly similar in the lymph node cell systems and sulphydryl-exchange reaction, though the di-t-pentyl substituted chromone (FPL52806) was less active in the sulphydryl-exchange reaction than the di-t-butyl substituted chromone (FPL52806). The reverse was the case with the lymph node cell systems, the di-t-pentyl substituted chromone was more active than the di-t-butyl substituted chromone. The activity of the other chromones FPL56112, 55618, 56294, 55990 and 52816 of miscellaneous substitute grouping and position of substituents tested in the lymph node systems did not correspond markedly with their activity in the sulphydryl-exchange reaction.

The activity of 6,8-di-alkyl chromones and established anti-inflammatory drug in the two tests agreed well, with the exception of chloroquine which was inactive in the sulphydryl-exchange reaction. Chloroquine is an anti-rheumatic anti-inflammatory agent (Duthrie, 1971) and this suggests that the lymph node cell system is better than the sulphydryl-exchange reaction at detecting chloroquine-like activity.

The results of a further in vitro test, the hypotonic erythrocyte haemolysis test, using 10, 100 and 1000μM compound are shown also in Table 27. The acidic non-steroidal
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**Means inactive - means compound was hemolytic**
<table>
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50% ID50 found by extrapolation of lines at several concentrations.
anti-inflammatory drugs had a similar order of potency in the lymph node cell systems and erythrocyte haemolysis tests, though ibuprofen was more active than phenylbutazone in the erythrocyte haemolysis in contrast to the reverse order with the lymph node cell systems. Both prednisolone and chloroquine were active in the erythrocyte haemolysis test, but while prednisolone was very potent in both the erythrocyte haemolysis tests and lymph node cell systems the potency of chloroquine was weak in erythrocyte haemolysis in contrast to its very great potency in the lymph node cell systems.

The order of activities of the 6,8-di-alkyl substituted chromones was very similar in both the erythrocyte haemolysis and lymph node cell systems, the larger the size of the substituents the more potent the compound was as an inhibitor of the synthetic processes of lymph node cells, or as an inhibitor of erythrocyte haemolysis. FPL50670, disodium cromoglycate, was of low potency in both the lymph node and erythrocyte haemolysis systems. The activity of other chromones tested in the erythrocyte haemolysis system was similar to their order of activity in the lymph node system, for example FPL56112, 55618 and 55990 (100μM) caused 5, 17 and 60% inhibition of haemolysis and their potencies, as the compound concentration causing 50% inhibition of the rate of $[^3H]$-thymidine incorporation in the lymph node cell system, was 400, 200 and 70μM respectively.

The results presented in Table 27 show considerable similarities between the three in vitro test systems. The lipophilicity of the 6,8-di-alkyl substituted chromone controls their potencies in the lymph node cell system. It is suggested that lipophilicity has a similar importance in the sulphydryl-exchange and erythrocyte haemolysis tests, at least for the 6,8-di-alkyl substituted chromones. The low potency of chloroquine in the sulphydryl-exchange and erythrocyte haemolysis tests contrasts greatly with the very high potency of the compound in the lymph node cell system. This suggests that the lymph node cell system is
a better screen for anti-rheumatic anti-inflammatory compounds than the sulphydryl-exchange or erythrocyte haemolysis tests.

6.4.2 Effect Of Compounds On DNA And Protein Synthesis By PHA-Stimulated Guinea-Pig Lymph Node Cells, Rat Adjuvant Arthritis, Rat Carrageenin Oedema and Rat Passive Cutaneous Anaphylaxis

In the previous section the similarities of the effects of established anti-inflammatory drugs and novel chromones on the rates of $[^3H]$-thymidine and $[^14C]$-leucine incorporation by PHA-stimulated lymph node cells, and their effects on two in vitro screens for anti-inflammatory activity, were described. The agreements between the three in vitro tests was not complete, however; the sulphydryl-exchange and erythrocyte haemolysis tests underestimated the potency of the anti-rheumatic anti-malarial compound chloroquine compared with its high potency in the lymph node cell system. In this section the effects of compounds on the lymph node cell systems and the effect of some of the compounds on two in vivo anti-inflammatory screens, rat adjuvant arthritis (Newbould, 1963) and rat carrageenin oedema (Winter, 1962), are compared (Table 28). The effect of chromones on a test for anti-allergic activity, the rat intravenous passive cutaneous anaphylaxis test, PCA, (Goose and Blair, 1968), is also shown in Table 28. The rat adjuvant arthritis, carrageenin oedema, and rat passive cutaneous anaphylaxis data in Table 28 was determined by other colleagues at Fisons Ltd. I would like to thank especially Mr. H. Radzivonik and Mr. H. Cairns.

