LYMPHOCYTE TRANSFORMATION AND POTASSIUM TRANSPORT

by

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To my dear wife, Eva

who bore three children

Daniel, Aaron and Benjamin

not excluding Jonas

(and endured)

while I bore

this

*

ABSTRACT

This work demonstrates a critical factor operative in the control and regulation of small lymphocyte transformation at the cellular level. This factor is associated with the transport function of the plasma membrane, and in particular, with the uptake and intracellular level of potassium.

The work described herein starts with an investigation of the binding of phytohemagglutinin (PHA) to the cell, and is followed by studies on the inhibition of cellular synthetic processes by interference with transport using the cardiac glycoside, ouabain. Pronounced sensitivity of the transforming lymphocyte to the glycoside is demonstrated. The synthetic responses of lymphocyte transformation to agents modifying the transport and intracellular distribution of cations and to compounds affecting bioenergetics are examined. A study on the cellular uptake of potassium using the isotope K^{42} as a tracer is then presented. Evidence is given for conformational changes in the cell membrane associated with the early action of PHA, leading to activation of potassium transport, and very likely to increased intracellular potassium levels. A mathematical analysis of the results is included.

Preliminary studies of cellular respiration, ATPase activity,

of ouabain effects on leukocytes obtained from patients with leukemia and cystic fibrosis, and of homograft rejection are also included. It is suggested that the pronounced sensitivity of lymphocyte transformation to substances which affect membrane transport may be useful in investigations of immune function.

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LSIT OF ABREVIATIONS

ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
cAMP	Cyclic adenosine monophosphate
dcAMP	Dibutyryl cyclic adenosine monophosphate
ATPase	Adenosine triphosphatase
AL	Acute leukemia
ALL	Chronic lymphocytic leukemia
CML	Chronic myeloid leukemia
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
PHA	Phytohemagglutinin
PHA-FITC	Phytohemagglutinin - fluorescein isothiocyanate
NEM	N-ethylmaleimide
PCMB	P-chloromercurobenzoate
TCA	Trichloracetic acid

For other symbols, see page 470.

1. INTRODUCTION

1.1. GENERAL CONSIDERATIONS

1.1.1. Nature of Problem

The small lymphocyte of the peripheral blood and the lymphoid organs is a small round mononuclear cell characterized by a low cytoplasm: nucleus volume ratio. Almost ubiquitous in body tissues in its differentiated state, it is but rarely seen in mitosis and is considered to be a repressed non-dividing cell. The numerous concepts which have arisen concerning its origin, function and fate have been strongly influenced by recent findings that this cell will consistently undergo a pronounced enlargement and change to a mitotic cell under certain conditions in vitro. These conditions include exposure to various plant substances such as phytohemagglutinin (an extract of the red kidney bean, Phaseolus vulgaris), bacterial extracts, antigens to which the donor of the cell had been sensitized, and when mixed with leukocytes from an unrelated donor. Such observations demonstrated that the lymphocyte was not necessarily an 'end cell' but had a functional potential related to body defence reaction. The observation that at least some small lymphocytes are extraordinarily long

lived (up to at least 20 years in the human) and the demonstration of its role in immunological 'memory', delayed hypersensitivity, graft rejection, cytotoxicity and other aspects of cellular immunity, as well as in the elaboration of humoral antibody, have now implicated this cell as a key to the understanding of immune phenomena.

The induction of lymphocyte transformation and mitosis by phytohemagglutinin has been widely used for the reliable demonstration of mammalian chromosomes; this has probably become the most widely used technique for human cytogenetics since its original development by Hungerford et al (1959) and Nowell (1960). Karyotyping of lymphocyte chromosomes has found use, not only for the demonstration of certain hereditary or congenital conditions, but also for the assessment of the genetic effect of environmental agents such as radiation (Quastel et al, 1970). However, knowledge of the significance and mechanism of the production of chromosomal aberrations in lymphocytes is dependent on an understanding of the process of transformation induced in this cell.

The process by which the 'blastoid' response or transformation of the small lymphocyte is induced remains poorly understood. Evidence based on the effects of antigens,

antilymphocyte antibody, PHA and anti-PHA antiserum, and of the mixed lymphocyte reaction (which will be described) suggest that events on the surface of the transforming cells, at least at an early stage, are of importance in the induction of transformation. Recent evidence from both bacterial and mammalian cells has suggested that the cell membrane is involved in the regulation of DNA replication and transcription. The present study was initiated with a view to elucidating the role of the cell membrane in lymphocyte transformation.

1.1.2. Scope and Purpose of the Investigation

In view of the multifaceted nature of this cell, a wide variety of potential studies was possible. The work described herein is based almost entirely on short-term culture, that is, maintenance of cells for less than one week in vitro. Early experiments showed that phytohemagglutinin (PHA) fluorescein isothiocyanate rapidly became associated with the cell membrane, this prompted further studies on the role of cell membrane function.

In this study, the hypothesis is advanced that the cation transport function of the plasma membrane is a critical factor in lymphocyte transformation; initial experiments using ouabain, a specific inhibitor of coupled sodium and potassium transport, support this possibility. The

initial purpose of the study was to characterize the action of ouabain and similarly acting drugs, since the action of the compound on blastoid cells suggested a new method for the inhibition of lymphocyte transformation. The sensitivity of the cells to ouabain suggested a control of transformation by cation transport; hence it became important to investigate the kinetics of potassium uptake in unstimulated and stimulated lymphocytes. It was shown that early lymphocyte transformation is associated with a stimulated uptake of K^{42} by the cells. These studies were further supplemented by work on the respiration of the lymphocyte, measurements of ATPase activity, on the action of ouabain on lymphocytes from patients with various diseases and on the binding of fluorescein-labelled PHA to lymphocytes and blast cells.

1.1.3. Historical Notes

These brief notes of a general nature are supplemented by further historical data where of interest in the review of the literature.

Probably the earliest mention of the lymphatics was made by Herophilus and Erasistratus about 300-250 BC. Two millenia later, they were noted by Gaspari Aselli in Paris who, about 1622, pricked distended lacteals in the mesentery of the gut of a well fed dog, and observed a milky fluid to

gush out. Pecquet (1651) described the thoracic duct leading from the gut to the neck, through which the "Liquor of the Milkie Veins throws itself headlong into the Whirlpool of the Heart". Bartholin (1653) first used the term 'lymphatics'.

The first observation of cells in the lymphatic fluid appears to have been made by the Hunter brothers in the late 1700's, who claimed that they came from lymph glands. William Hewson, a pupil of William Hunter, noted that lymph glands (or nodes) were absent in fish and the turtle; although there were a few in birds, they were well developed only in mammals. He also described the lymphatic fluid, and thought that it came from the lymph glands to enter the blood.

The first warm stage observations of fresh blood were apparently made by Schultze (1865), who observed a class of small (5 μ) mononuclear leucocytes that did not exhibit amoeboid movements. However, Ranvier (1888) later observed rabbit blood lymphocytes and described their considerable vitality in vitro at 37° C.

Concepts regarding lymphocyte origin and function have undergone considerable evolution and change in emphasis during the period ranging from the nineteenth century until the present. By the early 20th century, a dichotomy of opinion had developed

between (a) the proponents of the Renault (1888) and Ranvier (1875) concepts of a multipotential lymphocyte as a mobilized connective tissue cell - into which the lymphocyte would transform under certain conditions, and (b) the ideas developed by Metchnikoff (1905), Buchner (1894)and Bordet (1895) that lymphocytes featured in body defense by secreting soluble substances.

It has been maintained that the lymphocyte is a multipotential cell, capable of development into other cells of the blood and connective tissue. On the other hand, it has been considered to be an 'end cell' or a 'trephocyte'. Carrel and Eberling developed the theory of the lymphocyte as a trephocyte, bringing 'nourishment' to fixed cells of the body. They stated (1922) "Leucocytes may be regarded as mobile uncellular glandular bodies which set free their secretions in the humors of the organism. But little is known of the nature and function of the substances they secrete".

These ideas were reviewed by Coulson (1967), who emphasized the fact that, before the advent of phytohemagglutinin as a lymphocyte stimulant, considerable evidence had accumulated to indicate that the mammalian blood lymphocyte was not an 'end cell', like the mature granulocytes, but had an ability to undergo striking enlargement and

transformation in structure. But the conditions required to trigger this transformation, or the cell type into which the lymphocyte transformed, were never clearly established.

Controversy regarding the theory of blood cell development and the role of lymphocytes has continued; both the source of development and interrelation of lymphocytes with other cells remain matters of dispute and speculation. Wintrobe (1946) emphasized that a major source of disagreement between the various proponents of different theories was the question as to what actually are lymphocytes, and whether they represent a homogenous or heterogeneous class of cells.

It has long been held that the lymphocyte is involved in body defence mechanisms. This was based on familar observations of changes in the level of circulating lymphocytes in infectious disease, their accumulation in areas of subacute and chronic infection such as in tuberculosis and syphilis, and their appearance as a mantle around tumors and in areas of trauma. The anatomical distribution of lymphoid tissue at the throat and in the intestinal tract have been regarded as evidence of their protective role. Nevertheless, until recently the role of the small lymphocyte in the lymphoid tissue has been almost completely obscure.

Maximow proposed that lymphocytes could transform in vitro into 'polyblasts' or large mononuclear cells, and thence into fibroblasts or cells of the myeloid series. In support of this concept, Margaret Lewis (1925) claimed to observe transformation of leukocytes into macrophages, giant cells and epitheloid cell in hanging drop cultures. Calista Eliot (1926) made similar observations. Bloom (1926) cultured lymphocytes from the thoracic duct of the rabbit and found that after one day, the culture became dominated by numerous large amoeboid cells. However, the many cell types which were observed to develop variably in vitro did not lend themselves to identification. Their source was unknown and they did not necessarily arise from lymphocytes; for example, Rhodes and Parker (1928) were unable to show changes in lymphocytes cultured for as long as 2 weeks in-vitro.

Timofewski and Benowolenskaja (1927) observed lymphocytes and monocytes to transform into 'polyblasts' and epitheloid cells when cultured in the presence of tubercle bacteria. This was probably the first example of lymphocyte transformation in vitro in response to an antigen.

Chrustschoff (1935) utilized hanging drop cultures of leukocytes to obtain 'polyblasts' which were found to be mitotic after 314 days of culture. Using hypotonic medium, he prepared Feulgen stained chromosomes thus anticipating the work of Hungerford et al (1959).

Jean Medawar (1940) accounted for the polyblasts which developed in leucocyte cultures in terms of the initial monocyte content of the white cell culture, since serial photographs of lymphocytes did not provide evidence for enlargement of these cells.

The reproducible and reliable induction of lymphocyte transformation, with which this work is concerned, began with the finding by Hungerford et al (1959), and Nowell (1960) that transformation was consistently induced by phytohemagglutinin (PHA), an aqueous extract of the red kidney bean, Phaseolus vulgaris. However mitogenic extracts of other plants have since been discovered. Carstairs (1961) provided evidence that it was the small lymphocyte that was activated by PHA to form blast cells. Shortly after this, Bain et al (1964) and Bach and Hirschhorn (1964) demonstrated that when leukocytes from the blood of the individual were cultured for several days with leukocytes from another person, there occurred mutual stimulation and blast transformation. It was also shown by Ling et al (1965) that bacterial endotoxin would cause a 'nonspecific' stimulation similar to that induced by PHA. Pearmain et al (1963) showed that antigens would induce the transformation in vitro of lymphocytes obtained from a donor who had been first immunized with the antigen.

More recently, evidence has been obtained for the release of physiologically active, soluble substances from stimulated lymphocytes, thus supporting some of the early speculations of Carrel and Eberling (1922).

1.2. Lymphocytes and Lymphoid Tissue: Some Considerations of Development, Classification and Lymphocyte Circulation

1.2.1. General

In the present investigation, the cell types primarily under consideration have been the unstimulated and stimulated forms of the human small lymphocyte obtained from the peripheral venous blood and cultured under *in vitro* conditions. In order to interpret the observed responses of the small lymphocyte under highly artificial culture conditions, in relation to its normal physiology *in vivo*, the normal physiology of lymphocytes and lymphoid tissue must be kept in mind. I have therefore included, in sections 1.2 and 1.3 of this literature review, a brief summary of lymphocyte physiology *in vivo*.

The large amount of data which have accumulated on this subject during the past decade render it difficult to present a complete up-to-date picture. Indeed the origin, function and fate of the small lymphocyte has long been a matter of conjecture. Yoffey (1964) wrote that: "the history of lymphocyte research activity abounds in hasty and plausible generalizations which may take a long time to expunge from the literature". For reviews of some of the less recent

literature used in this and other sections, the books by Yoffey and Courtice (1956), Elves (1967) and Ling (1968) and the reviews by Yoffey (1964), Coulson (1967) and Daniels et al (1969) have been very useful.

1.2.2. Morphology and Cytology of Lymphocytes

As they appear in the peripheral blood stream, or following separation from lymphoid tissues, these 'small round' mononuclear cells are seen to be about 6-10 μ in diameter. The nucleus is densely pachychromatic (Whitby and Britton, 1963) and occupies the main volume of the cell; the cytoplasm is characteristically relative slight in volume. Lymphocyte cytology has been described in detail (Maximow and Bloom, 1957, Wintrobe, 1965, Braunsteiner and Zucker-Franklin, 1962).

Phase microscopy of flattened cells reveals mitochondria adjacent to the nuclear indentation or encircling the nucleus (Wintrobe, 1965). One or more highly refractile lipid structures about 0.5 μ in diameter occur in the cytoplasm of about 1/3 the lymphocyte population (Gall, 1936). In the living state, the nucleus tends to be located at one pole of the cell, giving it a 'hand mirror' appearance (Ebert et al, 1940). This cell is highly mobile and has

been observed to enter the cytoplasm of other cells to move about therein, the phenomonon being termed emperipolesis (Humble et al, 1956). Pulvertaft (1959) has emphasized the semi-directional movements of lymphocytes in close physical association with other cell types.

Cytochemical studies have shown traces of glycogen (Wachstein, 1949), lactate dehydrogenase (Starkweather et al, 1966) and cytochrome-C (Hoffman et al, 1951). Variable activities of nuclease, adenosinase, cathepsin, amylase, lysozyme and lipase have been demonstrated (Barnes, 1940, Elves, 1967) and stimulated lymphocytes often show increased enzyme levels (Gough and Elves, 1967).

1.2.3. Phylogeny, Ontogeny and Anatomical Distribution

Small lymphocytes are almost ubiquitous throughout the tissues of the body and are closely associated with the lymphatic vascular system, although there is some evidence that the cellular components of the lymphoid system evolved separately from the lymphatic vessels (Yoffey and Courtice, 1956).

In the adult human, lymphocytes account for 0.5-1% of the total body weight (Yoffey, 1960), and normally are present in the peripheral blood, in concentration of 1500-3000 cells/mm^3

(or about 25-70% of the total leucocyte count). According to Osgood (1954), about 3 g of lymphocytes are in the peripheral blood, about 70 g in the marrow, 100 g in the lymphatic tissues, and further 1300 g distributed through most of the tissues of the body.

Abnormal numbers and types of lymphocytes commonly occur in various diseases conditions. Lymphocytopenia occurs in Hodgkin's and other lymphoid diseases (Aisenberg, 1965), in Cushing's Disease (De la Balze et al, 1946), in uremia (Riis and Stougrard, 1959), following irradiation and in a variety of infectious, cardiac and steroid therapy problems (Wintrobe, 1965). Lymphocytosis is well known to occur in acut infections and leukemias. Atypical lymphocytes are seen in infectious mononucleosis (Downey and McKinley, 1923) and in other conditions.

The total mass of lymphoid tissue is a function of age. In the human, it has been estimated that the lymphoid system reaches maximum development in terms of unit weight toward the end of the prepubertal years and later undergoes a relative shrinkage (Braunsteiner and Zucker-Franklin, 1962).

The first lymphoid tissue to develop in the human is the thymus, and lymphocytopoiesis is recognizable in this organ

after the 9th week of gestation (Hammar, 1936). The lymphocytes appear to originate from epithelium (Harrison et al, 1970). Lymph nodes are observed by the 3rd month, after the lymphatic vascular system has become fairly well organized, and are well developed by the 5th month (Hellman, 1930). Their formation continues after birth. The lymph nodes first appear as a twisting network of lymphatic capillaries. Blood vessels then enter the lymphatic reticulum which subsequently becomes populated with lymphocytes.

Lymphoid tissue is found throughout the vertebrates. However, in fish, amphibia and reptiles, it has not become differentiated from the tissues concerned with the production of myelocytes and granulocytes, and has thus been termed 'lymphomyeloid' tissue.

In fish and amphibians, Drzewina (1905) found lymphomyeloid masses to occur in various tissues, including the kidneys, liver, heart, generative organs, spleen, alimentary canal and skull. Lymphoid tissue was closely associated with the intestine early in the phylogenetic scale, and occurs in the cyclosomes (e.g. the lamprey), in which the spleen has not developed. Animals above the lamprey have a thymus. Spleens are generally present in fish, and bone marrow appears in amphibians and reptiles, but differentiation of the

lymphomyeloid tissues into lymphoid and myeloid compartments does not occur until the birds and mammals.

Lymph nodes occur in some birds. Avian lymph nodes have a different structure than those of mammals, and birds also have important lymphoid structures which are not seen in mammals (such as the bursa Fabricii).

1.2.4. Classification of lymphoid tissue

The classification of lymphocytes by morphological criteria as large, medium or small, by mitochondrial number, nucleolar size, cytoplasmic granules, etc. have not been particularly meaningful for what is now recognized as very heterogeneous cellular population. Criteria based on functional activity such as enzymatic, synthetic and particularly immunological responses appear to be promising (see Section 1.3).

The lymphoid organs may likewise be classified by morphological or functional criteria. For example, Ehrich (1929) suggested a classification based on the presence of or absence of afferent or efferent lymphatic vessels. A useful functional classification by Fichtelius et al (1969) speaks of central or first level lymphoid organs, and peripheral or second level lymphoid organs. The 'first level'

organs would include the thymus and the bursa Fabricii (or its mammalian equivalent), since removal of each of these organs early in ontogenesis leads to an altered development of 'second level' lymphoid organs. In birds, the bursa Fabricii seems to be responsible for that part of the lymphoid system required for immunoglobulin production, whereas the thymus is required for cellular immunity (Cooper et al, 1967).

While all animals phytogenetically above the lamprey have a thymus and the thymus-dependent function of cellular immunity (Good et al, 1966), and all animals above the lamprey display the bursa-dependent function of immunoglobulin production, only birds have the bursa. The search for a bursa-equivalent in bursa-less vertebrates has led to evidence that the Peyer's patches and appendix of the intestine may have this function (Cooper et al, 1967, Good et al, 1966). Fichtelius (1967) has suggested that the epithelium of the gut in bursa-less vertibrates is a 'first level' lymphoid organ, having the same influence on the development of lymphocyte and lymphoid tissue as does the bursa Fabricii in birds.

1.2.5. Lymphocyte circulation, life time and fate

It was an early observation that, when the lymph passes through lymph nodes, it accumulated lymphocytes and other cells

Heyfelder, 1852). Cellular lymph from the lower part of the body joins the thoracic cervical and sub-clavian ducts and empties into the circulating blood. Extensive work by Drinker and Yoffey (summarized by Yoffey and Courtice, 1956) showed that the number of lymphocytes added to the blood stream each day from the thoracic duct was so large that the cells must be replaced in the blood stream 6-20 times each day (for man) and 23 times per day (for the rat). Since the grave yard of such a large number of cells has not been found, there must be recirculation, as postulated by Sjovall (1936). The problem therefore arose as to the origin, fate and life-time of these cells.

More recently, the elegant studies of Gowans and his colleages have provided important evidence that the small lymphocytes which enter the lymphatic ducts are part of a large pool of cells which circulate from the blood into the lymph and lymphoid tissues (except the thymus) and thence back into the blood stream (Gowans, 1966).

The recirculation of the lymphocyte provides a rational for the concept that at least some of these cells survive in the body for periods of years. Current experimental evidence suggests that there exist at least 2 populations of lymphocytes with widely differing life spans. Some of the evidence is as follows;

Continuous infusion of H^3-thymidine in rats indicated a lymphocyte survival for more than 100 days, since some of the peripheral blood lymphocytes remained unlabelled (Little et al, 1963). Hamilton (1956) found that lymphocyte pre-labelled with H^3-adenosine persisted as long as 300 days in patients with chronic lymphocytic leukemia. In persons exposed to radiation for ankylosing spondylitis (Buckton and Pike, 1964), chromosome aberrations in lymphocytes were observed for years after exposure. The nature of the aberration found indicated that the cells could not have undergone mitosis between the time of irradiation and chromosome analysis up to 10 years later.

The apparently long lifespan of the lymphocyte was suggested by Fitzgerald (1964) to provide a basis for long term immunity and immunological 'memory'.

However, there is also evidence that at least some lymphocytes have a relatively short lifespan. This was indicated by the extensive lymphocyteopoiesis which occurs in lymphoid tissues (Yoffey et al, 1961). In the rat, there appears to be a rapidly labelled lymphocyte population with a circulating lifespan of less than 2 weeks (Gaffrey et al, 1962). Ottesen (1954) studied the decay of P^{32} in the DNA of peripheral human blood lymphocytes. One population,

representing about 20% of the cells, had a mean age of about 2-3 days. The remaining lymphocytes had a mean age of 100-200 days.

1.3. Lymphoid Tissue and Lymphocytes: Functional Considerations

1.3.1. General

Although the lymphocyte has long been considered to be involved in body defence mechanisms, this association has not always been clear. Ehrlich (1878) considered it to be a cell sui generis, unrelated to other cell types. Kelsall and Crabb (1959) have proposed that the lymphocyte is essentially a trephocyte, transporting purine and pyrimidine bases, potassium and other substances to certain tissue sites such as areas of wound healing. Loutit (1962) has suggested that the cell may have a trophic as well as an immunological role. A recurring theme is that at least some lymphocytes may represent a class of fixed or free macrophages (Metchnikoff, 1888; Boak et al, 1968).

Since the localization of antibody formation in the lymph nodes (McMaster and Herdack, 1935), the participation of lymphoid tissue and small lymphocytes in various kinds of immune phenomena has been frequently demonstrated. In this section, the participation of lymphocytes and lymphoid tissues in immune phenomena will be briefly outlined, as a background to the phenomenon of lymphocyte transformation which will later be examined in some detail. The 'unusual' lymphocytes of the thymus and bone marrow are discussed in sections 1.3.2.2. and 1.3.2.3.

1.3.2. Lymphoid tissue

1.3.2.1. Central and peripheral lymphoid tissues

The discovery by Glick (1965) that the bursa of Fabricius is essential to the development of those lymphoid cells required for antibody production has led to a useful, though perhaps overly explicit separation between different aspects of lymphoid function. A classification based on central and peripheral, or primary and secondary lymphoid organs has been proposed (Fichtelius et al, 1969). For example, the thymus has been described as a 'primary lymphoid organ (Good et al, 1962), and it has been proposed that the intestinal lymphoid tissue (Peyer's patches and appendix) may play a role in animals similar to that of the bursa Fabricii in birds (Fichtelius, 1967).

The peripheral lymphoid tissues, such as the spleen and lymph nodes appear to contain lymphocytes which are dependent on the thymus for development. These cells are considered to be responsible for delayed hypersensitivity, graft rejection and graft versus-host reactions. On the other hand, another class of lymphoid cells may give rise to germinal centers and plasma cells (Cooper et al, 1966; Oort and Turk, 1965; Parrot et al, 1966). These two cells which are discussed further in section 1.3.3, may be located in different parts of the lymph node follicle (Meuwissen et al, 1969).

The role of peripheral lymphoid tissue of the lymph nodes in cellular immunity is illustrated by the large, pyroninophylic cells which develop in regional nodes draining the sites of homografts (Scothorne, 1957; Turk, 1967).

1.3.2.2. Thymus and thymic lymphocytes

Unknown in function until recently, this lymphoid organ is relatively large in size during fetal and early life, so much so that this was at one time given as a reason for unexplained death in children (Status Lymphaticus). It is composed of thymocytes, which are morphologically undistinguishable from lymphocytes, supported by a reticulum cell network. Each lobe of the thymus contains few numerous lobules, held together by fibrous tissues. The lobules contain several follicles, each with cortical and medullary areas. The former contain mainly thymocytes and some larger cells, whereas the medulla is more loosely packed and contains deeply stained cells. Cell proliferation and DNA synthesis in the thymus is, in the young animal considerably more active than in any other lymphoid tissue. This organ is particularly sensitive to radiation, adrenal steroids and 'stress', responding by involution and cellular depletion of the cortex (Burnet, 1962; Arnason, et al, 1962).

The thymus has been described by Good as the 'pacemaker of the lymphoid complex in animals and man, and as a 'cradle' for the initial commitment of 'stem cells', which may migrate initially from the bone marrow. The thymus appears to be essential in early life for the maturation of the immunological system, and possibly in the adult for maintenance of cellular immunity. Good and his colleagues (1954, 1955) noted an association of agammaglobulinemia with thymoma, and suggested that there was an association between this organ and the immune system. Some years later, Gitlin et al (1959) noted that small lymphocytes and Hassel's corpuscles were absent from patients with congenital sex-linked agammaglobulinemia. Certain primary immune deficiencies have now been classified with respect to the presence or absence of thymus dependent or thymus independent lymphocytes (Fudenberg et al, 1970).

A soluble production, termed 'thymosin', has been isolated from calf thymus tissue. Thus factor appears to stimulate lymphocytopoiesis and cause a peripheral absolute lymphocytosis (Goldstein et al, 1966). The relative heat stability of this preparation appears to differentiate it from the so called 'lymphocytosis stimulatory factor (Metcalf, 1958) which is also involved in the stimulation of lymphoid tissue (Osaka and Mitter, 1963), and which may be required for the maturation of immunocompetent cells (Osaka and Miller, 1964; Levey et al, 1963).

Hence, the function of the thymus appears to involve both the seeding of thymic lymphocytes to the periphery (Sainte-Marie and Leblond, 1958; Nossal, 1964) and the production of non-cellular diffusable factors which are involved in the maturation of lymphocytes and lymphocytopoiesis. According to this view, final maturation occurs in the peripheral lymphoid organs; thymocytes have not yet reached their full state of immunocompetence and thus show less immunological reactivity than do 'mature' small lymphocytes. This is illustrated by the following evidence.

Thymocytes react differently than do peripheral lymphocytes. They do not respond in a hyperplastic manner (Wiseman, 1931), nor by antibody formation (Bjorneboe et al, 1947) after the administration of antigen in vivo. They have a reduced response to phytohemagglutinin (McIntyre and Segal, 1966; Winklestein and Craddock, 1965), a reduced ability to transfer a secondary response (Thorbecke and Cohen, 1964) and a diminished graft-versus-host reaction (Miller, 1964).

Neonatal thymectory leads to poor development of lymphoid tissue and incompetence of cellular defence mechanisms, such as impaired allograft rejection (Martinez et al, 1962) and diminished delayed hypersensitivity (Miller, 1961; Waksman et al, 1962; Miller, 1964).

These effects are not associated with defects of the humoral defence capability. Hence these properties of the thymus can be differentiated from those of the bursa Fabricii, the removal of which prevents development of the humoral defence system, but permits the cellular defence mechanism to function unimpaired.

1.3.2.3. Bone Marrow Lymphocytes

The presence of numerous lymphocyte-like cells in this organ was observed at the beginning of this century (Dominici, 1902. Nevertheless, the origin, fate, and function of these cells still remains essentially obscure. It is not clear, for example, whether these cells originate elsewhere, or if they are formed endogenously in the marrow. The relationship of these cells to the erythroid and myeloid precursors is also a matter of some conjecture. The early descriptive literature has been reviewed by Yoffey and Courtice (1956).

One important finding has been that the lymphocytes present in mammalian bone marrow are mainly scattered among the other cell types, rather than in organized nodules as in other lymphoid tissues. Yoffey et al (1965) report that they are characteristically found concentrated within the sinusoids (lymphocyte loading). The proportion of the total lymphocyte

mass present in marrow has been estimated by different observers using different species, and values ranging from 7 to 14% have been estimated for human adults, with higher values for small children).

There has been some confusion regarding the differentiation between the 'micromyeloblast' of Naegeli (1900) and the small lymphocyte. Downey (1938) considered that there were recognizable differences in that the chromatin structure of the micromyeloblasts was characterized by a "diffuse and rather uniform distribution of the chromatin which occur in the form of fine dust-like particles". Nevertheless, morphological differences with peripheral lymphocytes are minimal. A myelopoietic function of the bone marrow lymphocyte therefore remains a possibility.

The use of chromosome markers has provided evidence that bone marrow cells can repopulate the thymus, spleen and lymph nodes (Ford et al, 1956; Ford and Micklem, 1963). These workers suggested that 'the thymus is continuously being seeded from the bone marrow'. Transplantation experiments in lethally irradiated mice have also suggested that the proliferative capacity of donor bone marrow cells may be correlated with the number of marrow lymphocytes (Cudkowicz et al, 1964).

In this respect, it is interesting to note that Kaplan et al (1954) observed that mouse thymus recovered from X-ray depletion much more quickly if a portion of marrow was shielded or if normal marrow was given intravenuously.

Electron microscopic evidence (Yoffey et al, 1966) does not provide morphological data that would distinguish these cells from lymphocytes derived from other tissues. In the latter study, a range of cell types from the small lymphocyte to blast cells were observed, but only the intermediate and larger cells were found to incorporate H^3-thymidine.

Autoradiographic observations by Osmond and Everett (1964) and Osmond (1964) suggest that the bone marrow is a site for lymphopoiesis. Keiser et al (1964) found that lymphocytes may also enter the marrow from the blood stream. Bond et al (1964) observed that labelled lymphocytes passed from the blood stream to the marrow of the rat. By electron microscopy, an interchange of lymphocytes between marrow and blood was observed in the sinusoidal endothelium of guinea pig bone marrow (Hudson and Yoffey, 1966).

There are some immunological data regarding the properties of bone marrow lymphocytes. Tyan and Cole (1965) suggested that the bone marrow is a major source of potentially

immunologically competent cells in the adult mouse. It is interesting that injected bone marrow cells will appear in the thymus, unlike cells from other lymphoid tissues (Ford and Micklem, 1963). Miller et al (1964) suggested that 'marrow cell suspensions lack adequate numbers of immunologically competent cells', but that cells mature under the influence of the thymus. On the other hand, Singhal and Richter (1968) have recently provided evidence suggesting that the bone marrow supplies the immunocompetent 'antigen recognition' cells in the mature adult rabbit, and are capable of initiating a primary immune response in vitro, unlike thymus, spleen and lymph node cells.

Craddock (1965) has studied the uptake of H^3-thymidine by bone marrow lymphocytes, and reported that they are self sustaining, have a turnover similar to that of thymus lymphocytes, and exhibit a low intake of the isotope into progenitor cells. A large proportion of the small lymphocytes in the bone marrow appear to have a fast turnover (a few days), but a significant fraction of the highly labelled cells do not decline rapidly with time.

In summary, the bone marrow lymphocytes appears to contain both short and long lived varieties of lymphocytes, some of which may function as the erythoid or myeloid precursor cells. These cells, which may be self sustaining are to a

larger extent immunologically 'incompetent' and are transferred to the thymus, unlike lymphocytes from other sources. An interchange of lymphocytes between bone marrow and blood stream has been demonstrated morphologically. The participation of the bone marrow, in the immune response has been postulated and may involve the transfer of 'antigen recognition' or potentially 'immunocompetent' cells to other parts of the lymphatic system.

1.3.3. Lymphocyte populations

It is probably no longer correct, to speak of the lymphocyte, or of the function of the lymphocyte. As emphasized by Meuwissen et al (1969), these cells, which appear by classical histological techniques to be a homogeneous population, probably represent several lines of differentiation with different specialized functions; these 'separate'lines may be under the influence of separate regulatory mechanisms, and thus may have different distributions in the body.

The evidence for this comes from labelling experiments (see section 1.2) which have indicated varying lymphocyte lifetimes from several days to years. Nowell (1965) has also presented evidence that radiation induced, unstable chromosome

aberrations in tuberculin-stimulated cells are removed rapidly from the population (unlike PHA-stimulated cells), one explanation for this was that both long-lived and short-lived lymphocytes exist.

The classification of lymphoid tissue into thymus-dependent and bursa-dependent tissue has led to a functional classification of lymphocytes along similar lines. This has recently been summarized by a WHO committee for Primary Immunodeficiencies, chaired by H.G. Kunkel (Fudenberg et al, 1970). Part of this report is summarized below.

The immunological responses, which are classically divided into those mediated by humoral antibodies, and those by cells, depend ultimately on the activity of small lymphocytes. Neonatal thymectomy prevents the development of cell mediated immune reactions (such as delayed hypersensitivity or homograft rejection) as well as the humoral antibody response to certain antigens. In birds, antibody production, but not cell mediated immunity, is dependent on the bursa of Fabricius. Gut-associated lymphoid tissue may provide a mammalian analog of the bursa.

It was therefore suggested that stem cells for lymphocytes, which probably reside in the marrow during post-natal

life, differentiate and form at least 2 distinct lymphocyte populations, one dependent on the thymus (T-lymphocytes) and the other independent of the thymus (B-lymphocytes. Both are postulated to contain antigen-sensitive cells.

According to this scheme:

1.3.3.1. The T-lymphocyte contributes the greater part of those present in the circulation, and can be stimulated non-specifically (such as by PHA), and also by contact with a specific antigen, possibly after macrophage preprocessing. These cells may:

(i) divide further to form an expanded, long lived population of primed, antigen-sensitive cells, which are the basis of the immunological 'memory';

(ii) have cytotoxic properties to foreign cells such as those of grafts;

(iii) release a number of soluble factors which (a) inhibit macrophage migration, (b) are chemotactic for mononuclear cells, (c) can be mitogenic and (d) increase vascular permeability;

(iv) cooperate, during the immune response to certain 'thymus dependent' antigens, by stimulating B-lymphocytes to antibody production.

1.3.3.2. The B-lymphocytes appear to be more restricted to lymphoid tissues than are the T-lymphocytes. These cells can differentiate, proliferate and mature into plasma cells which synthesize humoral antibody.

1.4. Transformation of Lymphocytes: Modes of Induction

In the historical notes of section 1.1.3, it was shown that the capacity of the lymphocyte to undergo transformation to another kind of cell is not a recent concept. However, when lymphocyte transformation *in vitro* became simply and reliably induced, the physiological significance of this phenomenon for the role of the lymphocyte in the immune response became quickly recognized, and in addition culture techniques for the rapid and simple preparation of human chromosomes were rapidly developed.

Two major findings have been responsible for much current lymphocyte research. Although phytohemagglutinin (PHA) was first used as a material that would accelerate the rate of sedimentation of red cells from the peripheral blood, Hungerford et al (1959) and Nowell (1960) observed the development of blastoid cells and mitoses in leukocyte cultures that had been cultured for 2 or more days *in vitro* with PHA. The second and equally important finding was that small lymphocytes could be induced to undergo transformation and mitosis by exposure *in-vitro* to specific mitogens to which the donor of the cells had been sensitized (Pearmain et al, 1963).

These observations demonstrated the mitogenic potential and the 'immune capability' of the small lymphocyte and thus

markedly altered an older alternate view that the lymphocyte was an 'end cell' or a trephocyte. Since then a number of ways by which lymphocyte transformation can be induced have been found. The following discussion will consider some of the different modes which cause the small lymphocytes to undergo transformation *in vitro*. A subsequent section will consider the events taking place within the cell during stimulation.

Reviews of the subject have been published by Robbins (1964), Cooper and Amiel (1965), Bach and Hirschhorn (1965), Gowans (1966), Heath (1966), Ling (1968) and Daniels et al (1969).

1.4.1. Phytohemagglutinin (PHA)

PHA is probably by far the most widely used 'non-specific' mitogen for the study of lymphocyte blast transformation. The characteristic morphology of cells treated with PHA has been described in detail by Yoffey et al (1965) and also in section 1.5.1. Section 1.6. is concerned with some aspects of the properties and effects of this substance.

The extract of the red kidney bean, (*Phaseolus vulgaris*, was first used in 1916 to accelerate the separation of red cells in the preparation of anti-hog cholera serum (Dorset

and Henley, 1916) and later was used by Li and Osgood (1949) to separate erythrocytes from white blood cells. Osgood and Krippaehne (1955) developed a tissue culture method for the study of leucocytes using PHA as a separatory agent. When Hungerford et al (1959) and Nowell (1960) subsequently observed the development of large blastoid cells in PHA-treated cultures of human leukocytes, the origin of the large cells was not clear. However, it was subsequently shown that the small and medium lymphocytes were the cells that underwent blastogenesis (Elves and Wilkinson, 1963; Carstairs, 1961, 1962).

PHA-induced lymphocyte transformation and mitosis has been used extensively for the preparation of human chromosomes, following the development of spreading techniques initiated by Moorhead et al (1960); lymphocytes have probably become the most frequently used source of chromosomes for human karyotyping.

1.4.2. Response to 'specific' antigens

In a short note, Pearmain et al (1963) introduced the discovery of the blastoid response of lymphocytes *in vitro* to the purified protein derivative of old tuberculin antigen (PPD). This phenomenon occurred when the donor of the cells was sensitive to tuberculin. Independently, Schrek (1963),

Roberts (1963), Cowling et al (1963) and Witten et al (1963) observed the same effect. Also about the same time, Elves et al (1963) and Ling and Husband (1964) reported that polio virus, diphtheria toxoid, smallpox vaccine and tetanus toxoid caused blastogenesis in lymphocytes from donors sensitized to these antigens. The same phenomenon was reported for pertussis vaccine and penicillin by Hirschhorn et al (1963).

The antigen-induced blastoid cells that appear in leucocyte cultures are morphologically indistinguishable from PHA-induced blasts, but like the mixed leukocyte reaction (MLR), their apparent response to antigen (in terms of macromolecular synthesis) is much slower in that due to PHA, usually becoming marked after 4-5 days. In addition, the blastoid cells formed are fewer in number than those caused by PHA. Usually, less than 5% of the lymphocyte population respond (ling and Husband, 1964; Cowling et al 1963). However, Cowling et al (1963) reported that after 3 days, 1-5% of the cells were activated, but after 7 days, as many as 20% of the surviving lymphocytes were blastoid. These observations do not, however, rule out the possibility that a few cells may respond at the same rate as observed for PHA stimulation.

The degree of transformation caused by PPD was found to be dependent on the nature of the mycobacterial antigen used (Heilman and McFarland, 1966 a and b; McFarland and Heilman, 1966). Using different PPD preparations, these workers

correlated the intensity of the delayed skin hypersensitivity reaction to the tuberculin with the respective effects of different preparations on the lymphocyte blast response. They found that the mitogenic potential depended on the amount of biologically active protein present, but that the polysaccharaide fractions of PPD were less effective.

An interesting interpretation of the tuberculin response was made by Nowell (1965), using cultures of leukocytes obtained from therapeutically irradiated patients. He found that 6 months after irradiation, unstable chromosome aberrations were present in mitotic cells induced by PPD, but that after 11 months such aberrations were no longer present. However the same samples showed the presence of unstable aberrations in cells which were induced to divide by PHA. He suggested that either the tuberculin-sensitive cells were frequently exposed to antigens, resulting in a relatively early removal of the tuberculin-sensitive cells containing unstable aberrations, or that separate long-lived and shorter-lived lymphocyte populations were present.

The question whether each small lymphocyte simultaneously carried a responsiveness to several different antigens, or whether it is immunologically committed to respond only to one antigen does not appear to have been satisfactorily answered.

However, the former would seem to be more logical, since it is otherwise hard to see how as many as 5% of the cells could respond to a single antigen. On the other hand, the possibility that unsensitized cells may be 'recruited' into transformation has been suggested by Larralde (1970).

1.4.3. 'Non-specific' Stimulation

1.4.3.1. Plant substances

In addition to PHA, other substances have been found to have the property of causing large numbers of the lymphocyte population to undergo transformation. These substances include other plant mitogens, such as the pokeweed mitogen (PWM) from Phylolacca americana (Farnes et al, 1964), extracts from legumes such as Robinia pseudoaccacia and Vica Faba (Hirschhorn, 1965), and most recently, Concanavalin-A (Douglas et al, 1968) from the jack bean, Canavalia ensiformis.

1.4.3.2. Bacterial extracts

Bacterial extracts of the staphylococcus and streptococcus have been found to cause strong stimulation (Ling et al, 1965), like that caused by PHA, although it is not certain whether the pronounced response of lymphocytes to these substance represents a form of antigen 'recognition' in the same sense as that occurring

with PPD. In this respect, it is of interest that Oppenheim and Leikin (1967) reported that human cord blood lymphocytes were markedly activated by staphylococcal filtrate (SF), presumably before the fetus had been exposed to these bacteria. Ling and Holt (1967) also found that maximal responses to SF occurred on the 4th day of leukocyte culture with the material, and that higher doses tended to give a more pronounced and rapid rise of DNA synthesis. These results were considered to favor the 'push' rather than the 'trigger' hypothesis of transformation induction (Ling, 1968).

1.4.3.3. Anti-leukocyte antisera

Another type of 'non-specific' stimulation has been found following the interaction of lymphocytes with human anti-leukocyte antisera (Grasbeck et al, 1963, 1964) and with non-specific antisera to anti-immunoglobulin and subunits, and by anti-allotype serum (Sell and Gell, 1965, Sell, 1967). Sell (1965) suggested that a common mechanism for the specific and non-specific induction of lymphocyte transformation may be the ability of the stimulant to react with protein present in, or manufactured by, the lymphocyte. Many of the agents that induced transformation react with proteins; for example, antisera react with allotypic determinants, IgG or other serum proteins; antileukocyte serum may react with proteins on the lymphocyte; in addition, PHA and other bean stimulants react

with serum proteins to form precipitates (Beckman, 1962; Harris and Robson, 1963). However, SF has not been shown to act in this way.

Antigen-antibody reactions, in the presence of complement, may cause a non-specific stimulation of the non-immunized lymphocyte (Bloch-Stacher and Hirschhorn, 1969).

1.4.3.4. Mercuric chloride

At a concentration of 0.85-3.5 x 10^{-5} M, $HgCl_2$ was found to cause transformation of guinea pig lymph node cells, (Pauly et al, 1969). Concentration greater than 4 x 10^{-5} M were cytotoxic. This was shown first by Schöepf et al (1967) and by Caron and Provost (1970) for peripheral lymphocytes. The degree of transformation was of the same order of magnitude though somewhat less than that due to PHA.

1.4.4. The Mixed Leukocyte Reaction (MLR)

The activation of the lymphocyte by various antigens led to the prediction that the different histocompatibility antigens present on lymphocytes from unrelated individuals would lead to mutual stimulation when the cells were cultured together. Schrek and Donnelly (1961) had noticed that after 5 days incubation of mixed culture, there developed a small

number of large cells with large clear nuclei and prominent nucleoli, some of the cells being in mitosis. This chance observation prompted Bain et al (1964) to study mixed cultures and to demonstrate that large immature mononuclear cells developed in mixed leukocyte cultures from two separate donors, except from identical twins. Independently, Hirschhorn et al (1963) made very similar findings. These observations led to the postulate that the MLR depended on a genetic difference between the donors of the cells.

The subsequent application of the MLR to test potential donors of homografts became a subject of considerable investigation; one of the resultant problems has been the differentiation of the reactivity of each set of lymphocytes, since it is difficult to differentiate between the origin of the blastoid cells in mixed culture. Another problem has been to obtain an earlier response than the 5 days required for the MLR to develop. The problem of the number of histocompatibility antigens has also not been clarified.

1.4.4.1. Characteristic features of the mixed leukocyte reaction

The blastoid cells seen in the MLR are similar morphologically to those which develop in the presence of PHA, except that fewer transforming cells are seen and their maximum response

does not occur until about 5 days have passed. Morphological studies *in vitro* were carried out by McFarland and Heilman (1965). By time-lapse, phase contrast photography, they observed the interaction of the living cells *in vitro* and showed that, in contrast to control cultures, antigen-treated lymphocytes tended to gather in the vicinity of macrophages after the first day; both peripolesis and emperipolesis were observed. After a day had passed, 10 or more lymphocytes were observed to have attached to each macrophage, held to its surface by a 'foot appendage', the 'uropod'. This attachment withstood vigorous cell movements. On the third day, some of the attached cells were seen to have enlarged to several times the size of surrounding lymphocytes. By day 4, further enlargement of these blastoid cells were observed. During the next few days, some blast cells became detached and their places were taken by small lymphocytes. Cytoplasmic extensions between lymphocytes and other cells were noted.

These observations suggest a close functional interrelationship between the macrophage and the lymphocyte, and several studies have been carried out to determine the nature of the response with macrophages. It is interesting to note that Moorhead et al (1967) found that both the MLR and the antigen response became enhanced as the cell density increased;

they interpreted this as potentiating the recruitment of otherwise non-reactive lymphocytes through the mechanism of uropodiapedesis.

1.4.4.2. 'One-way-reactions'

Because of the MLR's potential importance on histo-compatibility testing, considerable attention has been directed to determining the individual reactivity of leukocytes derived each of the donors. The products of distrupted cells have been found to be blastogenic, and in some cases the degree of stimulation have been as in the MLR. Bach and Hirschhorn (1964) freeze-thawed cells five times for the preparation of leukocyte extracts and obtained stimulation. Moynihan et al (1965) found that leukocytes from grafted individuals had responded to freeze-thawed (X5) leukocytes of the donor. Kasakura and Lowenstein (1965) found that the cell-free medium and micro-particulate-free supernatant obtained for 5 days had blast-ogenic activity which was resistant to RNAase and DNAase. They also showed (1967) that this blastogenic factor was probably not produced by blastoid cells, and that a stimu-lation factor was present in the plasma of rabbits with chronic lymphocytic leukemia (1967).

Gordon and McLean (1965) reported that freshly drawn

leukocytes, after having been subjected to various disruptive procedures, were not stimulatory. On the other hand, centrifuged leukocyte cultures, disrupted after 24 hours of incubation yielded a centrifugate and supernatant which were active. The longer the incubation period, the more active was the disrupted material, and the most active component was in the supernatant.

The above mentioned experiments illustrate that a mitogenic material is actively produced by leukocytes in cultures, but is not present immediately after the cells are obtained from fresh blood. If antigens on the lymphocyte membrane are responsible, some factor associated with the culture at initiation must prevent them from being expressed.

One way reactions have also been obtained by rendering one of the lymphocyte populations non-dividing by the use of drugs (such as mitomycin-C), irradiation or by sex typing. Leucocytes when irradiated (400-1500 rad) remained able to stimulate blastogenisis (Kasakura and Lowenstein, 1966), 4000 rad was later found to be the optimum dosage. Bach and Voynow (1966) reported that mitomycin-C prevented the response of lymphocyte to the MLR but did not affect their capacity to stimulate homologous untreated lymphocytes.

1.4.5. The Role of Macrophages in the Response to Antigens and the Mixed Leukocyte Reaction

1.4.5.1. Antigen Response

As already mentioned, lymphocytes may communicate with macrophages by means of a foot process or uropod (McFarland and Heilman, 1965). Removal of most of the phagocytes from a suspension of leukocytes markedly reduces the blastogenic response to antigen by the remaining lymphocytes in the culture (Oppenheim et al, 1966). However this response to antigen can be restored by culturing the lymphocytes with autochthonous macrophage mono-monolayers (Kinoseta and Mori, 1968, Hersh and Harris, 1968). The requirement for macrophages (glass-adherent cells) in the blastogenic antigen response was also shown by McFarland (1967). Increasing the cell density of phagocyte-free cultures has been shown to cause some degree of restoration of the antigen response, possibly by increasing the degree of contact between macrophages and lymphocyte (Leventhal and Oppenheim, 1967; Moorehead et al, 1967). Hersh et al (1970) showed that the concentration-dependence of antigen-stimulated cultures did not occur with PHA.

The mechanism of the macrophage-lymphocyte interaction is not clear. It has been suggested (Fishman and Adler, 1967;

and by Askonas and Rhodes, 1965) that the macrophages take up and 'process' antigen *in vitro*, couple it to an RNA; the resultant immunogenic RNA is assumed to be transferred to lymphoid cells in which it induces an immune response. In support of this concept, Mosier (1967) has demonstrated that a primary antigen response *in vitro* can be induced by non-glass-adherent mouse spleen cells (i.e. lymphocytes) only when glass adherent cells (i.e. macrophages) are present.

1.4.5.2. Role of Macrophages in the Mixed Leukocyte Response

Gordon (1968) demonstrated that the mixed reactivity of lymphocytes obtained from different donors was eliminated when the cells were isolated from other leukocytes, but that their reactivity was restored when purified monocytes were added. Gordon isolated the monocytes by eluting glass bead columns (through which the lymphocytes had already been passed) with EDTA. The resultant mixture of leukocytes, after incubation for a further 4-7 days, was almost a pure macrophage population since the neutrophils had degenerated over this period of time. Gordon suggested that the role of the macrophage in the MLR is to process antigen for the lymphocytes. Jones (1966) and McFarland et al (1966) also concluded that the macrophages played a significant role in the MLR.

Oppenheim et al (1966) came to similar conclusions. They found that the incidence of blast cell transformation in purified lymphocyte cultures was considerably lower than that observed in 'unpure' cultures. However, restoration of reactivity was not brought about by use of a 'feeder-layer' of cells.

An association of the lymphocyte with macrophages is also thought to be significant in the development of primary immune response (Askonas and Rhodes, 1965; Fishman and Adler, 1967).

1.4.6. Stimulation caused by anti-leukocyte serum

Gräsbeck et al (1963, 1964) first showed that leukocyte antibodies were mitogenic. The investigations by Gräsbeck et al were carried out on the reasonable though unproven assumption that the effects of PHA and antigen were due to a cell surface action, and they reasoned that a principle which would attach itself to leucocytes in a manner comparable to PHA might have a similar effect. It is interesting, in this respect, to note that Perlmann (1959) obtained a stimulation of parthenogenetic division in sea urchin eggs exposed to anti-sera. Gräsbeck et al (1964) also could not separate the mitogenic activity of antileukocyte antiserum from its leuk-agglutinating property.

Antibodies against various human antigens have since been tested on leukocyte cultures but only antisera against whole leucocytes were found to be blastogenic (Holt et al, 1966). It is of interest that antibodies against human IgG and IgM were also ineffective, although antisera against rabbit IgG were found by Sell and Gell (1965), and Sell (1967) to be highly mitogenic in rabbit lymphocyte cultures.

Holt et al (1966) reported that the mitogenic effect of anti-leukocyte serum was dependent on the absence of complement. In the presence of complement, the cells were lysed by the antibody. In addition, the blastogenic effect of the antisera was not specific to lymphocytes derived from any single donor. The active principle was shown by Stanworth (1968) to be an IgG.

It may be relevant here to note that antibodies both to whole cells or to surface membranes have been shown to produce lysosomal activation (Darling and Loewi, 1965; Dumonde, et al, 1965). This effect was not complement dependent, and it was suggested by Hirschhorn et al (1965) and Allison and Mallucci (1964) that lysosomal activation is required for the induction of lymphocyte transformation.

1.4.7. Cell-free Factors Causing Lymphocyte Stimulation or Modification of Lymphocyte Function

Interaction of sensitized lymphocytes with antigens results in blast transformation, but a number of 'secondary' effects also have recently been demonstrated. Some of these effects can be correlated with the *in vivo* phenomenon of delayed hypersensitivity. These secondary effects include the elaboration of various cell-free factors including (a) the elaboration of mitogenic substances in the supernatant of leukocyte cultures (b) the formation of a cell-free material which inhibits macrophage migration, and (c) the stimulation or recruitment of non-sensitized leukocytes. In addition, (d) the formation of a 'lymphocyte growth promoter', (e) an effect of cell-free leukocyte extracts (transfer factor) and (f) a cytotoxic action of stimulated leukocytes have also recently become the subject of considerable investigation. The following summary concerns some of the work that has been carried out on these effects with particular reference to lymphocyte transformation.

1.4.7.1. Blastogenic factors in culture medium

The mixed leucocyte response discovered by Bain et al (1964) prompted Kasakura and Lowenstein to investigate whether

factors released into the medium from cultured peripheral blood leucocytes might initiate DNA synthesis in leucocyte cultures from another individual. In 1965, they and Gordon and MacLean reported a blastogenic effect of medium in which leucocyte had been cultured, and that medium from mixed cultures was more active than from unmixed leucocytes. In 1967, Kasakura and Lowenstein described a blastogenic effect both in the supernatant and precipitate of ultracentrifuged medium in which leucocytes had previously been cultured. DNA and RNA synthesis began after 2 days and became maximal between 5 and 8 days after the start of incubation with the medium. They also (1967) described a blastogenic plasma factor in chronic lymphocytic leukemia which passed through a millipore filter of 0.22 microns pore size, and retained its activity after 1 hour at 60°C, after repeated freezing and thawing, or after treatment with RNAase and DNAase.

Hashem and Rosen (1964) reported that subcellular extracts of lymphocyte culture would induced mitoses in vitro in homologous lymphocyte cultures but that RNAase would prevent this effect. In this respect, it is of interest that Weiss and Mayhew (1966, 1967) found that treatment of cells with RNAase caused a reduction of electrophoretic mobility (see section 1.10) and suggested that the loss of charge was associated with

the loss of ionized phosphate groups at the cell periphery caused by RNAase. The possible role of RNA on the cell surface in the transfer of information has been discussed by Weiss (1968).

1.4.7.2. Lymphocyte Mitogenic Factor (MF) in Antigen-treated Cultures

Maini et al (1969) have described a 'lymphocyte mitogenic factor', also termed 'lymphocyte transforming factor' (LTF) in the medium of antigen (PPD)-stimulated lymphocytes. This is a labile, non-dialyzable material that causes normal lymphocytes to become antigen responsive and undergo transformation, repeated cell division and clonal proliferation. This factor caused DNA synthesis to occur in cultures of lymphocytes from both Mantoux positive and negative individuals, and also stimulated autologous lymphocytes, so that the expression of this factor was not dependent on the presence of soluble transplantation antigens.

It is possible that the results of Larralde (1970) may be explained on this basis. It has been observed (see section 1.4.3.) that antigen stimulation leads to a blastogenic and mitotic response from 0.1% to as high as 40%; these values appear rather high for a strictly clonal organization of

immunological memory. Larralde examined the possibility that unsensitized cells were 'recruited' into transformation by sensitized cells in the presence of antigens. Using sensitized lymphoid cells from a male rat, he showed that unsensitized female cells responded to a much greater extent than would be expected on the basis of a mixed culture response, and postulated on this evidence that recruitment of unsensitized cells had occurred.

Spitler and Fudenberg (1970) carried out further studies to characterize the mitogenic factor. They concluded that MF was not transplantation antigen, because it would cause stimulation of normal leukocyte cultures derived from the same strain of animals. Its activity was not enhanced by additional antigen. MF was found to be active on leukocytes from various lymphoid tissues and was heat stable.

1.4.7.3. Lymphocyte Growth Promoter (LGP)

Imrie and Mueller (1968) found that replacing of medium of PHA-stimulated or mixed leukocyte cultures with fresh medium considerably reduced the ability of the cultures to initiate DNA synthesis. When the medium was removed at 3 hours, growth of the cultures could be restored to normal by adding a protein

isolated from the β-globulin fraction of human serum (LGP) or medium from leukocyte cultures. The properties of the factors from either sources were functionally similar. Since leukocyte cultures were used, it was not certain which type of leukocyte produced the factor. The LGP was not mitogenic as was the factor of Kasakura and Lowenstein (1965).

1.4.7.4. Macrophase Migration Inhibiting Factor (MIF)

Bloom and Bennett (1966) and David (1966) simultaneously found that an interaction between sensitive lymphocytes and specific antigen induces the synthesis of a protein which caused normal macrophages to stick to each other, this preventing their migration from capillary tubes. The evidence from this has been reviewed by David (1968). Mixed populations of macrophages and sensitive lymphocytes were required for the effect, macrophages alone were unaffected by antigen. Inhibition of macrophage migration was achieved using a cell-free containing supernatant of sensitized lymphocytes stimulation with antigens. Preliminary treatment of lymphocyte or the supernatant with trypsin rendered it inactive, but RNAase and DNAase did not affect it.

Bloom and Bennett (1968) reviewed the evidence that the migration inhibiting factor could be correlated with a delayed

type hypersensitivity. It was noted that MIF was produced 6 hours to 2 days after exposure of lymphocytes to antigen such as PPD, before lymphocyte blastogenesis occurred, thus implying that lymphocyte transformation is not a requirement for the production of MIF. But normal lymphocytes, cultured in the presence of MIF, appear to undergo enhanced blast cell transformation in comparison to control cells. They suggested that not every transformed cell was 'sensitized' cell, and that the interaction of a few antigen stimulating cells may give rise to the production of a mitogenic substance which could cause the transformation of non-sensitized lymphocytes (see also section 1.4.6.2). Trypsin treatment of lymphocytes abolished their capacity to produce MIF, but they eventually regained this capacity. Thus, there may be a 'receptor' on the surface of lymphocytes which is removed by trypsin but which is eventually resynthesized (see also section 1.10.1.5).

MIF is (a) produced by lymphocytes only from animals showing delayed type hypersensitivity, (b) it is a non-dialyzable, heat stable protein that is probably not an immunoglobulin (c) it inhibits macrophage migration without killing the cells (unlike the cytotoxic action of stimulated lymphocytes), and (d) it is produced by sensitized lymphocytes only upon interaction with specific antigens. After intradermal reaction, MIF produces an accelerated 'delayed type' of

cutaneous hypersensitivity (Bloom and Bennett, 1970).

Thor et al (1968) showed that the MIF was not species specific. For example, MIF from humans would inhibit guinea pig macrophages.

1.4.7.5. Transfer Factor (T.F.)

It was demonstrated in 1955 by Lawrence that delayed hypersensitivity to tuberculin can be passively transferred to a previously tuberculin-negative individual using cell-free leucocyte extracts (transfer factor) from tuberculin-sensitive donors. It was shown that the lymphocyte, rather than the glass adherent cell, is the vehicle for transfer factor. Fireman et al (1967, 1968) demonstrated that the culture of human leukocytes in the presence of this transfer factor resulted in an increased transformation into blasts and stimulation of mitosis. Indeed, in the presence of transfer factor, leukocytes from tuberculin negative individuals showed transformation with PPD. Lawrence (1970) has recently reviewed data on L.T.F. It is a heat stable (56°C), non-dialyzable material that confers delayed sensitivity but not capacity for antibody synthesis. Its elaboration by leukocytes may be very important in the mechanism of the immune response (Lawrence, 1970).

1.4.8. Stimulation of Lymphocytes by Antigens Associated with Neoplastic Tissue

The notion that the body may attempt to reject neoplasms by reacting against antigens unique to the tumor has received increasing attention during recent years (Cooper, 1970). It has been suggested that much 'surveillance' activity is continuous, and that tumor growth may signify a failure of this mechanism. Nevertheless, the extreme complexity of the immune response to the growth of neoplastic tissue must be kept in mind when the apparent stimulation by some tumor extract on lymphocytes is evaluated.

Tumor specific antigens have been identified in a number of different neoplasms induced by chemicals and viruses in laboratory animals (Symposium, 1968) and in human cancer (Hellstrom et al, 1968; Fridman and Kourilsky, 1969).

The findings of cutaneous hypersensitivity to autologous tumor cell extracts (Hughes and Lytton, 1964; Stewart, 1969; Fass et al, 1970) as well as circulating antibodies to melanoma cells (Martin et al, 1968; Lewis et al, 1969) led Jehn et al (1970) to evaluate the effect of melanoma extracts on autologous lymphocytes (from the patients with the disease). These authors presented evidence of sensitization of the patients'

lymphocytes to the melanoma extracts. The antigen(s) responsible for the lymphocyte transformation had the mobility of beta globulins and were also found in the serum and urine. The response was rather greater than expected from the usual response to antigens.

Savel (1969) reported on *in vitro* lymphocyte transformation as a measure of autosensitization to various tumor extracts. Seven out of 44 patients with various tumors gave slight responses to autologous tumor extracts.

1.5. Morphological and Biochemical Aspects of Lymphocyte Transformation

1.5.1. Morphology

1.5.1.1. Gross morphological changes at light microscope magnification

The gradual transition of the small or medium lymphocyte to a 'blast' cell involves intermediate stages which are not clearly defined. Yoffey et al (1965) have detailed some of the morphological changes occurring in PHA-treated cells. After 24 hours, they noted the appearance of transitional (intermediate) lymphocytes with a high nucleus-cytoplasm ratio, but with nuclear chromatin less pachychromatic than seen with the unstimulated cell. After 48 hours, blast cells were present in addition to transitional cells. The blast cells, with diameters of 15-30 μ, contained large or ovoid nuclei with several nucleoli or one large nucleolus, a reduced nucleus/cytoplasm ratio, and basophilic cytoplasm which sometimes contained a perinuclear halo. Many blasts were vacuolated, particularly in older cultures. Considerable variation in the size of blast cells has been observed; this is probably due to uneven spreading of cells on glass slides after drying in air.

1.5.1.2. Ultrastructure of small lymphocytes

'Resting' or 'inactive' lymphocytes usually contribute about 90% of the lymphocytes in the normal human peripheral blood. Electron dense heterochromatin tends to be distributed along the inner aspect of the nuclear membrane. The nucleolus is relatively small. The lighter 'euchromatin' of the nucleus, which is presumed to represent the genetically active chromatin, extends to the nuclear membrane where it is associated with a "fusion" of the inner and outer nuclear membranes. A perinuclear space is often seen to be in continuity with the scanty rough endoplasmic reticulum (in protein secreting cells).

Ribosomes are not clustered and a relatively small Golgi apparatus is seen. Some lymphocytes have a larger Golgi and prominent endoplasmic reticulum suggesting that some lymphocytes may differ in origin or functional state. Inclusion bodies of various kinds have been seen in the cytoplasm, some of which are claimed to be characteristic of disease states. Since they are higher in frequency in patients with severe or chronic illness, Zucker-Franklin (1969) suggests that they may reflect non-specific 'stress' reactions. Several small mitochondria are usually seen.

'Intermediate' cells, seen particularly in dysproteinuria

and some virus infections, are characterized by more endoplasmic reticulum, a larger Golgi apparatus, clustered ribosomes and larger nucleolus.

1.5.1.3. Ultrastructure of blast transformation

The ultrastructure of blast transformation has been studied by Tanaka et al (1963), Marshall and Roberts (1963), Johnson and Roberts (1963), Inman and Cooper (1964, 1965), Elves et al (1964), Parker et al (1965), Chapman (1967a,b) and others.

PHA-cells pass through the ultrastructural changes characteristic of mitosis described for other cells. (Fawcett, 1966; Robbins and Gonatas, 1964; Murray et al, 1965). Most conspicuous in the blast cell are the heavy cytoplasmic concentrations of ribosomes which appear in clusters. Nucleoli may be multiple. However, the endoplasmic reticulum remain sparse in comparison to the plasma cell. PHA cells generally contain lysosome-like electron dense bodies about 0.3 - 0.5 μ in diameter near the Golgi, and stain positively for acid phosphatase. Mitochondria are stated to become numerous (Douglas et al, 1967) and are often swollen, elongated and bizarre (Tanaka et al, 1963; Inman and Cooper, 1963; Parker et al, 1965), although definitive measurements of changes in mitochondrial

number and volume do not appear to have been made.

Douglas et al (1967) and Chessin et al (1966) have cliamed that the response of lymphocytes to pokeweed mitogen (PWM) differs from that to PHA. The developed PWM cells include some distinct cell types, larger than the PHA cell, having nuclei which are eccentric in position. The endoplasmic reticulum is better developed than in the PHA-cell and frequently dilated.

1.5.2. Macromolecular Synthesis Associated with Blast Transformation

Among the most pronounced changes associated with lymphocyte transformation are the increased syntheses of RNA, DNA and protein. Measurements of these parameters using radioactive precursors, particularly H^3-thymidine for the measurement of DNA synthesis have been widely used for the quantitative evaluation of transformation.

1.5.2.1. RNA Synthesis

(a) Effect of PHA on the incorporation of RNA precursors

The cytoplasm of 'resting' lymphocytes contains relatively few ribosomes, when compared to the dense clusters of these organelles in blast cells (Inman and Cooper, 1963).

The increased RNA synthesis is easily detected by an augmented incorporation of radioactive precursor, and it has been well established that RNA synthesis in a lymphocyte population increases within hours of the addition of PHA (Epstein and Stohlman, 1964). The incorporation of H^3-uridine was quite marked at least 1 hour after the addition of PHA-P (Pogo et al, 1966). Actinomycin-D (10 µg/ml) inhibited the incorporation of tracer suggesting that DNA-dependent RNA synthesis was taking place.

Mueller and le Mahieu (1966), using H^3-cytidine, observed RNA synthesis to increase exponentially during a 24 hour period of PHA treatment. The latter authors also noted that the co-carcinogen, croton oil, prevented the induction of DNA synthesis, although permitting RNA synthesis to continue. Kay (1966) observed H^3-uridine incorporation to have increased by about 20% one hour after PHA administration with a more pronounced stimulation after 5-6 hours. In 1969, Kay and Cooper noted that a stronger but variable degree of stimulation could often be observed after 20 minutes to one hour with PHA. The uptake of H^3-cytidine into RNA was stimulated to about the same extent (Parenti et al, 1966).

As pointed out by Kay (1966) the interpretation of initial rises of H^3-uridine incorporation into stimulated

lymphocytes is not simple. This is because of possible effects of PHA-stimulation on the stability of the RNA-synthesized, the reutilization of endogenous nucleotides and the size of the intracellular nucleotide pool.

(b) Possible breakdown of RNA soon after PHA administration: lysosomal activation

Cooper and Rubin (1965) observed a small fall in total RNA, together with an increased incorporation of H^3-uridine into RNA during the first hour after giving PHA, although Monjardino and MacGillivray (1966) could not confirm this.

Hurvitz and Hirschhorn (1965) suggested that the early breakdown of RNA soon after PHA administration reported by Cooper and Rubin (1965) could be due to the release of activation of a ribonuclease, brought about by a PHA-induced lysosomal proliferation (Allison and Malluci, 1964; Hirschhorn et al, 1964). As mentioned in section 1.4.6, antigens to whole cells, which can induce lymphocyte transformation, have been shown to induce lysosomal activation (Darling and Loewi, 1965; Dumonde et al, 1965).

(c) Types of RNA synthesized; centrifugal studies

'Resting' lymphocytes appear to maintain a slow rate

of 45 S precursor transcription and a slow rate of 45 S processing to form 28 S and 18 S rRNA (Rubin, 1970). Cooper and Rubin (1965, 1966) reported that the largest fraction of rapidly synthesized RNA in lymphocytes immediately following PHA administration was non-ribosomal. This was because its sedimentation profile in a sucrose gradient was unlike ribosomal RNA or precursor, it was relatively unstable to chase with actinomycin-D, and its nucleotide component was unlike that of ribosomal RNA.

Torelli et al (1968) found that the unstimulated small lymphocyte synthesized an RNA of high molecular weight which was primarily not a ribosomal precursor, and which was partly complementary to DNA. Stimulation by PHA brought about a cell population in which a large amount of ribosomal RNA was produced.

Cooper (1969) proposed a regulatory system for the lymphocyte based on the formation of ribosomal molecules. This was based on the finding that in growing lymphocytes, there was an equal amount of 19 S and 32-28 S rRNA present. However, in resting lymphocytes, there was 32-28 S rRNA, but about 50% less 18 S RNA. The deficiency was found to be the result of nuclear retention of newly synthesized 32S and 28S rRNA without corresponding 18 S molecules. Cooper proposed

that half of the 18 S molecules are lost, presumably through degeneration, and that this 'wastage' of rRNA in resting lymphocytes is part of a control mechanism which regulated lymphocyte growth by limiting the accumulation of ribosomes.

Rubin (1970) proposed that ribosomal RNA production in cultured lymphocytes may be regulated by the rate of 45 S RNA transcription and also by the rate of 45 S processing to 28 S and 18 S components of mature ribosomes. The lack of 18 S components in 'resting' lymphocytes, as a result of selective degradation, would reduce ribosomal formation. The initiation of lymphocyte growth and the maintenance of the growing condition might thus depend on the efficiency of 45 S rRNA precursor utilization for the assembly of ribosomes.

(d) Stability of RNA synthesized soon after PHA treatment

Kay and Cooper (1969) reported that the early stimulation of incorporation of H^3-uridine into cytoplasmic RNA was greater than the stimulation of formation of total cellular RNA. The rapidly labelled cytoplasmic RNA was mostly not rRNA but was predominantly 4 S. PHA stimulated the labelling of cytoplasmic RNA components uniformly. The authors suggested that PHA stimulated the formation of an unmethylated tRNA precursor.

Darzynkiewicz and Piankowski (1969) showed, by autoradiography, the existence of 2 pools of pulse labelled RNA with different turnover rates. The T1/2 survival rates and respective proportions of the total pulse labelled RNA were characterized by values of 25 min (75%) and 300 min (25%). The most rapid pool appeared to be located in the nucleus. These results were confirmatory of the reports by Kay and Korner (1966) and Rubin and Cooper (1964) as to the instability of a large portion of the pulse labelled RNA. This fraction is not preserved after 1 hour of chase with actinomycin-D and unlabelled uridine. The role of this fraction is unknown but it might be messenger RNA. Rapid degradation of pulse-labelled RNA has also been reported for chronic and acute leukemic cells (Sinks and Hayhoe, 1967; Pileri et al, 1968).

(e) Induction of Uridine Kinase

Lucas (1967) showed that the PHA-stimulated incorporation of H^3-uridine into lymphocyte RNA coincided with a pronounced increase (by 20 fold) in the specific activity of uridine kinase. This increase was subject to end product inhibition by cytidine. Lucas suggested that the initial rate limiting factor determining the incorporation of uridine into RNA may be the enzymatic phosphorylation of uridine by uridine kinase, and that the loss of cytidine metabolite from the cell might derepress on the cellular mechanism required for the synthesis of uridine and cytidine kinase.

Hausen and Stein (1968) independently demonstrated the induction of uridine kinase in lymphocytes treated with PHA. They also found that the enzyme activity decreased if the cells were treated with antibodies against PHA. Their data indicated that the rate of uridine incorporation into TCA-insoluble material was influenced by the processes which transfer the external uridine into the UTP pool of the cell. Furthermore, the rate of uptake of uridine was dependent on the external uridine concentration. They caution that the actual stimulation of RNA synthesis after PHA may be much smaller than that indicated by the uridine incorporation.

1.5.2.2. DNA Synthesis

(a) Kinetics of DNA synthesis

It has been shown that for human lymphocytes, the induction of DNA synthesis is delayed for at least 24-30 hours after the start of culture with PHA (Bender and Prescott, 1962; Cooper et al, 1968; MacKinney et al, 1962; Tormey and Mueller, 1965), after which period the rate of DNA synthesis increases markedly. It was also shown that the cells which undergo DNA synthesis are the large 'blast' cells and not the small lymphocytes. However, a much more rapid response to PHA (compared to human cells) has been observed with kangaroo lymphocytes (Burton, 1968).

Bender and Prescott (1962) found that mitoses were absent in PHA-treated cultures prior to 36 hours with the mitogen, but that numberous mitoses accumulated after 42 hours of culture. After 72 hours, 74/75 metaphase figures showed labelling of both chromatids, indicating that the majority of the cells were undergoing first division. However, this information on the timing of the first divisions has been questioned by Buckton and Pike (1964), who reported a larger number of second divisions after 72 hours with the mitogen.

Sasaki and Norman (1966) found that not more than 30% of the mononuclear cells in PHA cultures began DNA synthesis by 72 hours. However after transformation had occurred, the cells proliferated with a generation time of about 22 hours and by 72 hours, at least 70-80% of the cells were in second division, as indicated by experiments of chromatid labelling with H^3-thymidine. The possibility that human lymphocytes may show variations in the time of onset of PHA-induced division, for different donors, does not appear to have been examined.

Michalowski (1963) studied the course of DNA synthesis for a 80 hour period following the stimulation of human leukocytes with PHA-P. He labelled the cells with C^{14}-thymidine

for 2 hour periods at 6 hour intervals. The uptake of thymidine was extremely low during the first 30 hours and then it increased to a maximum at 60 hours, dropping after about 66 hours. Using colcemid, he found two different peaks of synthesis at 65 and 77 hours.

(b) Patterns of synthesis

Cooper et al (1963) examining DNA synthesis of PHA-treated leukocytes, added DNA precursors (H^3-deoxycytidine, C^{14}-formate, H^3-thymidine) to a population of cells. Autoradiographs showed that only a proportion of cells were labelled and there was considerable variation in nuclear grain counts. Direct DNA measurements by microdensitometry of the Feulgen stain showed DNA values ranging between 2 n and 4 n for the labelled cells, whereas unlabelled cells were either 2 n or 4 n.

Prescott and Bender (1963) described a diffuse and a localized pattern of labelling with H^3-thymidine interphase nuclei. Lima-de-Faria and Reitalu (1963) demonstrated the incorporation of H^3-thymidine on the heterochromatin situated around the nucleolus of the cultured leukocytes. Ribas-Mundu (1966) found three patterns of DNA replication in the interphase nuclei of short term leucocyte cultures. These included

(1) a light, homogeneous labelling in the whole nucleus without labelling over the nucleolus, (2) heavy labelling over the whole nucleus and nucleoli, and (3) heavy labelling over the nucleoli and moderate labelling at the periphery of the nucleus. The author concluded that the nucleolar labelling corresponded to DNA synthesis of intranucleolar and nucleolar-associated chromatin, the existance of which was previously demonstrated by Bernhard and Granboulan (1963).

Asynchrony of chromosome replication was observed by Bader et al (1963) who observed by autoradiography that some of the large chromosomes had completed duplication whereas small ones were still synthesizing DNA.

(c) Relationship of DNA synthesis to dose of mitogen

The relationship of DNA synthesis to the dose of mitogen is of considerable interest. Ling and Holt (1967) found that increased amounts of PHA led to a more rapid onset of DNA synthesis, but the maximum response to PHA occurred on the 3rd day of culture. The response of the cells to staphylococal filtrate (also a 'nonspecific' mitogen) was similar but the maximum response occurred on the 4th day. It was suggested that the overall rate of entry of the cell population into DNA synthesis is a function of dose of the mitogen.

(d) Synchronization of DNA synthesis

The degree of synchronization of DNA synthesis in lymphocytes exposed to PHA is rather low, although the cells are all exposed at the same time to the stimulant. This is illustrated by some of the autoradiographic data already described (this section part b). Partial synchronization has been achieved in a number of ways. These have included the use of excess thymidine by Kasten and Strasser (1966) and of amethopterin (methotrexate), an antagonist of tetrahydrafolic acid which is required for the biosynthesis of thymidylic acid. Petersen (1964), incubated cells in amethopterin for 10-18 hours. After removal and culture in original medium, a degree of synchrony was achieved. Tormey and Mueller (1965) treated the cells for 48 hours with PHA, after which time amethopterin was added. After a further 16 hour period, C^{14}-labelled thymidine was added. Addition of the thymidine caused a surge of DNA synthesis in all cells of the population.

A problem in the use of tritiated thymidine to measure DNA synthesis in these cells has been the capacity of these cultures to break down, or degrade the nucleoside. The breakdown seems to be more pronounced in the presence of polymorphonucleocytes (Cooper and Milton, 1964). In addition,

the breakdown products of DNA may be utilized by other cells (Hill, 1967).

(e) Enzyme induction

DNA polymerase was shown to undergo a slight increase in activity during the first day of lymphocyte culture with PHA, and then a pronounced increase in parallel to the timing of the onset of DNA synthesis during the 2nd and 3rd days of culture (Loeb et al, 1968; Rabinowitz et al, 1969). Loeb et al suggested that induction of DNA polymerase is a factor in the control of DNA synthesis during lymphocyte transformation.

In a study of the thymidine salvage pathway, Rabinowitz et al (1970) found that in culture of PHA-stimulated lymphocytes, thymidine kinase activity increased in parallel to DNA synthesis.

1.5.2.3. Protein synthesis

(a) Kinetics of Protein Synthesis

Like the stimulation of nucleic acid synthesis, protein synthesis has been found to increase following the administration of PHA and other mitogens to lymphocytes in vitro. This increase is to be expected in view of the pronounced increase in cytoplasmic volume and organelles (Carstairs, 1962; Johnson and Roberts, 1964; Marshall and Roberts, 1963; Tanaka

et al, 1963). Kay (1966) found that the incorporation of C^{14}-leucine was increased 3 or more hours following the administration of PHA, and became pronounced after one and 2 days, after which a decline in incorporation was noted. C^{14}-phenylalamine was incorporated into protein as new RNA appeared (Hirschhorn et al, 1963). Similar results were obtained by Trowell (1965), Cooper (1961) showed that the large lymphocytes were the cells that labelled most heavily with H^3-leucine. Sell et al (1965) found considerable turnover of C^{14}-lysine in cultured rabbit lymphocytes although this did not become markedly increased until 24 hours after the stimulant (staphylococcal filtrate or PHA) had been added.

Cooper et al (1967) showed that the degree of protein synthesis and DNA synthesis by human lymphocytes in the presence of PHA was strongly influenced by the amount of human serum present in the culture medium. Synthetic activity was maximal at approximately 5-15% serum, but became markedly reduced at higher serum concentrations. Stimulation of protein and DNA synthesis was greatest in the presence of autologous serum; other human sera or calf serum reduced the degree of stimulation.

Bach and Hirschhorn (1963) found that if actinomycin-D was added to lymphocytes grown in the presence of PHA,

an initial burst of protein synthesis occurred during a 2 hour period and then stopped. Since this drug blocks DNA-dependent RNA synthesis, the evidence suggested that small lymphocytes contain endogenous messenger RNA.

(b) Nature of proteins formed

Soon after the response of lymphocytes to specific antigens was discovered, considerable work was carried out to determine whether stimulated lymphocytes produce immunoglobulins. Several workers have indeed reported that PHA-induced transformation is associated with a stimulated synthesis of Ig (Hirschhorn et al, 1963; Turner and Forbes, 1966; Paresti et al, 1966).

Some of the protein formed appear to be gamma globulin (Bach and Hirschhorn, 1963). Fluorescein-conjugated anti-globulin was taken up by 'blast' cells but not by unstimulated lymphocytes, suggesting antibody production by the blast cells (Hirschhorn et al, 1963). The types of protein produced by lymphocytes appear to include all three classes of gamma-globulin and perhaps alpha globulin (Hirschhorn et al, 1963; Cooperband et al, 1964).

Detailed immunofluorescent studies of immunoglobulin content from peripheral cells was made by van Furth et al (1966).

He found that some of the medium sized lymphocytes stained for IgA, IgG, and possibly for IgM.

Forbes and Turner (1965) found that PHA caused a stimulation of globulin synthesis. But lymphocytes from patients with chronic lymphocytic leukemia and macroglobulin anemia failed to produce more globulin under PHA stimulus. The increase due to PHA was observed after 24 hours, but not 6 hours after administration of the mitogen. In another paper, Forbes (1965) reported that the leucocytes from patients with Hashimoto's disease synthesized precipitating antithyroid globulin, and that the synthesis of this antibody was stimulated by phytohemagglutinin.

Turner and Forbes (1966) calculated that mononuclear cells synthesized 3 micrograms/10^7 cells of IgG-like protein in 48 hours.

Quaglino et al (1962) pointed out that since the synthesis of new protein by blast cells is mainly structural and enzymatic, only a fraction of the total protein synthesized would be expected to represent Ig synthesis. With the exception of lymphocytes from Hashimoto's disease, Greaves (1966) was unable to detect an enhancement of Ig by PHA, and suggested that almost all the protein synthesized under the influence of PHA for 72 hours was required for cell growth and division.

Asofsky and Oppenheim (1966) reported that the proteins formed in PHA treated lymphocyte cultures depended on the time of incubation. Some globulins were not detected until after 60 hours of culture.

There is some evidence for specific antibody production following antigen stimulation, Elves et al (1963) found that lymphocytes stimulated with specific antigen produced specific antibodies. These workers found that lymphocytes from diphtheria sensitive donors produced anti-diphtheria antibody when stimulated with diphtheria antigen.

Immunofluorescent studies showing an increased reaction to human antiblobulin were carried out by Mazzei et al (1966), who showed that immunofluorescence was considerably greater with antigen-stimulated cells in comparison to PHA-stimulated cells, although the latter were much more numerous and developed earlier in time.

Girard also studied the production of immunoglobulin by antigen-stimulated lymphocytes. In 1967 he observed that anti-penicillin could be obtained by stimulation of peripheral lymphocytes from sensitive subjects after 6-8 days of culture in the presence of the antibiotic. In 1968, he examined the relationship between the production of antibodies and the

induction of blastogenesis, and found that antigen-induced transformation became maximal after 6 days of incubation. The antigen caused an increased incorporation into protein of labelled amino acid, which also became maximal after 5-6 days of culture. Hemagglutinating and skin sensitizing antibodies in patients allergic to penicillin or pollen were obtained *in vitro* after about the same period of time.

Chessin et al (1968) provided evidence that circulating human lymphocytes can synthesize immunoglobulins *in vitro*, by demonstrating the incorporation of C^{14}-amino acids into protein which were identified by radio-immunoelectrophoresis. There were two functionally distinct populations of immunoglobulin synthesizing cells in peripheral blood; one of these populations included small lymphocytes, and the other included other leukocytes. The immunoglobulins were isolated from the culture medium.

In summary, at least part of the peripheral lymphocyte population would appear to be capable of synthesizing Ig. Most of the increased protein synthesis following PHA stimulation is probably structural or enzymatic, but stimulation of cells from pre-sensitized donors by specific antigens appears to cause production of specific antibody production *in vitro*.

Other proteins produced by stimulated lymphocytes include interferon (Wheelock, 1965). Apparently, interferon production by lymphocytes requires the presence of PHA in the medium, since its production ceased upon the removal of PHA (Cooper and Friedman, 1966).

1.5.3. Cellular changes associated with transformation

1.5.3.1. DNA-protein (DNP) complex

In an essay on the biochemistry and significance of the histones, T'so and Bonner (1964) discussed the regulation of DNA synthesis and RNA synthesis by histones inside the cell, and suggested that a suitable experimental system "should be one in which the activity of the cell goes through a drastic change, a dramatic awakening from a dormant to an ultra-active state". They suggested that it should be possible, given such a system, to determine and compare the molecular state of the histone and DNA after the change from inactivity to activity. The transforming lymphocyte system is clearly of this type.

(a) Acelylation and phosphorylation of histones

Until 1966, little was known of the initial changes of the nuclear histones associated with the stimulation of the

small lymphocyte from the 'resting' state. Pogo et al (1966) demonstrated that within the first 15 minutes after phytohemagglutinin administration, acetate-methyl-H^3 was taken up at an increased rate, apparently into the arginine-rich histones. However, the lysine-rich histones showed only a very small rise in specific radioactivity. This uptake was shown to occur well in advance of the increase in RNA synthesis. They concluded that acetylation of the histones precedes the increase in nuclear RNA synthesis. The phenomonon was not observed with some lymphocytes or with non-lymphocytic leukocytes, as also shown by Mukherjee and Cohen (1969).

During 1966, Kleinsmith et al, in an investigation concurrent to that of Pogo et al (1966), showed that the onset of RNA synthesis in PHA-stimulated lymphocytes was preceded by an early stimulation of the phosphorylation of nuclear proteins. They concluded that this finding was consistent with a role of phosphoproteins in the modification of chromatin structure and in the modulation of DNA template activity. A stimulation of P^{32} and C^{14}-acetate incorporation into histones was also observed during the first 20-30 minutes of PHA treatment (Cross and Ord, 1970).

(b) Binding of acridine orange, fast-green, bromphenol blue and actinomycin

Since histone occurs in combination with the chromosomal DNA, a change in chromosomal fine structure or function is presumably initiated, and it is interesting to note the finding of Killander and Rigler (1965), that lymphocytes stimulated by phytohemagglutinin rapidly increase by more than five fold their capacity to bind acridine orange to DNA.

Black and Ansley (1965) stained lymphocytes for histone using both the alkaline-fast-green method and the ammoniacal-sliver technique. This work, done with lymphocytes in vivo, was performed following the injection of antigen (tetanus-toxoid) into rats that had been previously immunized against the substance. It was found that a significant reduction in the intensity of histone staining occurred in lymphocyte nuclei within hours after injection of the antigen. The reduced staining of histone found after the injection of antigen was interpreted by the authors as indicating an altered DNA-dependent RNA synthesis.

Similar observations have been made by Burton (1968) and by Zetterberg and Auer (1969) on the binding of Bromphenol blue to histones of lymphoid cells activated by PHA. Surprisingly, it appears that the DNP change occurs not only in the lymphocytes but also in the PMNs, although the latter population has not been observed to undergo transformation.

Darzynkiewiez et al (1969) have shown that PHA-stimulation of lymphocytes results in an increased ability of the chromatin to bind H^3-actinomycin. Like the acetylation, phosphorylation and staining changes of the nucleohistone, this phenomenon was observed to occur before RNA synthesis is initiated. The same authors (Ringertz et al, 1969) showed by a double labelling technique that the same cells took up C^{14}-acetate also were stimulated to incorporate H^3-uridine.

(c) Role of histones

Frenster et al (1963) found that repressed and active chromatin could be isolated in mass from interphase calf thymus lymphocytes, and that up to 80% of the interphase lymphocyte DNA was found in the repressed chromatin fraction. (Repressed interphase DNA appeared as condensed heterochromatin, where active interphase DNA appeared as extended euchromatin fibrils). The latter fraction was observed to contain most of the rapidly labelled RNA of the nucleus. They found that the active extended euchromatin fibrils had a slight deficiency of histone in relation to their DNA content. The role of histone in the maintenance of the chromatin structure of lymphocytes was also examined by Littau et al (1965) who concluded that the dense inactive chromatin of thymus lymphocyte nuclei is formed by lysine-rich histone cross-linking the chromatin fibrils.

The arginine-rich histone was considered to be bound to the chromatin fibrils but not to be involved in their cross-linking. These observations, taken in conjunction with those of Pogo et al (1966), indicate that the histones may have both a structural and repressor function. That is, the arginine-rich histone could be more involved as a repressor substance, and the lysine-rich fraction could have a structural function.

Ringertz (1969) discussed the problem of cytochemical changes of nucleoprotein in relation to nuclear function. He considered that the nucleoprotein changes observed in the PHA-activated lymphocytes (Bolund et al, 1969) may represent a 'chromatin activation reaction'. This would be part of a genetic control system operating by the activation or deactivation of genes or extra nuclei. Ringertz suggested that rather simple signals such as an altered ionic environment around the deoxynucleoprotein complex may be capable of inducing the reaction, and that the transport of ions and other low-molecular material across nuclear and cell membranes may be important factors in controlling the reaction.

The distribution of internal ions, both divalent and nonovalent, may be of significance in the mechanism of chromatin derepression and the unmasking of phosphate groups in the DNA molecule. In this respect, it is of interest that Kroeger (1966)

found the activation of a specific gene locus in giant insect chromosomes to be controlled by the Na^+-K^+ balance within the nuclear compartment.

1.5.3.2. Early effects of PHA on cell membrane function

In 1.5.2. and 1.5.3.1., some of the effects of PHA on lymphocytes were considered, and early stimulating effects on RNA synthesis, including the possible degradation of pre-existing RNA, stimulation of RNA and protein synthesis, acetylation of nucleoprotein and increased turnover of phosphoproteins were described. Such effects resemble the changes occurring in regenerating liver (Pogo et al, 1966). The following section examines certain early effects of the mitogen which alter the dynamic state of membrane structure and function.

(a) Endocytosis

In 1968, Hirschhorn et al described an effect of PHA on the redistribution of acid hydrolases in lymphocytes; previous work by these authors had indicated that lymphocyte lysosomes may mediate some of the metabolic alterations of the transforming lymphocytes (Hirschhorn and Hirschhorn, 1965). They showed that cytoplasmic granules which were isolated from lymphocytes and which had the characteristics of lysosomes

showed changes in the distribution of acid hydrolases. This was associated with an increased permeability of stimulated lymphocytes to neutral red in the surrounding medium. This effect occurred about one hour after PHA administration. The results were compatable with the hypothesis that PHA induced early changes in the lymphocyte surface which led secondarily to lysosomal changes. The authors showed that these changes were energy-dependent.

(b) Phospholipids

Fisher and Mueller (1968) studied the incorporation of Pi^{32} into the phospholipids of the PHA-treated lymphocyte in order to obtain direct evidence for the interaction of PHA with the membrane. They found that the rate of phosphate incorporation into phosphatidyl inositol was markedly accelerated within 10 minutes of adding the PHA. However, incorporation into phosphatidyl choline and a phosphatidyl serine ethamolamine mixture were at first only slightly affected. The degree of activation of $P^{32}O_4$ incorporation into phosphatidyl inositol was approximately proportional to the dose of PHA, with a plateau and half maximum concentration similar to that required for the stimulation of DNA synthesis. Incorporation into phosphatidyl inositol and phosphatidyl thamolamine became pronounced after 4 to 12 hours with PHA.

The stimulation also occurred in cells in which protein synthesis was blocked, indicating that PHA caused activation of existing enzymatic machinery in the phosphatidyl inositol pathway. The results could be explained by increased synthesis and turnover of the phospholipid, or by an increased specific activity of a phosphorylated precursor. The latter was unlikely because an increase in the specific activity of ATP had not been observed by Kleinsmith et al (1966).

These workers suggested that PHA might stimulate phosphatidyl inositol synthesis by enhancing the synthesis of phosphatatic acid, possibly by stimulating the interconversion of phosphatidic acid and phosphatidyl inositol (Atherton et al, 1966; Friedal et al, 1967).

A similar phospholipid response was reported by Hokin and Hokin (1958) for the effect of acetylcholine on the pancrease. They suggested that the acceleration of Pi^{32} uptake into phosphatidyl inositol and phosphatidic acid is associated with a stimulation of membranal transport (1965).

Fisher and Mueller (1969) also have shown that the incorporation of label from choline and C^{14}-labelled methionionine into phosphatidyl choline is stimulated by PHA. This early increase was unaffected by the presence of cycloheximide or actinomycin-D activity in later stages of transformation.

Only after six hours of stimulation did the addition of cycloheximide prevent further acceleration of choline incorporation. Kay (1968) also has reported that PHA-induced a stimulation of the incorporation of C^{14}-choline into the lipid fraction of lymphocytes.

(c) Amino acid transport

Little work has so far been done on the study of transport by lymphocytes. However, Mendelsohn et al (1970) has recently described an early and pronounced stimulation by PHA of the transport of α-amino-isobutyric acid into the cell. This stimulation was characterized by an increase in the V_{max} without change in the K_m determining the kinetics of transport.

1.5.3.3. Effect of PHA on cell size

It is unfortunate that little accurate information is available on the early changes of cell volume associated with the early effects of PHA, although later increases of volume (after more than one day with PHA) are obvious. One of the reasons for this is the agglutinating effect of PHA on the cells, which makes it difficult to estimate cell volume by direct observation or with the electronic cell counter.

Volume measurements have been carried out for the unstimulated lymphocyte and appear to agree reasonably well. Westring et al (1968) obtained a mean volume of 250 μ^3. The mean volume estimated by Lichtman and Weed (1969) was 269 μ^3.

Michalowski (1967, 1968) made careful measurement of cell size in air dried methanol fixed and May Grunwald-Giemsa stained smears in the presence and absence of PHA at various doses. He showed that the size distribution of unstimulated lymphocytes was skew from normal and suggested the existance of two populations based on cell size. The skew was markedly increased in 71 hour cultures, because of the increased number of blastoid cells.

A rough idea of the time course of events is given by Yoffey et al (1965). At zero time, lymphocytes were about the size of red cells (6-8 μ diameter) with densely pachychronatic nuclei occupying most of the cell volume. After 24 hours, transitional (intermediate) lymphocytes observed. These had a high nucleus:cytoplasm ratio. The cytoplasm was polar in distribution and the cells were slightly larger than small lymphocytes. A few blast cells were also observed, but these formed a very small proportion of the cell population, in comparison to the appearance of the cultures after 48 hours.

At 48 hours, there were numerous large cells, 15-30 μ in diameter, as well as transitional cells 10-20 μ or more in diameter. Mitoses were present. By 72 hours, mitoses and larger blast cells were numerous. Cell surfaces were irregular and many of the cells were vacuolated and appeared fragile.

Robinieaux et al (1968) studied the morphology of lymphocytes treated with PHA by continuous time lapse cinematography. The main volume changes in the cell that he studied occurred between 20-44 hours in the presence of PHA. During this time, the nucleus/cytoplasm ratio decreased from 0.7 to 0.3.