The order of potency of the acidic non-steroidal anti-inflammatory agents ibuprofen, phenylbutazone and flufenamic acid was similar in the lymph node cell system and the rat adjuvant arthritis test. Prednisolone was more active than these three acidic non-steroidal anti-inflammatory drugs in both tests, but the potency
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**Hypotheses:**
- Outcomes: Cytotoxic Activity of PHA-stimulated
- In vitro: Inhibition of DNA synthesis
- In vivo: Induction of lymphocytes

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**Results:**
- The effect of anti-interferon therapy on the synthesis of interferon activity was studied.
- The interferon concentrations were measured at various times post-treatment.
- The data shows a significant increase in interferon activity over time.

---

**Conclusion:**
- Anti-interferon therapy significantly enhances interferon activity in vitro.
- Future studies are needed to investigate the clinical implications of these findings.
difference was greater in the lymph node cell test to that in the adjuvant arthritis test. Chloroquine was active in both tests, but it was less active in adjuvant arthritis than ibuprofen, whereas it was the most active anti-inflammatory agent in the lymph node cell test. A similar difference was seen in the activity of indomethacin, which was the most active compound tested in the rat adjuvant arthritis test, considerably more active than prednisolone, but was only as active as phenylbutazone in the lymph node cell test.

The only chromone, FPL52806, for which a precise result was available, was of very low potency in the rat adjuvant arthritis test, but was more potent than flufenamic acid in the lymph node cell system, though less potent than prednisolone and chloroquine.

In the rat carrageenin oedema test prednisolone was relatively inactive ($ID_{50%} = 170\text{mg/kg}$) compared with its high potency in the lymph node cell system and adjuvant arthritis ($ID_{50%} = 5.2\text{mg/kg}$). Chloroquine was highly active in the carrageenin oedema test ($ID_{50%} = 2.4\text{mg/kg}$) as it was in the lymph node cell systems and in contrast to its low potency in rat adjuvant arthritis ($ID_{50%} = 62\text{mg/kg}$). Indomethacin was very active in the carrageenin oedema test, as it was in adjuvant arthritis, but not in the lymph node system. Sodium salicylate was weakly active in carrageenin oedema and of low potency in the lymph node cell system. It was not tested in adjuvant arthritis.

None of the established anti-inflammatory drugs was tested in the rat passive cutaneous anaphylaxis test. This test detects compounds which inhibit the release of mediators from reagin-sensitized mast cells on exposure to antigen, that is compounds which have anti-Type 1 allergy properties, and is not a test for anti-inflammatory activity. The correspondence between the activity of chromones in this test and their activity in the lymph node cell systems was poor, for example, FPL55618 was a potent inhibitor of the passive cutaneous anaphylaxis reaction ($ID_{50%} = 0.008\text{mg/kg}$).
and FPL56294 was a poor inhibitor (ID$_{50}$ = 10mg/kg), but their relative potencies were reversed in the lymph node cell system with ID$_{50}$s for the inhibition of rates of [${}^{3}H$]-thymidine incorporation, of 200 and 75/min respectively. The activity of the 6,8-di-alkyl substituted chromones in the lymph node cell systems increased with the increasing size of the alkyl substituents. A somewhat similar trend was seen in the passive cutaneous anaphylaxis test, but the di-t-pentyl chromone (FPL56220) was considerably less active than its di-t-butyl analogue (FPL52806). The activities of FPL55689 and 55731 (the di-ethyl and di-n-propyl analogues) were similar in the passive cutaneous anaphylaxis reaction, but their relative potencies (5.6 and 6.4mg/kg) were reversed compared with their potencies in the lymph node cell systems (360 and 77/min) respectively for inhibition of the rate of [${}^{3}H$]-thymidine incorporation.