1.5.4. Respiration and glycolysis in lymphocytes and the effect of PHA

Determination of the respiratory and glycolytic activity of lymphocytes became possible when methods for the separation of these cells from other blood elements were developed. While a large amount of information relating to the metabolism of the circulating leukocyte is available (Cline, 1965), it is only marginally useful in the case of lymphocytes, since most work until recently has been carried out without separating the different types of leukocytes.

Nowell (1960) demonstrated that lymphocyte transformation can occur in the absence of oxygen. It was therefore suggested that anaerobic glycolysis is the cell's primary source of energy, since glucose is utilized and lactic acid produced (Cooper et al, 1963; Parker et al, 1965).

It has been shown, by periodic acid-Schiff staining reactions, that stainable glycogen increases in blastoid cells during the first day after PHA treatment, but later disappears as dehydrogenase activity rises during the 30-32 hours of culture with PHA (Quaglino et al, 1962; Rosenszajn and Fischer, 1969). However these authors could not correlate accurately the degree of blastogenesis with glycogen synthesis in PHA-treated cells.

Quaglino et al (1962) suggested that since PHA-induced blast cells do not normally appear in cultures until 30-48 hours, the raised level of glycogen observed may be required for subsequent transformation. Quaglino and Cowling (1964) found that lymphocytes from chronic lymphocytic leukemia responded poorly to PHA and there was an absence of the rise of the glycogen content of the cells in the early stages of culture.

Lymphocytes treated with PHA were found to show a marked increase in glucose consumption and lactic acid production (Cooper et al, 1963; Parenti et al, 1966). Hedeskov (1968) has studied early effects of phytohemagglutinin on the glucose metabolism of human lymphocytes. After a period of 4 1/2 hours with PHA, glucose utilization and lactate products were both doubled. Pyruvate formation and fatty acid synthesis were also increased. The pentose cycle and glycogen synthesis were also slightly enhanced, but less than that of glucose utilization. Puromycin inhibited the PHA-induced stimulation of glycolysis and lactate production.

Rabinowitz and Dietz (1967) therefore examined the production of lactic dehydrogenase (LDH) and malate dehydrogenase (MDH) by stimulated lymphocytes. Stimulation of LDH (M-type) was observed within 4 hours of stimulation, increasing linearly over a 48 hour period, and an increase

in MDH also was seen within 24 hours. However, there was a change in the LDH isozyme pattern following PHA. The authors concluded that PHA must somehow alter the activities of the genetic loci for H and M type LDH subunits, and suggested that PHA may act at allosteric sites on protein repressors or inducers of operator genes for the H and M type polypeptides in accordance with the theories of Monod et al (1963). An increase of M subunits was also found by Bloom et al (1966). Hellung-Larsen and Anderson (1968) also studied LDH isoenzymes in PHA-treated lymphocytes, showing that the system was affected by oxygen. At low pO_2, there was a marked increase in the M-containing isoenzymes, whereas high pO_2 tended to increase the H-containing isoenzymes.

N-acetyl-D-galactosamine, which inhibits the leucoagglutinating and mitogenic action of PHA (Silber et al, 1966) also inhibited the early PHA-induced stimulation of glycolysis. Hedeskov (1968) suggested that the most likely cause of these effects is an increased synthesis of hexokinase, but that enhancement of glucose transport, or of the intracellular concentrations of certain nucleotides or phosphate levels which affect glucose phosphorylation had also to be considered.

Polgar et al (1968) evaluated respiration and glycolysis as energy sources for PHA-induced lymphocyte transformation.

However these workers were unable to demonstrate an increase in the respiratory activity of lymphocytes, even after 4 days of PHA treatment. Moreover, dinitrophenol, which inhibits oxidative phosphorylation, had no significant effect on DNA formation at a concentration of 2.5×10^{-4}M. On the other hand, deoxyglucose, which inhibits glycolysis at the phosphohexoisomerase step, strongly inhibited DNA, RNA and protein synthesis to the level of unstimulated cultures. They admitted that while mitochondrial oxidative phosphorylation would provide a much greater supply of ATP per molecule of glucose utilized, it was necessary to conclude that the contribution of mitochondrial activity was minimal.

The respiration and glycolysis of normal and PHA stimulated lymphocytes was also investigated by Pachman (1967). Using horse lymphocytes, she reported that not only was there a marked increase in glucose consumption and leutate production, but unlike Polgar et al (1968), found that the oxygen consumption by the cells was also increased. Pachman cautions that the evaluation of glycolysis and respiration on a cellular level must be interpreted with reserve because of the problem of accurate evaluation of cell number.

Stjernholm and Falor (1970) have studied relatively early (4-5 hours) metabolic changes in PHA-treated lymphocytes

and found elevated Krebs cycle activity as well as increased glucose utilization. More than 90% of glucose was metabolized via the glycolytic pathway. There was a slight increase in lipid production and in pentose cycle activity with PHA as found by Hedeskov (1968). The increment in the oxidation of acetate $C^{14}O_2$ indicated that more ATP was being produced via oxidative phosphorylation, in apparent disagreement with the results of Polgar et al (1968). Stjernholm and Falor suggest that a control mechanism for the metabolism of acetyl-Co-A to CO_2 via the tricarboxylic cycle may be the availability of oxalacetate as a 'sparker', and the conversion of aspartate to oxalacetic by transamination may be regulating factor. Hence absence of aspartate from the medium, or failure to transport it into cells, might lead to a poor respiratory response such as that found by Polgar et al (1968).

A marked 'crowding effect' was demonstrated by Hedeskov and Essman (1966) to be characteristic of the respiratory activity of lymphocytes in vitro. The more the cells were crowded, the lower was the oxygen consumption per cell. A similar but weaker crowding effect was noted for lactic acid but not for the utilization of glucose. They also demonstrated small Pasteur and Crabtree effects in cultured lymphocytes.

1.6. PHYTOHEMAGGLUTININ (PHA)

The effects of the substance present in the aqueous extract of the red kidney bean (Phaseolus vulgaris) were reviewed by Naspitz and Richter (1968), but it will be appropriate here to consider briefly the nature of its interaction with lymphocytes and other cells, since the use of PHA has been the primary mode for the induction of lymphocyte transformation in this study.

It is now clear that lymphocyte transformation can be brought about by a number of diverse agents of very different chemical structures, and that PHA is only one of such substances. Hashem and Kabarity (1966) found that extracts from broad beans, lentils, fenugreek and peas were active mitogens. An extract from the pokeweed was found to be highly potent (Farnes et al, 1964; Borjeson et al, 1966). The jackbean has yielded Concanavalin-A, also a mitogen. Various antigens, antisera and mercury are effective (Section 1.4).

1.6.1. Erythroagglutination and Leukoagglutination by PHA

Landsteiner discovered the hemagglutinating properties of PHA in the early 1900's (see his 1936 paper). Dorset and Henley (1916) used PHA to remove red cells in the preparation of hog cholera antiserum. In 1940, Li and Osgood utilized PHA

to obtain red cell-free leukocytes, and Osgood and Krippaehne (1955) used it in the preparation of leukocytes for in vitro culture. However, it was not until 1959 that Hungerford and his colleagues reported the blastogenic effect of the bean extract. Soon after, Haskings et al (1961) observed that mitoses occurred in white cell clumps and suggested that leukagglutination might be a necessary condition for the onset of mitotic activity.

Phytohemagglutinin causes agglutination of blood cells from a wide variety of animal species, and the property of red cell agglutination can be removed (by incubation of the substance in the presence of red cells) without affecting its leukoagglutinating property (Barkham and Ballas, 1963; Borjeson et al, 1964; Kolodny and Hirschhorn, 1964; Nordman et al, 1964; Robbins, 1964). However the agglutination of white cells was not separated from the mitogenic property until the work of Borjeson et al (1967), who demonstrated that Salmonella typhosa Vi polysaccharide (an N-acetyl galactosamino-sucric acid polymer) would dissociate leukagglutination from the transforming property of PHA, The substance inhibited leukag-glutination by PHA but not mitogenesis.

Leukagglutination was shown by Boberg et al (1966) to be prevented by N-acetyl-D-galactesamine. These workers suggested

that since this sugar is a constituent of the cell surface, it might act as a hapten and participate in the interaction between PHA and the cell surface. Presumably, the sugar would bind to PHA and prevent attachment to chemically similar sites on the cell surface. Unfortunately, these workers did not examine the mitogenic potential of PHA in the presence of N-acetyl-D-galactosamine.

Inhibition of agglutination was also obtained with EDTA (ethylenediamine tetraacetic acid), the effect of which was reduced at higher Ca^{++} or Mg^{++} levels (Tunis, 1965).

The capacity of PHA to precipitate various sera has been proposed as a mechanism by which the mitogens would remove a growth inhibitor from the sera (Beckman, 1962). However, Holland and Holland (1965) showed that the blastogenic activity of PHA could be separated from the serum precipitating ability of the substance.

1.6.2. PHA: Preparation and Analysis

Rigas and Osgood (1955) obtained the preparations known as PHA-M (PHA-mucoprotein) and PHA-P (PHA-protein) from aqueous extracts of Phaseolus vulgaris or Phaseolus communis by ethanol treatment or $(NH_4)_2SO_4$ precipitation. PHA-M (the type used in the present study) was found by Robbins and Wachtel (1963)

by electrophoresis to contain at least 13 components as well as a major component. More recently, Dahlgen et al (1970) have isolated 2 proteins with molecular weights between 83,000 and 109,900, with transforming properties, and which were apparently not heterogeneous.

Because of the dramatic effect of the bean extract on lymphocytes, there has been considerable effort directed towards the isolation of the active component(s). Table 1.6.1. lists some of these results, and unfortunately illustrates the lack of clear cut results obtained.

In summary, there seem either to be several mitogenic substances in PHA, or the same mitogenic substance may be attached to other fraction migrating electrophoretically or by chromatography at different rates. The most potent preparations have consisted mainly of protein but have always contained some carbohydrate, and have had a molecular weight of approximately 100,000. Purely chemical procedures have not separated the leukagglutinating from the mitogenic properties, although the results of Borjeson et al (1967) indicate that the two are probably separable.

Table 1.6.1.

Analyses of PHA

Rigas and Osgood 1955	Prepared PHA-M (mucoprotein) and PHA-P (protein) by ethanol separation and $(NH_4)_2SO_4$ precipitation. The mucoprotein was soluble in water, dissociated into protein and an inactive polysaccharide at pH<5.8. PHA-P was a euglobulin, insoluble in water but soluble in saline or buffer. Both preparations were potent agglutinins and heterogeneous by electrophoresis.
Punnett et al, 1962 Punnett & Punnett, 1963	Separated at least 4 hemagglutinins and 3 protein free polysaccharides devoid of blastogenic activity. Fractions with blastogenic activity stable to 50-70° C.
Genest, 1963	Mitogenic and hemagglutining activities were concentrated in fractions precipitating after addition of 39% ethanol. Removed agglutinating activity by Seitz filtration.
Barkham and Ballas, 1963	Attempted to separate the mitogenic and agglutinating principles by starch block electrophoresis, but could only recover hemagglutinating activity.
Spitz, 1963	Observed 8 lines of precipitation on immunoelectrophoresis of PHA using anti serum from an immunized rabbit.
Borjeson et al, 1964	Removed inert proteins by heating to 80° C and subsequent precipitation of inert proteins. Further chromatographic purification yielded a protein or polypeptide containing 6% carbohydrate which was mitotically active at 0.2 µg/ml.
Rigas and Johnson, 1964 Rigas et al, 1965	Obtained a protein which was homogeneous by ultracentrifugation and electrophoresis MW = 128,000 approx. Possessed both agglutinating and mitogenic potency. Fractions from chromatography suggested the existence of more than one mitogenic substance, since it dissolved into 8 subunits in 8 M urea at neutral pH.
Hurn, 1966	Isolated a potent PHA extract which was active at concentrations as low as 0.06 µg/ml, maximally at 2-3 µg/ml. A water soluble protein of MW = 118,000, lacking sulfur but containing about 10% carbohydrate. Remained active after 30% ethanol or acetone. Found to be heterogeneous by immunoelectrophoresi
Rivera and Mueller, 1966	Using various separation techniques, isolated an active protein with MW = 100,000. Remove erythroagglutinating and part of leukagglutinating properties. Possible separation of factors apparently stimulating RNA and DNA synthesis separately.
Weber et al, 1967	Chromatographic fractionation of PHA-P yielded 3 peaks. Lymphocyte stimulation material was present in 2 peaks indicating that there may be at least 2 different lymphocyte-stimulating substances.
Dahlgren et al, 1970	Isolated 2 protein phytohemagglutinins with Sephadex A-50 and G-150. Molecular weight 91,200 and 109,900 chemically, and 83,000 by ultracentrifugation - lower than previous reports. Affinity to carbohydrate. No dissociation between mitogenic and agglutinating properties. No sialic acid or N-acetylgalactosamine. Apparently not heterogeneous.

1.6.3. Cellular Localization of PHA

Michalowski et al (1964) studied the cellular localization of phytohemagglutinin in white blood cell cultures using fluorescein isothiocyanate-labelled PHA, and observed fluorescence in lymphocytes near or in the nucleus. FITC added to blood cultures caused fluorescence in dead or dying cells. The same authors (1965) used anti-PHA immune serum labelled with FITC. They observed strongest fluorescence associated with dead and dying cells. After 10 minutes to several hours of incubation, label was observed on the surface of the cells. In cultures exposed to PHA for a period of 24 hours, PHA was observed to penetrate the cells. However, PHA was never observed to be associated with the chromosomes. The authors concluded that cellular penetration of the cells may have been associated with the enhanced pinocytosis observed electron microscopically in PHA treated white cells (Inman and Cooper, 1963; Elves et al, 1964; Tanaka et al, 1963).

Using fluorescein-conjugated PHA, Razavi (1966) found label inside all white cells, including polymorphonucleocytes, with 15 minutes of administration. Fluorescence was confined to cytoplasm and was not assoicated with nuclear structures or the cytoplasmic membrane.

Autoradiographic analyses of H^3-labelled PHA were carried out by Conard (1967). He obtained the preparation by growing Phaseolus vulgaris plants in a nutrient solution containing tritiated water. His results showed most leukocytes to be labelled after 4-6 hours of exposure. After 2-3 days, labelling in large blasts was predominantly nuclear.

PHA labelled with I^{131} was found inside lymphocyte cytoplasm by Rieke (1965). When PHA was labelled with a mercury diazonium salt, it appeared in the cytoplasm of stimulated lymphocytes (Greenland and Oppenheim, 1966). Tuberculin PPD labelled with fluorescein was found in the cytoplasm of lymphocytes derived from tuberculin positive blood donors (Witten et al, 1963).

An intracellular action of PHA was postulated by Coulson (1969) in a speculative article, based on the apparently greater time required by PHA to cause activation when compared with antigen. This data is, however, questionable.

The mere appearance of label within the cytoplasm is not 'hard' proof that the site of activity of the mitogenic principle is in the cytoplasm. On the contrary, there is considerable evidence for a surface action of PHA, based on consideration of the different ways that lymphocyte transformation can be induced, since a common denomenator would appear to

involve membrane events. Some of this evidence will now be described.

1.6.4. Indirect Evidence that the Location of Action of PHA and some other Mitogens is on the Cell Membrane

1.6.4.1. Agglutination of lymphocytes

As already described, one of the primary initial effects of PHA is that of agglutination (Borberg et al, 1966). It is hard to see how this can represent anything but a surface effect. As shown by Tunis (1965), EDTA prevents aggutination and Ca^{++} and Mg^{++} both suppress this action of EDTA. If PHA were to act entirely from within the cell, some form of secondary surface change would be required to cause the effect noted. Alford (1970) found that EDTA prevented transformation although excess Ca^{++} and Mg^{++} were not sufficient to suppress this effect.

1.6.4.2. N-acetyl-D-galactosamine (NAGA) inhibits the early phospholipid response to PHA

Inhibition by NAGA of the leukagglutination caused by PHA was demonstrated by Borberg et al (1966). Borberg et al (1968) also showed that NAGA prevented the binding of I^{131} labelled PHA to lymphocytes. Fisher and Mueller had shown that one of the earliest lymphocytic changes caused by PHA

was to enhance the turnover of the phosphatidyl inositol of the cell membrane. The fact that NAGA was subsequently shown to inhibit phosphatidyl inositol turnover in the presence of PHA (Fisher and Mueller, 1969) is evidence that the binding of PHA to the cell is important in the activation of the turnover of phosphatidyl inositol.

1.6.4.3. Effect of PHA and antigen-antibody reaction on the surface charge of the lymphocyte

Vassar and Culling (1964) found that PHA altered the electrophoretic mobility of the cells, indicating a change of the surface charge. Alteration of surface charge by PHA was also reported by Currie (1967). It is of interest that Sundaram et al (1967) observed that an antigen-antibody reaction of lymph node cells produced a change in membrane charge density and thus a decrease of electrophoretic mobility. Bert et al (1968) showed that treatment of human lymphocytes with anti-human immunoglobulin caused a reduction of electrophoretic mobility. (Anti-immunoglobulin will also cause lymphocyte transformation, as discussed in section 1.4.3.3.).

1.6.4.4. Mixed leukocyte reaction

The blastogenic response of leukocytes mixed from separate individuals (Bain et al, 1964; Hirschhorn et al, 1963)

appears to represent a response of cells to different histo-compatability loci, presumably located on the cell surface. However, the mechanism of this effect is not yet clear.

1.6.4.5. Antileukocyte antiserum

Grasbeck et al (1964) showed that the antiserum caused mitogenic activity in leukocyte cultures comparable and similar to that caused by PHA. The mitogenic action was accompanied by and was related to the agglutinating capacity of the anti-serum. Adsorption with leukocytes removed both the mitogenic and leukagglutinating factors. This effect seems to be initiated by events on the cell surface, as discussed in Section 1.4.5.

1.6.4.6. Binding of PHA to mucoproteins

The data of Borberg et al (1966) with NAGA and of Chessin et al (1966) with Vi polysaccharide indicate that PHA may cause its effect through a binding to sugar groupings which are known to be present in the cell surface. Lindahl-Kiessling and Petersen (1967) have also suggested this poss-ibility.

1.6.4.7. Data obtained with anti-PHA antibody

Kay (1969) evaluated the role of PHA in lymphocyte stimulation by studying the time that surface-bound PHA was

required for activation. Subsequent lymphocyte activation became maximal after a brief early exposure of the cells for 20 minutes, but activation did not become refractory to inhibition by anti-PHA antisera until 6 hours after PHA addition. Kay therefore suggested that the PHA was taken up by lymphocytes rapidly, but that its role in the initiation of stimulation was not complete until at least 6 hours after its addition, during which time it remained accessible to neutralizing antibody. Hausen and Stein (1968) found that anti-PHA antisera prevented the stimulating effect of PHA on the induction of uridine kinase activity.

In both studies, the argument may be made that since antibodies normally do not enter living cells, the induction of transformation by PHA must have taken place on the cell surface.

1.6.4.8. Binding of PHA to other cell types

Steck and Wallach (1965) studied PHA with respect to its ability to agglutinate red cells, endothelial cell types and mouse tumour cells, and showed that these cells may have surface receptors in common which are responsible for their agglutination. Their data indicated that PHA binding sites are located mainly on the cell surface, since binding was not

increased by cell lysis. The number of PHA binding sites for whole cells was about 10^{-16} moles/tumor cell.

Evidence for PHA 'receptors' on the cell surface, according to the data of Steck and Wallach (1965), are (a) the rapid binding of PHA, (b) the alteration of PHA absorption with variation of the ionic strength of the medium, since the intracellular milieu should not have been affected, and (c) that pinocytosis does appear to be significant as a route for intracellular penetration.

1.7. THE LYMPHOCYTE IN LYMPHOCYTIC LEUKEMIA

1.7.1. Clinical Aspects

In this study, an examination was carried out of the effects of ouabain on leukemic cells from some leukemic patients. It was therefore desirable to include a brief note on the responses of these abnormal blood cells.

1.7.1.1. General characterization of disease

In leukemia, there is a pronounced increase of white cells which accumulate in abnormal amounts at both normal and unusual sites, and appear in large numbers in the circulating blood. In many ways the disease resembles and is generally considered to be a neoplasm (Anderson, 1953).

The increase in white cell number of the peripheral blood is typical of all the different varieties of leukocytes although aleukemic types of the disease are known. Most commonly seen are myeloid leukemias (in which granulocytes or their precursors are increased in number), lymphocytic leukemias (involving the lymphocyte) and monocytic leukemias (monocytes and their precursors). Rare forms include eosinophilic, plasma cell and megakaryocytic leukemias. The lymphocytic form is more frequent during the first 3 decades whereas the myeloid condition may be present at all ages.

All forms of the disease are fatal but may be acute, subacute or chronic in nature. The acute forms often present with symptoms of acute infection with acute inflammatory gangrenous lesions of the oral mucosa, and bleeding tendencies. Splenic and lymph node enlargement are frequent but not invariable. The chronic forms are usually insidious in onset with gradual enlargement of the spleen and lymph nodes.

1.7.1.2. Chronic lymphocytic leukemia (C.L.L.)

Peripheral white blood cell concentrations generally vary from 50,000 to 200,000/mm^3, as compared with normal values of 7,000/mm^3. Of these cells, usually the great majority are small lymphocytes. Occasionally normal and subnormal counts are seen at different stages of the disease. Lymph nodes and lymphoid tissues are usually much enlarged, mainly in the cervical region. Other organs are seen to be infiltrated with lymphocytes.

1.7.1.3. Acute lymphocytic leukemia (A.L.)

The increased number of white cells is associated with a high proportion of primitive cells. Sometimes, the total white cell count is relatively low. It may be difficult to differentiate this type from acute myeloid leukemia since primitive cells from various sources are similar in appearance.

Infiltration is not as pronounced as with chronic leukemia and splenic enlargement is not as marked.

In the following, we will discuss lymphocytes from lymphocytic leukemias.

1.7.2. Properties of Leukemic Lymphocytes

1.7.2.1. Nucleic acid and protein

Gahrton and Foley (1966) studied the nucleic acid and protein content of normal and leukemic cells. Leukemic cells from AL showed a greater intercellular variation of total nucleotides and proteins but only a few cells in DNA synthesis. This suggested that the average AL population proliferates slowly and contains a considerable fraction of non-DNA synthesizing, non-dividing, but RNA and protein forming cells. In CLL however, there was less intercellular variation of nucleotides and proteins, and very few cells in DNA synthesis, indicating that such cells were 'mature' and non-dividing.

The mean Feulgen DNA content per cell in AL and CLL was about 10% greater than that of mature normal cells. The RNA content in CLL was very small, like normal lymphocytes, but was greater in AL. The lowest mass values (measured by interferometry) occurred in CLL and normal lymphocytes, but larger values were found in AL. Biochemical studies (Kiss, 1963, 1965,

Seitz, 1965) have also suggested that RNA and protein synthesis are increased in leukemic cells, as measured by the incorporation of RNA and protein precursors, despite the low DNA synthesis frequency. Most of the CLL cells are mature, non DNA synthesizing and non proliferating (Craddock and Nakai, 1962) although increased mass values have been reported (Christopherson, et al, 1963) in comparison to normal lymphocytes.

Swierenger and Basrur (1968) reported the existence (up to 30%)of DNA synthesizing lymphocytes in the peripheral blood of cattle with lymphosarcoma. It has been reported that the fraction of lymphocytes engaged in DNA synthesis in the peripheral blood of normal humans is low, but is elevated in leukemic patients (Bond et al, 1958, 1959; Cooper, Rubin, 1965; Epstein and Brecher, 1965; MacKinney et al, 1962).

Henry et al (1967) investigated RNA synthesis in vitro by lymphocytes from chronic lymphocytic leukemia. This was of particular interest because, as already mentioned, the cells synthesize RNA but not DNA appreciably (Henry et al, 1964, 1967; Hamilton, 1957; Lewis et al, 1964). Only a small fraction of CLL lymphocytes from patients with WBC counts greater than $100,000/mm^3$ could be transformed with PHA, in comparison to the normal small lymphocyte (Oppenheim et al, 1965). Considerable amounts of RNA were, however, synthesized in vitro by these cells,

in contrast to normal small lymphocytes. There was a delay in the conversion of the rapidly sedimented RNA into material with sedimentation velocities corresponding to ribosomal and soluble RNA, when compared to thoracic duct lymphocytes and cultured fibroblasts.

Silber et al (1968) has also reported experiments on RNA synthesis in normal and leukemic lymphocytes. These authors had already described the increased RNA synthesis in the cells of patients of leukemia. However, like the PHA increase this is not specific for leukemia since patients with infectious mononucleosis or leukemoid reactions also showed a similar effect (Cline, 1966). The latter worker found that RNA synthesis was extremely sensitive to actinomycin-D which, in low concentrations, inhibits DNA-dependent RNA synthesis. He used this as evidence to rule out the hypothesis that RNA viruses are responsible for the increased RNA synthesis in leukemic cells.

1.7.2.2. Cytoplasmic globules in leukemic lymphocytes

Laszlo et al (1967) described these globules on a cytologic and ultrastructural basis and noted their similarities to the Russell bodies of plasma cells. Special stains revealed that the most likely content of the globules was glycoprotein.

Electronmicroscopy indicated that the material lay within the dilate cysternae of the rough endoplasmic ritoculum. They proposed that the globules, which could represent stages in the formation of Russell bodies, were indicative of a developmental relationship between lymphocytes and plasma cells.

1.7.2.3. Respiration and glycolysis

There have been a number of studies on the metabolism of leukemic cells (Beck, 1958; Valentine, 1960). Ohta (1965) found that the presence of bacteria caused an increase of respiration and possibly of glycolysis in all white cells; the stimulation occurring in lymphocytic leukemias was relatively low.

1.7.2.4. Glycogen

Gahrton and Zetterberg (1966, a & b) studied the PHA reaction in leukemic cells. The PAS effect on cellular glycogen was slight or negative for normal lymphocytes, but larger amounts of glycogen were found in lymphocytes from CLL, except after successful therapy. Since augmented PAS scores are also found in lymphoid blood cells as well as in other lymphoproliferative disease (Jones et al, 1962) and diabetes mellitis (Mahr, 1959) as well as in acute leukemia, their diagnostic values is uncertain.

1.7.2.5. Effect of phytohemagglutinin (PHA)

(a) Glycogen

When normal leukocytes are cultured in vitro with PHA, a prominent cytochemical change is the increase in PAS reactive material of the lymphocytes, becoming maximal during the first 24-48 hours, and more prominent in some cells than in others (Quaglino et al, 1962). Increased amounts of PAS material are also found in lymphoproliferative disorders. However, the significance of this glycogen increase has been a matter of controversy.

Quaglino and Cowling (1964) tested the effect of PHA on lymphocytes from CLL, on the assumption that the presence of glycogen would assist leukemic lymphocytes to divide under the effect of PHA. In sharp contrast to normal cells, there was a marked reduction in the PAS reactive material in these cells during the first 24 hours. Blast transformation was also markedly reduced. They postulated an abnormality of glycogen metabolism in CLL cells.

(b) Morphological response

Authors who have reported a reduced blastogenic effect of PHA on CLL lymphocytes include Nowell (1960), Schrek and Rabinowitz (1963), Schrek and Stefani (1964), Bernard et al

(1964), Winter et al (1964), Hirschhorn et al (1964), Fitzgerald and Adams (1965), and Oppenheim et al (1965). However, Elves and Wilkinson (1963) did not find differences between their case of CLL and normals.

Astaldi et al (1966) reported that peripheral blood lymphocytes from patients with AL yielded a high proportion of blast cells after PHA treatment. Kourilsky et al (1966) confirmed this observation.

Astaldi et al (1965, 1966) showed that PHA caused normal lymphocyte transformation in chronic myeloid leukemia CML, but had very little effect in CLL. He also confirmed that transformation occurred in AL. In another communication Astaldi et al (1966) demonstrated that, in CLL, the low degree of blast transfomration in the presence of PHA was enhanced following splenic irradiation.

(c) DNA synthesis

Sharman et al (1966) found that lymphocytes from CLL had a markedly lower mitotic index and reduced uptake of H^3-thymidine after 3 days in PHA *in vitro* than did normals. An inverse relationship between the *in vitro* mitotic index and the *in vivo* blood count was also noted by Bernard et al (1964).

(d) Cause of reduced CLL lymphocyte response to PHA

The impaired response might be due to an immunological deficiency associated with the cell (Robbins, 1964, Elves and Wilkinson, 1963), although Astaldi et al (1965) have suggested a plasma factor. In support of the former possibility, Holm et al (1967) have demonstrated a decrease in the cytotoxicity of leukocytes from cases of CLL. The poor PHA effect could also be due to a metabolic deficiency in glycogen metabolism, with a resulting failure to carry on normal synthetic activities (Quaglino and Cowling, 1964). Winter et al (1964) suggested that cell damage caused by therapy, rather than by any intrinsic defect of the leukemic cells. However, Sharman et al (1966) demonstrated the lack of transfomration in untreated subjects. Indeed, they noted, in support of Astaldi et al (1966), that therapy improved the PHA response of CLL lymphocytes.

1.8. TRANSPORT OF CATIONS AND OTHER SUBSTANCES ACROSS CELL MEMBRANES

The present investigation was concerned primarily with the relationship between membrane transport and the synthetic processes of lymphocyte transformation. It was therefore desirable to summarize concepts of transport as they relate to the present problem. However, the considerable volume, both of experimental data and of theoretical models concerning this important cellular function, which have accumulated over the past 20 years, preclude more than an overall and relatively superficial consideration of membrane transport. In this section, primary emphasis has been directed to the transport of sodium from and of potassium into cells. The imbalance which will be clearly apparent upon consideration of the included material is deliberate, since only certain aspects of transport considered relevant to the present work have been discussed.

1.8.1. Maintenance of Intracellular Cation Levels

More than half a century ago, Macallum (1905) showed that potassium was the dominant intracellular ion by studying its precipitation with chloroplatinic acid. The mechanism by which intracellular potassium and sodium are respectively concentrated within or removed from the cell, have been the

subject of much investigation (see reviews by Kernan, 1965; Glynn, 1964; Whittam, 1967).

There are several theories. One theory identified mainly with Ling (1960, 1962) and Troshin (1961) has it that the cation content of the cell is determined by the physiochemical binding to potassium by intracellular macromolecules. These authors argue that an active 'pump' in the cell membrane is in fact non-existent. Thus, adsorption of certain cations would occur because of a specific affinity for these intracellular anions. Szent-Gyorgi (1957) proposed that selective K^+ accumulation might be related to the structure of water within the cell. He suggested that water molecules are arranged in a kind of crystalline state to a depth of several hundred molecules around the larger protein molecules of the cell. Because of its spacial dimension, K^+ would fit into this structure and thus be 'preferred' to Na, which would disrupt the structure due to its greater charge density and the resultant re-orientation of water around it. Recent findings of unusual states of water (superwater), as discussed by Erlander (1969), may revitalize this concept if it can be shown that such states of water exist under biological conditions.

In 1867, Traube suggested that cells were surrounded by membranes containing pores, the size of which determined permeability. Using a similar concept, Boyle and Conway (1941) accounted for the fact that some ions, including K^+, pass into muscle fibers, wheras others, including Na^+, are relatively impermeable because their hydrated diameters are greater than the pore diameter. This possibility was supported by evidence that ions with diameters greater than a certain critical value were impermeable. The application of pore models to problems of cellular transport requires that a hydrophilic region extends through the membrane from the inner to the outer interface. Penetrating molecules would diffuse through these regions rather than between the hydrophilic lipid side chains. However, the simple pore model alone is inadequate to account for the dynamic and energetic properties of cation flux. It is therefore of interest that Skou (1964) has developed a model of cation transport by a membrane ATPase which also forms a pore through the membrane.

One of the most useful theories of cation transport by cells is based on a pump mechanism by which cations are 'actively' transported into or out of the cell against electrochemical gradients. The idea of 'active transport', introduced by Verzar in 1935, was defined by Ussing (1949) as the movement of a substance against its electrochemical gradient, requiring

the expenditure of metabolic energy. Thus, both Na^+ extrusion and to some extent K^+ uptake are examples of active transport.

Active transport of cations was placed on a firm enzymatic basis by Skou (1957) who found in crab nerve an ATPase, the activation of which required both Na^+ and K^+. This ATPase, which subsequently was found in almost all tissues, is activated in vitro by cations and inhibited by glyocsides in a way which is remarkably similar to the effect of these agents on the active flux of cations across cell membranes (Schatzman, 1953).

Before considering the problem of transport in further detail, it will be useful to consider briefly some of the different mechanisms by which substances are understood to cross cell membranes.

1.8.2. Movement of Substance Across Cell Membranes

Park (1961) and Bresnick and Schwartz (1968) have summarized some of the recognized mechanisms of by which substances penetrate into cells. These include various types of diffusion, as well as energy requiring processes. Because of the use of K^{42} as a measure of potassium uptake by cells in this study, these considerations of transmembrane cation movement are relevant.

1.8.2.1. No Metabolic Energy Required

(i) Simple diffusion: The substance passes through aqueous pores in the cell membrane by random molecular motion and without interaction with other molecules. The movement of water in and out of cells is an example of this mechanism.

(ii) Solvent drag: A bulk flow of water sweeps the substance through aqueous pores. This mechanism has been demonstrated in the movements of thiourea and water flow in toad skin.

(iii) Diffusion restricted by membrane charge: Small anions such as chloride, will pass through positively charged aqueous channels which restrict cations.

(iv) Diffusion restricted by a lipid barrier: In this case, the penetrating molecule can enter the cell only if it can dissolve first in the membrane lipid and subsequently in the intracellular aqueous phase. This mechanism does not require aqueous pores.

(v) Facilitated diffusion (mediated transport): The transported molecule combines reversibily with a 'carrier' in the membrane. The carrier moves or oscillates between the inner and outer surface of the membrane to release or push up molecules. This movement may be quite slight and accounted for by thermal agitation or molecular deformation caused by binding and release of the transported molecule. An example

of this is the transport of glucose in red cells. Uphill transport may occur when facilitated diffusion is induced by counterflow of a transport competitor. The mechanism is reviewed by Stein (1967).

(vi) Exchange diffusion: The concept of exchange diffusion was introduced by Ussing (1949) to explain the large efflux of labelled sodium from frog muscle fibres without a corresponding consumption of energy. If it is assumed that Na^+ moves inward only by diffusion, and that the outward movement preceeds only at the expense of metabolic energy, then the energy requirements would exceed half of the total metabolic energy production of muscle in the resting state. Ussing proposed that the carrier may make a round trip with Na^+, so that no net change in the concentration of Na^+ in either side of the membrane develops, but isotopically labelled sodium would be moved. Keynes and Swan (1959) demonstrated that about half of the Na^+ efflux forced cells was dependent on the presence of Na^+ ions in the external medium. Tosteson and Hoffman (1959) reported that close to 80% of the sodium output from the sheep red cell was attributable to this process.

Exchange diffusion is usually seen in connection with active transport, and can account for the increased flux of a labelled compound as the concentration of the unlabelled compound on the opposite side of the membrane is increased.

Thus, amino acids concentrated in tissue cells will exchange rapidly with amino acids in the cell exterior. Exchange diffusion may occur more rapidly than the process of active transfer (Heinz and Walsh, 1958; Heinz, 1954) but no energy is expended.

1.8.2.2. Metabolic Energy Required

(i) Active carrier transport

The penetrating molecule combines with carrier. The carrier or carrier-complex is modified by a reaction requiring metabolic energy, such as from ATP, which affects the affinity of the carrier for the penetrating molecules. The carrier transports the molecule to the other side of the membrane where its affinity is changed by a second reaction. The carrier then binds another molecule or returns empty to its original state and the cycle is repeated. This mechanism is considered in greater detail in Section 1.8.4.

(ii) Pinocytosis, phagocytosis, rhopheocytosis, and endocytosis

The membrane of the cell develops invaginations on an ultrastructural level (rhopheocytosis or micropinocytosis) or on a light microscopic scale (pinocytosis) which are then pinched off as intracellular vesicles. Extracellular substances such as proteins may thus be trapped and released within the cell

upon dissolution of the vesicles. Phagocytosis involves the engulfing of relatively large aggregates such as bacteria. Endocytosis is the general term referring to the process by which the cell ingests parts of its external environment, enclosing them within invaginations of its plasma membrane. The process is reviewed by North (1970).

1.8.3. Active Transport of Cations by Cells

The problem of potassium accumulation and sodium extrusion by cells (Macallum, 1901; Osterhout, 1931) prompted Boyle and Conway (1941) and Dean (1941) to postulate an active ion transport mechanism or 'pumping device' by cells. Huff (1935) recognized the dependency of transport on cell metabolism, and Krough and Ussing (1937) suggested a specific transport system for sodium. Such a system is probably located within the cell membrane (Ussing, 1960; Glynn, 1959).

Following the work of Harris and Maizels (1951) many studies on cation transport have been carried out using erythrocytes. In these cells, the magnitude of K^+ influx depends on the external K^+ concentration, a Km value of approximately 2 mM being required for 1/2 maximal flux (Glynn, 1956). The efflux of Na^+ also depends on the internal Na^+ concentration, about 20 mM for 1/2 maximal flux (Post and Jolly, 1957).

The K^+ influx can be inhibited competitively by external Na^+ (Post et al, 1960). These workers found that in the presence of Na, the K influx was 1/2 maximal at 2 mM. However, when choline was substututed for Na, the 1/2 maximal K^+ influx occurred at an external K^+ concentration of 0.4 mM. Conversely, Hoffman (1962) showed that internal K inhibits Na efflux. The lower the internal K concentration, the higher was the Na^+ efflux.

Detailed studies of K^+ transport in red cells were carried out by Shaw (1955) and Glynn (1956) who found that potassium enters red cells by two pathways. In one case, the rate of entry varied linearly with the external potassium concnetration. The other pathway showed a saturation effect characterized by Michaelis-Menten enzyme kinetics. The latter route appears to be 'active' for it alone was affected by depriving the cells of glucose and was inhibited by cardiac glycosides.

Sodium efflux from red cells also seems to consist of two components. A portion of this varies with the external potassium concentration as with potassium influx (Glynn, 1956). This portion is suppressed by cardiac glycosides and glucose lack (Glynn, 1957). The remaining part of the Na efflux is not affected by glucose lack, but still is partly inhibited by digoxin.

1.8.4. Enzymatic Basis of Active Transport

It has been shown (a) that Na^+ and K^+ are moved against electrochemical gradients, representing active transport as defined by Ussing (1949), (b) that the energy for this system came from ATP, and not ITP, GTP or UTP (Hoffman, 1960, Skou 1965), (c) that there was a coupling between the movement of Na^+ and K^+ in many tissues (Glynn, 1956; Harris, 1954; Post and Jolly, 1957; Steinbach, 1952), and (d) that the transport of cation was inhibited by cardiac glycosides (Schatzman, 1953).

Based on these experimental findings, Skou (1965) foumulated certain requirements for a Na^+ and K^+ transport system. This system should

1) be located in the cell membrane.
2) have an affinity for Na^+ that is higher than for K^+ at a site located on the inside of the cell membrane.
3) have an affinity for K^+ that is higher than for Na^+ at a site located on the outside of the cell membrane.
4) contain an enzyme system that can catalyze the hydrolysis of ATP and thus convert energy from ATP into the movement of cations.
5) be capable of hydrolyzing ATP at a rate dependent on the concentration of Na^+ inside the cell and of K^+ outside the cell.

6) be found in all cells in which an active linked transport of Na^+ and K^+ occurs.

The Na^+-K^+-activated ATP hydrolyzing enzyme system found by Skou (1957) met these conditions and also met two additional requirements, namely that

7) there should be a correlation between the effect of cardiac glycosides in the cation transport system of the intact cell and on the ATPase system; and

8) the enzyme system should have the same quantitative relation to Na^+ and K^+ as the transport system in the intact cell.

It may be noted that although Skou's elegant demonstration of a sodium-potassium dependent enzyme system located in the membrane was convincing evidence for an ATP splitting system associated with cation transport, it did not shed much light on how the cations were transported, except to indicate the existence of the postulated membrane-located enzyme system.

1.8.4.1. Characteristics of the Na-K-ATPase

The enzyme system is found to hydrolyze ATP to ADP and Pi in the presence of Mg^{++}, but both Na^+ and K^+ are also required for maximum activation. If Na^+ is present in the medium, the enzyme will also respond to NH_4^+, Rb^+, Cs^+ and Li^+ in that order (Skou, 1960). Although kinetic analyses suggest

the existence of two sites, each having a high and low affinity respectively for Na^+ and K^+ (Skou, 1960), the possibility of a shifting, single site has not been ruled out. K^+ can displace Na^+ from the first site at increased concentrations of K^+, resulting in an inhibition of enzymatic activity (Schwartz et al, 1963; Skou , 1957).

Na-K-activated ATPases have since been located in numerous tissues (Bonting et al, 1962; Katz and Epstein, 1968), with highest activities found in the nervous system and secretory tissues. The concentration of K^+ required for 1/2 maximal activity in the presence of Mg^{++} and Na^+ has been found to vary from 0.5 to 2.2 mM (Skou, 1957, 1962; Autidore, 1962; Whitham and Ager, 1965), but this depends on the Na^+ content of the extracellular medium.

Attempts to solubilize and isolate the ATPase have proven very difficult. Skou (1961) found that treatment of crab nerve with phospholipase A removed the activating effect of sodium and potassium, suggesting that the presence of lipids was essential. Similar observations were made with the erythrocyte system by Schatzman (1962). In contrast, Wheeler and Whittam (1964) reported that extraction with organic solvents, presumably removing most of the lipid material, left enzymatic activity intact.

Over the past few years, evidence has accumulated that the transport ATPase splits ADP and Pi by a mechanism which involves at least two steps ;(a) a Na^+ dependent transfer of the terminal phosphate of ATP to a membrane protein, and (b) a K^+ activated dephosphorylation (Albers et al, 1963; Whittam et al, 1964; Post, Sen and Rosenthal, 1965; Ahmed and Judah, 1965; Hokin et al, 1965; Nagano et al, 1965; Rodnight et al, 1966; Blostein, 1968).

The affinity of the enzyme system for Na^+ and K^+ appear to be influenced by phosphorylation. The results of experiments using a P^{32}-labelled ATP suggest that an intermediary step in the hydrolysis of ATP is the formation of a phosphorylated compound by the transfer of an energy rich bond from ATP to the system (Post and Rosenthal, 1962; Skou , 1964). Post et al (1965) found that an acceptor group in erythrocyte stroma is phosphorylated when ATP is split in the presence of Na^+ and K^+ and the kinetics of phosphorylation paralled that of the membrane ATPase. Heinz and Hoffman (1965) and Skou (1966) reported that incorporation of phosphate by a purified preparation occurred only when K^+ is absent. When K^+ is present, the terminal phosphate group is not transferred to the enzyme.

Several kinetic studies have indicated that the Na-K-ATPase has allosteric properties (Ahmed et al, 1966; Squires, 1965; Robinson, 1967, 1968). Albers et al (1968) have proposed a model for transport based on allosteric transitions mediated by phosphorylation.

1.8.4.2. Relationship between cation transport and the Na-K-ATPase

The remarkable parallelism between the response to various agents of the enzyme system already described and the transport of cations into cells has led most workers to accept that the Na-K-ATPase plays a fundamental role in cellular cation transport. The subject is reviewed by Skou (1965).

Support for the concept is based on the resemblance of the enzyme system to the transport system in several important characteristics.

(i) Post and Jolly (1957) have shown that the presence of potassium outside the cell is essential if sodium efflux is to occur. They found the Km of potassium for 1/2 maximal Na efflux to be 2.2 mM at 37°C.

(ii) At different K^+ concentrations, the ouabain sensitive Na-K-ATPase and the active K^+ influx show a marked proportionality (Whittam and Ager, 1965).

(iii) The rate of Na^+ extrusion is dependent on the internal Na^+ concentration up to a level of about 50 mM and apparently saturates thereafter (unlike the extrusion of Na^+ from Na^{24}-labelled squid axons -- Hodgkin and Keynes, 1956). The concentration of Na^+ half maximal for enzyme activity is 24 mM, for transport 20 mM (Post and Albright, 1961).

(iv) The rate of potassium influx is dependent on the external potassium concentration and is characterized by a Michaelis-Mention relationship with the value of 2.2 mM for the Km term for half maximal stimulation, equal to that for both the half maximal stimulation of Na^+ efflux, and for the stimulation of ouabain-sensitive ATPase activity (Glynn, 1956).

(v) The distribution of enzymes in different tissues parallels the intensity of cation transport (Szaky, 1965).

(vi) Both cation transport and the Na-K-ATPase are inhibited by Ca^{++} (Hoffman and Ryan, 1960; Skou, 1957; Soloman et al, 1956).

(vii) The cardiac glycoside ouabain, when present at 10^{-7}M, causes half-maximal inhibition of the enzyme, whereas transport is inhibited at 3-7 x 10^{-8}M (Post and Albright, 1961).

The presence of an ATPase in the cell membrane was first shown by Libet (1948) who found that ATP splitting activity of the squid axon remained after removing the axoplasm.

That ATP has a significant role in transport was shown by Caldwell et al (1960), who found that cyanide inhibited the active transport system of squid axons, but that intracellular injection of ATP restored this process. The requirement for ATP in transport by red cell ghosts has also been demonstrated (Gardos, 1954).

Intracellular K^+ concentrations have been shown to depend on the activity of the enzyme system. Certain sheep are known to have red cells with high K^+ content, whereas certain other sheep have red cells with low K^+ and high Na^+. In the latter animals, the Na-K-ATPase is less active by a factor of four (Tosteson, 1963). The cat red cell which also has low K^+, shows less enzymatic activity by a factor of eight in comparison to the higher K^+ human red cell (Bonting et al, 1961).

1.8.4.3. Models for transport

Skou (1965) proposed a 'fixed site ATPase model', suggesting that the transport of Na and K is contingent on the affinity of Na^+ and K^+ for two separate sites in the enzyme system. When ATP and Mg^{++} are present, Na^+ and K^+ are shifted to opposite positions, as ATP is hydrolyzed to ADP and Pi. If no external K^+ is present, the outer K^+ site becomes occupied by

Na^+ and no net transfer of Na^+ can take place. These changes of affinity for Na^+ and K^+ are explained by postulated local changes in the elctron density of the enzyme system caused by the action of ATP.

This model is, of course, only one of a series of different models of both 'fixed site' or 'mobile carriers' involved to explain the transport of cation and other substances. It would be beyond the range and purpose of this brief review to consider all these models, which have been described in detail by Bresnick and Schwartz (1968). Furthermore, the elegance of the demonstrated role of the Na-K-ATPase system in transport in animal cells does not rule out the concurrent existence for animal cells of inducable permease systems similar to that of bacterial system, such as the beta-galactosidase permease of E Coli (Kepes, 1964).

1.8.5. Role of Calcium in Membrane Function and Transport

The fundamental importance of calcium metabolism is well known and it would be inappropriate to dwell on more than a limited aspect of the manifestated role of this cation, that is, in association with the cell membrane and active transport. However, interpretation of the results of the present work and the effects of PHA require some consideration of the role of calcium on the cell membrane.

The influence of calcium on the membrane function of cells is profound, affecting surface change, adhesiveness, permeability (Mannery, 1966; Rasmussen and Tenhenhouse, 1968) and the interaction between cells (Schoenberg et al, 1964; Okada and Murayama, 1966). Both calcium and magnesium also have been observed to stimulate the passage of rat thymocytes into mitosis (Whitfield and Youdale, 1966; Whitfield et al, 1969).

1.8.5.1. Membrane permeability

Calcium is reported to decrease the permeability of cells and cell layers (Curran et al, 1963; Herrara and Curran). Curran and Gill (1962) suggested that calcium exerts its action on the outside of the membrane causing a non specific 'tightening' in some regions, leading to a change in permeability or alteration of pore size.

Whittenburg et al (1960) studied the effect of changes in the external Ca^{++} concentration on the equivalent pore radius. Although the pore radius was independent of Ca^{++} concentrations over a range of 1-10 mM, removal of Ca^{++} caused the radius to increase. Solomon (1961) considered the action of pitressin on the permeability of kidney tissue to be mediated by Ca^{++}.

1.8.5.2. Na-K-ATPase

Considerable work has been carried out on the relationship of Ca^{++} to the function of this transport enzyme, since Skou's (1957) initial demonstration that calcium ion inhibited its activity; the effect of Na^{+} was proportional to the Mg^{++}/Ca^{++} ratio, since the requirement for Mg^{++} was increased as the Ca^{++} concentration increased. The inhibition of the enzyme system by calcium is reminiscent of the action of the cardiac glycosides; similar effects of calcium have been observed in intact cellular cation transport systems, as well as in the heart in vivo.

Calcium and magnesium apparently compete for ATPase (Skou, 1960) and the inhibition by calcium ions of Na^{+}-K^{+}-ATPase has been confirmed by others (Whittam and Blond, 1964; Gore, 1951; Hess, 1962; Epstein and Whittam, 1966). Indeed, the inverted ratios for K^{+} and Na^{+} observed in the extracellular and intracellular fluids are paralleled by the Mg^{++}/Ca^{++} ratios (Wacker and Parisi, 1968). In brain tissues, absence of calcium leads to an increased influx of Na^{+} (Frankenhauer and Hodgkins, 1955; Ames et al, 1967), with resulting increase in ATPase activity, and this increase is suppressed by tetrodotoxin (Chan and Quastel, 1967).

1.8.5.3. Calcium mediated membrane effects and cell metabolism

It has been considered that much of the cellular energy metabolism is controlled by the rate of transport of monovalent cations across cell membrane, the control being mediated by intracellular ADP, which in turn is governed by the activity of Mg^{++}-dependent Na^{+}-K^{+}-activated ATPase in the membranes (Whittam, 1961, 1962; Whittam and Blond, 1964; Blond and Whittam, 1964).

A number of workers have found that ouabain inhibits respiration in the absence of Ca^{++}. However, in the presence of this cation respiration may be stimulated by ouabain at concentrations greater than 10^{-5}M. (Whittam, 1961, 1962; Whittam et al, 1965; Bourke and Tower, 1966). Similar results were obtained by Swanson and Ullis (1966) who also found that the ouabain-induced decrease of intracellular K^{+} and increase of intracellular Na^{+} were less marked in media containing Ca^{++}. The results were interpreted as indicating that Ca^{++} and membrane acidic groups help to maintain the physical integrity of the cell membrane structure and tend to prevent ouabain from combining with the Na^{+}-K^{+}-ATPase.

To explain the aformentioned respiratory stimulation by ouabain at very low Ca^{++} levels, Bourke and Tower (1966)

and Tower (1968) provided evidence that it was due to a stimulation of mitochondrial respiration consequent to a ouabain induced mobilization and increase of tissue calcium, and its entry and sequestration within mitochondria. They postulated an additional effect of ouabain, namely, that of altering the tissue levels and distribution of calcium.

A possible mechanism for metabolic control involving membrane Ca^{++} was proposed by Epstein and Whittam (1966). They showed that the inhibition by calcium of Na^{+}-K^{+}-activated ATPase in membranes of kidney cortex and red cells was due to a competition with MgATP. They suggested that since some glycolytic enzymes such as phosphofructokinase and pyruvate kinase are inhibited by calcium, cells require mechanisms for maintaining low levels of calcium in the cytoplasm and at the inner surface of the cell membrane. Such mechanisms might serve to regulate cell metabolism in conjunction with the active transport of adenosine nucleotides, citrate, magnesium and orthophosphate (Gever and Krebs, 1966). The role of metal cations as in the regulation of cell metabolism has also been discussed by Bygrave (1967).

Antagonistic effects of K^{+} and Ca^{++} have been observed for a number of tissue and metabolic effects (Kleinzeller, 1961). The stimulation of respiration in brain tissue has been found to depend on the K^{+}/Ca^{++} ratio and depends on the integrity

of the brain cell membrane (Quastel, 1961).

1.8.5.4. Role of Calcium and other Metallic Cations in Lymphocyte Transformation

Alford (1970) examined the role of several divalent meeal cations on lymphocyte transformation. He found that EDTA and sodium citrate inhibited DNA synthesis at concentrations greater than 12 mM, and the effect of citrate was reversed by calcium and magnesium, more effectively by the former. DNA synthesis of PHA-treated cells was prevented by omission of calcium but much less by the omission of magnesium. Ni^{++}, Zn^{++}, and Fe^{++} were effective in preventing the EDTA inhibition which could be only slightly reversed by Ca^{++} and not by Mg^{++}. These effects suggest that other cations beside Mg^{++} and Ca^{++} may be required for transformation; the author suggests that both Zn^{++} and Fe^{++} may be essential since both are essential to tissue growth (Thomas and Johnson, 1967), presumably for oxidative enzymes (Bentler, 1957), and for metalloenzymes associated with RNA synthesis (Wacker, 1962) and DNA (Fujioka and Lieberman, 1964). Unfortunately, Alford did not examine the effect of high Ca^{++} levels on lymphocytes without EDTA. It would be interesting if such levels would inhibit transformation.

It may be noted that PHA-induced red cell agglutination is inhibited by EDTA, and this effect is prevented by Mg^{+} or Ca^{++} (Tunis, 1965).

1.8.6. Cation Transport in the Maintenance of Cell Volume

The movement of cations against an electrochemical and concentration gradient appears to be a fundamental property of animal cells. Speculation has it that this primitive function may have originated as a mechanism for the maintenance of cell volume by cellular structures surrounded by water environment. The presence of various organic molecules (proteins, nucleic acids and various smaller anions) which cannot pass the membrane would otherwise tend to cause infinite cellular swelling due to a Gibbs-Donnan effect.

Swelling induced in this way may be avoided by the cell having a rigid membrane (as with plant cells and some bacteria, Rothstein, 1959), or by impermeability of the cell wall to water (which however has not been demonstrated. A third way that cell volume may be maintained appears to be the method evolved in animal cells and involves the active, linked transport of cations (specifically Na^+ and K^+) against a gradient into and out of the cell which contains a high internal K^+/Na^+ ratio. Interference with this system by metabolic inhibition or use of strophanthin alters the cell volume and leads to swelling. The efficiency of this system is controlled both by the pumping action and in the rate of leakage of these cations, as shown particularly by the data of Tosteson and Hoffman (1960)

and Tosteson (1964) on the 'high K' and 'low K' red cells of sheep, which differ genetically in this respect.

The way that the cell senses a volume change theoretically may involve (i) a stretch sensor in the membrane, (ii) a sensor responding to the total concentration of solute in the cytoplasm, or (iii) a sensor responding to changes in the concentration of a specific solute such as Na^+. The third mechanism would seem most likely, but only if the internal solute concentration is maintained at a very different level from that of the environment. This possibly is supported by the evidence of the allosteric activation of Na-K-ATPase (Squires, 1965; Rotman, 1967; Monod et al, 1965; Tobin et al, 1970).

The mechanism by which cells maintain their volume in this way has been considered by Robinson (1968). Two major functions are proposed for the regulation of pump fluxes. (a) The pump mechanism may involve cooperative allosteric interaction (Monod et al, 1965) by which small concentration changes of an allosteric activator (such as a leaky ion) may produce augmented changes in rate. (b) The sensitivity to ion leaks may be magnified if the major external ion (giving the major initial flux leak) is kept at a low concentration within the cell.

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Pathological effects of ouabain, interpreted as being due to cell swelling caused by inhibition of the Na-K-ATPase have been described by Ginn et al (1968).

1.9. ACTION OF THE CARDIAC GLYCOSIDES IN VITRO

It is almost 200 years since Withering was first told of the old woman who treated dropsy with a herbal mixture, and began his studies which led to the clinical introduction of digitalis. The cardiac glycosides have since become indispensable in cardiac therapeutics, but their mode of action at the cellular level is only recently being elucidated.

This section will be restricted to the effect of cardiac glycosides and similar compounds on the transport of cations by the cell.

The reviews by Glynn (1964) and Repke (1963) were found useful for reference to less recent work.

1.9.1. Effect of Glycosides on Movement of Sodium and Potassium

In 1953, Schatzman reported that the active movements of sodium and potassium in red cells were prevented by low concentrations of strophanthin-K, one of the cardiac glycosides found in Strophanthus gratus. Since the compound did not affect lactic acid production, it seemed unlikely that energy yielding reactions were also being affected. An effect of the compound on the movement of cations from red cells placed in the cold did not occur, and it therefore seemed unlikely that the glycoside altered the passive permeability of the cells. Later, Joyce and

Weatherall demonstrated that the uptake of K^{42} by red cells was blocked by these compounds. More recently, however, a small ouabain sensitive efflux of potassium has been described (Glynn and Luthi, 1967, 1968; Glynn and Lew, 1969). Glynn has suggested the possibility that this part of the potassium efflux may represent a reversal of the pump mechanism. A small ouabain sensitive passive influx of sodium has also been observed in the absence of external potassium.

Half maximum inhibition by ouabain of cation transport in red cells was found to occur at concentrations of 3-7 x 10^{-8}M (Post and Albright, 1961; Gill and Solomon, 1959; Kahn and Acheson, 1955; Solomon et al, 1956), similar to the concentration about 10^{-7} required for the inhibition of the Na-K-ATPase (Bonting et al, 1962). In general, the sensitivity of a transport system to the glycosides has been observed to parallel the sensitivity of the corresponding enzyme preparations to the same glycoside.

The site of action of ouabain on cation transport appears to be at the cell surface but not inside the cell. Caldwell and Keynes (1959) found that ouabain injected into the giant squid axon had no effect on sodium extrusion, but was markedly effective when administered on the outside of the cell.

As previously mentioned in section 1.8, potassium influx could be characterized by two modes, one obeying saturation kinetics, and one in which the influx was proportional to the extracellular potassium concentration. It was shown that the portion of the potassium influx which was characterized by Michaelis-Menten kinetics was inhibited by ouabain, and that the inhibition was reversed by increased levels of extracellular potassium. (Glynn, 1957; Bonting et al, 1964; Dunham and Glynn, 1961; Kinsolving et al, 1963; Schatzman, 1963). These effects could not, however, be characterized by simple competitive kinetics, possibly because the effect of potassium was strongly influenced by the sodium concentration.

The antagonism between K^+ and cardiac glycosides appears to take place at the site of the Na^+-K^+-exchange pump in the cell membrane (Hoffman, 1966) but the mechanism of antagonism is obscure. Baker and Willis (1970) have recently examined this problem by studying the binding of H^3-ouabain to cells. Binding of the glycoside was both non-linear and K^+ sensitive, but also linear and K^+ insensitive at high ouabain concentrations. The degree of binding of the non-linear component was strikingly parallel to the extent of inhibition of the sodium pump. Binding of the glycoside took place with 12-16 min (T 1/2) at an external K^+ concentration of 6 mM. K^+ concentrations of 1-2 mM (at 140 mM Na^+) were required to reduce the

binding of the glycoside by a half, but lowering the Na^+ concentration to 50 mM increased the effectiveness of K^+ as an antagonist of glycoside binding. The results were associated with an apparently competitive relationship between ouabain and potassium in which the K_i for ouabain in the absence of potassium was about 10^{-8}M. The non-competitive nature of the inhibition to ouabain which was found in some cases was explained on the basis of a partial irreversibility of glycoside binding.

1.9.2. Effect of Cardiac Glycoside on ($Na^+ + K^+$)-Activated ATPase

This enzyme system is sensitive to glycosides; the inhibition of ATP-splitting activity also has the same dependence on potassium concentration as does the inhibition of potassium influx into intact cells.. The effect depends on the amount of sodium present (Dunham and Glynn, 1961; Kinsolving et al, 1963; Schatzman, 1963).

Matsui and Schwartz (1966) found that the K_i for cardiac glycoside induced inhibition was markedly affected by the Na^+ : K^+ ratio. At a 'physiological' Na, K ratio of about 29, they obtained a K_i of 2.2×10^{-7}M for heart muscle ATPase. In 1968, these authors reported on the binding of H^3-digoxin binding to a heart Na^+-K^+-ATPase. The binding, which required ATP and Mg^{++}, was stimulated by Na^+ and depressed by K^+. The binding

was reduced by active glycosides, but not by inactive glycosides or by other steroids such as prednisolone. Their evidence suggested that digoxin binds with the phosphorylated conformation of the enzyme. Moreover, it may be the presence of Na^+ that inhibits the binding of H^3-digoxin to non-phosphorylated forms of the enzyme (Matsui and Schwartz, 1968 b).

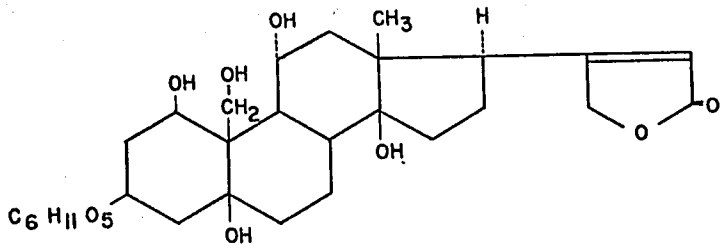
Albers et al (1968) have investigated the binding of H^3-ouabain to Na-K-ATPase. The hydrolysis of ATP by Na-K-ATPase proceeds through several steps (Albers, 1967), which were incorporated into a model. In this model, the phosphorylated enzyme mediates vectorial transport by allosteric transitions. They also suggested that the combination of the glycoside with the enzyme is irreversible under physiological conditions in opposition to earlier studies and to the work of Baker and Willis (1970). This would, however, invalidate the concept of a K_i, such as that determined by Ahmed et al (1966) who found a K_i of 6.5×10^{-8}M and by Matsui and Schwartz (1966).

At low concentrations of ouabain, a stimulating effect on the Na-K-ATPase has been observed (Palmer et al, 1966; Repke, 1963). Repke (1963) noted that at concentrations between 10^{-10} and 10^{-7}M, there was a distinct stimulation of the ATPase activity, but higher concentrations led to inhibition.

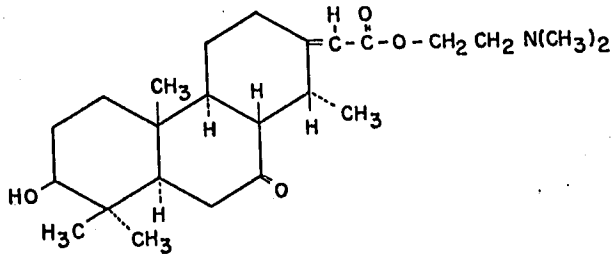
It is of interest that the low, stimulating concentrations of ouabain are, in fact, within the therapeutic range. Lee and Yu (1963) stimulated heart microsomal Na^+-K^+-ATPase with low concentrations of strophanthin-K. Bonting et al (1964) caused a stimulation of rabbit brain enzyme at 5×10^{-8}M, and Palmer and Nechay (1964) caused stimulation of ATPase activity and electrolyte transport at concentrations ranging from 10^{-8} to 10^{-11}M. However, Kinsolving et al (1963) failed to observe this effect. Palmer et al (1966) found a stimulation between 10^{-8} and 10^{-11}M which was not affected by varying the K^+ concentration, and aging of the enzyme preparation caused loss of stimulation although the inhibitory effect of the glycoside was not affected. These authors suggested, therefore, that ouabain may have 2 different effects.

1.9.3. Effect of other inhibiting substances on transport and Na-K-ATPase

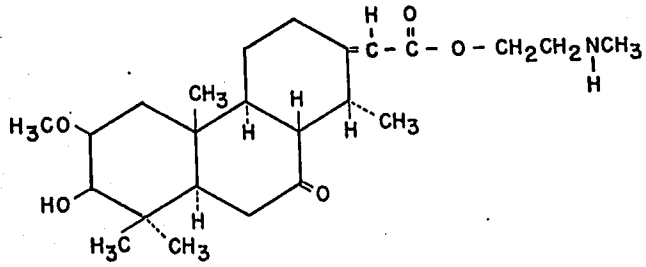
Kahn (1962 a,b) showed that the erythrophleum alkaloids, which resemble the cardiac glycosides pharmacologically (Dalma, 1954) but differ from them in some aspects of structure (Fig. 1.9.1), inhibit the influx of K^+ into erythrocytes. The compounds (erythrophleine, cassaine) also inhibit Na-K-ATPase obtained from various tissues, and the effect of erythrophleine was reversed by increasing the potassium concentrations (Bonting et al, 1964).



OUABAIN



CASSAINE



ERYTHROPHLEINE

Fig 1.9.1.

Structural formulae for ouabain, erythrophleine and cassaine.

Ethacrynic acid, a new diuretic agent reported by Cannon et al (1963) was shown by Hoffman and Kregenow (1966) to inhibit sodium outflux in red cells when the agent was superimposed on cardiac glycosides. They termed the effect of the compound as acting on 'pump II' (in contrast to pump I which is supposed to be inhibited by the glycoside alone). Smith et al (1967) suggested that the two pumps may be dissociated. Sachs and Welt (1968) used ethacrynic acid to evaluate the possible requirement by transport of 2 external sites to be occupied by potassium. While they could not distinguish between the sites by the technique used they found that this compound strongly inhibited the active potassium influx and demonstrated competition with potassium.

The sensitivity of the Na-K-ATPase to oligomycin raises problems about the relationship of ion transport to oxidative phosphorylation (Glynn, 1963; Jarnefelt, 1962; Jobsis and Vreman, 1963; Van Gronnigen and Slater, 1963; Matsui and Schwartz, 1966).

Matsui and Schwartz (1966) and Schwartz and Laseter (1964) have characterized the inhibiting properties of p-chloromercurobenzoate, N-ethylmaleimode, tributezinehloride, octylguanidine and oligomycin on the Na-K-ATPase. The sensitivity of the enzyme to the first two preparations indicates the specific requirement for SH groups on the enzyme.

1.10. THE SURFACE OF THE LYMPHOCYTE

To state that the cell surface is important is trivial, and this is particularly true for the lymphocyte, the functional response of which is almost certainly mediated by events associated with the cell membrane. However, many significant cellular properties such as contact inhibition or associated with immune reactions have not been related to biochemically defined surface entities, with the exception perhaps of the virus receptor sites and their susceptibility to the vibrio cholera extract (Burnet, 1947). The effects of this substance, generally known as neuraminidase (n-Acetyl neuraminidate glycohydrolase) and of other surface acting enzymes on lymphocytes are of great interest in the elucidation of the role of the plasma membrane in lymphocyte function.

The present discussion will be concerned with a limited aspect of the lymphocyte surface, that is the cell's charge, its relation to function, and the effects of surface acting enzymes on both charge and functional response. These aspects are specially considered since they are involved in the interpretation of some of the experimental results of Section 3.4. No attempt has been made to review the important matter of the antigenicity of the cell surface (Amos and Koprowski, 1963).

1.10.1. Origin and possible role of surface charge of mammalian cells

1.10.1.1. The surface charge of mammalian cells is one of several factors which affect the interaction between the cell membrane and other surfaces (Pethica, 1961; Weiss and Mayhew, 1967). It is recognized that the surface charge density of the leukocyte and other cells may be important in surface properties such as pseudopod formation, adhesiveness, deformability and contact inhibition. Such responses are probably significant in the function and circulation of leucocytes in the marrow, lymphoid tissue and in the response of these cells to foreign (non-self) substances.

Most mammalian cells carry net negative charges (at physiological pH) which cause them to migrate towards anodes *in vitro* (Robineaux and Bazin, 1951). Since the mobility of various cells including leukocytes is reduced by neuraminidase (Rubenstroth-Bauer et al, 1961), part of the surface charge is considered to be due to the ionized carboxyl groups of n-acetyl neuraminic acid (NANA, sialic acid) in the electrokinetic (sheer) zone of the cell periphery as first suggested by Klenk (1958). The residual charged groups may be ionized phosphate groups of ribonucleic acid located at the surface of some cells (Weiss and Mayhew, 1966; Mayhew, 1967, 1968). Haydon and Seaman (1967) suggested that the residual charge on neuraminidase treated red cells may be due to an α-carboxylic acid of a protein bound

amino acid Weiss (1960) has postulated the existence of 'cationic clouds' surrounding negatively charged cells, and suggested that calcium reduced the depth of the cationic layer.

Sialic acids occur as part of a carbohydrate-rich glycoprotein found on the surfaces of normal and tumor cells. Cell electrophoretic studies have resulted in the detection of sialic acid in many types of cells (Simon-Reuss et al, 1964) and have demonstrated that the increased negative mobilities of some cells are caused by increased amounts of sialic acids on the cell surface. Glycoproteins containing NANA are abundant on the erythrocyte surface, conferring an overall negative charge (Eylar et al, 1961; Madoff and Eylar, 1901). A sialomucopeptide has been demonstrated at the surface of Ehrlich Ascites tumor cells (Wallach and Eylar, 1961; Cook et al, 1962; Langley and Ambros, 1964, 1967) and has been shown to make a major contribution to the surface charge of these cells.

1.10.1.2. Role in membrane structures and deformatility

Gottschalk (1960, 1962a,b) pointed out that charged carboxyl groups of terminal N-acetylneuraminic acid could form part of the carbohydrate prosthetic groups of the mucoidal material of the membrane and confer rigidity to the underlying proten core. Weiss (1965, 1966) provided experimental evidence for this by demonstrating an increased 'deformability' of

neuraminidase treated monocytes by micropuncture experiments, and found an alteration of phagocytic cell function, characterized by an increase in the contact and engulfment stages of phagocytes after treatment with this enzyme.

1.10.1.3. Role of sialic acids in cation transport and receptors

Wooley and collaborators (1964) postulated that sialic acid, present in the cell or on the membrane, functions as a specific receptor for serotonin. As components of gangliosides, sialic acids have been implicated in transport mechanisms, particularly in neural tissues where the content is relatively high.

McIlwain (1963) has suggested that membrane 'pores' may be lined with electronegative charges conferred by sialic acid compound molecules. This concept is utilized in McIlwain's (1963) model of a transport system characterized by a 'fixed site ATPase'. Glick and Githens (1965) have implicated sialic acid in potassium transport by leukemic cells.

1.10.1.4. Cell contact and surface antigen recognition

The overall negative charge can act as a repulsive mechanism preventing cellular contact, and the ability of cells to distinguish self from non-self may depend on the ability to undergo mechanical contacts. It has been proposed (Currie and

Bagshawe, 1967; Currie et al, 1968; Sanford, 1967; Linderman and Klein, 1967) that sialic acid groups in the cell periphery can mask cell surface antigens from detection by the immune system. Thus, Bagshawe and Currie (1968) found that treatment with neuraminidase revealed histocompatibility antigens on the cell surface of trophoblasts. They considered that the high negative charge on fetal trophoblasts is caused by a peri-trophoblastic coat of sialomucin which, by repelling negatively charged lymphocytes, prevents contact and an immune response to these otherwise foreign cells.

It is therefore of interest that Inbar and Sachs (1969) described an increase in the number of concanavalin-A binding sites after treatment of hamster cells in vitro with trypsin.

1.10.1.5. Effect of Surface Acting Enzymes

Trypsin has been used for many years to disrupt cell monolayers in vitro for subsequent reseeding and reculture. It is a matter of faith that the enzyme will digest only non-viable cells and living cells are not prevented from under-going subsequent mitosis and growth. Nevertheless, there is considerable evidence that the enzyme does effect the cell surface.

Older work had shown that trypsin removed an extraneous coat from the surface of cells (Weiss, 1958, 59). Weiss (1961) states 'it is a common observation that a mucoid material is liberated from mammalian cells by trypsin treatment' and, from studies of the effect of neuraminidase on rat dermal fibroblasts, inculdes that sialic acid is an important structural component of the cell surface. Makela et al (196) and Pesola (196) found that trypsin caused the release of 50% of the sialic acid and 15% of the hexosamine from ted cells. Cook et al (1961) found that trypsin released a sialomucopeptide from red cells.

The action of the enzyme neuraminidase, isolated from vibrio cholerae, is to liberate sialic acid moieties by cleaving their O-glyoside links with underlying amino sugars (Gootschalk, 1957), and acts in this way on cell surfaces (Wallach and Eyler, 1961; Eyler et al, 1962; Rubenstroth-Bauer, 1962). The enzyme has been shown to cause pronounced changes in cell electrophoretic mobility (Cook et al, 1960, 1962) indicating the contribution of sialic acid as a charge determinant.

1.10.1.6. Metabolic activity

Ben-Or et al (1960) found an increased surface-charge density in regenerating rat liver cells as compared to adult cells and suggested that the increased surface charge density of tumor cells (Ambrose et al, 1956; Purdom, 1958; Forrester

et al, 1962) may be a manifestation of growth. Heard et al (1961) have also described a high charge density in embryonic as compared to adult mouse fibroblasts.

Brent and Forrester (1967) and Mayhew (1966) have described an increased surface charge density at the time of mitosis. This may be due to conformational changes in macromolecules containing NANA during mitosis (Kraemer, 1967).

Spangler and Cassen (1967) observed an increase in mobility following the irradiation of lymphocytes.

1.10.2. Possible Role of Sialic Acids in the Charge, PHA Response and In Vivo Circulation of Lymphocytes

1.10.2.1. Charge

Robineaux and Bazin (1951) studied the electrophoretic mobility of various types of leukocytes and found that the negative charge of lymphocytes was higher than that for other varieties of leukocytes. A greater negative surface charge on lymphocytes relative to other leukocytes was also described by Hannig and Krussman (1968) and Waler et al (1969) using cell electrophoresis.

Lichtman and Weed (1970) were unable to show a charge difference between normal and leukemic cells.

1.10.2.2. Effect of PHA and antigen antibody reactions on lymphocyte surface charges

Vassar and Culling (1964) had tested the possible surface action of PHA by examining the electrophoretic mobility of cells treated with the mitogen. The mitogen invariably caused electrophoretic slowing of both lymphoid cells and of latex particles used as controls. Thoracic duct lymphocytes showed the highest mobility and the most pronounced slowing by PHA.

More recently, Currie (1967) examined the effect of PHA on the surface charge of human and mouse lymphocytes. They also observed the slowing caused by PHA and concluded that it reduced the net surface charge. Mouse lymphoid cells had higher negative charges than human cells, and PHA abolished this difference.

Recently Bert et al (1968, 1969 a, b) have reported that antigen-antibody reactions on the lymphocyte surface caused a lowering of the cell surface charge, as measured by electrophasis. They proposed this as a method to measure immunoglobulin on the cell surface. Sundaram et al (1967) showed that antigen-antibody reaction in lymph node cells lowered their charge density. In 1970, Bert et al showed that prednisolone and antilymphocyte serum also decreased the mobility and charge of lymphocytes.

1.10.2.3. Effect of surface acting enzymes on charge and PHA response of lymphocytes

Thomson and Mehrishi (1969) found that the adhesiveness of lymphocytes to polystyrene was abolished by trypsin, which also reduced the electrokinetic properties of these lymphocytes. This was consistent with the possibility that the enzyme removed the sialic acid-bound protein (Selman and Heard, 1960; Cook et al, 1960; 1961; Ponder, 1951). Neuraminidase-treated leukemic lymphocytes also showed a lowering of mobility, suggesting that the enzymes affected bound sialic acid.

Lindahl-Kiessling and Petersen (1969a) found that trypsin, neuraminidase, pronase and amylase affected lymphocytes so they could no longer respond to phytohemagglutinin. In the case of trypsin and neuraminidase, the enzyme treatment removed a factor necessary for PHA stimulation, but the cells remained viable and regained their capacity to respond to PHA after 2-3 days in culture. They suggested that sialic acid may be a common target for enzymes that deepens the PHA response and that sialic acid components are involved in the initial stages of PHA stimulation.

The latter authors (1969b) also found that the mixed lymphocyte reaction was unaffected by lymphocyte treatment

with neuraminidase or trypsin. They proposed that sialic acid is important for the PHA effect but that different receptors may be involved in the mixed lymphocyte reaction.

1.10.2.4. Effect of surface acting enzymes on the in vivo circulation of lymphocytes

A number of recent studies have been carried out on the effect of trypsin and neuraminidase on the electrophoretic mobility, PHA effect and in vivo circulation of small lymphocytes.

David et al (1964) found that trypsin treatment of lymphoid cells interfered with their immunological capability, as evaluated by the in vitro migration test for delayed hypersensitivity.

Woodruff and Gesner (1968) measured the circulation and fate of lymphocytes after treatment with this enzyme for a 5 minute period. By labelling cells with Cr^{51}, these workers demonstrated that the selective accumulation of labelled, trypsin treated lymphocytes in lymph nodes was initially abolished, but returned after a period of more than 22 hours after tranfussion. They concluded that since the cells were viable (heat-killed cells did not subsequently accumulate in the lymph nodes and Cr^{51} bound to lymphocytes was poorly

reincorporated into other cell types), the alteration in the cells responsible for their initial inability to 'home' to lymph nodes must be repaired in vivo. The most likely explanation was that trypsin cleaves certain constituents from the lymphocyte surface required for selective emigration into the lymph nodes, and that these constituents are eventually replaced by the cells.

It is of interest that Gesner and Ginsburg (1964), Woodruff and Gesner (1967, 1969) and Gesner (1966) found that prior treatment of lymphocytes with glycosidase and neuraminidase also alter their fate, although the cells were viable prior to transfusion. Woodruff and Gesner (1969) injected lymphocytes treated for 15 minutes with neuraminidase. There was marked initial decrease in the homing of the cells to the lymph nodes and spleen, replaced by increased trapping in the liver. Subsequently the cells migrated to the lymphoid tissue.

These workers suggest that surface of the cells was probably altered because they agglutinated in sera in which they did not normally agglutinate, and because the electrophoretic mobility of the cell was reduced, indicating that neuraminidase reduced the electrokinetic charge density of the lymphocyte surface. The results were consistent with the hypothesis that cell surface sugars play a physiological role in ensuring the

normal in vivo distribution of lymphocytes and might be involved with cellular reactions which influence the fate and behavior of these cells (Gesner, 1966).

1.11 POTASSIUM TRANSPORT, POTASSIUM LEVELS AND MEMBRANE FUNCTION IN THE CONTROL OF CELL METABOLISM

1.11.1 Role of Cation Pump

The cation pump associated with the cell membrane has been postulated to have a significant role in the regulation of metabolism (Whittam and Wheeler, 1970). For example, lactate production which is the only energy source for red cells may be divided into a part which is insensitive to the pump, and a second part, the activity of which is both proportional to the operation of the pump and inhibited by ouabain (Whittam, 1967). This implies that active transport can regulate metabolism. The latter part was dependent on the availability of external K^+ and internal Na^+, and regulated by the concentrations of these ions, which determine the rate of pump operation.

Whittam suggested that the intracellular ADP level might regulate metabolism, since the cation pump splits ATP to ADP and Pi. Hoffman and Parker (1967) proposed that in red cells the pump might regulate the conversion of glucose to lactate as follows;

$$\text{1, 3-diphosphoglycerate} + \text{ADP} \underset{\text{phosphoglycerate kinase.}}{\rightleftharpoons} \text{3 phophoglycerate} + \text{ATP}$$

a reaction which occurs in the cell membrane. By this model the sodium pump should determine the rate of lactate production

whether the metabolite was glucose or a purine nucleoside, and it was therefore predicted that ouabain would decrease the rate of lactate production from deoxyribonucleosides. This prediction was verified by Wiley (1967).

Oxidative metabolism also appears to be related to the cation pump, and is probably mediated by the level of ADP. Landon, 1967) among other factors. Levinson and Hempling (1967) suggested that ATP from glycolysis is used to produce a high energy intermediate of the electron transport system which would energize the Na^+ pump. The results of Gordon and de Hartog (1968) also indicated that potassium transport regulated glycolysis in these cells.

The influence of external K^+ on the respiration of nerves was demonstrated by Rang and Ritchie (1968), who observed that ouabain abolished the increase in O_2 consumption following electrical stimulation. Brain cortex respiration is also very sensitive to the level of external K^+ which stimulates and to ouabain, which causes inhibition (Ruscak and Whittman, 1967).

1.11.2. Intracellular Potassium and Cell Metabolism

As the dominant intracellular cation, K^+ would be expected to have a significant influence on cellular function. It has become evident that cell respiration, glycolysis, enzymatic activity, macromolecular synthesis and possibly fat metabolism

are affected by changes in K^+ concentration. The following discussion will summarize some of the known requirements for this cation by different aspects of cell metabolism.

1.11.2.1. Respiration

The relationship of potassium to respiration was demonstrated in 1934 by Hegnauer et al who found, using isolated frog muscle, that the respiration rate depended on the external potassium concentration. Dickens and Greville (1935) and more recently Ruscak and Whittman (1967) showed that K^+ stimulates respiration in brain tissue. Using rabbit kidney cortex slices, Whittam and Willis found that the degree of oxygen consumption above a basal level was controlled by the rate of Na^+ and K^+ transport (1963), which was considered to be a 'pacemaker' of respiration.

A K^+ effect on mitochondria might be anticipated since these organelles contain most of the respiratory enzymes. Mitochondria have been shown to take up K^+ in preference to Na^+ (Ulrich, 1959). However, a fraction of the mitochondrial potassium is bound, since it is not removed by repeated washing with sucrose and NaCl (Stanburg and Mudge, 1953; Gamble, 1957; Ulrich, 1960). ATP increased the non-exchangeable fraction (Ulrich, 1959). However, uncoupling agents, such as dinitrophenol, inhibited the accumulation of potassium by the mitochondria.

The requirement of oxidative phosphorylation for the K^+ ion was first demonstrated by Pressman and Lardy (1955) and more recently by Krall et al (1964). Suranyi and Avi-Dor (1965) provided evidence that both K^+ and ATPase were involved in the regulation of the permeability of the mitochondrial membrane. Loss of intramitochondrial K^+ caused by valinomycin was shown by Kimmich and Rasmussen (1967) to lead to a marked inhibition of O_2 consumption which could then be reactivated by the addition of K^+. This was evidence that mitochondrial electron transport was sensitive to the K^+ level, rather than to the rate of K^+ movement across the mitochondrial membranes.

Pena et al (1969) reported that the stimulation of respiration and glycolysis in yeast caused by K^+ was associated with a reduction of intracellular ATP and an increase in ADP, which stimulated glycolysis and respiration.

1.11.2.2. Carbohydrate Metabolism

Early work by Hastings and his colleagues (1942) showed the requirement for K^+ and Mg^{++} in glycogen synthesis, and for Na^+ in glycogenolysis, which is usually associated with a loss of cell K^+(Buchanan et al, 1949).

As already mentioned, glycolysis is affected by the state of active transport and ADP level may be a primary

modulating factor. However, it is not simple to differentiate between the effects of changes in the activity of the cation pump, the effect of the actual level of cation within the cell, and the effect of these cations on the transport of glucose and other substances into the cell. The data of Penna et al (1969) and Clausen (1966) showing K^+ dependency of glycolysis in yeast and rat diaphragms do not clearly differentiate between these possibilities. However, by affecting certain key enzymes such as pyruvate kinase, changes in intracellular K^+ would be expected to cause profound changes in carbohydrate metabolism.

1.11.2.3. Potassium requirements of some enzyme systems

Ussing (1960) listed a number of enzyme systems which require K^+ for activation, many of which are concerned with transphosphorylation. For example, acetyl kinase, which catalyzes the fusion of acetate and Co-A in the presence of ATP was found to be strongly activated by K^+ but inhibited by Na^+ (Korff, 1953). The transfer of phosphate from phosphopyruvate to ADP, catalyzed by pyruvate kinase, also requires K^+ for activation (Boyer et al, 1943). In some cases, Na^+ is a competitive inhibitor of K^+ (Dixon and Webb, 1965). Adenosine-5-phosphate deaminase is activated allosterically by K^+ (Smiley and Sueller, 1967).

More recently, Sueller (1970) has classified a large number of enzymes requiring monovalent cations and obtained a characteristic pattern for reactions activated by monovalent cations. Evans and Sorger (1966) made an extensive compilation of enzymes requiring K^+. In general, those requiring K^+ are poorly activated by Na^+ and vice-versa. Since the conformation of pyruvate kinase is affected by cations, Evans and Sorger (1966) suggested that the monovalent cations exert a role by maintaining a specific protein conformation.

Of particular interest has been the possibility that the formation of cyclic AMP may be dependent on potassium. Shimizu et al (1970) reported that formation of this substance was greatly stimulated with K^+, provided that Ca^{++} was also present. Endogenous levels of cyclic AMP were elevated in brain slices after incubation with high concentrations of potassium ions (Saltin and Rall, 1967). In a study with rat diaphragms, Lundholm et al (1967) found that high K^+ increased markedly the production of cyclic AMP and potentiated the stimulating action of adrenalin.

These effects might result from a reduction of the breakdown of cyclic AMP by phosphodiesterase or by a stimulation of the adenylcyclase system in the presence of K^+.

Ho et al (1967) found that removal of K^+ from incubation medium caused a marked decrease in adenyl cyclase activity of fat cells. The administration of ouabain also led to an inhibition of cyclic AMP production. Similar inhibitary effects of ouabain on cyclic AMP formation in adipose tissues were obtained by Vulliemoz et al (1968).

1.11.2.4. Macromolecular synthesis

The requirement of K^+ for synthesis of proteins and nucleic acids was considered by Lubin. He showed that the intracellular potassium level governed the rate of protein synthesis in bacteria (Lubin, 1964; Lubin and Ennis, 1964). Using sarcoma cells (1967), Lubin found that inhibition of K^{42} uptake by the cells, caused by an enhancement of leakage out caused by amphotericin-B, thus leading to lowered intracellular K^+ levels, was associated with a reduction in the incorporation of leucine, uridine or thymidine into protein, RNA and DNA. Excess K^+ caused a reversal of the effect of amphotericin-B.

The activity of DNA dependent RNA polymerase was studied by So et al (1967), who found that the synthesis of RNA in a cell-free system was stimulated by the presence of potassium. Furthermore, KCl effectively reversed end product inhibition by newly synthesized RNA. The authors suggest that enzymes may

be caused to dissociate from active 21S dimer units to two active 13S monomers.

The role of the K^+/Na^+ ratio in RNA synthesis and the regulation of gene action, is also clearly illustrated by the puffing phenomenon of the giant chromosomes of the insect order, Diptera (Kroeger and Lezzi, 1966). Extensive investigation on the role of ions in puffing have shown that increased K^+ caused an acceleration of puffing patterns, and Na^+ a reversal. In C thummi, a series of puffing patterns can be evoled at will merely by adjustment of Na^+/K^+ ratios in the explant medium. The ionic balance in the nuclear sap was considered to be a critical controlling factor. It was proposed that the action of the stimulating hormone (ecdysone), which measurably altered the membrane potential, caused a stimulation of the sodium pump → decrease of intracellular Na^+ → increase of intracellular K^+ → changing of puffing pattern → release of a specific selection of RNA molecules to the cytoplasm → changes in cell biochemistry.

1.11.3. Cation Levels in Lymphocytes

Very few accurate measurements of cation levels in lymphocytes have been carried out although a number of attempt using non-separated leukocytes have been made.

Studies of the cation content of mammalian leukocytes are summarized in Table 1.11.1. Most workers have found the potassium to exceed the sodium content, although not by more than a factor of 2-3. Almost all the studies were carried out with a preponderance of polymorphonucleocytes so that the results are meaningless for lymphocytes.

The only measurements of cations in small lymphocytes which appear to have been published were carried out by Lichtman and Weed (1969). Following lysis of red cells with isotonic ammonium chloride, they incubated the cells for various periods of time, after which they were washed in choline chloride and potassium and sodium content measured. Immediately after isolation, the potassium levels were quite low but after allowing the cells to incubate for a few hours, there occurred an increase in the total potassium content and a lowering of the sodium levels. The potassium-sodium ratio was approximately 3.5 after 19 minutes of incubation in vitro. Total molarity of potassium was of the order of 28 n moles/10^6 cells and of sodium about 8 n moles/10^6 cells. The mean lymphocyte volume was measured to be approximately 269 cubic microns which corresponded relatively well to the value of 250 cubic microns reported by Westring et al (1969). The apparent concentration of potassium in the cells was of the order of 120 mM.

Table 1.11.1.

Potassium and Sodium Content of Leukocytes

Cells	Cation Content Na meq/l	K meq/l	K/Na	Author
Rabbit leukocytes 85% PMN	80 ± 8	106 ± 10	1.3	Wilson & Mannery (1949)
Human leukemic cells(type not specified) meq/kg	37 - 54	96 - 98	1.8-2.6	Waterhouse et al (1955)
Rabbit leukocytes (mainly PMN)	98 ± 21	95 ± 17	10	Elsbach & Schwartz (1959)
Chronic lymphocytic leukemia	80 ± 9	107 ± 15	1.3	Rigas (1961)
Normal human leukocytes (not separated	60 ± 6	100 ± 6	1.7	Baron & Roberts (1963)
Acute and chronic myeloid leukemia	Cell volume and number not stated		2.3-2.6	Block & Bonting (1964)
Lymphocytes after incubation	34 ± 3	120 ± 3	3.5	Lichtman & Weed (1969)
Granulocytes after incubation	30 ± 4	118 ± 3	3.9	Lichtman & Weed (1969)

A criticism of these measurements is that they were carried out after a variable period of incubation and a choline chloride wash. Hence, 'surface bound potassium', as has become apparent from the present study, would not have been removed. It is therefore not certain that the measured potassium values actually represented the true intracellular cation level.

1.11.4 Associations between Membrane and Nuclear Function

Control of DNA synthesis by the bacterial cell membrane has been proposed by Marvin (1968) and Ryter (1968) who observed attachment of the chromosome to the nuclear membrane.

It has long been realized that cell division and cell movement are associated (see Abercrombie, 1957 and Swann, 1957). Tyler (1957) showed that an antibody prepared against a polysaccharide on the surface of fertilized and unfertilized sea urchin eggs caused inhibition of cell division. Harnsa (1958) found that cell to cell contact by amoebae caused inhibition of culture growth.

The role of cell contact in the control of cell division has been a subject of increasing study recently because of the observation that neoplastic cells show a loss of 'contact inhibition'. More recently, the phenomenon of contact inhibition

by neoplastic and non-neoplastic cell lines (Abercrombie et al 1957; Abercrombie and Ambrose, 1962) has been demonstrated for cells transformed by carcinogenic hydrocarbons, X irradiation and polyoma virus (Borek and Sachs, 1966). Fisher and Yeh (1967) suggested that contact between cells initiates a sequence of intracellular events that interfere with cellular proliferation. However, a diffusible factor may also be involved in the mechanism (Yeh and Fisher, 1969; Kruse and Miedema, 1965) of contact inhibition.

2. MATERIALS AND METHODS

2.1 TECHNIQUES FOR CELL AND LYMPHOCYTE CULTURE

2.1.1. Source of Cells

For almost all experiments, human lymphocytes were obtained following venipuncture of the peripheral anticubital veins of presumably healthy, adult, male and female volunteers. In some cases, blood was taken from hospital patients having various types of leukemia, and a group of patients with cystic fibrosis was also examined.

Blood samples were obtained in volumes of 10-200 ml at a time and were placed in culture after red cell separation, about an hour following venipuncture. Although larger volumes of blood could have been obtained from blood banks or abattoirs, fresh human or animal blood was always used. The methods used for obtaining animal cells are outlined in Section 2.1.7.

In some experiments, BSC-1 were used. These cells, which were kindly given to me by Dr. Furesz of the Virus Laboratory,

Department of National Health and Welfare, were originally derived from Green Monkey kidney cells, and had been passaged over a hundred times by the time they were placed into a culture for continuous maintenance during these studies.

2.1.2. Method for Lymphocyte Separation

The separation of lymphocytes from other leukocytes of the peripheral blood has been a matter of considerable experimentation in different laboratories. In general, it is probably true that the more painstaking and gentle one is with the cells, the better is the separation achieved. A number of different methods have been used. One of the most popular principles of separation is based on the 'stickiness' of all the leukocytes, except the small lymphocyte, for glass and certain other substances such as plastic, nylon, etc. Thus, separation of small lymphocytes has been achieved by placing leukocytes in contact with glass or polystyrene beads, nylon mesh, glass wool or glass bottomed 'T-flasks' for varying lengths of time and under varying conditions (Garvin, 1961; Cudkowicz et al., 1964; Rabinowitz, 1964; Morrison, 1967). Mills and Chandler (1968) described a method based on the trapping of non-lymphocytes in thrombi. Another commonly used technique is based on density gradient separation. Thus, albumin, dextran, gelatin or 'Ficoll' have been used, with varying centrifugal 'g'

forces (Ling et al., 1965; Coulson and Chalmers, 1964; Boyum, 1968; Perper et al., 1968; Noble and Cutts, 1968). The capacity of polymorphonucleocytes (PMN's) and macrophages to engulf small particles has been used as a principle for cell separation; if iron filings are used, the phagocytic cells can be separated by the use of a magnet (Cassen et al., 1958; Kuper et al., 1961; Thierfelder, 1964). All of these techniques involve manipulation of the cells either physically or chemically over varying period of time, and some degree of cell damage probably occurs in most cases.

In the present experiments, it was desired to separate the cells without substantially altering the surrounding medium or causing much cellular damage, keeping the temperature at 37°C, and at the same time, utilizing an easily constructed, simply packaged and sterilizable system with an accurately measured rate of outflow.

Several systems were tested. The apparatus finally developed and used was modified from the glass bead system originated by Rabinowitz (1964). A picture of the system used is shown in Figure 2.1.1; and Figure 2.1.2 is a schematic diagram of the system. In principle, a 50 ml syring barrel was packed with 25 ml of 200 μ diameter glass beads (Superbrite, #100-5005, 3 M Co., Minneapolis, Minn.), bounded above and below by 4 ml layers of

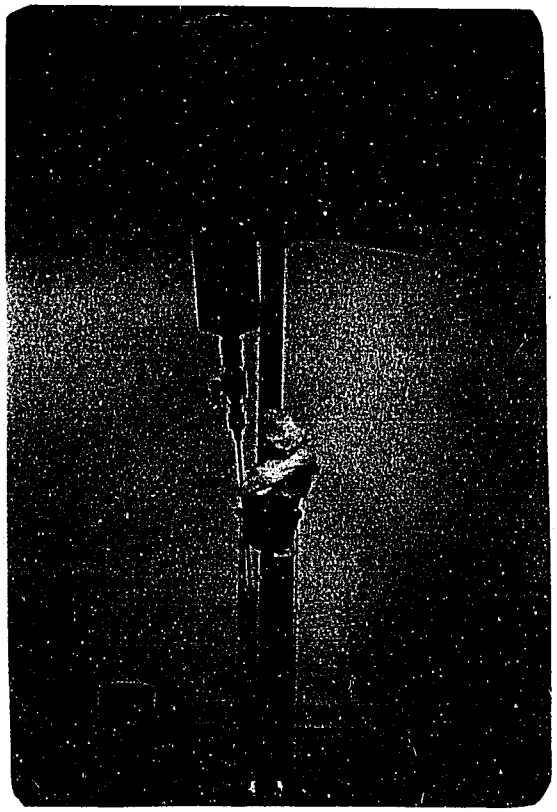


Fig. 2.1.1.

Photograph of apparatus designed to separate lymphocytes from leukocytes. The schematic diagram is shown in Fig. 2.1.2. Vial at base of stand was used to separate red cells from leukocytes using dextran.

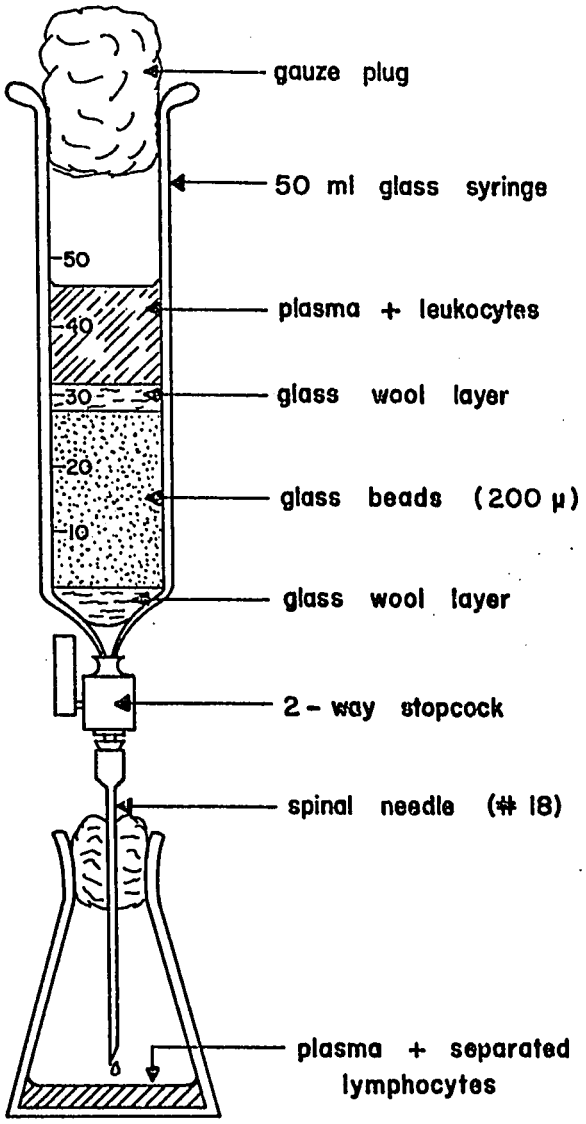


Fig. 2.1.2.

Schematic diagram of lymphocyte separation system. The system is simply assembled, sterilized and stored for use. The efficiency of separation is shown in Fig. 2.1.3. and in Table 2.1.1.

glass wool which served to keep the beads in place. A spinal needle was attached by a stopcock to the syringe after assembly of the package. The entire system could be sterilized by autoclaving in the usual way and was rapidly and simply assembled.

The system was operated as follows; 10-20 ml of normal fresh culture medium (37°C) was first completely run through the system at full speed. After closing the stopcock, 20-30 ml of the leukocyte suspension which was obtained following sedimentation of the red cells in dextran was added. The stopcock was then opened and the medium run almost completely through so that about half of the plasma and cells had passed into contact with the glass beads. This could be seen clearly by the movement down the syringe of the line of demarcation between the pink medium and yellowish plasma following. However, some medium was left in the bottom of the syringe at the level of the lower glass wool packing.

After the syringe was filled with the leukocyte suspension, it was plugged with the sterile cotton wad with which it had been sterilized and then incubated for about ½ hour at 37°C. The spinal needle of the syringe was subsequently inserted into a graduated cylinder, (see Figure 2.1.1) and the 'rate-control' stopcock opened to allow a flow of not more than 1 drop per second. (The slower the flow, the better was the separation, but if the flow was

too slow, the cell yield was reduced to unacceptably low values.) After the flow of plasma and cells had stopped, another 20 ml of plasma or medium was passed through to remove additional lymphocytes. However, further addition of plasma usually resulted in the appearance of PMN's. The characteristics of the system were tested and are shown, for one experiment, in Figure 2.1.3.

When these directions were followed, the resultant plasma usually contained about 96-98% small lymphocytes. Too rapid a passage of cells led to a less satisfactory separation. Experiment 403 is an example of an experiment in which contamination by PMN's was pronounced due to too rapid flow or poor technique (first row of Table 2.1.1).

Leukocyte preparations were obtained simply by adding the fresh blood to a solution of 6% dextran in saline to give a final dextran concentration of 0.6 to 0.8%. The dextran used for this purpose (INTRADEX, Glaxo Lab Ltd., Middlesex) was obtained originally by fermentation by strains of Leuconostoc. The mean molecular size of this preparation, after partial hydrolysis, was not dissimilar to that of plasma albumin, i.e., about 110,000 (with a range of 50-150,000). Ethylenediamenetetraacetic acid (EDTA) was not used, as it was desired not to alter the concentration of bivalent cations in the plasma.

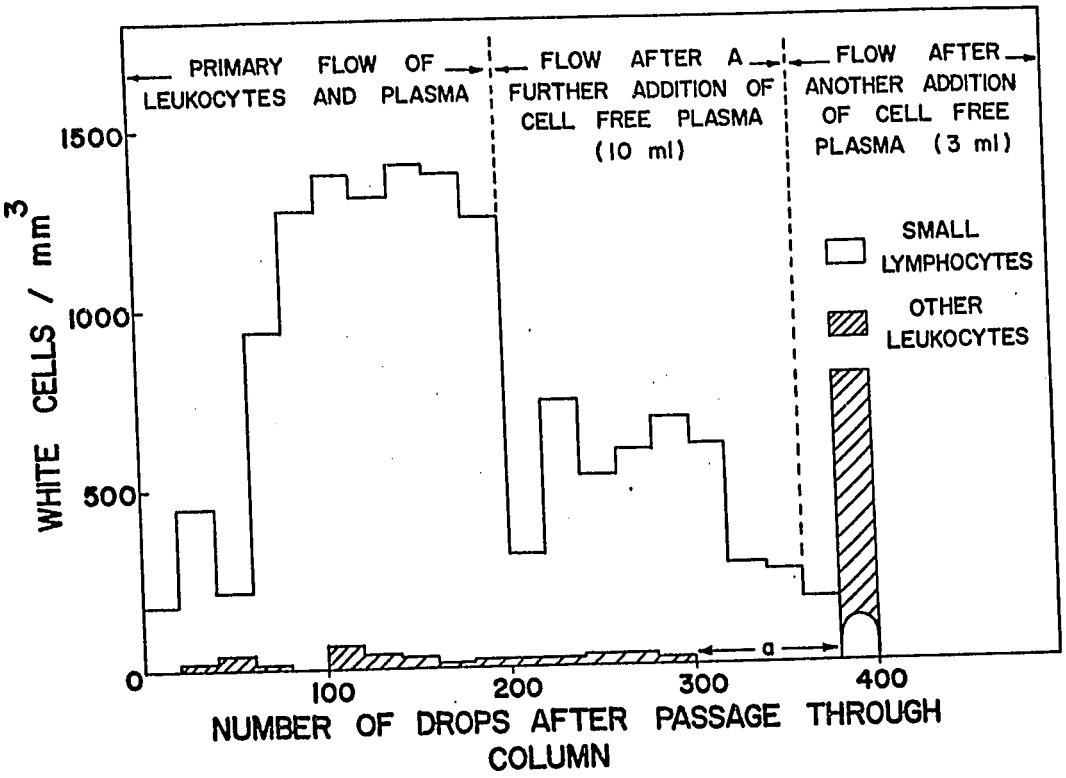


Fig. 2.1.3.

Histogram illustrating separatory efficiency of system shown in Fig. 2.1.1. and 2.1.2. A leukocyte suspension, obtained following dextran-enhanced red cell sedimentation, was added to the column. Cell counts and differentials were carried out on cumulative 20 drop aliquots as the cell suspension was passed through the system.

Table 2.1.1.

Typical Cell Counts and DNA Measurements

Exp't	No. of white cells initially present	% small lymphocytes	No. of lymphocytes	DNA µg	DNA: µg per 10^6 lymphocytes
403 *	10.7×10^5	55%	5.9×10^5	5.9	10.0
4.6	6.6×10^5	97%	6.4×10^5	8.2	12.8
451	8.4×10^5	98%	8.2×10^5	9.0	10.9
452	8.2×10^5	96%	7.8×10^5	9.2	11.7
455	2.6×10^5	100%	2.6×10^5	2.8	10.6

* Separation attempted but accident caused rapid bypass of glass beads by leucocytes. Note that DNA readings (carried out after one day *in vitro*) are meaningful only if lymphocytes only are considered. After one day, PMN were usually observed morphologically to have undergone considerable disintegration.

Complete removal of red cells from the lymphocyte cultures was sometimes desirable, although this was unnecessary in most of the experiments described, since the red:white cell ratio of 2-3:1 was usually acceptable. Removal of red cells by known methods requires lysis of the erythrocyte membrane and some degree of concurrent damage to the lymphocyte surface membrane is therefore probable. However, in some experiments on the measurement of total lymphocyte cation content, the erythrocytes were removed by suspending the cells for 2 minutes in 0.83% NH_4Cl. The remaining lymphocytes were shown to be 'viable' by the Trypan Blue exclusion test for at least 2 days and could also be stimulated by PHA.

2.1.3. Culture Conditions and Medium

When the white cells had been separated from most of the erythrocytes using dextran, and in a large number of experiments, from other leukocytes such as polymorphonuclear cells (PMN's) and monocytes, the resulting cell suspension was added to medium 199 (115H, Grand Island Biological Co., Grand Island, N.Y.), which is a readily available commercial medium originally formulated by Morgan et al. (1950). There were some disadvantages to the use of this medium, since its constituents could not be varied at will. However, a major advantage to its use was that uniformity of the nutrient medium in different experiments was

TABLE 2.1.2.

GIBCO Medium-199 (Morgan et al, 1950)

Components

Component	Gm/L
NaCl	8.000
KCl	0.400
$MgSO_4 \cdot 7H_2O$	0.200
$Na_2HPO_4 \cdot 7H_2O$	0.090
KH_2PO_4	0.060
Dextrose (anhyd.)	1.000
Phenol Red	0.020
$CaCl_2$ (anhyd.)	0.140
$NaHCO_3$	0.350

	mg/L
L - arginine	70
L - histidine	20
L - lysine monohydrochloride	70
DL - tryptophan	20
DL - phenylalanine	50
DL - methionine	30
DL - serine	50
DL - threonine	60
DL - leucine	120

TABLE 2.1.2.

GIBCO Medium-199

Components (Cont.)

Component	mg/L
DL - isoleucine	40
DL - valine	50
DL - glutamic acid monohydrate	150
DL - aspartic acid	60
DL - alpha - alanine	50
L - proline	40
L - hydroxyproline	10
Glycine (aminoacetic acid)	50
L - glutamine	100
Sodium Acetate Trihydrate	50
L - cystine	20
L - tryosine	40
L - cysteine hydrochloride	0.1
Adenine	10
Guanine	0.3
Xanthine	0.3
Hypoxanthine	0.3
Uracil	0.3
Thymine (5-methyl uracil)	0.3
Disodium Alpha Tocopherol Phosphate	0.01
Thiamine	0.01
Pyridoxine Hydrochloride	0.025

TABLE 2.1.2.

GIBCO Medium-199

Components

Components	mg/l
Riboflavin	0.01
Pyridoxal Hydrochloride	0.025
Niacin	0.025
Niacinamide	0.025
Clacium Pantothenate	0.01
i - inositol	0.05
Ascorbic Acid	0.05
Folic Acid	0.01
p - Aminobenzoic Acid	0.05
Ferric Nitrate	0.1
Biotin	0.01
Menadione	0.01
Glutathione	0.05
Vitamin A	0.1
Calciferol	0.1
Cholesterol	0.2
Tween 80 (Atlas Powder Co.)	5.0
Adenosinetriphosphate (sodium salt)	1.0
Adenylic Acid	0.2
Desoxyribose	0.5
D - ribose	0.5
Choline Chloride	0.5

maintained better than would have been possible otherwise. Before the addition of cells, plasma, and heparin, the medium contained the constituents shown in Table 2.1.2.

The final plasma concentration was generally kept at 15% by volume. This concentration has been found by many workers in this field to be suitable (Ling, 1968). Medium alone, without added serum or plasma, is usually inadequate for the maintenance of cell in vitro. In these experiments autologous plasma was almost always used, although fetal calf serum was also employed under some circumstances. The use of autologous or homologous plasma, or of human, bovine or fetal calf serum introduced unknown constituents and undoubtedly caused variability with respect to cell growth and maintenance condition. However, it was sometimes necessary to accept this. For example, the use of serum, rather than plasma, has been found to be important if rat lymphocytes are cultured (Metcalf, 1966).

For these reasons, autologous plasma from the donor of the cells was used when possible, nonetheless recognizing that variations in the plasma of individuals do occur. In general, experiments were planned whereby control and experimental data were obtained from the same cultures, obtained from the same person at the same time under the same culture conditions. All of the experiments here reported were done under such conditions, unless stated otherwise.

In addition to plasma (or serum in some cases), heparin was also added to the culture medium. It became apparent that preserved heparin was not appropriate for cell culture, probably because of the presence of phenol. A preservative-free heparin was therefore used for culture purposes and was added to a final concentration of 20 μ/ml. When heparin was not used, fibrin formation often occurred. Although cells remained visible and radioactive counting could still be carried out under such conditions, the formation of a fibrin matrix probably altered the conditions of cell culture leading to changes in the diffusion of oxygen and metabolites, and furthermore, a serious restraint was imposed on morphological studies. Moreover, any fibrin in the culture made it impossible to pipette cells quantitatively.

In some experiments, the use of plasma in the medium was disadvantageous because precise knowledge of the amounts of certain constituents, such as of the cation content, was essential. Also, it was sometimes desirable to vary the potassium level to values below those used in commercial preparations of medium 199. In such cases, (a) a modified 'Hank's solution' was made up without potassium, the pH of which was 7.2 - 7.4 by measurement, or (b) a specially prepared potassium-free 'medium 199' was used.

The constituents of the defined potassium-free, balanced salt solution are shown in Table 2.1.3. It was prepared from the

Table 2.1.3.

Constituents of potassium free, balanced salt solution

Compound	Concentrations (mg/liter)
NaCl	8300
Na_2HPO_4	48
$NaH_2PO_4 \cdot H_2O$	52
$MgCl_2 \cdot 6H_2O$	100
$CaCl_2$ (anhydr.)	140
$NaHCO_3$	350
Glucose	1000

inorganic salts usually present in medium 199, plus glucose, but replacing KCl with NaCl and KH_2PO_4 with NaH_2PO_4.

Antibiotics were used only rarely, as for the response of rat cells when there was a realistic possibility of infection. The reason for not using antibiotics for this was threefold; it was desirable (a) to avoid 'unphysiological' substances in the medium, (b) to avoid the possibility of antigenic responses of the cells which sometimes occur with antibiotics such as penicillin, and (c) to use the same defined medium as much as possible for all experiments.

2.1.4. Cell Counting

For most experiments, leukocyte counts were carried out in the usual way using a hemocytometer, after suspending a known volume of cells in 3% acetic acid. A plasma smear of the cells to be counted was also made and the differential count was thus obtained for a quantitative estimate of the relative proportion and numbers of the different types of white cells. In all cases, together with the white count, a 'total cell' count was also obtained in order to estimate the relative number of red cells present in the cell population. The accuracy of cell counting was about 10% of the actual count, as shown in Table 2.1.4.

Table 2.1.4.

Accuracy of Counting With the Hemocytometer

No. of Experiments	No. of Separate Counts	St. Dev. per Measurement *
22	59	0.10

$$* S = \sqrt{\frac{1}{mn-1} \sum_{i=1}^{n} \sum_{j=1}^{m} \left(1 - \frac{x_{ji}}{x_j} \right)^2}$$

Where x_{ij} = individual cell count # i for experiment # j

$\bar{x}_i$ = mean of all cell counts reported for particular experiment # i

n = number of experiments.

m = number of repeated measurements in each experiment.

For many experiments, an estimate of the total number of cells present at the time of the experimental procedure was essential. However, this was hard to do by direct cell counting using the hemocytometer because of the impossibly large numbers of counts that would have been necessary, the clumping of cells and their 'stickiness' to plastic culture tubes in the presence of PHA, and the loss of cells during washing or when they had been treated by agents such as 5% trichloracetic acid. Measurement of total protein was again fallacious, since it was not possible to separate the protein of lymphocytes from that of the red cells present.

After some investigation, an apparently adequate method was obtained, based on the work of Tedesco and Mellman (1966) relating total DNA content to cell number. However, it is clear that this procedure can only be used if the lymphocytes have already been separated from PMN's and macrophages. Since PHA is known not to cause an increase in total lymphocyte DNA for at least one day, this technique was employed for the evaluation of cell number during the first day of PHA treatment. The actual technique used for the estimation of DNA in the cultures is given in Section 2.4.

Figure 2.1.4 shows the relationship between the number of lymphocytes added (as determined by hemocytometer count) and their DNA content, as determined by the diphenylamine method.

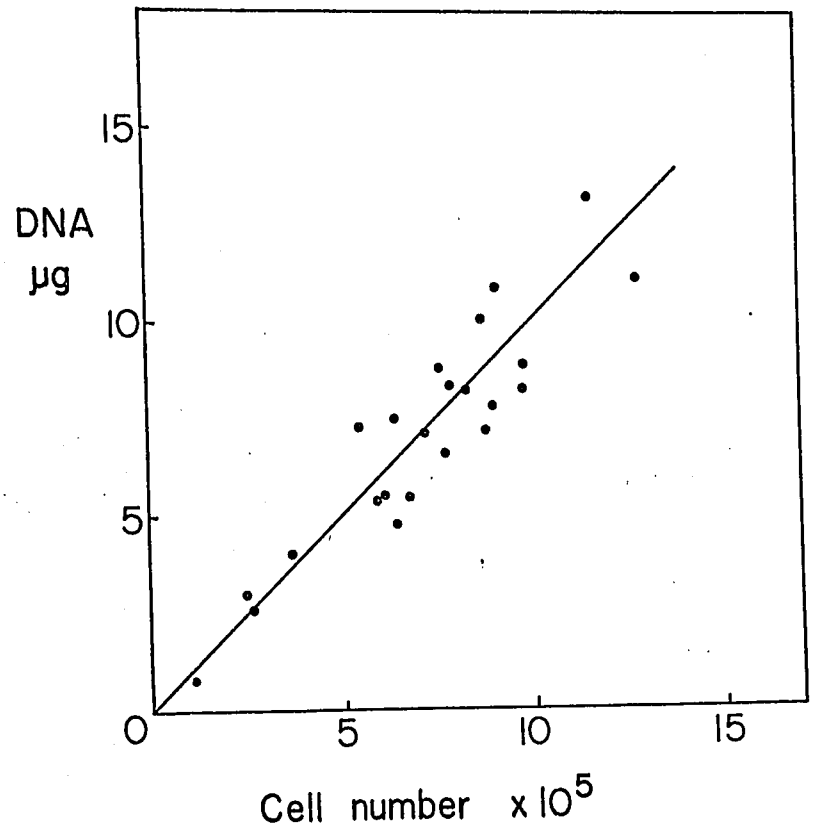


Fig. 2.1.4.

Relationship between lymphocyte number and DNA content. Each point represents the mean cell count and total DNA of separate lymphocyte cultures, obtained from different donors.

The mean value was 10.6 ± 0.2 (S.E. of the mean) μg DNA/10^6 lymphocytes. However, it is likely that after one day, the PMN DNA was not measured due to disintegration of the PMN's. For example, the DNA content of the cells in Experiment S-403 (Table 2.1.1, first row) can only be explained if the DNA contribution of the PMN's was no longer available for measurement.

2.1.5. Culture Vessels Employed and Their Preparation for Use

2.1.5.1 Choice of Culture Vessels

It was realized at an early stage of these experiments that the type of culture vessel used was of critical importance to the maintenance of cells in vitro and for the measurements of their biological responses. A number of different methods and a large range of vessels were tested, such as Leighton tubes, Petrie dishes, vials, centrifuge tubes and various kinds of glass and plastic test tubes and flasks. Of significance was the fact that, because of the lengthy cleaning procedures used for non-disposable items, the entire time available for experimentation could easily be taken up only with the washing of dishes. Therefore plastic disposable ware were used as much as possible. A word of caution is given here because some plastic culture tubes can give trouble (Bain et al., 1968). The types of vessels listed in Table 2.6.3 were found to be satisfactory.

Leukocytes (and lymphocytes) are dependent not only on the amount and kind of nutrient and to some extent, the oxygen available, but their biological responses are particularly dependent on the concentration of cells present and on the degree of crowding (Hedeskov and Essman, 1966). However, early experiments showed that there was no particular advantage in keeping the cells continuously agitated and reproducible results could be obtained in stationary cultures. On the other hand, the height of the medium above the cells was important, and the tightness of the vessel cap or stopper was critical.

In order to obtain sufficient mitoses in phytohemagglutinin-treated lymphocytes for the preparation of chromosomes (Fig. 3.1.9) it was found useful to employ glass Ehrlenmeyer flasks (50 ml) which were tightly stoppered with white rubber (West Co.). The total volume of culture medium for adequate lymphocyte transformation was 6-12 ml. In this culture system, the lymphocytes did not adhere to the bottom surface of the vessel but existed as a thin layer of leukocytes and red cells in the first millimeter above the bottom glass surface. In the presence of PHA, the cells formed clumps (Fig. 2.1.5). The advantage of this system was that since the cells did not adhere to the glass, they could be easily removed without cell loss or the application of agents such as trypsin. This culture method was therefore used for morphological examination and for autoradiography.

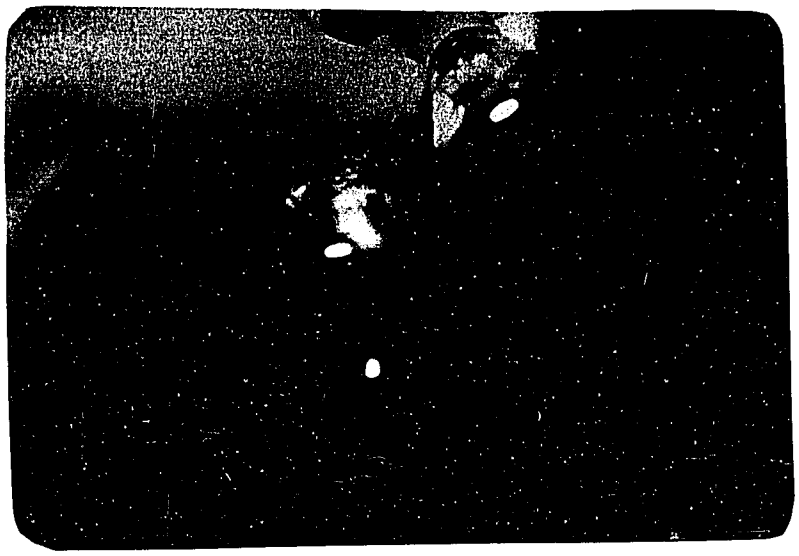


Fig. 2.1.5. External appearance of leukocyte cultures while maintained in 50 ml Ehrlenmeyer flasks. Left: No PHA; Right: PHA for 3 days.

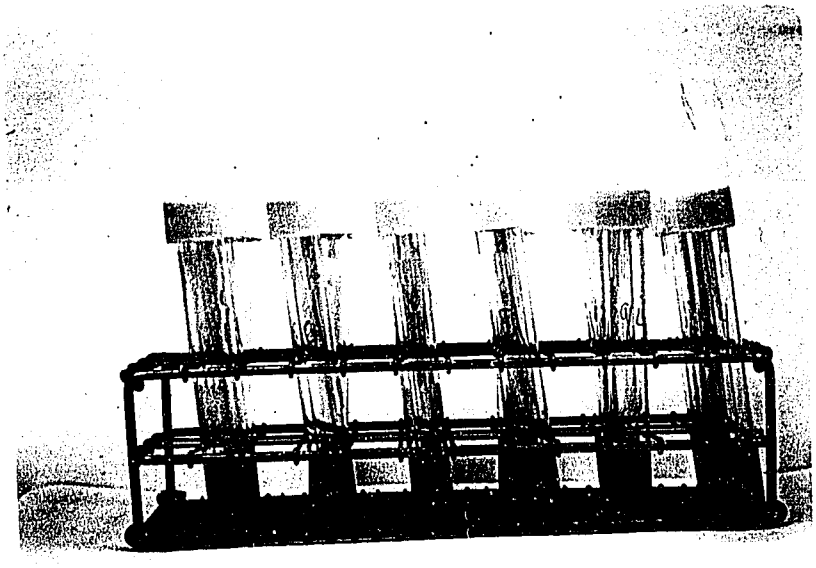


Fig. 2.1.6. External appearance of cell cultures while maintained in plastic culture tubes.

Although the presence of clumps caused problems of cell enumeration and of morphological observation of individual cells, the clump size and degree of cellularity was found to be a useful parameter for the quantitative description of some effects of ouabain (see Section 3.2.2).

When labelled precursors were used for the measurement of transport or the measurement of RNA, DNA or protein synthesis, large numbers of multiple cultures were required in order to obtain statistically reliable results. The Erlenmeyer flask system was inadequate for this because impossibly large numbers of flasks would have to have been used (hundreds each week). It was found that the 'Falcon' plastic test tubes, #3033, supported lymphocytes under in vitro conditions and that transformation could be assessed quantitatively by the incorporation of radioactive precursors into acid insoluble material, using replicate cultures. The disadvantage of this system was that the cells tended to adhere to the plastic bottom of the tube, particularly after PHA was applied. This did not allow concurrent morphological studies to be made; but this adherence to the bottom of the tube in the presence of PHA was also an advantage, since direct measurements of cellular constituents such as for DNA or total cations could be made on the same cells, using the same culture tubes with which radioactive counting was previously carried out, with very little cell loss. Figure 2.1.6 is a picture of the cultures in a typical experiment.

The continuous maintenance of cells such as the BSC-1 line was carried out successfully using disposable 100 ml T-flasks (Falcon Plastics; Becton, Dickinson & Co., Clarkson, Ont.). The purpose of culturing these cells was threefold. Firstly, successful maintenance of the culture in the absence of antibiotics was a very useful indication of the sterility and adequacy of the methods used for cell culture and glassware preparation. Secondly, these cells were useful for the testing of cytological and autoradiographic techniques, and for measuring the uptake of radioactive tracer before carrying out the often more complicated procedures for lymphocytes. Thirdly, these cells could be studied as a continually dividing cell line, in comparison to the usually non-dividing lymphocyte cultures.

2.1.5.2. Washing Procedures for Glassware

All used glassware was first washed in detergent (MICROSOLVE, Microbiological Associates Inc., Bethesda, Maryland). Glassware in which cells were cultured, rather than merely transferred, were soaked for 2-4 days in concentrated nitric acid After a thorough washing in water, followed by distilled water, they were soaked for one hour in 0.1% Na_2CO_3 solution. After this they were washed at least 6 times in distilled and then twice in double distilled water. After insertion of a fitted cotton plug, the vessel was wrapped about the mouth or completely with autoclave paper, tied with a string, and autoclaved for one hour at 270°C.

Finally, the vessels thus sterilized were placed in an oven at 80°C for several hours until dry. Such vessels were serviceable indefinitely.

New glassware which had not been used was first placed for 1-2 hours in 0.6% hydrochloric acid and then the same procedure was used (without the nitric acid soak).

Glassware which was used in contact with cells but which was not used for cultures and rubber stoppers was washed thoroughly in detergent, followed by 0.1% Na_2CO_3 and then 6 rinses in distilled and 2 rinses in double distilled water before culture wrapping and autoclaving. These items included glass pipettes, test tubes, syringes, needles, graduated cylinders, etc. Rubber stoppers were treated similarly but without Na_2CO_3.

Glass beads used for lymphocyte separation were washed thoroughly in 0.1% hydrochloric acid, followed by much washing with distilled water. Passage of medium through the beads showed no acidic residue that caused color change of the phenol red in the medium.

Because awkwardly shaped glass utensils had to be immersed in concentrated nitric acid, the hazard to persons carrying out this procedure was not inconsiderable, and a special acid bath was therefore designed. A photograph and plan of the system is shown in Figure 2.1.7.

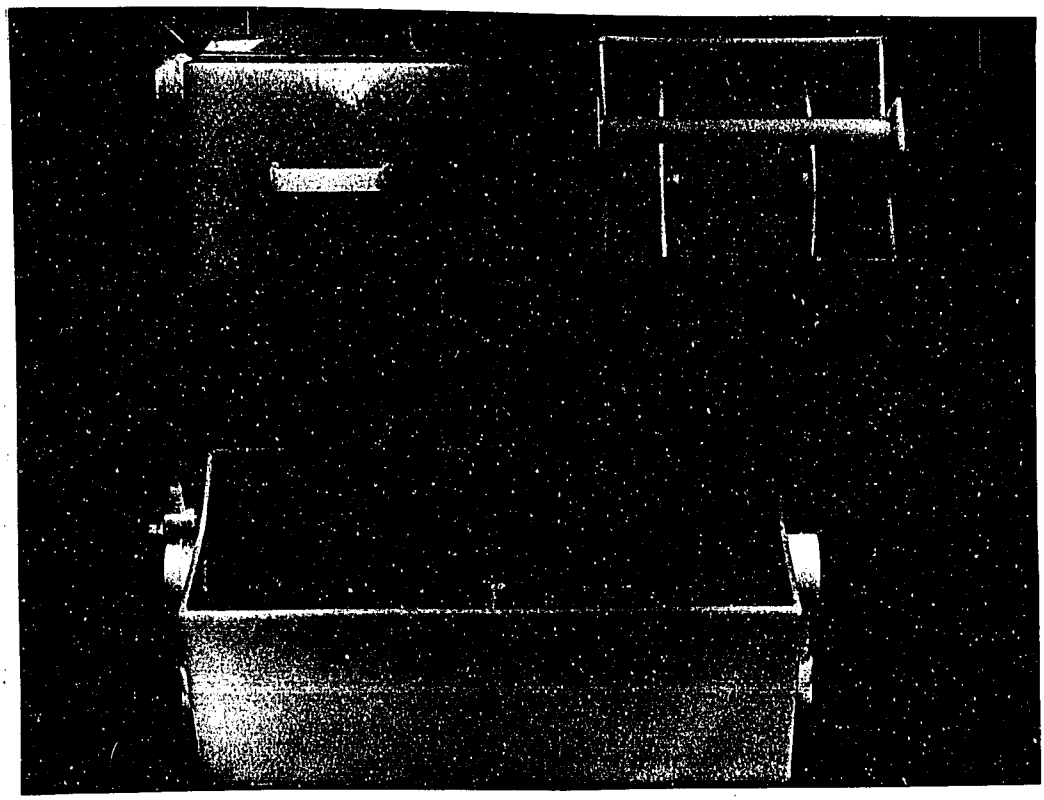


Fig. 2.1.7.

Acid bath. Specially constructed for the soaking and rinsing of awkwardly shaped glass utensils in concentrated nitric acid. Section of left holds acid which can be slowly removed from bath by a siphon. Section on right holds water for continuous rinse by a siphoning system. Container on upper right holds glassware in place to permit transfer from one section to the other with minimal splashing of acid thus reducing danger to the person handling the apparatus. Cover on upper left covers acid section.

2.1.6. Culture Maintenance Procedures

Most of the experiments described were 'short term', lasting not more than a few (3-4) days, with occasional exceptions. When cells were maintained for periods of a week, the medium was changed after 3 or 4 days of culture.

When lymphocyte or leukocyte cultures were maintained in test tubes, they were usually cultured in 3 ml amounts; the height of the medium being about 2-3 cm. It was essential that the plastic screw-capped tubes were tightly closed.

Mixed leukocyte cultures were prepared simply by mixing equal numbers of leukocytes (by hemocytometer count) in equal volumes of plasma. The final mixture was added to medium-199 and the cells cultured in plastic culture test tubes as described for single donor cells. These cultures were, however, maintained *in vitro* for 5-6 days or longer.

BSC-1 cells were cultured in 100 ml Falcon plastic flasks. Each week after seeding, they were changed to fresh medium fortified with 2% fetal calf serum. Every second week, the cells were harvested by incubation in 5% trypsin, resuspended in medium-199 containing 5% fetal calf serum, and replaced in a new set of flasks which were satisfactory for this purpose.

2.1.7. Animal Cells

In some experiments, blood from Wistar rats and from inbred red line Minnesota pigs was used in order to examine the response of the leukocytes and lymphocytes of these animals.

Rat blood, obtained from the inferior vena cava, was a particular problem because the red cells did not sediment in the presence of dextran. Furthermore, rat lymphocytes did not survive in vitro with rat plasma. Similar results have been reported by other authors (Metcalfe, 1966). A modified technique was therefore used in which the blood was defibrinated with glass beads. The cell suspension was then added in small amounts as a whole blood culture (Hungerford, 1965) using 15% fetal calf serum by volume. Blast transformation and DNA synthesis was obtained by this method (see Section 3.5.6.2).

Pig blood was obtained by cardiac puncture and was cultured in the same way as for human cells. The leukocyte cultures responded strongly with the same techniques used for human blood.

2.2. MICROSCOPIC TECHNIQUES

2.2.1. Preparation and Staining of Cells for Light and Phase Contrast Microscopy

It was often necessary to examine the morphological appearance of cells after various times in culture or after different kinds of treatment. This was done both by the examination of living cells under phase contrast microscopy, and by the staining of preparations of fixed or air dried cells.

Unlike blood films, white cells suspended in plasma smeared on glass and allowed to dry in air may undergo considerable distortion. Such cells often seem small in size and their nuclei stain densely if left to dry for longer than ½ hour. Considerable variation in morphology may also occur if the suspending plasma or serum is not applied evenly to the glass slide or coverslip.

2.2.1.1. Phase Contrast Microscopy

The sample to be examined was usually deposited as a drop on a glass slide in its own medium, and a coverslip applied with slight pressure so as to press some of the cells out on the slide in single layers. The coverglass was usually surrounded

by vaseline or silicone grease to prevent evaporation of water from the medium under the coverslip. Examination under phase contrast at objective magnifications of X 40 and X 100 was usually satisfactory for morphological examination and photography.

2.2.1.2. Light Microscopy

A Zeiss photomicroscope was used, with objectives of 16X, 40X, and 100X magnification. Photography was carried out by standard procedures, using ADOX KB-14 or Kodachrome film.

2.2.1.3. Fixation of Cells

The methods included:

(i) fixation with glacial acetic acid:ethanol (1:3)

(ii) air drying

(iii) methanol

(iv) formalin

(v) acetone

When the cells were fixed in acetic-alcohol (1:3), they were dropped onto cold wetted slides and allowed to dry. This procedure caused sufficient spreading of nucleus and cytoplasm to permit the examination of intracellular morphology after staining. The best kind of fixation depended on the type of preparation desired. For example, the preparation of chromosomes, of autoradiographs or of cells with sufficient spreading for nuclear size analysis was found best using an acetic-ethanol fixative. Fluorescence was best with acetone but

autofluorescence was often a problem. Air drying was useful for the least disruption of the normal cell structure, although spreading of the cell was often inadequate. Fixation for electronmicroscopy is described in Section 2.2.2.

2.2.1.4. Staining

Staining procedures were restricted to standard stains such as Wrights, Giemsa or Orcein. The following methods were used:

Giemsa Stain (BDH Code 3055): After fixation in absolute methyl alcohol for 3 minutes, the slides were stained for 3-10 minutes, washed with buffer at pH 6.8 (BDH #5003) for 30 seconds, then with distilled water. The smears were then dehydrated in ethanol and xylene, and finally mounted with Eukit.

Wright's Stain (BDH 3375): Essentially, the same method was used, except that the cells were not prefixed with methanol. After 2-3 minutes in the stain, buffer was added to the stain for a further 5 minutes, and after washing with distilled water, the slides were dried and mounted in Eukit.

Aceto-Orcein (BDH 46868, 3% w/v): After acetic acid: ethanol fixation, the cells were stained for 20-35 minutes, depending on the condition of the stain. After dehydration in 50%, 95%, and 100% ethanol and xylene for 2 minutes each, the slides were mounted in Eukit.

2.2.2. Preparation of Cells for Electron Microscopy, Including Staining for Na-K-ATPase

Various methods (Inman and Cooper, 1963; de Harven et al., 1967; Chapman et al., 1967; Jamuar et al., 1968; Clarke et al., 1968; Smith and O'Hara, 1968) have been employed for the electron microscopy of leukocytes and lymphocytes in the peripheral blood. The method used here was as follows:

1. Fixative: 2.5% glutanaldehyde in 0.1M cacodylate (pH 7.4) 0°C for 2 hours. In some cases, the cacodylate was dissolved in isotonic sucrose.

2. Wash: 0.1M cacodylate 2-3 times for 2 hours at 4°C.

3. Post-Fixation: 1% OsO_4 in 0.1M cacodylate, 7-8 hours at 0°C (overnight).

4. Further Staining: 1% uranyl acetate in water. One hour at 0°C.

5. Dehydration: 50%, 70%, 90%, 100% acetone at 0°C, the last overnight 100% acetone, room temperature for 1 hour.

6. Embedding: Epon acetone (1:1), 1 hour, room temperature.
Epon-1, 1 hour, room temperature

7. Polymerization: Epon overnight, 45°C.
One day, 55°C.
One day, 60°C.

2.2.2.1. Cytochemical Procedure for ATPase

Incubating Medium:

0.2M tris-maleate buffer (pH 7.2)	20 ml
125 mg/100 ml ATP	20 ml
2% lead nitrate	3 ml
0.1 M $MgSO_4$	5 ml
water	2 ml

Incubate in this medium for 30 minutes at 35°C before fixation.

In certain experiments, this incubation was carried out in the presence and absence of sodium, potassium and/or ATP. The aforementioned procedure for electron microscopy was then used.

2.2.3. Labelling of PHA with Fluorescein-Isothiocyanate (FITC) and Fluorescence Microscopy

2.2.3.1. Labelling of PHA

The method used was that described by Marshall et al. (1958), as adapted by Michalowski et al. (1964).

83.6 mg of PHA-M was added to 4.3 mg of fluorescein isothiocyanate, to give a weight ratio of 20:1, and dissolved in 836 ml of 0.15M NaCl containing 10% carbonate:bicarbonate buffer (0.5M, pH 9.6). The 0.5M buffer contained 0.42 ml each of 0.5M Na_2CO_3 and 0.5M $NaHCO_3$. The mixture was agitated overnight in a

Dubnoff shaker at 4°C.to ensure uniform distribution of the dye. On the following day, the clear solution was transferred to a cellulose dialysis casing and dialyzed against 0.01M phosphate buffered saline solution at pH 7.0 at 4°C, until no fluorescence was seen in the dialysate when examined with UV light. This took about 3 days of dialysis. The resultant solution was kept at 4-5°C until use.

The PHA-FITC complex was filtered through a millipore filter for sterilization before adding aliquotes to the culture.

2.2.3.2. Microscopy

A Zeiss photomicroscope equipped with a UV source was used for microscopy and photomicroscopy. Different combinations of fixatives, mounting media and glass coverslips were tried. Good results were obtained when the cells were fixed in acetone and mounted in glycerine, or with a special non-fluorescent mounting medium. However, useful results were also obtained when the cells were examined in vitro, surrounded by ordinary culture medium. Although total fluorescence was markedly reduced under such conditions, autofluorescence was minimal, while fluorescein showed up clearly.

2.2.3.3. Effect of Fixatives on Fluorescence Associated with PHA-FITC

Certain experiments were carried out to determine the optimum fixation conditions for the observation of cellular

fluorescence following the administration of PHA-FITC. It was found that after Wright's stain, or after acetic:ethanol (1:3) fixation, there was no fluorescence whatsoever. Fixation with 70% ethanol permitted fluorescence of PHA-FITC-treated cells, as did methanol, but the most fluorescent specimens were observed after suspension of the cells in acetone, or simply by observing the unstained and unfixed cell smears.

Unfortunately, both acetone fixation or air drying caused serious distortion of the cells, and in addition, was associated with an 'autofluorescence' with reddish-green light of many of the cellular elements (such as disintegrating cells) in the absence of FITC, so that it was often difficult to distinguish FITC treated cells from autofluorescent artifacts.

The most useful results were obtained simply by observing the cell in vitro on a simple glass mount, the cells being suspended by their own medium, and the coverglass sealed with a gelatin or silicone grease to prevent evaporation. While this method had the disadvantage of cutting down very severely on the degree of fluorescence observed, autofluorescence was minimal and FITC labelled material was clearly distinguishable, as shown by the absence of fluorescence in non-FITC-treated controls.

2.3. METHODS WITH RADIOACTIVE TRACERS

2.3.1. Measurement of the Incorporation of Labelled Precursors Into RNA, DNA and Protein

The degree of incorporation of H^3-labelled uridine and thymidine into material precipitated with 5% trichloracetic acid was generally used for the quantitative measurement of RNA and DNA synthesis respectively. C^{14}-labelled L-methionine and L-leucine were employed for the measurement of protein synthesis. The same procedure, with some modifications, was used for each system. The sources and specific activities of the compounds used are given in Table 2.6.3.

When synthetic activity was to be measured, the cells were cultured in the Falcon plastic test tubes described earlier in Section 2.1.5.1. Measurements of incorporation were carried out as follows: The labelled precursor was added to a solution of 0.01M phosphate buffered isotonic saline, and then distributed among the test tubes. Generally, 0.2 ml or 0.5 ml of the solution containing the precursor was added to the 3 ml cultures. Control measurements showed that the resulting small dilution of the culture medium during the period of incubation with the precursor solution did not affect the rate of precursor incorporation by the cells. In all cases, however, control and experimental groups were treated in the same way.

For most of these measurements, the cells were incubated at 37°C with the radioactive medium for a period of 2 hours. However, pulse doses of 20 minutes or longer were sometimes used, and 24 hour cumulative incorporations of H^3-thymidine into DNA were carried out in some experiments. As shown in Section 3.1, the rate of uptake of labelled precursors into RNA, DNA and protein was apparently linear over 2 hours, a time-period which was therefore useful for the determination of changes in the rate of synthesis.

After the desired incubation period had elapsed, the tubes were centrifuged directly at 2,000 rpm (500 g) for 2 minutes and the clear radioactive supernatant was immediately removed with a Pasteur pipette connceted to a vacuum line and trap. Four ml of ice-cold phosphate buffered saline was then added to the cells, mixed, centrifuged and the supernatant then removed. This was repeated twice with ice-cold 5% trichloracetic acid (TCA).

When the last TCA wash was done, 0.5 ml of hyamine-X (Packard Instrument Co., Downers Grove, Illinois) was added and after mixing, the tubes allowed to stand overnight. On the following day, the radioactive hyamine solution was transferred to counting vials. This was done either with Pasteur pipettes, or by directly pouring the counting solution into the test tubes

containing hyamine, in which the hyamine solution rapidly dissolved, and back into the counting vial. In the former case, 0.3 ml of absolute ethanol was added to take up any remaining hyamine solution. However, the latter method was found to be much more rapid and to give equivalent results.

The vials were counted in a Packard TRICARB scintillation counter which had previously been calibrated for H^3 and C^{14}. Some degree of chemoluminescence was often present in the samples, and it was generally necessary to allow this to die down for 24 hours before quantitatively useful counting could be done. Sufficient counts were obtained so that the standard deviation of a measurement was less than 3%. Background counts were obtained by carrying out the same procedure with the radioactive tracers, but without cells present in the culture tubes.

In order to reduce the degree of experimental variation between cultures in the measurement of incorporation, it was necessary to have replicate tubes. Between 4 and 6 were used for each experimental point. The standard deviation of the mean of the replicates was usually about 5-10% of the mean count rate obtained.

For leukocyte cultures, expression of incorporation results in terms of the total DNA, phosphorus, or protein led to error

because of the presence of granulocytes and red cells. Expression in terms of the final cell count was inaccurate due to the clumping of cells in the presence of PHA, resulting in gross errors in cell counting. Expression in terms of the initial cell count was sometimes useful, but did not take into account the degree of cell death or inactivation in the cultures, nor the extent of cell multiplication that occurred after the second day of culture. Incorporation into DNA, RNA, or protein is expressed simply in terms of the total 3 ml test tube cultures, which sufficed for many of the experiments described in Sections 3.1, 3.2, and 3.3.

2.3.1.1. A Typical Experiment

The technique just described may be illustrated by a study of the early effects of PHA on RNA and protein synthesis in purified lymphocytes, which was carried out as follows:

190 ml of whole blood was obtained from a healthy male volunteer using 3 disposable syringes moistened with 0.1-0.2 ml of non-preserved heparin. The red cells were sedimented in the presence of 0.8% dextran, and the granulocytes and macrophages removed by passage through a separatory syringe (Figs. 2.1.1 and 2.1.2) partially filled with glass beads. The resultant 60 ml cell suspension was finally resuspended in 335 ml of medium-199. After adding non-preserved heparin, the cells were distributed among 128 plastic, disposable, screw-cap, sterile test tubes (Falcon #3033). The final plasma

concentration in the completed culture medium was 14.2%. There were 4.9×10^5 white cells per culture tube, and the ratio of red cells to white cells was 3:1. Ninety-five per cent of the white cells present were small lymphocytes. The cells were then incubated overnight at 37°C. On the following morning, PHA-M (200 µg/ml) was administered to half of the cultures, followed 5-10 minutes later by the tracer (H^3-uridine or C^{14}-leucine). At intervals of 2, 4, and 6 hours after adding the tracer, the cells were washed twice in ice-cold phosphate buffered saline and then twice in ice-cold 5% trichloracetic acid. After solubilization in 0.5 ml hyamine, the mixture was added to 15 ml of scintillation solution (a dioxane, PPO, POPOP, and naphthalene mixture) and counted in the TRICARB, for 10 minutes each. (The counting was repeated several cycles to make sure that the count ratio was stable, since non-radioactive scintillation (i.e., chemoluminescence) sometimes occurred. In the latter case, the count rate decreased with time.)

2.3.2. Measurement of the Total Uptake of Labelled Tracers and Potassium-42

The method used was to incubate the cells with the radioactive tracer, the uptake of which was to be measured, and after a variable period of time, to measure the amount in the total cell mass with or without further addition of trichloracetic acid (TCA).

For example, K^{42} was incubated with cells for periods of 20 minutes to 24 hours. The cells were washed in the desired wash medium (such as ice-cold isotonic KCl), and after 3 washes, the

supernatant was removed and the cells measured by counting of the tubes plus cells directly in the well of a Packard 256 Channel Na I well type scintillation counter (Technical Measurement Corporation, New Haven). When the cells were also treated with cold 5% TCA (or ethanol), 95-97% of the total radioactivity appeared in the supernatant (Table 2.3.1). The 3-5% residual activity in the TCA or alcohol precipitate was probably due mainly to some potassium-42 present in a small amount of residual wash medium with the cells, although some radioactivity conceivably may have been bound to the precipitate.

In the case of the amino acids or nucleosides used to measure incorporation into protein or nucleic acid, the difference between the amount bound to TCA precipitable material and the total cell context was considered to represent the amount of the substance taken up by the cell but not incorporated into protein or nucleic acid.

These experiments gave results that could be expressed in terms of the total tracer taken up per million cells or per 10 μg of DNA. Due to the small number of cells generally present, no attempt was made to carry out concurrent measurements of actual cell volume. Moreover, the contamination with red cells would have led to serious error. For this reason, direct measurements of the intracellular concentration of the substances examined were not done, and therefore no attempt was made to evaluate active transport against a gradient,

Table 2.3.1.

Residual K^{42} in precipitate after 5% TCA or ethanol treatment of cells 'preloaded' with K^{42}

Cells	Treatment	cpm in Supernat.	ppt.	% K^{42} in ppt.
Red Cells	5% TCA	110	8	5.2
	Ethanol	1518*	60	3.8
Lymphocytes		165*	6	3.5

* 2 different experiments.

except on the basis of theoretical calculations.

The procedure used to measure K^{42} uptake into the cell was contingent on washing the cells with ice-cold isotonic KCl. The use of other wash media such as isotonic saline or isotonic sucrose gave different results. Because of the experimental significance of such differences, the experimental problem of cell washing is considered in greater detail in Section 3.4.3.

2.3.3. Localization of Radioactive Tracers in Cells by Autoradiography

Kodak NTB-3 emulsion was used in all cases. The glass slides had been rendered scrupulously clean by soaking in concentrated sulfuric acid and chromate and after lengthy washing in distilled water, were then 'subbed' with gelatin and chrome alum and dried. The cells were fixed in acetic:alcohol (1:3). After several changes of fixative (mainly in order to remove radioactivity from the supernatant), the cell suspension was dropped onto cold wetted 'subbed' slides and allowed to dry. This procedure caused the cell to flatten out on the slides.

After the slides were dry, the emulsion was placed in a small 50 ml beaker suspended in a warm constant temperature bath at 45°C. The emulsion was then spread directly on the slides using a liquid emulsion spreader (after the design of Dr. L. Belanger), and the slides then placed on a slide warmer at 40°-42°C for about 10 minutes. When it could be seen from the safelight that the emulsion

was dry, the slides were placed in a light tight box and kept at $4^{o}C$ for the required period of exposure. This period depended on the amount and type of radioactivity present, but was usually one or more weeks.

After the desired period of exposure had elapsed (determined by experiment), the cells were placed in D-19 or Dektol developer for 10 minutes at room temperature, washed in water for 1 minute and fixed for 15 minutes. After washing, the emulsion was dried. Staining with Giemsa through the emulsion was found to give satisfactory results. Finally, the slides were mounted using DPX or Eukit.

Although autoradiographs with thymidine-H^3 generally were characterized by a clear localization of grains over the cell nucleus, slides of cells treated with H^3-uridine often showed much diffusion of label into surrounding emulsion, leading to a non-specific background between and within clumps of cells. This was partially avoided by several washes in fixative before placing the cells on the slides. There was a distinct impression that the cells had accumulated an amount of H^3-uridine which was unbound and diffusable from the cell. This was supported by direct evidence (see Section 3.1.4.4) of an unbound pool of radioactive uridine.

2.4. MEASUREMENT OF CELLULAR CONSTITUENTS, ATPase AND RESPIRATION

2.4.1. DNA

The method of Burton (1956), as adapted for leukocytes by Tedesco and Mellman (1966), was used. The reaction of DNA with diphenylamine in a mixture of acetic and sulfuric acids has been widely used for the quantitative estimation of this nucleic acid (Dische, 1930). Burton found that if acetaldehyde was added, a more intensive color would be produced if the mixture were allowed to stand overnight at 30°C, instead of boiling and observing the color 10 minutes later. Tedesco and Mellman (1966) allowed the reaction mixture to stand at room temperature. The method used is described in Sections 2.4.1.1 and 2.4.1.2.

2.4.1.1. Reagents

A highly polymerized calf thymus DNA preparation (Schwarz BioResearch Inc., Orangeburg, N.Y.) containing 82% DNA by weight was used. A stock solution was prepared by dissolving DNA in 5 mM NaOH to a concentration of 0.4 mg/ml. This stock solution was used in 0.2 ml amounts when measuring the DNA content of lymphocytes. Acetaldehyde was stored at 4°C in a glass stoppered flask as an aqueous solution containing 16 mg/ml. Diphenylamine reagent was made by dissolving 1.5 g of diphenylamine in 100 ml of glacial acetic acid and adding 1.5 ml of concentrated sulfuric acid. The reagent was stored in the dark.

On the day that DNA was to be measured, 0.1 ml of aqueous acetaldehyde was added for every 20 ml of reagent used. The reagent was invariably colorless when used, although in the presence of acetaldehyde control samples developed a greenish tinge after 2 days of standing at room temperature in the light.

2.4.1.2. Measurement of DNA in Lymphocytes

After lymphocytes had been cultured and analyzed for various purposes, such as for the measurement of K^{42} content by gamma scintillation counting, the cells were usually present at the bottom of the plastic test tubes used for culture, and could be observed adhering to the wall of the tubes in a thin and barely visible layer. The tubes and cells were kept at $-20^{o}C$ until DNA measurements were to be made.

No attempt was made to remove the cells from the tubes. Instead, 0.5 ml of 5% perchloric acid was added and the tubes placed in hot water at $65-70^{o}C$ for 20 minutes and then cooled. To the hydrolysate, 1 ml of diphenylamine reagent was added. After mixing, the tubes were stoppered and allowed to stand overnight at room temperature. A similar technique was used for distilled water blanks and the DNA standard. On the following day, the DNA content of the cells in each culture tube was estimated by reading the absorbance at 600 mμ with a Bausch and Lomb 505 spectrometer.

2.4.2. Protein

The phenol method of Daughaday, Lowry, Rosebrough, and Fields (1952) was used. Commercially prepared Folin-Ciocalteu phenol reagent (HARLECO, Philadelphia) was diluted and added in 1 ml aliquots both to the unknowns and to the 0.2 ml protein standard, which had previously been treated with 10 ml of alkaline copper reagent. The latter was prepared by adding 45 ml of alkaline tartrate reagent to 5 ml of 0.1% $CuSO_4 .5H_2O$. Alkaline tartrate reagent was made by dissolving 20 g of Na_2CO_3 and 0.5 g of Na tartrate in one litre of 0.1 M NaOH. After allowing the reaction mixture to stand for at least 30 minutes, the absorbance of the solution was read at 750 mμ.

2.4.3. Potassium and Sodium

The cells were lysed either in 1-3 ml of distilled water or in 1-3 ml of 5% trichloracetic acid, using the cultures grown in the plastic culture test tubes previously described. After centrifugation, the clear supernatant was removed and diluted with 2 ml of 2.4% $CsNO_3$ solution and the contents of potassium and sodium determined by flame photometry using a Jarrel Ash flame photometer. For sodium measurements, particular care had to be taken not to use glass in the procedure, which was therefore carried out using plastic vessels. Use of glass often led to falsely high sodium values and the determinations often had to be carried out at the lower limits of detection by the apparatus.

The problem of cell preparation prior to the measurement of total potassium was the subject of some study because of the findings, using K^{42}, of more than one potassium compartment associated with the cells. Preparation of the cells by different methods led to differing results. This problem is discussed later in Section 3.4.11.

2.4.4. Measurement of Respiration

Cells were cultured in Ehrlenmeyer flasks as already described in Section 2.1.5.1. When respiratory measurements were to be done, the cells were transferred to a Clarke Oxygen Electrode which was fitted to a glass cell of 1.8 ml volume, the latter being surrounded by a water jacket to maintain the temperature at 37°C.

The polarograph cell was first filled with 0.9% NaCl which had been equilibrated with the oxygen of the outside air; the apparatus was then calibrated for 100% oxygen saturation. The cells were added directly to the electrode monitored polarograph cell after removing the saline solution; the respiratory rate was measured from the rate of decrease of the oxygen tension within the medium bathing the cells. The concentration of cells present was kept as close as possible to 10^7 ml, since the rate of oxygen utilization was dependent on the degree of cell crowding (Section 3.5.2.1). While a greater concentration may have been slightly

more useful because of the reduced rate of change of respiration with cell concentrations, the limited availability of cells determined the number of measurements that could be made.

The respiration of the cells was measured either in the medium in which they were cultured (which had sometimes been partially depleted of glucose) or in fresh medium (thereby abruptly changing the environment of the cell). Cultures included both leukocytes or purified lymphocytes, and red cells were inevitably present (usually in a ratio of 2-3:1 erythrocytes:leukocyte).

Protein measurements were carried out by the technique already described in Section 2.4.2. Estimates were made of the relative numbers of small lymphocytes, blast cells, granulocytes and of red cells present in the culture from smears of the cell suspension which was subsequently added to the polarograph for respiratory measurement.

2.4.5. Measurement of ATPase Activity

In order to measure the Na-K-ATPase activity of leukocyte and lymphocyte cultures following stimulation, analyses were first carried out for red cells using the method of Post, Merrit, Kinsolving and Albright (1960), and the technique then applied to leukocytes with some modifications. The method used is described in Section 2.4.5.1 and 2.4.5.2.

2.4.5.1. Preparation of Cells

Leukocyte cultures grown in 50 ml Ehrlenmeyer flasks were treated with PHA for various periods of time. Each culture was centrifuged at low speed (approximately 50 g) and the supernatant removed. The cells were then washed once in normal saline and then all the cells present in 5 culture flasks were pooled After recentrifugation, the saline supernatant was removed and each group of cells resuspended in 10 ml of ice-cold TRIS (5×10^{-4}) at pH 7.0 After suspension and recentrifugation, the pink supernatant was removed and the whitish thick precipitate (swollen leukocytes by microscopy) resuspended in fresh TRIS (5×10^{-4}M) buffer.

Each 10 ml suspension thus prepared was placed in 20 ml beakers, surrounded by ice, and ultrasonicated 3 times for 5 seconds, each exposured to ultrasound separated by one minute. The resultant cloudy suspensions were centrifuged at 16,000 rpm for 15 minutes. The precipitate was resuspended in 5 ml of fresh TRIS buffer (5×10^{-4}M) and kept frozen until use.

2.4.5.2. Measurement

The constituents of the incubation medium are shown in Table 2.4.1. The preparation was added to the medium and incubated at 37°C for several hours. After various times of incubation, 0.5 ml of the mixture was removed and added to 0.5 ml of 10% perchloric acid.

Table 2.4.1.

Composition of Incubation Medium for ATPase Measurement

Component	Stock conc. (mM)	Volume added (ml)	Final conc. (mM)
$MgCl_2$	10	0.5	2.5
NaCl	150	0.5	37.5
KCl	150	0.5	37.5
TRIS (pH 7.0)	20	0.5	5
ATP solution	5	0.8	1.25
Water or enzyme	varied with experiment	1.2	varied with experimen

One ml of water was then added and the resultant mixture allowed to stand until phosphate measurements could be made. Countrol samples were incubated (a) without ATP, (b) with ATP but no cell preparation, and (c) with ATP plus boiled cell preparation. Phosphate measurements were carried out by the method of Lecocq and Inesi (1966).

2.5. SURGICAL TECHNIQUES FOR SKIN GRAFTING

2.5.1. Method for Skin Grafts

Pigs of a partially inbred strain (red pigs of the Minnesota line), 10-20 kg. in weight, were used. Grafting was carried out as follows. After cleaning the dorso-lateral aspect of the thorax with PhisoHex, followed by shaving and sterilization with alcohol, 2-4 graft sites were selected on pairs of anaesthetized animals (see Fig. 2.5.1). A special skin cutting device was used to incise a circle approximately one cm. in diameter to a depth of approximately 2mm. The grafts were cut out with a scalpel, leaving part of the subcutaneous connective tissue. The skin graft, kept wet in a sterile saline solution, was then scraped on the underside, removing fat and connective tissue until a pearly white glistening surface was reached which was resistant to further scraping, and which contains 20-30 dark spots which were the bases of hair follicles.

Grafts of this type were either replaced in their original sites (autografts) or regrafted in corresponding beds on the other animal of the pair (homografts). The grafts were held down by 8 stitches of 5-0 silk on the periphery, as shown in Figure 2.5.2, thus anchoring the graft to the bed site and holding the edges together.

After sewing the grafts in place, they were protected by a layer of sterile vaseline in gauze; this was covered by a pressure

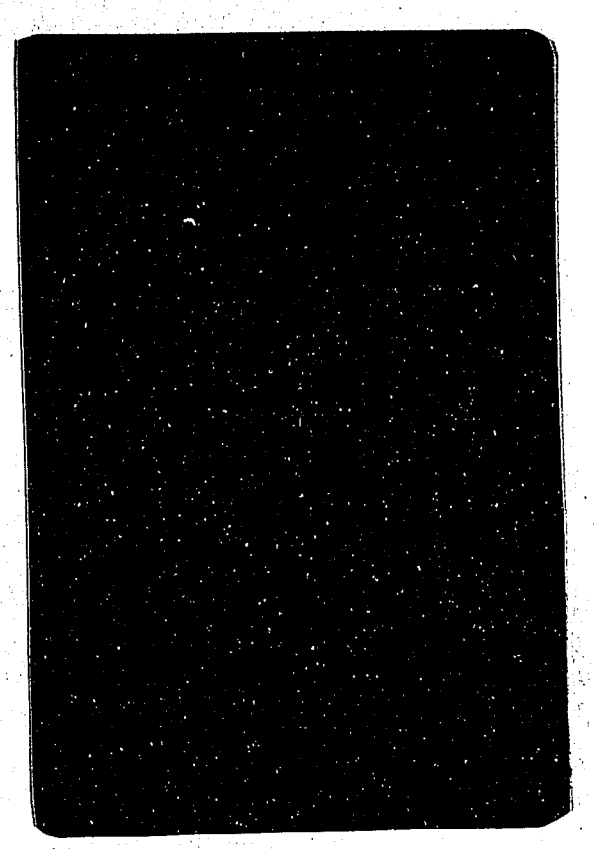


Fig. 2.5.1.

Graft sites on skin of dorsolateral aspect of shoulder of experimental pig. These grafts were initiated 5 days prior to the photograph, and show the hyperemic flush characteristic of both homografts and autografts after this period of time. See also Fig. 3.5.22.

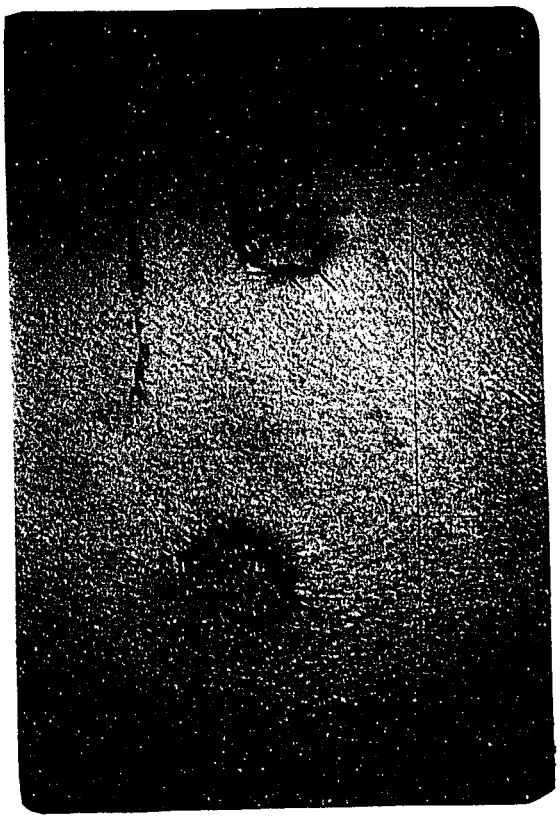


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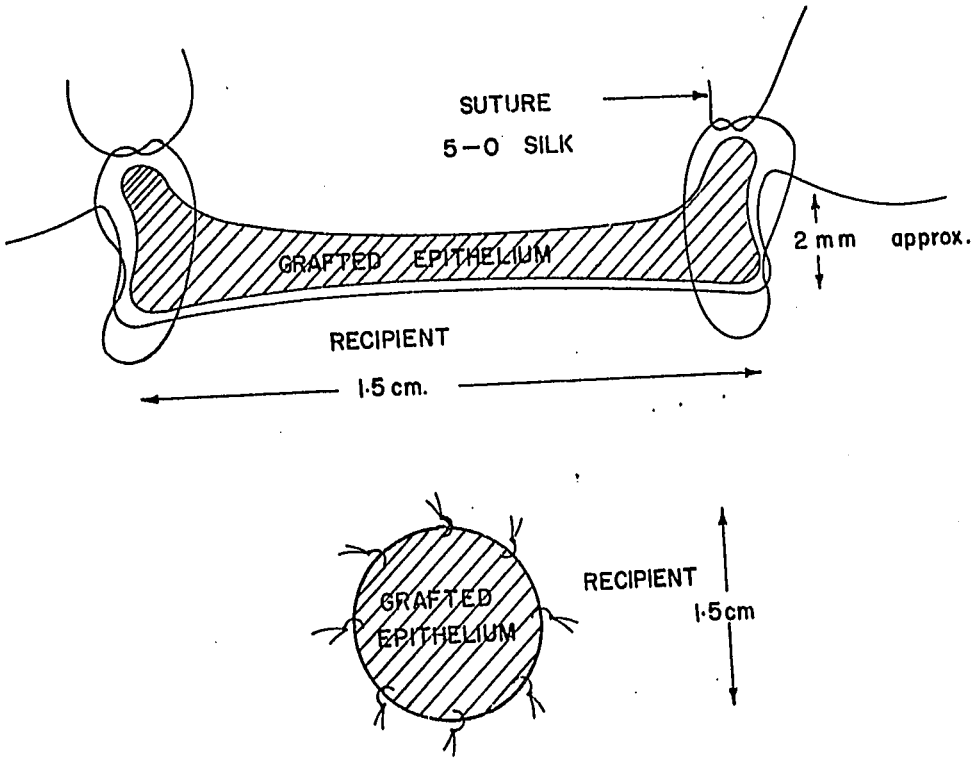


Fig. 2.5.2.

Procedure used for skin grafting. For description of technique, see text of Section 2.5.

bandage held on by elastic tape bound around the thorax of the animal. After 4 days, a dry gauze dressing was used.

The grafts were inspected and removed at various times following transplantation, and color photographs were taken when necessary. Histological sections of the graft site were obtained when required after formalin fixation.

The method used for rat skin was similar, except that a more delicate technique had to be used. It was difficult to apply pressure bandages to these animals and revascularization may have been hindered because of this. While colloidin helped to keep the grafts adherent to the recipient site, the substance also tended to obscure observation of the grafts.

2.5.2. Animal Handling

Pigs were caged individually since they tended to fight and to remove the pressure bandages applied over the grafts of their fellows. Rats were caged 2-3 to a cage. The animals were fed on standard diet, but when evaluating drug toxicity, they were weighed daily and their water and food intake was assessed.

2.6. DRUGS AND REAGENTS USED

The various drugs, compounds and reagents employed in different aspects of this investigation are listed in Tables 2.6.1. The doses and reasons for use have been described separately in greater detail in the relevant sections. The name, source, catalog and lot numbers for the various substances used are provided in this table, although many commonly used materials are not included.

Table 2.6.2 is a list of certain apparatus and supplies found to be particularly useful for this type of investigation and are included for easy reference.

Table 2.6.3 lists details relating to radioactive substances.

TABLE 2.6.1. DRUGS AND REAGENTS

Substance	Catalogue #	Lot #	Source	Remarks
Adenosine-5' monophosphate	1181	801270	Calbiochem	2-1/2 hydrate A grade
Adenosine-5' diphosphate	1171	900256	Calbiochem	2-1/2 hydrate A grade
Adenosine-5' triphosphate disodium	1191	900635	Calbiochem	A grade
Adenosine-3'-5' cyclic phosphate (cyclic AMP)	116801	900693	Calbiochem	A grade monohydrate
Adenosine-3'-5' monophosphate N6, O2 cyclic dibutyryl (dibutyryl cyclic AMP)	28745	920266	Calbiochem	A grade
Actinomycin-D	114666	80733	Calbiochem	200 µg final A grade Stat pack
Cassaine base	9704880		E. Merck	
p-Chloromercurobenzoate-Na	2209	35533	Calbiochem	C grade

Substance	Catalogue#	Lot #	Source	Remarks
Cycloheximide			Upjohn	Gift from Dr. Singhal
Deoxyribonucleic acid	211011	6605 A	Schwartz Bio-Research Inc.	100 gms 1 bottle approx. 82% by weight, from herring or salmon
Dextran	4471	Many	Abbott Glaxo Lab Ltd Middlesex, U.K.	Dextran 75 6gm/100ml of 0.9% NaCl. No longer made by this company. MW 110,000
2,4 Dinitrophenol	317422	73278	Calbiochem	B grade
Erythrophleine sulphate	9704880		E. Merck	
N-ethylmaleimide	E3876		Sigma	
Fluorescein isothiocyanate	119-F	2876920	British Drug Houses	
Gramicidin	U-1784	3325	Mann Research Lab.	974 µg activity/mg
Heparin (sodium salt)	50770	86715 9313 C	General Biochemicals	Grade USP XVI P 317 approx. 120 units/mg
Heparin sodium injection USP	LYRE	407-1	Connaught Med. Res. Labs.	1000 µ/cc
Hydrocortisone sodium succinate (solucortef')	8693	16,982	Upjohn Co. of Canada	Mix-o-vials contain dry 100 mg hydrocortisone, 0.8 mg sodium biphosphate, 8.37 mg sodium phosphate, 2.48 mg methylpara, 0.28 mg pro

Medium 199	115 H	Various Lots	Grand Island Biological Co.	With Hank's salt
Medium HB-597	CRY	120-2 and other lots	Connaught Med. Res. Lab.	Modified Medium 199 and antibiotics
Methotrexate	4567-89	162-423	Lederle Cyanamic Corp. of Canada, Ltd.	Contains methyl-pavaban, 1.6 mg/vial; pro pavaban 0.4 mg/vial; NaCl, 17.2 mg/vial Na to approx. pH 8.4
Nigericin			Commercial Solvents Corp., Terra Haute, Indiana.	Dr. Roger Harned, Research and Development Dept.
Oligomycin	63118	115 b-150	Sigma	15% 'A' 85% 'B' No 'C'
Ouabain	445	845,969	Armaud Lab. Nationelle, Paris	0.25 mg/ml sterile water
Ouabain	4995	900113	Calbiochem	Dry Powder
Phenol Reagent (Folin-Ciocalteu)	2690	0043 P	Harleco	
Phytohemagglu-tinin-M	0528 code 3110	537083	Difco, Detroit	Dessicated muco phytohemagglutinin Method of Rigas and Osgood
Phytohemagglu-tinin	MR10	K9880	Burroughs Wellcome Co., UK	Sterile freeze-dried extract of seeds of Phaseolus vulgaris
Prednisolone sodium hemisuccinate meticortilone		7 ACX 2	Schering	50 mg lyophilized per vial

Substance	Catalogue #	Lot #	Source	Remarks
Prednisolone-21-phosphate disodium salt		L 555333-1-33	Merck, Sharpe and Dohme	
Prednisolone	6723	L-1017	Mann Research Labs.	
Tetrodotoxin	584411	73564	Calbiochem	A grade, lyophilized in 5 mg dry citrate buffer at pH 4.8-4.9
Trypan blue	525	18012 A	Grand Island Biological Co.	0.4% in balanced salt solution
Trypsin	509L	00098D	Grand Island Biological Co.	2.5% lyophilized
Valinomycin	676377	860031	Calbiochem	25 mg lot A grade

TABLE 2.6.2. Special Materials

Item	Catalogue #	Lot #	Source	Remarks
Glass beads 'Superbrite'	100-5005	UR-683	Minnesota Mining + Manufacturing Co., Reflection + Products Divs.	#1 weight
Plastic disposable culture tubes	3033	Various lots	Becton-Dickinson Falcon Plastics (Bio Quest)	
Plasti-Pak sterile disposable syringes	8045 820L/s 810L/s 802L/s 850L/s	Various lots	"	Sizes 1-50 ml.
Tissue culture flasks 30 ml, 75 ml	3012	Various lots	"	
Sterile disposable plastic pipettes 1, 5, 10 ml.	7506 7259 7530	Various lots	"	
Sterile disposable needles (discarded)	F0493	Various lots	"	20, 22 1-1/2"
Microsolve detergent for tissue culture washing	18-908		Microbiological Associates	Biodegradable Active agent is alkyl-aryl-sulphosate
Liquid photographs emulsion	NTB3	LSB-9-9 KP-56900	Kodax	
Stoppers white rubber	1922		Bellco	

Table 2.6.3

Radioactive Tracers

Substance	Source	Catalog #	Lot#	Spec. Activ. Ci / mole
L-alanine-1-C^{14}	NEN	NEC-266	333-138-1	125.0
L-methionine-methyl-C^{14}	NEN	NEC-165	361-107	14.7
α-aminoisobutyric acid-1-C^{14}	NEN	NEC-212	305-073	4.0
L-leucine-1-C^{14}	NEN	NEC-169	471-083	25.6
			408-057	31.0
Uridine-5-H^3	NEN	NET-174	444-68	24.0 x 10
			444-269	23.7 x 10
			438-190	24.9 x 10
			398-88	19.5 x 10
			317-264	19.4 x 10^3
Thymidine-methyl-H^3	SB	1533-87	6606	20.0 x 10^3
	SB	2532-90	13602	15.0 x 10^3
	NEN	NET-027	555-020	6.7 x 10^3
			444-268	6.7 x 10^3
			319-210-5B	16.5 x 10^3
			368-179	6.7 x 10^3
Thymidine-methyl-C^{14}	SB	3523-99E	6806P	12.0
K^{42}	ICN	475	Various	~ 400mCi/gm K

NEN New England Nuclear Corp., Boston, Mass.

SB Schwarz BioResearch, Inc., Orangeburg, N.Y.

ICN International Chemical and Nuclear Corp., Buffalo, N.Y. Chemical and Radioisotope Division.

3. RESULTS

The experimental results presented in this chapter are arranged in the following way.

The first section (3.1) describes confirmatory experiments on morphology and the quantitative measurement of RNA, DNA and protein synthesis during lymphocyte transformation, under the experimental conditions of this investigation. These data have been included to provide a background to the subsequent presentation of experimental results.

The main body of original work is presented in the next three sections (3.2, 3.3 and 3.4). These studies are concerned with the responses of unstimulated and stimulated lymphocytes to various conditions and agents, particularly those which affect cation transport, and with radiopotassium flux dynamics in these cells. Attention is drawn to the central nature of these results.

The final section (3.5) contains supplementary material, some of which is presently in progress. Although these results are preliminary, some of the data were considered of sufficient interest to merit inclusion. The subdivisions of this section are representative of different directions of follow-up of the main results and of potentially fruitful future activity.

3.1. LYMPHOCYTE•STIMULATION: CONFIRMATORY STUDIES RELATING TO MORPHOLOGY AND MACROMOLECULAR SYNTHESIS

3.1.1. Introduction

In most of the experiments of this investigation, it was necessary to examine cells at the light microscope level of magnification, and in some cases at the ultrastructural level. It was also necessary to carry out measurements of synthetic activity. It was therefore essential to obtain qualitative and quantitative descriptions of lymphocyte transformation as it occurred under the experimental conditions of this investigation.

This section provides a description of morphological changes associated with lymphocyte transformation, and examines the effect of PHA administration of PHA administration on the mitotic activity and synthesis of RNA, DNA and protein by these cells. The experiments include measurements of the dose response to PHA and autoradiographic studies of H^3-uridine incorporation and turnover in the cell. Some unusual patterns of DNA synthesis, observed in autoradiographs of H^3-thymidine uptake into the nuclei of transforming lymphocytes are described.

3.1.2. Morphology of Unstimulated and Stimulated Lymphocytes

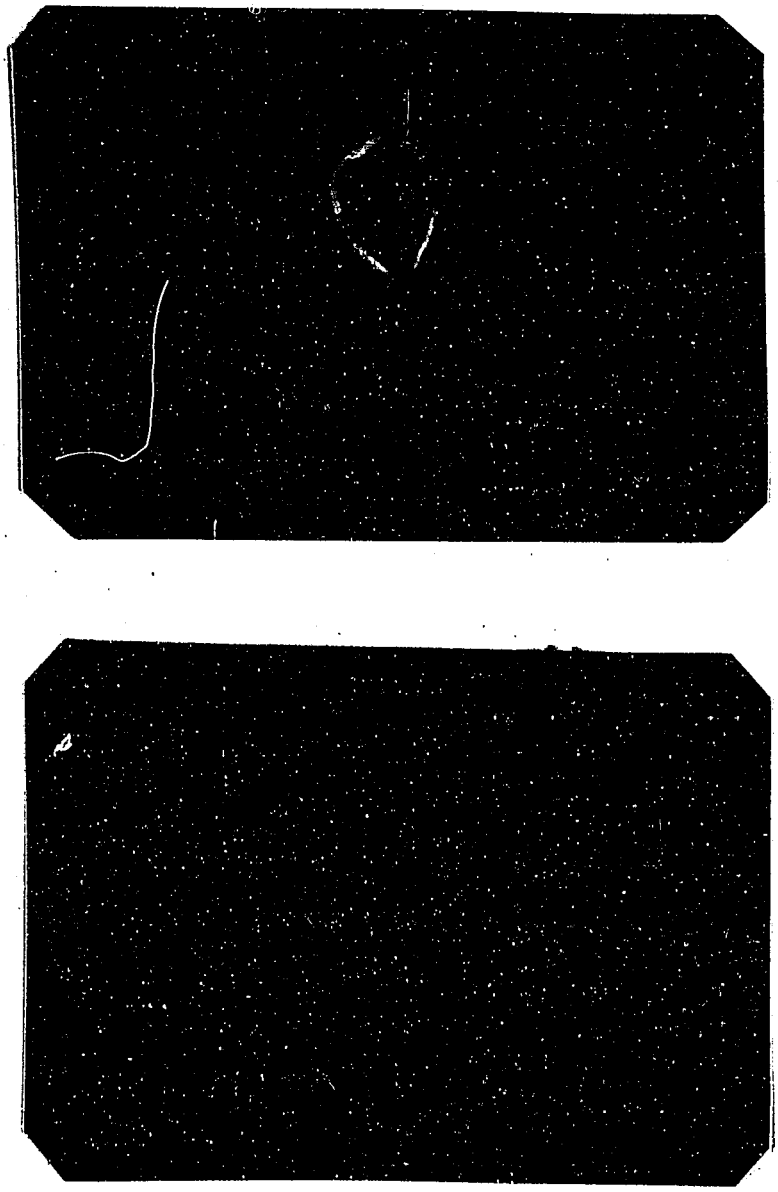
3.1.2.1. Unstimulated Lymphocytes

Small lymphocytes in vitro were observed as mononuclear

cells about 6-8μ in diameter, sometimes somewhat larger. The nuclei of these cells were usually round or slightly bean-shaped, and were often characterized by an indentation which could be seen both by phase-contrast microscopy and in stained smears (Fig. 3.1.1).

In the living state in vitro, phase contrast observations of the small lymphocyte revealed a rounded granular nucleus, usually surrounded by a pronounced optical halo which made direct visualization of the nuclear and cytoplasmic boundaries very difficult. Infrequently, the cell attached to the surface of the coverglass, and under such circumstances, the nucleus appeared rounded but somewhat flattened and oval, and some internal structures such as the nucleolus could be seen. The cytoplasm was seen to have a delicate undulating membrane in which a slow movement of organelles could be detected. The cytoplasm sometimes extended as a tail, the whole cell then giving a 'hand mirror' appearance. The cell was characteristically motile and altered shape and position relatively rapidly (Fig. 3.1.2).

When air-dried smears were stained with standard blood cell stains (e.g. Wright's or Giemsa), the cytoplasm of the small lymphocyte appeared relatively meagre in relation to nuclear size, in comparison to the characteristic appearance of many other cell types, and appeared as a thin rim around the nucleus, although extended tails or wisps of cytoplasm were sometimes seen. Small



(a)

(b)

Fig. 3.1.1.

Small lymphocytes: (a) phase contrast *in vitro* (b) Giemsa-stained air-dried smear. x 1250.

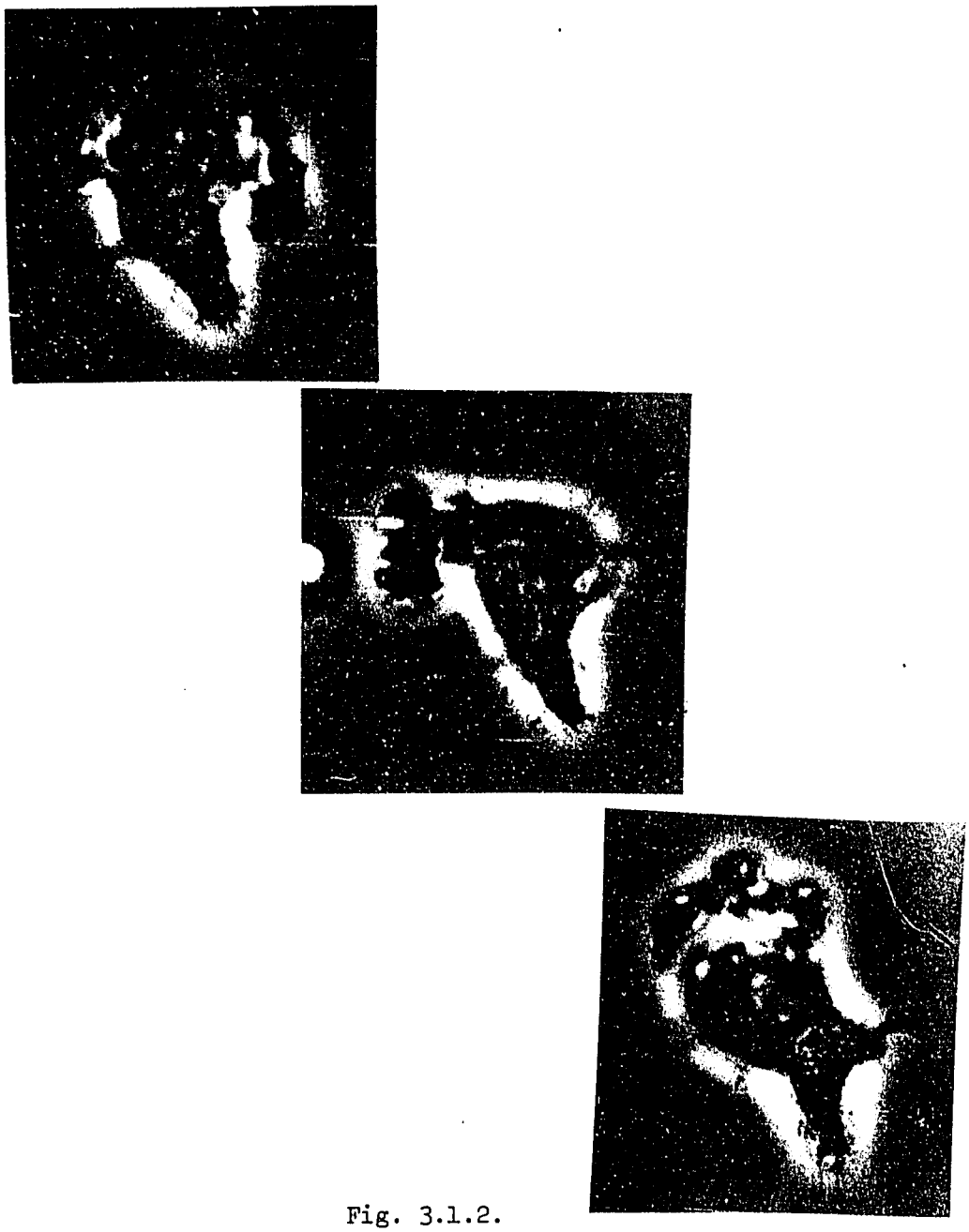


Fig. 3.1.2.

Motility of the lymphocyte. Photographs taken one minute apart.

round unstained vacuoles were seen in the cytoplasm, usually near the nuclear indentation. Nuclei tended to be densely stained, particularly in the perinuclear region adjacent to the nuclear membrane. Nucleoli were usually difficult to visualize in air dried cells.

When cultures were fixed in acetic acid : ethanol (1:3) and stained with orcein or the previously mentioned stains, cytoplasmic and nuclear structure was altered; they became more homogeneous in appearance and internal detail was partly lost. On the other hand, the spreading of the cells led to better visualization. This fixative was advantageous for certain morphological and autoradiographic observations because of the cell spreading and the lysis of erythrocytes.

3.1.2.2. Stimulated Lymphocytes

It should be pointed out that the following description is restricted to the action of PHA, since most of the experimental work of this investigation was carried out with this mitogen, although some studies were also done with the mixed lymphocyte reaction. As already documented, lymphocyte transformation *in vitro* can be induced by a number of different agents, and the cellular events associated with transformation may not be the same for the different modes of stimulation.

Shortly after PHA administration, agglutination of cellular elements in the culture was observed. This took place within 20 minutes of the administration of phytomitogen (Fig. 3.1.5), and involved both red and white cells. Red cells were equally susceptible to the aggluinating action of PHA (Fig. 3.1.4).

Changes in cellular size were not apparent visually within the first 6-8 hours after giving PHA. After one day with the mitogen, a variable number of the total cell population were recognizable as enlarged 'blasts' but mitotic figures were not present. The nuclei of these cells were relatively large and clear with abundant vacuolated cytoplasm (see Fig. 3.1.5 & 6). Rod-shaped mitochondria and small round vacuoles could sometimes be seen by phase contrast within the cytoplasm. Most of the blast cells were present in clumps of 2-4 to several hundred after 1 day or more in vitro with PHA.

Air dried specimens were often difficult to evaluate due to clumping, but the cells, when separated from the clumps, were characterized by large rounded nuclei, prominent nucleoli and abundant cytoplasm which stained densely blue with Giemsa or Wright's stain (Fig. 3.1.7). Non-staining, clear round vacuoles were often seen mainly in the cytoplasm. Mitotic figures did not appear until at least 30 hours had passed in the presence of PHA.

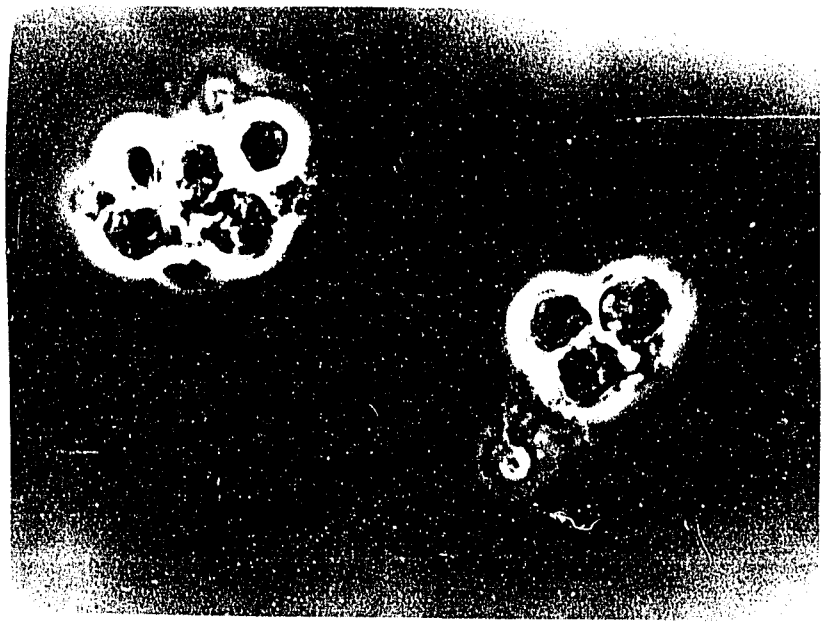


Fig. 3.1.3.

Agglutination of lymphocytes 1/2 hour after PHA administration.

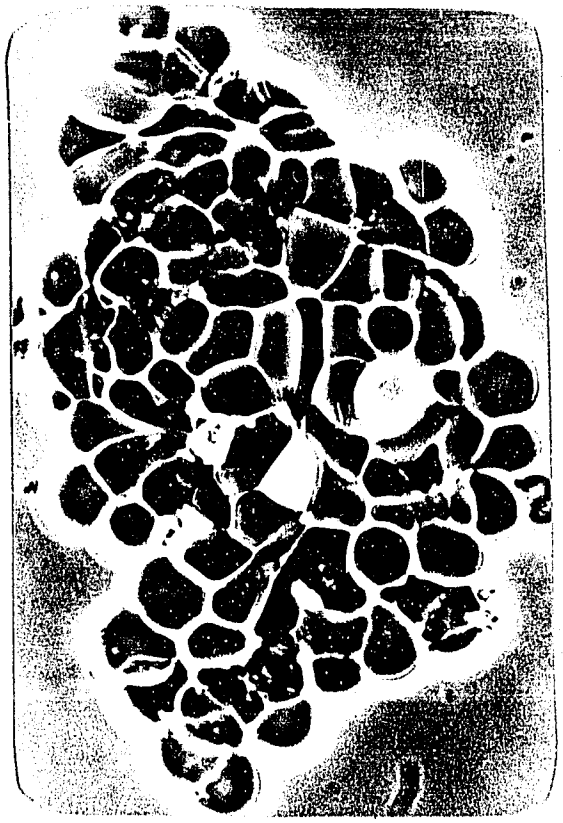


Fig. 3.1.4.

Agglutination of red cells shortly after the administration of PHA. x 1250.

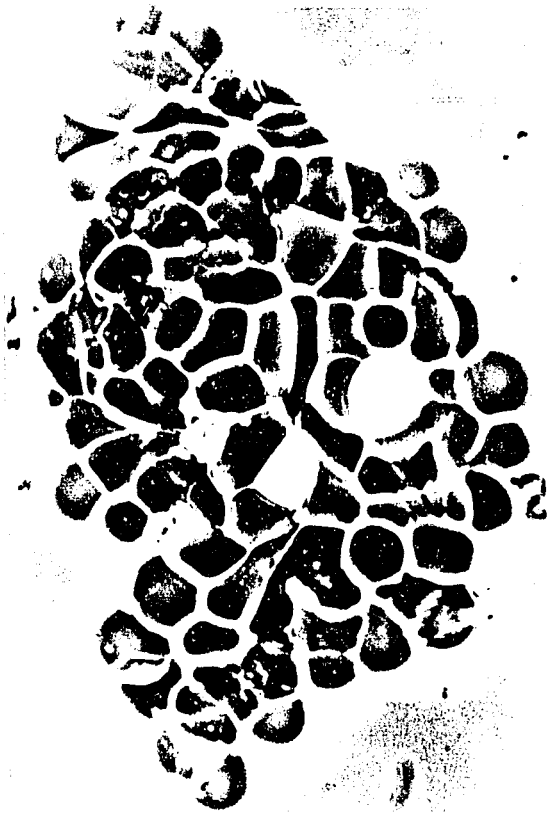


Fig. 3.1.4.

Agglutination of red cells shortly after the administration of PHA. x 1250.