The increase in the synthetic activities of lymphocytes on exposure to an antigen to which the cells are sensitized is an *in vitro* manifestation of a Type 4 allergic reaction, or cellular immunity. The increased synthetic activity of lymph node cells stimulated with PHA is a similar system. It has been shown that FPL52806 inhibits [${}^{3}H$]-thymidine incorporation in both PHA- and antigen-stimulated cells. The effects of compounds on the rate of incorporation of [${}^{3}H$]-thymidine, etc., by PHA-stimulated lymph node cells is therefore indicative of activity against cellular immune reaction. The poor correspondence between the effect of chromones on the lymph node cell systems and their inhibitory effect in the passive cutaneous anaphylaxis reaction indicates that the lymph node cell system has no predictive value for the identification of anti-type 1 allergy compounds, of the disodium cromoglycate type. This conclusion also indicates that identical mechanisms to those occurring in the mast cell which disodium cromoglycate type compounds inhibit, are not involved in lymphocyte transformation.

The differing rates of absorption, metabolism and elimination of the various compounds tested in rat adjuvant arthritis and carrageenin oedema will affect their observed
potencies compared with their intrinsic potencies. (In the rat passive cutaneous anaphylaxis reaction the compounds were administered intravenously and only 30 mins. elapsed between administration of the compound and sacrifice of the rat. Variation in the rates of absorption of the compound are eliminated and if the excretion and metabolism of the drug in 30 mins. is assumed to be minimal the measured potencies of the compounds should be similar to their intrinsic potencies). In the adjuvant arthritis one oral dose of compound was given per day. If two compounds possessed similar rates of absorption and intrinsic potency, but had different rates of elimination, so that animals treated with one of the compounds had pharmacologically active levels for 24h. and animals treated with the second compound for only 6h., it would be reasonable to expect considerable differences in their observed potencies. Similar difficulties apply to the carrageenin oedema test, especially with regard to the rate of absorption of compound.

It is therefore difficult to compare the potencies of compounds in an in vivo test involving oral absorption and an in vitro test, such as the lymph node cell system, in which the differences in compound absorption into the cells are small compared to those into the animal and elimination of the compound by excretion, at least, is not present. A low observed potency of a compound in adjuvant arthritis may indicate either poor absorption or rapid metabolism and elimination, in addition to low intrinsic potency.

The great difference in the observed potency of indomethacin in the rat adjuvant and carrageenin oedema tests and in the lymph node cell system can be explained to some extent at least by the long half life of indomethacin in the rat and re-absorption of indomethacin excreted in the bile, which result in high plasma indomethacin levels, (Hucker et al, 1966). Prednisolone was very active in the lymph node cell system and adjuvant arthritis but only a weak inhibitor of carrageenin oedema. Chloroquine, in contrast, was very active in the lymph node cell system and carrageenin oedema, but a poor inhibitor of adjuvant arthritis.
Chloroquine accumulates in many tissues, e.g. spleen and liver (Rollo, 1970), and it is possible that the tissue storage sites must be filled before pharmacologically active levels can be achieved at the site of inflammation. This could explain the low potency of chloroquine in the adjuvant arthritis test, but cannot be reconciled with the high potency of chloroquine in the carrageenin oedema test.

The inconsistencies in the activities of chloroquine and prednisolone in the adjuvant arthritis and carrageenin oedema tests suggest that comparison of the activity of compounds in these two tests and the lymph node cell system is not a satisfactory way to validate the lymph node cell system as a screen for anti-inflammatory drugs. The lymph node cell system can be considered to be a superior screening system to either of the in vivo anti-inflammatory screens, for the detection of intrinsic activity since both chloroquine and prednisolone are highly active in the screen. The lymph node cell system does not, however, give any indication of the rates of absorption and elimination of the compounds in vivo.
6.5 RELATIONSHIP BETWEEN THE EFFECT OF DRUGS ON LYMPH NODE CELLS AND THEIR THERAPEUTIC ACTIVITY IN HUMAN DISEASES

In the previous section of this chapter the effect of anti-inflammatory drugs and chromones on the synthetic activities of stimulated guinea-pig lymph node cells were compared with the effect of the compounds on various \textit{in vitro} and \textit{in vivo} systems which are considered to be predictive for anti-inflammatory agents. The lymph node cell system gave orders of drug potencies in the other \textit{in vivo} and \textit{in vitro} tests, with the exception of the potency of chloroquine in some of the tests. In this section the order of potency of anti-inflammatory drugs in the lymph node cell system is compared with the order of potency of these drugs in human inflammatory disease, such as rheumatoid arthritis.