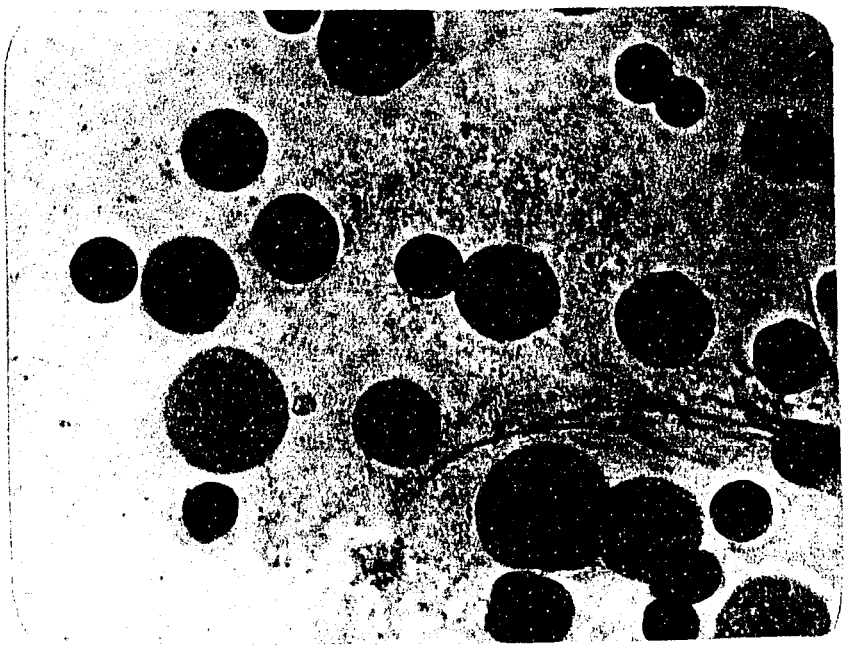


Fig. 3.1.5

Appearance of nuclei of small lymphocytes and PHA-induced blast cells after acetic acid showing variations in nuclear size: ethanol fixation and orcein stain.

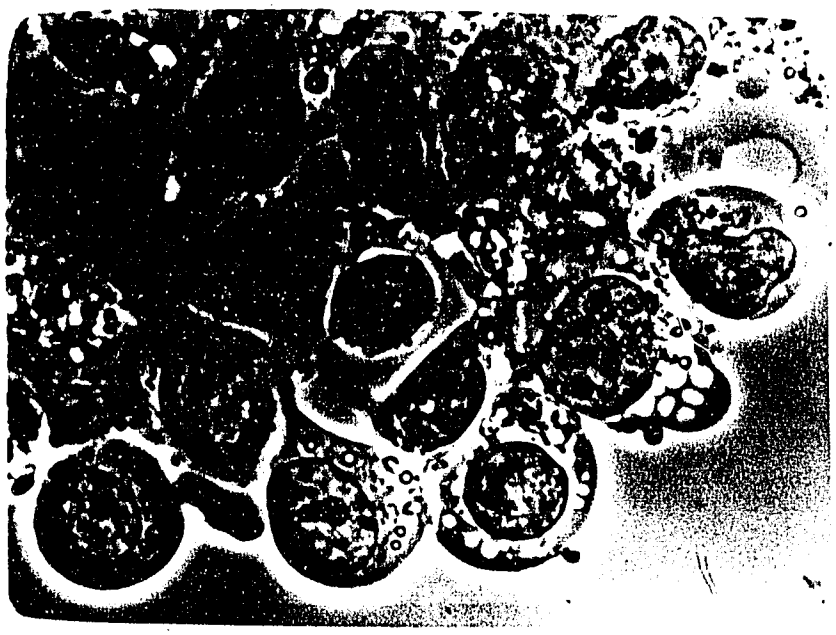
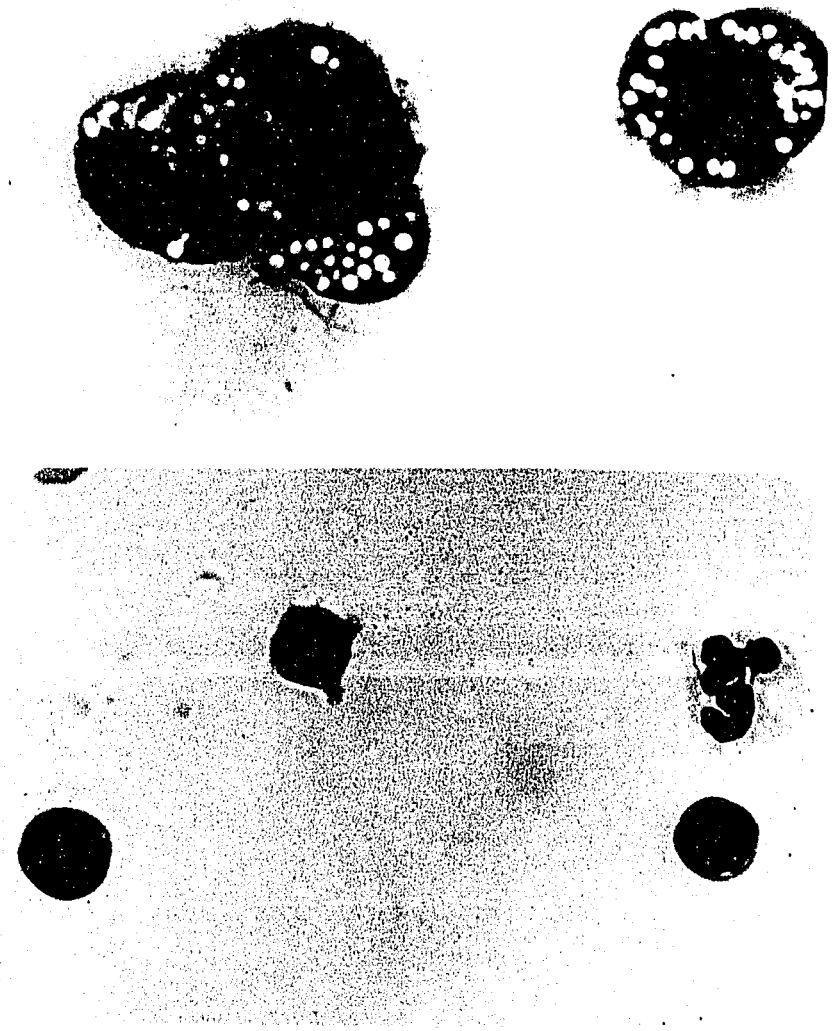


Fig. 3.1.6.

Clumps of transformed and transforming cells after one day in PHA. Phase contrast. x 1250.



(a)

(b)

Fig. 3.1.7.

(a) Blast cells and (b) unstimulated lymphocytes. Giemsa stain. x 1250.

Fixation of blast cells with acetic acid:ethanol (1:3) caused increased clarity due to better spreading, but caused a loss of cytoplasmic fine structure, such as the cytoplasmic vacuoles, seen in the air-dried specimens.

Osmium oxide stained, glutaraldehyde-fixed blast cells, when examined by electron microscopy, showed abundant ribosomes in the cytoplasm as well as vacuoles (Fig. 3.1.8). The endoplasmic reticulum was, however, poorly, if at all developed. A few mitochondria were present. The cytoplasmic border tended to have a corrigated, ridged appearance with many fringes, projections and internal channels. The nucleus, which was irregularly oval or elliptical in shape, was often indented, and characterized by electron opaque material about the nuclear membrane which projected in thick 'bridges' towards the centre of the nucleolus which was also surrounded by electron opaque material. Large areas of the nucleus were less electron dense, sometimes giving the entire nucleus a rather mottled appearance. The nucleus was separated from the cytoplasm by a double membrane which communicated with channels extending into the cytoplasm. Some small areas along the nuclear membrane which were not electron opaque, often seemed to be directly connected to the cytoplasm.

Fig. 3.1.8.

Ultrastructure of PHA-stimulated lymphocyte.

cm - CYTOPLASMIC MEMBRANE

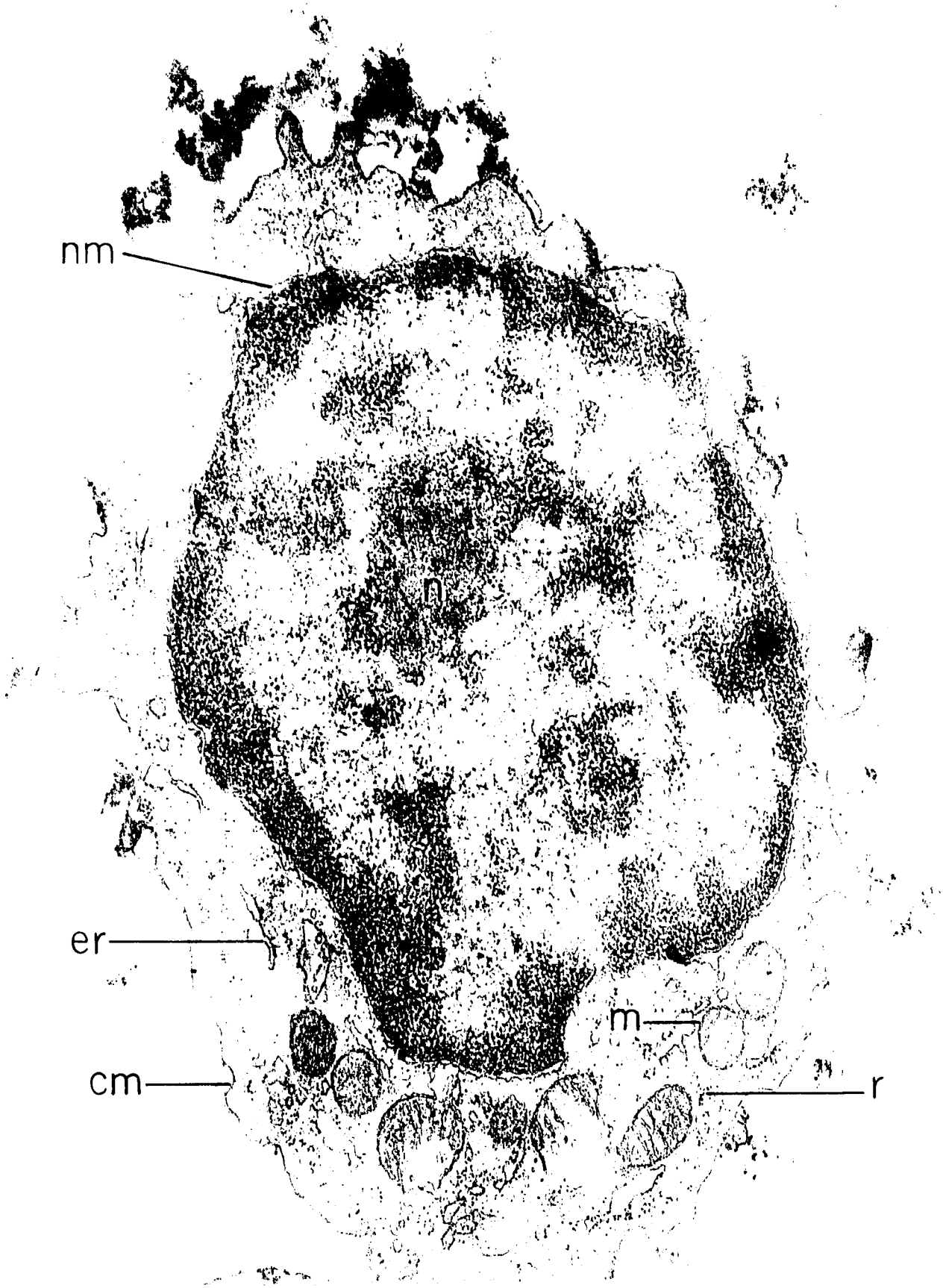
er - ENDOPLASMIC RETICULUM

m - MITOCHONDRIA

n - NUCLEOLUS

nm - NUCLEAR MEMBRANE

r - RIBOSOMES


nm
n
er
m
cm
r

Lymphocyte or leukocyte cultures treated with PHA for 2-4 days showed intense mitotic activity. Arrest of metaphase with colcemide, followed by swelling with hypotonic KCl (Hungerford et al, 1965) allowed chromosome preparations to be made, as shown in Figure 3.1.9.

3.1.3. Synthetic Activity During Lymphocyte Transformation: Incorporation of Labelled Precursors into Nucleic Acid and Protein

3.1.3.1. General Considerations

It was not desired in this study to repeat in detail the results reported by other workers during the past few years. However, it was important to obtain data for characterization of the lymphocyte culture system used. The parameters of lymphocyte transformation to be described here are those relating to nucleic acid and protein synthesis. Other observations, such as measurements of potassium transport, were of an original nature and have therefore been described in detail at a later stage of this report (Section 3.4).

3.1.3.2. Nucleic Acid and Protein Synthesis in Lymphocyte Cultures During the First Six Hours After PHA Administration

RNA and protein synthesis, as measured by the incorporation of H^3-uridine and C^{14}-leucine into TCA-precipitable material, were stimulated soon after the addition of PHA, as shown in Figure 3.1.10.

Fig. 3.1.9.

Chromosomes derived from human mitotic cells induced by PHA in a leucocyte culture. Orcein, x 1250.

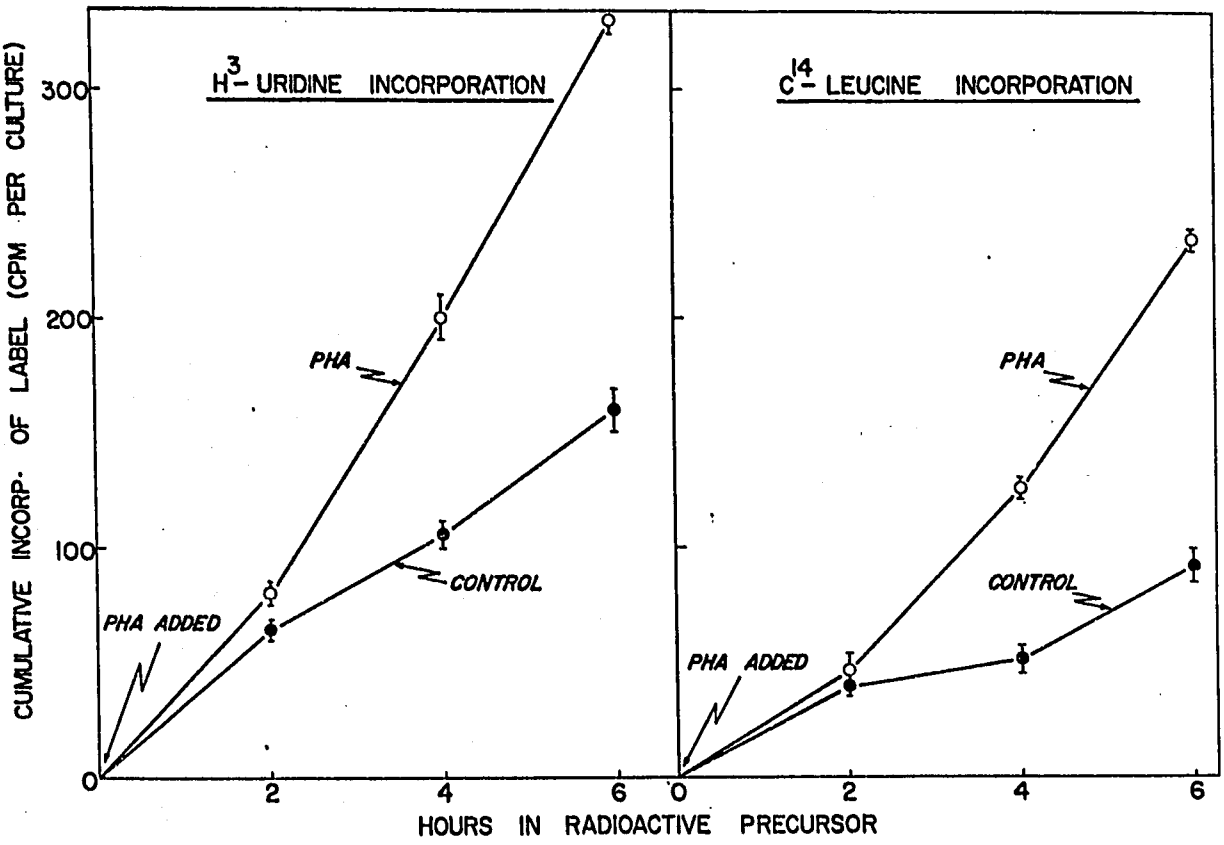


Fig. 3.1.10.

Effect of PHA on RNA and protein synthesis of lymphocytes in vitro. Radioactive precursors were given 5-10 minutes after PHA. Ordinate represents cumulative incorporation of radioactive tracer into TCA-insoluble material over the period of time in the abscissa. Vertical error bars are standard deviations of the mean in this and subsequent graphs.

The cumulative incorporation of these tracers into purified lymphocyte cultures was slightly increased above control levels after 2 hours with PHA (the earliest measurements made for this purpose), reaching about twice control levels after 4-6 hours with the mitogen.

3.1.3.3. Nucleic Acid Synthesis During the First Few Days of Transformation Caused by PHA

The measurements made in Section 3.1.3.2 were extended over a larger time scale. The purpose of these measurements was to evaluate, under the experimental conditions used, the changes in the rate of incorporation of radioactive precursors into the nucleic acid and protein which occurred in lymphocytes undergoing transformation.

As shown in Figure 3.1.11, the rate of H^3-uridine incorporation into RNA of PHA-treated cells accelerated with elapsed time; the rate of increase was approximately exponential over the first 2-4 days. The incorporation rate of radioactive amino acid into protein also increased (for example, 2-hour incorporation values for 10^6 lymphocytes in the presence of 0.1 μCi/ml C^{14}-l-leucine were: unstimulated, 240 ± 90 cpm; 1 day PHA, 890 ± 240 cpm; and 2 days PHA, 1680 ± 300 cpm). As shown in Figure 3.1.14, the degree of incorporation generally diminished after several days. DNA synthesis was maximal after 3-4 days and then decreased in rate. After 7 days of culture, little, if any, DNA synthesis was measurable.

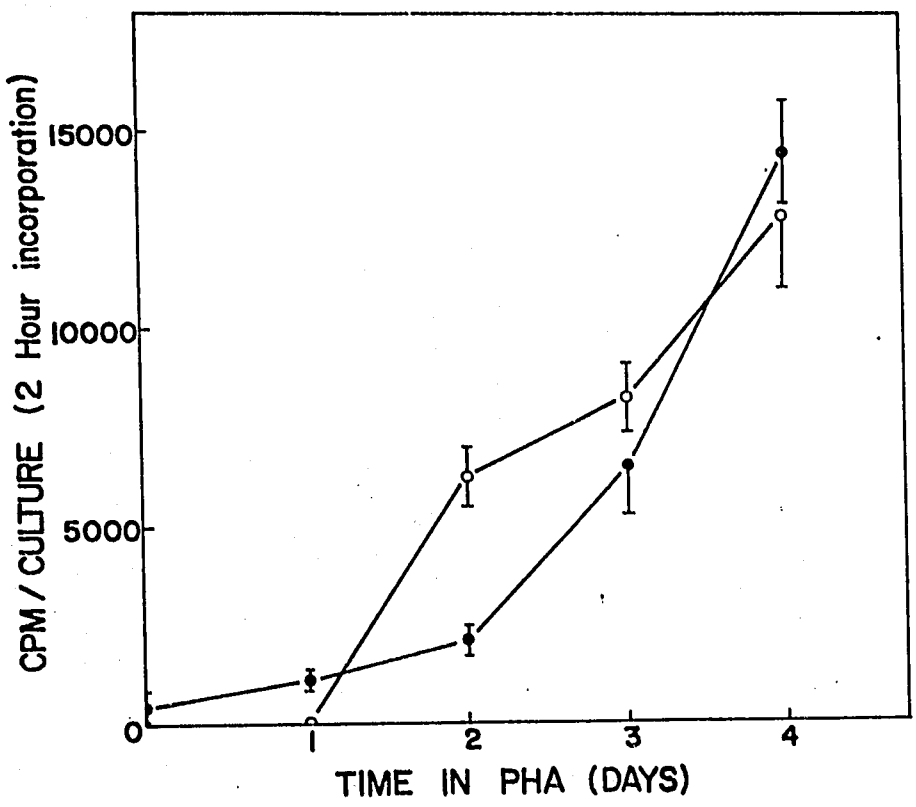


Fig. 3.1.11.

RNA and DNA synthesis during first 4 days after PHA administration to leucocyte cultures. H^3-thymidine (circles) and H^3-uridine (solid points) were added for 2 hour incubation periods.

The incorporation of labelled thymidine into DNA increased only after more than one day of PHA administration. This was in agreement with observations of others (Bender and Prescott, 1962; Sasaki and Norman, 1966) who found that in cultures obtained from healthy donors, DNA synthesis usually started not less than 24-30 hours after PHA administration, and remained minimal in unstimulated lymphocytes.

3.1.3.4. Kinetics of Radioactive Tracer Incorporation

Before investigating the effect of various agents on synthetic activity, it was necessary to characterize the 'normal' uptake of tracer, and to determine a period of incubation with the precursor which would provide an accurate indicator of the rate of synthesis of acid insoluble nucleic acid and protein. If the rate of uptake were to change during the period of incubation with the labelled precursor, the degree of uptake would not necessarily be representative of the rate of synthetic activity.

For these reasons, cells were incubated with precursors for various periods of time (20 minutes to 4 hours) and the degree of incorporation measured at various periods of time. In most experiments, the cells were extracted with trichloracetic acid, but in some, the unextracted cells were counted directly.

The rates of uptake of H^3-uridine into TCA precipitable material are shown in Figure 3.1.12. In these experiments, the cells

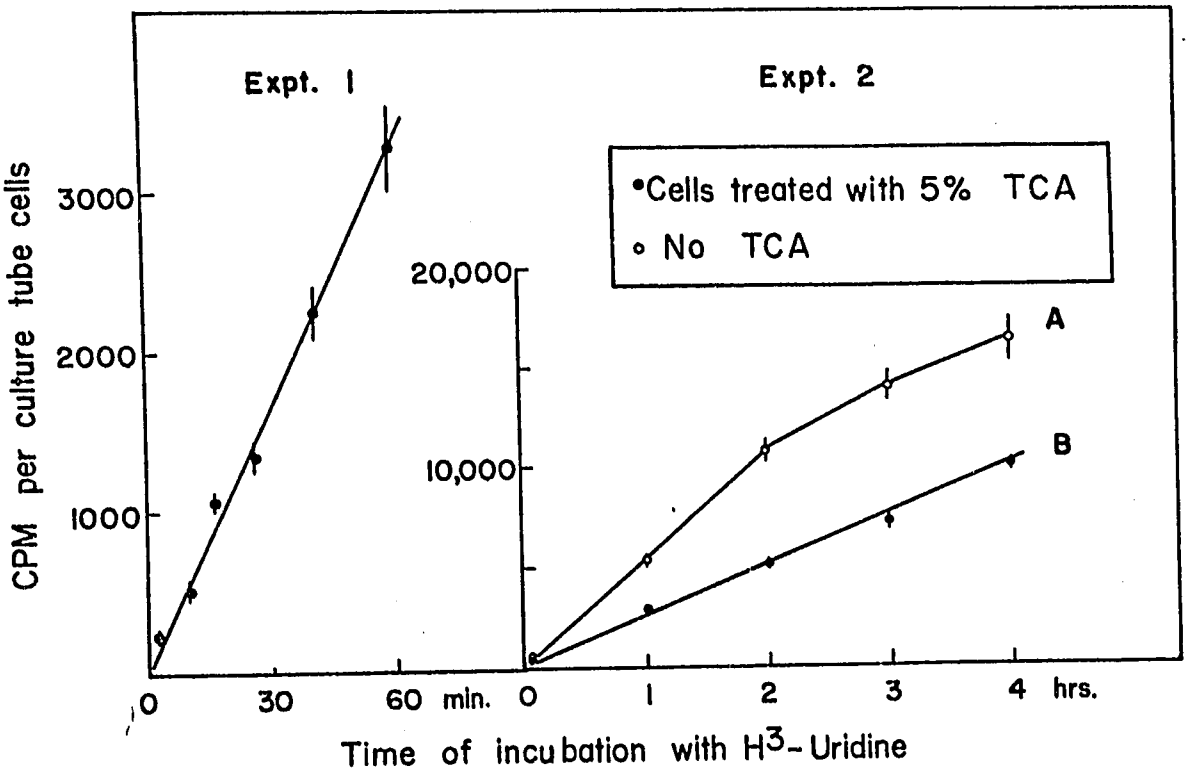


Fig. 3.1.12

Cumulative uptake of H^3-uridine into TCA-insoluble and TCA-soluble fractions from leukocyte cultures treated for one day with PHA. Expt 1 and curve B, Expt. 2 represent incorporation into TCA-insoluble material. Curve A, Expt 2, shows uptake of label into washed cells which were untreated with TCA.

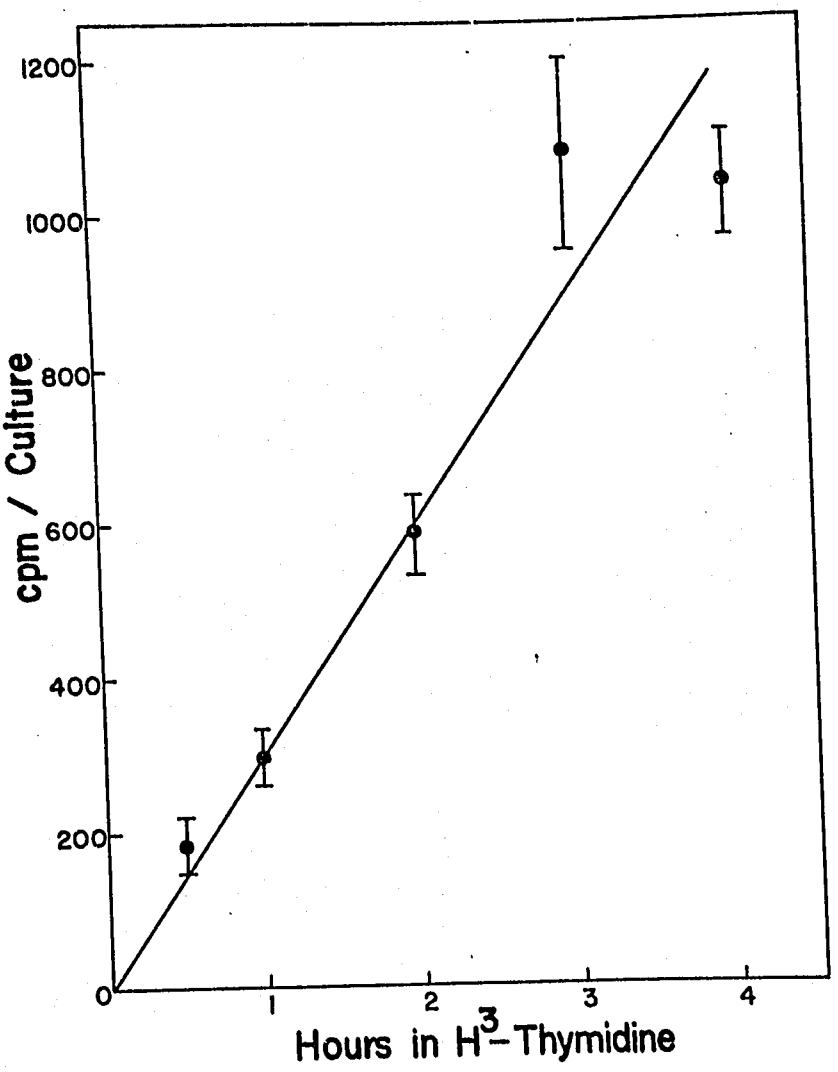


Fig. 3.1.13.

Cumulative incorporation of H^3-thymidine into the DNA of cultures stimulated with PHA for 2 days. Ordinate represents the uptake into TCA-insoluble material during the time indicated on the abscissa.

had been treated with PHA for one day. The uptake of label was found to be linear with time (Expt. 1 and Expt. 2, Curve B). A linear cumulative uptake over a 2-hour period was also observed when H^3-thymidine was administered to cells treated with PHA for 2 days (Fig. 3.1.13). However, over incubation periods of longer than 2 hours, the cumulative uptake sometimes was not linear but increased with time of incubation. A 2-hour incorporation was therefore generally used in the experiments described, except where otherwise stated.

In cultures to which TCA was not added, the cellular uptake of tracer was considerably higher (Fig. 3.1.12, Curve A). The difference between the two curves was considered to represent H^3-uridine taken up by the cells but TCA soluble, and was assumed to be tracer in an intracellular pool which had not been incorporated into RNA. The acid soluble pool observed using H^3-uridine is considered further in Section 3.3.14.

In the present studies, the incorporation of radioactive precursors into TCA insoluble material has been assumed to be a measure of the rate of macromolecular synthesis. Such an assumption, is recognized as being not necessarily always valid. Intracellular pool size and its specific radioactivity must be considered. In addition, DNA 'repair' as differentiated from

'synthesis' may also be associated with the incorporation of H^3-thymidine.

3.1.3.5. Mixed Lymphocyte Reaction

This phenomenon was shown by Bain et al. (1964) and Bach and Hirschhorn (1964) to occur when leukocytes from unrelated individuals are mixed together, although the response takes a longer period of time than is characteristic of PHA, and fewer blast cells develop.

The technique used was already described. Specifically, leukocytes from two different individuals were obtained after dextran sedimentation of erythrocytes, without any further separation of the white cells, and the cells made up to approximately the same concentration of 5×10^5 cells/ml. They were then either (a) cultured with PHA; (b) without PHA; or (c) together as a mixture.

The results in Figure 3.1.14 show the degree of DNA synthesis after PHA, as measured by the 2-hour incorporation of H^3-thymidine. In the absence of PHA, no DNA synthesis was observed. In the mixed culture, DNA synthesis was maximal after 5-6 days but was much lower than that caused by PHA.

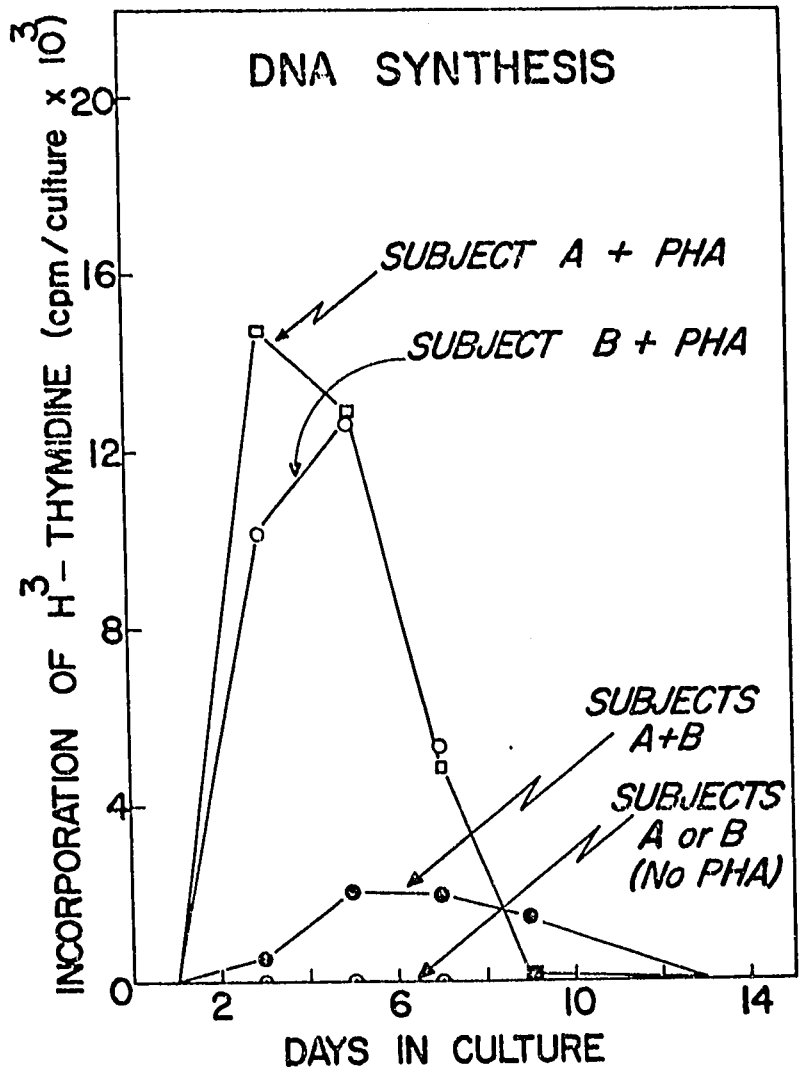


Fig. 3.1.14.

The mixed leukocyte reaction: DNA synthesis was measured over a 13 day period by the incorporation of H^3-thymidine during 2 hours incubation times. Open circles and squares are of the leukocytes from each individual alone, cultured with or without PHA. Solid circles represent DNA synthesis in mixed cultures without PHA.

3.1.4. Autoradiographic Analyses of Nucleic Acid Synthesis and Turnover

3.1.4.1. DNA Synthesis

Stimulated lymphocytes exposed to 'pulse' doses of H^3-thymidine for periods of 20 minutes to 2 hours took up the label only into relatively few of the 'blast' cells, as shown in Figure 3.1.15. In some cells, the thymidine was taken up in a patch-like pattern (Fig. 3.1.16). In these cells, patches of DNA synthesis (i.e., observable H^3-thymidine incorporation) were seen at intervals along the circumference of the nucleus and within it. Such cells represented about 5-10% of the labelled cells present. Since the remaining labelled cells showed heavy concentrations of radioactivity, either the cells in question may have been labelled during early or late stages of DNA synthesis, or there may be a non-uniform rate of DNA synthesis in different parts of the nucleus of the transformed blast, or the cells may have been abnormal.

3.1.4.2. RNA Synthesis and Turnover

The objective of these measurements was primarily to provide data for subsequent autoradiographic studies of the effect of ouabain on RNA synthesis, as described in Section 3.2.3.7.

Autoradiographic demonstration of RNA labelling required a somewhat more complex technique than that for DNA labelling. For the latter, it was sufficient to incubate the cells with the tracer,



Fig. 3.1.15.

Uptake of H^3-thymidine into PHA-treated leukocytes

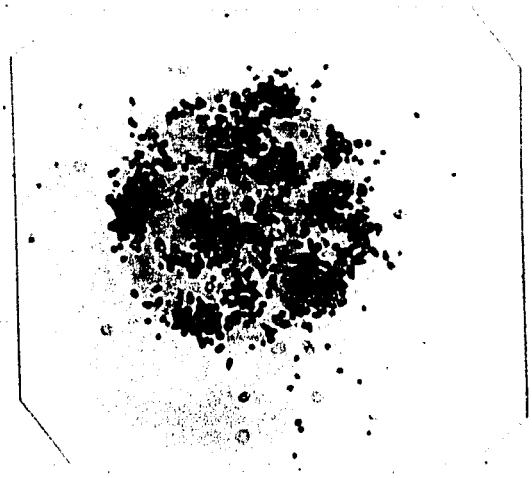


Fig. 3.1.16.

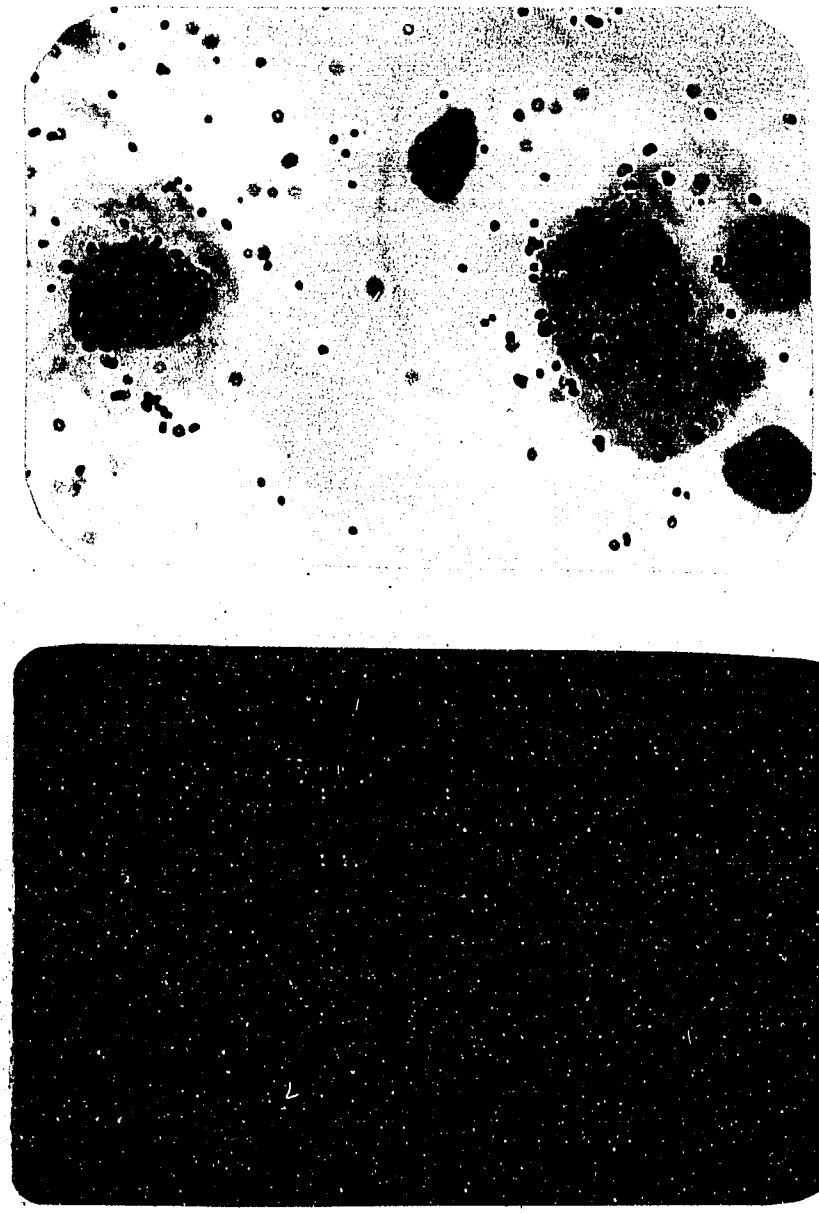
Patchy or irregular DNA synthesis which was seen in blast cells. Grains represent incorporation of H^3-thymidine into nucleus of a blast cell. Stain is too faint to be seen in this picture. Acetic acid: ethanol fixative. x 1250.

fix in acetic alcohol, make smears, and coat with photographic emulsion.

In the case of H^3-uridine incorporation into RNA, the relatively simple technique used for DNA labelling resulted in a grossly high 'background' around the cells, as if tracer were leaking out of the cell into the surrounding emulsion. This may have been 'unbound' H^3-uridine, since parallel studies had shown that a sizable fraction of the tracer taken up into PHA-treated lymphocytes was acid soluble and assumed to be 'unbound' (Fig. 3.1.12).

The labelled cultures were therefore washed three times in acetic acid/ethanol fixative following incubation in medium containing the tracer and the cells were dropped onto ice-cold wet slides for subsequent autoradiography. This procedure removed most (although not all) of the anomalous labelling by allowing acid soluble tracer to diffuse out of the cells.

The time during which the cells were cultured in the presence of H^3-uridine was an important factor, as shown in the photographs (Fig. 3.1.17). Labelling usually appeared at first in a local area within the nucleus, probably the nucleolus, although however, in some cells the tracer was not 'focussed' over a specific nuclear area, but was diffusely scattered



(a)

(b)

Fig. 3.1.17.

Autoradiographs of PHA-induced blast cells incubated with H3-uridine for (a) 40 min., and (b) 1 hr. 40 min. In (b), labelling extends to the border of the cytoplasmic membrane.

throughout the nucleus. For exposure times between 20-40 minutes, labelling was exclusively nuclear; no tracer was seen in the cytoplasm.

When the cells were incubated with H^3-uridine for 40 minutes, then washed and cultured for further periods of time, label appeared in the cytoplasm during the 2nd hour following the addition of the tracer.

In summary, radioactive label from H^3-uridine in acetic acid: ethanol fixed cells did not appear in the cytoplasm before one hour after the first pulse of H^3-uridine was administered. The label first appears mainly over a small area in the nucleus which is presumed to be the nucleolus and only later appeared in the cytoplasm. (These results taken alone strongly suggest, but do not prove that the cytoplasmic label which later appeared was originally nuclear. This is because some 'unbound' H^3-uridine could conceivably have been taken up into a cytoplasmic pool, remaining there after removal of the cells from the radioactive medium, and only slowly incorporated into cytoplasmic RNA.)

Label from H^3-uridine was seen only in slight amounts in small lymphocytes from unstimulated cultures, or in non-transforming cells in PHA-treated cultures. Labelling of PMN's was never observed.

3.1.5. PHA Dose-Response

The usual concentration of PHA-M used was 200 µg/ml. This value is larger than that required (MacKinney, 1964; Tormey and Mueller, 1965) for a maximal response by other types of PHA (such as the Burroughs Wellcome preparation, or Difco PHA-P). Since all phytohemagglutinin studies herein reported were carried out with PHA-M, it was desirable to have quantitative data on the dose response of the cells to this particular mitogen preparation.

In characterizing the dose-response of the lymphocyte culture system to PHA-M, several parameters of transformation were examined. These included (a) DNA synthesis, and (b) RNA synthesis (as measured by the incorporation of tritiated uridine and thymidine), (c) the incidence of 'blast' cells, and (d) mitotic frequency.

The results obtained are plotted together in Figure 3.1.18. It is clear that for all parameters studied, the greatest (though not maximal) response obtained was at 200 µg/ml (the greatest dose used), while progressively reduced values for all parameters were observed at lower PHA-M concentrations. Higher doses of PHA may have increased the effect some more, but were not considered necessary for this study; the PHA dose-response has been characterized by others (MacKinney, 1964; Tormey and Mueller, 1965; McIntyre and Cole, 1969). Morphological criteria used for the microscopic identification of 'blast' cells were found to be somewhat subjective;

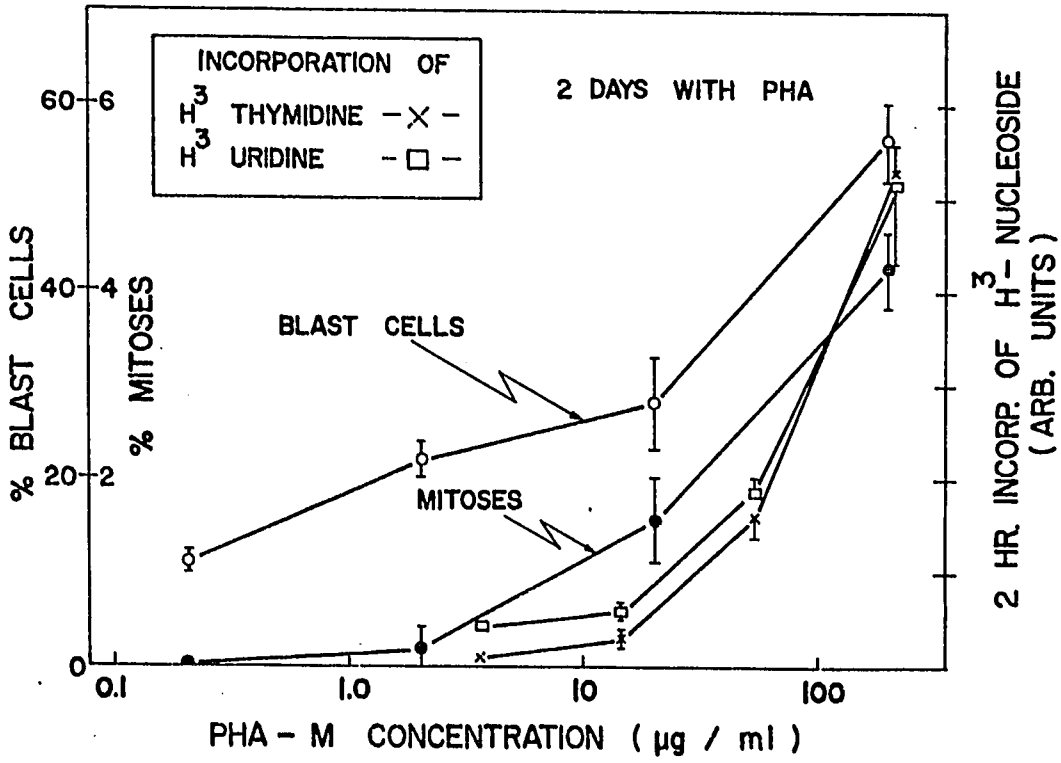


Fig. 3.1.18

Dose-response of leukocyte cultures to PHA-M. RNA and DNA synthesis, and the incidence of blast cells and mitoses, were measured after a 2 day period of culture with various doses of the mitogen.

there was a wide margin of error in assessing their frequency, particularly at the lower doses of PHA.

In later experiments, it was sometimes desirable to use PHA in 'suboptimal' amounts. PHA concentrations of 20 µg/ml were then used for comparison with effects at 200 µg/ml.

3.1.6. Summary of Chapter

This chapter has been concerned primarily with parameters of lymphocyte transformation as observed under the experimental conditions used. Consequences of PHA administration on the cellular morphology, mitotic activity, RNA, DNA, and protein synthesis were examined, both on a short-term basis (20 minutes to 6 hours with PHA) and over several days (or as long as 7 days with the mixed leukocyte reaction).

While the main body of the work is intentionally confirmatory of previous studies, there were some original observations.

The confirmatory work may be summarized as follows:

(a) Lymphocytes will undergo 'blast' transformation when cultured in the presence of phytohemagglutinin. This transformation is characterized by marked morphological changes in the cell, starting with an acceleration of RNA and protein synthesis during the first few hours after culture with PHA.

DNA synthesis starts after 24, but before 48 hours of culture with phytomitogen. Cell enlargement and mitoses are observed after the second day of culture with PHA.

(b) Leukocytes from unrelated donors will transform if cultured together for several days. Under such conditions, in the absence of PHA, DNA synthesis is stimulated, occurs after a longer period of culture than with PHA, and reaches a maximum at about 5-6 days after initiation of the mixed culture.

(c) The effect of PHA is related to its dose; the degree of blast transformation and mitotic activity (from morphological observations) corresponds to the degree of synthetic activity induced by the stimulatory agent.

The original observations are as follows:

(a) The dynamics of H^3-uridine turnover and labelling of blast cells suggest that the time taken for the nucleoside label to be taken up into the nucleolus and subsequently releasted into the cytoplasm is of the order of one hour.

(b) Patterns of DNA synthesis, apparently unreported for transforming lymphocytes, were observed in autoradiographs. These were characterized by alternate patches of intense uptake within the nucleus alternating with areas devoid of labelling.

(c) After administration of H^3-uridine to PHA-treated cells, a significant amount of unbound, acid-soluble tracer was observed, comparable in amount to the amount of tracer actually incorporated into RNA (Fig. 3.1.12).

3.2. EFFECTS OF OUABAIN ON UNSTIMULATED AND STIMULATED LYMPHOCYTES IN VITRO

3.2.1. Introduction to Section

The initial experiments carried out in this work were done on the working hypothesis that lymphocyte transformation was brought about by an early change in plasma membrane activity, possibly associated with the function of active transport. It will be appropriate to mention, at this stage, some early experiments that were done on the binding of fluorescein-labelled PHA to lymphocytes.

These results, more fully described in supplementary Section 3.5.7, indicated that PHA bound to fluorescein-isothiocyanate would induce lymphocyte transformation, but that fluorescence was observable only on the cell membrane or in the cytoplasm. No nuclear fluorescence occurred, although early biochemical events following PHA administration have nuclear localization (Killander and Rigler, 1965; Pogo et al., 1966; Kleinsmith et al., 1966). This suggested (but did not prove) that PHA causes lymphocyte activation by a direct effect on the surface of the cell or within the cytoplasm, but not within the nucleus. Similar conclusions were reached by Michalowski et al. (1965), Kay (1969), and Hausen and Stein (1968), who used anti-PHA antiserum (see discussion of this matter in Section 1.6).

It was considered that if PHA were to act 'primarily' on the lymphocyte membrane, it would be likely to affect (directly or indirectly) the transport of ions and other substances across the cell membrane, and in addition, lead to an increased availability to the cell of high energy compounds which are presumably required during the transforming process for the synthesis of protein and RNA. Although the mechanism for such changes might be very indirect as far as the primary action of PHA is concerned, the events associated with activation could involve alterations in the activity of sodium-potassium dependent ATPase during the transforming process. In order to test the sensitivity of PHA-activated cells to changes in the activity of the cation transport system, ouabain was administered to non-stimulated and PHA-treated leukocyte cultures, and the effects of the glycoside on parameters of transformation (Section 3.1) were evaluated. Separation of the neutrophils and monocytes from the lymphocytes was not necessary at this stage of the work.

In the first experiments, gross morphological changes were noted and evaluated on a semi-quantitative basis. The effect was then characterized more accurately using radioactive tracers of nucleic acid and protein synthesis.

It was shown that the blast transformation of lymphocytes is strongly inhibited by ouabain. Quantitative descriptions of

this effect are obtained by measurement of RNA, DNA, and protein synthesis. The inhibitory effect of ouabain on macromolecular synthesis was partly or completely prevented by excess extracellular potassium levels. The effect was found to be independent of PHA stimulation since the mixed lymphocyte reaction was inhibited in an identical way.

3.2.2. Morphological Effects of Ouabain

3.2.2.1. Special Aspects of Experimental Procedure

The general methods used have already been described, but some special aspects must be described here. Leukocyte cultures were prepared as described previously (Section 2.1). Leukocyte-rich plasma was separated from heparinized blood samples by dextran sedimentation and added aseptically to Medium-199, in a volume ratio of about 1:9. Ehrlenmeyer flasks (50 ml) were employed, using room air as the gas phase, and PHA-M was added to give final concentrations varying from 0.2 to 200 µg/ml. Cultures were maintained at 37°C from several hours to five days. Ouabain was added in concentrations varying from 4.4×10^{-8}M to 4.4×10^{-6}M at different times and for varying periods after the cultures were initiated. In some experiments, ouabain and PHA were added successively, the cells being washed between the addition of the different compounds.

When PHA was added, cultures showed agglutination of both red and white cells and colonies of a few to some hundreds of cells were later observed. (See Figure 3.2.1.) The number of recognizable cells per unit size of clump is referred to in the following as 'cellularity'.

A semi-quantitative measure of the morphological effects of ouabain was obtained by counting the number of cells present in clumps. In some of the cultures treated with ouabain, cellular disintegration had occurred, resulting in an undefined stained mass containing a variable number of cellular entities of abnormal shapes, size, and consistency (Fig. 3.2.2). The reduced number of cells per unit clump size, i.e., the cellularity, was taken as a measure of the gross morphological damage.

Staining of the preparations was carried out with orcein because changes in nuclear morphology caused by PHA or ouabain were easily observable with these stains and mitoses were clearly defined.

It must be pointed out that the method of quantitation described in this section (3.2.2) was based on subjective morphological criteria, and also suffered from the inherent disadvantage in that it was difficult to follow the development of the cellular changes induced by the glycoside, or to estimate accurately the length of time that the cells had been affected.

3.2.2.2. Appearance of Control Cultures

This is described in detail in Section 3.1.2. Some special notes are given here. Gradual increases in the size of some mononuclear cells were usually observed after about one day in the presence of PHA, and mitoses appeared by the second day, usually in the periphery of the clumps. However, no size changes of lymphocytes were observable by light microscopy during the first 4-6 hours of culture. Leukocyte cultures, when examined one day after the administration of PHA, contained polymorphonucleocytes (PMN's) which accounted for about 20-50% of the total cell number. The rest of the cells were mononuclear, of which perhaps 10-60% had larger and paler nuclei than the remaining small lymphocytes. Two days after the initiation of culture, the PMN's were usually much reduced in number and appeared pycnotic and disintegrating. In some cultures, however, PMN's persisted for longer periods of time. After 2 days in the presence of PHA, there was a relative increase in the number of large, lighter stained cells (compared to small lymphocytes) whose nuclei had a reticulated appearance with prominent nucleoli. Mitoses appeared on the periphery of the clumps. By the third day, the incidence of large cells had increased to about 60-90% of the total cell number. Figure 3.1.23 shows the fraction of 'blast' cells and of mitoses at different PHA concentrations after three days in vitro and Figure 3.2.1 shows a typical clump including mitotic cells and blasts.

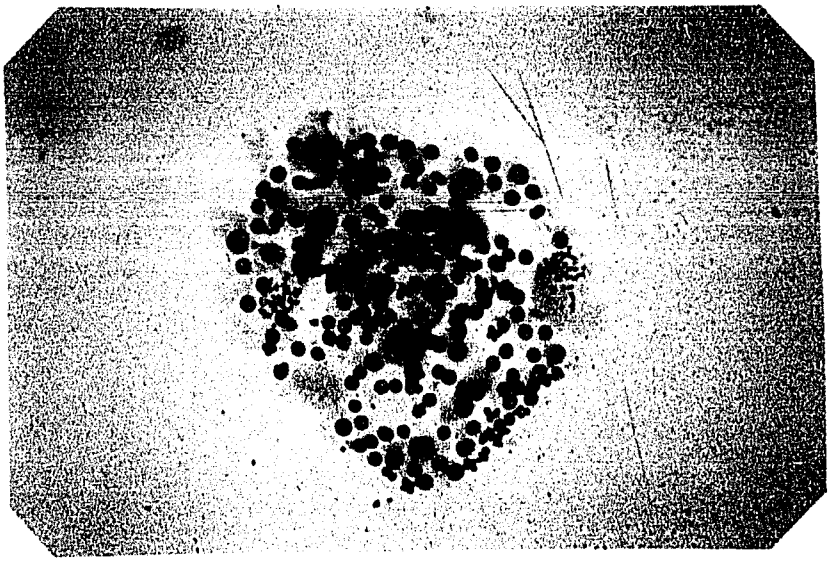


Fig. 3.2.1. Leukocyte clump after culture with PHA for 2 days. 20 µg/ml, PHA; acetic acid : ethanol fixation, aceto-orcein stain; x 100.

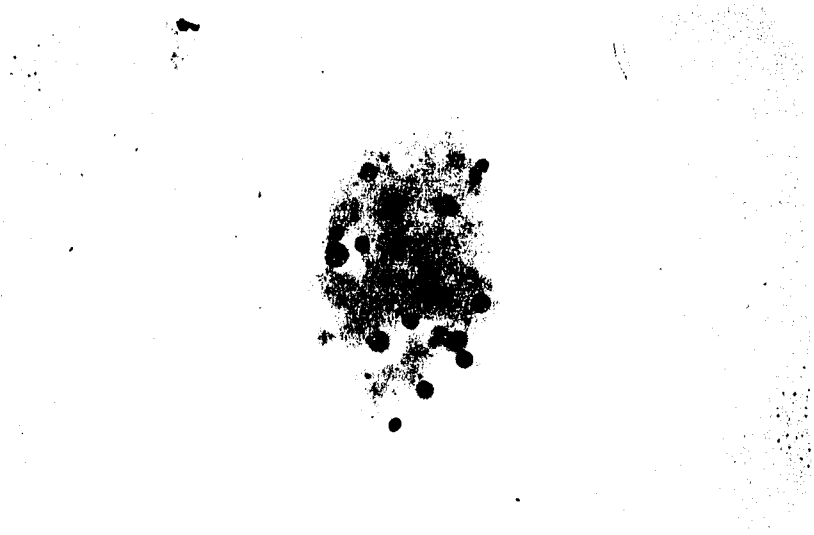


Fig. 3.2.2. Leukocytes cultured with PHA for 2 days in same experiment as in Fig. 3.2.1, but also in the presence of ouabain at 4.4×10^{-8}M; x 320.

3.2.2.3. Effect of Ouabain on Morphology

In the cultures which were not treated with ouabain, the clumps were full of normal appearing small lymphocytes and blastoid cells. When ouabain was added to the cultures in the presence of PHA at the start of the culture period, using a concentration of 4.4×10^{-6}M, pronounced cellular damage and disintegration was observed after 24 hours in culture (see Figures 3.2.1 and 3.2.2). The damage was so severe that remaining cellular structures were recognizable only as nuclear-sized, darkly stained masses in various stages of fragmentation. These structures were surrounded by a granular, pink-staining material (using orcein as the stain). At lower ouabain concentrations, the damage was less marked in that more intact cellular elements were present, but it was apparent that all the cells were much distorted in morphology, and were unlikely to have been viable. At the lowest ouabain dose used for morphological study, 4.4×10^{-8}M, the appearance of the cells approached that of the control cultures, in that blast cells were present, but the cells were characterized by a complete inhibition of mitoses after 2 or 3 days of culture. The nuclei of many mononuclear cells showed peripheral vacuolization at this dose.

The degree of damage, for a given concentration of ouabain, was related to the PHA concentration. The higher the dose of PHA,

the more pronounced was the damage. This relationship is shown in Figure 3.2.3. Thus, at a ouabain dose of 4.4×10^{-6}M, the damage was much less pronounced at low than at high PHA levels. In Figs.3.2.4 & 5 , it is shown that the degree of cellularity loss, for the same dose of ouabain, was related to the amount of PHA with which the cells were treated.

In some experiments, leukocyte cultures were incubated in the presence of PHA, or in medium only, for two days. During the final 24 hours of this two-day period, some cultures were treated with ouabain, using a dose (2.2×10^{-7}M) which caused profound inhibition of nucleic acid and protein synthesis (as in Fig. 3.2.15). As shown in Figure 3.2.8, ouabain caused a marked change in the microscopic appearance of both untransformed lymphocytes and blast cells. In acetic acid/ethanol fixed cells, there was a redistribution of nuclear chromatin away from the nuclear membrane, and the formation of fine or coarse dark-staining rod-like bodies within the nucleus that sometimes resembled chromosomes. In these cells, it was difficult to confirm the existence of nucleoli or to distinguish the nucleolus from other material (Fig.3.2.6&7). These effects were seen better with orcein than with giemsa stain, and were most pronounced in the large 'blast' cells. It would be desirable to carry out ultrastructural observations of this phenomenon, although these were not done in this study.

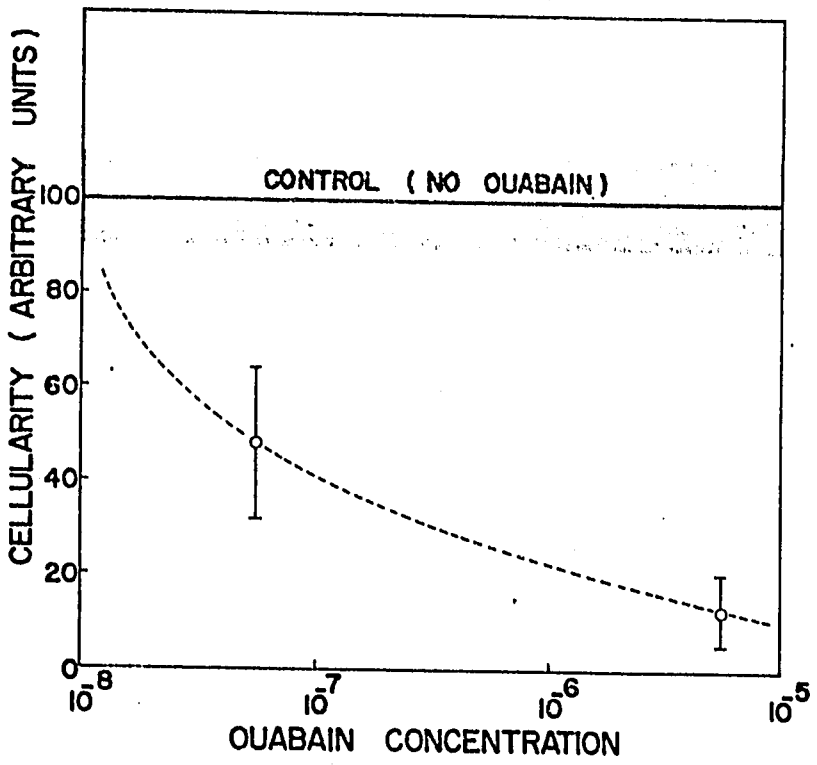


Fig. 3.2.3.

Effect of ouabain at different concentrations on the 'cellularity' of PHA treated leukocyte cultures. Ouabain was present together with PHA for the entire 2 day period of culture. the term 'cellularity' is defined in the text in section 3.2.2.1. PHA-M concentration, 20µg/ml.

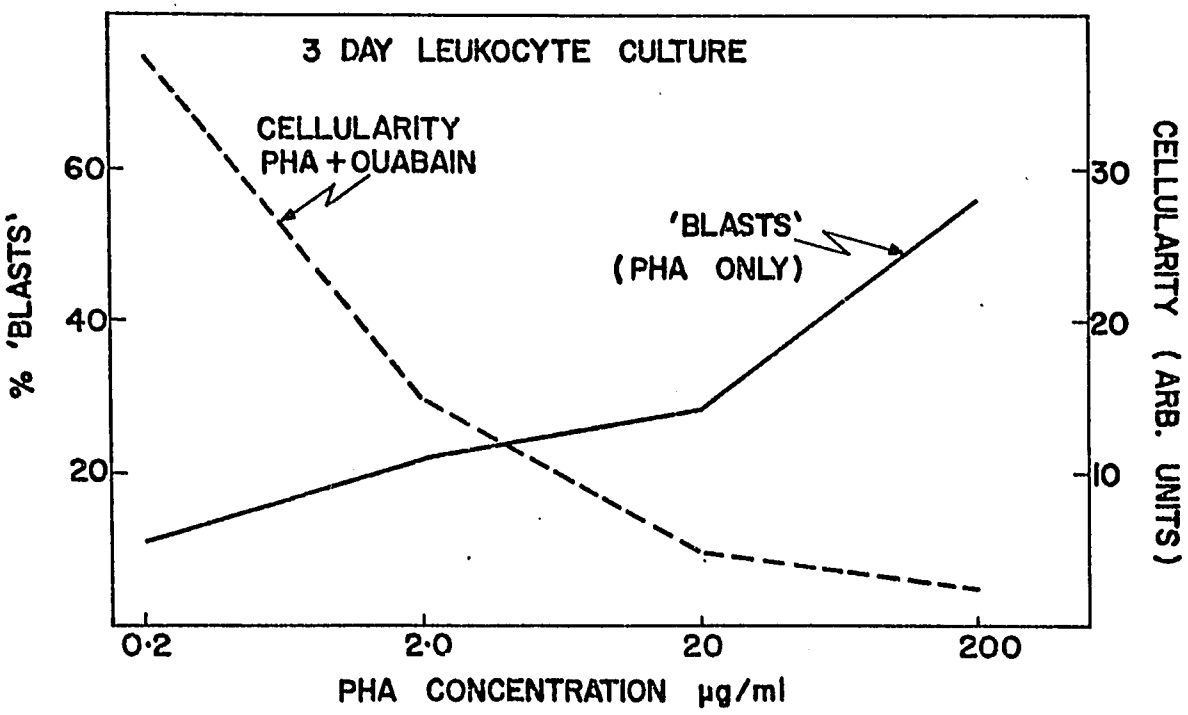


Fig. 3.2.4.

Effect of varying PHA concentrations on the incidence of blast cells in non ouabain-treated controls, and on the cellularity of leukocyte clumps cultured for 3 days in the presence of both PHA and ouabain (4.4×10^{-6}M).

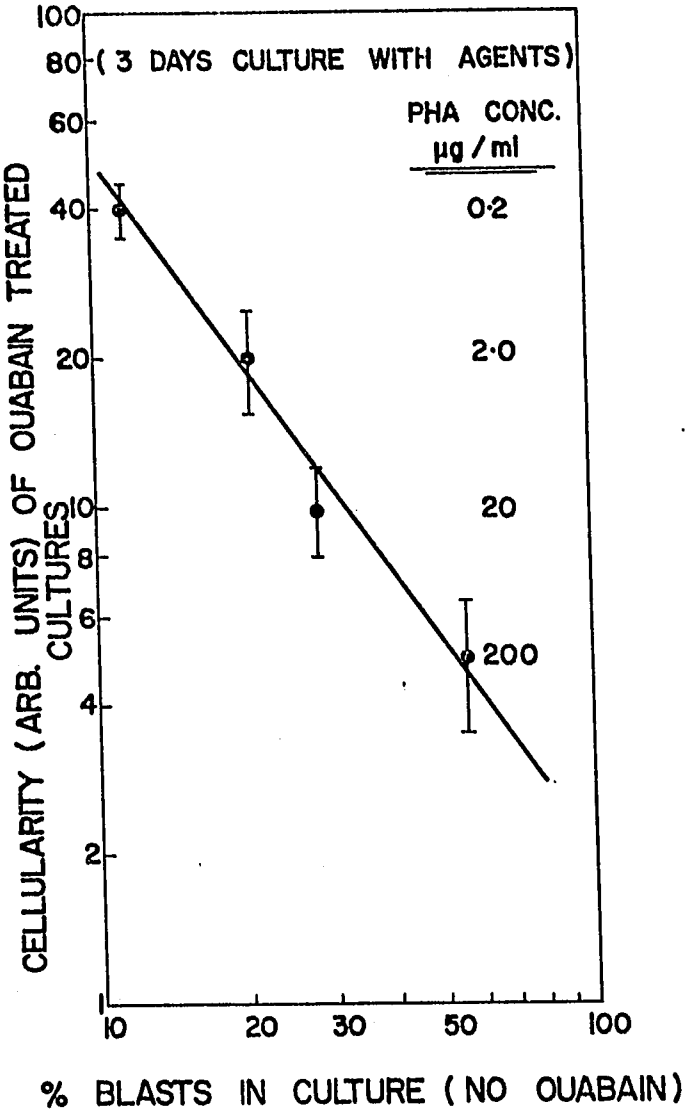


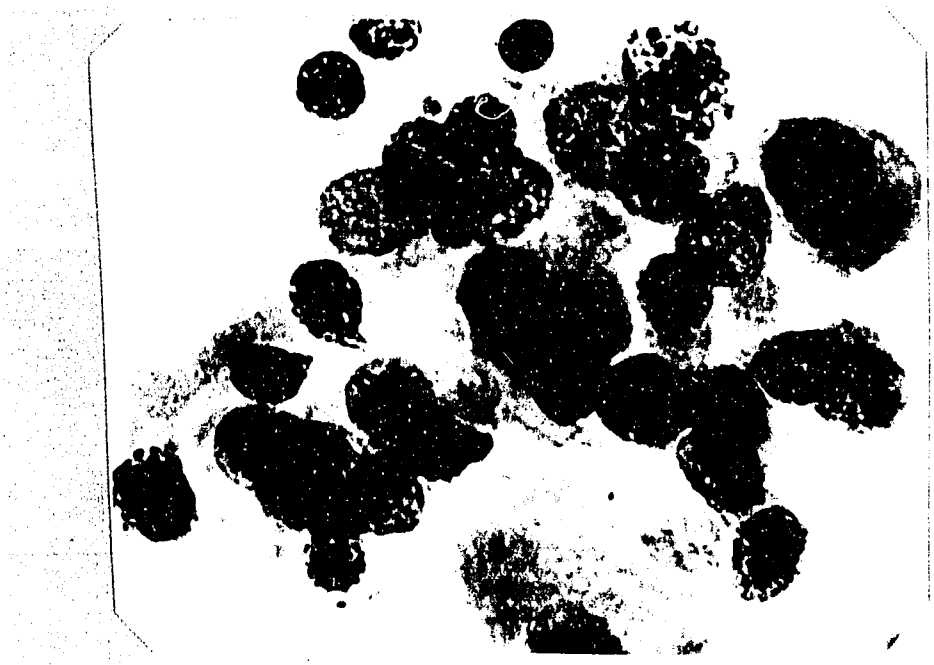
Fig. 3.2.5.

Relationship between the degree of transformation induced by varying concentrations of PHA, and the degree of reduction of cellularity induced by a fixed dose of ouabain (4.4×10^{-6}M) in parallel, PHA-treated cultures. The same data are used as shown in Fig. 3.2.4.



(a)

Fig. 3.2.6.



(b)

Fig. 3.2.7.

Morphological changes induced by ouabain (2.2×10^{-7}M) in the nuclei of PHA-treated lymphocytes in vitro. Acetic acid : ethanol fixation, accto-orcein stain; x 500.

(a) No ouabain.

(b) Ouabain treated.

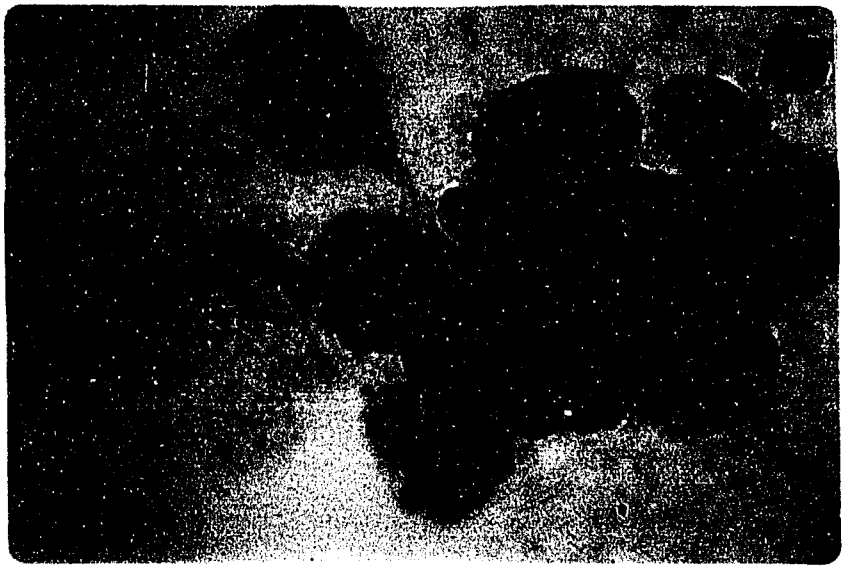


Fig. 3.2.8.

Changes in the nuclear chromatin of large blast cells cultured in the presence of ouabain (2.1×10^{-7}M). The cells were cultured for a total period of 2 days with PHA and then with PHA and ouabain for the final 24 hours. x 1250
Acetic acid : ethanol fixation, aceto-orcein stain.

3.2.2.4. Comparative Action of Ouabain on Stimulated and Unstimulated Cells

As already mentioned, the cellular disintegration caused by the glycoside was reduced when lower doses of PHA were used. Thus, at a dose of 4.4×10^{-6}M, the relative degree of cellularity of the clumps was considerably greater when lower PHA concentrations were used. Experiments were carried out on the appearance and survival of unstimulated cells. In the presence of ouabain, unstimulated small lymphocytes retained their nuclear structure by phase contrast, but a swelling or 'ballooning' of the cytoplasm became evident after one day of ouabain treatment. When the cells were resuspended in fresh medium for another day, the cells appeared to be normal in gross appearance, and did not take up trypan blue. However, PHA-treated cells showed poor survival in the presence of ouabain. Later evidence (Section 3.2.3.5) will show that ouabain-treated small lymphocytes retain their capacity to be stimulated by PHA.

When PHA-stimulated cells were treated with ouabain (2.2×10^{-7}M) and then reincubated in fresh medium for one more day, no blast cells were seen in the remaining suspension. There was a marked reduction in cellularity, and the only cells seen by phase contrast or after staining, were some bizarre shaped cells of unidentifiable origin, highly motile cells which may have been macrophages, and apparently normal small lymphocytes. When

unstimulated lymphocytes were treated with ouabain for a day, and then recultured in fresh medium for another day, the remaining suspension was very cellular and contained many small lymphocytes which appeared normal in number and in appearance by phase contrast microscopy or in stained smears (although often their nuclei showed clumped chromatin). These results suggested that the unstimulated small lymphocyte is less sensitive than is the stimulated lymphocyte to the same concentration of ouabain.

3.2.2.5. Summary

1. Ouabain in concentrations of 4.4×10^{-6}M caused marked cellular damage to leukocytes cultured in vitro in the presence of phytohemagglutinin (PHA).

2. Doses of the same compound at a concentration of 4.4×10^{-8}M led to a partial reduction in blastoid transformation and inhibition of mitosis.

3. In the absence of PHA, the morphological effects of ouabain on small lymphocytes were temporary and less obvious by gross visual examination. For the same ouabain level, the degree of damage appeared greater with higher PHA concentrations.

These observations suggest that ouabain has an inhibitory and toxic action on lymphocytes in which transformation had been induced, and that the glycoside may affect a process in the

transforming lymphocyte to which unstimulated small lymphocytes are less sensitive.

The evaluation of the influence of various agents on the lymphocyte culture system is inadequate when carried out only on a morphological basis. Numerical counts of 'blast' cells, though necessary, are also subjective, tedious, subject to gross errors caused by the qualitative definition of 'intermediate' cells or 'blast' cells, and are affected by variations in size and shape of cell structures in air-dried smears, by the presence of mononuclear cells not derived from small lymphocytes, as well as by the possible transformation of non-lymphocytic cells in the culture to cells resembling macrophages or fibroblasts (see Coulson's paper (1970) for a discussion of the latter possibility).

Because of the difficulty in quantitation and of controlling the experimental conditions required for obtaining reproducible morphological results, a more precise method for evaluating changes in cell function was needed. The measurements of gross damage described in this section were felt to be too crude to permit characterization of the ouabain effect. For this reason, subsequent studies were based on the incorporation and uptake of radioactive precursors, after a suitable and reproducible culture technique had been devised.

3.2.3. Effects of Ouabain on the Nucleic Acid and Protein Synthesis of Unstimulated and PHA-Stimulated Lymphocytes

For the reasons just mentioned, the uptake of radioisotope labelled precursors was investigated as a method for quantitation, and in this study, the technique was found to be extremely useful. It was, of course, necessary to characterize the 'normal' response of lymphocytes to stimulation in vitro, and the kinetics by which the labelled precursors were taken up by the cells. These normal kinetics of lymphocyte transformation have been described separately in Section 3.1. For each experiment, comparison between treated and controls was carried out only with cultures treated together under the same conditions and at the same time.

3.2.3.1. RNA Synthesis

One of the first experiments to be carried out involved the effect of ouabain on the incorporation of H^3-uridine, because RNA synthesis is known to be accelerated soon after the start of PHA addition to a lymphocyte or leukocyte culture. As shown in Figure 3.2.9 , ouabain caused a pronounced decline in the incorporation of H^3-uridine, as measured by this technique. This ouabain-induced decline usually was found to reach about 5-10% of the non-inhibited incorporation rate.

The relative effects of the glycoside on stimulated and unstimulated cultures are shown in Figures 3.2.10 and 3.2.11. In

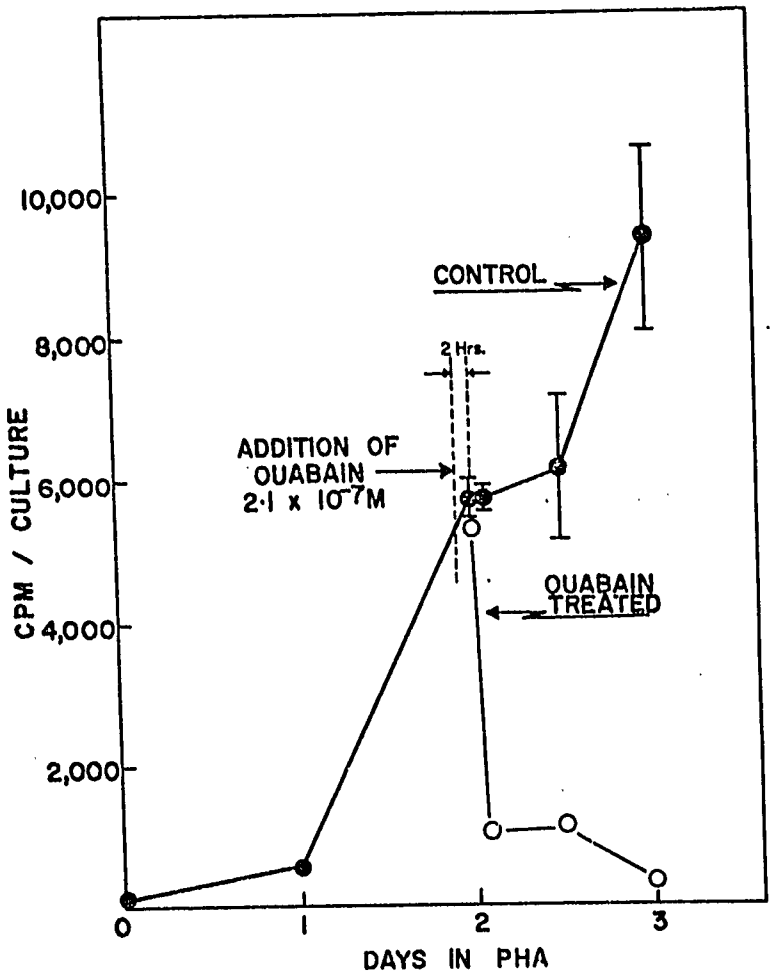


Fig. 3.2.9.

Effect of ouabain on RNA synthesis in PHA-stimulated lymphocytes. Ordinate represents the incorporation of H^3-uridine into TCA-insoluble material after a 2 hour period of incubation with the labelled nucleoside. PHA-M concentration was 200 µg/ml in this and other figures where not specifically mentioned otherwise.

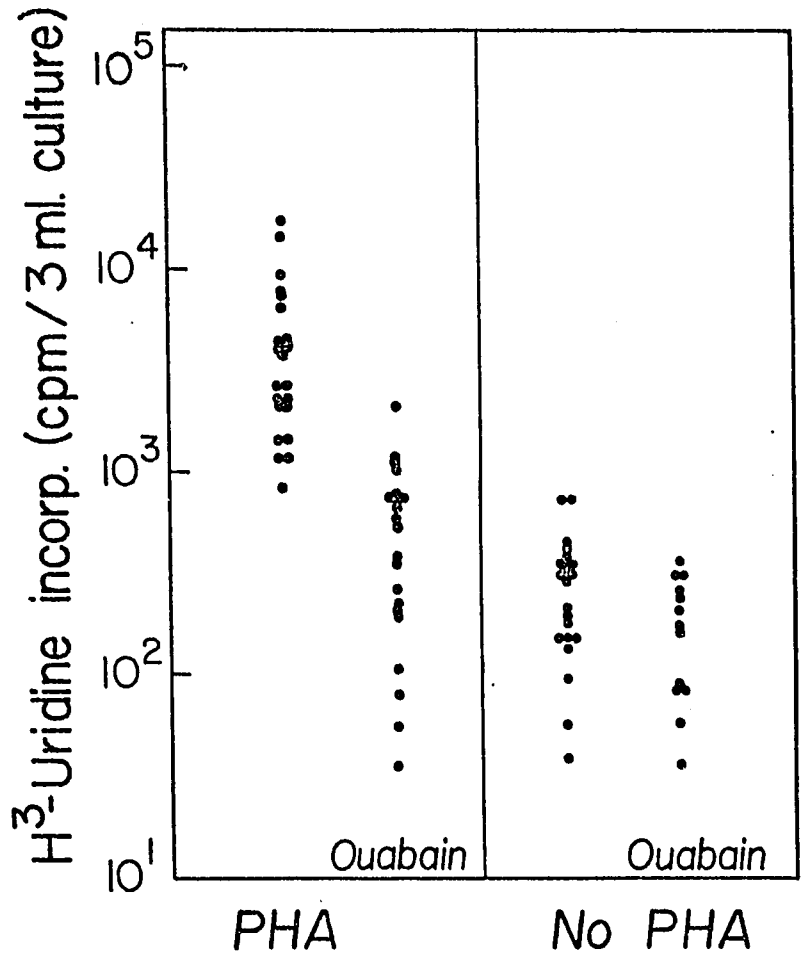


Fig. 3.2.10.

Effect of ouabain on H^3-uridine incorporation; a comparison between paired PHA-stimulated and control leucocyte cultures from different subjects.

A log scale is used to show the extent of variation and the degree of inhibition observed in absolute counts. The non-specific background of about 30 cpm was subtracted from the measured values. The stimulated cells had been treated with PHA for 1 or 2 days, and ouabain was added during the final 17-24 hours. Control cells were treated similarly but without PHA. The ouabain concentrations were 2.2×10^{-7}M (PHA-treated cells) and $2.2 - 56.0 \times 10^{-7}$M (control non-PHA-treated cells). Leukemic cells are not included.

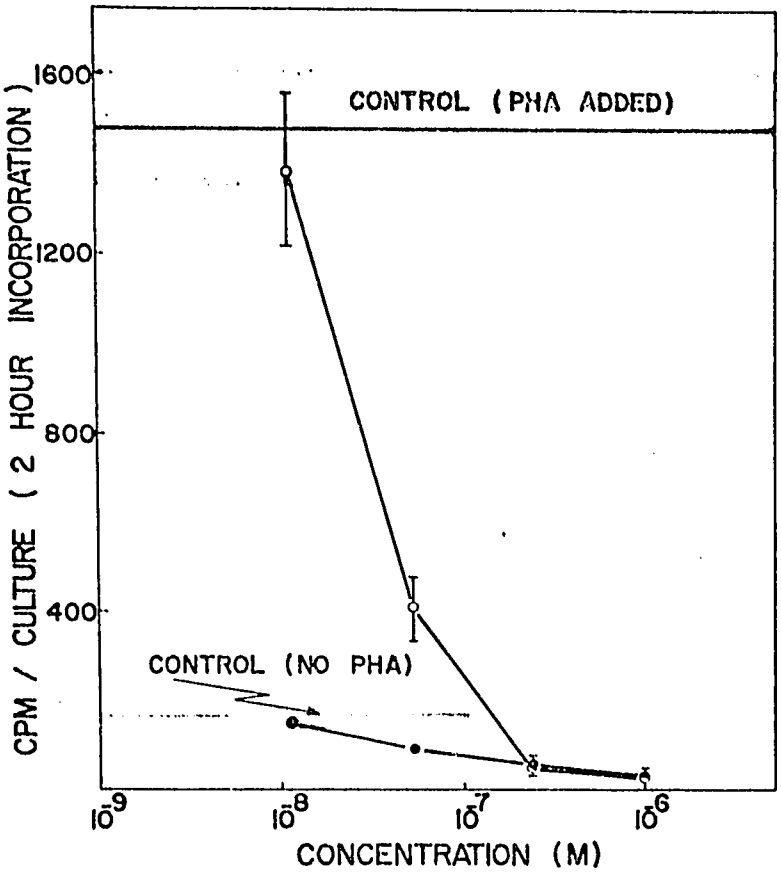


Fig. 3.2.11.

Effect of ouabain at different concentrations on the incorporation of H^3-uridine into unstimulated (solid circles) and PHA-stimulated (open circles) purified lymphocytes. All cell cultures were prepared from blood samples obtained from the same human donor, and were tested at the same time.

Ordinate shows uptake of tracer into TCA-precipitable material after a 2 hour period of incubation. Abscissa gives the molar concentration of ouabain.

Figure 3.2.10, the relative effect on cultures for many individuals is given. In Figure 3.2.11, the effects of different ouabain doses are shown using 'purified' lymphocyte cultures, with and without PHA-induced stimulation.

When the effect of ouabain was measured in the absence of PHA, a smaller decrease in ^{3}H-uridine incorporation was observed to occur at the same ouabain concentrations; however, the maximal levels of incorporation could not be reduced below 25-50% of the control values. Thus, the increment of ^{3}H-uridine incorporation induced by PHA was extremely sensitive to ouabain; the residual incorporation following ouabain treatment of PHA-activated cells was quantitatively about the same as the residual incorporation of the cells which had not been treated with PHA. This is shown graphically in Figure 3.2.12, in which results from some leukemic patients are also included. This figure shows the degree of inhibition of RNA synthesis caused by ouabain in cultures from different subjects, in relation to the degree of stimulation caused by PHA in parallel cultures.

3.2.3.2. Effective Concentrations of Ouabain

The effects of different concentrations of ouabain (present for 24 hours) on RNA, DNA, and protein synthesis in transforming lymphocytes are plotted together in Figure 3.2.13.

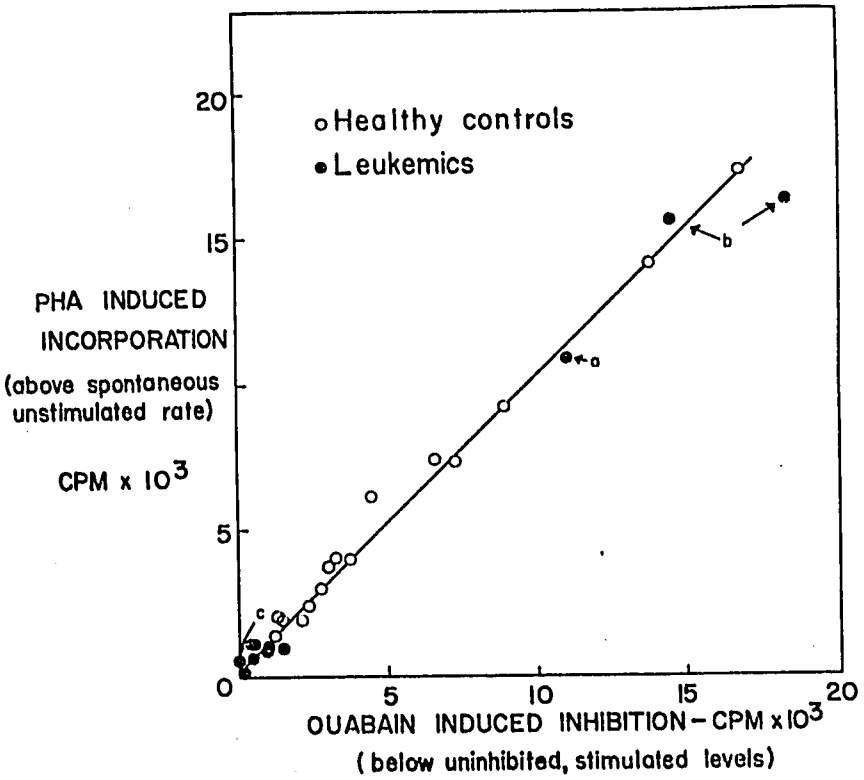


Fig. 3.2.12.

PHA-stimulation of H^3-uridine incorporation, plotted against the inhibition caused by ouabain, in duplicate cultures obtained from healthy (open circles) and leukemic (solid circles) subjects.

Line was fitted by the method of least squares.

Patient 'a' had chronic myeloid leukemia.

Patient 'b' had chronic lymphocytic leukemia, and the test was repeated twice a month apart.

Patient 'c' had acute myelomonocytic leukemia.

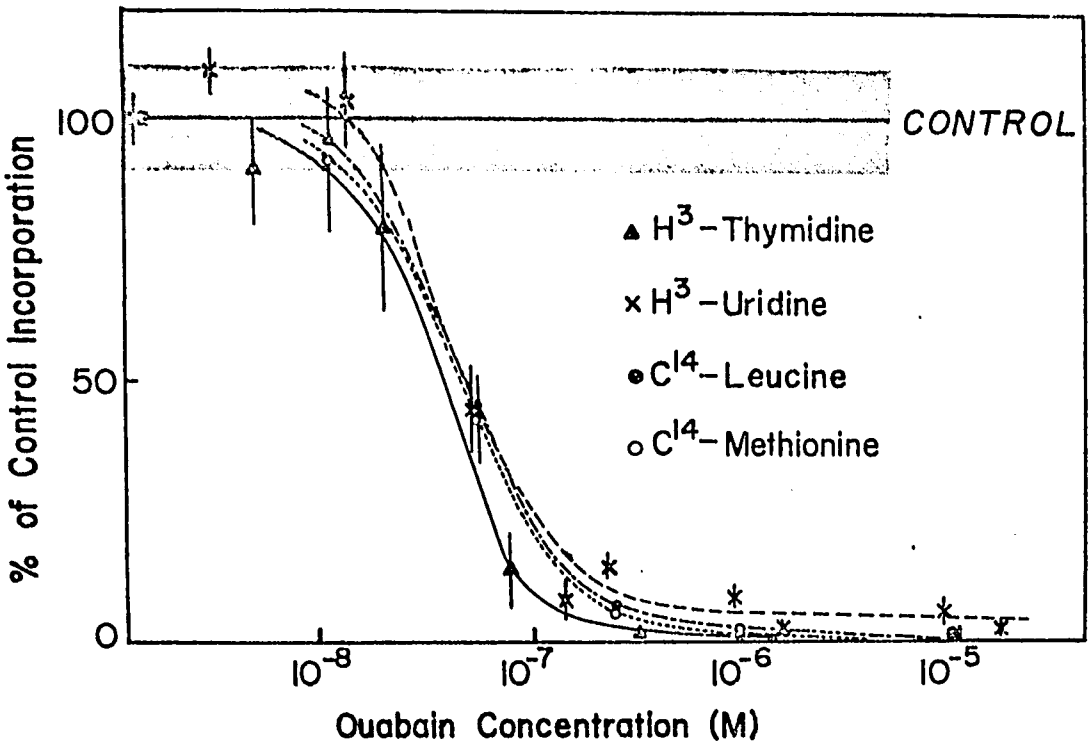


Fig. 3.2.13.

Effect of different ouabain concentrations on the synthesis of RNA, DNA and protein by PHA-stimulated lymphocytes.

PHA was added at the inititation of the cultures, and ouabain was added to obtain the indicated concentrations after one day of culture in the presence of PHA. H^3-uridine and thymidine and C^{14}-leucine and methionine were added after 2 days in culture (i.e., after one day in the presence of ouabain). After a further 24 hours in the presence of both PHA and ouabain, the incorporation of tracer into TCA-precipitable material was measured after a 2 hour incubation period. The labelled nucleosides and amino acids were added separaely to parallel cultures. Each point gives the mean and S.D. mean of 4 - 6 cultures.

The curves are remarkably similar; in all three cases, maximal inhibition occurred at concentrations between 2-5 x 10^{-7}M, 50% inhibition being observed at 3-5 x 10^{-8}M. At concentrations greater than 10^{-8}M, the glycoside caused a progressive decrease in the incorporation of these precursors. Concentrations greater than 2 x 10^{-7}M resulted in a pronounced inhibition of ^{3}H-uridine incorporation (95%) and an almost complete inhibition of ^{3}H-thymidine (>99%) incorporation. Similar dose-effect curves were obtained when the ouabain was given 1 or 3 days after PHA (Figure 3.2.14).

In the absence of ouabain, incorporation of ^{3}H-uridine was stimulated by a factor of ten to fifteen times after one day of treatment with PHA and by thirty to seventy times after two days' treatment. At low concentrations of ouabain (such as 5.5 x 10^{-8}M), RNA synthesis was reduced, but nevertheless, continued to increase with time (Fig. 3.2.15).

3.2.3.3. Time Required for Inhibitory Action to Become Manifest

Nucleic acid synthesis was not significantly reduced during the first two hours of incubation with ouabain (Fig. 3.2.9 and 3.2.16). A variable degree of inhibition was observed after 4 hours in the presence of ouabain. In some cases, there was a pronounced fall, as seen in Figure 3.2.9. Use of an expanded time scale as in Figure 3.2.16, shows that in the case of nucleic acid synthesis, there was a lag of approximately two hours before

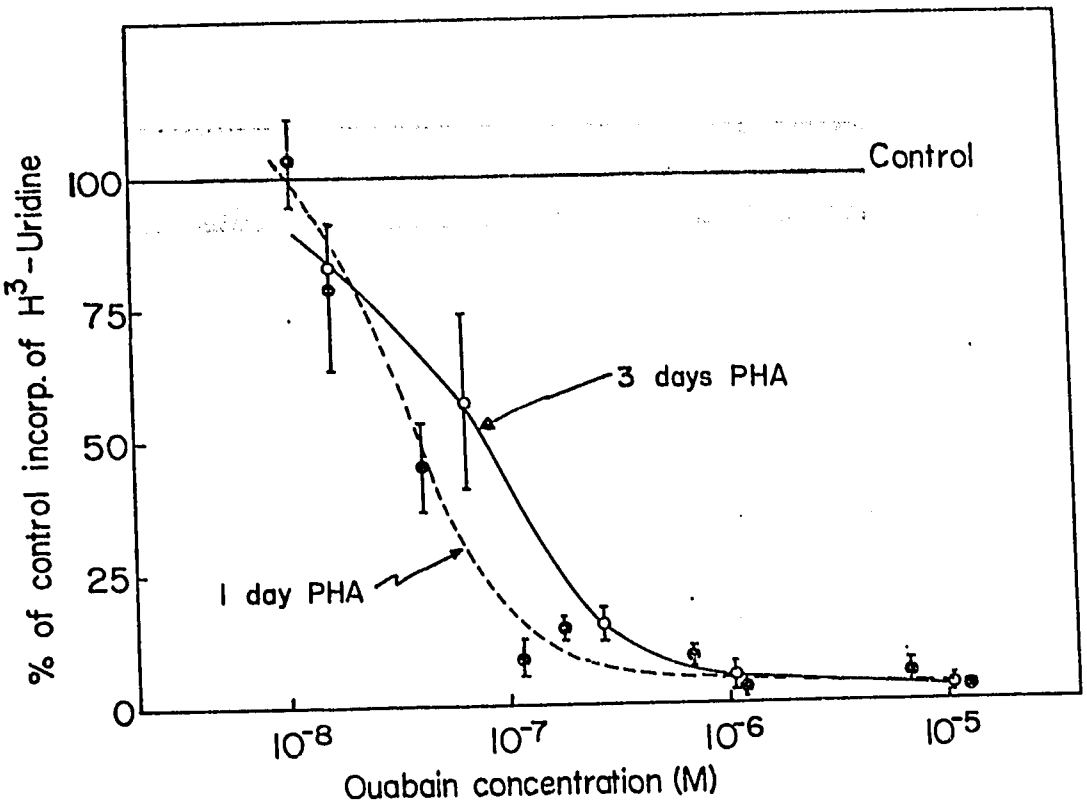


Fig. 3.2.14.

Comparison of the effect of ouabain on RNA synthesis during the first day of PHA administration, with its effect when blastogenesis was advanced after 2 days of PHA.

In the former case, ouabain at varying concentrations was given at the same time as PHA, at the initiation of the cultures, and measurement of the incorporation of H^3-thymidine carried out after one day.

In the latter case, ouabain at varying concentrations was given after 2 days of culture with PHA, and incorporation of H^3-uridine was measured on the third day.

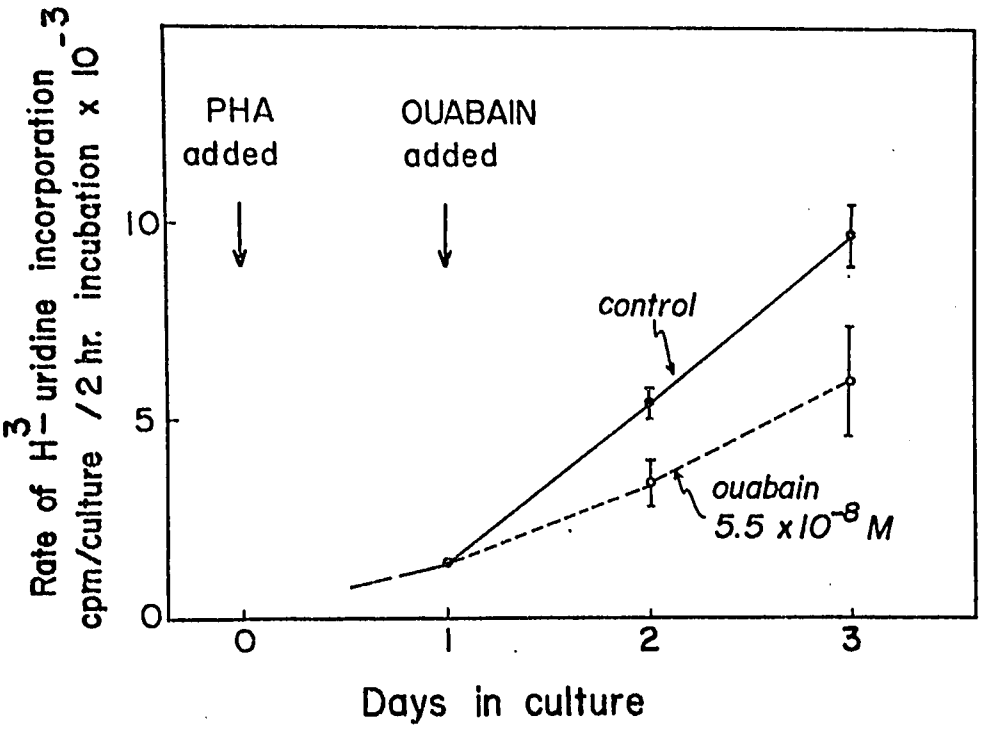


Fig. 3.2.15.

Effect of a relatively low concentration of ouabain on RNA synthesis of PHA-stimulated lymphocytes, as measured by the incorporation of H^3-uridine over a 2 hour incubation period. By 'low concentration' is meant a concentration of ouabain that does not cause full inhibition of RNA synthesis.

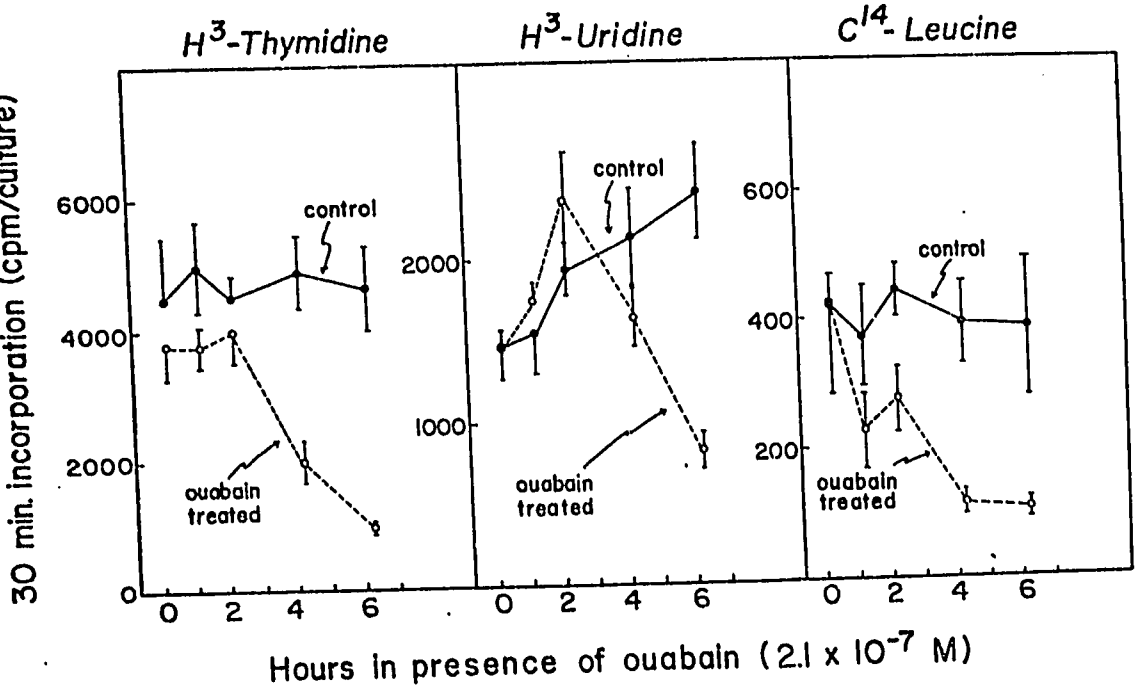


Fig. 3.2.16.

Time required for ouabain to cause inhibition of RNA, DNA and protein synthesis in PHA-stimulated cultures

Labelled nucleosides and amino-acid were incubated with the cultures for 30 minute pulses before measuring incorporation into TCA-precipitable material. The leukocytes were cultured in the presence of PHA for 2 days before addition of ouabain at a concentration of 2.1 x 10^{-7}M.

the inhibitory effect became apparent. Inhibition of protein synthesis appeared to be somewhat more rapid and was observed during the first hour of ouabain administration.

3.2.3.4. Effect of Potassium on the Inhibition Caused by Ouabain

In order to test the hypothesis that ouabain caused its effect by inhibiting metabolically active ion transport, isotonic KCl was added to leukocyte cultures to give extracellular potassium concentrations above normal values. The ouabain concentrations used were $1.7 - 2.1 \times 10^{-7}$M, which caused a marked depression of ^{3}H-uridine incorporation at PHA concentrations of 0.2 mg/ml.

Figure 3.2.1.7 shows that the inhibition by ouabain of uridine and thymidine incorporation was reduced or prevented at higher potassium concentrations. This effect was rather like that observed by Lubin (1967) with respect to the effect of potassium on the inhibition of protein synthesis in sarcoma cells caused by amphotericin B.

The quantitative relationship between the concentrations of ouabain and potassium required to inhibit transformation was therefore examined. As shown in Figure 3.2.18, the concentration of ouabain required to cause a given degree of inhibition of DNA synthesis was increased at higher potassium levels. In these experiments, the controls and treated cultures were both given

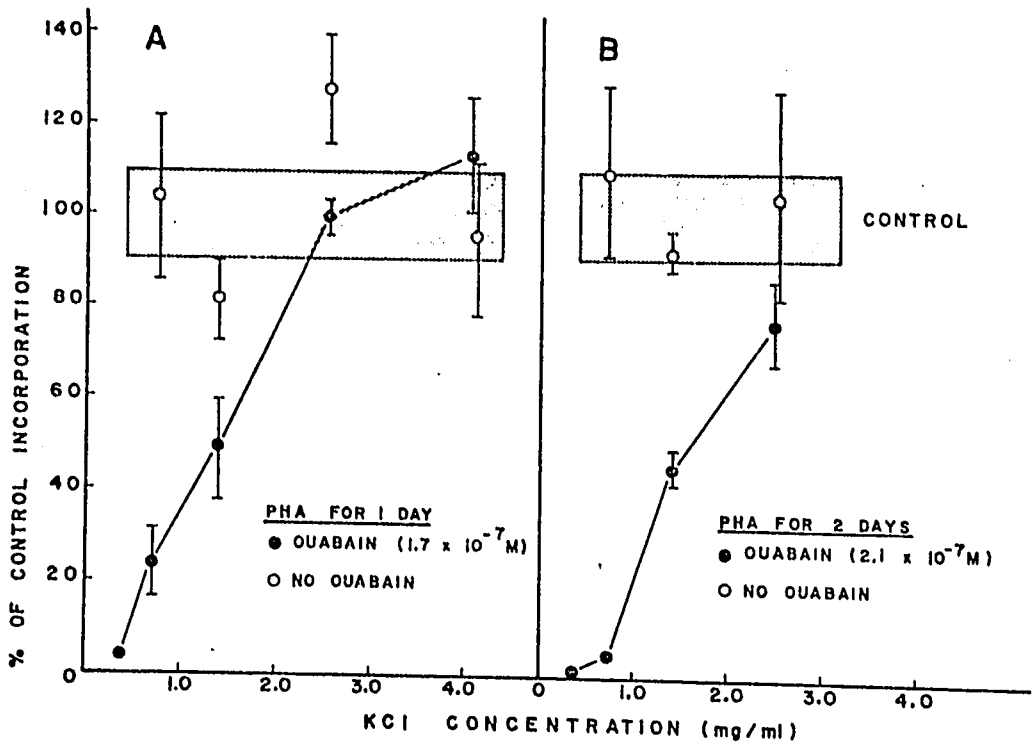


Fig. 3.2.17.

Effect of increasing the potassium concentration in the culture medium on the inhibition of RNA and DNA synthesis induced by ouabain.

A. H^3-uridine incorporation. PHA (20 µg/ml) was added after one day in culture. Isotonic KCl added 2 hours after PHA, and ouabain at the concentration indicated was added ½hour after KCl administration.

B. H^3-thymidine incorporation. PHA (20 µg/ml) was added after one day in culture. Isotonic KCl was added after 1 day of culture with PHA. Ouabain was given ½ hour after KCl administration.

Control cultures were treated with the same amounts of KCl but were not given ouabain. Each point is mean of 4 duplicate cultures.

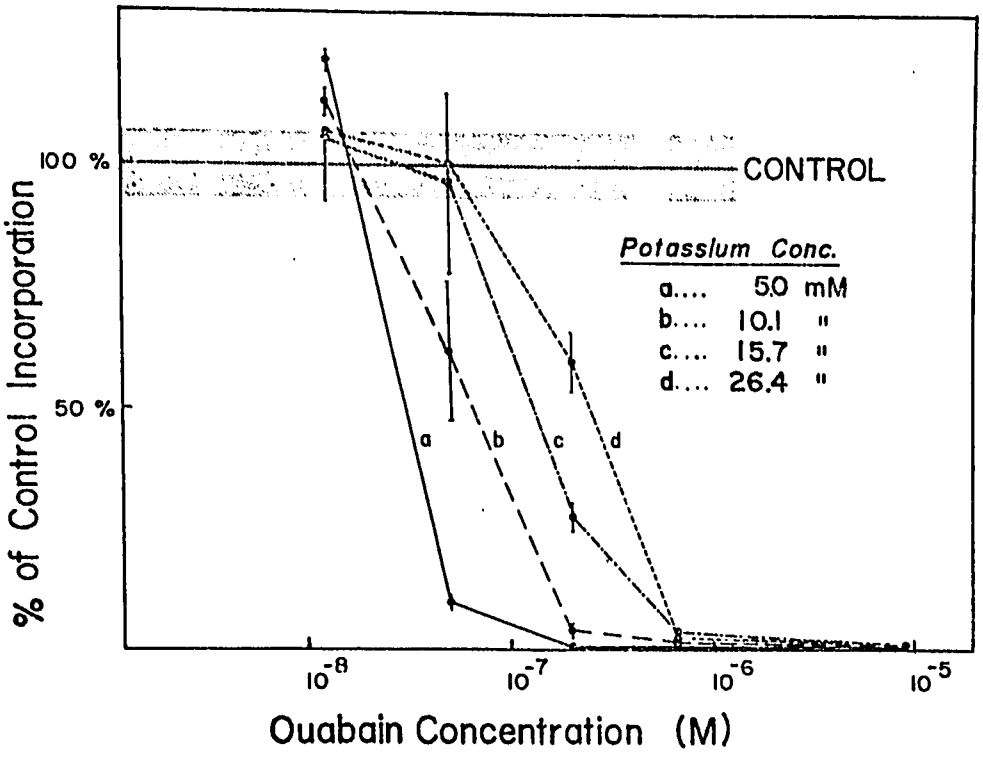


Fig. 3.2.18.

Effect of ouabain on DNA synthesis of PHA-treated lymphocytes at different levels of potassium in the culture medium.

PHA was added at the time of initiating the culture, and ouabain was given after one day in 0.06 ml aliquots per 3 ml culture. The desired volumes of isotonic KCl were added ½ hour after ouabain administration. The medium of all cultures including controls were brought to the same degree of dilution by the further addition of appropriate amounts of isotonic NaCl. The total volume of combined isotonic KCl and NaCl added to each culture was 0.5 ml.

The ordinate represents the 2 hour incorporation of H^3-thymidine by ouabain-treated cultures, divided by the degree of incorporation by controls, for each potassium concentration. The different potassium levels used did not significantly affect the 2 hour H^3-thymidine incorporation of the control cultures.

Incorporation was measured 1 day after the addition of ouabain.

various amounts of potassium, which was added as an isotonic solution of KCl in NaCl, and adjusted so as to cause the same degree of medium dilution in both ouabain-treated and control cultures.

When the concentrations of the two agents, ouabain and potassium, together required to cause 50% inhibition were examined, an approximate proportionality was observed, as shown in Figure 3.2.19. The slope of the line drawn through the points indicates that 1.05×10^5 equiv. of K^+ were required to overcome by 50% the inhibition of DNA synthesis caused per mole of ouabain over the concentrated range tested.

3.2.3.5. Reversibility of Ouabain Action

The reversibility of the inhibitory effect of ouabain on lymphocytes was tested in two types of experiments. The principle behind these experiments was to incubate lymphocytes, with or without PHA, and for various periods of time in the presence of ouabain, and after changing to fresh medium, to test their stimulatory response to PHA by the amount of H^3-uridine incorporated in a 2-hour period. These results are illustrated in Figure 3.2.20.

(i) The cells were incubated in the presence of both PHA and ouabain (2.2×10^{-7}M) for 1, 2, or 3 days (upper graph, Fig. 3.2.20). The medium was then removed, the cells washed

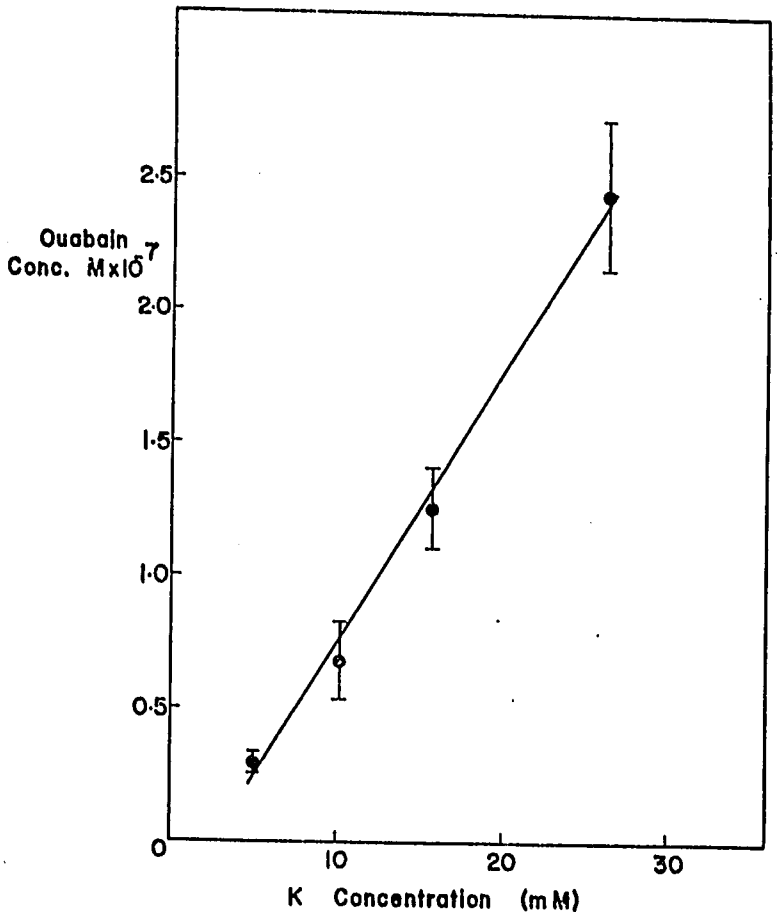


Fig. 3.2.19.

Ouabain and potassium concentrations which, when present at the same time, resulted in 50% inhibition of DNA synthesis. The values used are from the same experiment illustrated in Fig. 3.2.18. Line is plotted by least squares, and indicates that 1.05×10^5 equiv. of K^+ are required to overcome by 50% the inhibition of DNA synthesis caused per mole of ouabain, over the concentration range tested.

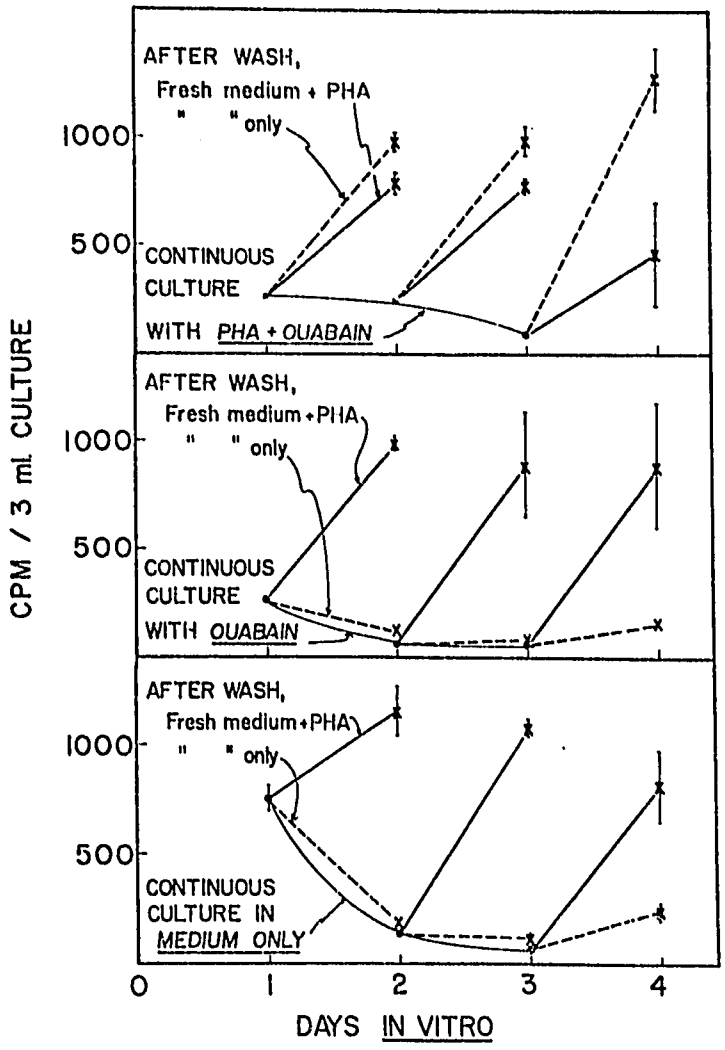


Fig. 3.2.20.

Reversibility of inhibition due to ouabain, measured by responsiveness of the cells to PHA after washing the cultures.

At daily intervals, the cells were washed in medium-199 and recultured in fresh medium only (dotted line) or in both fresh medium plus PHA (heavy line) for one day. The 2 hour incorporation of H^3-uridine was then measured.

(a) Cells cultured in both PHA and ouabain (2.2×10^{-7}M) for up to 3 days. At intervals of 1,2 or 3 days, cells were washed and responsiveness to PHA tested.

(b) Cells cultured in the presence of ouabain but no PHA; then same procedure as in (a).

(c) Cells cultured without PHA or ouabain; same procedure as in (a).

twice in Medium-199 and finally resuspended in either fresh Medium-1-9 (plus autologous plasma) or in medium plus PHA. The 2-hour incorporation of H^3-uridine was measured as a parameter of stimulation.

When the cells were reincubated in fresh medium only, there was a marked stimulation of H^3-uridine incorporation, equivalent to that of control cultures (lower graph, Fig. 3.2.2$_0$). Surprisingly, in this experiment, addition of fresh medium alone, after removing the old medium, was somewhat more effective than the addition of fresh medium plus PHA.

(ii) The cells were incubated in medium containing ouabain (2.2×10^{-7}M) but no PHA for 1, 2, or 3 days. The same procedure was used as described in the preceding paragraph. When the cells were incubated in fresh medium only (middle graph of Fig. 3.2.2$_0$), a marked stimulation of H^3-uridine incorporation occurred, equivalent to that of the control cultures (lower graph of Fig. 3.2.2$_0$), which were treated in the same way but without ouabain.

It might be noted that the base-line of H^3-uridine incorporation of unstimulated cultures gradually decreased with time, as seen in Figure 3.2.2$_0$ (lower graph), and was inhibited by ouabain to 40-50% of control levels (compare with middle graph) similar to the values given in Table 3.2.1 (first row, column B).

Treatment (A)		Healthy Subjects (B)	Chronic Lymphocytic Leukemia (C)	Acute Leukemia (D)	Chronic Myeloid Leukemia (E)
Treated with ouabain for one day (8.8×10^{-7}M) (Cells without ouabain - 100%	% H^3 -uridine incorporation	46 ± 9% mean of 7 cases	54 ± 7% mean of 5 cases	91 ± 20% 91 ± 12% 59 ± 20%	89 ± 15% 98 ± 13%
	% H^3 -thymidine incorporation	Insignificant incorporation by control and treated cells	Insignificant incorporation by control and treated cells	57 ± 23% 41 ± 10% See also section 3.5.5.1	64 ± 9% 66 ± 16%
Degree of stimulation* after 1 or 2 days with PHA (Cells without PHA - 1.0)	After 1 day	7.1 ± 1.1 mean of 10 cases	1.5 2.3 3.1	3.1 1.0	not done
	after 2 days	35.5 ± 7.9 mean of 10 cases	1.5 19.2, 23.7 †	not done	3.9 1.4

*

* $\frac{H^3\text{-uridine incorp. after PHA}}{H3\text{-uridine incorp.. of untreated cells}}$

† same patient repeated after one month

Table 3.2.1. Effect of PHA and/or Ouabain on Spontaneous Nucleic Acid Synthesis in Normal and Leukemic Leucocyte Cultures

These results indicate that ouabain-treated cells remain potentially capable of responding to PHA. They also suggest that some initial effect of PHA must have been irreversible and was not prevented, but was masked by the presence of ouabain; increased RNA synthesis only occurred after the ouabain was washed out.

3.2.3.6. Effect of Ouabain on the Mixed Leukocyte Reaction

From the results obtained with PHA-stimulated lymphocytes, it was important to know whether the action of ouabain was to interfere with changes specifically induced by and requiring the presence of PHA, or whether it acted instead on the subsequent process of transformation initiated by this mitogen, but not specific to its interaction with the cell.

Transformation is known to occur in mixed cultures of leukocytes derived from unrelated individuals in the absence of PHA, or in the presence of specific antigens (Section 1.4.2). If ouabain were to inhibit one of these reactions, its effect would necessarily be independent of the specific interaction of PHA with the cell.

In prior observations of the mixed leukocyte reaction under the conditions used in this study, RNA and DNA synthesis generally became maximal about five days after the initiation of mixed cultures (Fig. 3.1.12). Administration of ouabain was therefore carried out on the fourth day of mixed culture, and

measurements of H^3-thymidine incorporation carried out on the fifth day. As shown in Figure 3.2.2[1] (compare with Fig. 3.2.1[3]), ouabain inhibited DNA synthesis to the same extent and at precisely the same concentrations as were effective in PHA-stimulated cells.

3.2.3.7. Autoradiographic Studies of Ouabain-Treated Leukocytes

Autoradiographic analyses were carried out on the PHA-induced 'blast' cells to determine whether the uptake of H^3-uridine was prevented in all cells by the glycoside, or whether a fraction of cells remained unaffected. It was also important to know whether there was uptake of RNA precursor by cells other than lymphocytes and 'blasts' which might explain the residual incorporation observed at strongly inhibiting concentrations of ouabain.

Pulse doses of H^3-uridine were given to cultures incubated with PHA for two days. After 20 minutes, the tracer was removed, the cells washed twice and then reincubated with fresh Medium-199 (plus 15% autologous plasma) for 0 to $1\frac{1}{2}$ hours. This was followed by autoradiography as described in Section 2.3.3.

Tritium labelling of blast cells was seen at first in the nucleus, with concentration over the nucleolar area. About an hour after administration of the tracer, grains appeared over the cytoplasm which became heavily labelled during the second hour (see Section 3.1.5.2).

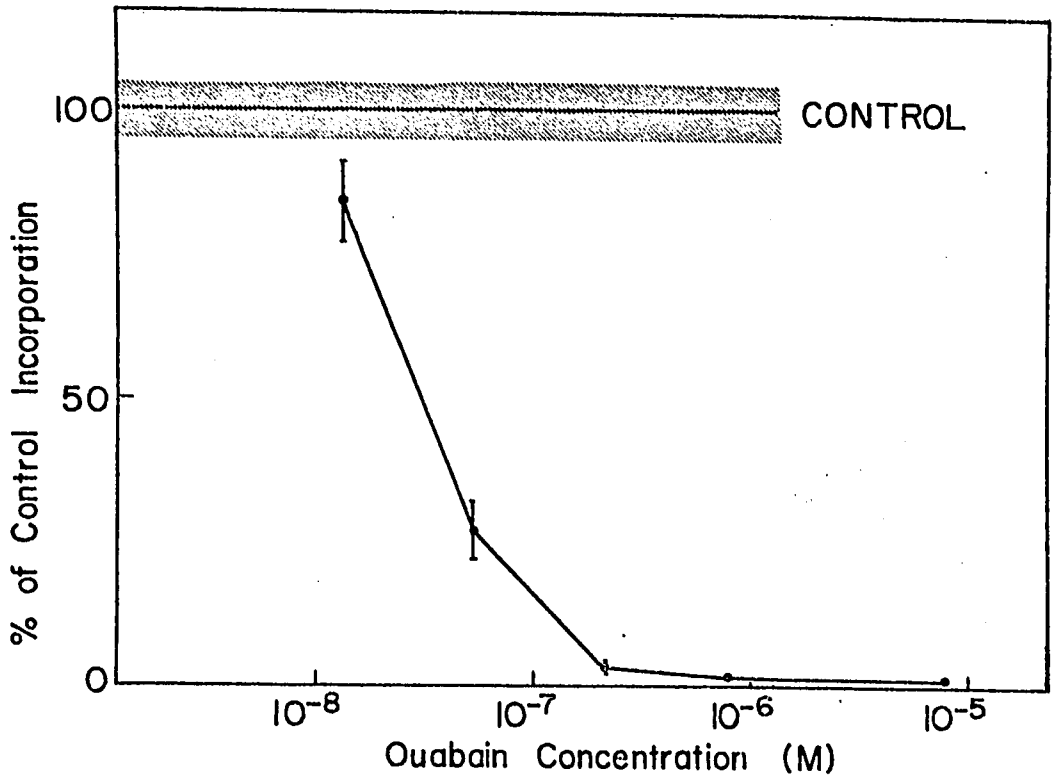


Fig. 3.2.21.

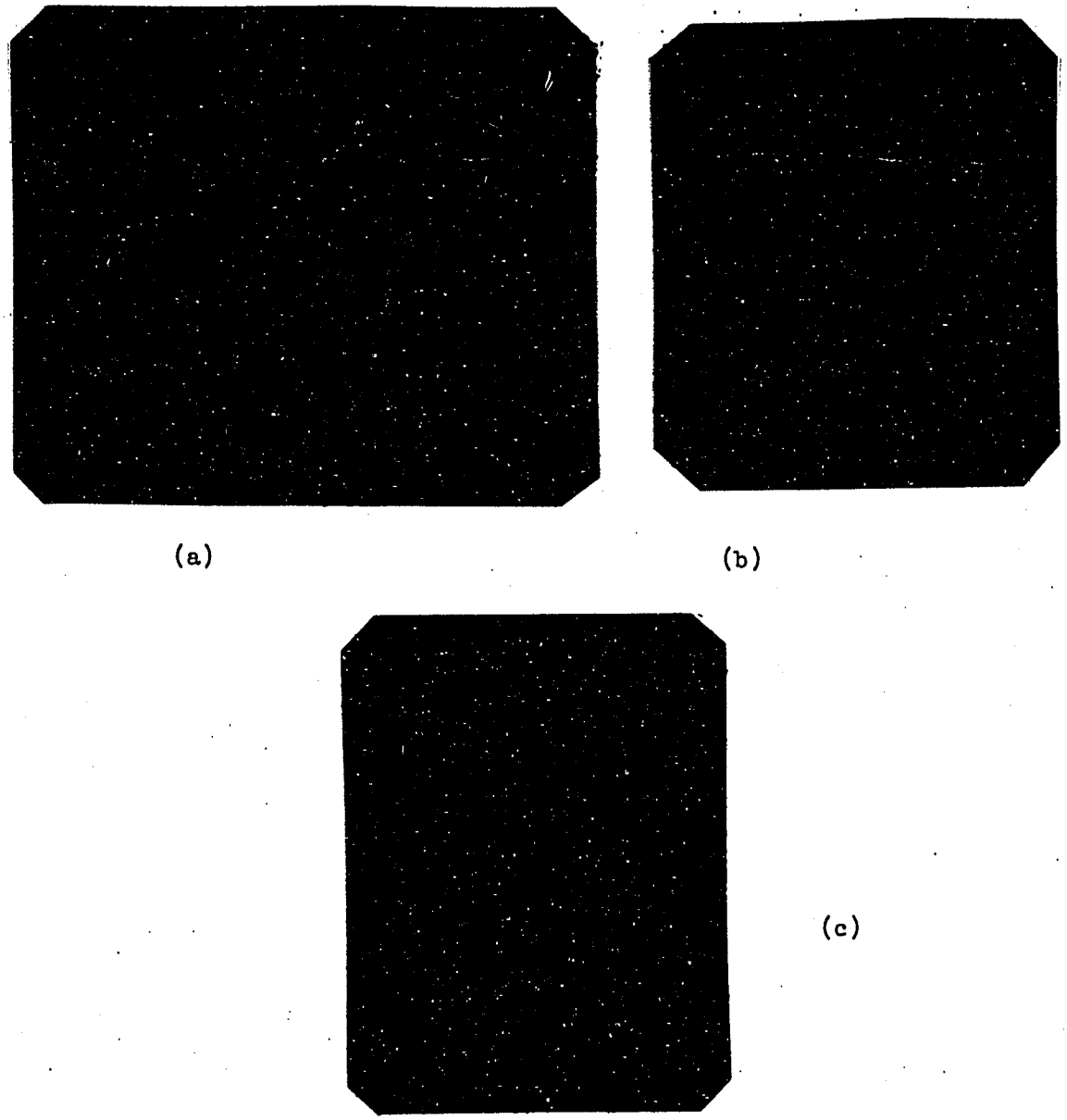
Effect of ouabain on DNA synthesis in the mixed leukocyte response. Leucocytes from unrelated donors were cultured together for 4 days, when ouabain was added at various concentrations. After one more day of culture, the incorporation of H^3-thymidine during a 2 hour incubation period was measured.

Other leukocyte cultures were also treated with PHA for a total of two days, but in addition, ouabain was given at various concentrations during the final 24 hours of incubation. H^3-uridine was then administered in 20-minute pulse doses. The cells were then washed twice in medium and reincubated for a further 1½ hours in fresh medium containing autologous plasma, followed by autoradiography.

The results are illustrated in Figure 3.2.22. The glycoside, at concentrations of 5.2×10^{-8}M, caused marked reduction in cytoplasmic labelling and a partial inhibition of nuclear labelling. At this dose, an inhibitory effect was observed uniformly with all blast cells in the culture. At higher ouabain doses, such as 2.2×10^{-7}M, labelling of the nucleus became confined to a few grains, which tended to concentrate over unstained areas between the discrete nuclear bodies already described. Labelling of the cytoplasm was reduced to background levels.

3.2.3.8. Acid Soluble 'Unbound' Precursor in Transforming Lymphocytes

The inhibition of incorporation of labelled precursors in the presence of ouabain could have been due to (a) inhibition at the level of macromolecular synthesis; (b) metabolic changes involving the phosphorylation of precursors prior to incorporation,



(a) (b)

(c)

Fig. 3.2.22.

Effect of ouabain on the appearance of label in the nucleus and cytoplasm of PHA-induced blast cells. Cultures were treated with PHA for 2 days and with PHA plus ouabain for one more day. H^3-uridine was given as a pulse dose for 20 min, and the cells reincubated in non-radioactive medium for a further 1½ hours. Fixed in acetic acid : ethanol. Autoradiographs prepared with NTB-3 Kodak emulsion. One week exposure. Stained with Giemsa through emulsion. x 1250

(a) Control - no ouabain.
(b) Ouabain treated (5.2×10^{-8}M)
(c) Ouabain treated (8.4×10^{-7}M)

as a result of ouabain induced alteration in the levels of certain intracellular factors; or (c) might have resulted from an inhibition of the movement of the radioactive tracers across the cell membrane. It was therefore desirable to separate, if possible, the effect of ouabain on the uptake of precursors into the cells from an effect of ouabain on synthetic activity.

Studies on the uptake of H^3-uridine into stimulated leukocyte cultures have already been described (Section 3.1.4.4), showing that when the cells were incubated with the labelled precursor, but not subsequently treated with 5% trichloracetic acid the content of tracer with the cells was higher than after TCA administration. The difference between the TCA treated and untreated values was considered to represent the amount of 'unbound' or acid-soluble tracer in the cell.

The uptake of H^3-uridine into the acid soluble pool can be differentiated from its incorporation into acid-insoluble material (i.e., RNA) by the use of actinomycin D. Figure $3.2.2_3$ shows that this drug (at 4.4×10^{-7}M) almost completely inhibited the incorporation of H^3-uridine into RNA, without affecting its uptake into the acid soluble pool.

When ouabain was given over a period of six hours, it was found to cause partial inhibition of RNA synthesis (upper graph on right, Figure $3.2.2_3$) without causing any noticeable effect on

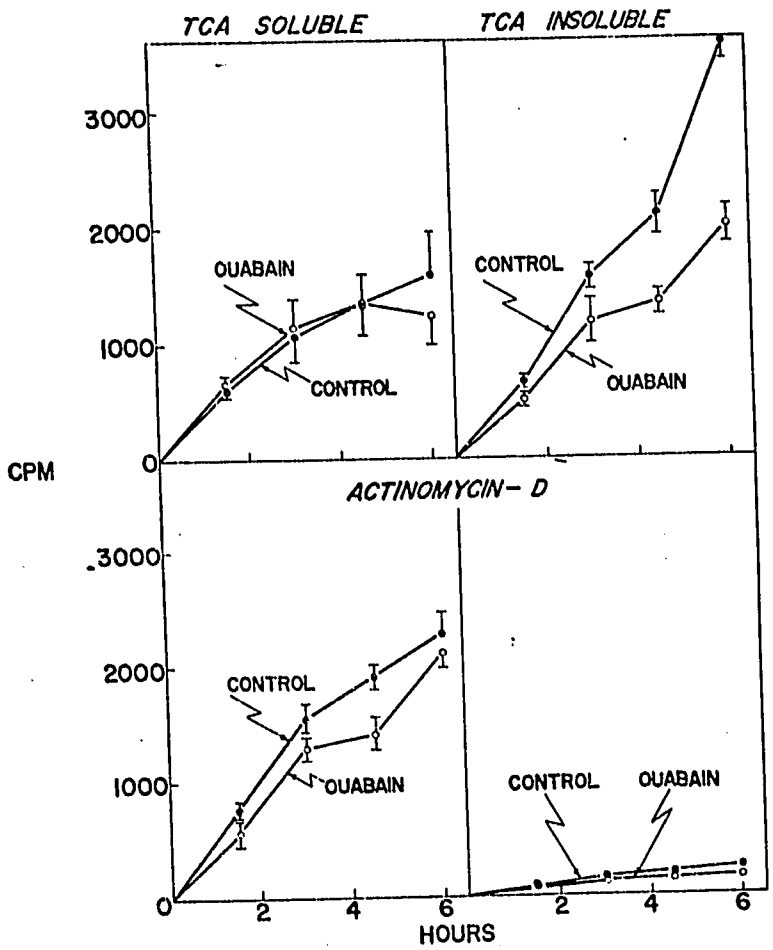


Fig. 3.2.23.

Effect of ouabain on the uptake of H^3-uridine into the TCA-soluble pool (left side), and on its incorporation into TCA-insoluble material (right side), of PHA-stimulated lymphocytes.

The graphs represent cumulative uptake or incorporation over a six hour period. Uptake into the TCA-soluble pool was measured from the difference between the amount of radioactivity taken up into non-TCA treated cultures and the amount taken up into TCA-precipitable material. The cells were cultured with PHA for 19 hours before addition of ouabain and/or actinomycin-D (4.4×10^{-7}).

the uptake of tracer into the acid soluble pool. Actinomycin-D treated cultures showed only a slight effect of the glycoside on uptake into the acid soluble pool. As shown in Figure 3.2.25, the effect of ouabain on the pool of leucine was not observable, with experimental error, over a 6-hour period, in contrast to the inhibitory effect of the glycoside on protein synthesis.

When ouabain was given over a period of 24 hours, it had pronounced inhibitory effect on RNA synthesis, but did not have any obvious effect on the size of the acid-soluble pool (Fig. 3.2.24).

3.2.3.9. Effect of Ouabain on Nucleic Acid Synthesis in Monkey Kidney Cells

It was desirable, in view of the results on the effect of ouabain on transforming lymphocytes, to determine whether the sensitivity of mitotic activity and macromolecular synthesis transforming lymphocytes to ouabain was also demonstrable using other mammalian cells. The only reference to this was found in a report by Mayhew and Levinson (1968), who observed that the glycoside prevented the onset of mitosis in Ehrlich Ascites cells, at the relatively high concentrations of 10^{-4}M.

In the present study, the response of monkey kidney cells (BSC-1) to the glycoside was examined. Synthesis of DNA was completely unaffected by concentrations of ouabain up to 10^{-4}M,

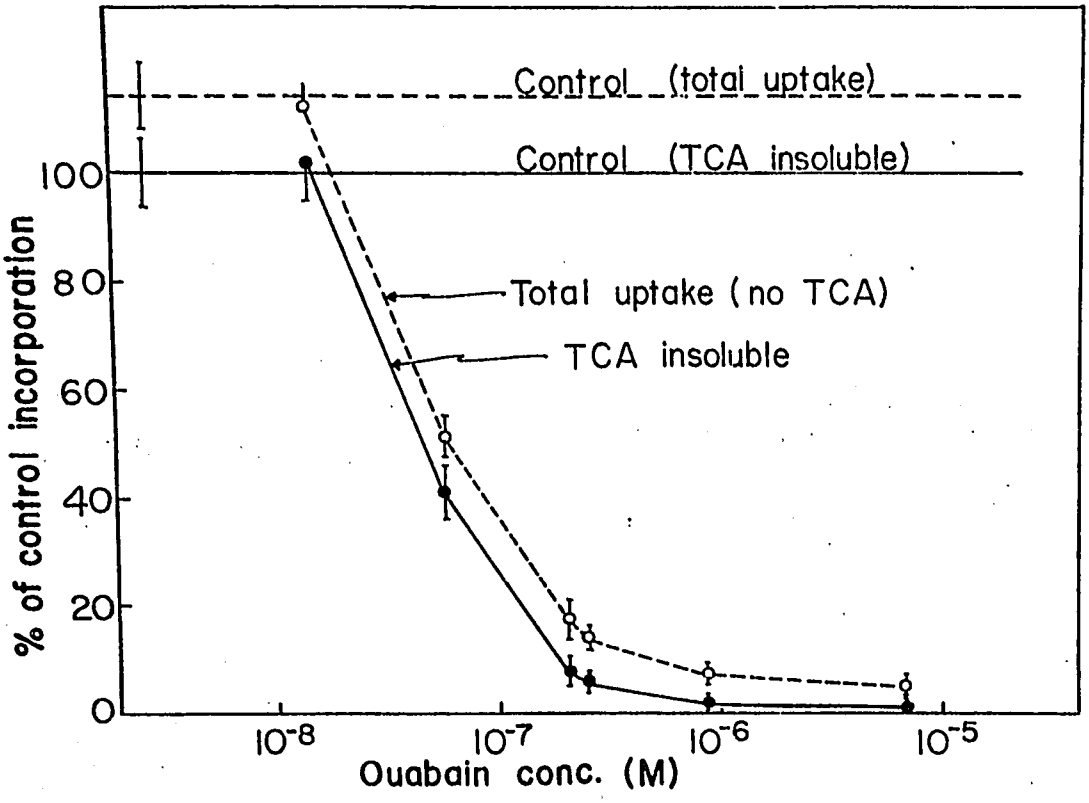


Fig. 3.2.24.

Effect of ouabain, given for a 24 hour period, on the subsequent 2 hour uptake of H^3-uridine into TCA-soluble and TCA-insoluble fractions. The cultures had been treated with PHA for a period of 2 days before culture in the presence of both PHA and ouabain. 'Total uptake' means the radioactive content of the cells after washing them with phosphate-buffered saline, but without treatment with TCA.

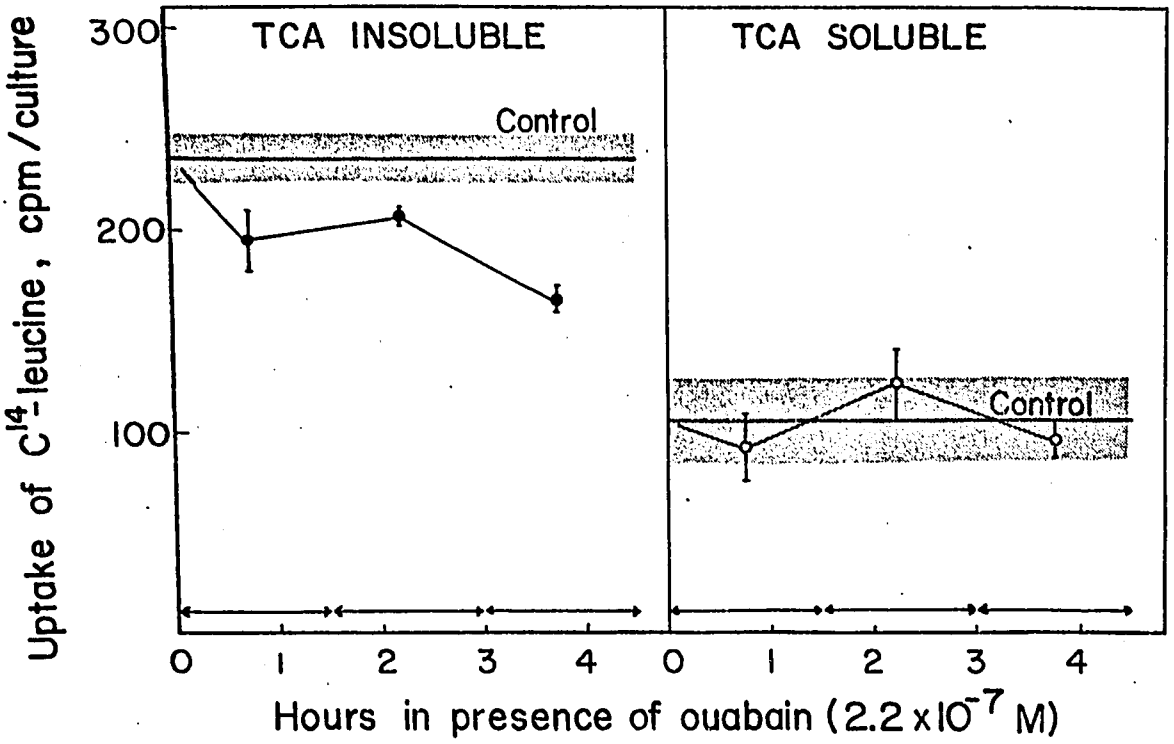


Fig. 3.2.25.

Effect of ouabain, present for a period of up to 4½ hours, on the uptake of C^{14}-1-L-leucine into TCA-soluble and TCA-insoluble fractions of PHA-stimulated cells.

Calculation of uptake into TCA-soluble fractions was done as stated in Fig. 3.2.23. Each point represents uptake results during a period of incubation with the precursor shown by the horizontal arrows. The cells were cultured for a period of one day with PHA before adding ouabain at the concentration shown.

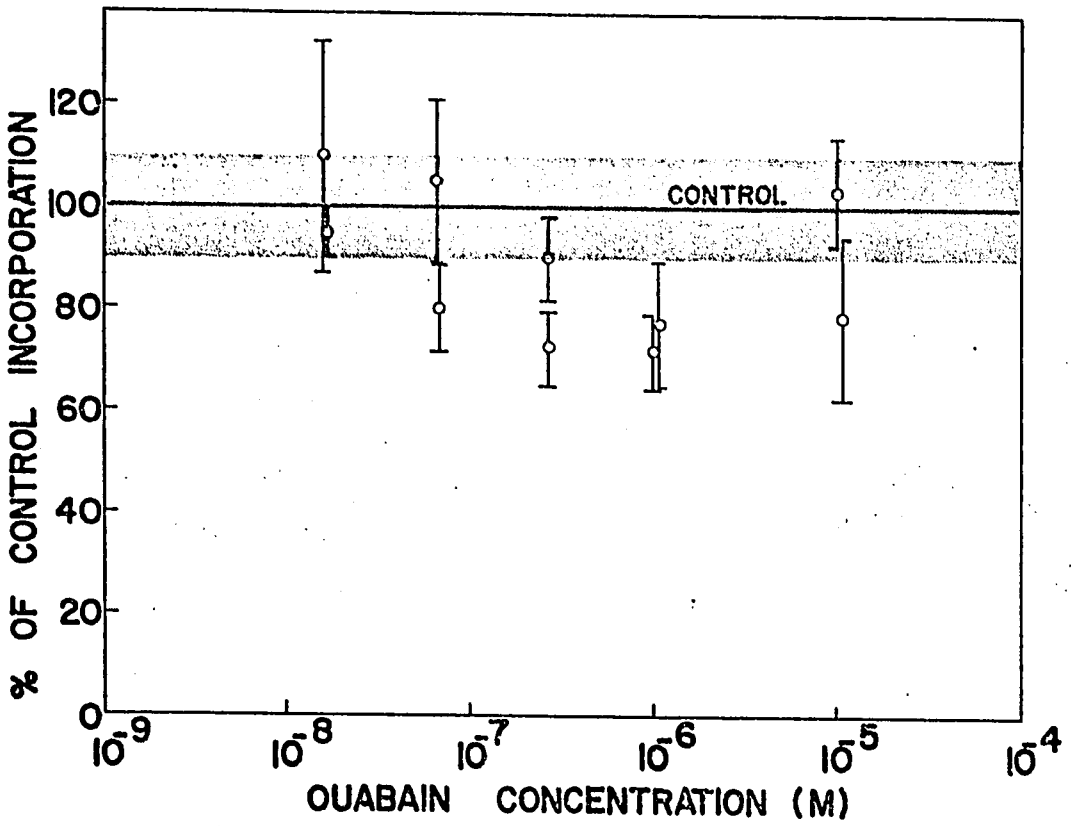


Fig. 3.2.26.

Effect of ouabain at various doses on DNA synthesis of monkey kidney BSC-1 cells. Ouabain was added at the start of initiation of the culture, and measurements of the incorporation of H^3-thymidine over a 2 hour period of incubation were carried out 24 hours later.

present for 24 hours, as shown in Figure 3.2.26. In addition, no gross morphological changes were seen following administration of the glycoside at the doses used.

3.2.3.10. SUMMARY OF CHAPTER

1. The syntheses of RNA, DNA and protein by PHA-stimulated human lymphocytes were each inhibited to almost the same extent by ouabain at concentrations greater than 10^{-8}M.

2. The cells were equally sensitive soon or late after PHA administration.

3. Inhibition of nucleic acid synthesis was delayed by about two hours, but inhibition of protein synthesis took place more rapidly.

4. The action of ouabain on unstimulated cells at low concentrations (about 2×10^{-7}M) appeared to be reversible.

5. In cultures obtained from both healthy and almost all leukemic donors, ouabain inhibited the increment of nucleic acid synthesis induced by PHA. However, spontaneous RNA synthesis was only partly suppressed by ouabain.

6. By autoradiography using H^3-uridine, it was shown that the glycoside uniformly reduced or prevented RNA synthesis in blastoid cells throughout the culture, and not just in certain sensitive cells.

7. Excess potassium in the culture medium prevented the effect of ouabain; about 1.05×10^5 equiv. of K^+ per mole of ouabain together caused 50% inhibition of DNA synthesis.

8. The glycoside inhibited DNA synthesis in the mixed lymphocyte reaction at the same concentrations and to the same extent as it did PHA stimulation, indicating that its inhibitory effect on lymphocyte transformation was not specific to PHA activation.

9. Clumping and redistribution of nuclear chromatin were the major morphological changes observed to occur in the nuclei of lymphocytes and blasts caused by adding relatively low doses of ouabain to the culture medium. At higher doses, pronounced cell disintegration was observed.

10. The acid soluble pools of H^3-uridine and C^{14}-leucine were unaffected by ouabain, within experimental error, although synthetic activity as measured by the incorporation of these tracers into nucleic acid and protein was strongly inhibited by the same doses of the glycoside.

3.3. EFFECT OF CERTAIN PHARMACOLOGICAL AGENTS ON LYMPHOCYTE TRANSFORMATION

3.3.1. Introduction to Section

The investigation described in Chapter 3.2. characterized the sensitivity of the transforming lymphocyte to ouabain in vitro, and it was inferred from these results that the activity of the sodium-potassium dependent transport ATPase of the plasma membrane, which affects and is probably required for the transport of cations (and other substances) into and out of the cell, was an important determinant of lymphocyte transformation. It was thus desirable to test the effect on lymphocyte transformation of other agents known to inhibit the Na-K-ATPase; inhibition of transformation by these substances would provide further evidence in support of the hypothesis that adequate function of this transport-enzyme system was essential for transformation. The effects of compounds including erythrophleine, cassaine, N-ethylmaleimide (NEM) and p-chloromercurobenzoate (PCMB), known to inhibit the membrane of Na-K-ATPase, were therefore examined. Tetrodotoxin was also tested, as was valinomycin, since both agents are known to have profound effects on sodium and potassium metabolism on various cellular and organ systems.

The effect of ouabain on transforming lymphocytes was compared to the action of hydrocortisone and prednisolone, since the

steroid-like moiety of ouabain (see Fig. 1.9.1) suggested that the mechanism of its inhibiting effect might be similar to that of the adrenal steroids.

The action of ouabain might reflect a pronounced sensitivity of the cell to ATP availability and changes in oxidative metabolism, and for this reason, the effect of the metabolic inhibitors dinitrophenol and oligomycin were tested. These agents were also tested on the respiratory activity of the cells (see Chapter 3.5).

The sensitivity of lymphocyte transformation to various inhibitors of RNA, DNA and protein synthesis was examined; these included the effect of the folic acid antagonist, methotrexate, and the antibiotics actinomycin-D and cycloheximide.

Since the transformation of lymphocytes appeared to be dependent on membrane function, it was important to investigate whether the adenyl cyclase-cyclic AMP system was involved. Therefore, the effects of cyclic AMP and other adenosine nucleotides were tested.

In these studies, only the short-term effects of these agents (over a period of 1-2 days of culture) was generally tested and comprehensive analyses of their effects have not yet been done. Thus, the very interesting differential effect of some of these agents

on RNA and DNA synthesis in the lymphocyte in vitro system has not yet been followed up. The effects of the drugs described in this Chapter are, however, useful in the overall interpretation of the results.

3.3.2. Note on Methods

The methods used have already been described in general (Section 2). Very briefly, cells were obtained from venous blood after red cell sedimentation was assisted with dextran, and the leukocytes or separated lymphocytes subsequently cultured in Medium-199 fortified with 15% autologous plasma. PHA was added at the time of initiation of the culture. The substances whose effects were to be tested were generally added either at the time of culture initiation, or after one day in the presence of PHA.

Measurements of the incorporation of radioactive precursors (H^3-thymidine, H^3-uridine or C^{14}-leucine) were usually carried out during a 2-hour incubation period, one or two days after administration of the substance whose effect was to be examined. The test cell system used was that of the PHA-treated leukocyte culture; changes in DNA and RNA syntheses were generally used as criteria of drug effects. Further specific variations in technique are described where necessary in the appropriate subsection.

3.3.3. Erythrophleine Sulfate

The erythrophleum alkaloids have been reported to have a digitalis-like effect *in vivo*, and cause inhibition of the sodium-potassium dependent ATPase (see Section 1.9.3). Since these authors have reported the alkaloid to have an effectiveness of the same order of magnitude as that of ouabain, it was highly desirable to see whether this alkaloid would also inhibit lymphocyte transformation.

Erythrophleine sulfate was dissolved in water and subsequently administered to transforming lymphocytes, which had been cultured for the previous 17 hours in the presence of PHA. On the following day, the incorporation of tritiated thymidine and uridine were examined.

The results (see Fig. 3.3.1) indicated that erythrophleine strongly inhibited transformation; 50% inhibition of RNA synthesis was obtained at concentrations of the order of 3×10^{-8}M, but at somewhat reduced concentrations for DNA synthesis. On a molar basis, the cells were equally sensitive to this drug as they were to ouabain. In all cultures tested, the degree of inhibition of DNA synthesis was more pronounced than that of RNA synthesis.

3.3.4. Cassaine Base

This substance, also one of the erythrophleum alkaloids, (see Fig. 1.9.1) was tested for its effect on transforming

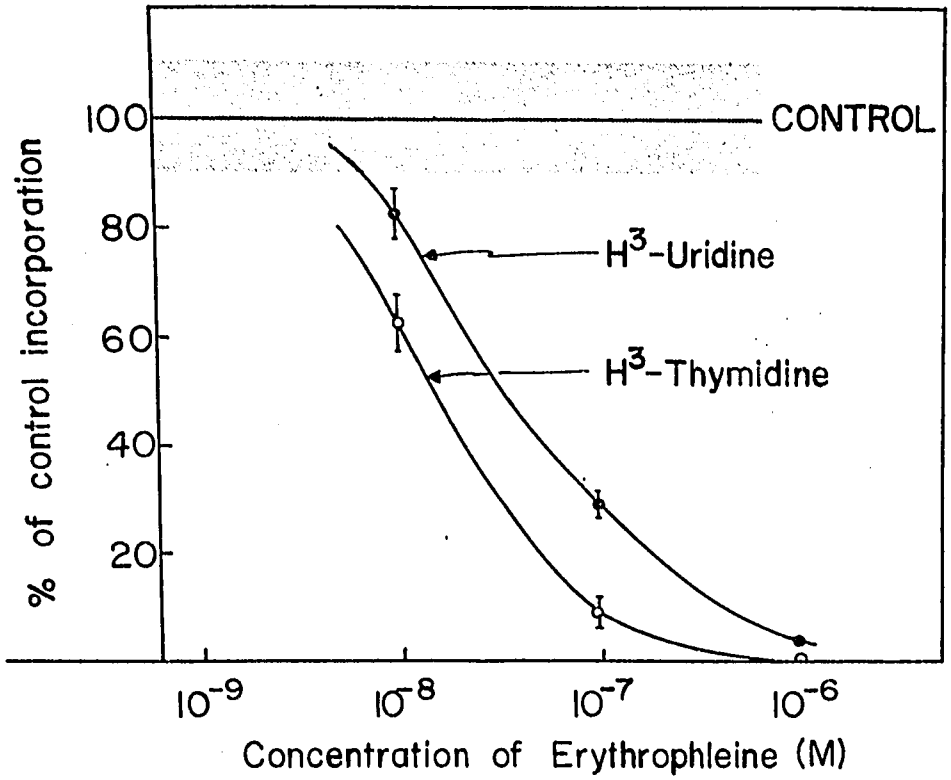


Fig. 3.3.1.

Effect of erythrophleine sulfate on RNA and DNA synthesis of PHA-stimulated lymphocytes.

The cells were cultured for one day in the presence of PHA. The compound was dissolved in water and added to the cultures. On the following day, measurements of the incorporation of H^3-thymidine and H^3-uridine into TCA-precipitable material during a 2 hour incubation period were carried out.

lymphocytes at the same time and under the same conditions as with erythrophleine sulfate. The inhibitory action of cassaine was found to be considerably less than that of erythrophleine on a molar basis; the differential sensitivity of RNA and DNA synthesis was again present, as shown in Figure 3.3.2. Inhibition of DNA synthesis was observed at the lowest concentrations used (10^{-8}M), but marked suppression of either DNA or RNA synthesis required concentrations greater than 10^{-6}M. It was therefore difficult to specify a value for the 50% inhibitory concentration of DNA synthesis since the dose-effect curve was rather flat (Fig. 3.3.2). In this case, 50% inhibition occurred at a concentration as low as 10^{-8}M; however, cells were inhibited to 30% of control values at 10^{-6}M. A small amount of H^3-uridine incorporation into RNA was noticed at doses as high as 10^{-4}M.

The response to cassaine was considerably different from that of the ouabain in that it took place over a much wider concentration range, and a larger differential between the effects of the drug on RNA and DNA synthesis was present.

3.3.5. N-ethylmaleimide (NEM)

The inhibitory effect of this compound occurred over a relatively short concentration range greater than 10^{-5}M. The 50% inhibitory value was of the order of 3.5×10^{-5}M in the case of

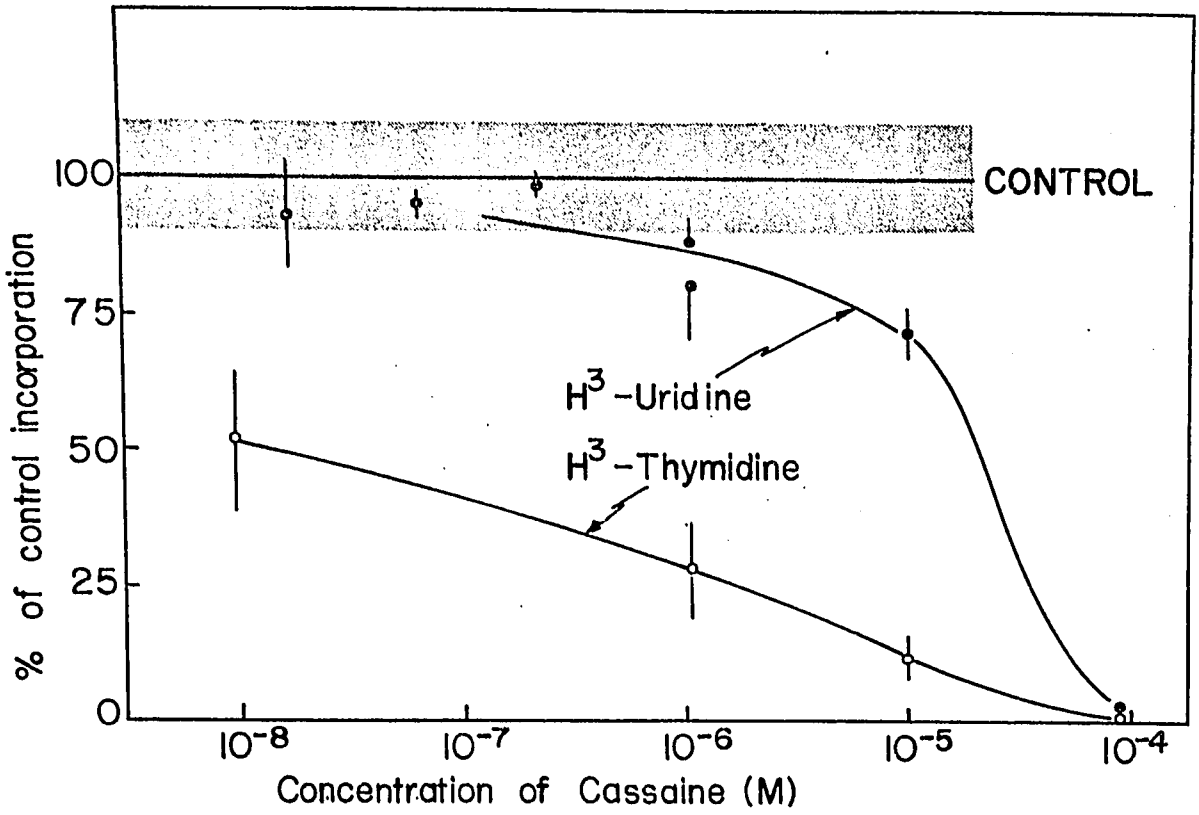


Fig. 3.3.2.

The effect of cassaine base on RNA and DNA synthesis of PHA-stimulated lymphocytes.

The same procedure was used as described for the results presented in Fig. 3.3.1. Experiments with cassaine were carried out in parallel to studies with erythrophleine.

both RNA and DNA synthesis. These results were highly reproducible within limits of technical error, and the sharpness of the drop (see Fig. 3.3.3) was more pronounced than that due to ouabain. For example, in one experiment, normal uptake rates were observed up to 3×10^{-5}M, but at 8×10^{-5}M, complete inhibition of DNA synthesis occurred.

3.3.6. P-chloromercurobenzoate (PCMB)

Like NEM, this compound was effective at concentrations greater than 10^{-5}M, and had a similar dose response. The 50% inhibitory value for RNA and DNA synthesis was about 4×10^{-5}M. This effect of the compound also became maximal over a fairly narrow concentration range (see Fig. 3.3.4).

3.3.7. Oligomycin

This substance was a highly effective inhibitor of DNA synthesis but less so of RNA synthesis in the transforming leukocyte cultures. As shown in Figure 3.3.5, inhibition of DNA synthesis occurred at concentrations greater than 10^{-10}M. DNA synthesis was inhibited by 50% at a concentration of about 2×10^{-9}M. The inhibitory 'efficiency' of the compound was different from that of ouabain, since a 100-fold increase in concentration was required to cause the transition from minimal to maximal inhibition.

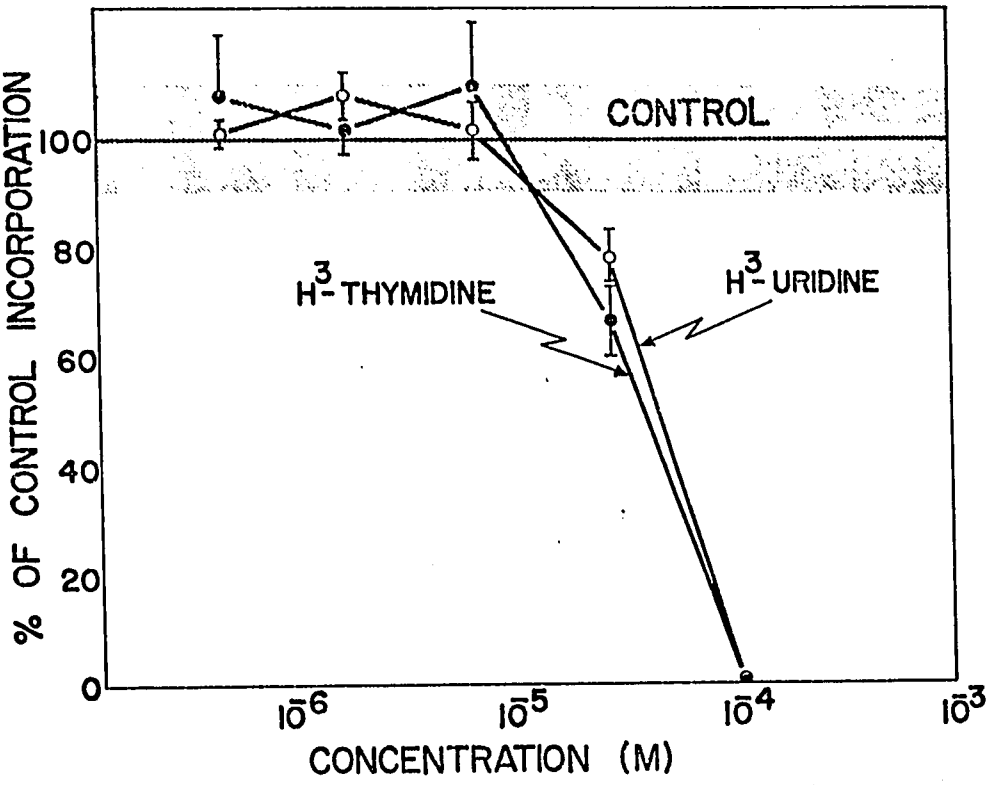


Fig. 3.3.3.

Effect of N-ethylmaleimide on RNA and DNA synthesis of PHA-stimulated lymphocytes.

The same procedure was used as described for the results presented in Fig. 3.3.1.

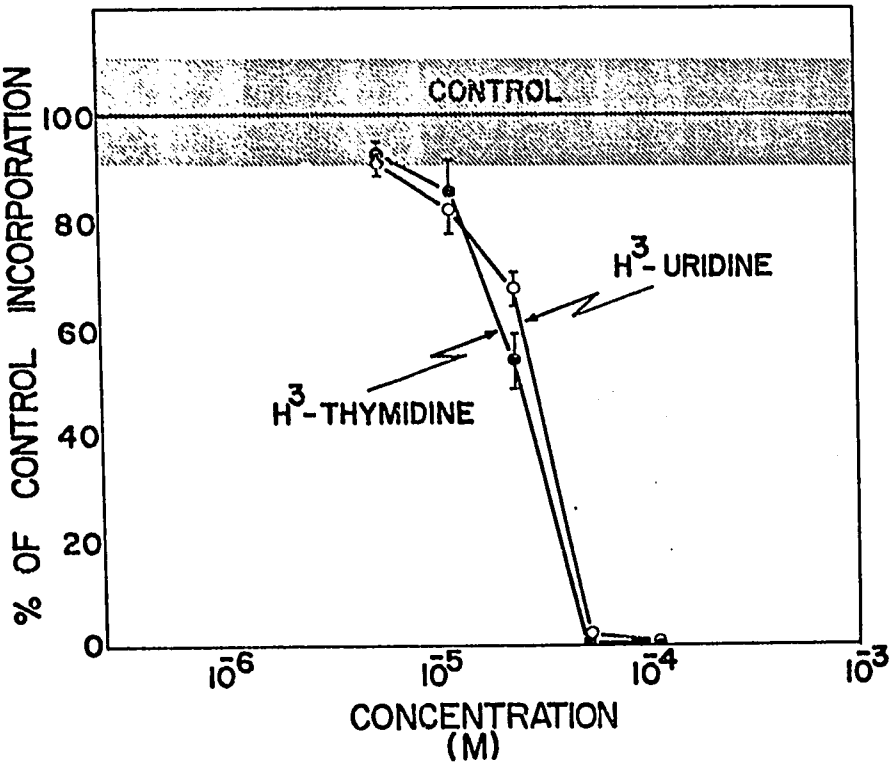


Fig. 3.3.4.

Effect of p-chloromercurobenzoate (PCMB) on RNA and DNA synthesis of PHA-stimulated lymphocytes.

The same procedure was used as described for the results presented in Fig. 3.3.1.

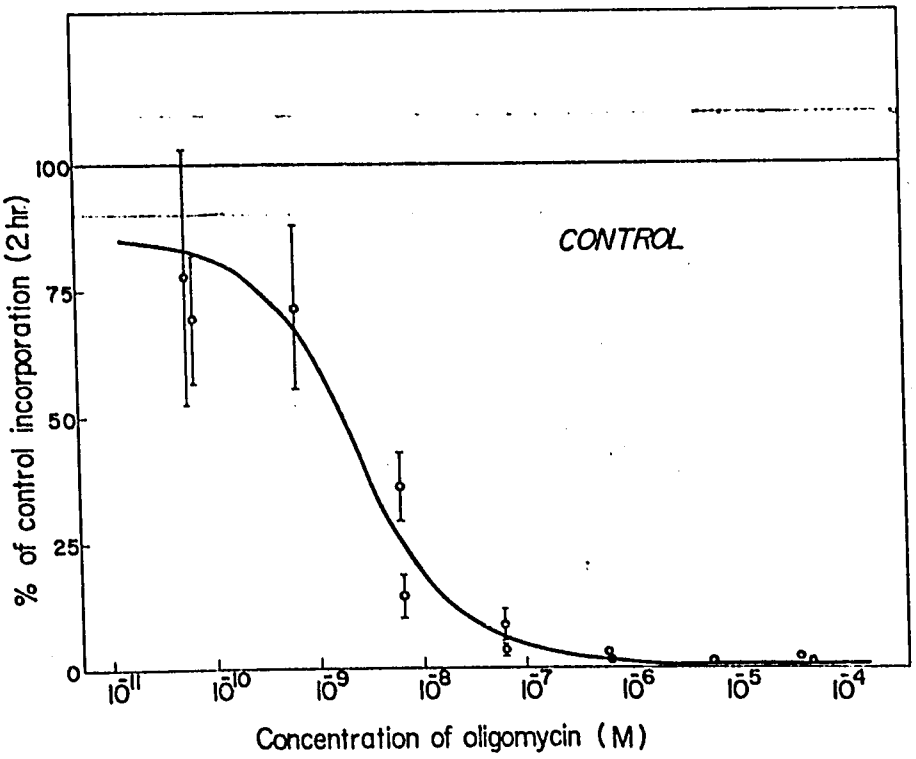


Fig. 3.3.5.

Effect of oligomycin on DNA synthesis of PHA-stimulated lymphocytes. Concurrent measurements of RNA and protein synthesis are presented in Table 3.3.5.

The cells were cultured in the presence of PHA for a period of 18 hours before oligomycin was added. Measurements of H^3-thymidine incorporation, during a 2 hour incubation period, were carried out 24 hours after the addition of oligomycin. The compound was initially dissolved in ethanol and subsequently diluted in water. The final ethanol concentration for all cultures (including controls) was 1%. This concentration of ethanol did not affect DNA, RNA or protein synthesis in these cells under the experimental conditions.

As indicated by Table 3.3.1, the inhibitory action of oligomycin on RNA synthesis was less pronounced than on DNA synthesis. At concentrations as high as 2-6 x 10^{-5}M, substantial RNA and protein synthesis was observed (see Table 3.3.1). It is apparent that the DNA synthesis of transforming lymphocytes is more sensitive to oligomycin than is RNA or protein synthesis.

3.3.8. Dinitrophenol (DNP)

The action of DNP on synthetic activities was minimal. Although substantial enhancement of oxygen uptake was caused at 2.8 x 10^{-5}M (see Section 3.5.1) by this classical 'uncoupling' agent, the DNP concentrations which affected respiration did not cause inhibition of DNA synthesis after the drug had been present in the culture medium for as long as one day. Concentrations of DNP greater than 10^{-4}M were required to cause a slight inhibition of DNA synthesis (see Table 3.32.) without effect on RNA synthesis.

3.3.9. Valinomycin

This compound was tested because of its high efficiency for promoting the movement of potassium across cellular membranes.

A marked differential effect between the effect of valinomycin on RNA and DNA synthesis was seen in this case. RNA synthesis was only slightly affected, but DNA synthesis was profoundly inhibited

Table 3.3.1.

Effect of oligomycin on RNA, DNA and protein synthesis of PHA-induced transforming leukocyte cultures

Oligomycin conc.	Incorporation (% of control)		
(M)	H^3-thymidine	H^3-uridine	C^{14}-leucine
0	100 ± 6.6	100 ± 7.8	100 ± 9.6
6.76×10^{-11}	69.0 ± 12.6	63.7 ± 9.2	109 ± 10.7
6.43×10^{-9}	36.1 ± 6.8	62.1 ± 4.3	55.3 ± 8.9
6.06×10^{-7}	6.1 ± 0.7	50.2 ± 8.2	61.0 ± 5.3
2.8×10^{-6}*	14.1 ± 4.3	72.4 ± 12.9	
5.72×10^{-5}	1.3 ± 0.7	7.3 ± 1.4	51.0 ± 8.9
2.7×10^{-5} *	8.9 ± 2.5	38.3 ± 4.1	

* Separate experiment.
At 7×10^{-5} M, some blast-like cells were observed microscopically (although mitoses were never seen).

Table 3.3.2.

Effect of dinitrophenol on the RNA and DNA synthesis of PHA-induced transforming leukocyte cultures. DNP was added after 2 days of culture with PHA and incorporation measurements were carried out on the 3rd day.

Conc. of DNP (M)	H^3 - thymidine incorp. (cpm)	H^3 - uridine incorp. (cpm)
0	34,885 ± 1546	8,077 ± 361
0 (0.1% ethanol)	37,251 ± 4043	7,576 ± 1336
9.5×10^{-6}	32,735 ± 6621	7,881 ± 820
1.4×10^{-4}	17,101 ± 3380	8,666 ± 1922

at concentrations higher than 10^{-9}M (see Fig. 3.3.6). The sensitivity of DNA synthesis, but not of RNA synthesis, to valinomycin suggests that redistribution of potassium within the transforming lymphocyte, possibly in mitochondria, may be an important consideration in the mechanism of action of this compound on the cells under study.

3.3.10. Tetrodotoxin

This highly poisonous material from the puffer fish has been shown by a number of workers to be a very efficient inhibitor of sodium movements in nerve and muscle fibres at minute concentrations (Kao, 1966). Since ouabain is presumed to act on cation transport, an effect of tetrodotoxin on transformation would clearly be of great interest.

As shown in Table 3.3.3, tetrodotoxin, at the concentrations ranging from 6.9×10^{-10} to 6.9×10^{-6}M, had no inhibiting effect whatsoever on lymphocyte transformation, although these drug concentrations used were effective on nerve and muscle preparations (Kao,1966). Slightly increased incorporation of H^3-thymidine at higher concentrations of the drug was observed, but is not statistically significant. Therefore, if any biological action of tetrodotoxin occurred in this system, it was not reflected by an effect on nucleic acid synthesis during transformation.

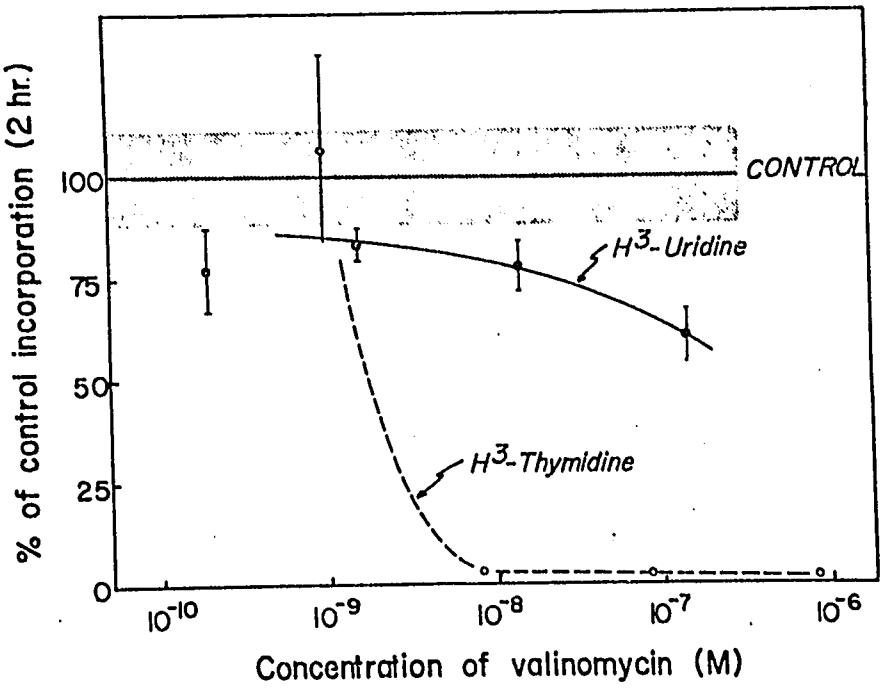


Fig. 3.3.6.

Effect of valinomycin on RNA and DNA synthesis of PHA-stimulated lymphocytes.

The same procedure was used as described in Fig. 3.3.5, except that the final ethanol concentration was 0.006%.

Table 3.3.[3].

Effect of Tetrodotoxin on RNA and DNA Synthesis of Unstimulated and PHA-Induced Transforming Leukocyte Cultures

Tetrodotoxin Conc. (M)	H^3-thymidine incorp. (cpm)		H^3-uridine incor. (cpm)	
	PHA	No PHA.	PHA	No PHA
0	29,370 ± 3840	37 10	13,930 ± 820	300 ± 40
6.9×10^{-10}	30,080 ± 3950	15 4	13,500 ± 1600	230 ± 20
6.9×10^{-8}	34,510 ± 5170	31 5	12,880 ± 1630	170 ± 60
6.9×10^{-6}	44,360 ± 2680	6^{15}_{0}	13,640 ± 420	200 ± 50

3.3.11. Cyclic AMP and Other Adenosine Nucleotides

The action of adenosine-3'-5' cyclic monophosphate (cAMP) and the dibutyryl derivative (dcAMP) were tested with other adenosine nucleotides to see whether the compound might initiate or enhance mitosis and cell division in lymphocytes. These substances were tested both on unstimulated cells, in cultures treated with 'suboptimal' amounts of PHA (20 µg/ml) and on cells treated with the usual concentrations of PHA (200 µg/ml). However, no stimulatory effect was observed, and it was found that cyclic AMP caused an inhibition at concentrations greater than 10^{-5}M (see Fig. 3.3.7.).

However, the other adenosine nucleotides, AMP, ADP, ATP, and particularly dcAMP, were even more effective in inhibiting transformation than was cyclic AMP (see Table 3.3.3a). This inhibitory action was observed when low PHA concentrations were used (20 µg/ml). At higher concentrations of PHA (200 µg/ml), the effects of the adenosine nucleotides were not observed. Fig. 3.3.8.

AMP, cAMP, and dcAMP were all ineffective in inducing or enhancing cell division or DNA synthesis (in 2-day cultures) at the concentrations used, even in the presence of high potassium concentrations. In this respect, it is of interest that Hirschhorn et al. (1970) have reported a small stimulatory effect of cAMP occurring in 3-6 day cultures. (Table 3.3.4).

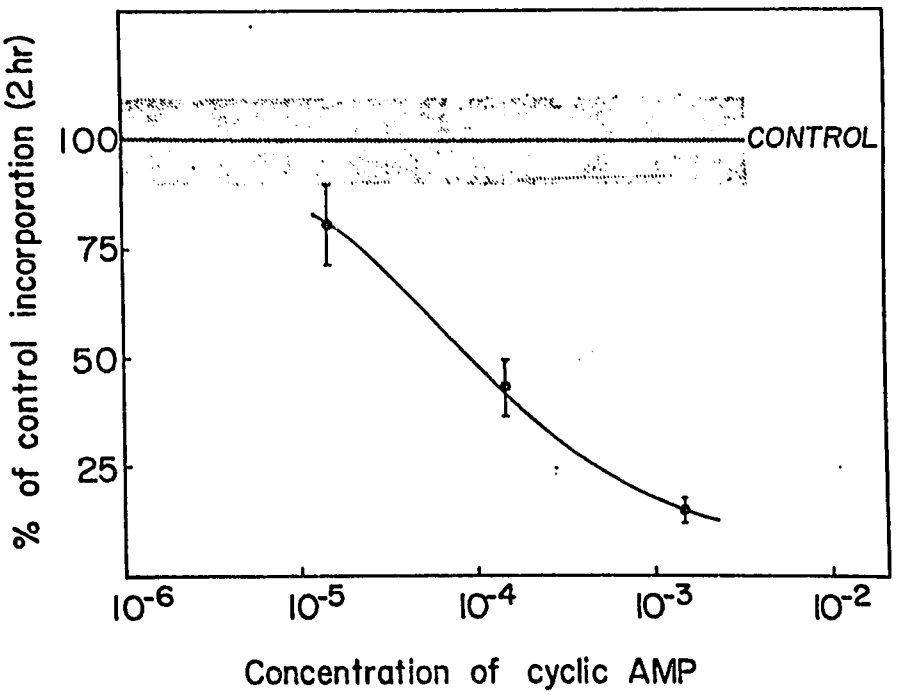


Fig. 3.3.7.

Effect of cyclic AMP on DNA synthesis of PHA-stimulated lymphocytes.

Cyclic AMP was added to the cultures at the same time as a relatively low dose of PHA (20 µg/ml). Measurements of the incorporation of H^3-thymidine, during a 2 hour period of incubation, were carried out after a 2 day period in culture with both PHA and cyclic AMP.

Effect of cAMP, dcAMP, AMP, ADP, and ATP on DNA Synthesis

in PHA-Stimulated and Unstimulated Lymphocytes

Experiment	Nucleotides Added		2-Hour Incorporation of H^3 - thymidine			
	Compound	Concentration (M)	PHA-M	cpm	PHA-M	cpm
#1	Control	0	200µg/ml	13,135 379	20µg/ml	2,144 190*
	cAMP	1.57×10^{-4}	"	11,928 ± 647	"	1,291 ± 126
	AMP	1.23×10^{-4}	"	14,114 ± 589	"	713 ± 124
	ADP	1.48×10^{-4}	"	12,552 ± 879	"	664 ± 88
	ATP	1.30×10^{-4}	"	11,030 ± 737	"	680 ± 74
#2	Control	0	0	31± 6	20µg/ml	644 ± 115*
	cAMP	5.0×10^{-5}	0	24± 5	"	499 ± 97
	dcAMP	4.7×10^{-5}	0	26± 3	"	50 ± 19
	AMP	$5.4 \quad 10^{-5}$	0	18± 3	"	446 ± 17

Table 3.3.3a.

* Two experiments involving different cultures from different donors, different lots of H^3 -thymidine and a different vial of PHA-M. In all cases, cell were cultured for 2 days in presence of PHA and /or nucleotide.

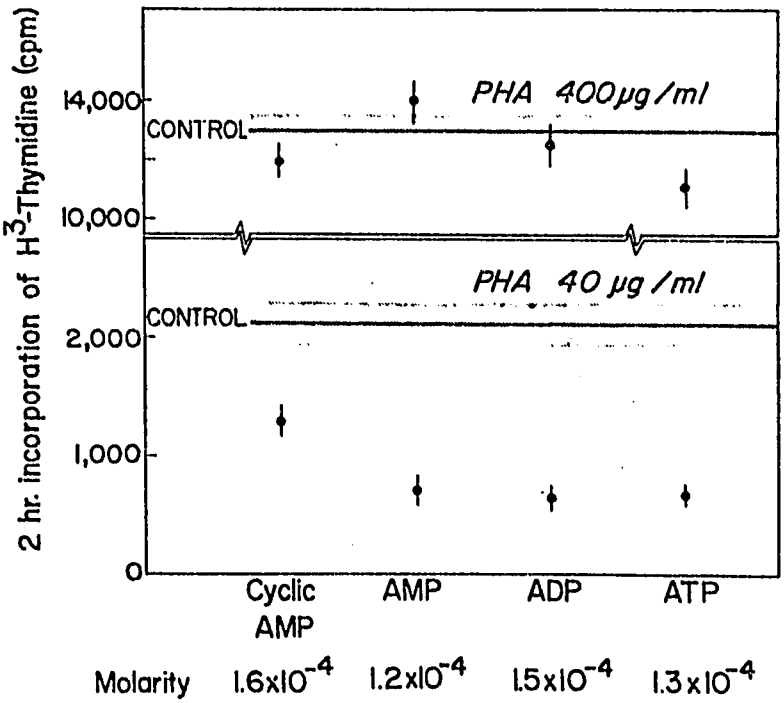


Fig. 3.3.8.

Effects of cyclic AMP, AMP, ADP and ATP on DNA synthesis of PHA-stimulated lymphocytes, at two PHA concentrations (20 µg/ml and 200 µg/ml).

The compounds were added after the cells had been cultured in the presence of PHA for one day. The incorporation of H^3-thymidine, following a 2 hour period of incubation, was measured 24 hours after the addition of the adenosine nucleotides.

Table 3.3.4.

Effect of Low Concentrations of cAMP* on DNA Synthesis Induced by 'suboptimal' PHA Concentrations

PHA Conc. µg/ml	Conc. (M)	Uptake of H^3-thymidine in 1 hour (cpm)
	1.25×10^{-6}	1306 ± 47
20	9.4×10^{-8}	1308 ± 217
	1.1×10^{-8}	1182 ± 164
	9.4×10^{-10}	1320 ± 291
	0	1712 ± 134
0	0	55 ± 15

* cAMP and PHA present during the total 2 days of culture

3.3.12. Prednisolone and Hydrocortisone

These two steroids were tested to determine whether the sensitivity of transforming lymphocytes to ouabain was similar to their sensitivity to adrenal steroids. It was found, however, that ouabain was considerably more effective in causing inhibition of the transforming cells than were the steroids (Fig. 3.3.9) . On a molar basis, the inhibitory action of ouabain was greater by about 2 orders of magnitude in comparison to that of prednisolone or hydrocortisone.

It has been shown (Elves et al, 1964) that prednisolone has its most pronounced effect when given prior to the onset of transformation, but that PHA appears to render the cells less sensitive to these compounds. The opposite seems to be true in the case of ouabain, since PHA appears to cause lymphocytes to become more sensitive than unstimulated cells to the glycoside.

3.3.13. Methotrexate (Amethopterin, N- {p- [2,4-Diamino-6-pteridylmethyl) methylamino] benzoyl }-glutamic acid)

This folic acid antagonist was tested for the following reasons:

(a) When the mechanism of action of ouabain was considered, it was thought that it conceivably might affect macromolecular synthesis indirectly, by reducing the activity of biosynthetic pathways which utilize tetrahydrofolate coenzymes, including those

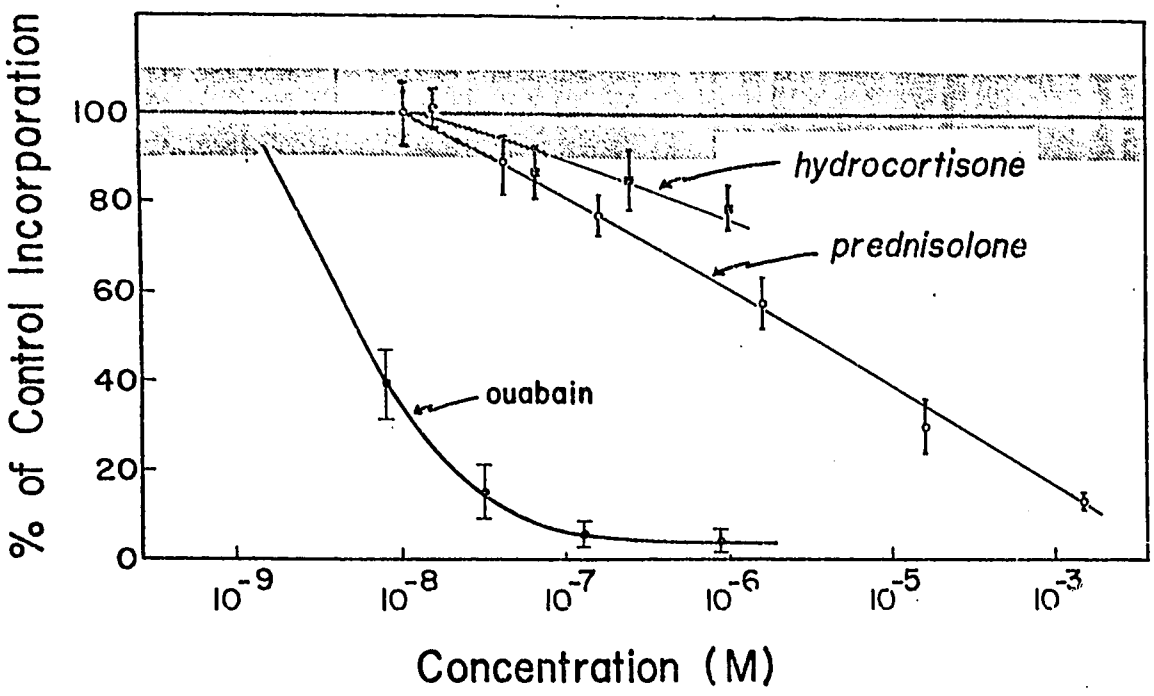


Fig. 3.3.9.

Effect of prednisolone and hydrocortisone on the synthesis of RNA by PHA-stimulated lymphocytes, compared with the effect of ouabain.

In this experiment, water-soluble sodium succinyl derivatives of the steroids were used, but very similar results were obtained when the ethanol-soluble compounds and prednisolone-21-phosphate were tested.

required for the formation of purines and methionine, as well as for the biosynthesis of thymidylic acid (Berlin et al., 1963). If so, one might also expect the transforming lymphocyte to be extremely sensitive to the action of folic acid antagonists, such as methotrexate, which would by this hypothesis cause a reduction in RNA and DNA synthesis.

(b) Another reason was as follows: It was brought to my attention by Dr. T.H.M. Stewart of the Ottawa General Hospital, that methotrexate may potentiate the defence mechanism of the body against certain tumours, particularly those which are accompanied by an influx of lymphocytes into the vicinity of the tumor. If methotrexate were to cause its therapeutic effect against certain neoplasms by potentiating the action of lymphocytes against the target tissue, the drug might have a 'stimulating' effect on DNA synthesis, which is known to occur during the in vitro cytotoxic effect of lymphocytes on target cells (Moller et al, 1966; Holm and Perlman, 1967).

Methotrexate was first tested on the African Green Monkey kidney cell line (BSC-1). As shown in Figures 3.3.10 and 3.3.11, there was a slight increase in the incorporation of H^3-thymidine into DNA after 2 days in the presence of the antagonist, but marked inhibition was noted after 7 days, 50% inhibition occurring at 10^{-8}M, a value similar to the ID_{50} concentration determined by Eagle and Foley (1956) for other cell types in vitro. These results were then compared with the effect of methotrexate on PHA-treated leukocyte cultures.

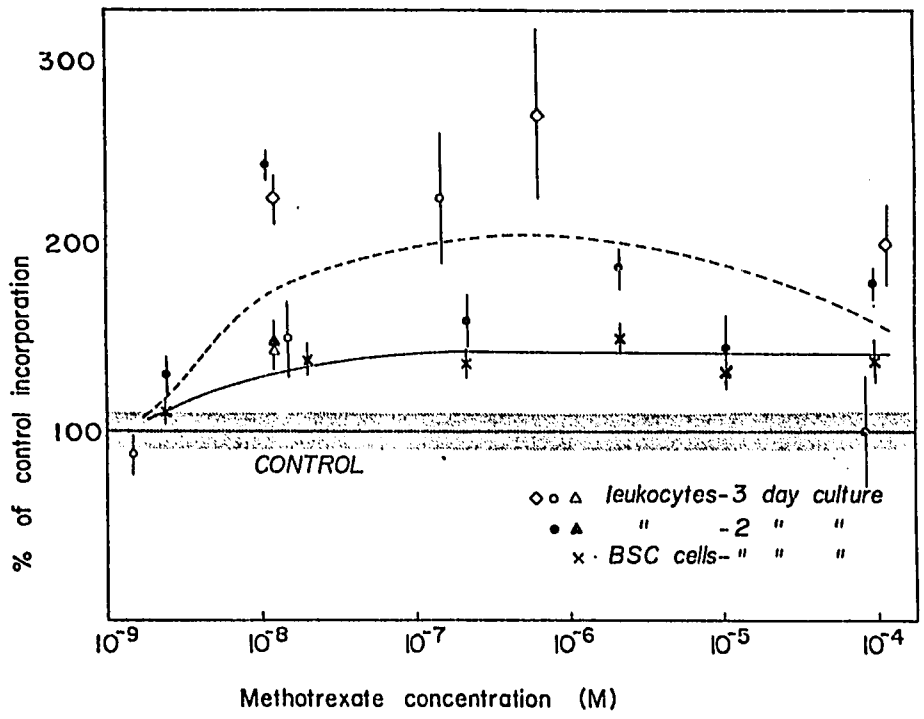


Fig. 3.3.10.