The concentrations of the anti-inflammatory drugs which inhibited the rate of incorporation of $[^{3}H]$-thymidine by stimulated guinea-pig lymph node cells by 50\% and the therapeutic total daily dose of these compounds (with the source of this data) are shown in Table 29. A given dose of drug in man can produce very different therapeutic (or toxic) effects. Man does not behave as consistently as a group of animals in a laboratory test. The cause of inconsistent action in man may be due to different body weights, rates of metabolism and excretion or interaction with other drugs. The stage of the disease also affects the degree of benefit obtained by a given dose of drug, for example 6mg of cortisone is only effective in mild cases of rheumatoid arthritis. Placebo effects may also be important when drugs are used in trials which are not double-blind. It is therefore not surprising that the literature contains many contradictory reports of the therapeutic value of the newer anti-inflammatory drugs when these are compared with
Examples of such contradictory reports for ibuprofen are Jasani et al (1968) and Dornan and Reynolds (1974), who report that the compound was as active as acetylsalicylic acid, and Dick-Smith (1969) and Brooks et al (1970), who report that the compound was less active.

The order of potency of drugs in the lymph node cell system is similar to their order of therapeutic activity in man, with the exception of chloroquine (Table 29). Chloroquine is a slow acting compound in contrast to the other drugs whose effect is rapid. Chloroquine accumulates in the tissue and it has been suggested that its storage sites must be saturated before its beneficial effects are revealed. Chloroquine is also one of the two drugs which have true anti-rheumatic activity (the other is gold) as opposed to the acidic non-steroidal drugs and steroids which only affect the symptoms of rheumatoid arthritis (Duthrie, 1971).

The high figure (250mg) for the therapeutic daily dose of chloroquine is the dose at the start of treatment and the low figure (40mg) that after one year's treatment. If this low figure is the true therapeutic daily dose once the body storage sites are filled, chloroquine is a very potent drug for the treatment of rheumatoid arthritis and the agreement between the potency of drugs in the lymph node cell system and their therapeutic activity is close. The lymph node cell system has predictive value for the detection of drugs useful in the treatment of rheumatoid arthritis.

All of the drugs in Table 29 cause side effects. The acidic non-steroidal anti-inflammatory drugs (sodium salicylate, indomethacin, etc.) and cortico-steroids induce gastro-intestinal bleeding; steroids can also induce sodium retention (oedema) and adrenal insufficiency. Treatment with chloroquine can cause blindness, due to toxic action on the melanin containing cells of the retina, where it accumulates.
<table>
<thead>
<tr>
<th>Concentration (pM)</th>
<th>Rate of Con A-induced Mitogenesis (%)</th>
<th>Rate of Superantigen-induced Mitogenesis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-250</td>
<td>70</td>
<td>70</td>
</tr>
<tr>
<td>250</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>300 - 750</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>1200</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>5000 - 6000</td>
<td>300</td>
<td>300</td>
</tr>
</tbody>
</table>

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- Incorporation of Anti-IL-4 antibody induces reduction in hyperproliferation.
- Incorporation of Anti-IL-4 antibody induces reduction in hyperproliferation.
- Incorporation of Anti-IL-4 antibody induces reduction in hyperproliferation.
It has been suggested that for the acidic non-steroidal anti-inflammatory drug at least, therapeutic potency is paralleled by toxicity, but this has been questioned on the basis of tests in animals by Wilhelm (1974). He summarised his results by the statement 'ulcerogenicity and anti-inflammatory potency need not necessarily parallel each other', which indicates that generally they did. It is possible that the inhibitory effects of drugs on the lymph node cell system is related more to the mechanism of their toxicity than to their therapeutic activities.
All the established anti-inflammatory drugs studied for their effect on $[^{3}H]$-thymidine incorporation in PHA-stimulated guinea-pig lymph node cells were found to be active and the order of their potencies agreed well with the therapeutic doses of the drugs in rheumatoid arthritis (Table 29). The non-anti-inflammatory analogue of sodium salicylate, sodium p-hydroxy benzoic acid, was nearly 5 times less active than sodium salicylate as an inhibitor of $[^{3}H]$-thymidine incorporation by PHA-stimulated lymph node cells (Table 10). The order of potency of established anti-inflammatory drugs as inhibitors of $[^{3}H]$-thymidine incorporation was superior, with respect to the therapeutic doses in rheumatoid arthritis, to the order of potency of the drugs in other tests considered to detect anti-inflammatory agents (Tables 27 and 28). These results indicate that study of the effects of compounds on the synthetic activity of PHA-stimulated guinea-pig lymph node cells is a useful screen for anti-inflammatory agents (including acidic non-steroidal compounds, steroids, and chloroquine-like agents) and supports the belief of Forbes and Smith (1967) that lymphocytes are a suitable system for the detection of anti-inflammatory agents.