Effect of methotrexate on DNA synthesis of PHA-treated leukocyte cultures and of monkey kidney BSC-1 cells.

Methotrexate was added together with PHA at the time of initiation of the leukocyte cultures and at the time of seeding the BSC-1 cells. Measurements of the incorporation of H^3-thymidine, following a 2 hour period of incubation, were carried out after 2 and 3 days of culture. The results are expressed as the % of control incorporation values. The dotted line is a rough approximation of the leukocyte results. The solid line is drawn through the results obtained from the BSC-1 cells (by eye). The different symbols (o,Δ,◇) represent results obtained from separate subjects.

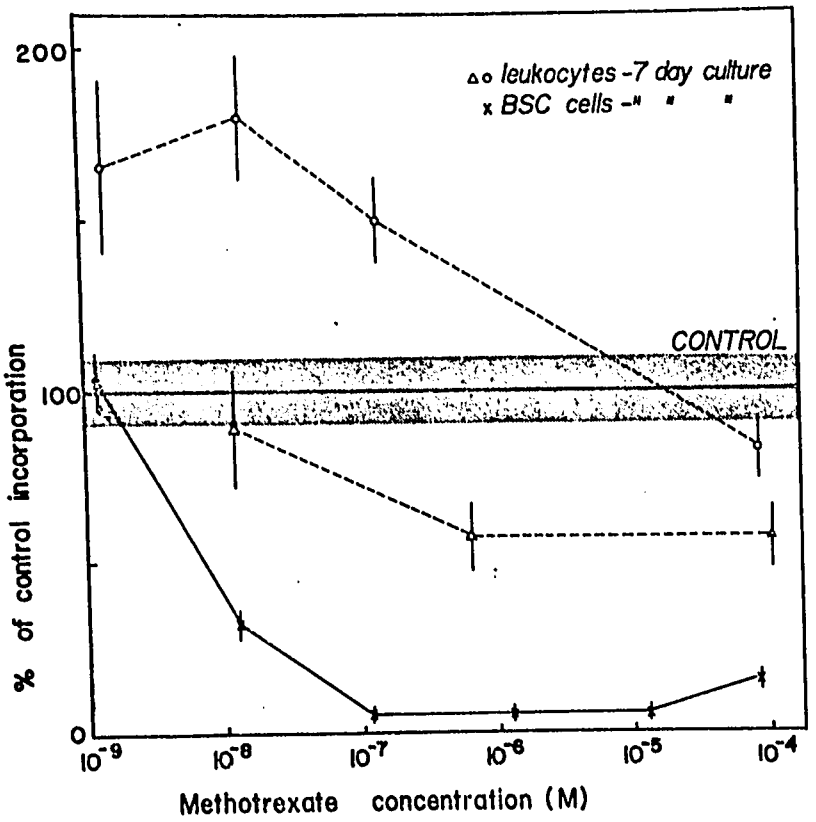


Fig. 3.3.11.

Effect of methotrexate on the DNA synthesis of leukocyte cultures treated with PHA and of BSC-1 cells, over a period with the drug of 7 days.

Dotted lines represent leukocyte cultures obtained from two different subjects, whereas the solid line represents BSC-1 cells. The incorporation of H^3-thymidine was measured after a period of 2 hours incubation with the tracer. PHA was added together with methotrexate at the start of initiation of the leukocyte cultures. Methotrexate was added to the BSC-1 cells at the time of seeding.

When methotrexate was given over the range of 10^{-8} to 10^{-4}M, increased rates of H^3-thymidine incorporation were observed, without corresponding changes in the incorporation of H^3-uridine (Figs. 3.3.10 and 3.3.11 . This interesting effect of methotrexate was particularly pronounced at low PHA concentrations. Moreover, the presence of methotrexate at concentrations as high as 10^{-4}M did not cause any substantial diminution in the rate of incorporation of H^3-thymidine into DNA of PHA-treated cultures when the drug was continuously present during one week of culture. The work is being followed up by measurements of the size of the thymidine pool.

3.3.14. Actinomycin-D

The effect of this drug on the synthetic activity of the cell and on the size of the uridine pool was of interest for the following reasons. It was important, (a) to evaluate the uptake of H^3-uridine into the acid soluble pool (Section 3.2.3.8) in the presence and absence of RNA synthesis; (b) to show that the method used to measure RNA synthesis was valid. Inhibition by actinomycin-D of H^3-uridine incorporation into TCA-precipitated material helped to confirm this; (c) to determine whether DNA synthesis in PHA-treated lymphocytes was affected by inhibition of DNA-dependent RNA synthesis; (d) to evaluate the effect of inhibiting RNA synthesis on cellular K^{42} uptake (see Section 3.4.10); (e) to obtain, for the experimental conditions used, a suitable inhibitory concentration of the drug.

Three types of experiments were done:

3.3.14.1. Effect of Actinomycin-D on RNA Synthesis

As seen in Figures 3.2.23 and 3.3.12, actinomycin-D strongly inhibited the incorporation of H^3-uridine into TCA-insoluble material, using lymphocyte cultures given the drug at the same time as PHA or after a day in the presence of PHA. The effect was more pronounced with increased dosage. The concentrations used were $2\text{-}4 \times 10^{-7}$M.

3.3.14.2. Effect of Actinomycin-D on DNA Synthesis

At concentrations greater than 10^{-8}M, the drug caused a pronounced inhibition of DNA synthesis, as measured by the incorporation of H^3-thymidine into cultures treated with PHA for two days (Table 3.3.5).

3.3.14.3. Effect of Actinomycin-D on the Acid-Soluble Pool of Uridine, With or Without Ouabain

As shown in Figure 3.2.23, actinomycin-D at a concentration of 4×10^{-7}M had no noticeable effect on the uptake of H^3-uridine into the TCA-soluble pool of the cell, although the incorporation of this precursor into RNA was profoundly inhibited.

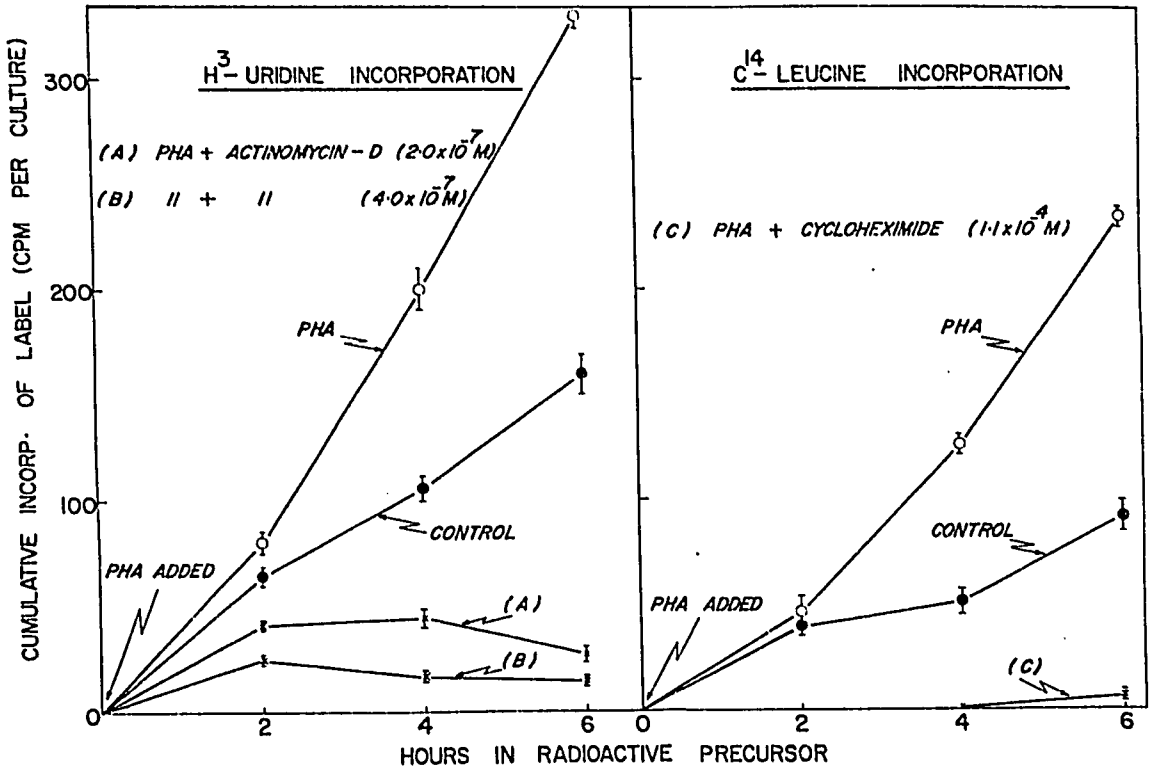


Fig. 3.3.12.

Effect of actinomycin-D and cycloheximide on RNA and protein synthesis of unstimulated lymphocytes and of lymphocytes treated with PHA for a 6 hour period.

PHA was added 5 minutes prior to the administration of the drugs at the concentrations shown, and radioactive precursors were given 5 minutes after the drugs. Ordinate represents the cumulative incorporation of H^3-uridine or C^{14}-leucine (in cpm per culture), after 2,4 and 6 hours of culture, into TCA-precipitable material.

Table 3.3.5.

Effect of Actinomycin-D on DNA synthesis

Actinomycin-D Conc. (M)	2 hr. incorporation of H^3-thymidine (cpm)	
	PHA present	No PHA
4.7×10^{-7}	31 ± 14	10 ± 7
6.6×10^{-8}	80 ± 11	50 ± 20
9.8×10^{-9}	605 ± 118	32 ± 17
0	18,390 ± 1,730	37 ± 4

3.3.15. Cycloheximide

As with actinomycin-D, it was desirable to inhibit protein synthesis in some experiments with K^{42} (Section 3.4.10), and it was therefore necessary to determine the inhibitory concentrations of this drug which were effective under the experimental conditions used. When the compound (1.1×10^{-4}M) was given to lymphocyte cultures at the same time as PHA, it completely inhibited the incorporation of C^{14}-leucine into the cultures.

When the drug was administered to cultures treated 2 days previously with PHA, or the same time with precursor, it caused pronounced inhibition of protein synthesis (Table 3.3.6). At the same time, RNA synthesis, measured by the incorporation of H^3-uridine, was much less affected, although the rate of incorporation into RNA decreased to about 42% of control values after 4.8 hours in the presence of cycloheximide.

Table 3.3.6.

Effect of Cycloheximide on Incorporation of Labelled Precursors into RNA and Protein of PHA-Stimulated Leucocytes

Time in cyclo-heximide (hrs.)	H^3-uridine incorp. (cpm)		C^{14}-leucine incorp. (cpm)	
	Control	Cycloheximide	Control	Cycloheximide
1.58	1545 ± 65	1077 ± 53 (70 ± 6%)*	854 ± 43	6 ± 2 (0.7 ± 0.2%)
3.20	2208 ± 88	1377 ± 39 (61 ± 4%)	1163 ± 85	21 ± 3 (1.8 ± 0.3%)
4.78	2757 ± 104	1171 ± 40 (42 ± 3%)	1146 ± 31	28 ± 4 (2.4 ± 0.4%)

* Percent of control value

3.3.16. SUMMARY OF CHAPTER

1. The enhanced DNA and RNA synthesis characteristic of PHA-induced lymphocyte transformation was strongly inhibited by the erythrophleum alkaloids, erythrophleine sulfate and cassaine base, the former being most effective. The relative effects of the two alkaloids corresponded to reported relative effects on the isolated Na-K-ATPase (Bonting et al, 1964).

2. N-ethylmaleimide (NEM) and p-chloromercurobenzoate (PMB) both inhibited the nucleic acid synthesis associated with lymphocyte transformation. The effect of both drugs became maximal over a very short concentration range, with 50% inhibition at approximately $3\text{-}4 \times 10^{-5}$M. These drugs inhibited RNA and DNA synthesis equally for the same dose.

3. Oligomycin strongly inhibited DNA synthesis (50% of the maximal effect at 1.8×10^{-9}M), but RNA and protein synthesis were less affected.

4. Valinomycin strongly prevented DNA synthesis but, even more so than oligomycin, did not appear to affect the incorporation of label into RNA.

5. The relative or differential action of the drugs on RNA and DNA synthesis was in decreasing order of magnitude, as follows: valinomycin > oligomycin > cassaine > erythrophleine > ouabain

> NEM or PCMB.

6. Tetrodotoxin (6.9×10^{-6} - 6.9×10^{-10}M) had no apparent effect on the nucleic acid synthesis of transforming lymphocytes.

7. cAMP, dcAMP, AMP, ADP and ATP (at concentrations greater than 10^{-5}M) all inhibited the DNA synthesis associated with transformation. The effect was most pronounced at 'suboptimal' PHA concentrations (e.g., at 20 µg/ml). dcAMP was a more effective inhibitor than the other nucleotides. No stimulatory effect was observed under the experimental conditions used, even in the presence of excess extracellular potassium.

8. Prednisolone and hydrocortisone were less effective than ouabain (by about 2 orders of magnitude on a molar basis), in causing inhibition of nucleic acid synthesis in transforming lymphocytes.

9. Methotrexate caused an increase in the rate of incorporation of labelled thymidine into DNA. Transforming lymphocytes were relatively 'resistant' to the drug at concentrations as high as 10^{-4}M, in the sense that substantial levels of H^3-thymidine incorporation were observed after a week in the presence of the drug.

10. Actinomycin-D strongly inhibited RNA synthesis at concentrations of 2-4 $\times 10^{-7}$M, and markedly inhibited DNA synthesis at concentrations greater than 10^{-8}M. However, it did not affect the uptake of tracer into the acid-soluble compartment.

11. Cycloheximide strongly suppressed protein synthesis at concentrations of 1.1×10^{-4}M, and at the same concentrations caused a slow and partial inhibition of RNA synthesis.

Some of these results are summarized in Table 3.3.7.

Table 3.3.7.

Effect of Inhibitors on DNA and RNA Synthesis

of PHA-Stimulated Leukocyte Cultures

Substance	Concentration Required for 50% Inhibition of DNA Synthesis	RNA Synthesis	Na-K-ATPase Activity (Published Data)	Reference
Erythrophleine Sulfate	1.4×10^{-8}	3×10^{-8}	2.6×10^{-7}	Bonting et al.* 1964
Cassaine Base	1×10^{-8}	$\sim 1.7 \times 10^{-5}$	5.2×10^{-6}	Bonting et al.* 1964
N-ethylmaleimide	2.5×10^{-5}	2.5×10^{-5}	6×10^{-4}	Matsui and + Schwartz, 1966
p-chloromercuro-benzoate	4×10^{-5}	4×10^{-5}	5×10^{-4}	Matsui and + Schwartz, 1966
Ouabain	3×10^{-8}	4×10^{-8}	$10^{-7} - 10^{-6}$	Bonting et al. 1962; 1964 Matsui and Schwartz, 1966/
Valinomycin	$\sim 2 \times 10^{-9}$	$> 10^{-7}$		
Oligomycin	2×10^{-9}	$\sim 10^{-5}$		
Dinitrophenol	$> 10^{-4}$	$> 10^{-4}$		

* Rabbit brain Na-K-ATPase
\+ Calf heart muscle Na-K-ATPase
// Various Na-K-ATPase preparations

3.4. POTASSIUM UPTAKE AND TURNOVER IN UNSTIMULATED AND STIMULATED LYMPHOCYTES

3.4.1. Introduction to Section

The results described in Sections 3.2 and 3.3 indicated that lymphocytes were highly sensitive to ouabain during the process of transformation. This sensitivity was presumably associated with changes induced by the glycoside in the transport function of the plasma membrane. Furthermore, the transforming cell was relatively more sensitive to the glycoside than was the unstimulated cell, both in terms of the severity of morphological damage and with regard to the inhibition of macromolecular synthesis (Section 3.2).

The observed results could be explained if:

a) there was an increase of ouabain-sensitive transport activity in the stimulated cell, upon which transformation was dependent;

b) that the Na-K-ATPase and cation transport function associated with the plasma membrane did not change, but that lymphocyte transformation was more dependent on its function;

c) the Na-K-ATPase activity was reduced by the action of PHA and rendered more sensitive to ouabain.

In order to elucidate this problem, the turnover of K^{42} by lymphocytes in the unstimulated and PHA-treated state was examined. It was assumed that changes in the transport activity of the cell would be reflected by alterations in the uptake and turnover of K^{42}. However, the problem of technique had first to be explored.

3.4.2. Materials and Methods

A general description of the methods used has been given in Section 2.3. Further details are given here.

In these experiments, the white cells were 'purified' to 93-98% by passing them through a glass bead column, as described earlier (Section 2.1.2). The residual non-lymphocyte population consisted mainly of neutrophils. However, some red cells remained present in the cultures, usually at a ratio of 2-3 red cells per lymphocyte. It therefore was necessary to carry out parallel measurements of the red cell uptake of K^{42} to correct for the presence of these cells. Although the red cells could have been removed using an isotonic NH_4Cl solution (Section 2.1.2.), this was found to be unnecessary; it was also avoided, since it was desirable to subject the cells to as little trauma as possible.

Generally, experiments were carried out using about 180 ml of fresh blood from which 80-90 ml of heparinized plasma containing 'purified' lymphocytes could be obtained. About 120-140 plastic culture tubes were usually prepared containing 15% plasma and 85% medium-199, each tube initially containing 0.8 - 1.2 x 10^6 lymphocytes in a 3 ml volume. Non-preserved heparin was added to a concentration of 20 units/ml. Trypan-blue exclusion measurements of unstimulated cells indicated a cell 'survival' of about 95-99% after one day and 86-95% (usually 90-95%) after 2 days in vitro.

After receipt of potassium-42 by air-express from the manufacturer, the isotope was diluted in saline to give the desired stock concentration of potassium, i.e., a concentration which would result in the desired final potassium level after adding an aliquot (usually 0.5 ml) to the culture. In some cases, the K^{42} was added directly to a potassium-free balanced salt solution (Table 2.1.3.) or to medium-199 without potassium to give the final concentration required for the experiment.

The cells were incubated at 37°C in the radioactive medium and, after the desired period of time, were washed and the gamma emission of the cells counted in a 256 Channel NaI well-type scintillation counter. This was done simply by

placing the culture tubes containing the cells (from which the supernatant wash solution had been removed after centrifugation) directly in the well counter. After counting, diphenylamine reagent was added and DNA measurements carried out for estimation of the lymphocyte content of the culture tubes. (Section 3.4.1). The results were always expressed in terms of the total DNA present; in the figures, the potassium uptakes per 10^6 cells are calculated from the DNA measurements and the known specific activity of K^{42} in the medium.

The nature of the wash solution used was found to be of considerable importance in the results obtained. For example, the effect of washing with medium-199 or isotonic sucrose was considerably different from the effect of washing the cells with isotonic KCl. This problem is examined in the next section.

3.4.3. Removal of Extracellular K^{42}

3.4.3.1. Effect of Washing Cultures with Different Wash Media: Choice of Wash Solution

Ice-cold isotonic sucrose, isotonic NaCl, medium-199, isotonic choline chloride, and isotonic KCl were compared as wash solutions. As shown in Fig. 3.4.1., the amount of

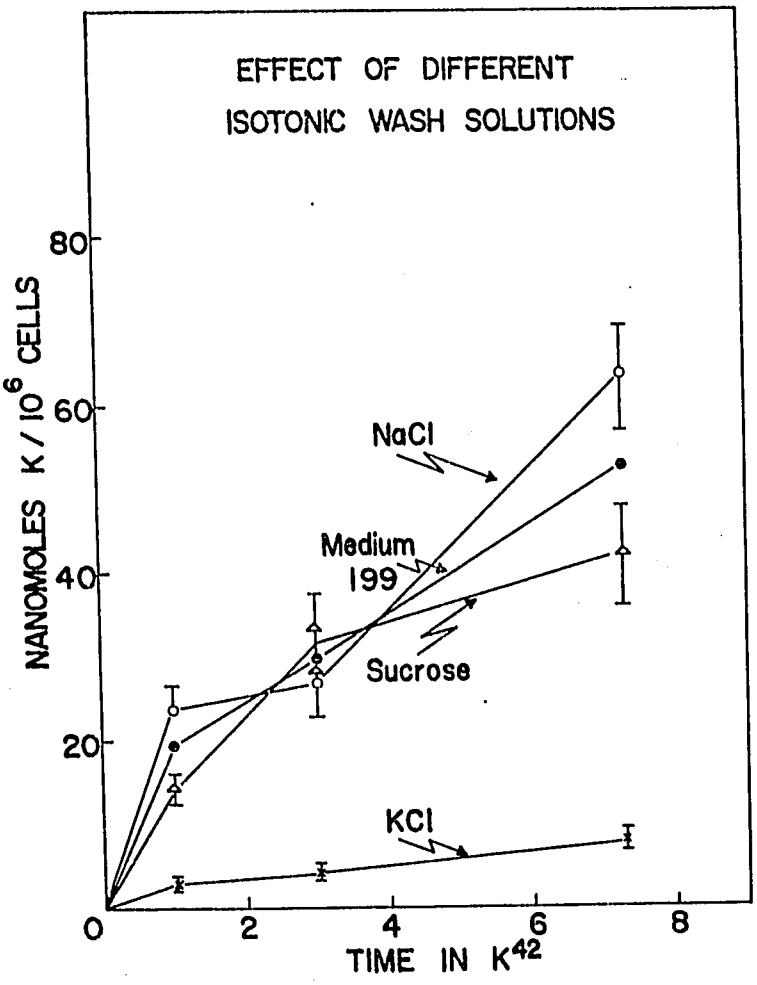


Fig. 3.4.1

Effect of different isotonic wash solutions on the measured uptake of K^{42} by lymphocytes in vitro.

All cells were treated in the same way with the same amounts of K^{42} except for 3 washes in the different ice-cold wash solutions as described in text, section 3.4.3. Ordinate represents measured cellular radioactivity divided by specific activity of K^{42} in culture medium.

K^{42} found to be associated with the cells was much greater when the first 3 solutions were used, in comparison to the much reduced amount seen in the KCl washed cells. The use of isotonic choline chloride led to identical K^{42} values as with the first 3 solutions. Microscopically, KCl-washed cells appeared 'normal' under phase contrast or in stained smears, although some increased flattening and attachment to glass surfaces was noted. There was no effect on the DNA measurements of the cells after using any of the wash media mentioned.

Fig. 3.4.2. compares the effect of washing the cells with ice-cold isotonic NaCl or isotonic KCl. Each point represents the amount of K^{42} associated with the cells after 1, 2...8 washes. It is clear that after an initial displacement of a relatively large amount of K^{42} by the first KCl wash, the rates of decrease of K^{42} were the same for both NaCl and KCl solutions, and were characterized by $T_{1/2}$ values of 61 ± 2 and 57 ± 3 minutes respectively. These values presumably represent identical rates of K^{42} leakage from the cell into the two cold wash media after removal of a labile fraction with isotonic KCl.

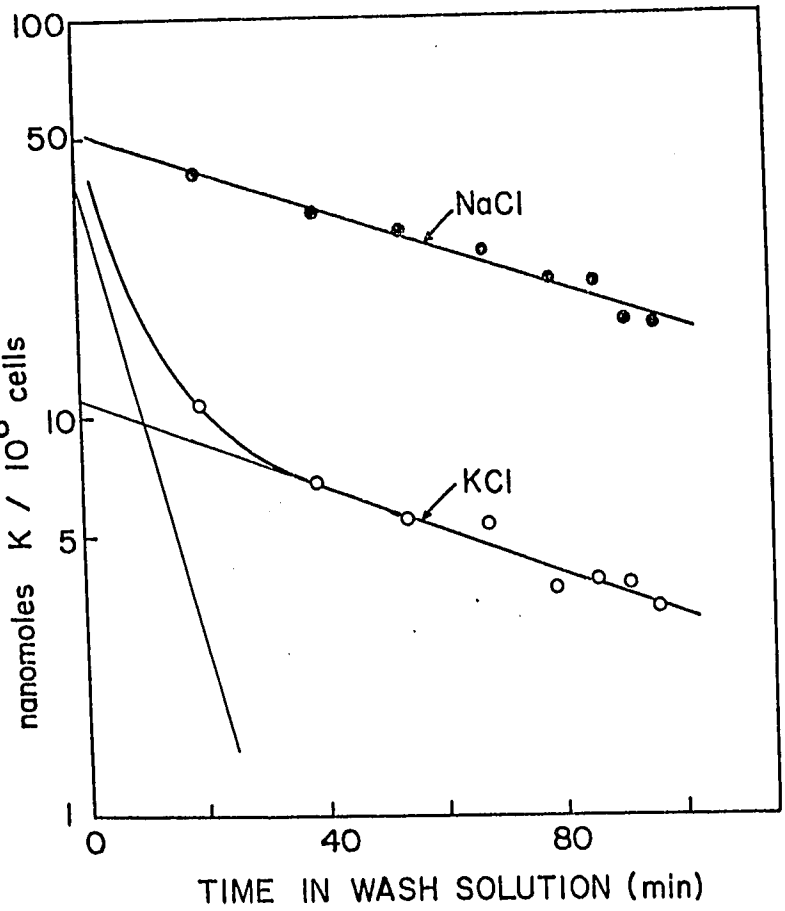


Fig. 3.4.2

Effect on cellular K^{42} content of washing cells repeatedly with ice-cold isotonic NaCl or isotonic KCl.

Ordinate units are the measured content of K^{42} of the cells divided by the known specific activity of K^{42} in the medium. For each graph, experimental points represent the cellular radioactivity after 1, 2 ..... 8 repeated washes.

Washing with isotonic ice cold solutions of CsCl, RbCl or NH_4Cl also caused a reduction in the K^{42} associated with the cells. As shown in Table 3.4.1, isotonic RbCl was as effective as the KCl wash, but isotonic CsCl caused much less displacement. Isotonic NH_4Cl caused a partial loss of K^{42}, but was not used as a wash medium because of possible damage to the cell membrane.

3.4.3.2. Efflux of K^{42} from Preloaded Lymphocytes washed with KCl, compared with cells washed with Medium-199, into a High [K] and Low [K] Medium

The previous results suggested the possibility that all of the K^{42} associated with the cells after the sucrose or saline washes was not necessarily completely 'within' the cells; that is, the high levels of K^{42} observed after sucrose, NaCl or medium-199 washes may not have truly represented the amount of potassium taken up into the cell. The very rapid displacement of a large fraction of the K^{42} using a KCl solution led to the hypothesis that much of the K^{42} was bound to a rapidly exchangeable, possibly extra-cellular compartment of the cells. This problem therefore was studied further.

It was important to determine whether there was any

Table 3.4.1.

Measured radioactivity of unstimulated lymphocyte cultures 'pre-loaded' with K^{42} after 2 washes in various salt solutions (same experiment as in Fig. 3.4.3.

Solution (isotonic, ice-cold)	K^{42} content (cpm/10^6 cells)
NaCl	1410 ± 40
KCl	308 ± 45
RbCl	270 ± 68
CsCl	1390 ± 24

Results corrected for variations in DNA content of culture.

difference between the efflux (or dissociation) of K^{42} from cells washed by either method, since two hypotheses could explain the data of Section 3.4.3.1.

Hypothesis 1 was that there existed two compartments of cellular K^+, of which one represented a rapidly exchangeable peripheral compartment in the presence of excess K^+. If so, the rate of K^{42} efflux from cells in ice-cold isotonic KCl would be expected to differ from the rate of efflux in ice-cold medium-199, provided the cells were not previously washed with isotonic KCl. On the other hand this hypothesis predicts that preloaded cells washed with isotonic KCl would have all their K^{42} 'inside', and hence would be expected to show no difference in the rate of diffusion out into isotonic KCl as compared to medium-199.

Hypothesis 2 was that there was actually no 'second compartment' of potassium, but the KCl wash merely accelerated the rate with which K^{42} diffused out of the cell. Hence there would be no difference between the KCl washed cells and the medium-199 washed cells, when the preloaded cells were re-incubated in the cold in either solution.

The results of the experiment are shown in Fig. 3.4.3.

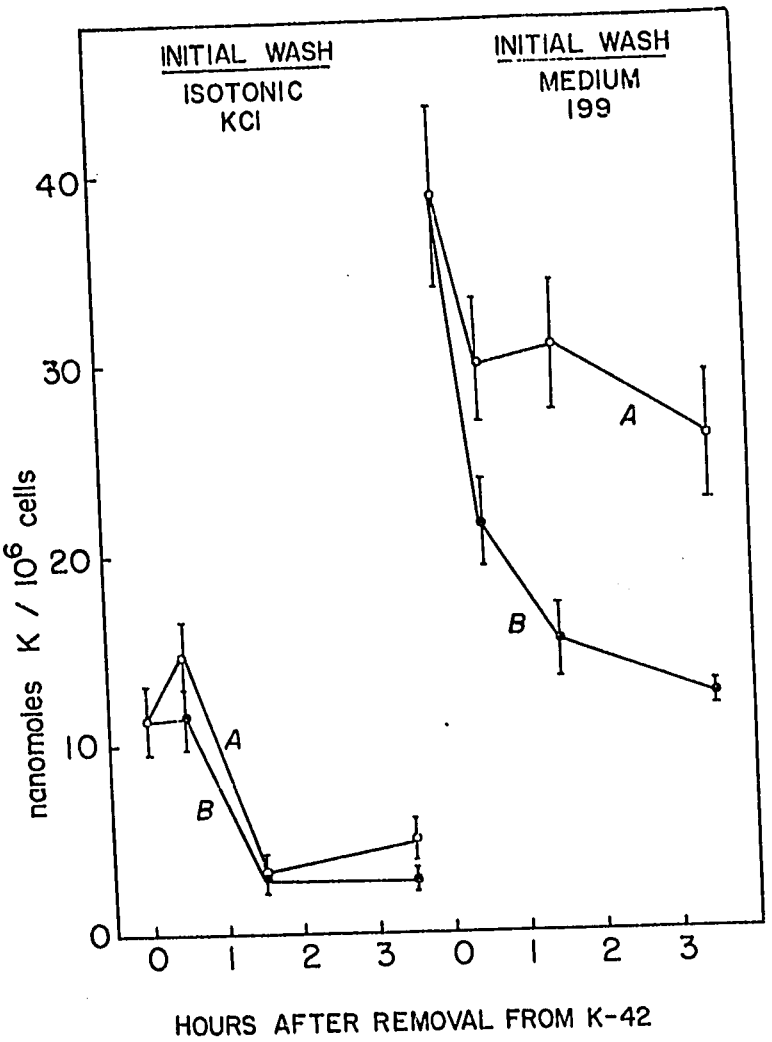


Fig. 3.4.3

Influence of K content of the ice-cold incubating medium on the loss of K^{42} from lymphocytes, the cells having been previously washed with isotonic KCl (left side) or with medium-199 (right side).

The slight increase after ½ hr. for the KCl washed cells is not considered to be significant. Unit of the ordinate cellular radioactivity divided by the specific activity of the culture medium. In this experiment, the cells were preloaded with K^{42} by incubation with the isotope for 3 hours.

A, Resuspension in ice cold medium 199.

B, Resuspension in ice cold isotonic KCl

At ice-cold incubating temperatures, there was no significant difference between the rate of diffusion of K^{42} out of cells washed with isotonic KCl, when they subsequently were re-suspended in KCl or in medium-199. However with the medium-199 washed cells, the finding was different. These cells contained a greater amount of associated K^{42}, as expected from the data of Figure 3.4.1. When resuspended in ice-cold isotonic KCl, there occurred a rapid and pronounced drop in the K^{42} content during the first hour. However, when re-suspended in ice-cold medium-199, or in balanced salt solution without potassium, the decrease of cellular K^{42} content was considerably less. These results clearly support hypothesis 1.

3.4.3.3. Effect of Trypsin on the K^{42} Content of Lymphocytes

The previous experiments suggested that there was a compartment of K^{42} associated with the cells which exchanged rapidly with ice-cold KCl; such a compartment could be extracellular, since the amounts of K^{42} associated with the cells after sucrose, saline, medium-199 or choline chloride washes was usually greater than the levels that might reasonably be expected in cells the size of lymphocytes (when taking into account the presence of red cells). For example, the uptake of K^{42} into medium-199 washed cells indicated a total potassium uptake of more than 50 nanomoles/10^6 cells (and the values

observed were still increasing). Assuming a lymphocyte volume of 250 μ^3,,this suggests the unlikely internal concentration of greater than 200 mM K^+. In the presence of PHA (Fig. 3.4.10), the estimated internal concentration of K^{42} may have been up to twice this amount if there was no early volume change induced by PHA, and assuming all the K^{42} were intracellular.

On the other hand, the K^{42} levels in the lymphocyte cultures washed with isotonic KCl were lower than expected, but the degree of lowering could be reasonably explained as being due to normal diffusional loss of K^{42} during the washing process, (as shown in Fig. 3.4.2). For example, an asymptotic value corresponding to 10 nanomoles/10^6 cells implies an internal concentration of 40 mM K^+, for a cell of 250 μ^3. But correcting for the considerable amount of outward leakage, an corrected internal concentration of 70-80 mM would seem to be more probable.

The working hypothesis was therefore put forward that the 'excess' K^{42} was actually bound loosely to an extracellular membrane-associated substance. Evidence for mucoproteins bound to the lymphocyte membrane have been suggested by others (see Section 1.10). An externally located protein

would presumably be subject to lysis by a proteolytic enzyme such as trypsin (Section 1.10.1.5).

The following experiment was therefore carried out. The cells were incubated in the presence of K^{42} for 3 hours. 15 minutes before the washing of cells, some cultures were treated with trypsin (added directly to the radioactive culture medium containing the cells) to give a final trypsin concentration of 0.25%. Some of the cultures were then washed with ice-cold isotonic KCl, and others with ice-cold isotonic sucrose. The results are seen in Table 3.4.2.

After trypsin treatment, KCl-washed cells did not show a significant change in their K^{42} content. However, sucrose washed cells showed a pronounced drop in their K^{42} content, almost to the level observed with the non-trypsin-treated, KCl-washed cells.

These results strongly suggest that the excess K^{42} is attached in some way to a trypsin-susceptible extracellular protein(s) bound to the cells in the culture, from which the K^{42} is rapidly removed by a KCl wash but not by a NaCl wash. The cells washed with KCl showed little if any change in K^{42} content when trypsin was added, indicating that the proteolytic enzyme had not ruptured the cell membrane, causing the internal K^{42} to leak out.

Table 3.4.2.

Effect of Trypsin (0.25%) on K^{42} Content of Lymphocytes.

Treatment	Uptake of K^{42} for a 3 hr. period in nanomoles K/10^6 cells	
	Washed with isotonic KCl	Washed with medium-199
No trypsin	11.2 ± 1.7	38.7 ± 4.7
0.25 trypsin for 15 min. before washing cells	9.0 ± 1.6	14.8 ± 2.7

Because of these observations, it was decided to wash the cells with ice-cold KCl. In this way, it was possible to be more certain that the K^{42} associated with the cells was actually intracellular. It was recognized that the amount of K^{42} actually measured within the cells was less than the amount originally taken up, because of outward leakage during the washing process. Nevertheless, relative measurements of turnover rates and their changes following PHA stimulation could be and were made. Parallel measurements using isotonic sucrose washes were also made in many experiments.

3.4.4. Potassium-42 Uptake into Red Cells

Since red cells were constant 'contaminants' of the lymphocyte cultures, it was essential to evaluate the incorporation of K^{42} into these cells in the absence of a contribution due to white cells. This was done both after KCl washes and also after sucrose washes.

The results (using a KCl wash) are shown in Fig. 3.4.4. Washing the red cells with sucrose yielded K^{42} values, higher by a factor of 3-4 in comparison to KCl-washed cells, Fig. 3.4.5. The rate of uptake was about 0.2 nanomoles K^{+} per hour/10^{6} red cells for the KCl wash. Assuming an intracellular red cell potassium concentration of about 130 mM, this gives

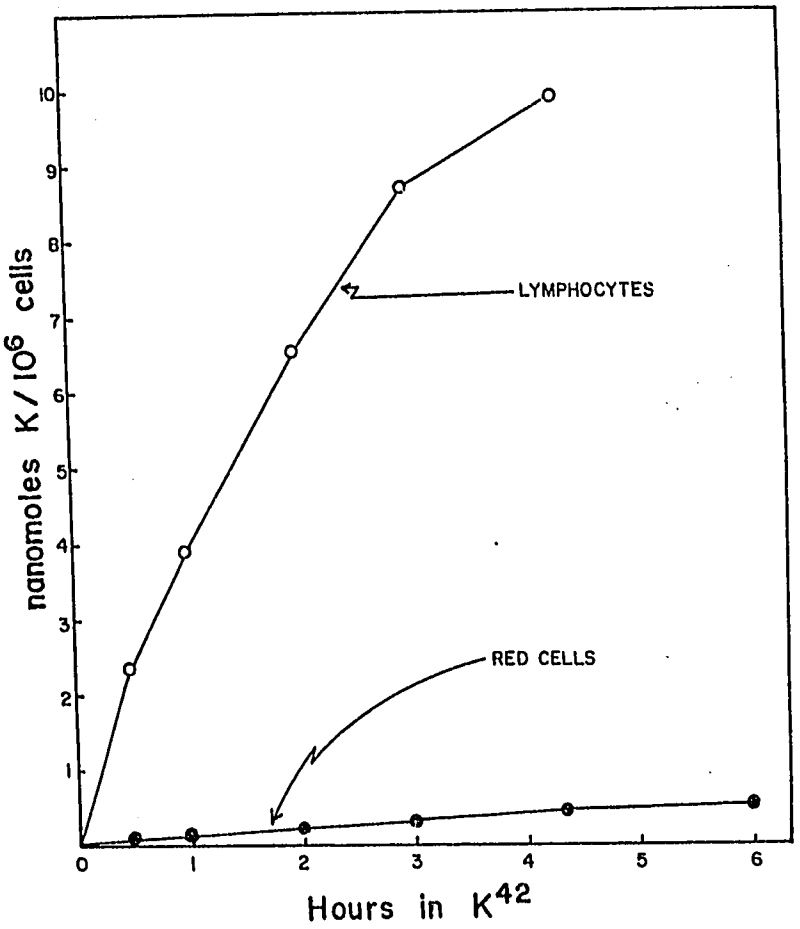


Fig. 3.4.4

Comparison of the rate of K^{42} uptake by red cells with that by lymphocytes. Isotonic KCl wash. Ordinate as with Fig. 3.4.1.

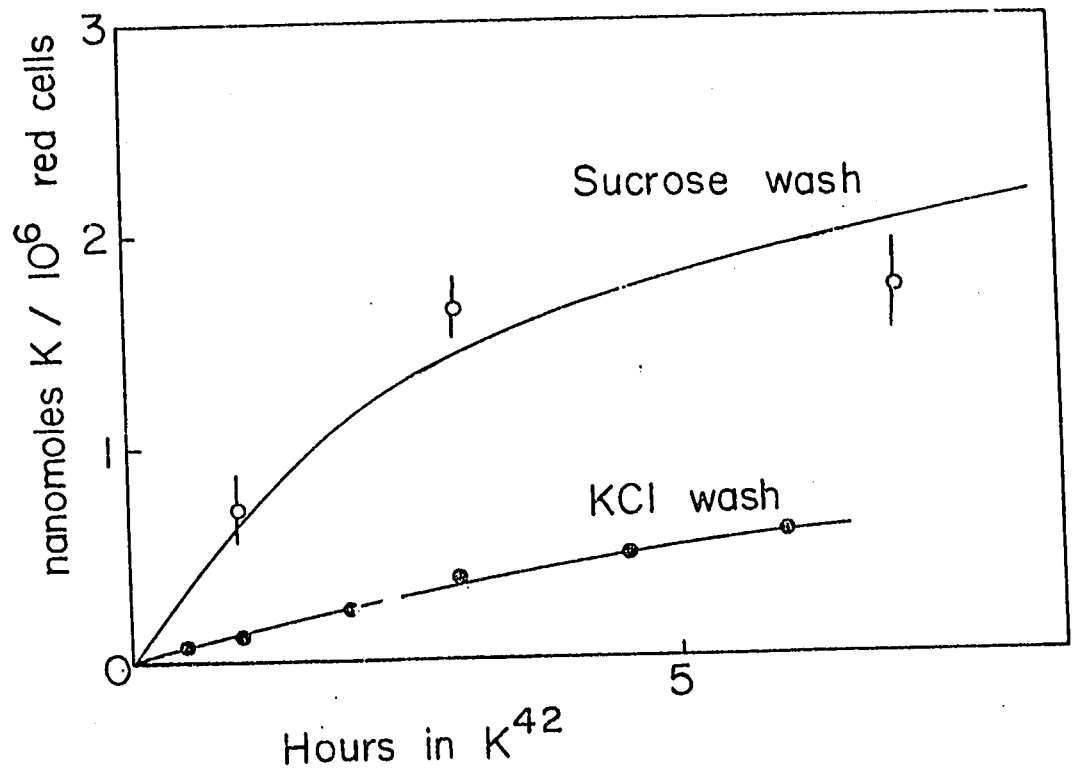


Fig. 3.4.5.

Comparison of the effect of ice-cold sucrose wash with an isotonic KCl wash on the uptake of K^{42} by red cells.

Ordinate as in Fig. 3.4.1. Upper curve continues to increase though measurement at 10 hours not shown.

a turnover of 1.8% of the total intracellular K^+ per hour. Very similar results were obtained by Joyce and Weatherall (1955).

These results were used to provide a correction factor for subsequent studies with lymphocyte cultures, for which the red cell content was always measured.

3.4.5. Potassium-42 Uptake into Unstimulated Lymphocyte Cultures

The uptake of K^{42} into lymphocyte cultures with respect to time (following a KCl wash) always appeared to be exponential in shape, reaching an asymptotic value of about 7-10 nanomoles/ 10^6 lymphocytes (Fig. 3.4.6). However, this value varied for different cultures and very probably was influenced by the degree of K^{42} leakage from the cells during the washing process. Hence the measured K^{42} values are probably less than the true uptake of K^{42} into the cell.

A consistent value for a time constant was obtained, however, for repeated measurements of K^{42} uptake into the cell. This time constant was characterized by a $T_{1/2}$ of 1.0 - 2.5 hours, averaging about 1.5 $\pm$0.2 hours. (The $T_{1/2}$ was defined as the time required for the K^{42} content of the cell to reach 1/2 of the maximum asymptotic value).

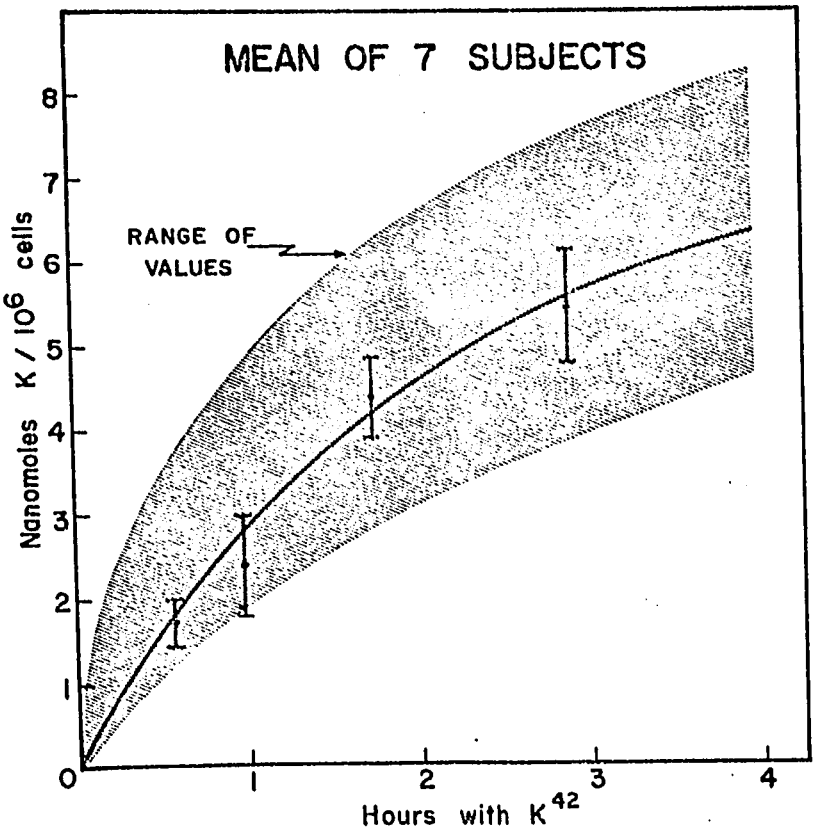


Fig. 3.4.6.

Uptake of K^{42} by unstimulated small lymphocytes in vitro.

Pooled results for 7 different subjects. The shaded area shows the extent of variation observed between cultures from the different subjects. The cells were washed with ice-cold isotonic KCl prior to measurement of cellular radioactivity. Units for ordinate as with Fig. 3.4.1. The $T_{\frac{1}{2}}$ was 1.5 ± 0.2 hours. In this and many of the subsequent figures, the results are expressed in terms of the estimated number of lymphocytes present, based on measurement of the initial cell number and the DNA content of the cultures.

As described in the theoretical section, (Section 4), this time constant is related to the permeability of the plasma membrane to potassium, the cell size and the membrane potential.

It may also be noted that the K^{42} uptake rate was markedly greater for lymphocytes than for red cells, indicating that in lymphocytes, the turnover rate of exchangeable potassium per hour is 35-50% of the total exchangeable potassium. 'Non-exchangeable' potassium, if it exists, would of course not be detected by this kind of experiment, without knowing the total intracellular potassium content of the cell.

3.4.6. Efflux of K^{42}

In the theoretical analysis of Section 4, it was predicted that the time constant determining the efflux of potassium from the cell would be the same as that for influx. This was tested experimentally as follows.

The cells were 'preloaded' with K^{42} by incubating them with the isotope overnight, and the rate of release subsequently measured after washing the cells in medium-199 and then incubating them for various periods of time in non-radioactive medium. The cells were washed with ice-cold

isotonic KCl solution before their K^{42} content was measured by gamma scintillation counting.

An exponential decrease was obtained over a 3 1/2 hour period, characterized by a $T_{1/2}$ of about 1.7 ± 0.1 hours (see Fig. 3.4.7). A slightly smaller value for the $T_{1/2}$ (1.3 ± 0.1 hours) was observed with cells that had been treated with PHA overnight. This decrease in the value of the $T_{1/2}$ may have resulted from a PHA-induced increase in cell size. PHA had no effect on the $T_{1/2}$ when the mitogen was given at the start of incubation of the preloaded cells in the non-radioactive medium.

Thus, the time constants determinging the uptake of K^{42} into lymphocytes and the rate of release of the isotope were very similar in value, as predicted by Section 4.

3.4.7. Effect of PHA on K^{42} Uptake and Turnover in Lymphocytes

In the presence of PHA, the uptake of K^{42} by the cells was increased. As shown in Fig. 3.4.8., this increase became apparent an hour after PHA administration, and was consistently present during and after the second hour. In the presence of ouabain, the uptake of K^{42} into both PHA-treated and unstimulated cultures was strongly inhibited. Indeed, the uptake of K^{42} into cells which had been in the presence of PHA

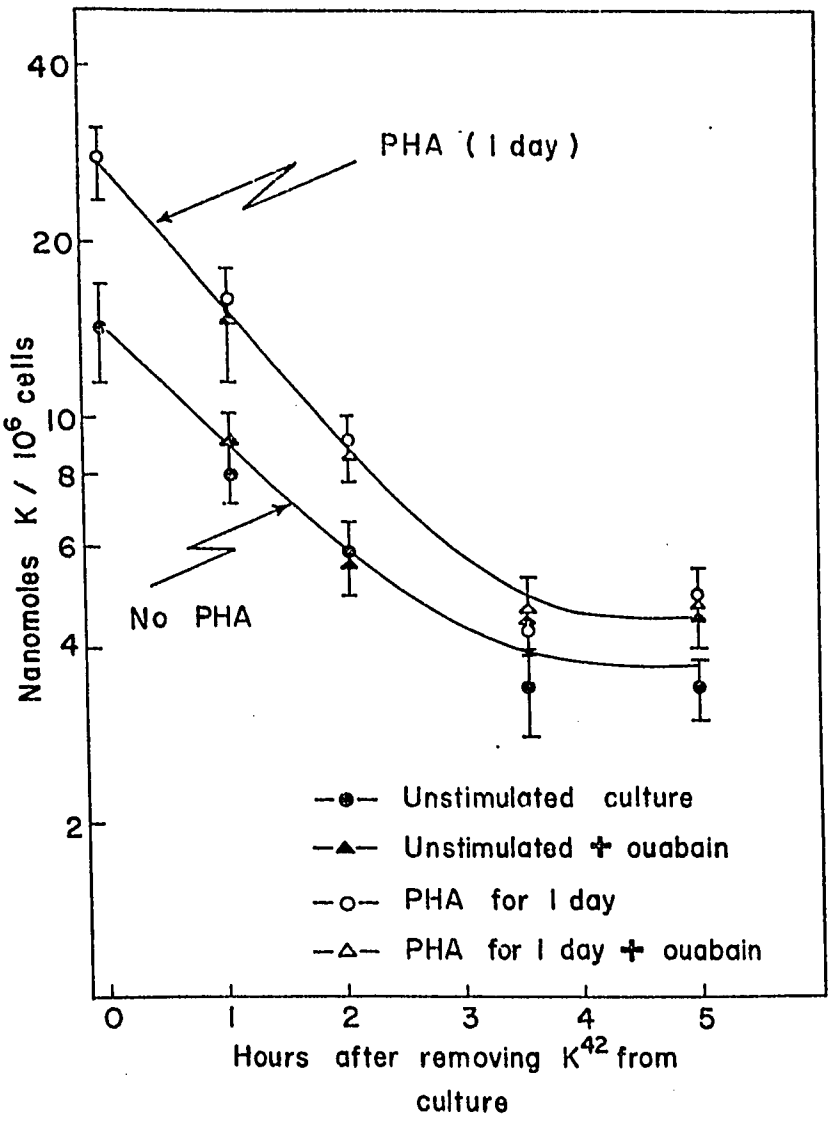


Fig. 3.4.7.

Efflux of K^{42} from 'pre-loaded' lymphocyte cultures.

Lines drawn by eye. $T_{\frac{1}{2}}$ was approximately 1.3 hours (19 hours with PHA) and 1.7 hours (no PHA). Initial value on ordinate represents the total uptake of potassium after a preincubation period in K^{42} of 12 hours, assuming that at the time of measurement, the total potassium inside and outside the cell is at equilibrium. Units of ordinate as in Fig. 3.4.1. Ouabain was added to the cultures (triangles) immediately after washing the cells and transferring them to non-radioactive medium.

Ouabain concentration: 6.2×10^{-7}M

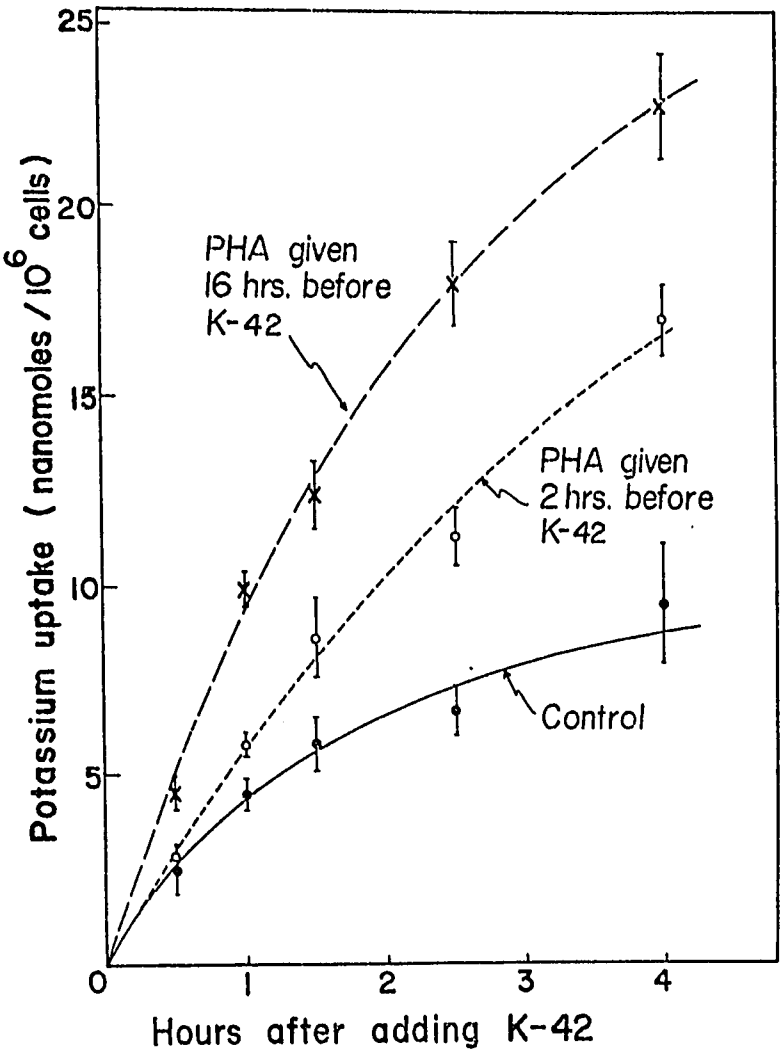


Fig. 3.4.8

Effect of PHA and ouabain on K^{42} uptake by small lymphocytes in vitro.

This experiment was similar to that shown in Fig. 3.4.9. but was carried out over a longer period of time. All cultures were incubated for 21 hours before the addition of K^{42}. Ouabain was added half of the cultures 2-5 minutes before the addition of K^{42}. Lines fitted by eye. Corrected for red cell contribution. Crosses: PHA present for 21 hours before adding K^{42}. Open circles: PHA added 2-5 minutes before K^{42}. Solid circles: No PHA added. Ordinate as in Fig. 3.4.1. PHA: 200 µg/ml.

was reduced by ouabain below the values seen in control cultures.

A similar effect is seen in Fig. 3.4.9. Here a somewhat larger uptake of K^{42} was observed, but the conclusion was the same; PHA caused a marked increase in the potassium uptake into the cell within 2 hours of its administration.

When the cells were washed with ice-cold isotonic sucrose or medium-199 instead of with isotonic KCl, the measured K^{42} associated with the cells was considerably higher. However, PHA also augmented the total bound K^{42} observed under these conditions. As can be seen from Fig. 3.4.10, the amount of K^{+} in the PHA-treated cells was more than 100 nanomoles/10^6 cells, so that there is serious doubt whether these values actually represented intracellular potassium. The experiments carried out to investigate this point have already been described in Section 3.4.3.

As shown in Table 3.4.3. PHA did not increase the rate of K^{42} uptake by red cells.

The results obtained suggested that PHA increased the active transport of potassium into lymphocytes, and further experiments were carried out to elucidate this finding.

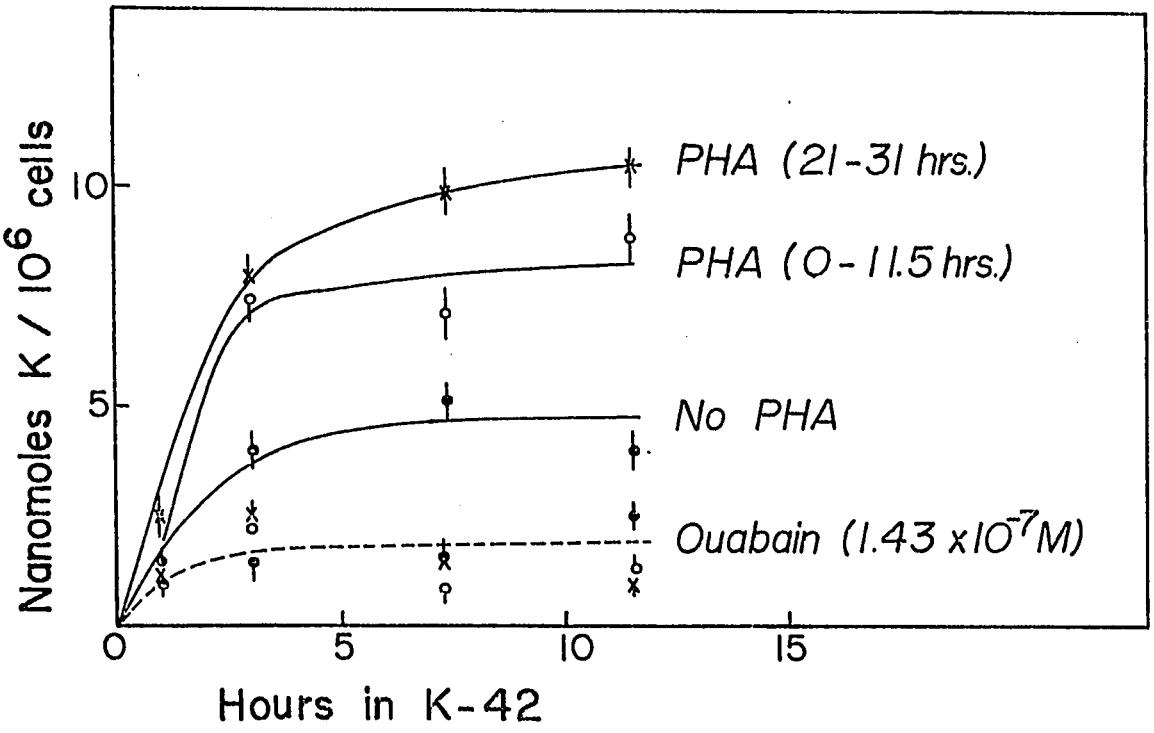


Fig. 3.4.9

Effect of PHA on K^{42} uptake by small lymphocytes in vitro.

All cells were cultured for a total period of 16 hours before adding K^{42}. Lines fitted by eye. Ordinate as in Fig. 3.4.1. PHA: 200 µg/ml.

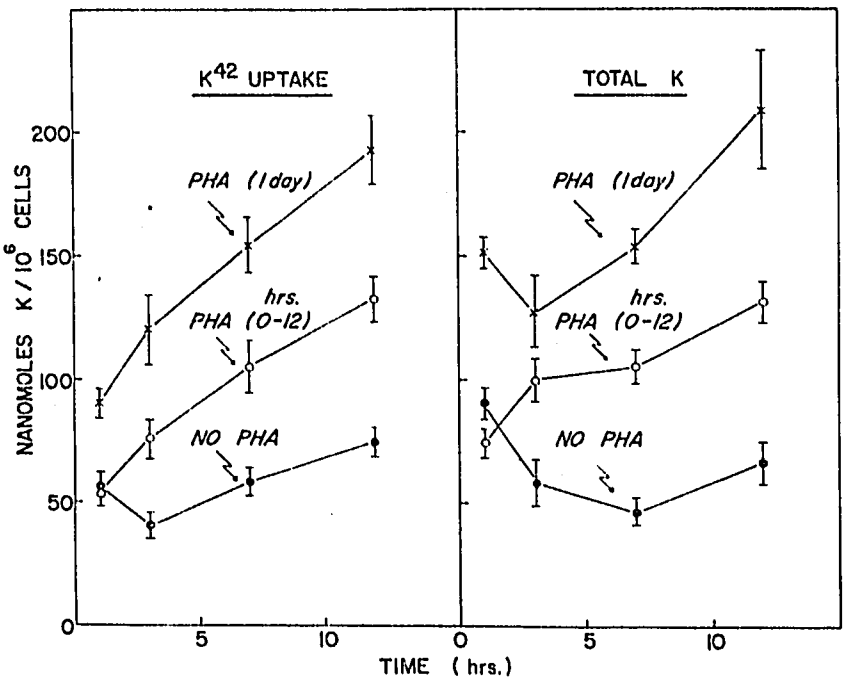


Fig. 3.4.10

Effect of PHA on the uptake of K^{42} and on the total potassium present in lymphocytes washed with ice-cold isotonic sucrose.

Upper curve; cells treated with PHA approx. 19 hours previously. Middle curve; cells treated with PHA 2-5 minutes before adding PHA. Lower curve; no PHA added. Ordinate (for radioactive measurements) as with Fig. 3.4.1.

Table 3.4.3.

K^{42} uptake by red cells, with or without added PHA

Time with tracer (hrs)	Uptake of K^{42} (nanomoles K/10^6 cells)	
	No PHA	PHA
0.53	0.08 ± 0.01	0.08 ± 0.01
1.0	0.11 ± 0.01	0.11 ± 0.01
2.06	0.24 ± 0.03	0.20 ± 0.01
3.0	0.31 ± 0.01	0.26 ± 0.01
4.53	0.46 ± 0.02	0.42 ± 0.05
6.0	0.57 ± 0.06	0.38 ± 0.05

3.4.8. Relationship of K^{42} Influx to the Extracellular Potassium Concentration

In order to determine whether the kinetics of K^{42} uptake by the cell resembled saturation kinetics, the rate of uptake of the isotope was measured in relation to the extracellular potassium concentration. One would expect a hyperbolic curve (see theoretical concepts in Section 4), superimposed on a linear relationship to the extracellular potassium concentration representing passive diffusion.

3.4.8.1. Low external potassium concentrations (1-6 mM)

The culture medium was replaced by a balanced salt solution which did not contain potassium. PHA was added to half of the cultures at the time of changing the medium. One hour later, K^{42} was added so that the final potassium concentration varied from 1 and 6 mM; the K^{42} had first been diluted appropriately with NaCl to obtain an isotonic solution, and 0.5 ml aliquots were added to each 3 ml culture. The reason for waiting an hour before adding K^{42} was to avoid rapid changes that might have occurred due to the possibly traumatic procedure of transferring the cells to the balanced salt solution after centrifugation and washing. This treatment did not affect the microscopic appearance, viability or PHA response of the cells.

Three hours later (4 hours following the addition of PHA), the cultures were washed three times in ice-cold isotonic KCl and counted for gamma activity.

As shown in Fig. 3.4.11, there was a gradual increase in the rate of K^{42} uptake by the cells which became progressively less pronounced with increasing extracellular potassium concentrations. The results could also be expressed graphically as a Lineweaver-Burke double reciprocal plot, assuming that the uptake of K^{42} after a 3 hour period was proportional to the influx rate of potassium uptake by the cells. A straight line could be plotted through the experimental points (Fig. 3.4.12), and it was found that while PHA caused a change in the slope and vertical intercept, the lines for control and PHA-treated cells almost met at the same intercept on the X-axis. Thus, from an operational point of view, PHA did not significantly change the K_M for K^+ transport, but did affect the V_{max}. The K_M for potassium was found to be 1.5 mM, and the V_{max} was found to have increased from 9.6 to 15.9 nanomoles $K^+/10^6$cells/3hours.

These results indicate that over the potassium concentration range of 1-6 mM, the uptake of K^{42} into lymphocytes can be described operationally by a relationship equivalent to that of simple saturation kinetics. A 'Hill' plot was made

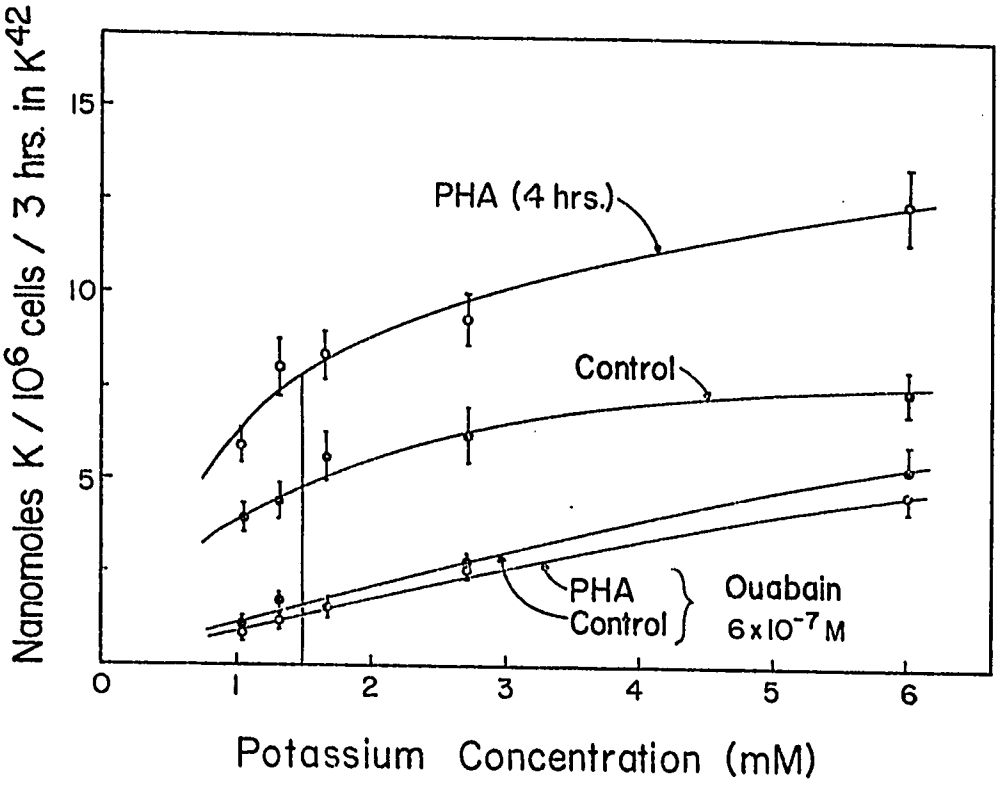


Fig. 3.4.11

K^{42} uptake into lymphocytes at different potassium concentrations.

Cells were cultured for one hour in potassium-free balanced salt solution containing glucose and PHA, before addition of K^{42}. Cells were washed with ice-cold isotonic KCl and radiactivity measured after 3 hours of incubation with the radioisotope. Vertical line is at estimated K_M = 1.5 mM for both control and PHA-stimulated cells.

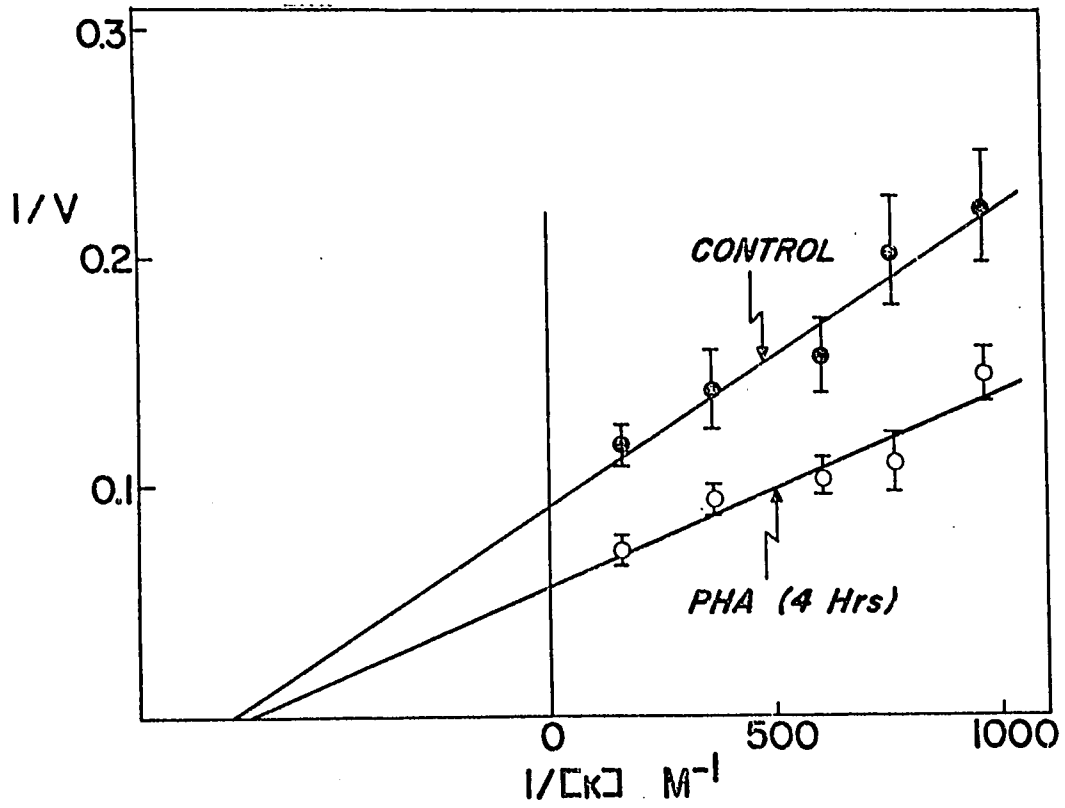


Fig. 3.4.12.

'Lineweaver-Burke' double reciprocal plot of data presented in Fig. 3.4.1. Lines are calculated by least squares. This calculation was based on an influx assumed to be proportional to the uptake of K^{42} over a 3 hour period.

to determine whether the exponent 'n' was equal to unity, as it should be if there was no interaction between the K^+-binding sites. When this was done, both PHA-treated and control results lay on the same line and the exponent 'n' was found to be 0.93 ± 0.07 by least squares calculation (Fig. 3.4.13).

The conclusions from these results were (a) that PHA altered the rate of potassium transport by inducing an increase in the V_{max}, but without causing a corresponding change in the K_M, and (b) that there was no interaction between the K binding sites of the transport system.

3.4.8.2. High external potassium concentration (6-21.9 mM)

Different results were obtained when a similar experiment was carried out at higher potassium concentrations. As shown in Fig. 3.4.14, control cells showed a pronounced uptake of potassium which appeared to be related linearly to the external potassium concentration. PHA-treated cells also showed a similar linearity, with the identical slope, but increased by a constant separation from the control slope by approximately 5.3 nanomoles/10^6cells/3 hours (by least squares calculations), a value very similar to the difference between the V_{max} values of a separate experiment in Section 3.4.8.1,

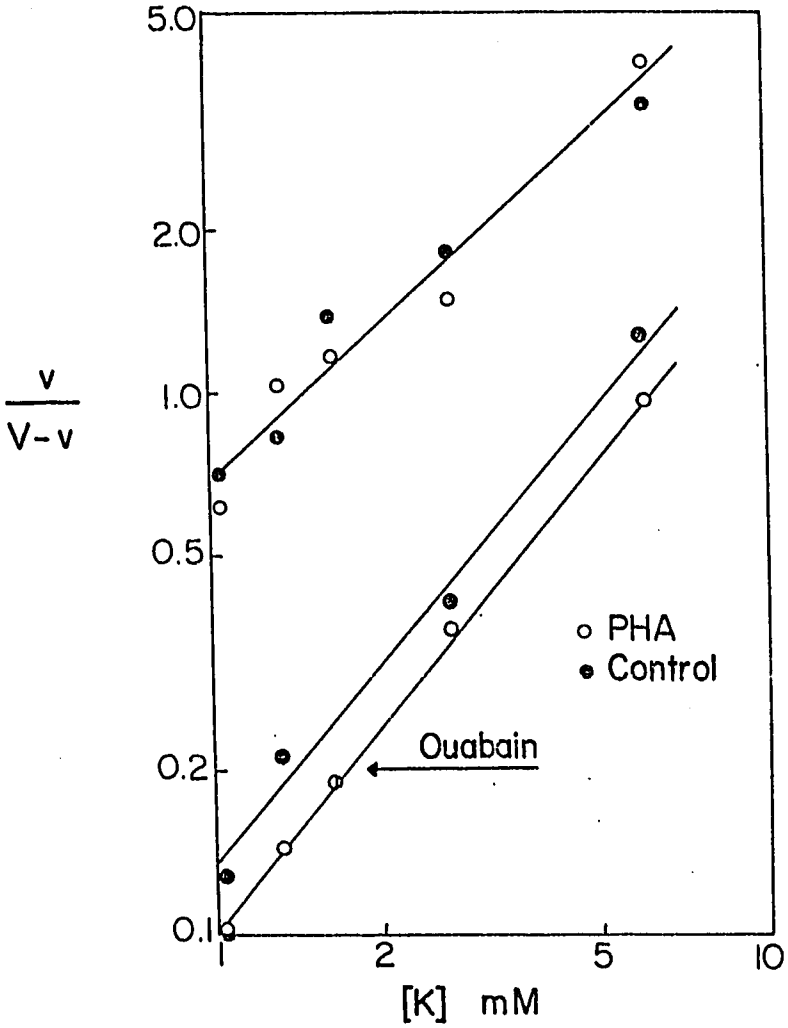


Fig. 3.4.13

'Hill' plot of K^{42} uptake data, with respect to the extracellular potassium concentration.

Upper plot: No ouabain. Least squares line was calculated by pooling one PHA and non-PHA-treated values. Slope n = 0.93± 0.7.

Lower plots: ouabain, 6×10^{-7} M. Least squares lines were calculated separately for PHA and non-PHA treated values. In each case, n = 1.16

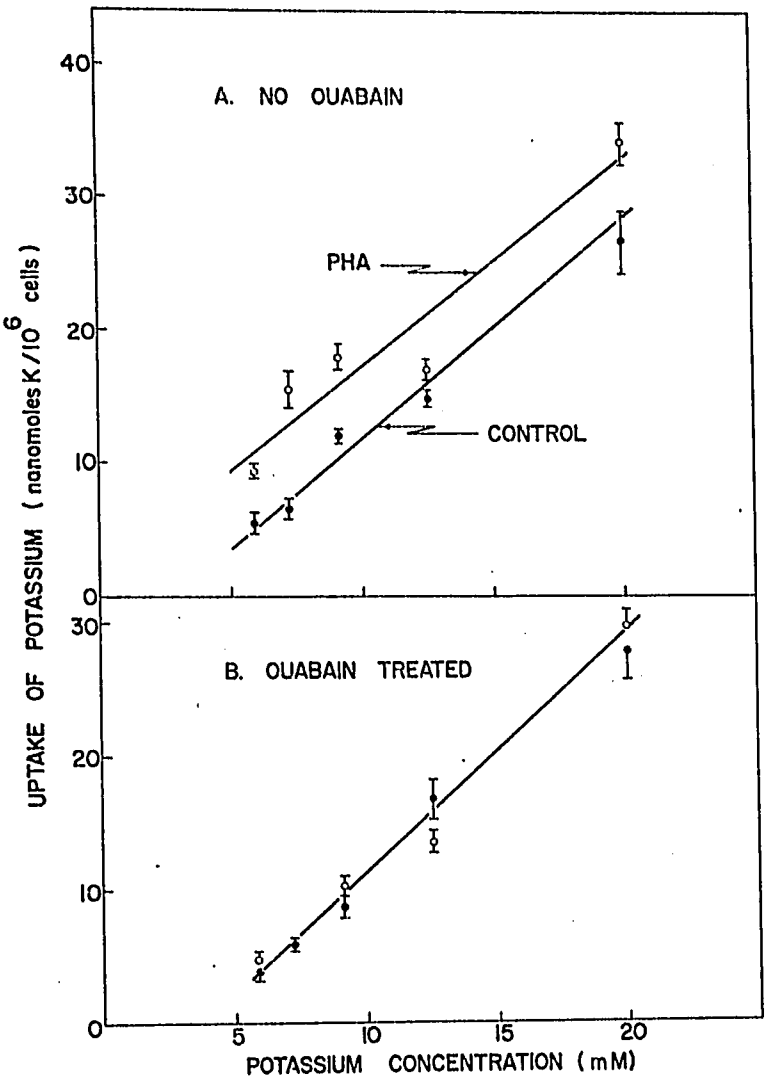


Fig. 3.4.14

Effect of varying the potassium concentration of the medium on the uptake of K^{42} by control, PHA stimulated and ouabain treated cells.

Ordinate as in Fig. 3.4.1. This figure differs from that of Fig. 3.4.11 by the higher range of potassium values, which were between 6 and 21.9 mM. Cells washed with ice-cold isotonic KCl.

Upper graph; no ouabain. Low graph: ouabain concentration was 9×10^{-7}M. In both graphs, solid circles are for unstimulated lymphocytes. Open circles are for PHA-treated cells.

which was 6.3 nanomoles/10^6cells/ 3 hours.

In the presence of ouabain at 9×10^{-7}M, the slope of the curve was the same (lower graph of Fig. 3.4.14), but in this case, PHA had absolutely no effect; the graphs for PHA-treated and control cells were identical. However, at the lowest potassium concentration, the K^{42} uptake by the ouabain-treated cells was less than that of the control cells.

It is also noted that the control and ouabain-treated cells yielded plots which if projected downwards, passed below the origin.

3.4.8.3. Low external potassium concentration (0.6-6 mM), and sucrose wash

The same experiment as in Section 3.4.8.1 was carried out, but the cells were washed with ice-cold isotonic sucrose instead of ice-cold isotonic KCl. As seen in Fig. 3.4.15, PHA caused a pronounced increase of K^{42} associated with the cells, but the total amount of potassium (when calculated from the known specific activity in the medium) was very much higher than obtained after washing with KCl (Fig. 3.4.11). While the curves were similar in shape to those of Fig. 3.4.11, the magnitude was much greater - as also seen with Fig. 3.4.10.

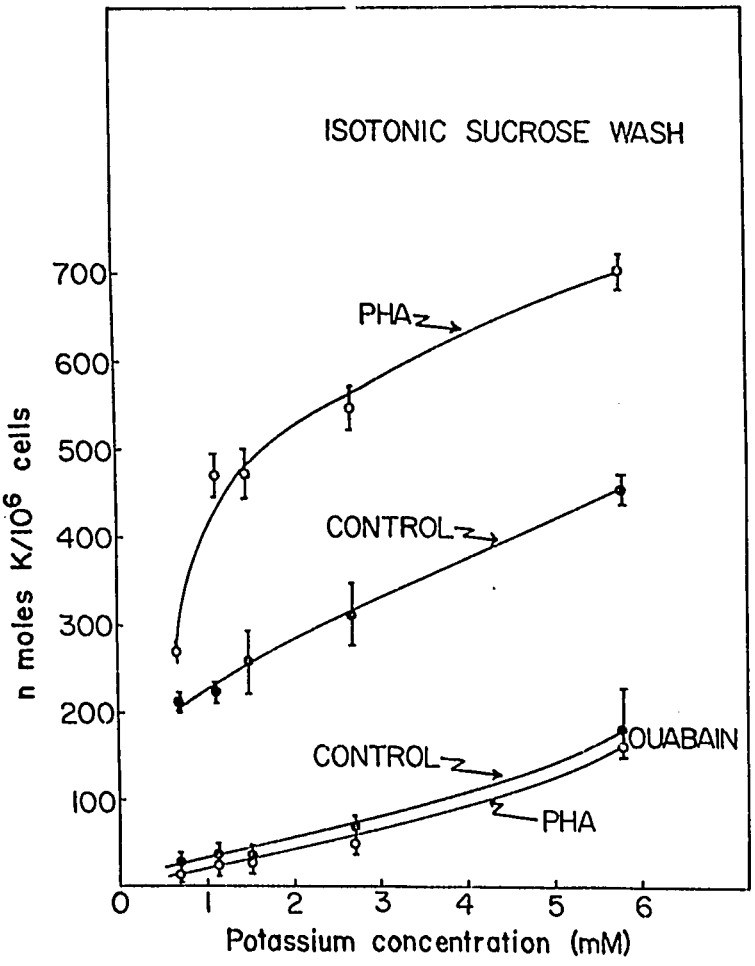


Fig. 3.4.15

Effect of varying the potassium concentration of the medium on the uptake of K^{42} by control, PHA stimulated and ouabain treated cells.

This experiment was the same as that shown in Fig. 3.4.11, except that the cells were washed with ice-cold sucrose instead of KCl. Ordinate as in Fig. 3.4.1.

Ouabain (2.2×10^{-7}) inhibited the binding of K^{42} but again, the amount of residual radioactivity associated with the cells was much greater than after the KCl wash.

3.4.9. Effect of Ouabain on the Uptake and Efflux of K^{42} by Control and PHA-Stimulated Cells

Ouabain has already been shown to be strongly inhibitory to the cellular uptake of K^{42}, both in control and PHA-stimulated lymphocytes (Fig. 3.4.8). Previous results had also shown that excess potassium could reverse the inhibitory action of ouabain on the nucleic acid synthesis of stimulated lymphocytes, (Section 3.2.3.4).

Although ouabain profoundly affected the uptake of K^{42} by the cells, it had no effect on the time constant characterizing the rate of efflux, either in control cells or after the addition of PHA (Fig. 3.4.7). The effect of excess extracellular potassium on the action of ouabain on K^{42} uptake was therefore examined.

In Fig. 3.4.11, it is shown that when the external potassium concentration was increased, the inhibitory action of ouabain on K^{42} uptake was reduced. A double reciprocal plot (Fig. 3.4.16) indicated that the effect of ouabain resembled competitive kinetics, but that neither of the

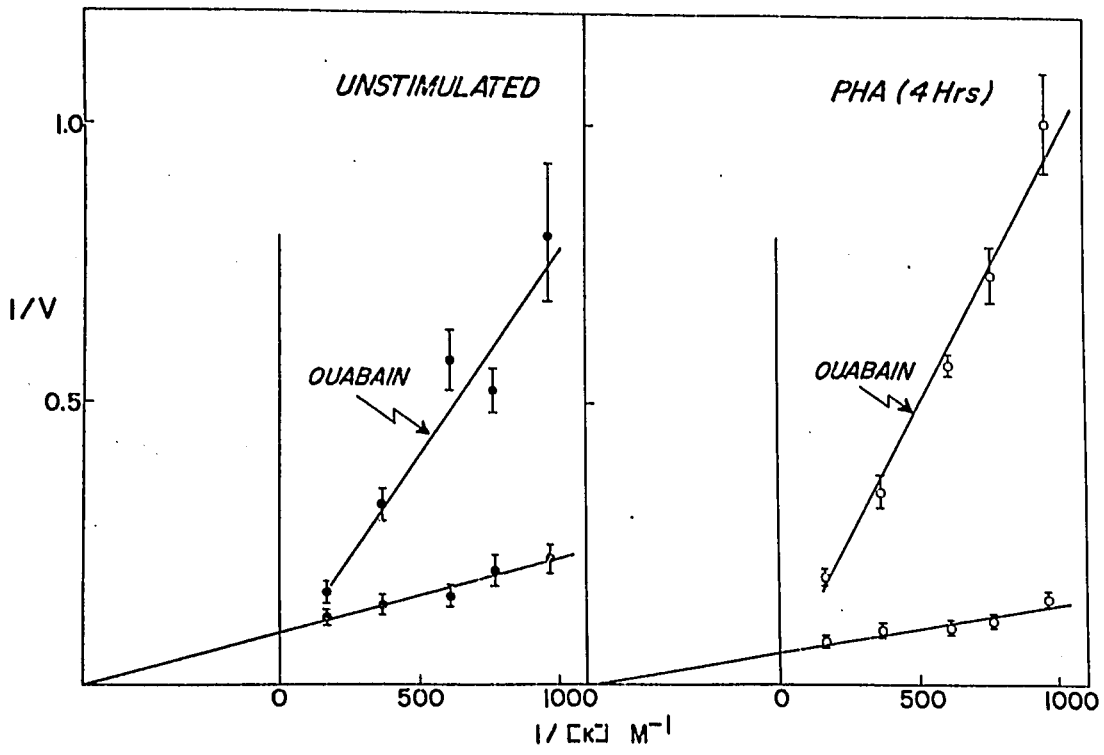


Fig. 3.4.16

'Lineweaver-Burke' double reciprocal plot of the effect of ouabain on the K^{42} uptake at different extracellular potassium concentrations.

These data are from the same experiment as that shown in Fig. 3.4.11.

curves for ouabain-treated cells, if extended, accurately passed through the intercept for non-ouabain-treated cells with the Y axis. A 'Hill' plot of these data is given in Fig. 3.4.13. In the experiments shown, a value of n greater than unity was obtained. Repeated experiments showed that the values of 'n' in the presence of ouabain varied between 1.0 to 1.2.

As shown in Fig. 3.4.15, ouabain also reduced the K^{42} associated with the cell, remaining following a sucrose wash, but the amount of radioactivity present was considerably greater than after washing with isotonic KCl.

Ouabain did not affect the marked uptake of K^{42} which was characteristic of high external potassium concentrations (Fig. 3.4.14).

3.4.10. Effect of Cycloheximide and Actinomycin-D on the Accelerated Influx of K^{42} Caused by PHA

The results described in 3.4.8. suggested that an early effect of PHA was to activate the enzymatic process of potassium uptake by the cell. Although the K_M did not appear to have changed following PHA administration, the V_{max} increased. This could happen if (a) more enzyme molecules were synthesized by the cell during the early phase of PHA

activation, or (b) if the same amount of enzyme were present, but some qualitative change was induced in the enzyme system (and in the cell membrane) which led to an increase in the rate of potassium uptake.

Possibility (a) was tested by administering actinomycin-D and cycloheximide in concentrations known to be effective inhibitors of RNA and protein synthesis in these cells (see Sections 3.3.14 and 3.3.15). The results are shown in Fig. 3.4.17. Neither drug had any effect on the stimulation of K^{42} uptake. The results indicated that when protein and RNA synthesis were suppressed, the stimulation of K^{42} uptake by PHA remained manifest. Hence, possibility (a) was rejected.

3.4.11. Measurements of Total Potassium in Lymphocyte Cultures

The results using K^{42} suggested the possibility that the total potassium content of the cell might increase following the administration of PHA. However, the possibility of more than one potassium compartment associated with the cells complicated the interpretation of such measurements.

Fig. 3.4.10 shows preliminary results of total potassium measurements carried out on sucrose washed cells; these measurements were carried out on cells which had previously been given K^{42}, and the figures show both total K and K^{42} uptakes.

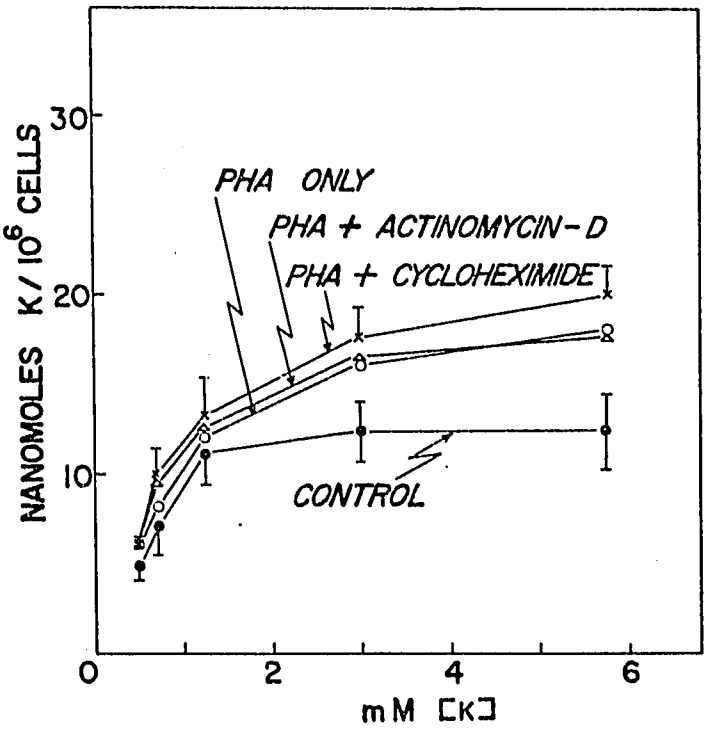


Fig. 3.4.17

Effect of actinomycin-D and cycloheximide on K^{42} uptake by control and PHA stimulated lymphocytes.

This experiment was similar to that illustrated in Fig. 3.4.1. The drugs were added 5 minutes before the addition of PHA. The concentrations of the drugs were for actinomycin-D, 4.0×10^{-7}M, and for cycloheximide, 1.1×10^{-4}M.

The results were remarkably similar; PHA caused both an increase in the K^{42} uptake, and also of the total potassium of the cultures. Not shown are sodium determinations which in all cases, were below detectible levels by the method used. In the presence of ouabain, the total potassium decreased, and sodium became detectable in the cells after 5-7 hours with the glycoside.

It is emphasized that these results obtained with sucrose washed cells may not represent the true intracellular potassium.

Assuming a mean lymphocyte volume of 250 μ^3 (Westring et al, 1968, Lichtman and Weed, 1969), the values in Fig. 3.4.10 would give an internal potassium concentration of about 200 mM, and possibly more after PHA administration. On the other hand, preliminary measurements made after washing the cells with isotonic RbCl suggest a more reasonable internal potassium concentration of 80-100 mM for unstimulated lymphocytes increasing to about 120 mM during the first few hours with PHA.

3.4.12. Relationship of Transformation to the External Potassium Concentration

The results already presented suggested that transformation was dependent on the function of the Na-K-ATPase,

and that an activation of the transport mechanism was an early consequence of PHA stimulation which resulted in an increased uptake of potassium. It was inferred from these results that transformation was dependent on the transport mechanism, and specifically on the transport of K^+ into the cell. A consequence of this hypothesis was that the degree or rate of transformation would be dependent on the external potassium concentration.

Accordingly, cells were treated with PHA and cultured in specially prepared medium-199 without potassium. Potassium was added to give concentrations ranging from 0.5 to 21.0 mM. DNA synthesis was measured after the first and second day of culture with PHA by administration of H^3-thymidine over a 24 hour period. The results are shown in Figures 3.4.18 and 3.4.19.

At low potassium levels, the DNA synthesis occurred but was very low. The degree of DNA synthesis was closely related to the external potassium concentration. The concentration of potassium required for half maximal DNA synthesis was about 1.7 mM, a value very close to that required for half saturation kinetics of the transport of K^{42} (Section 3.4.8.1). The shape of Fig. 3.4.19 suggests that DNA synthesis may be dependent on potassium site interaction. A Hill plot of the data in this curve gives a value for $n = 1.4 \pm 0.2$, as shown in Fig. 3.4.20.

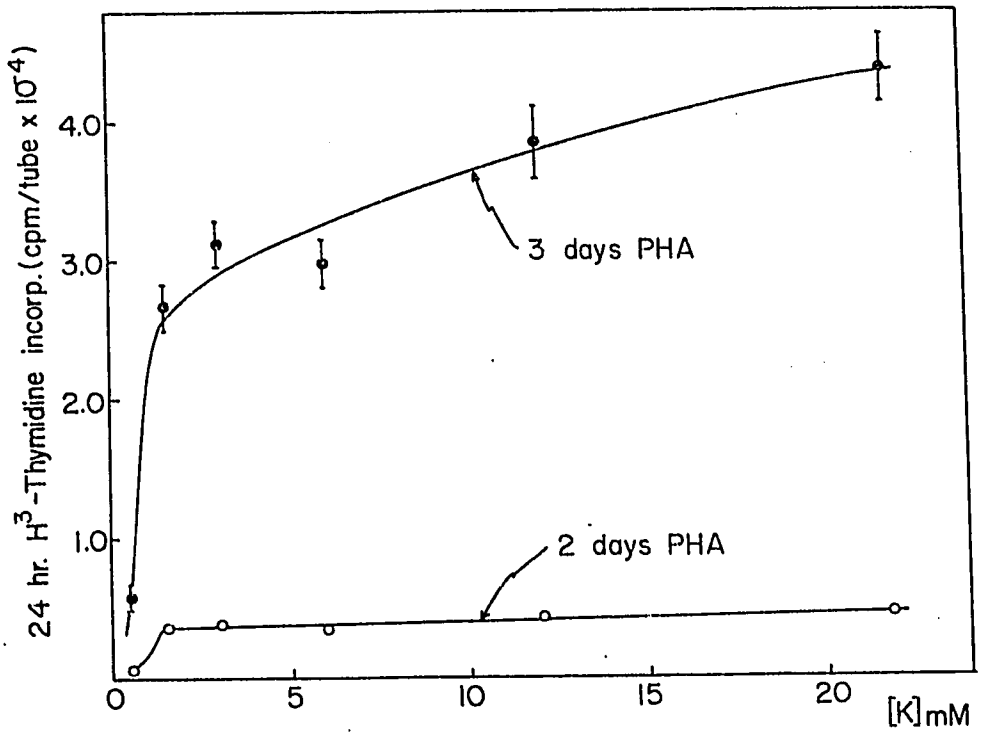


Fig. 3.4.18

Effect of varying and high concentrations of potassium on the degree of DNA synthesis induced by PHA.

PHA concentration was 200 µg/ml. Medium was prepared at various potassium concentrations, all other constituents being unchanged except for sodium, which was reduced by about 15% at the highest potassium levels (21.9 mM). H^3-thymidine was added after the second day of culture, and the degree of incorporation measured after a further 24 hour period of incubation.

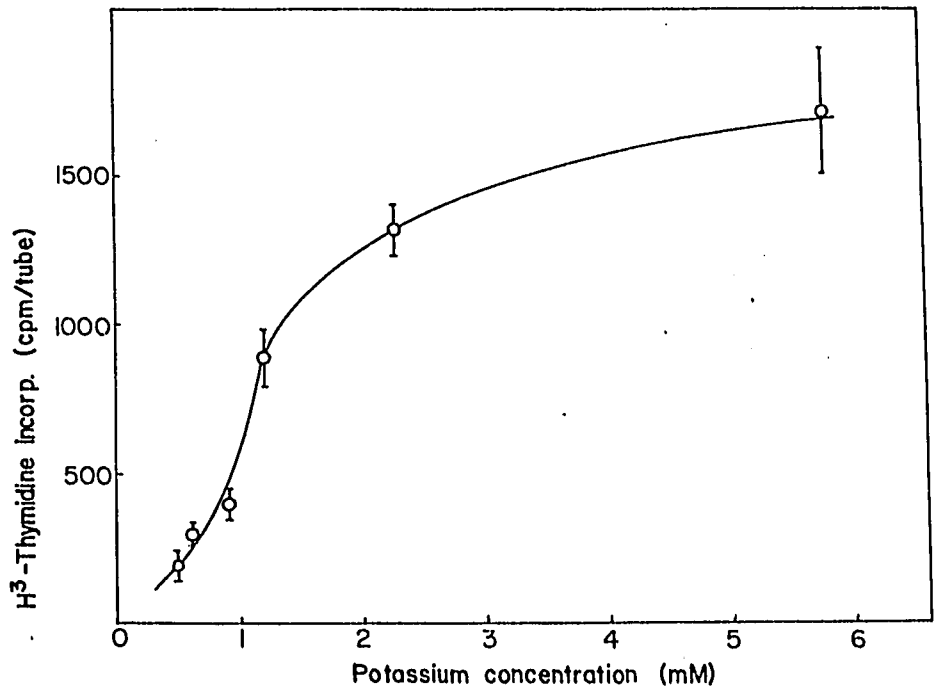


Fig. 3.4.19.

Effect of varying and low concentrations of extracellular potassium on the stimulation of DNA synthesis by PHA.

PHA concentration was 200 µg/ml H^3-thymidine was added after one day in culture with the mitogen, and the degree of incorporation into DNA measured after a further 24 hours of incubation period. At the lowest potassium concentrations, the sodium concentration was increased above the usual value (138 mM) by approximately 3%.

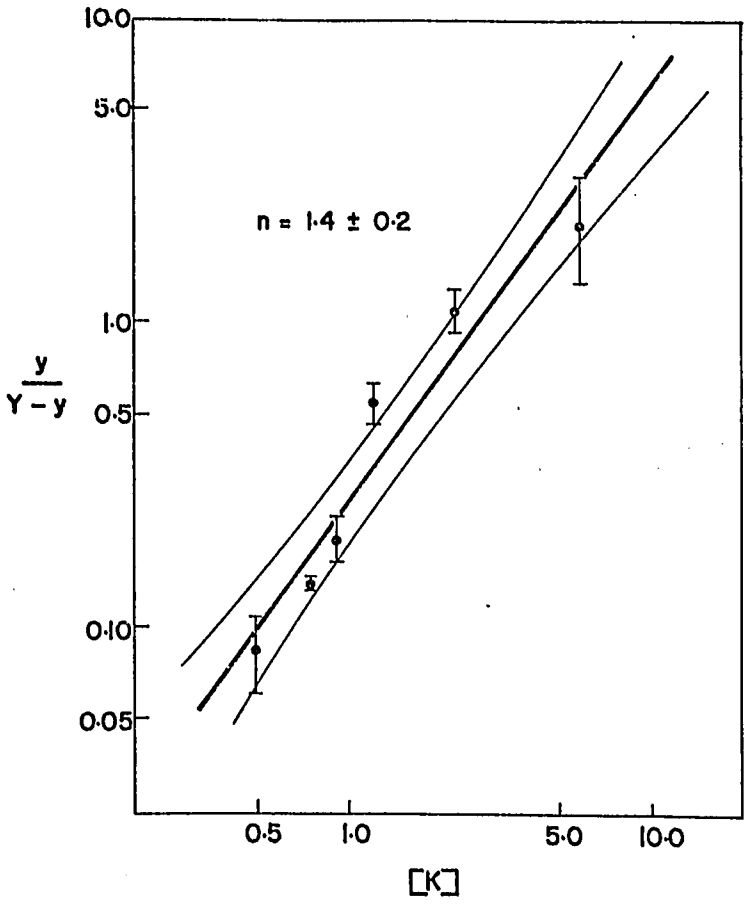


Fig. 3.4.20

Relationship between the rate of DNA synthesis and the extracellular potassium concentration. 'Hill plot'.

The data used in this plot were taken from the experiment illustrated by the results in Fig. 3.4.19. In deriving the units of the ordinate, 'y' was the total incorporation of H^3-thymidine over a 24 hour period, and 'Y' was the estimated maximum rate of incorporation over the same period of time. The value of 'Y' was estimated by a reciprocal plot (Lineweaver-Burke) of the H^3-thymidine incorporation versus the extracellular potassium concentration, extrapolating to the intercept on the vertical axis.

Error lines represent one standard deviation.

3.4.13. Uptake of Na^{22}

Some measurements were carried out on the uptake of Na^{22} into unstimulated and PHA-treated lymphocytes. The amount of radioactivity taken up by the cells was extremely low, but PHA-treated cells consistently showed slightly less cellular Na^{22} than did the controls. Over a 24 hour period, the Na^{22} values averaged 84 ± 6% of the control uptake measurements. At the very low levels of cellular Na^{22} it was not possible to measure an efflux rate. In the presence of ouabain 2.2×10^{-7}M, a marked increase in cellular sodium was observed, becoming pronounced after 7 hours with the glycoside.

3.4.14. Summary of Chapter

1. Potassium is taken into the cell and diffuses out at a rate characterized by a time constant varying (for lymphocytes from different donors) between 1.0 and 2.5 hours, with a mean of 1.5 ± 0.2 hours.

2. Ouabain profoundly inhibits the uptake but does not affect the efflux of potassium.

3. The rate of uptake of potassium may be considerably faster than that of red cells. Whereas in the latter, about 1.8% of the total cell potassium turns over per

hour, in the lymphocytes, about 35-50% of the total exchangeable potassium turns over per hour.

4. PHA causes an acceleration of the uptake of potassium into lymphocytes but not into red cells.

5. The rate of uptake of K^{42} by the cells was related to the external potassium concentration: at extracellular potassium concentration of 1-6 mM, the relationship was characterized by saturation kinetics in which the K_M was about 1.5 mM and was inhibited by ouabain. At higher potassium concentrations K^{42} was taken up by non-saturation kinetics. This mode of uptake was not affected by ouabain.

6. The effect of PHA was to increase the V_{max} without corresponding changes in the K_M. The V_{max} changed from 9.6 to 15.9 nanomoles $K^+/10^6$cells/3 hours.

7. The augmentation of K^{42} uptake by PHA was unaffected by cycloheximide and actinomycin-D at concentrations that prevented RNA and protein synthesis.

8. The relationship between K^{42} uptake and external [K] was characterized by a 'Hill' exponent 'n' of 0.93 ± 0.07.

9.

9.1 Washing the cells with medium-199, isotonic sucrose, choline chloride, or NaCl led to much higher K^{42} uptake values in comparison to washing with isotonic KCl.

9.2 The increment above the values obtained by the KCl wash was removed rapidly during the first 10 minutes after washing with KCl,

or

9.3 after adding 0.25% trypsin for 15 minutes.

9.4 The total K^{42} in medium-199 washed cells diffused out more rapidly when the cells were resuspended in isotonic KCl than with medium-199, but diffused out at the same rate when KCl-washed cells were resuspended in either solution.

10. Total potassium measurements indicated that after PHA administration, there was an increase in the amount of this cation associated with sucrose washed cells. A lesser increase was observed for KCl-washed cells.

11. DNA synthesis in transforming lymphocytes was found to be strongly dependent on the extra-cellular potassium concentration.

3.5. SUPPLEMENTARY STUDIES

3.5.1. Introduction to Section

The results documented in previous sections provided strong experimental evidence that small lymphocyte transformation is associated with, and probably dependent on, changes occurring in the cell membrane. These changes in the cell membrane function would appear to lead, directly or indirectly, to alterations in cellular function. Further studies were carried out because of the theoretical and practical significance of the initial findings.

This work was therefore supplemented by studies on (a) respiratory activity of lymphocytes, (b) ATPase activity, (c) effects of ouabain on lymphocytes obtained from patients with leukemia and cystic fibrosis, (d) the effect of ouabain on the immune response in vivo, and (e) the binding of fluorescein-labelled PHA to lymphocytes. It seemed desirable to present some of the results obtained here, although these investigations are continuing since some of them may represent potentially fruitful directions of future activity.

3.5.2. Oxidative Metabolism of Lymphocyte and Leukocyte Cultures

Among the metabolic functions of the cell which are affected by the intracellular cation level, respiration is of particular interest because of its importance in cellular energetics. The relationship

between respiratory activity and lymphocyte transformation is not yet clear (Section 1.5.4). Measurements of respiration in unstimulated and PHA-stimulated cells were therefore initiated. Information on the respiratory response of these cells to certain drugs was also obtained.

3.5.2.1. Respiration of Unstimulated Lymphocytes

In Fig. 3.5.1, the respiration rate of lymphocytes in vitro is plotted with respect to the concentration of cells present in the culture. It was apparent that 'crowding' of the cells was associated with a decrease in the respiratory activity per cell. The values obtained were similar to those of Hedeskov and Essman (1966) (dotted line on the graph). For most of the experiments described, the cell density used for respiratory measurements was close to 10^7 cells/ml, that is, about 10-20 times the concentration of the cells when they were cultured. This concentration was necessary since the respiration of lower numbers of cells was extremely low and difficult to measure.

3.5.2.2. Effect of PHA on Respiration of Lymphocytes

This experiment was carried out both with leukocytes and cultures of lymphocyte purified to contain 92-98% small lymphocytes. The effects of PHA on respiration are shown in Figs. 3.5.2 and 3.5.3. Pronounced increases in respiratory activity were observed in the leukocyte cultures after some hours of PHA. The increase in respiratory activity occurred during the first 12 hours after the administration of PHA to leukocyte cultures, but the values obtained after one

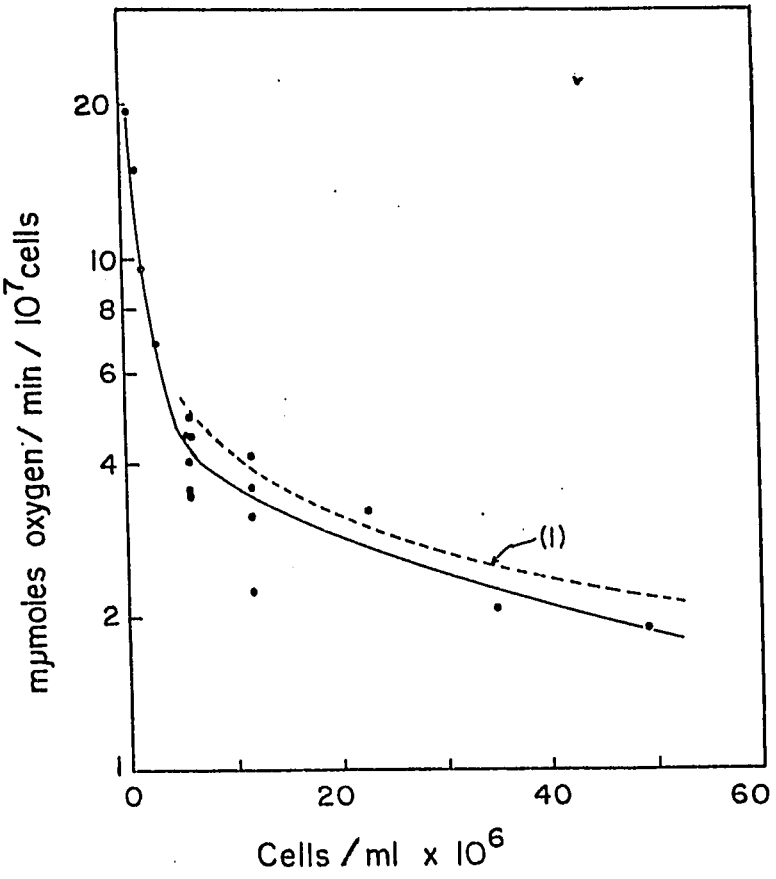


Fig. 3.5.1

Respiration of lymphocyte cultures; effect of cell concentration.

All measurements shown are of cells from the same culture of purified lymphocytes. Dotted line (1) represents results obtained by Hedeskov and Essman (1966). Lines fitted by eye. Cells were cultured in vitro for 18 hours before respiratory measurements were carried out.

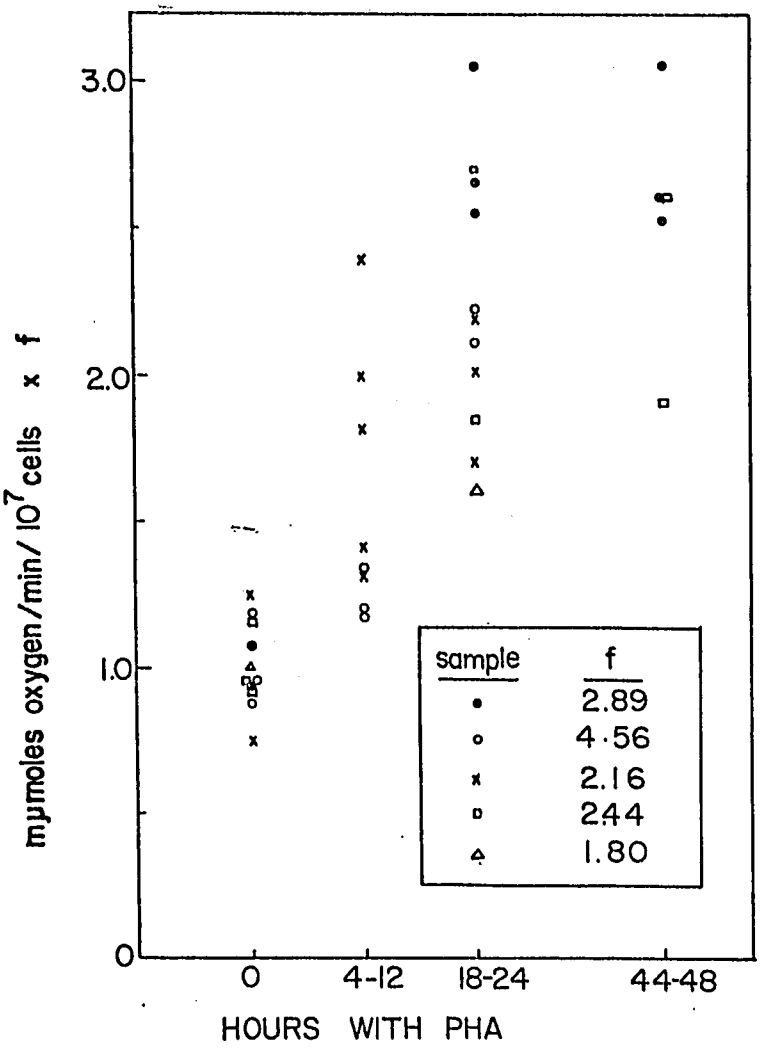


Fig. 3.5.2

Effect of PHA on respiration of leukocyte cultures.

Symbols express results obtained from 5 separate experiments. Results are normalized in graphical presentation to give a mean control (untreated with PHA) value of 1. All cells had been cultured in vitro for a total time of 48 hours before measurements were carried out. PHA was added at various times during the 48 hour culture period. To obtain actual respiratory rate, multiply by f = 2.89 (o); 4.56 (o); 2.16 (x); 2.44 (□); and 1.80 (△).

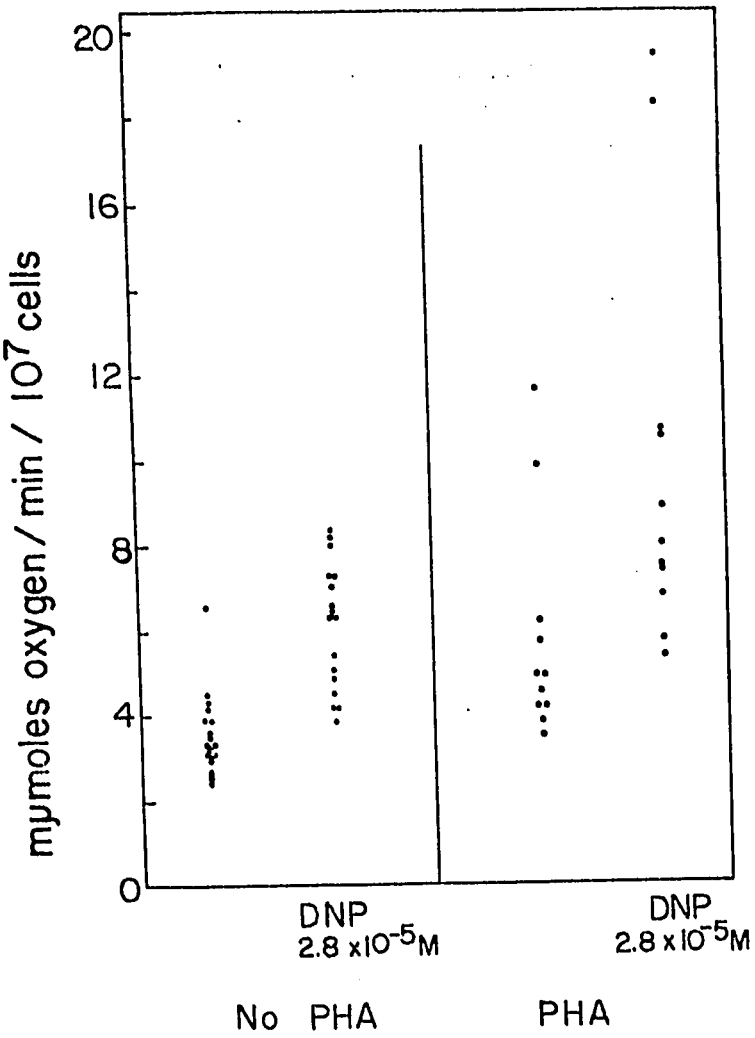


Fig. 3.5.3

Effect of PHA and dinitrophenol (DNP) on respiration of purified lymphocytes.

PHA (200 μg/ml) present for 17-24 hours and paired controls were cultured for the same period of time without PHA. DNP was added directly into the polarograph cell after obtaining a recording for the respiratory rate (as in Fig. 3.5.7). Statistical analyses showed that both coupled and DNP-coupled respiration was increased ($p<0.01$) in PHA-treated cultures.

day did not differ from those observed after two days in the presence of phytomitogen. Separated lymphocytes treated with PHA showed a lesser degree of respiratory stimulation which was, however, significantly increased above control levels ($P < 0.01$), as shown in Table 3.5.1.

3.5.2.3. Effect of Ouabain, and Ouabain plus Potassium on the Respiration of PHA-Treated Leukocytes

The glycoside had no immediate effect on the respiration of these cells, but an inhibition of the rate of oxygen uptake was noted after about 6 hours (Fig. 3.5.4). However, the reduced respiratory rate remained constant thereafter. Excess extracellular potassium (32.9 mM) tended to reverse the inhibitory effect of ouabain (2.2×10^{-7}M) on respiration (Figs. 3.5.5 and 3.5.6), although not when relatively high ouabain concentrations (2.2×10^{-6}M) were used. This effect was similar to the relationship between the effects of K^+ and ouabain on K^+ transport (Section 3.4.8) and nucleic acid synthesis (Section 3.2.3).

A parallel experiment was done on the same cultures in which ATPase activity was measured. This is described in Section 3.5.3.2.

Table 3.5.1. (a)

Statistical Analysis

Respiration Values (mμmoles $O_2/10^7$ cells) for PHA-Treated Lymphocyte Cultures.

Exper. No.	Control		Treatment 18-30 hrs.		Probability
	N	Mean	N	Mean	P
294	2	5.55	2	10.84	< 0.005
299	6	2.84	3	3.98	0.025
302	3	3.13	3	4.44	0.023
306	2	4.12	3	4.63	0.019
Overall	13	3.52	11	5.80	P < .005

Source of Variance	Degrees of freedom	Sum of Squares	Mean Square
Treatment	7	109.21	
Error	16	6.76	0.422
Total	23	115.97	

Table 3.5.1. (b)

Respiration Values (mμmoles $O_2/10^7$ cells) for
PHA Treated Lymphocyte Cultures
(DNP added)

Exper.	Control		PHA Treatment 18-30 hrs.		Probability
No	N	Mean	N	Mean	P
294	2	6.35	2	19.30	< .005
299	6	4.73	3	6.01	.005
302	3	6.04	3	7.95	.031
306	2	8.27	3	9.78	.029
Overall	13	5.83	11	9.99	< .005

Source of Variance	Degree of Freedom	Sum of Squares	Mean Square
Treatments	7	356.35	
Error	16	20.55	1.28
Total	23	376.88	

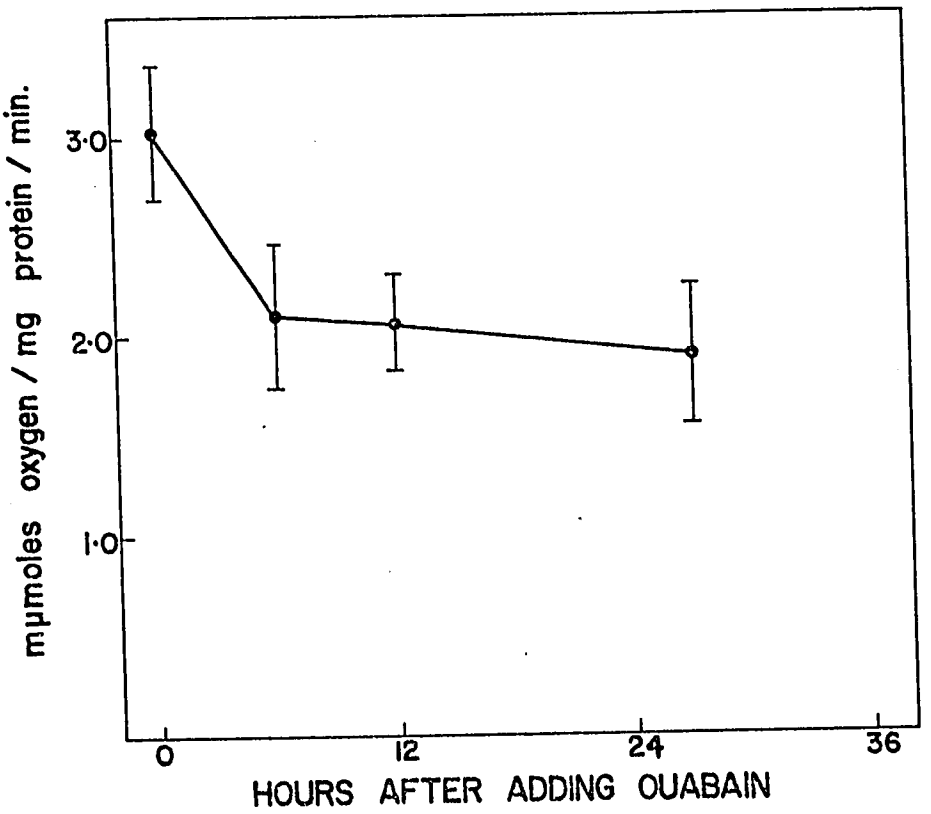


Fig. 3.5.4

Effect of ouabain, given for various times, on the respiration of stimulated leukocyte cultures that had been treated with PHA for 3 days. Ouabain was added 6 hours, 12 hours, and 27 hours before respiratory measurements were made. In other experiments, the degree of inhibition was usually about 50% of control values.

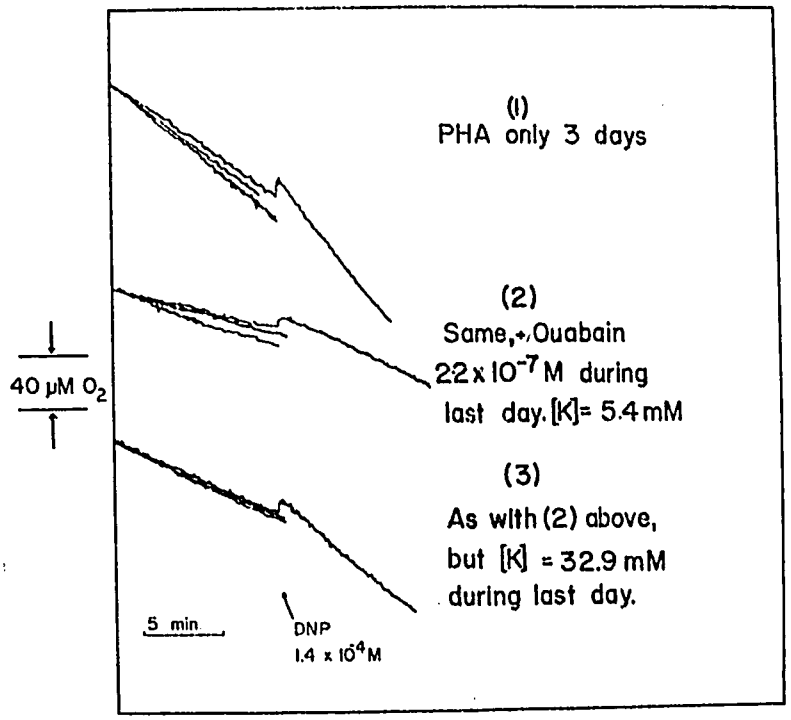


Fig. 3.5.5

Effect of ouabain, and ouabain plus excess potassium, on oxygen uptake by PHA-treated cells.

The polarograph tracings show the effect of adding DNP (1.4×10^{-4}M). Each measurement was carried out in triplicate as shown. Ouabain (2.2×10^{-7}M) was present for 24 hours. Results shown are for parallel cultures obtained from one subject.

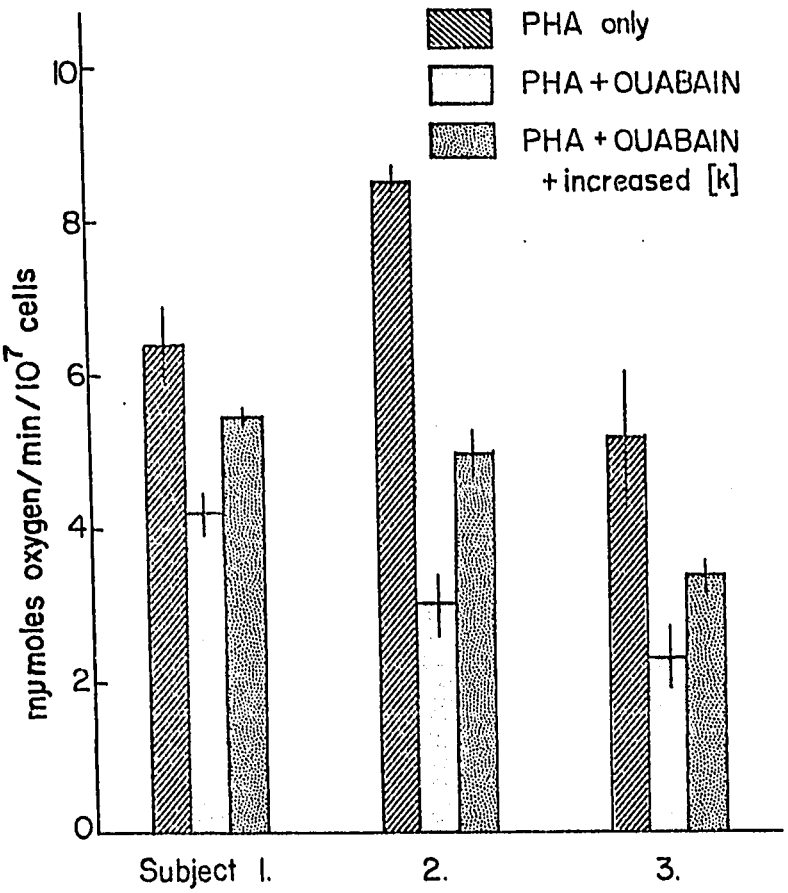


Fig. 3.5.6

Effect of ouabain, and ouabain plus excess potassium, on oxygen uptake by PHA-treated cells. This experiment was similar to that shown in Fig. 3.5.5, but repeated for cultures obtained from 3 different subjects.

3.5.2.4. Effect of Dinitrophenol (DNP) on the Respiration of Leukocyte Cultures

The uncoupling agent, dinitrophenol (2.85×10^{-5}M), invariably caused an immediate increase in the oxygen consumption by the cells (Figs. 3.5.5 and 3.5.7). The drug was administered to control cultures and to PHA-treated cells, as well as to ouabain-inhibited cells. It was also administered to cells previously treated with oligomycin, or with both oligomycin and valinomycin (see Section 3.5.2.5).

PHA-treated cells, in which an increase in respiratory activity was observed, immediately showed a further increase in oxygen consumption in the presence of DNP by the same fraction as observed for control cells. The relative increase of respiratory activity above non-DNP levels was 72 ± 18% for unstimulated cells, and 77 ± 3% for cells treated with PHA for 15-30 hours.

Ouabain-treated cells also showed a relative increase in respiration after the addition of DNP; the relative increase above non-DNP-treated values was very similar to that observed for unstimulated and PHA-treated cells.

3.5.2.5. Action of Oligomycin, Valinomycin, Nigericin and Cyanide

The concentrations of the various agents used are listed in the figure captions.

When oligomycin was added to medium containing PHA-stimulated cells, respiratory activity was markedly reduced (Fig. 3.5.7); respiration was reactivated again by the further addition of the respiratory uncoupling agents, dinitrophenol (DNP) or valinomycin (Fig. 3.5.8). It might be noted that although valinomycin promotes the energized transport of K^+ into mitochondria (Graven et al, 1966; Moore and Pressman, 1964), it did not further enhance the PHA-activated respiration when oligomycin was omitted (Fig. 3.5.7).

Respiration was immediately and progressively inhibited (Fig. 3.5.7) by the lipid soluble antibiotic, nigericin, which has been shown to act <u>in vitro</u> by abolishing the K^+ concentration gradient in mitochondria, thus promoting K^+ release (Estrada-O et al, 1967).

Sodium cyanide immediately stopped respiratory activity; this inhibition was not reversed by DNP.

It is of interest that DNP given after valinomycin did not stimulate respiration as it did in untreated cells (Section 3.5.2.4). In fact, it tended to inhibit respiration under this condition. For example, in one experiment, the following QO_2 values in mμ moles O_2/min/culture were obtained. Control rate 5.0; valinomycin 4.6; valinomycin, then DNP, 3.3. Here, the concentrations of valinomycin and DNP were 1.5×10^{-7}M and 2.8×10^{-5}M respectively. DNP

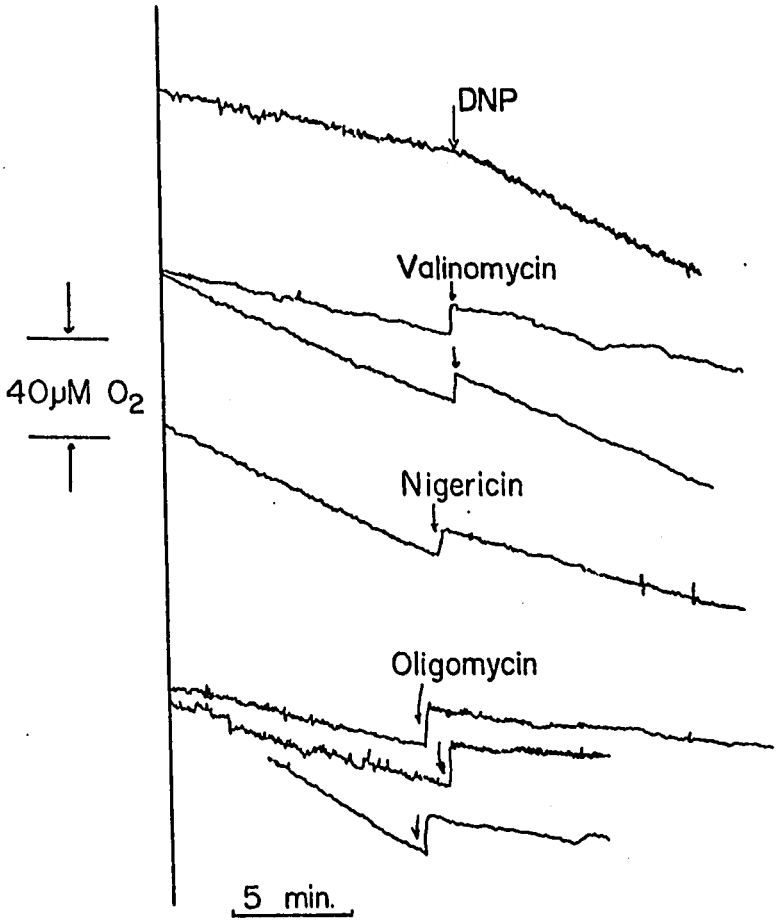


Fig. 3.5.7

The polarograph tracings show the effect of dinitrophenol (2.8×10^{-5}M), valinomycin 1.50×10^{-7}M), nigericin (1.51×10^{-7}M) and oligomycin (1.4×10^{-5}M) on the respiration of PHA-stimulated lymphocyte cultures.

also was found repeatedly to reverse the valinomycin effect on oligomycin-treated cells (Fig. 3.5.8), when given after valinomycin.

3.5.2.6. Effect of Cold Incubation on Respiration

A possible explanation (among others) for the fall in the respiration rate of ouabain-treated cells (Section 3.5.2.3) was a loss of potassium from the cell because of inhibition of active transport. A loss of potassium is also achieved by reduction of the incubation temperature. An experiment was therefore carried out in which PHA-treated cells were placed at 4°C for various periods of time, so that they would lose potassium and gain sodium. The cells were then rewarmed to 37°C and their respiratory rate measured. The results are shown in Fig. 3.5.9.

When the cells were rewarmed, respiratory activity began almost immediately. In the cooled cells, there was generally a delay of about 2 minutes before oxygen consumption commenced, in contrast to control (uncooled) cells that started to utilize oxygen almost immediately. The cooled, then rewarmed cells showed a reduced respiration in comparison to controls. After ½-1 hour at 4°C, the respiratory rate fell to 92% of the control rate. After 2-3 hours at 4°C, the respiratory rate had fallen to 64-95%, and by 18-22 hours, to 37-49%. Hence, there was a decrease in respiratory rate which was related to the time during which the cells were in the cold.

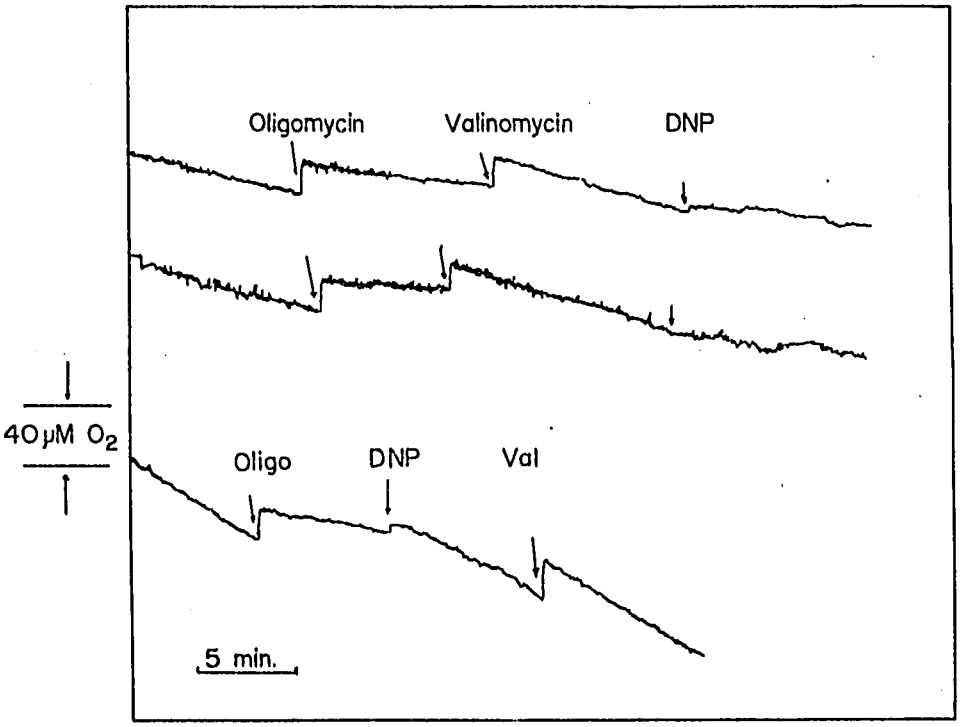


Fig. 3.5.8

The polarograph tracings show the effect of valinomycin (1.5×10^{-7}M) and DNP (2.8×10^{-5}M) given after the addition of oligomycin (1.4×10^{-5}M) on the respiration of PHA stimulated lymphocyte cultures.

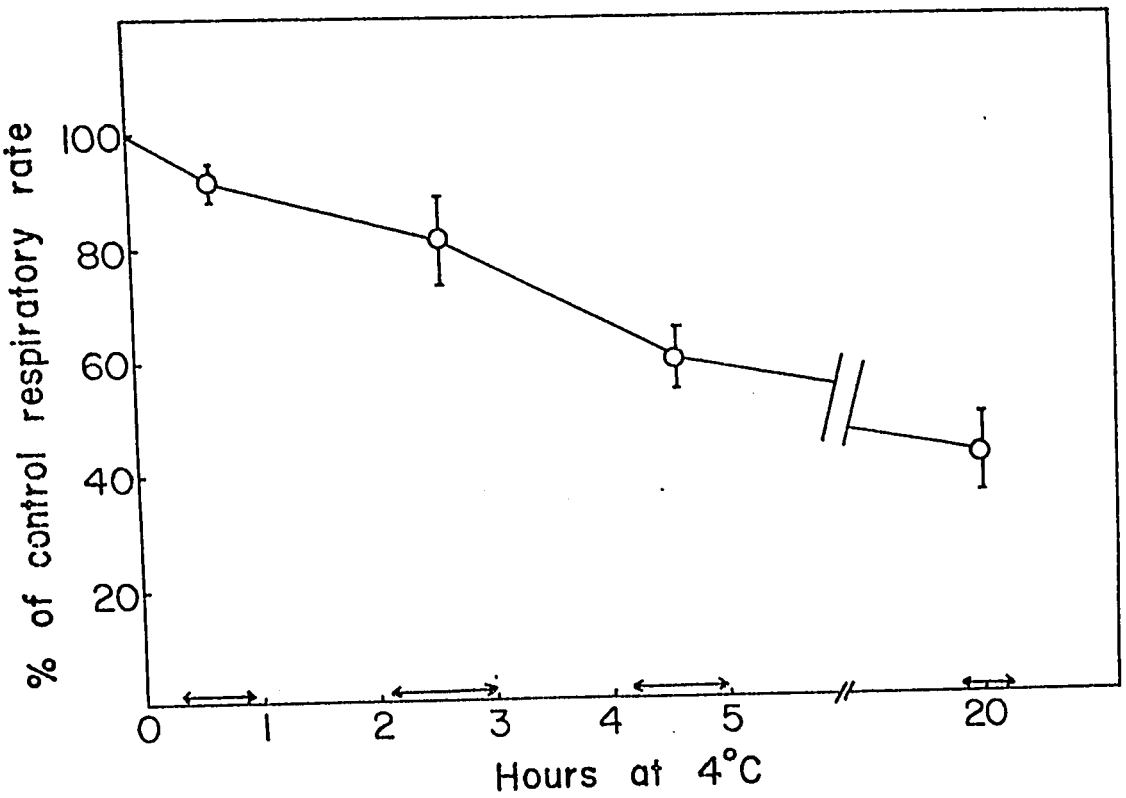


Fig. 3.5.9

Effect of cooling PHA-treated leucocytes to 4°C for various times on subsequent respiratory activity.

After obtaining an initial value for the rate of oxygen consumption, the cells were cooled to 4°C for varying periods of time up to 22 hours. They were then rewarmed to 37°C and their respiratory activity remeasured. No reduction in respiration was observed for uncooled cells.

3.5.2.7. Summary of Results

(1) PHA caused a significant increase in respiration, both of leukocyte cultures and, to a lesser extent, of lymphocyte cultures purified to 92-98%. The increased respiratory rates were the same after 1 or 2 days of PHA. The effect was more pronounced in the non-purified leukocyte cultures.

(2) DNP caused an increase in the respiratory activity of unstimulated controls to values approaching or greater than the PHA-treated rate. This activation was immediately suppressed by cyanide, as was the respiration of control and PHA-stimulated cells. DNP activated the PHA-stimulated respiration to the same extent it did control unstimulated cells (by 72-77%). However, DNP tended to reduce the respiration rate when it was given after valinomycin.

(3) Ouabain caused partial reduction of respiration at concentrations used to inhibit transformation, and to an extent dependent on the duration of incubation with the glycoside. This inhibition was largely prevented by an increased extracellular K^+ level (32.9 mM) at a ouabain concentration of 2.2×10^{-7}M, but not at a ouabain concentration of 2.2×10^{-6}M.

(4) Oligomycin caused a marked and immediate reduction of the respiration of control and PHA-treated leukocytes. Both valinomycin and DNP reversed the inhibitory effect of oligomycin on respiration.

(5) Valinomycin caused little, if any, change in the respiratory activity of control cultures, but it did reverse the respiratory inhibition by oligomycin. DNP prevented this effect of valinomycin, when given after the latter drug.

(6) Nigericin caused an immediate and increasingly progressive reduction of oxygen consumption.

(7) Cold treatment led to a progressive reduction of respiration (measured after rewarming the cells to 37°C), depending upon the time that the cells were kept at 4°C. However, even after 24 hours at near freezing, the rewarmed cells respired at substantial rates (40-50% of control values).

3.5.3. Measurements of ATPase Activity in Stimulated Leukocyte Cultures

3.5.3.1. Reasons for Measurement

Previous work (Section 3.2) had shown that stimulated lymphocytes were very sensitive to ouabain, a specific inhibitor of coupled Na and K transport. Furthermore, the transport of potassium into the cell was found to increase in the presence of PHA (Section 3.4). Hence, it was inferred that transforming lymphocytes were highly sensitive to inhibition of the Na-K-ATPase system, and that PHA-induced transformation was associated with a stimulation of the activity of this transport-enzyme system. It therefore

became highly desirable to make direct measurements of the Na^+-K^+-dependent-ATPase, and to evaluate the effects of PHA on it.

A number of preliminary experiments were carried out to measure the total ATPase activity of the cells. These results were of interest, and for this reason are briefly included here. The methods used have already been described (Section 2.4.5), but still require further development to obtain satisfactory measurements of the Na^+-K^+-dependent-ATPase. The cells were first ruptured by washing 3 times with a hypotonic TRIS solution in water, followed by ultrasonic treatment for 5 seconds. The resultant hazy suspension was centrifuged at 20,000 RPM and kept frozen until the desired enzymatic assay.

3.5.3.2. ATPase Measurements

The preparations obtained showed ATP-hydrolysing activity as shown in Table 3.5.2. When boiled, the preparations had no enzymatic activity.

Table 3.5.2 shows the effect of PHA on the ATPase activity of leukocytes with respect to the period of culture with the mitogen. The results are expressed in terms of the total protein present in the enzyme preparation. Under the influence of PHA, a marked increase was observed, starting from very low initial values obtained from preparations of unstimulated cultures.

Table 3.5.2.

ATPase activity in PHA-treated leucocyte cultures

Time in PHA	Rate of phosphate liberation from ATP
days	mg P_i/hr/µg protein
0	0.3 ± 0.3
1	5.4 ± 0.5
3	7.3 ± 0.7
4	14.3 ± 1.5

Table 3.5.3 shows the effect of ouabain (2.2×10^{-7}M), which was given during the last 24 hours of culture with PHA, on the amount of ATPase activity in preparations of cells that had been cultured for a total of 3 days with PHA. The enzyme activity of the ouabain-treated cells was found to be considerably lower than that of the controls which had been given PHA only. This inhibition was prevented by excess potassium (32.9 mM) when present during the same period of time as the ouabain. It is emphasized that since the ATPase assay was carried out in the absence of ouabain, the observed reduction in activity must have been due to an effect of ouabain on the total intact cell population, prior to the assay. The latter results may be compared with a similar effect of ouabain, and ouabain plus potassium, on respiration (described in Section 3.5.2.3). The two experiments were done in parallel, using cells from the same donor which were cultured at the same time and under the same conditions.

Further studies are being carried out in order to elucidate more precisely the changes in enzymatic activity herein reported.

Table 3.5.3.

ATPase activity in leucocytes treated for 3 days with PHA

Effect of ouabain and excess potassium.

Treatment	Rate of phosphate liberation from ATP
	µg Pi/hr/µg protein
PHA (3 days)	7.8 ± 0.6
PHA (3 days) plus ouabain during final 24 hours	1.5 ± 1.5
PHA (3 days) plus ouabain and K^+ ** during final 24 hours	9.7 ± 0.5

* ouabain concentration 2.2. x 10^{-7} M.

** same ouabain concentration.

final potassium concentration 32.9 mM.

3.5.4. Possible Ultrastructural Demonstration of an (Na^+-K^+) - ATPase Located on the Cell Membrane of Blast Cells

Preliminary results were obtained in which a Na^+-K^+-dependent-ATPase on cell membranes was demonstrated. The method used has already been described (Section 2.2.2).

In one experiment, PHA-stimulated or unstimulated cells were placed in special incubation medium in the presence or absence of sodium and potassium, and in the presence or absence of ATP, prior to fixation and examination in the electron microscope. Only cells in PHA-stimulated cultures, for which ATP, sodium and potassium were all present in the reaction mixture, showed deposits of electron opaque grains on the cell membrane (Fig. 3.5.10). Unstimulated cells, or PHA-stimulated cells incubated in the reaction mixture without sodium or potassium, gave negative results (Fig. 3.5.11). Fig. 3.5.12 shows some membrane preparations, obtained after hypotonic lysis of the cell which contain peripheral electron-opaque deposits in the presence of ATP and Na and K, but not in the presence of ATP alone. While these results do not yet prove that there was an activation of the transport-enzyme system of the cell membrane, they do provide a useful beginning to ultra-structural studies of a membrane-located ATP-splitting enzyme system associated with lymphocyte transformation.

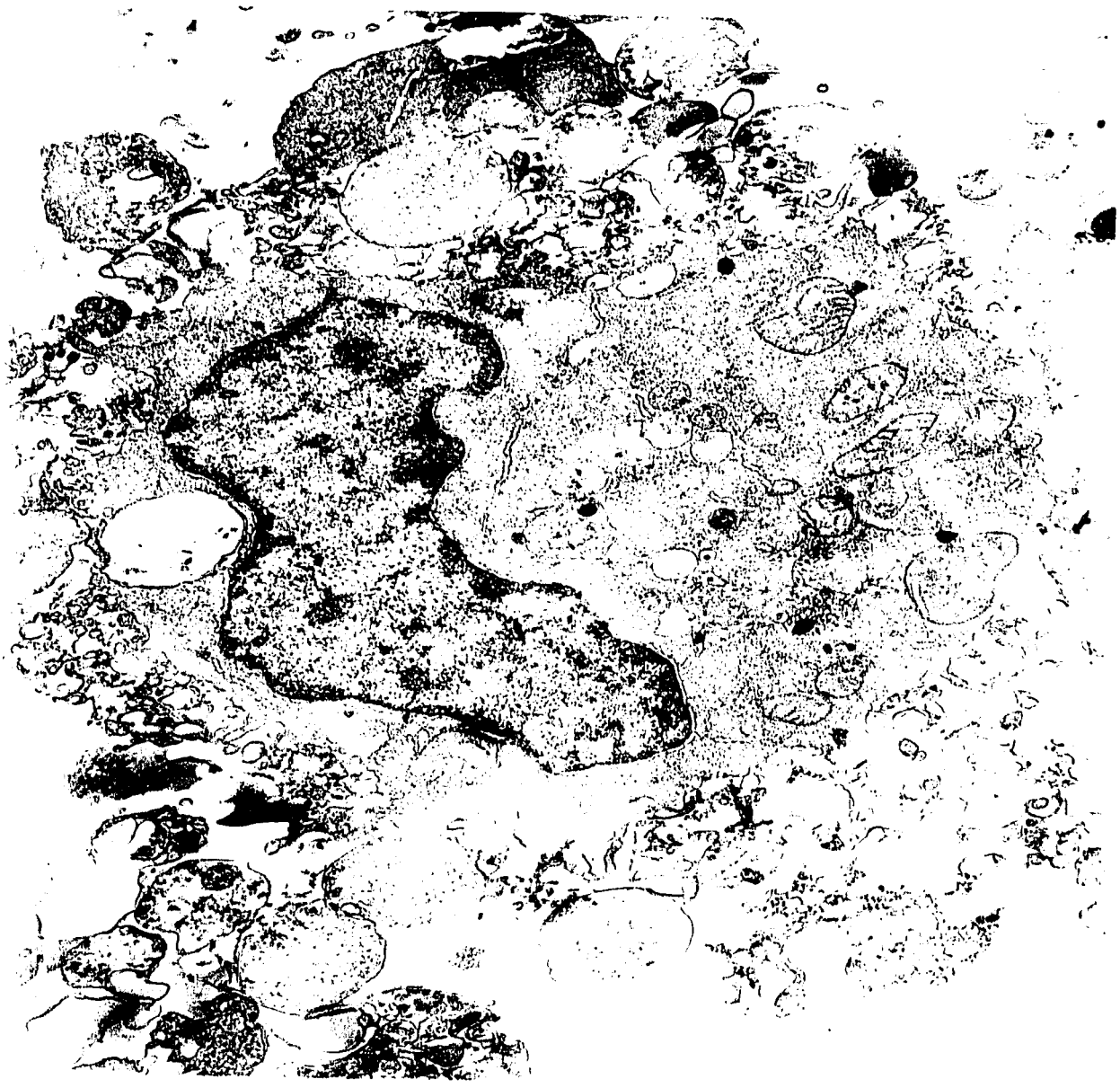
Fig. 3.5.10

Electron opaque material deposited in the membrane of a blast cell incubated in the presence of ATP, Mg^{++}, $Pb(NO_3)_2$, buffer, Na^+ and K^+ (see sections 2.2.2 and 3.5.4).



Fig. 3.5.11

Blast cells in culture parallel to that shown in Fig. 3.5.10. Incubated in the presence of $Pb(NO_3)$, ATP, Mg^{++}, but no Na^{+} or K^{+}. Note absence of electron opaque material around the cell membrane.



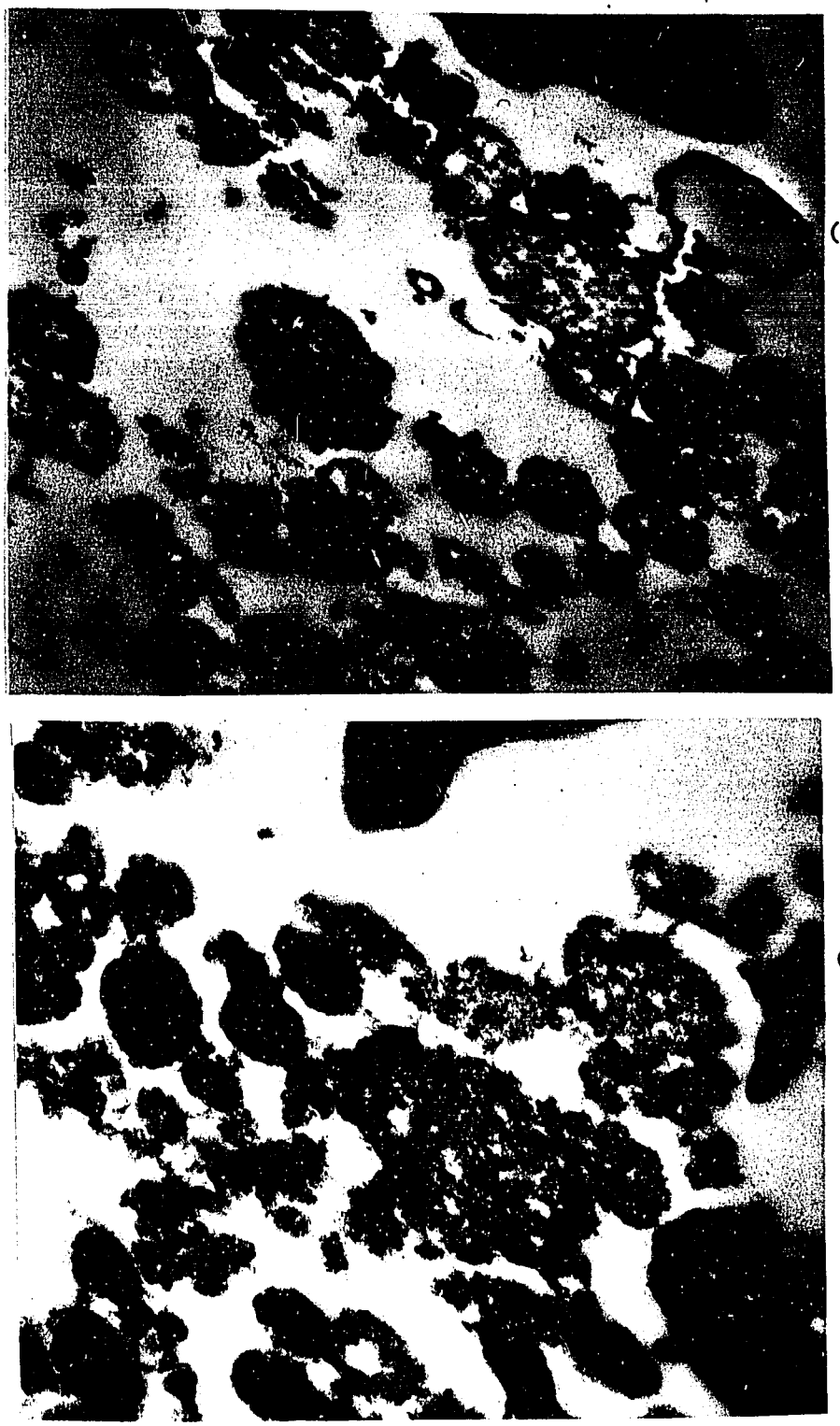


Fig. 3.5.12. Membrane preparations of PHA-treated leucocytes

(a) ATP + Na + K

(b) ATP (No Na or K)

3.5.5. PHA-induced Transformation and Ouabain Response of Lymphocytes Obtained from Patients with Leukemia and Cystic Fibrosis

The findings of the sensitivity of transforming lymphocytes to ouabain (Section 3.2) made it important to evaluate the response to ouabain of cells from different diseases, in which there occurred abnormal lymphocyte or leukocyte transformation, or in which there was a possible change in the mechanism of cation transport. Some clinical conditions for which it was thought that such a study might yield useful results included leukemias (particularly acute leukemias) infectious mononucleosis, and cystic fibrosis, which is a condition probably gene-linked involving the abnormal transport of sodium (Bensley and Patrick, 1969; Balfe et al, 1968, Dallaire and Destiné, 1969).

The following results describe some initial studies carried out with leukemic patients and with some patients diagnosed as having cystic fibrosis or as 'carriers' of this condition.

3.5.5.1. Leukemia

The methods used were almost identical to those described for the measurement of incorporation of radioactive nucleosides into DNA or RNA (Section 2.3.1). The usual procedure used was to obtain about 10 or 20 ml of whole blood from the patient, to sediment the red cells with dextran, and to culture the leukocytes with medium-199 fortified with 15% autologous plasma (the patient's plasma), after

having diluted the leukocytes to obtain a concentration of about 1,000 lymphocytes/ml. In cases of chronic lymphocytic leukemia, considerable dilution of the leukocyte preparation often had to be carried out.

(a) Chronic lymphocytic leukemia (CLL)

As shown in Table 3.2.1, ouabain caused inhibition of lymphocytic RNA synthesis to the same extent as observed with cells obtained from healthy donors, that is, by about 50% of the untreated control values.

The stimulatory response to PHA of CLL lymphocytes, in terms of the incorporation of precursors into RNA and DNA, was usually less than the PHA-response of cells from healthy individuals. However, this was not always true. The PHA-response of CLL cells could be normal (Case 'b', Fig. 3.2.14). However, as shown in Fig. 3.2.14 and Table 3.2.1, the response of CLL lymphocytes to PHA was usually relatively low. In all cases ouabain reduced the uptake and incorporation of the nucleoside to a fraction corresponding to the degree of stimulation (Fig. 3.2.14). In some circumstances, the spontaneous synthetic activity was unaffected by the ouabain, but in all cases the increment of stimulation caused by PHA was inhibited by ouabain at concentrations greater than 2.2×10^{-7} M.

(b) Acute leukemia (AL)

Two cases of AL havebeen examined, in which the patients who werein relapse,were not already being treated with various drugs. In these cases,ouabain caused a profound depression of the spontaneous DNA synthesis characteristic of peripheral leukocytes from this condition (Fig. 3.5.13). These results are being followed up by clinical trials and further studies on more acute leukemia patients.

It is likely that the concurrent chemotherapy used for the 3 other acute leukemia patients tested has already reduced the spontaneous DNA and RNA synthesis of their leukocytes to such low levels that the effect of ouabain was only poorly manifest. Therefore, insufficient results have been obtained, particularly with the acute lymphocytic leukemias to determine whether the increased spontaneous synthesis of neoplastic lymphoblasts is always inhibited by the glycoside.

In only one case was the PHA-induced stimulation not affected by ouabain. This was a patient diagnosed as having acute myelomonocytic leukemia. The degree of leukocyte stimulation caused by PHA in this case was extremely low; the values of H^3-uridine incorporation obtained are shown in Table 3.5.4.

In conclusion, it was found that, while lymphocytes from or leukemic patients showed highly variable degrees of stimulation

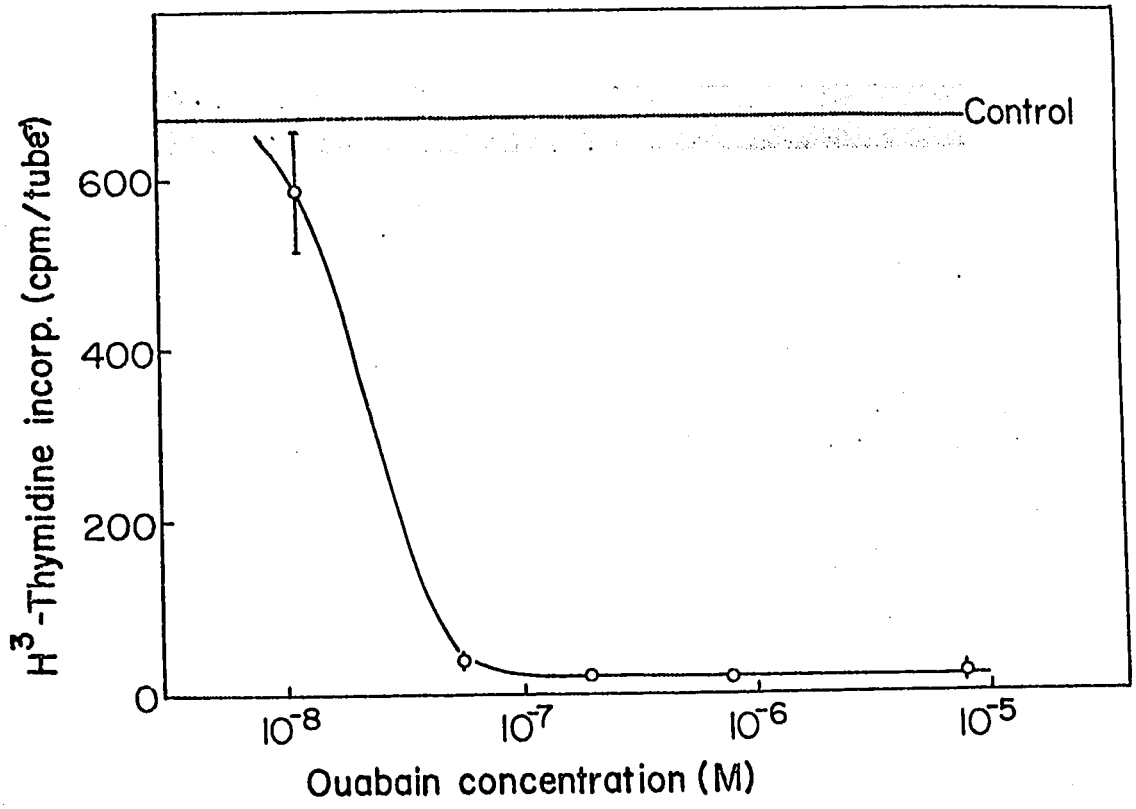


Fig. 3.5.13

Effect of ouabain on the spontaneous DNA synthesis of cells obtained from a case of acute leukemia. The cells were cultured without the glycoside for 24 hours. Ouabain was then added, and the cells cultured for a further 24 hours. After this time the incorporation of H^3-thymidine during a 2 hour incubation period was measured.

Table 3.5.4.

Effect of ouabain on leukocyte cultures from a patient with acute myelo-monocytic leukemia

Treatment	H^3-uridine incorp. (cpm)
Control	183 ± 30
PHA (1 day)	794 ± 44
PHA + ouabain (2.2×10^{-7} M), both for one day.	933 ± 44

in the presence of PHA, ouabain inhibited the increment of stimulation caused by the PHA. Spontaneous DNA synthesis of leukocytes from acute leukemia could be very strongly inhibited by the glycoside. It is to be expected that there will be much variation of response depending on the disease, clinical condition, type of therapy, etc., and further work is clearly necessary.

3.5.5.2. Cystic Fibrosis

The other clinical condition examined was that of cystic fibrosis. A total of 8 subjects who were patients of Dr. Park, Ottawa Civic Hospital were examined. All of them had the disease or were considered to have been 'carriers'. Siblings were also examined as controls. Almost all of the patients were children. The results are shown in the Figure 3.5.1.4. It is interesting to note that there was a somewhat decreased response to ouabain at inhibitory concentrations of the drug, although in all cases, PHA-induced stimulation was strongly suppressed. It will be necessary to obtain data from more patients before the significance of these results can be evaluated. However, if true, the results would imply that PHA-stimulated lymphocytes from cystic fibrosis are somewhat less affected than cells from normal subjects by inhibition of cation transport.

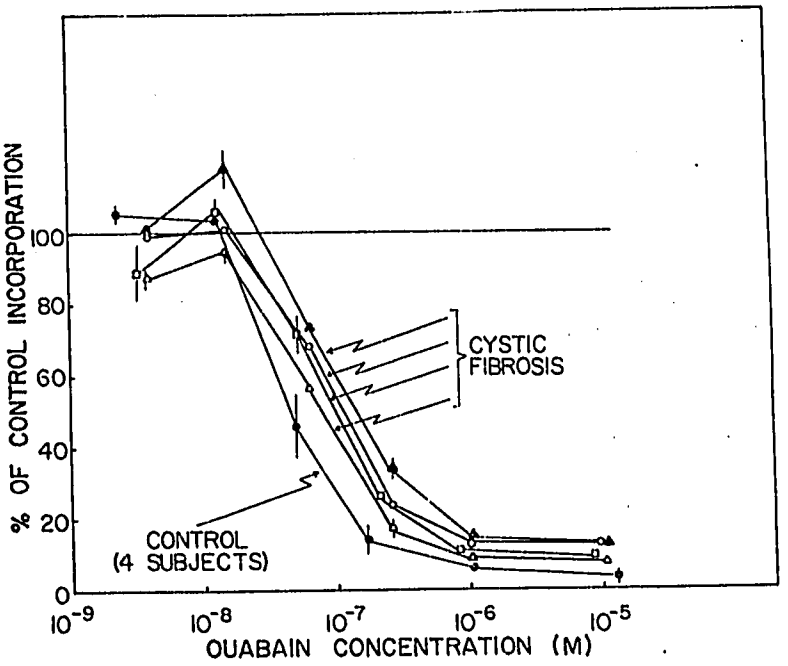


Fig. 3.5.14

Response to ouabain of RNA synthesis in PHA-treated leucocyte cultures from cystic fibrosis patients.

Cultures treated in the same way as in Fig. 3.2.15. Controls were paired from healthy subjects of the same age, and cultured and treated in parallel with the leucocyte samples of the patients. Ordinate represents the H^3-urodine incorporation compared to non-ouabain-treated cells for each subject.

3.5.6. Studies of Ouabain Effects on the Homograft Response

3.5.6.1. Introduction to subsection

During the past few years, it has become apparent that the transformation of human small lymphocytes *in vitro* from small, non-dividing, 'repressed' cells into large blast cells with accelerated synthetic and metabolic activity, in the presence of antigens or certain other agents, represents a type of immune phenomenon *in vitro*. The observed 'blast' cells are thought to be analogous to the large pyroninophilic cells which are seen in lymph nodes draining the sites of homograft transplantation and in delayed hypersensitivity states (Turk, 1967). Recent reports have also appeared which indicate that transforming leukocyte cultures under certain circumstances produce specific gamma globulin (see summary by Ling, 1968; and Section 1.5.2.3.).

A number of agents which are active immunosuppressants have been found to inhibit *in vitro* blast transformation. These include radiation, the adrenocortical steroids, and substances that specifically prevent nucleic acid and protein synthesis, such as 5-fluoro-uracil, actinomycin-D and puromycin. However, the cortico-steroids and their synthetic derivatives, radiation and radiomimetic agents such as mustards are most effective prior to the onset of transformation has been initiated. Antilymphocyte serum will variably inactivate small lymphocytes to PHA, but does not appear to suppress the progres-

sion of transformation, when given after transformation has been initiated by PHA (Greaves et al, 1967; Mosedale et al, 1968). With many of these agents, there are problems of toxicity which have not yet been resolved.

The results described in Chapter 3.2 indicated that ouabain would strongly inhibit lymphocyte stimulation caused by PHA or in the mixed lymphocyte reaction. A particularly useful property of this glycoside (at least *in vitro*) is that its action is reversible and does not cause lymphocyte death at inhibitory concentrations, but only prevents or inhibits their transformation. Thus, removal of the drug permits transformation *in vitro* to take place (Section 3.2.3.5). However, it is not known whether this action also occurs *in vivo*.

It was therefore decided to initiate a pilot study on the effect on homograft transplantation of ouabain and other inhibitors having a related action on lymphocyte transformation, to determine whether the *in vitro* inhibitory effects would correspond to a suppression *in vivo* of the homograft response. There were several difficulties in evolving an experimental procedure for this purpose. For example, it was necessary to select a suitable experimental animal, to choose an appropriate homografting procedure, to evolve the most suitable dosage schedule, to determine toxicity, to characterize the changes in grafts associated with rejection, etc. The following

account will describe the experimental procedures and results obtained as they evolved. The work is presently being continued, but it seemed appropriate to include the initial results which were obtained.

3.5.6.2. Initial Study using Rats

Because ouabain is not retained in the body but is excreted relatively rapidly, at least in the human, it was necessary to give repeated doses of the compound. Since the rat is particularly resistant to ouabain toxicity (Repke, 1961) it was first decided to use this animal, because relatively large doses of ouabain could then be administered. It was recognized that should the rat lymphocyte also be insensitive to ouabain as a reflection of this animal's general insensitivity to the drug, one might not get inhibition of the immune response. In this pilot study, however, it was decided to use the rat since the latter considerations were only possibilities and awaited experimental observations.

Accordingly, it was planned, in the pilot study, to investigate the toxicity of ouabain in the rat, the effect of ouabain on skin homograft rejection in the rat, and the *in vitro* response of rat leukocyte cultures to ouabain. At a later stage of this work, pigs were used.

(i) Toxicity of Ouabain in the Rat

This first experiment was carried out to determine the effect of intraperitoneal injections of a ouabain solution in saline (using male Wistar rats weighing approximately 150 gm). Injections of ouabain ranging in concentrations from 0 to 50 micrograms in saline were given intraperitoneally three times a week for a period of two weeks (on Mondays, Wednesdays and Fridays). The parameters by which 'toxicity' was measured were (i) the rate of ingestion of water, (ii) the rate of intake of solid food, (iii) the gain in weight, and (iv) subjective impressions of animal behavior. For each dose, 5 animals were used.

It was found that even at the highest dose of ouabain administered, no unusual change in weight gain could be observed. Experimental animals did not differ from control animals with regard to behavior, general appearance or the parameters noted above, with the exception of water intake. Rather consistently, those rats that received the highest dose of ouabain showed the highest rate of water intake; the rate of water intake was related to the dose given (Fig. 3.5.15 and 3.5.16). It is also probable that the urinary volume was increased but this was not measured.

(ii) Effect of Ouabain on Skin Homograft Rejection in the Rat

In view of the observation that ouabain doses as high as 50 micrograms given intraperitoneally three times weekly failed to

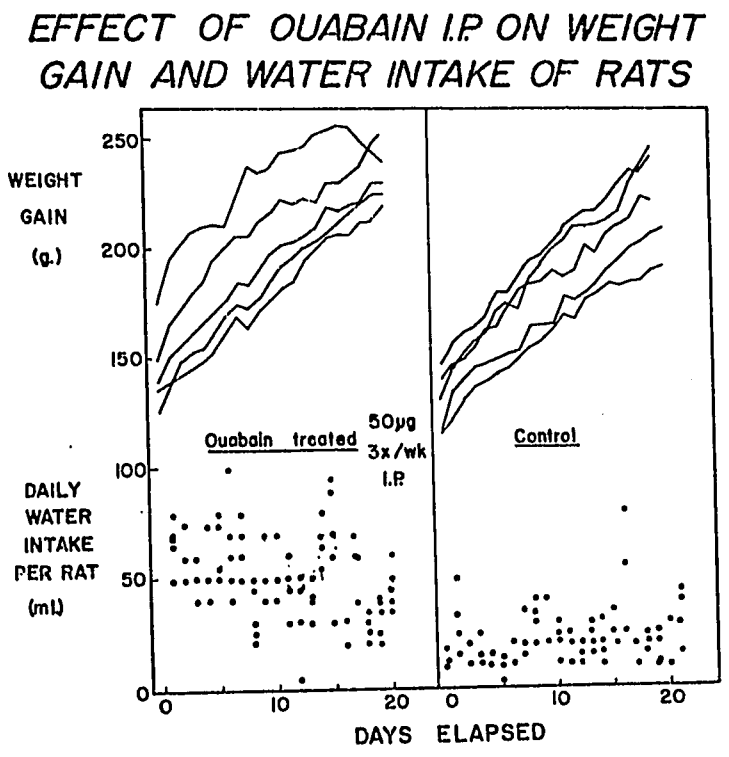


Fig. 3.5.15

Water intake and weight gain of rats treated with ouabain. The glycoside was administered in doses of 50 µg three times a week intraperitoneally. Controls received saline injections.

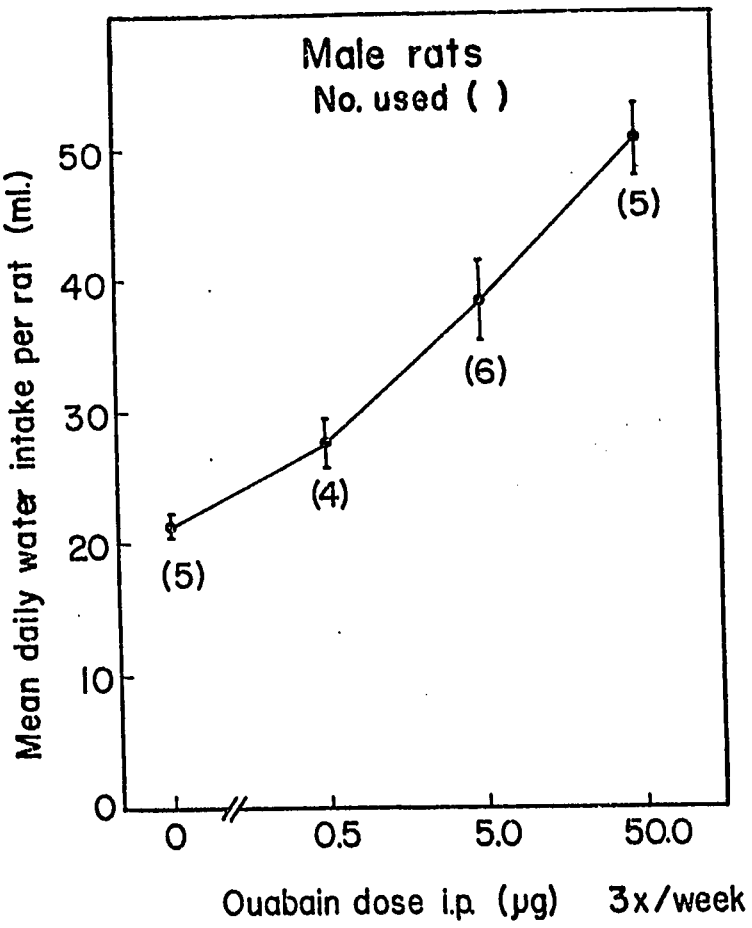


Fig. 3.5.16

Relationship of mean daily water intake to dose of ouabain.

cause any noticeable toxicity to the animal within a two-week period, it was decided to compare the rate of rejection of skin homograft by ouabain treated with control animals. The grafts were placed dorsally over the pelvic region and were 0.7 - 0.8 cm in diameter.

Using six pairs of Wistar rats, skin grafts were interchanged between individual animals of each pair. One animal of each pair was given ouabain intraperitoneally three times a week, on Mondays, Wednesdays and Fridays, the total injection of dose being 50 micrograms. The control animals were injected at the same times with normal saline. The results of the experiment were essentially negative; there was apparent difference between control and ouabain - treated animals in their rate of graft rejection. The experiment was repeated again treating a second group of 6 pairs of rats, and using the same dosage schedule. However, this time larger grafts were used in an effort to clarify the visual criteria for rejection. Again, the same results were obtained; the grafts were all rejected within 15 days. It was therefore necessary to determine whether rat lymphocytes themselves when treated with ouabain in vitro were sensitive to the glycoside.

(iii) The response of PHA-stimulated rat lymphocytes to ouabain

Several experiments were carried out to determine the degree of sensitivity to ouabain of stimulated rat lymphocytes in vitro.

This was at first found to be difficult because of the negligible transformation of these cells by the usual technique employed.

It is interesting to note that a number of other workers have commented on the difficulty of culturing rat lymphocytes in vitro and obtaining transformation with PHA. Marshall and Roberts (1963) failed to obtain transformation with PHA. Matsaniotis and Tsenghi (1964), Pearmain et al (1963) and Schrek and Rabinovitz (1963) had similar difficulties. Ling and Husband (1964) found that rat lymphocytes did not respond to staphylococcal protein and Metcalf (1965) also reported negative results.

More recent studies have shown, however, that the problem of rat lymphocyte culture is one of technique. Successful results were obtained by Rieke and Schwartz (1964) who washed the cells and then used rat serum rather than plasma. Elrod and Schrek (1965) obtained successful results with medium-199 containing 50% rabbit serum which was 'inactivated' by heating to 50°C for 30 min. Williams and Ray (1965) found that the addition of human serum led to some lymphocyte transformation and mitosis. Wilson (1967) found that culture of rat lymphocytes with PHA was best in media containing fresh rat serum, although fetal calf serum or human plasma was satisfactory. Metcalfe (1966) reported that rat plasma was inhibitory to PHA-induced transformation of rat lymphocytes, possibly because of the platelets, but that rat serum was permitted growth. In summary, the common factor for successful culture of rat lymphocytes has been the use of serum rather than rat plasma.

The experiments with rat cells were therefore repeated using small amounts of defibrinized whole blood cultured in medium - HB597, which contains antibiotics, fortified with 20% fetal calf serum. Measurements of H^3-thymidine incorporation and autoradiographic exposures of the cells were carried out in parallel.

The PHA-treated cultures of rat lymphocytes showed a positive incorporation of H^3-thymidine indicating that some degree of transformation had taken place. However, the addition of ouabain up to concentrations of 9×10^{-7} M did not significantly affect DNA synthesis, indicating that the transforming rat lymphocytes were insensitive to the glycoside at the doses used, unlike the lymphocytes of humans. The onset of DNA synthesis and incorporation of H^3-thymidine was verified by autoradiographs which showed heavy uptake of radioactive label into the nuclei of blast cells; no effect of ouabain was noted (Fig. 3.5.17).

These observations were of considerable interest since they suggested the existence of phylogenetic differences in the plasma membrane transport enzymes, depending on the species of animal investigated. This subject would be worthy of further investigation, but since it was desired to assess the effect of ouabain on the immune response *in vivo*, it was important to use an animal whose lymphocytes would show sensitivity to the glycoside under *in vitro* conditions. The pig was therefore selected as the second experimental animal.



Fig. 3.5.17

Autoradiograph of DNA synthesis in rat lymphocyte after after 2 days PHA including the last day with ouabain and a concentration of 9×10^{-7}M.

3.5.6.3. Studies on Pigs

(i) Lymphocyte transformation

A small pig was obtained and 60 ml of whole blood obtained by cardiac puncture. Culture of the pig leukocytes with PHA was carried out in the same way as for human cells. It was found that when pig leukocytes were cultures *in vitro* for 1 to 2 days there was marked transformation and chromosomes could be prepared from mitotic cells.

(ii) Sensitivity of pig lymphocyte transformation to ouabain

Blood from the above animal was obtained and the leukocytes cultured in the presence of phytohemagglutinin. Ouabain was added in various concentrations. Marked inhibition of transformation occurred at the same concentrations of glycoside found to be effective for human lymphocytes (Fig. 3.5.18).

Hence, these experiments show that, on the basis *in vitro* results, suppression of the capacity to initiate an immune response using ouabain may be possible in the pig.

(iii) Toxicity of ouabain to pigs

Ouabain was made up to a concentration of 2 mg/ml in sterile saline. The animals, approximately 10 kg in weight, were treated with doses of 0.1, 0.2, 0.4 and 1.0 mg per day by sucutaneous injection into

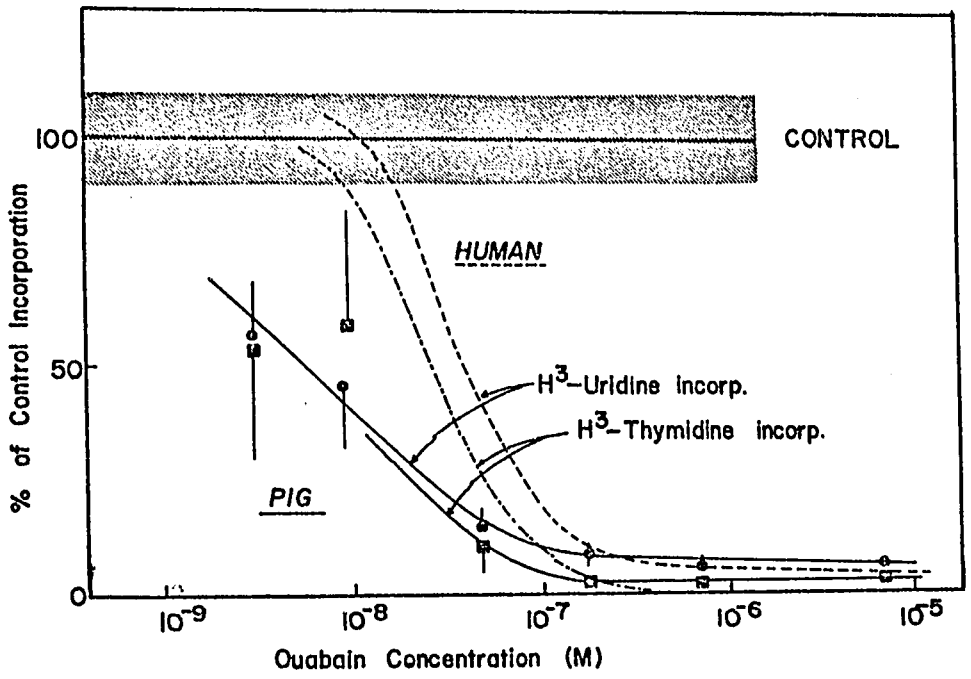


Fig. 3.5.18

Effect of ouabain on RNA and DNA synthesis of PHA stimulated pig leucocyte cultures, compared to the effect on human cells. Conditions used were the same as described in the experiment illustrated in Fig. 3.2.15.

the abdominal skin. Control animals received the same volume of saline only. The estimated initial doses were 8.6×10^{-8}M to 8.6×10^{-7}M, assuming complete mixing of the ouabain in the extracellular fluid, (the volume of which was taken to be 20% of the body weight). The results are shown in Table 3.5.5.

The immediate cause of death of the animals was not clear. However, toxicity was initially manifested by a rapid fall-off in weight gain. Autopsies showed that, in each case, the lungs and right auricle of the heart were engorged with blood. One animal also showed some hemorrhage into lymph nodes.

Based on this information, it was decided to use a dose of 10 µg/kg body weight of ouabain per injection, which would give an initial ouabain concentration of 8.6×10^{-8}M. It might be noted that the human therapy level of 3.5 µg/kg corresponds to a tissue level of 3.0×10^{-8}M (again assuming rapid equilibrium in extracellular tissue fluides). There was some doubt whether this tolerable level would be enough for the postulated inhibition of lymphocyte transformation *in vivo*, but since strong *in vitro* inhibition was obtained at 5×10^{-8}M (see Fig. 3.5.18), graft testing was carried out.

(iv) Graft survival in control pigs (primary response to homografts)

A series of experiments were carried out to determine the best grafting procedure with these animals. The method finally used

Table 3.5.5.

Survival of 10 kg young pigs treated with ouabain

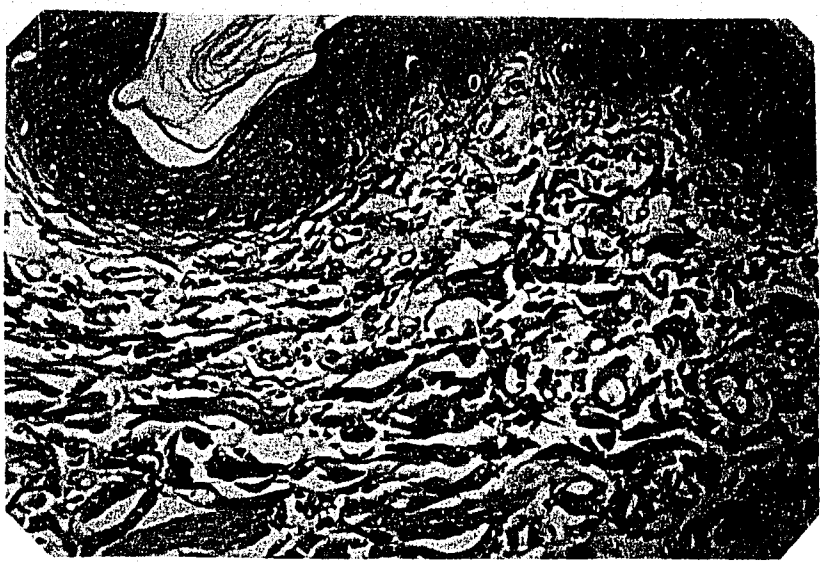
Dose (daily mg/10kg	No. of animals	Survival (days)
0.1	Many	Indefinite
0.2	6	3, 5, indefinite
0.4	2	9, 11
1.0	3	2, 2, 2

These animals weighed about 10 kg. Larger pigs appeared be more resistant to doses more than twice as great. Thus, 20 kg pigs did not lose weight over a 9 day period of daily treatment with 1.6 mg ouabain.

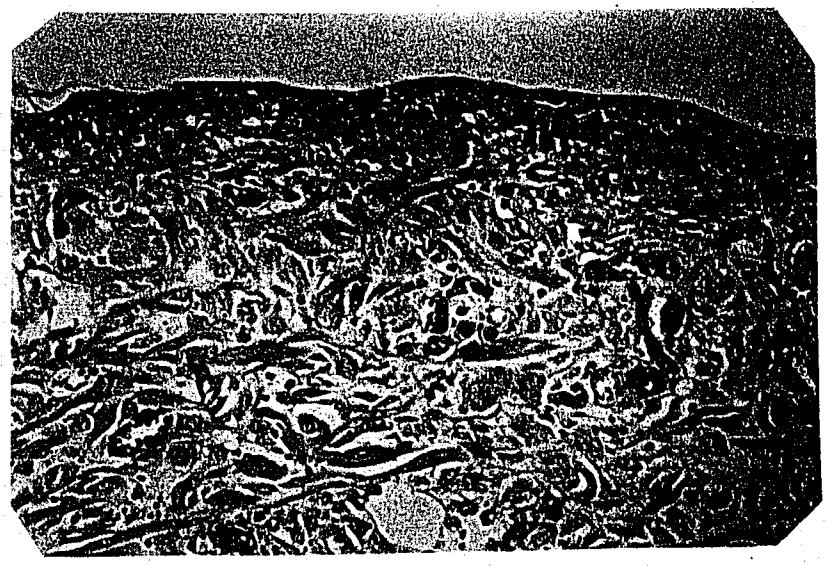
is described in detail in Section 2.5. The grafts were cut out with a special circular instrument and replaced on the same animal (autograft) or on the other animal of a pair (homograft). After careful stitching, a sterile vaseline gauze was placed over the graft, covered by a pressure bandage. These were left on for at least 4-5 days, except when photographs were taken. The condition of the graft was evaluated by its appearance, tactile consistency and histological state. Grafts were removed after 5 and 9 days for histological purposes.

5-Day Grafts (Fig. 3.5.19). All autografts had 'held'. They were yellowish-white in color and each had pink flush in the centre which presumably represented capillary dilation and revascularization. Most homografts also appeared to have taken by this time. Microscopically, local areas of inflammation were present. All of the grafts showed pronounced or moderate capillary cascularization and much invasion of spongy connective tissues and capillaries. All grafts characteristically showed a decreased thickness of the epidermal epithelium, although the number of epithelial cell layers remained numerically at about four. Hence, the decrease in thickness must have been due to a loss in the volume of the epidermal cells. There was no microscopic difference between homografts and autografts.

9-Day Grafts (Fig. 3.5.20). After this period of time, homografts had progressed from a hyperemic stage to a condition of



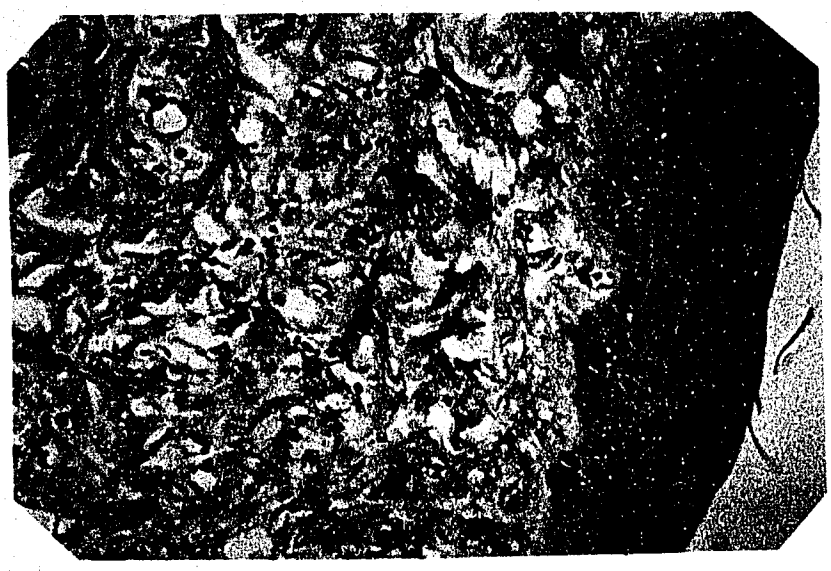
control



homograft

Fig. 3.5.17

Appearance of primary skin graft 5 days after transplantation. Note the thinness of epithelial layer and hyperemia. The animal was not treated with any immunosuppressive agent.



control



homograft

Fig. 3.5.20

Appearance of primary skin graft 9 days after transplantation. Note the ingrowth of normal epithelium from the sides of the graft below the rejected mass.

gradual hardening and darkening, beginning after about a week or later, and which became marked after 8-9 days had passed. The homografts showed loss of grafted epithelial cells and an ingrowth of host epithelium from the sides of the graft; part or all of the graft had developed a cheese-like appearance with a bluish infiltration of mononuclear and polymorphonuclear cells. Homografts were usually characterized microscopically by a pronounced infiltration of mononuclear cells into subcutaneous connective tissue. These cells often included large cells with globular, clear nuclei and a relatively large volume of eosinophilic cytoplasm.

Autografts generally had a relatively soft and pink appearance, but sometimes were partially or completely covered with a hard scab. Microscopically, the autografts showed some thickening and growth of the grafted epithelium, and a pronounced growth of the ungrafted epithelium adjacent to the graft site. When a scab had formed over the graft site, apparently healthy epithelium was seen beneath it. Sometimes only local 'pockets' of epithelium had survived and were seen to be spreading out over the surface under the scab.

(v) Graft Survival in Untreated Pigs (Secondary Response)

The same technique was used as described above, using the same pairs of animals. Autografts were usually retained but some (about 10%) eventually came off. The period of time required for

rejection of skin homografts was considerably shorter than the first set. In general, this period for transplant rejection was somewhat shorter than the first set. In general, this period averaged from 4 to 6 days, with an average value of 5.2 ± 0.3 days, (±SD of mean). Rejection was characterized by darkening and hardening of the transplanted skin. Microscopically, homografts were characterized by pronounced infiltration of mononuclear cells, sometimes resembling small lymphoid nodules, as in Figure 3.2.22. Visual criteria for graft rejection are shown in Figure 3.5.23.

(vi) Effect of Ouabain on Homograft Survival

Both primary and secondary homograft responses were tested in animals treated with the glycoside. No apparent change was noticed for animals given daily ouabain doses of 20 µg/kg. Daily photographs were carried out to determine the time required for the secondary response to become manifest. The results (based on visual criteria) are shown in Table 3.5.6. At the ouabain dosage used, there was an unexpected acceleration of the rejection rate. Thus, the treated animals rejected homografts more rapidly than the control animals. All homografts were rejected by the sixth day. Histological preparations were made on the ninth day because it was thought remotely possible that some islands of skin cells might be present. However, this was not observed to be the case. No difference was observed between the microscopic appearance of treated and untreated homografts after this period of time.

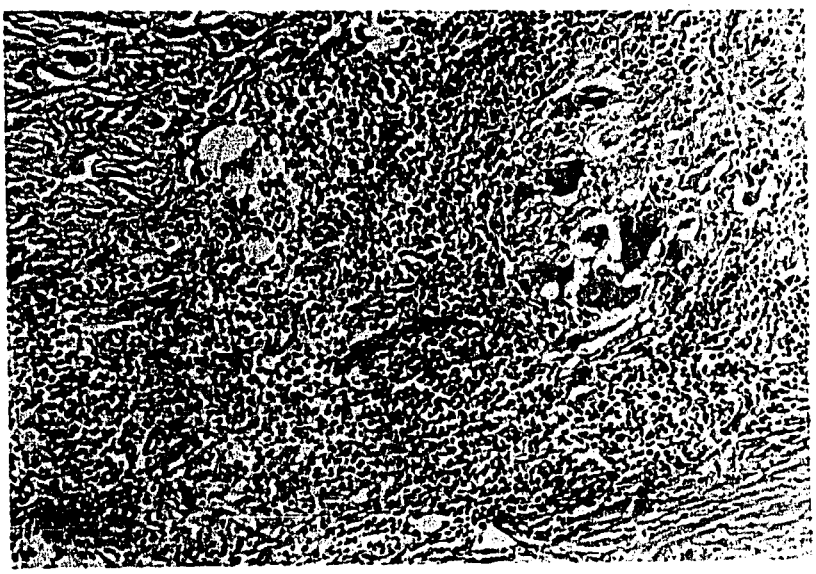


Fig. 3.5.22

Mononuclear cell infiltration into connective tissue underlying site of a homograft. Secondary response after a period of $8\frac{1}{2}$ days.