Advantages of the lymph node cell system compared to other screens for anti-inflammatory agents, such as rat adjuvant arthritis, include the small amount of compound required to assess the activity of the compound (this is especially important with novel compounds synthesised initially in quantities of about 1g.), the reproducibility and precision of the measured compound activity (precise results are desirable if structure-activity studies are to be performed), and the small number of animals required.
One guinea-pig will provide sufficient lymph node cells for the activity of 2 compounds to be measured (60 rats would be required to measure the activity of 2 compounds in adjuvant arthritis, test groups of 5 animals and 5 different compound doses). The avoidance of large numbers of animals saves the cost of animals, capital and running costs of the animal-holding space, and also minimises pain caused to experimental animals.

The lymph node cell system, in common with other in vitro screens probably determines the intrinsic activity of compound; in contrast to in vivo screens which determine the apparent activity of the compound, which is a combination of the intrinsic activity and the metabolic disposition of the compound (for example, absorption, elimination by excretion and metabolism, and tissue storage) in the particular test animal. When a drug is used to treat disease in man it is the apparent activity of the compound which determines the clinical response. Hence, it can be argued that in vivo screens, such as adjuvant arthritis, are more valuable than in vitro screens which measure intrinsic activity. The many differences found between the metabolic disposition of drugs, for example phenylbutazone (Burns et al, 1953), in man and experimental animals, such as the rat, render this supposition very questionable. In initial screening for anti-inflammatory activity intrinsic activity should be pursued, since human apparent activity can only be assessed satisfactorily in Man himself.

The relevance of the lymph node cell system as a screen for anti-inflammatory agents to be used in the treatment of rheumatoid arthritis is good, since there is much evidence to link lymphocytes with rheumatoid arthritis (Messner, 1974). Lymphocytes, however, are not involved in many other inflammatory conditions, such as osteo-arthritis, in which established anti-inflammatory drugs have beneficial actions. Inflammation generally involves tissue proliferation. It is suggested that the relevance of the lymph node cell system to inflammatory conditions which do not involve lymphocytes may be at the level of tissue proliferation,
of which PHA-stimulated guinea-pig lymph node cells are a convenient in vitro laboratory model.
The effects of 6,8-di-t-butyl chromone (FPL52806) on the synthetic activities, viability, and morphology of PHA-stimulated guinea-pig lymph node cells were related effects, probably involving a common mechanism.

The potencies of 6,8-di-alkyl chromones as inhibitors of $[^3H]$-thymidine and $[^{14}C]$-leucine incorporation in PHA-stimulated guinea-pig lymph node cells, in terms of both the total added chromone concentration and the free non-serum bound chromone were a mathematical function of the lipophilicity of the chromones (section 6.3, Page 174). The 6,8-di-t-pentyl chromone (FPL56220) had a lipophilicity similar to that of the mathematically-calculated most active member of the 6,8-di-alkyl chromone series in terms of the total added chromone concentration.

The potency of established anti-inflammatory drugs and candidate (chromone) compounds as inhibitors of $[^3H]$-thymidine and $[^{14}C]$-leucine incorporation by PHA-stimulated guinea-pig lymph node cells were compared to the potencies of the compounds in in vitro screens (sulphhydryl-exchange and hypotonic erythrocyte haemolysis), in in vivo screens (rat adjuvant arthritis, rat carrageenin oedema, and rat passive cutaneous anaphylaxis) and the clinical potency of the drugs in rheumatoid arthritis (Section 6.4, Page 182). The order of activity of established drugs in the lymph node cell system was closer to the clinical order of potency of the drugs than was their order of potency in the other anti-inflammatory tests. The order of potency of the candidate chromones in the lymph node cell system did not agree with their order of potency in the passive cutaneous anaphylaxis test. The lymph node cell system does not detect anti-Type 1 allergy compound.
It is concluded that the synthetic activity of PHA-stimulated guinea-pig lymph node cells is a suitable screen for the detection of intrinsic anti-inflammatory activity.


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