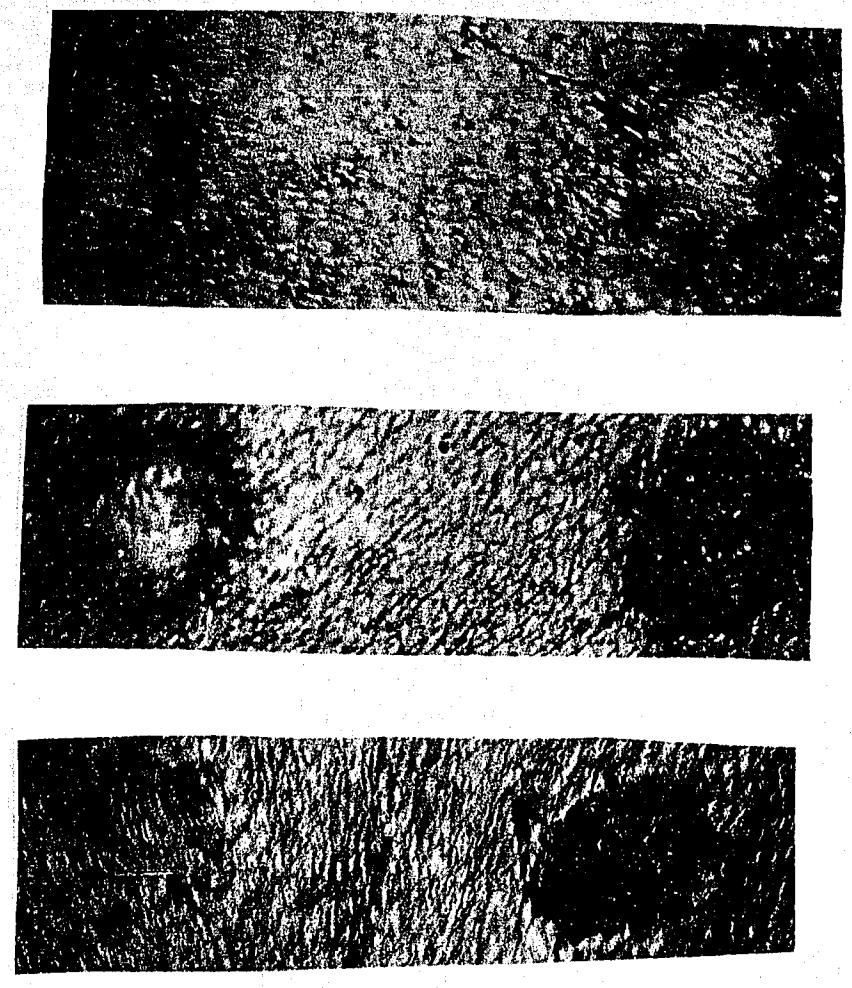


Fig. 3.5.23

Visual criteria for development of skin graft rejection in the secondary response. These photographs of homografts and autografts were obtained from the same animal at intervals of 2, 4 and 7 days after transplantation. Grafts rejected in the primary response were similar in appearance, but took longer periods of time to show gross evidence of rejection.

Table 3.5.6.

Skin homograft* survival for control and ouabain-treated 20 kg pigs. Ouabain dose 0.8 mg daily subcutaneously

Untreated		Ouabain treated	
Animal #	Survival (days)	Animal #	Survival (days) of graft
1	5-6** 4-5	2	4-5 4-5
3	5-6 5-6	4	5 4-5
5	4-5 4-5	6	3-4 3-4
7	5-6 6-7	8	4-5 4
Mean ± SE mean	5.3 ± 0.3		4.3 ± 0.2
Significance $p < 0.02$ by Student's t test			

* based on visual and tactile criteria

** assume rejection time of 5.5 days for calculation

Two skin homografts were interchanged between the paired animals in each row (1 and 2, 3 and 4, etc.).

3.5.6.4. Summary of Animal Data

(i) Pig leukocytes shown are strongly inhibited by ouabain at doses of 5×10^{-8}M, whereas the response of rat cells is negligible at doses up to 9×10^{-7}M.

(ii) Rats are very resistant to ouabain doses which, in terms of weight, would be lethal to pigs. The former animals respond to ouabain treatment by increased water consumption.

(iii) Neither pig nor rat homografts were delayed in rejection rate by the glycoside doses used.

(iv) Pig skin homograft grafts were rejected slightly more rapidly in the presence of daily injected doses of ouabain of 20 µg/kg.

In retrospect, it must be said that the visual criteria used for determining graft rejection were very crude and subjective; histological examination is useful but requires removal and termination of the graft. The appearance of the graft can be deceiving because crusts of exudate, or loss of viability can occur with autografts, giving an appearance of active rejection. Furthermore, it is known that skin transplants though conveniently carried out, are difficult to retain even with known immunosuppressants. It might be remarked here that a more accurate means of assessing rejection would be highly desirable. A method based on the use of radioactive tracer carried to the rejected graft by lymphocytes or released with the graft site would probably be an improvement.

3.5.7. Uptake of Fluorescein-Labelled PHA by Lymphocytes

3.5.7.1. Introduction to Subsection

The purpose of administering PHA labelled with fluorescein-isothiocyanate PHA was to observe the site of attachment of the mitogen to the cell, and to determine whether membrane binding occurred, and whether the mitogen was taken up into the cytoplasm and/or into the nucleus. It was recognized at the outset of these experiments, that definite conclusions as to the site of action, could not be obtained in this way, because the presence of fluorescence within the cell would not necessarily prove that PHA caused its initial mitogenic action by an effect within the cell. Conversely, if the fluorescence were to be seen only at the periphery of the cell, it would still not be known whether an active fraction of the mitogenic material became disocated from the fluorescein-labelled material, and entered the cell to induce cellular events leading to lymphocyte activation.

Nevertheless, the observation of early fluorescence associated with the cell surface, soon after the administration of fluorescein-labelled PHA, would be supportive evidence for a surface action as opposed to an intracellular effect of PHA. This is because the mitogen is known to cause marked changes in cellular metabolism during the first few hours of its administration (Section 1.5.3). For example, this present work has described increases in K^{42} transport into the cell after the first hour of treatment with PHA (see Section 3.4).

During the time when the results to be described were obtained, the very similar studies by Michalowski (1965) and Razavi (1966) were published. It was therefore decided to proceed directly to the work reported in Sections 3.1 to 3.4. However, the results obtained using fluorescein-labelled PHA are included in this supplementary section.

3.5.7.2. Capacity of PHA-M-fluorescein-isothiocyanate (PHA-FITC) to Induce Transformation

Blood from a healthy male volunteer was obtained and the leukocytes were separated in the usual way. PHA-FITC was prepared as already described (Section 2.2.3) and sterilized by suction through a millipore filter. The preparation was then mixed with the cells at a PHA concentration of 200 µg/ml.

Two days later, the morphological appearance of the cells was observed by phase contrast microscopy and observation of cell smears. Many 'blast' cells were observed (see the phase contrast photograph in Fig. 3.5.24) showing either that the PHA-FITC preparation was capable of inducing transformation or that the bond between PHA and fluorescein was cleaved in the culture medium.

3.5.7.3. PHA-FITC Binding to Small Lymphocytes

In the same experiment as that described above, cells were treated with PHA-FITC for 1-1½ hours. They were then suspended in a

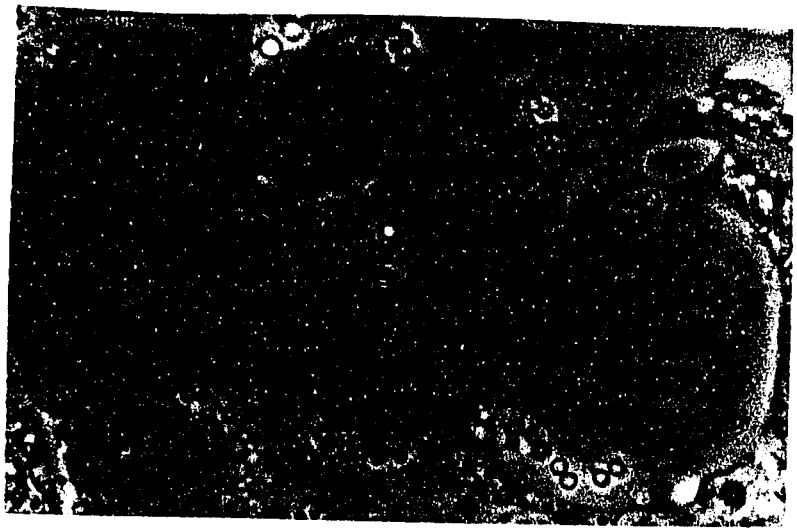


Fig. 3.5.24

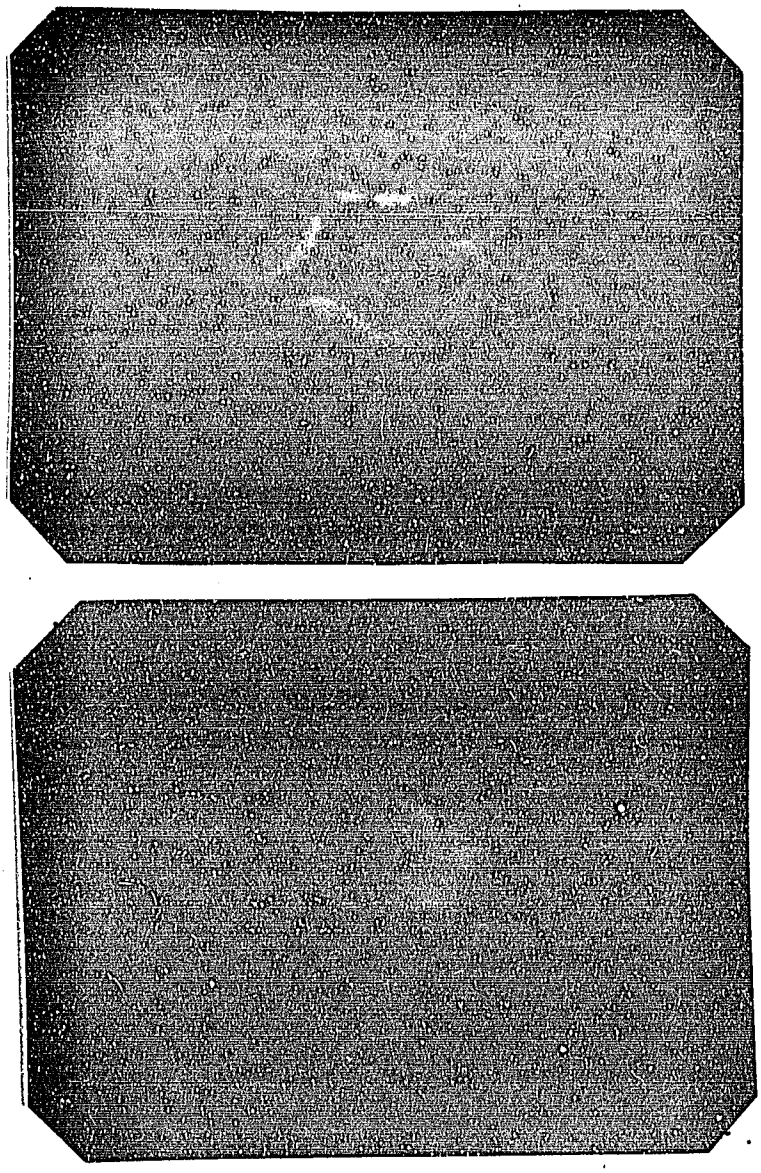
Blast cells formed after treatment of leukocyte cultures for 2 days with PHA-M labelled with fluorescein isothiocyanate (PHA-FITC). x 1250.

drop of medium and examined with ultraviolet light for fluorescence. Most lymphocytes so treated were observed to fluoresce around their circumference, unlike those treated with PHA only. In some cases (Fig. 3.5.25), fluorescence was seen around the circumference of the cell and also over and possibly within the cytoplasm. However, it was not possible to differentiate between cytoplasmic fluorescence and surface membrane localization of the fluorescence in these simple experiments. Fluorescence was never intranuclear.

3.5.7.4. PHA-FITC Binding to 'Blast' Cells

As well as binding to small lymphocytes, the fluorescein-labelled PHA was observed to associate with blast cells. It is of interest that the binding of PHA-M to red cells was relatively slight in comparison to that seen with the white cells. In cultures treated for 3 days with PHA-FITC, white cells embedded within the red cell clumps were seen to glow strongly in the presence of UV light in comparison to very slight or negligible fluorescence of the red cells.

A clump or group of blast cells is shown in Figure 3.5.26. Fluorescence was clearly peripheral and possibly cytoplasmic, occurring mainly between the junction of cells, but was definitely no intranuclear. It is possible that the fluorescence seen was present in cytoplasmic areas associated with the vacuole seen more clearly in Figures 3.1.7 and 3.5.24. Some 'blast' cells showed definite pronounced cytoplasmic fluorescence after a day or longer in PHA-FITC. This was particularly true for damaged or dying cells.

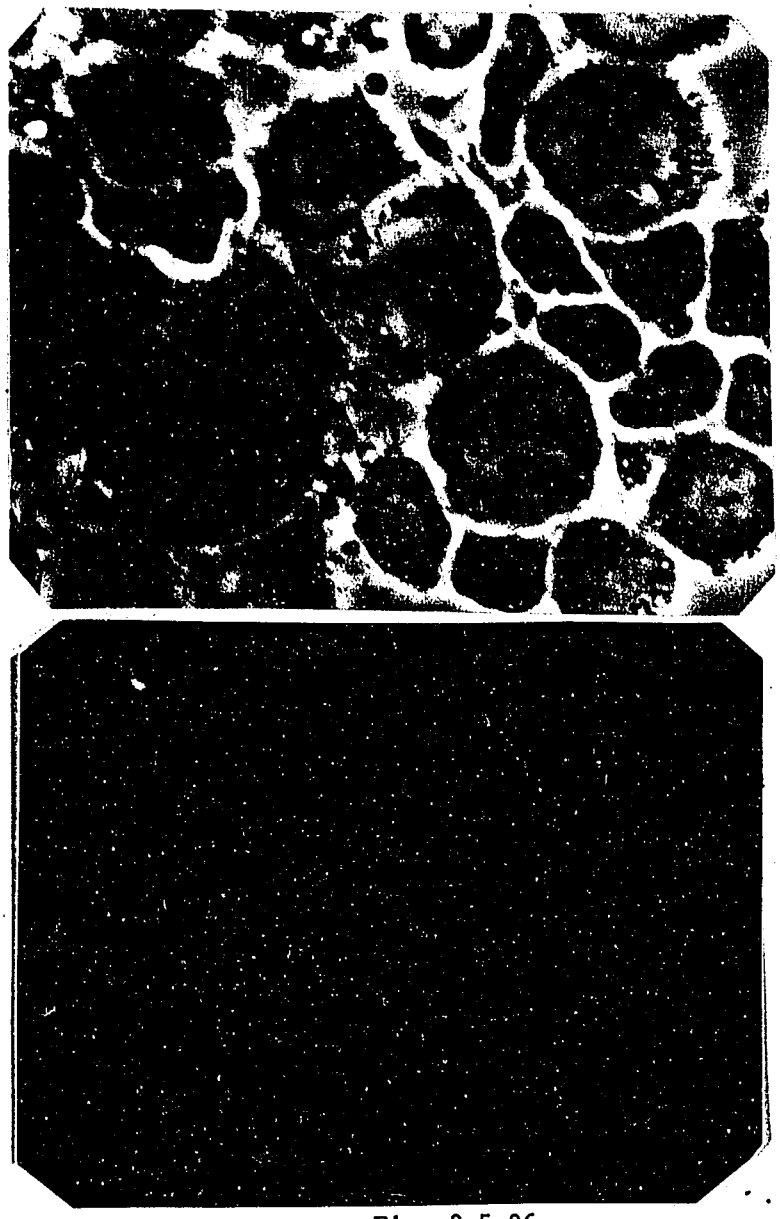


(a)

(b)

Fig. 3.5.25

Fluorescence of lymphocytes treated for $1\frac{1}{2}$ hours with PHA-FITC. (a) phase contrast (b) same cell, UV fluorescence. x 1250.



(a)

(b)

Fig. 3.5.26

Fluorescence of blast cells treated with PHA-FITC for 2 days. (a) phase contrast (b) same field, UV fluorescence. x 1250.

Note that fluorescence is non-nuclear and tends to concentrate at the periphery of the cells in localized areas. It was not possible to tell whether this fluorescence was within the cytoplasm or on the surface of the cells.

3.5.7.3. Summary of Results

(1) PHA-FITC was shown to be capable of inducing transformation.

(2) After 1½ hours with PHA-FITC, fluorescence on small lymphocytes was primarily peripheral but an intracellular component could not be ruled out.

(3) Fluorescence was never observed in the nucleus.

(4) When present for a day or more in PHA-FITC, strong cytoplasmic fluorescence was often observed.

(5) The degree of fluorescence was dependent on the type of fixative used. Thus, acetic acid:ethanol led to a complete absence of fluorescence, but ethanol alone did not affect it.

In view of the non-nuclear and possibly membrane localization of fluorescence, an action of PHA on the cell membrane or in the cytoplasm was likely. Later experiments were carried out to examine certain cellular effects of inhibiting the membrane cation transport, as described in Section 3.2.

4. THEORY

The objectives of this theoretical section are;

(a) to formulate mathematical relationships governing cation fluxes in unstimulated and stimulated lymphocytes, and from these relationships, to predict values for the uptake, efflux and steady state levels of potassium with which experimental results using radio-potassium may be compared;

(b) to construct models by which macromolecular synthesis may be related to cation content and fluxes, and to compare the predictions resulting from these models with experimental data.

4.1. FLUXES OF POTASSIUM AND SODIUM

The total cation flux across a membrane is assumed to be due to 3 main factors;

(a) simple diffusion across a concentration gradient;

(b) movement of the charged ions in an electric field within the membrane; and

(c) active directed transport requiring the expenditure of metabolic energy.

In the following formulation, the fluxes of other anions, cations and uncharged substances which may affect the overall cellular uptake of sodium and potassium are not considered.

4.1.1. Derivation of Formulae Describing 'Passive' Fluxes

The following symbols correspond to those in Figs. 4.1.1. and 4.1.2.

D = Diffusion constant (cm^2/sec).

P = Permeability (cm/sec).

C = Concentration of cation at a distance x cm within the membrane. Subscripts, such as C_1, C_2, C_i, C_o, refer to the concentration at side 1, side 2, inside and outside respectively. Superscripts refer to the ion considered; thus C_i^k, C_o^n refer to the inside potassium concentration (moles/ml) and the outside sodium concentration respectively.

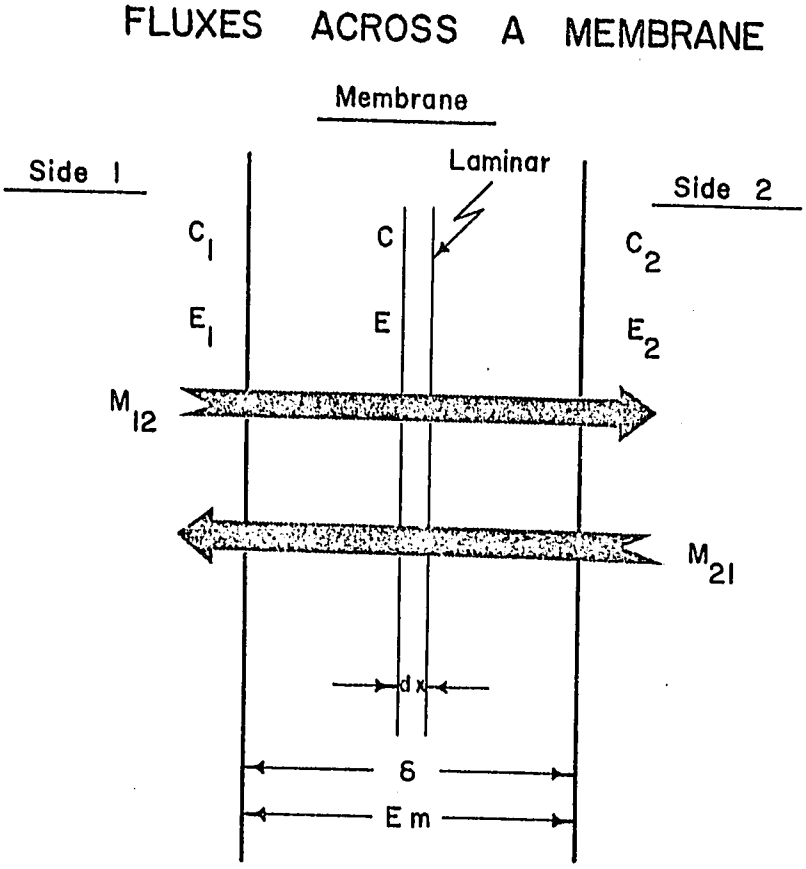


Fig. 4.1.1.

Fluxes across a membrane. The terminology used is defined in the text on page 470.

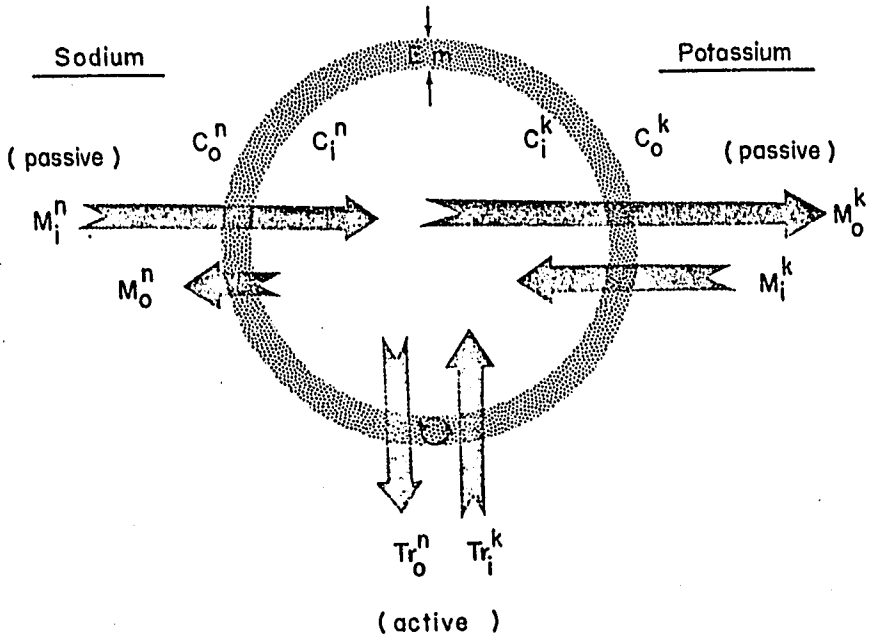


Fig. 4.1.2.

Cation fluxes in a model cell. The terminology used is defined on page 470 of the text.

E = Electrical potential (volts) in the membrane at any distance from the surface x. E_m is the transmembrane potential.

F = Faraday constant, i.e., the charge per mole of univalent ion (96,501 coulombs).

R = Gas constant = 8.314×10^7 ergs degrees^{-1} (cm mole)$^{-1}$.

T = Absolute temperature (degrees Kelvin).

M = Flux of cation (moles/cm^2/sec). Superscripts and subscripts are the same as for concentration C.

z = Valency of cation.

ν = zEF/RT

x = Distance into membrane (cm).

δ = Thickness of membrane (cm).

Tr = Active transport across membrane (moles/cm^2/sec). Superscripts and subscripts as for concentration C.

V_{max} = Maximum active transport flux (moles/cm^2/sec).

K_m = Michaelis constant for potassium transport, i.e. the concentration of potassium for half maximal rate of transport.

K_i = Inhibition constant for inhibitor (ouabain).

I = Concentration of inhibitor (ouabain) in moles/ml.

γ = Stochiometric relationship of number of sodium cations transported to the number of potassium ions transported.

S = Specific activity of radiopotassium (curies/mole). Subscripts are the same as for concentration C.

V = Cell volume (ml). Assumed to be very close to total cell water.

A = Cell surface area (cm^2).

$T_{1/2}$ = Time to reach half maximum asymptotic values or to decrease to half of initial value. Used for exponential relationships (secs).

u = Deduced amount of potassium entering or leaving the cell (moles/cell); it is determined as the amount of radioactivity measured in the cell (cpm) divided by the specific activity of the radiopotassium in the medium (cpm/mole).

b = Time constant = $\ln 2/T_{1/2}$ (sec^{-1}) = AM_o/VC_i.

t = Time (sec).

τ = Threshold. Used as a subscript. Thus C_τ is a threshold concentration.

Y = Rate of synthesis.

Y_{50} = 1/2 rate of synthesis.

In the analysis of 'passive' forces acting on cations in a membrane, 'passive' is defined as the absence of a metabolically-driven, energy requiring force on the substance undergoing transition across the membrane. Some of the foregoing quantitative aspects have been summarized by Patlak (1961).

The initial equations derived are in part similar to those of Tosteson and Hoffman (1961) except that the relationship between transport flux and leak influx is not assumed to be independent of the extracellular potassium concentration.

4.1.1.1. Diffusion Across a Concentration Gradient

In the absence of an electrical field, the unidirectional fluxes across a laminar (Fig. 4.1.1.) are given by

$$M_{12} = PC_1$$
$$M_{21} = PC_2 \qquad (1.1)$$

The net flux across a thin laminar area may also be expressed as

$$M_{net} = -D \frac{dC}{dx} \qquad (1.2)$$

since the permeability (P) of the membrane is determined by the diffusion constant (D) and inversely by the thickness of the membrane, among other things.

4.1.1.2. Movement of Ions in Electrical Field

If a concentration gradient is not present, the force acting per mole of ion is

$$\text{Force} = -zF \frac{\vec{dE}}{dx} \qquad (1.3)$$

The work done moving one mole of charged solute through an electric field is given by

$$dw = -zF \frac{dE}{dx} \cdot dx$$

But this must be equal to the loss of potential energy per mole of the solute

$$dw = -RT \frac{dC}{C}$$

Since the membrane would be expected to present the same resistance to the movement of particles whether their source of energy was thermal agitation or electrical potential, we may calculate the net flux by solving the above two equations for the concentration gradient $\frac{dC}{dx}$ induced by the electric field to give

$$M_{net} = -D \frac{dC}{dx} = \frac{-DzFC}{RT} \cdot \frac{dE}{dx} \qquad (1.4)$$

4.1.1.3. Movement of Ions in a Combined Concentration Gradient and Electrical Field

We previously considered the motion of ions in a concentration gradient and an electrical field separately. It is now assumed that these forces are additive in a combined concentration gradient and electrical field. From the addition of equations (1.2) and (1.4) for flow across a laminar, we obtain a net flux

$$M_{net} = -D \left(\frac{dC}{dx} + \frac{zF}{RT} C \frac{dE}{dx} \right) \qquad (1.5)$$

Expression (1.5) may be rewritten, without any loss in generality,

$$M_{net} = -D\, e^{\frac{-zEF}{RT}} \frac{d}{dx} \left(C\, e^{\frac{zEF}{RT}} \right) \qquad (1.6)$$

or $$M_{net} = -D\, e^{-\nu} \frac{d}{dx} (Ce^{\nu})$$

In order to obtain the flux of the ion across the membrane, it is necessary to integrate this expression for a laminar across the thickness of the membrane. This can be done if the simplifying assumptions are made that the electrical field across the membrane is constant (i.e., that $\frac{dE}{dx}$ is constant) and that the diffusion constant D is invariant with the distance into the membrane. It is also assumed that the inward and outward fluxes are independent and do not interfere with each other.

The unidirectional fluxes may then be solved as follows;

$$\begin{aligned} M_{12} \int_1^2 \frac{e^{\frac{zEF}{RT}}}{D} dx &= -\int_1^2 C\, e^{\frac{zEF}{RT}} \\ M_{21} \int_2^1 \frac{e^{\frac{zEF}{RT}}}{D} dx &= +\int_2^1 C\, e^{\frac{zEF}{RT}} \end{aligned} \qquad (1.7)$$

Solving by integrating across the membrane, under the constant field assumptions, we obtain the unidirectional fluxes,

$$M_{12} = PC_1 \frac{\nu}{1-e^{-\nu}}$$
$$M_{21} = PC_2 \frac{\nu}{e^{\nu}-1} \qquad (1.8)$$

4.1.1.4. Nernst Relationship

If an equilibrium is set up across the membrane, the fluxes $M_{12} = M_{21}$. In this case, we obtain from equations (1.8),

$$PC_1 \frac{\nu}{1-e^{-\nu}} = PC_2 \frac{\nu}{e^{\nu}-1}$$

or
$$C_1 e^{\frac{zE_mF}{RT}} = C_2$$

This may be rearranged into the more familiar form,

$$E_m = \frac{RT}{zF} \ln \frac{C_2}{C_1} \qquad (1.9)$$

4.1.1.5. Ussing Criterion for Active Transport

The ratio of the fluxes gives the relationship,

$$\frac{M_{12}}{M_{21}} = \frac{C_1}{C_2} e^{\frac{zE_mF}{RT}} \qquad (1.10)$$

If this relationship does not hold, part of the ion flux is assumed not to be 'passive'.

4.1.2. Potassium and Sodium Fluxes in Cells

Consider the situation in Fig. 4.1.2. We will assume that the active transport of the ions is independent of the diffusion of the ions in the electrochemical gradient. A voltage gradient across the membrane will be assumed, relatively negative inside the cell; the origin of this transmembrane potential is probably due both to the presence of indiffusible electronegative anions within the cell and to the relative difference in permeability of the membrane to sodium and potassium. In this derivation, the mechanism for the origin of the transmembrane potential is not assumed.

4.1.2.1. Sodium

From (1.8), we see that the passive sodium fluxes can be expressed as,

$$M_i^n = p^n C_o^n \frac{\nu}{1-e^{-\nu}}$$

$$M_o^n = p^n C_i^n \frac{\nu}{e^{\nu}-1} \qquad (1.11)$$

It is almost certain that a metabolically active transport of sodium compensates for the inward leakage of sodium in animal cells, thus maintaining a cation

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gradient. Under steady state conditions,

$$Tr_o^n + M_o^n = M_i^n \qquad (1.12)$$

It might be noted that since the sodium level is normally low in the cell, relative to the outside concentration and the electrical potential retards the movement of positive ions outwards, M_o^n is small, and

$$Tr_o^n \doteq M_i^n \qquad (1.13)$$

The function Tr is a complex factor depending on the external potassium concentration as well as intracellular factors including ATP availability and sodium concentration.

4.1.2.2. Potassium

An analogous set of equations may be used. However, the passive diffusion of potassium out of the cell is large, rather than small as in the case of sodium, owing to the high levels of potassium in the cell. From equations (1.8), the passive potassium fluxes can be expressed as;

$$M_i^k = p^k C_o^k \frac{\nu}{1-e^{-\nu}}$$

$$M_o^k = p^k C_i^k \frac{\nu}{e^{\nu}-1} \qquad (1.14)$$

Under steady state conditions, and including a term for active transport, we have

$$Tr_i^k + M_i^k = M_o^k \qquad (1.15)$$

The same argument as in 1.13 cannot be used for potassium, because the membrane potential enhances rather than suppresses the inward leak of potassium.

4.1.2.3. Coupled Sodium and Potassium Transport

The system is generally considered to require the coupled transport of n sodium ions for m potassium ions. Let the ratio $n/m = \gamma$.

For coupled transport, it is clear from the definition of γ that

$$\gamma\ Tr_i^k = Tr_o^n \qquad (1.16)$$

If a 1:1 relationship exists, then $\gamma = 1$, and

$$Tr_i^k = Tr_o^n \qquad (1.17)$$

Hence, under normal, steady state conditions, where internal sodium levels are low compared to those outside the cell, we have, from equation (1.13),

$$Tr_i^k \doteq M_i^n \qquad (1.18)$$

or, the transport of potassium into the cell is equal, on a molar basis, to the leakage of sodium into the cell. However, this will not necessarily be true under non-steady state conditions.

4.1.3. Membrane Potential

If we combine equations (1.12) and (1.15), we have

$$Tr_i^k + M_i^k = M_o^k$$

$$Tr_o^n + M_o^n = M_i^n$$

But, from (1.17),

$$Tr_i^k = Tr_o^n$$

Therefore, we may eliminate the transport term from the above equations by subtracting one from the other, and we obtain,

$$M_i^k + M_i^n = M_o^k + M_o^n$$

Inserting the values for fluxes determined in equations (1.14) and (1.11), and rearranging, we obtain,

$$e^{\nu} = \frac{p^k C_i^k + p^n C_i^n}{p^k C_o^k + p^n C_o^n} \qquad (1.19)$$

or the Goldman (1943) Hodgkin and Katz (1949) equation in a more familiar form

$$E_m = \frac{RT}{zF} \ln \left\{ \frac{p^k C_i^k + p^n C_i^n}{p^k C_o^k + p^n C_o^n} \right\} \qquad (1.20)$$

This equation will hold for a constant field across the membrane, provided a steady state exists, and the coupled

transport is a 1:1 exchange of sodium and potassium. This relationship does not necessarily determine the membrane potential, but only expresses how the cations rearrange themselves in the presence of the membrane potential. Chloride is presumed to distribute passively according to the Nernst equation and therefore does not have to be included in this equation as it is in the generalized field equation of Hodgkin and Katz (1949). If the value for $\gamma \neq 1$, it is easily shown that

$$E_m = \frac{RT}{zF} \ln \left\{ \frac{\gamma p^k C_i^k + p^n C_i^n}{\gamma p^R C_o^k + p^n C_o^n} \right\} \qquad (1.21)$$

which was used by Hodgkin and Horowicz (1959) in the form (where z = 1)

$$E_m = \frac{RT}{F} \ln \frac{C_i^k + \alpha C_i^n}{C_o^k + \alpha C_o^n} \qquad (1.22)$$

where $$\alpha = \frac{p^n}{\gamma p^k}$$

By equating the exponents of the logarithm term of (1.21) and the chloride equilibrium potential, a Donnan steady state condition is obtained directly, as follows:

$$(C_o^k + \alpha C_o^n)\, C_o^{cl} = (C_i^k + \alpha C_i^n)\, C_i^{cl} \qquad (1.23)$$

4.2. FLUXES OF K^{42}

It will be assumed in this discussion that;

(a) the cell is homogeneous and surrounded by a membrane, through which cations may diffuse at various rates determined by the permeability of the membrane to the cations, and which causes them to be transported actively;

(b) that the active and passive fluxes are separate and additive;

(c) that stable and radioactive potassium are biochemically identical.

Since only radiopotassium is considered throughout the following, the superscript k has been left off the symbols for concentration, flux and transport.

4.2.1. General Formulation

The rate of accumulation of radiopotassium by the cell is the difference between the influx and the outflux. Thus, we have (taking $Tr = Tr_i^k = Tr_o^n$ from 1.17),

$$\frac{d}{dt}(VC_iS_i) = AS_o(M_i + Tr) - AS_iM_o \qquad (2.1)$$

4.2.1.1. The Initial Rate of Uptake is

$$\frac{d}{dt}(VC_iS_i)_{t=o} = AS_o(M_i + Tr) \qquad (2.2)$$

or, since from the definition,

$$u = \frac{VC_iS_i}{S_o}$$

and from equation (1.14) equation (2.2) may be rewritten as

$$\left(\frac{d}{dt}\,u\right)_{t=o} = A\left(C_o P\,\frac{\nu}{1-e^{-\nu}} + Tr\right) \qquad (2.3)$$

4.2.1.2. Uptake and Efflux of K^{42} at Steady State

At a steady state, from equation (1.15)

$$M_i + Tr = M_o$$

Substituting this into equation (2.1) and re-arranging we obtain,

$$VC_i\,\frac{d}{dt}\,S_i = AM_o(S_o - S_i) \qquad (2.4)$$

or

$$\frac{d}{dt}\,S_i = b(S_o - S_i)$$

where

$$b = \frac{AM_o}{VC_i}$$

Integrating over a time, t, this may be solved simply for the two useful cases: (a) when the unlabelled cell is placed in radioactive medium, and its accumulation of K^{42} measured, or (b) when the 'prepacked' cell is replaced in non-radioactive medium, and the rate of K^{42} loss measured. For case (a), we have

$$u = VC_i\,(1-e^{-bt}) \qquad (2.5)$$

For case (b), we have

$$u = (u_{t=o})e^{-bt} \qquad (2.6)$$

where

$$b = \frac{\ln 2}{T_{1/2}} = \frac{AM_o}{VC_i}$$

For each of these cases, the time constant b is the same, and

$$b = \frac{AM_o}{VC_i} = \frac{A}{V} p \frac{\nu}{e^{\nu}-1} \qquad (2.7)$$

4.2.1.3. Determination of Membrane Potential

I have devised a method of determining the membrane potential experimentally from potassium flux data.

The value for b can be determined directly by measuring the rate of uptake of K^{42} into the cell, or its efflux from the cell. If the time constants obtained either way are equal, validity of the simple theoretical expression is supported.

In addition, if the transport term can be made invariant with respect to the external potassium concentration by (a) saturating the active transport system, or (b) by inhibiting it with an inhibitor, then the value for $\frac{d}{dt}(u)_{t=o}$ in equation (2.3) will be a function of C_o; hence, plotting $\left(\frac{du}{dt}\right)_{t=o}$ against C_o should give a slope $\sigma = Ap \frac{\nu}{1-e^{-\nu}}$. Thus the value σ may be determined experimentally.

Dividing b into σ, we have

$$\frac{\sigma}{b} = Ve^{\nu}$$

and since

$$\nu = \frac{zEF}{RT}$$

$$E_m = \frac{RT}{zF} \ln \frac{\sigma}{Vb} \qquad (2.9)$$

4.2.1.4. Estimates of Permeability

A value for the permeability constant for the lymphocyte membrane to potassium may be obtained from expression (2.7) and (2.9). If the membrane potential is calculated from equation (2.9), it can be used to complete equation (2.7) which may be rewritten as

$$p = b \cdot \frac{V}{A} \cdot \frac{e^{\nu}-1}{\nu} \qquad (2.10)$$

Values for the volume and area of the lymphocyte may be calculated on the assumption that it is globular in shape. Some error may be caused by the fact that a wrinkled or corregated surface would considerably increase the surface area of the cell without affecting its volume.

4.2.1.5. Estimates of Flux

An estimate of the total outflux, which equals the total influx at steady state, may be made from equation (2.7), provided that the internal concentration of potassium is known. Thus,

$$M_o = b \cdot \frac{V}{A} \cdot C_i \qquad (2.11)$$

The passive influx is given, from (1.14) and (2.8) as

$$M_i = \sigma C_o \qquad (2.12)$$

4.3. TOTAL CELLULAR CONTENT OF POTASSIUM AND EFFECT OF OUABAIN

In the following formulation, I have derived expressions for the total cation content of the cell and shall examine the effect of ouabain from a theoretical point of view. After this, I will propose models by which the rate of macromolecular synthesis will be related to the intracellular potassium concentration or to the rate of transport, both with or without thresholds. These 4 theoretical models will be compared to the observed effects of ouabain on macromolecular synthesis.

4.3.1. Total Intracellular Cation Content

Under steady state conditions, the rate of cation entering the cell equals the rate of its efflux. For potassium we have

$$\frac{d}{dt}(VC_i^k) = A(M_i^k + Tr_i^k - M_o^k) \tag{3.1}$$

and for sodium, we have,

$$\frac{d}{dt}(VC_i^n) = A(M_i^n - Tr_o^n - M_o^n) \tag{3.2}$$

At equilibrium, we have, from (1.11) and (1.15),

$$C_i^k = C_o^k e^{\nu} + \frac{e^{\nu}-1}{\nu}\frac{T_r}{p_R} \tag{3.3}$$

$$C_i^n = C_o^n e^{\nu} - \frac{e^{\nu}-1}{\nu}\frac{T_r}{p_n} \tag{3.4}$$

and

$$T_r = \frac{V_{max}\, C_o^k}{C_o^k + K_m} \qquad (3.5)$$

since $Tr = Tr_i^k = Tr_o^n$ from (1.17), and is assumed to be characterized by saturation kinetics.

4.3.2. Effect of Ouabain on Total Cellular Potassium

Since it can reasonably be assumed that ouabain inhibits the coupled transport of potassium and sodium in this system, the formula for transport (3.5) must be modified to take into account the presence of the ouabain.

We shall assume that ouabain acts as a simple competitive inhibitor of potassium for sites on the enzyme system. Support for this, functionally, is given by the results shown in Fig. 3.4.16. This assumption is known not to be strictly true for red cells, but it will be used here as an approximation of reality. Under such conditions, we have a new transport term* as follows:

---

*This is obtained by the following reasoning analogous to that used for simple competitive enzyme kinetics. E is the enzyme system required for active transport, and E' its form after releasing K^+ into the cell,

$$E + K \rightleftarrows EK \xrightarrow[\text{membrane}]{\text{across}\ \ j} E' + K$$

$$E + I \rightleftarrows EI$$

then $([E]-[EK]-[EI])\,[I] = K_i[EI]$

and $([E]-[EK]-[EI])\,[K] = K_m[EK]$

$$Tr = j\,[EK]$$

Solving these equations for Tr as a function of [I] gives equation (3.6).

$$T_r(I) = \frac{V_{max}}{1 + \frac{K_m}{C_o}(1 + \frac{I}{K_i})} \qquad (3.6)$$

After addition of ouabain at a given concentration I, and allowing the cells to reach equilibrium, the new potassium concentration of the cell interior becomes;

$$C_i(I) = C_o e^{\nu} + \frac{e^{\nu}-1}{\nu} \cdot \frac{1}{P} \frac{V_{max}}{1 + \frac{K_m}{C_o}(1 + \frac{I}{K_i})} \qquad (3.7)$$

4.4 ALTERNATIVE MODELS

In order to relate macromolecular synthesis to the effect of ouabain, models based on known effects of the glycoside will be considered in turn. It is assumed that the ouabain causes its effect by inhibiting coupled cation transport, resulting in a readjustment of internal cation levels. These models will only consider the transport rate or the cation levels, and the movements of substances such as amino acids have not been considered.

We will assume 4 possible circumstances;

(a) that macromolecular synthesis is directly proportional to the internal potassium concentration;

(b) that macromolecular synthesis is directly proportional to the internal potassium concentration above a certain threshold concentration;

(c) that macromolecular synthesis is directly proportional to the rate of cation transport across the cell membrane;

(d) that macromolecular synthesis is directly proportional to the rate of cation transport across the cell membrane above a threshold value.

The analytic procedure is as follows. First, expressions for the potassium concentration or transport

are formulated, with particular regard to the action of ouabain and of varying the concentration of potassium in the culture medium. These models are then fitted to the experimental data obtained from measurements of the effect of potassium and ouabain on macromolecular synthesis, and values for the K_m and K_i are deduced from this information, according to the model used.

Finally, these constants are compared to the same constants observed by direct measurement with K^{42}. If they agree with the values obtained using the model, the data are considered to support the validity of the model.

4.4.1. Model 1. Assume that the Rate of Macromolecular Synthesis is Directly Proportional to the Internal Potassium Concentration

From equation (3.7), an expression for the intracellular potassium concentration was obtained in terms of the external potassium concentration, the membrane potential and the concentration of ouabain; that is,

$$C_i(I) = C_o e^{\nu} + \frac{e^{\nu}-1}{\nu} \cdot \frac{1}{P} \cdot \frac{V_{max}}{1 + \frac{K_m}{C_o}(1 + \frac{I}{K_i})} \qquad (4.1)$$

This expression, when plotted against ln I gives a series of curves for different values of the extracellular potassium concentration, C_o, as shown in Fig. 4.4.1.

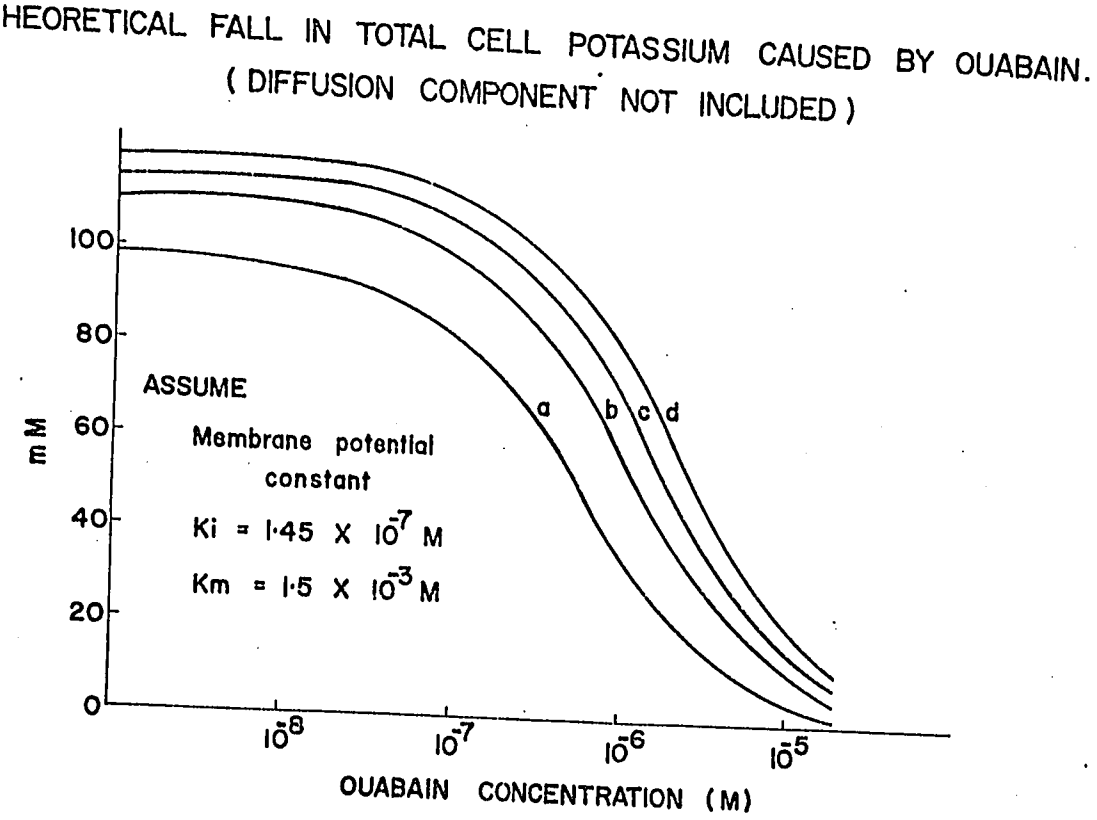


Fig. 4.4.1.

The diffusion component is not included because it depends on the membrane potential which is not known.

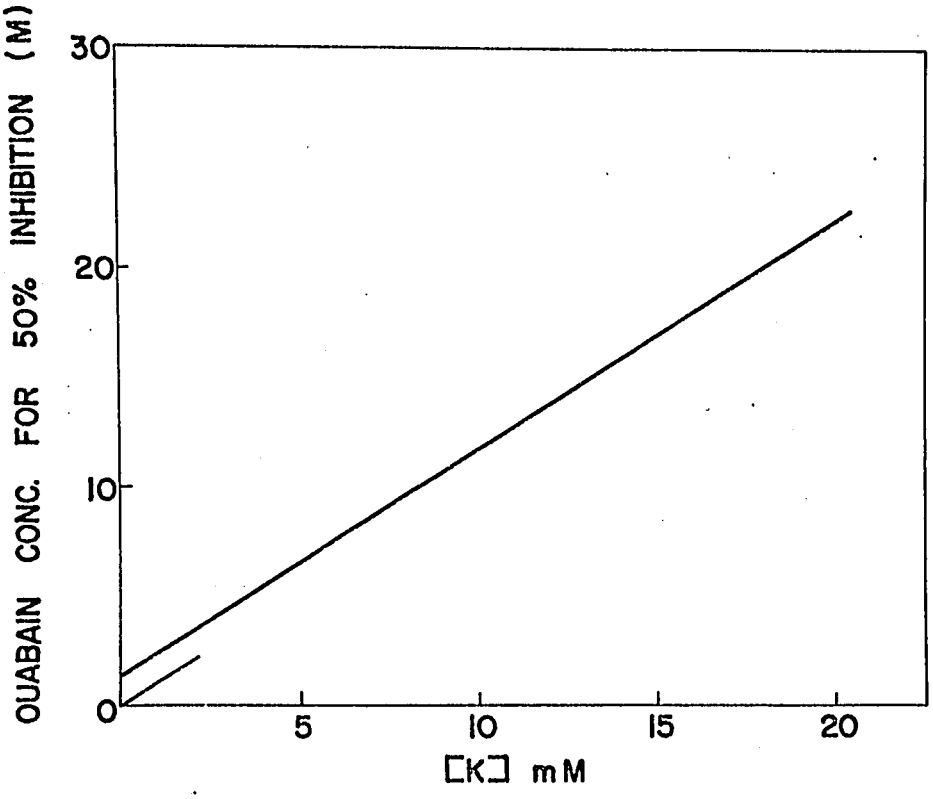


Fig. 4.4.2.

Theoretical plot for equation (4.2). Relation between ouabain concentration for half maximal reduction of potassium of the cell, and the extracellular potassium concentration.

The slopes of these curves are maximal at a value $\bar{I}$, where

$$\bar{I} = K_i(1 + C_o/K_m) \tag{4.2}$$

At this concentration, the equilibrium internal potassium concentration is,

$$\bar{C}_i = \frac{C_o + C_i}{2} \tag{4.3}$$

If one plots the value of $\bar{I}$ corresponding to the amount of potassium in the external medium, C_o, one obtains a linear relationship with slope

$$K_i/K_m \tag{4.4}$$

and intercept K_i as shown in Fig. 4.4.2. These values can be compared with those determined kinetically - see section 4.4.5.

4.4.2. Model 2. Assume that the Rate of Macromolecular Synthesis is Directly Proportional to the Internal Potassium Concentration above a Certain Threshold Concentration.

Let the threshold intracellular potassium concentration be C_τ. By definition, the rate of macromolecular synthesis R is given by

$$Y(I) = k\,(C_i(I) - C_\tau) \tag{4.5}$$

A 50% synthetic rate can easily be shown to be

$$Y_{50}(I) = k\left[\frac{C_i - C_t}{2}\right] \qquad (4.6)$$
$$= k(\bar{C}_i - C_\tau)$$

From equation (3.7), we obtain

$$C_t = C_o e^{\nu} + \frac{e^{\nu}-1}{\nu} \cdot \frac{1}{P} \cdot \frac{V_{max}}{1 + \frac{K_m}{C_o}(1 + \frac{I_\tau}{K_i})} \qquad (4.7)$$

The threshold concentration of ouabain I_τ is that which will bring the internal potassium concentration to the threshold C_t (Fig. 4.4.3.)

The ouabain concentration $\bar{I}$ which is required to obtain $\bar{C}_i$ is obtained simply by inserting equations (3.7) and (4.7) into (4.6), to get;

$$\frac{1}{\bar{I}} = \frac{2}{I_\tau} + \frac{1}{K_i(1 + C_o/K_m)} \qquad (4.8)$$

or alternatively

$$\bar{I} = K_i(1 + \frac{C_o}{K_m}) \frac{I_\tau}{I_\tau + 2K_i(1 + \frac{C_o}{K_m})} \qquad (4.9)$$

or, again alternatively,

$$\bar{I} = K_i(1 + \frac{C_o}{K_m}) \left\{ \frac{\frac{\beta}{C_t - C_o e^{\nu}} - (1 + \frac{K_m}{C_o})}{\frac{\beta}{C_t - C_o e^{\nu}} + (1 + \frac{K_m}{C_o})} \right\} \qquad (4.10)$$

where $$\beta = \frac{e^{\nu}-1}{\nu} \cdot \frac{1}{P} \cdot V_{max} \qquad (4.11)$$

Equations 4.8 to 4.10 are equivalent expressions. It is clear that they are the same as the non-threshold case (4.3) when I_τ is very large or when $C_\tau = C_o e^{\nu}$.

The relationship of $\bar{I}$ to C_o is a complex one, particularly because the effect of varying the external potassium concentration on the membrane potential is not obvious. However, inspection of (4.10) shows that the slope of $\bar{I}$ plotted against C_o becomes maximal when C_o is large, and then has the value K_i/K_m. However, the slope of this plot decreases as the C_o is lowered, and the intercept with the Y axis, when C_o approaches very small values is negative, lying below the origin, and has a value of $-K_i$, (Fig. 4.4.4.).

4.4.3. Model 3. Assume that the Rate of Macromolecular Synthesis is Directly Proportional to the Rate of Cation Transport Across the Membrane

From equation (3.6), we have

$$T_r(I) = \frac{V_{max}}{1 + \frac{K_m}{C_o}(1 + \frac{I}{K_i})}$$

By this model it follows that the rate of synthesis R(I) is given by

$$Y(I) = kT_r(I) = \frac{kV_{max}}{1 + \frac{K_m}{C_o}(1 + \frac{I}{K_i})} \qquad (4.12)$$

The value for $R_{50}(\bar{I})$ is given by

$$Y_{50}(\bar{I}) = \frac{Y(I=o)}{2} \qquad (4.13)$$

and it is easily shown that $\bar{I}$ depends on the external potassium concentration by the relationship

$$\bar{I} = K_i(1 + C_o/K_m) \qquad (4.14)$$

The kinetics for this situation are therefore very similar to those of model 1, in subsection 4.4.1. That is, the slope of the plot of $\bar{I}$ against C_o is K_i/K_m and has a positive intercept at on the Y axis at K_i. However, the family of curves found in Sect. 4.4.3. differ from those in 4.4.1. because they are not superimposed on the passive diffusion term $C_o e^{\nu}$.

4.4.4. Model 4. Assume that the Rate of Macromolecular Synthesis is Directly Proportional to the Rate of Cation Transport Across the Membrane Above a Threshold Value

In analogy to subsection 4.4.2. we have, by definition, the rate Y(I) given by

$$Y(I) = k(Y - Y(I_\tau)) \qquad (4.15)$$

and $$Y_{50}(I) = \frac{Y + Y(I_\tau)}{2} \qquad (4.16)$$

but $$Y(I_\tau) = kTr_\tau = \frac{kV_{max}}{1 + \frac{K_m}{C_o}(1 + \frac{I_\tau}{K_i})} \qquad (4.17)$$

where the threshold concentration of ouabain I_τ is that required to bring the transport rate to a threshold value Tr_t. The ouabain concentration $\bar{I}$ required to obtain $Tr_{50}(I)$ is obtained simply by inserting equations (3.6) and (4.17) into (4.16) to obtain

$$\bar{I} = K_i(1 + \frac{C_o}{K_m}) \frac{I_\tau}{I_\tau + 2K_i(1 + \frac{C_o}{K_m})} \qquad (4.18)$$

These equations are identical to those obtained for model 2 in subsection 4.4.2. and give the same relationship for the slope of the plot of $\bar{I}$ against C_o and the same intercept with the Y axis below the origin at $-K_i$.

Hence these equations taken alone do not allow us to distinguish between the threshold potassium concentration case and the threshold transport case. But other independent data, such as the time taken for ouabain to have an effect on macromolecular synthesis, provides further information for a choice between the hypotheses.

Equation (4.18) is also equivalent to

$$\overline{I} = K_i \left(1 + \frac{C_o}{K_m}\right) \frac{\frac{V_{max}}{Tr_\tau} - \left(1 + \frac{K_m}{C_o}\right)}{\frac{V_{max}}{Tr_\tau} + \left(1 + \frac{K_m}{C_o}\right)} \qquad (4.19)$$

This equation is very similar to (4.10) except that the passive diffusion term involving the membrane potential does not occur. This graph intersects the Y axis at $I = -K_i$.

4.5 CALCULATED PARAMETERS FROM THEORY AND EXPERIMENTAL DATA

4.5.1 Potassium fluxes; effect of PHA

Values for passive, steady state potassium fluxes, membrane permeability to potassium and transmembrane potential will be estimated from the following data:

(a) the time constant for K^{42} influx and efflux;

(b) the rate of K^{42} influx in the presence of ouabain;

(c) the measured intracellular potassium.

In these calculations, the cell will be assumed to be spherical and to have a volume of 250 μ^3, from the data of Westring et al (1969) and Lichtman and Weed (1969). The loss of K^{42} from the cell during the washing procedure is taken into account by corrections based on the data shown in Fig. 3.4.2.

An estimate of active transport is obtained from the difference between the passive influx and outflux, and the Vmax is then to be calculated using equation 3.5. This indirect estimate of the Vmax is compared with the value of Vmax measured by experiment (Figs. 3.4.11 and 3.4.12).

It is shown (see Table 4.5.1) that the Vmax required to account quantitatively for the observed increase in cell potassium caused by PHA agreed quite well with the observed increase in Vmax.

4.5.2. Calculation of Ki and Km from K^{42} flux data

Km was obtained simply from the data presented in Fig. 3.4.11, and was equal to 1.5 mM for both unstimulated and stimulated cells.

Ki was obtained from Fig. 3.4.16, by 'forcing' the values obtained for ouabain treated cells through the intercept of the control values with the Y axis. This gave a Ki of 1.45×10^{-7} M (equation 3.6). The fact that the curves obtained were not exactly representative of competitive inhibition leads to probable error in the value of Ki and, in fact, some question as to its precise meaning. However, it is convenient to use it as an approximation. The value of Ki for PHA-treated cells was not different from that obtained for control cells.

4.5.3 Application of models for calculation of Km and Ki from DNA - synthesis data

The plot of DNA synthesis vs. potassium concentration in the extracellular medium (Fig. 3.4.19) gave a half maximal rate of synthesis at a potassium concentration of about 1.7 mM. This value compares well with the Km found from flux data.

Table 4.5.1.

CALCULATED FLUX PARAMETERS FOR POTASSIUM:

UNSTIMULATED AND PHA-STIMULATED LYMPHOCYTES

Source of data	Symbol (Units)	Values	
		Both stimulated and unstimulated cells	
Measurement Direct	$T_{\frac{1}{2}}$ (hrs.)	1.5 ± 0.2	
	Km (mM)	1.5	
(see section 4,2,1,3)	σ (cm^3/cell/sec.)	$(6.0 \pm 0.4) \times 10^{-10}$	
Calculated from: definition of b	$b_{(hr^{-1})}$	0.46 ± 0.06	
equations 2.10	P (cm/sec)	$(4.2 \pm 0.8) \times 10^{-8}$	
2.8 2.9	Em (mV)	-41 ± 4	
2.12	Mi moles/cm^2/sec	$(0.50 \pm 0.05) \times 10^{-12}$	
		Unstimulated	Stimulated
Direct Measurement (assume constant cell vol.)	Ci^k mM	93 ± 4	125 ± 6
	Vmax moles/cm^2/sec.	1.29×10^{-12}	2.12×10^{-12}
Calculated from equations 2.11	Mo moles/cm^2/sec	$(1.54 \pm 0.23) \times 10^{-12}$	$(2.09 \pm 0.23) \times 10^{-12}$
1.15	Tr moles/cm^2/sec	$(1.04 \pm 0.25) \times 10^{-12}$	$(1.59 \pm 0.25) \times 10^{-12}$
3.5	Vmax moles/cm^2/sec	$(1.31 \pm 0.32) \times 10^{-12}$	$(2.01 \pm 0.32) \times 10^{-12}$

4.5.3.1. Model (a) - section 4.4.1.

The observed relationship of $\bar{I}$ vs. Co^k (Fig. 3.2.19) gave a slope of 0.95×10^{-5}.

Using this model, equation 4.4 predicts a Ki of 1.6×10^{-8}M. Therefore this model does not fit the hypothesis since Ki is 'out' by a factor of about 10. This can be visualized by comparing the slopes of the graphs in Fig. 3.2.19 and 4.4.2.

4.5.3.2. Model (b) - section 4.4.2.

This threshold model predicts that the plot of $\bar{I}$ vs. Co^k will intersect below the origin (Fig. 3.2.19).

Calculation of the theoretical graph is, however, not possible unless certain assumptions are made regarding the magnitude of the threshold potassium concentration and the membrane potential (equation 4.10).

However, if the graph of equation 4.10 is fitted to the observed curve of Fig. 3.2.19, the following values must be inserted

$C_\tau^{\sim}$ = 120 mM

Em = -41 mV at Co^k = 5.8 mM

= -36.7 mV 10 mM

= -31.5 mV 15 mM

Such a change of membrane potential with increasing extracellular potassium levels is seen for nerve preparations.

4.5.3.3. Model (c) - section 4.4.3.

The same situation holds as for model (a). Hence this model is unsatisfactory.

4.5.3.4. Model (d) - section 4.4.4.

Calculation of the slope at Co = 5.8 mM using the plot of I vs. Co (equation 4.19), leads to the prediction that Tr_τ is very close to the measured level of transport in stimulated cells, i.e. about 2×10^{-12} moles/cm^2/sec.

4.5.3.5. Conclusions from models

The non-threshold models (a) and (c) were not acceptable.

The threshold models (b) and (d) were both consistent with the experimental data. However, both models required that the threshold potassium level or the threshold transport level be very close to the measured level in stimulated cells. That is, 100 - 120 mM or at a transport rate of approximately 2×10^{-12} moles/cm^2/sec.

It should be noted that if K_i was overestimated, the threshold values for model (b) and model (d) would be lower than those mentioned.

5. DISCUSSION

5.1. General

This investigation has been primarily concerned with the mechanism by which the small lymphocyte, a 'repressed' cell, is stimulated to undergo transformation into a large dividing 'blast' cell. Several approaches were made to this problem, but major focus was directed to the role of cellular cation transport.

A hypothesis emerged by which a critical factor operative in the control and regulation of lymphocyte transformation was outlined. In this hypothesis it is proposed that the rate of synthesis of nucleic acid and protein is determined by the transport of potassium into the cell and the resultant level of intracellular potassium. A model is described which accounts quantitatively for the effects of ouabain on synthetic activity in the system, provided that synthetic activities are determined by potassium levels above a threshold intracellular concentration. In proposing this as a control mechanism, it is recognized that other related factors are probably also involved, such as changes in sodium permeability, calcium metabolism, or in some form of 'feedback' relationship between the membrane and nucleus. However, the theory of the potassium level as a critical factor *sine qua non* in the control of transformation has experimental

evidence in its favor which will be discussed.

The following discussion has not been arranged strictly according to the order in which the experimental results were presented. This is because logical discussion of the results obtained must take into account results obtained from diverse aspects of the work.

The following sections of the discussion will first consider the results obtained using ouabain and other inhibitors of the Na-K-ATPase; this will be followed by an evaluation of results obtained relating to the uptake of K^{42} by cells. Supporting data on the oxidative phosphorylation of the cell will be considered, and the action of certain drugs, found to be effective on the respiratory system, will also be discussed with respect to their action on synthesis during transformation. Finally, the general mode of action of PHA on lymphocytes will be considered, and the implications of the results obtained to the overall scheme of lymphocyte derepression and transformation will be examined.

5.2. ACTION OF OUABAIN

This glycoside was initially tested as part of a study of the role of membrane function in lymphocyte transformation. Investigation of its pronounced inhibitory effect on transformation led to subsequent studies of potassium transport.

5.2.1. Is the action of ouabain specific to the PHA induced transformation?

The inhibitory action of ouabain on the induction and subsequent process of lymphocyte transformation by PHA can be interpreted by means of two alternate hypothesis: (1) ouabain acts to prevent the stimulatory action specific to, and requiring the presence of, PHA; and (2) the effect peculiar to PHA is not affected by ouabain, which instead inhibits some critical step(s) subsequent to, or simultaneous with, the initiating effect of PHA. The mixed leucocyte reaction (Bain et al, 1964; Bach and Hirschhorn, 1964) involves an activation of the lymphocyte in the absence of PHA. Since ouabain was found to inhibit this response to exactly the same extent as in the case of the PHA-stimulated cultures (section 3.2.3.6), hypothesis (2) must be correct.

5.2.2. Effect of ouabain on leukemic cells

The inhibitory effect of ouabain on PHA-stimulated cells suggested that leukemic cells which spontaneously synthesized DNA might also be responsive to the glycoside.

Cultures from a variety of leukemic patients were treated with PHA and ouabain. Most leukocyte cultures obtained from patients with chronic lymphocytic leukemia (CLL) showed a reduced response to PHA as described by others (section 1.7.2.5). However, in one patient the CLL response to PHA was repeatedly normal, indicating a non-uniform *in vitro* PHA reactivity of CLL lymphocytes.

The spontaneous DNA synthesis observed in two acute leukemic patients was inhibited profoundly and repeatedly by ouabain (Fig. 3.5.15). This effect was seen with patients who had not already been treated for their condition. Patients who were treated showed a much lower rate of spontaneous synthesis which was only poorly inhibited by the glycoside. These studies of the *in vitro* responses of leukocytes from patients with acute leukemia have led to the possibility that the drug may prove useful in the study of the mechanism of mitotic control in this disease. Clinical trials are therefore being undertaken.

5.2.3. Effect of ouabain on the increment of RNA synthesis induced by PHA in normal and leukemic cells

For both leukemic and normal subjects, leukocyte cultures had identical responses in one important respect; ouabain (2.2×10^{-7}M or greater) inhibited the increment of RNA and DNA synthesis induced by PHA, regardless of the absolute magnitude of the increment. The only exception observed in the series of cases tested was a patient diagnosed as having acute myelomonocytic leukemia. A wide spectrum of responses would be expected in a larger patient series, based on the clinical state of the disease, therapy, etc. Further leukemia testing is therefore necessary and is being carried out.

5.2.4. Mechanism of action of ouabain

It was demonstrated that the effect of ouabain was not a nonspecific property of its steroid moiety, since the other steroids tested were much less effective as inhibitors, even at concentrations higher by two or more orders of magnitude, when they were given after PHA-induced stimulation had already taken place. Moreover, the action of ouabain differed from that of prednisolone (Elves et al, 1964; Tormey et al, 1965), and of chloroquine (Hurvitz and Hirschhorn, 1965) in that it was fully effective even when blast transformation was far advanced, such as after 2 or 3 days in the presence of PHA (Fig. 3.2.14).

The effect of ouabain at 50% inhibitory concentrations was not due to inhibition of a relatively small number of ouabain-sensitive cells, since the autoradiographic studies showed uniform inhibition of H^3-uridine incorporation in all cells of cultures treated with ouabain concentrations sufficient to cause 50% inhibition (5×10^{-8}M). Some morphological changes were also noticed (Fig. 3.2.8), characterized by the aggregation of nuclear material into rod-like clumps and the apparent disappearance of nucleoli.

Any mechanism proposed for the action of ouabain on PHA-treated lymphocytes would have to explain:

(a) The almost equal effectiveness of ouabain on RNA, DNA and protein synthesis, its suppression of the increment of synthesis assoicated with stimulation and the time (about 2 hours) taken for its inhibitory effect on nucleic acid synthesis to become manifest;

(b) The reversibility of the effect, since removing the ouabain from PHA-treated cells allowed the stimulation to take place (Fig. 3.2.20) even in the absence of newly added PHA;

(c) The partial or complete prevention of the ouabain effect inhibition by high extracellular levels of potassium; a linear relationship was noticed between the concentrations of ouabain and potassium which together caused 50% inhibition

of DNA synthesis over the dose range studied. This relationship (Fig. 3.2.19) was used in calculations for the threshold model (section 4.4.2).

An explanation compatible with these observations and the known biological effects of ouabain is that it affects the transformation of lymphocytes by inhibiting the action of the enzyme system responsible for membrane cation transport. Indeed, the effective concentrations of the glycoside (about 5 x 10^{-8}M for 50% inhibition) is within the range of effective concentrations (10^{-7} to 10^{-6}M) found for the inhibition of Na-K-ATPases from a variety of unrelated systems (Bonting et al, 1962). Table 3.3.7.

Cellular sensitivity to the glycoside might be associated with the degree of endocytosis. Cells with enhanced endocytosis, such as stimulated lymphocytes (Hirschhorn et al, 1968) might be highly dependent on the action of a ouabain-sensitive Na-K-ATPase for the control of cell volume and the maintenance of optimal internal cation levels (section 1.11). Hence it may be predicted that the RNA synthesis of some cells would be less sensitive to ouabain such as Ehrlich ascites cells (Mayhew and Levinson, 1968) or the BSC-1 cells in this study; the effect of ouabain is thus not a general one.

The data obtained with ouabain would be explained if (a) membrane Na-K-ATPase were activated during lymphocyte stimulation, (b) Na-K-ATPase activity remained constant but was more important for the metabolism of the stimulated cells, or

(c) if the activity of the Na-K-ATPase were reduced by PHA and thus more easily inhibited by ouabain. The cytochemical evidence of Fries et al (1967) and the preliminary enzyme measurements of the present study support the first possibility. Partly in order to choose between these hypotheses, studies of the uptake of K^{42} were later initiated.

5.2.5 Other inhibitors of the Na-K-ATPase

Agents besides ouabain known to be potent inhibitors of Na-K-ATPase in vitro such as erythrophleine, cassaine, p-chloromercurobenzoate (PCMB) and N-ethyl maleimide (NEM) (Bonting et al, 1964; Matsui and Schwartz, 1966) partially or completely inhibited DNA and RNA synthesis in transforming lymphocytes. These effects support the hypothesis that the function of the Na-K-ATPase is essential to the phenomenon of lymphocyte activation by PHA.

The results obtained with these inhibitors were related quantitatively to their effects on Na-K-ATPases of other tissues. As shown in Table 3.3.6, the inhibitory action of the respective agents on DNA synthesis in transforming lymphocytes corresponded to the degree to which they were observed to affect the enzyme (Bonting et al, 1964; Matsui and Schwartz, 1966).

The effects of these agents on lymphocyte RNA and DNA synthesis differed from each other in two ways. (a) One concerned the extent to which they caused inhibition of RNA and DNA synthesis respectively, and the other, (b) the concentration range over which they acted. Thus, NEM and PCMB both acted over relatively narrow concentration ranges, whereas cassein was characterized by a relatively wide range, in comparison to the effect of ouabain. This effect presumably resulted from the different affinities and modes of binding to the cell surface.

Marked differences between the effects on RNA and DNA synthesis were shown for erythrophlein and cassein, whereas the relative effect of PCMB and NEM on nucleic acid synthesis were not distinguishable. This matter will be discussed again in section 5.5 with respect to the action of valinomycin and oligomycin.

5.2.6. Does ouabain act by inhibiting the uptake into the cell of labelled nucleosides

In his recent interesting paper, Coulson (1969) speculates that our data on the effect of ouabain "may reflect an inhibition of transport of labelled nucleosides, rather than a specific anti-PHA effect". However, we have already concluded that the glycoside does not have a specific anti-PHA effect, since it

inhibits DNA synthesis in the mixed leukocyte reaction at the identical concentrations and to the same extent as it inhibits the PHA response. Moreover, the initial effect of the PHA was not prevented by ouabain, since washing away the glycoside permitted the stimulation of RNA synthesis to proceed, even in the absence of extra added PHA (Quastel and Kaplan, 1970).

With regard to the possible inhibition by ouabain of nucleoside transport, the glycoside probably affects the transport of a number of substances, such as cations, sugars and amino acids. (Bittner and Heinz, 1963; Smith, 1964). However, at the ouabain doses used (10^{-8}-10^{-6}M), we found that the inhibition of H^3-nucleoside incorporation was preceded by a lag period of at least 2 hours, after which inhibition of the rate of incorporation gradually developed, becoming maximal after about 12 hours or longer. On the other hand, K^{42} was strongly inhibited after one hour in the presence of the glycoside. Measurements carried out on the uptake of labelled uridine into the TCA-soluble pool indicate that ouabain did not significantly affect this parameter. (Fig. 3.2.23 and 3.2.24).

The similarity between the effective concentrations of the glycoside on the incorporations of nucleosides and amino acids (Quastel and Kaplan, 1970) suggests that a common factor

is involved in the inhibition of synthetic activity. The most likely common factor for all these inhibitory effects of ouabain, including that on respiration, is a decrease in the level of intracellular potassium, which is known to be required for nucleic acid synthesis, protein synthesis, respiration and transphosphorylation. A fall in the potassium level in the presence of ouabain must have occurred because the rate of efflux of K^{42} from the cell was unaffected by the glycoside, whereas the rate of uptake was strongly inhibited (see section 5.3).

5.3. POTASSIUM FLUX STUDIES

5.3.1. PHA-induced stimulation of K^{42} uptake

5.3.1.1. Nature of the Observations

The present work has described an increase in the rate of uptake of K^{42} into lymphocyt cultures following the administration of PHA. The stimulation occurred after about an hour of exposure to the phytomitogen; it was characterized by an increased V_{max} without obvious change in the K_m (or affinity for K^+). Such an effect could be due to the formation of new transport enzyme molecules within the cell or to the activation of preexisting sites. Culturing the cells in the presence of actinomycin-D or cylcoheximide, which are effective inhibitors of RNA and protein synthesis respectively, caused no change in the PHA-induced enhancement of K^{42} uptake. Hence, it may be concluded that the increased uptake of K^{42} caused by PHA represented a stimulation of the transport system and not biosynthesis of new transport proteins.

The uptake of K^{42} into both stimulated and PHA-treated cells was inhibited after one hour in the presence of ouabain, but efflux of the isotope was not affected by the glycoside.

It is interesting to compare these results with those of Mendelsohn et al (1970), who demonstrated an early increased transport of amino acid in the presence of PHA. This effect was also independent of protein synthesis and was characterized by an increase in the V_{max} but not in the K_m. It is possible that the transport of amino acid and cations are linked in this cell, as in some other systems (Christensen, 1955).

5.3.1.2. Significance of the PHA-induced changes in potassium influx

The present studies and previously published data point to certain conclusions. PHA must influence the cell membrane soon after it is bound to the cell. Some of the earliest events involved in PHA-induced stimulation occur at the cell surface; increased turnover of membrane phospholipids (Fisher and Mueller, 1968), increased endocytosis (Hirschhorn et al, 1968), the stimulation of the membrane-bound cation transport system described here and previously (Quastel and Kaplan, 1970 a,c), and the increased transport of a-aminoisobutyric acid (Mendelsohn et al, 1970). Since synthesis of new protein is not involved in the PHA-induced activation of the ouabain-sensitive K^+ transport, and since PHA affects the V_{max} rather than the K_m of the process, we may conclude that the signal(s) to the cell membrane result(s) in one or more of the following:

(a) appearance of new enzyme activity either by activation of previously formed but inactive enzyme protein within the membrane, or modification of membrane structure to permit such molecules to become active;

(b) exposure of K^+ binding sites previously inaccessible to external cation; or

(c) enhancement of the activity of already accessible and active transport enzyme.

All three hypothesis require that PHA induce, soon after binding to the cell, a conformational or structural change in the membrane or in site within the membrane. The recognition sites which bind PHA and provoke the rapidly ensuing changes of cell membrane function therefore seem likely to be located at the cell membrane.

5.3.2. Are there several compartments of potassium in the lymphocyte?

5.3.2.1. Possible binding of some K^{42} to the cell surface

An unexpected result was the observation (see Figs. 3.4.1. and 3.4.2) that when the cells were incubated with K^{42}, the measured amount of radioactivity associated with the cells was highly dependent on the wash medium used. If the cells were washed with ice-cold isotonic sucrose, choline chloride, medium-199 or sodium chloride, the K^{42} remaining associated with

the cells was substantially higher than that observed after washing the cells with isotonic KCl, RbCl, NH_4Cl, or to a lesser extent, with CsCl. Furthermore, when the cells were washed with the first group of solutions, the total estimated potassium associated with the cells, determined from the uptake of K^{42}, was often considerably higher than the total amount of potassium that would be expected to be in the cells.

For example, assuming an internal potassium concentration of 120 mM (Lichtman and Weed, 1969), and a lymphocyte volume of about 250 μ^3 (Lichtman and Weed, 1969; Westring et al, 1969), a total potassium content of 30 n moles per 10^6 cells may be calculated. As seen in Figs. 3.4.10 and 3.4.15, the estimated amount of potassium associated with the cells after washing with sucrose was usually considerably in excess of this amount. However, washing the cells with isotonic KCl or RbCl caused a rapid reduction of the amount of K^{42} associated with the cells (Fig. 3.4.2. and Table 3.4.1).

It seemed possible that the rapidly exchanged or 'excess' fraction of K^{42} was either (a) entirely intracellular, or (b) extracellular and bound to the surface of the cells. The results of experiments carried out to investigate this point supported possibility (b). For example, (see Fig. 3.4.3.) loss of cellular K^{42} occurred more rapidly when cells that had been prepacked with K^{42} and washed in medium-199, were resuspended

in ice-cold isotonic KCl (high K sol'n), in comparison to cells resuspended in ice-cold medium-199 (low K sol'n). However, when cells that had been prepacked with K^{42} were washed with a KCl solution (thus removing the 'excess' K^{42}) and then resuspended in high K solution, their K^{42} loss was not any more rapid than when they were resuspended in low K solution. In another experiment (Table 3.4.2), it was shown that the addition of trypsin for 15 minutes removed the 'excess' K^{42} in prepacked, medium-199 washed cells, without affecting the fraction of K^{42} remaining after the cells had been washed in KCl solution.

An attractive physical meaning to these observations would be that the 'excess' K^{42} represented isotope bound to certain sites on the external surface of the cell membrane. In subsequent discussions the term, K^{42} binding, will refer specifically to the 'excess' amount already discussed. However, the nature of this binding or 'excess' fraction remains to be elucidated.

5.3.2.3. Possible reason for the 'excess' K^{42}

The excess K^{42} binding may be to negative charges present on the surface of the lymphocyte. This cell is reported to be highly electronegative, when tested by cell electrophoresis (see section 1.10). Weiss (1960) has suggested

the presence of cationic 'clouds' surrounding the cell. The negative groups on the cell surface appear to be associated with a carbohydrate-rich glycoprotein on the cell surface, and are considered to be due partly to the presence of sialic acids. Binding of K^+ in the medium to the surface of cells at such negatively charged sites might result in the observed 'excess' fraction of K^{42} associated with the cells.

5.3.2.4. Significance of 'excess' K^+ binding

In this discussion, we shall assume as a working hypothesis that the excess K^{42} is in fact bound to the surface of the cell.

The surface coating of the lymphocyte is affected by both trypsin and neuraminidase. Both of these enzymes are known to affect temporarily the circulation of this cell in vivo, indicating that the enzyme-sensitive cell coat carries information affecting the function of the cell (see section 1.10.2.4). Indeed, Lindahl-Kiessling and Petersen (1969a) have suggested that sialic acid components are required for the initial stages of PHA-stimulation. Sialic acid has also been implicated in receptor structure (Wooley, 1964) and in the cation transport mechanism (see discussion in section 1.10.1.3). It is therefore of interest that (a) trypsin removed the excess K^{42} bound to the cell, and (b) PHA enhanced the amount of 'excess' K^{42}, which was presumably bound to the cell surface.

The results also suggest that the binding of 'excess' K^{42} to the cell surface, was of a highly specific nature. As shown in Fig. 3.4.2, there appeared to be two modes of release of K^{42}. One was unaffected by NaCl washing, but was rapidly removed by washing with KCl solution. The other, slower mode of release of K^{42}, was the same whether the cells were washed repeatedly in KCl or NaCl solution. The fact that PHA increased the 'excess' binding of K^{42} suggests the possibility that an increased number of highly specific potassium sites were exposed by the mitogen. Some of these sites may have been part of Na-K-ATPase complexes. One may also speculate that other sites for other membrane associated enzymes may have been exposed. For example, the activation of adenyl cyclase is reported to require the presence of potassium (see section 1.11.2.3). These considerations are discussed further in the proposed working model of PHA interaction with the cell surface (section 5.8).

5.3.3. Comparison of K^{42} uptake by lymphocytes and red cells

The rate of uptake of K^{42} by red cells was considerably lower than that for lymphocytes, but the values obtained for KCl-washed erythrocytes were reasonably in agreement with those obtained by Joyce and Weatherall (1955), who reported that about 1.8% of the red cell potassium was exchanged hourly. The values obtained in this study were between 1-2% per hour, the uncertainty

being due to a presumed loss of K^{42} during the washing process. However, larger values were measured when the red cells were washed with isotonic sucrose instead of KCl following incubation in K^{42} (Fig. 3.4.5). Assuming that all the red cells K^{42} after the sucrose wash was intracellular, these results would suggest a potassium turnover for red cells which is larger (about 6% per hour) than previously reported values. Hence the same situation holds as for lymphocytes; i.e., sucrose washed red cells appear to retain an 'excess' amount of the isotope, and it is tempting to consider this also as an extracellular K^{42} binding to the red cell surface.

The much faster uptake of K^{42} into lymphocytes, compared to red cells, might be due to a more active transport system in the former cells. Red cells are known to have a relatively low Na-K-ATPase activity, in comparison to other mammalian cells (Bonting et al, 1961, 1962).

5.3.4. Total potassium and K^{42} uptake

The increased uptake of K^{42} in PHA-stimulated lymphocytes should be accompanied by an increase in the total potassium of the cells. RbCl-washed cells, in culture, previously treated with NH_4Cl solution to remove red cells were used for total potassium measurements, in an attempt to remove the 'excess',

presumably surface-bound potassium. For example, washing the cells with isotonic saline or choline chloride, as carried out by Lichtman and Weed (1969) were found in this study not to remove this extracellular compartment. While RbCl-washed cells showed an apparent increase in cellular potassium levels, (from about 93 to 125 mM assuming no volume change), a larger increase would have been expected on the basis of K^{42} measurements. The following explanation may be given for this apparent discrepancy;

A fraction of the potassium in the cell is assumed to be poorly exchangeable. Under such circumstances, a doubling of the K^{42} uptake by the cell would not necessarily imply a doubling of the total potassium content of the cell, but mainly of the more rapidly exchangeable fraction. The flattening of the efflux curves in Fig. 3.4.7 could be due to a slow efflux of K^{42} from the poorly exchangeable compartment. Supportive evidence for a poorly exchangeable fraction comes from the work of Stanbury and Mudge (1953), Gamble (1957) and Ulrich (1960), who showed that mitrochondrial potassium is not removed by repeated washings with sucrose or NaCl.

Other hypotheses involving different rates of K^{42} uptake by active transport or during passive diffusion, or of increased potassium exchange diffusion, appear to be inadequate to account for the more pronounced changes in K^{42} equilibrium levels induced by PHA, in comparison to the total potassium levels. It should be noted that these hypothetical, slowly

and rapidly exchangeable fractions of intracellular potassium are to be differentiated from the 'excess' potassium which has been assumed to represent an extracellular compartment attached to the cell membrane.

5.3.5. Effect of ouabain on K^{42} fluxes

The glycoside markedly inhibited the uptake of K^{42} by both unstimulated and stimulated lymphocytes. As the extracellular potassium level was increased in the presence of relatively low ouabain levels, the flux of K^{42} into the cell increased according to saturation kinetics. However, at high ouabain concentrations, this did not occur (Fig. 3.4.11, 14 and 16), but the flux became proportional to the extracellular potassium concentration. This behaviour is highly reminiscent of the observations on red cells by Shaw and Glynn in the 1950's (see section 1.8.3).

Based on the values of the constants found from the results shown in Fig. 3.4.16, the ratio K_m/K_i could be measured. Since the K_m was obtained by the experiment shown in Fig. 3.4.11, a value for the K_i could be calculated, as in section 4.5.

5.3.6. Relationship of DNA synthesis to the extracellular potassium concentration

Results already discussed showed that (a) ouabain-inhibition of nucleic acid synthesis was reversed by increased extracellular potassium levels, and (b) that the flux of potassium into the cell was half maximal at an extracellular potassium concentration of approximately 1.5 mM. These results suggested that nucleic acid synthesis would bear a quantitative relationship to the rate of potassium transport across the cell membrane, or to the amount of potassium in the cell.

This was tested experimentally and, as shown graphically in Fig. 3.4.19, such a quantitative relationship was indeed apparent. DNA synthesis, measured by the incorporation of H^3-thymidine, was approximately half-maximal at a potassium concentration of 1.7 mM in the extracellular medium, which is close to the K_m for potassium transport (1.5 mM; see section 3.4.12). However, the shape of the curve obtained was sigmoid. Least squares calculation of the resultant 'Hill' plot led to a value for 'n' of 1.4 ± 0.2 (1 std. dev.), suggesting that some interaction of potassium binding sites may have taken place.

These results are strong supporting evidence that the synthetic processes of lymphocyte transformation are dependent on, and possibly mediated by, the transport of potassium and/or its level within the cell.

5.4. RESPIRATORY STUDIES

The effects of PHA and ouabain on respiration were tested, since oxidative metabolism is known to be sensitive to K^+ (Gonda and Quastel, 1962; Kimmich and Rasmussen, 1967; Levinson and Hempling, 1967). It was found that PHA caused an increase in the respiratory activity of leukocyte and to a lesser but significant extent, of lymphocyte cultures. The reduced response of purified lymphocyte cultures to PHA may explain the apparent lack of stimulation reported by Polgar et al (1968). Inhibition of respiration in the presence of ouabain occurred some hours after the glycoside was added, but this inhibition was partially prevented by the presence of excess K^+ in the extracellular fluid. A similar effect of excess extracellular K^+ has also been observed on the inhibition of nucleic acid synthesis (Quastel and Kaplan,1968b, 1970b) and of K^{42} uptake (Fig. 3.4.11) by ouabain. A reduction in intracellular K^+ availability caused by ouabain may therefore be a critical common factor involved in these inhibitory effects.

The pronounced and immediate inhibition of respiration caused by oligomycin indicates that respiration is tightly coupled to oxidative phosphorylation in stimulated lymphocytes. The rapidity of the effect of oligomycin on respiration argues against the possibility that its effect is only on the Na-K-ATPase

which it is known to inhibit at high concentrations (Van Groningen and Slater, 1963; Whittam et al, 1964). Both DNP and valinomycin caused reversal of the oligomycin inhibition of respiration, presumably due to the uncoupling of respiration from its control by phosphorylation. Immediately following the addition of DNP to the cells, respiration was stimulated to the same extent (by about 70-80%) in unstimulated, PHA-stimulated, and ouabain-inhibited cultures (see Fig. 3.5.5), indicating that in each case the respiratory potential exceeded the observed activity. On the other hand, valinomycin did not further enhance the PHA-activated respiration, although an uncoupling effect of this agent has been described for Erhlich ascites cells (Gordon et al, 1967).

Since valinomycin acts to promote uncoupling of respiration by energizing the transport of K^+, the above findings suggest that K^+ transport into the mitochondria by valinomycin cannot be further activated in PHA-stimulated lymphocytes. However, respiratory activity in these cells was markedly reduced by the addition of nigericin, which has been shown to promote the release of K^+ from mitochondria (Estrada-O et al, 1967). Nigericin is not a respiratory inhibitor when the extramitochondrial K^+ concentration is sufficiently high to prevent K^+ loss (Palmieri et al, 1967). These findings suggest that the cytoplasmic K^+ concentration is a limiting factor in the control of respiration in transforming lymphocytes, and that PHA causes an increase in the availability of this ion.

5.5. OTHER PHARMACOLOGICAL AGENTS

5.5.1. Valinomycin and Oligomycin

Both the respiratory uncoupler, valinomycin, and the inhibitor of oxidative phosphorylation, oligomycin, were found to be strong inhibitors of PHA-induced DNA synthesis, with smaller effects on RNA synthesis. It is therefore likely that transformation is dependent on intracellular ATP as well as on K^+ levels, since valinomycin, which specifically promotes mitochondrial K^+ uptake but inhibits ATP formation, also inhibits transformation. However, the ATP level is determined by ATP formation and utilization, which in turn is modulated by K^+. Intracellular K^+ concentration is dependent on the Na^+-K^+-ATPase pump and is thus affected by internal ATP availability. Thus, the regulation of the intracellular concentrations of K^+ and ATP are mutually interdependent. Since synthetic processes and kinase reactions are modulated by K^+ and ATP concentrations, the present findings suggest that the rate of transformation of PHA-activated lymphocytes is dependent on the degree of activation of the transport mechanism. The enhanced ATP dependent K^+ uptake may therefore be a necessary condition for the observed onset and increase in these synthetic activities.

The pronounced differential effects of both these agents on the RNA and DNA synthesis of transforming lymphocytes is not clearly explained, but the above considerations would suggest that DNA synthesis is more dependent than RNA synthesis on cytoplasmic ATP levels.

5.5.2. Cyclic AMP and other adenosine nucleotides

The observed inhibitory effect of the adenosine nucleotides, including cyclic AMP (Hirschhorn et al, 1970; Quastel et al, 1969), is not simply explained, but the possibility of the adenyl-cyclase and Na-K-ATPase systems being linked has been proposed by Mozsik (1969, 1970); he observed that both AMP and cAMP inhibited the Na-K-ATPase. These substances may therefore cause inhibition of DNA synthesis in transforming lymphocytes in a manner similar to that of ouabain. However, I was unable to confirm (for 2-day cultures) the observation by Hirschhorn et al (1970), (who used 3 to 6 day cultures) that a small stimulation of DNA and RNA synthesis occurred in the absence of PHA when cAMP was present in the culture medium.

These observations or the lack of positive effects caused by cAMP and dcAMP are of some interest in view of the recent report by Cross and Ord (1970) that cAMP can induce transformation in pig lymphocytes comparable to that of PHA. Furthermore, Smith et al (1970) have demonstrated an increase

in lymphocyte cyclic AMP levels after PHA administration. If the observation of Cross and Ord (1970) are correct, the negative results of this study and the borderline possible stimulation reported by Hirschhorn et al (1970) presumably are due to an unidentified difference in the media used or in the procedure employed. Preliminary results from this laboratory (not reported in the results section) did not shown any stimulatory effect of cAMP on pig leucocytes in vitro.

An incidental observation is of interest. This was that cAMP and the other adenosine nucleotides were most inhibitory at low PHA concentrations but not at relatively high PHA concentrations. This indicates that the inhibition caused by these compounds must be determined in part by the amount of PHA present or by a secondary effect quantitatively related to the amount of PHA used.

5.6. CONFIRMATORY AND SUPPLEMENTARY STUDIES

In view of the fact that this aspect of the work was carried out mainly in order to confirm the utility of the experimental system, these results obtained will be considered very briefly. Also included in this section are the results of experiments which were incidental to the main direction of this work.

5.6.1. Morphology and Transformation

The morphological effects of PHA on lymphocytes, were confirmatory of those published elsewhere. Blast cell formation was observed in lymphocyte and leukocyte cultures when the cells were cultured with PHA. Stimulation of RNA and protein synthesis, measured by the incorporation of labelled RNA and protein precursors into TCA-insoluble material, was observed after 2 hours or longer of incubation with PHA. DNA synthesis did not take place until a period of about one day of culture with PHA had passed. Mitoses were seen in substantial numbers after 2 days *in vitro* with the mitogen.

The mixed leukocyte reaction took place over the time periods and to the same extent as reported by others (section 1.4.4).

The appearance of the transformed lymphocyte by light microscopy or using the electron microscope, described in detail in section 3.1, was similar to that described by others (section 1.5).

5.6.2. Incorporation of Radioactive Precursors

The incorporation of H^3-uridine into TCA-precipitable material does not necessarily indicate the degree of RNA synthesis unless the size of the soluble pool of RNA precursors is accurately known. In this work, the terms 'RNA synthesis' and 'H^3-uridine incorporation into TCA-insoluble material' have been used in the same sense. But, strictly speaking, the size of the intracellular pool is important. For example, if the pool were to be reduced following PHA-administration, without any change in the absolute rate of total RNA synthesis, the incorporation of H^3-uridine would show an apparent increase. Measurements of the H^3-uridine pool have, however, been carried out by Forsdyke (1968) who was unable to demonstrate a change in pool size soon after the administration of PHA to lymphocyte cultures. While absolute pool size was not measured in the present study, an estimate of the relative rates of uptake of H^3-uridine into the pool were made (see Fig. 3.1.12). Evidence that this technique actually does provide an estimate of uptake into H^3-uridine pool is supported by the data presented in Fig. 3.2.23.

In this experiment, it was demonstrated that while actinomycin-D strongly inhibited the incorporation of H^3-uridine precursor into TCA-insoluble material (i.e. RNA), it had virtually no effect on the uptake of H^3-uridine into the intracellular pool of uridine.

That the early stimulation of H^3-uridine incorporation actually does represent an increase of RNA synthesis, is indicated by the work of Rubin (1970), who demonstrated rapid changes in the type of RNA formed by lymphocytes, after an hour of culture with PHA.

The use of a 2-hour period of incubation of radioactive precursors for the measurement of incorporation had both advantages and disadvantages.

(a) The advantages were that this is a convenient period of time for experimental determination of the rate of macromolecular synthesis. During this time, the rate of incorporation was linear for all practical purposes, whereas measurement of cumulative incorporation into the cells after incubation for longer periods of time usually showed a non-linear acceleration of incorporation.

(b) The disadvantage is using this time period is that small variations in rates of synthesis between duplicate cultures becomes exaggerated. Measurements of incorporation over longer

period of time, such as for 24-hours, provides a more accurate picture of the total amount of synthesis and avoids the problem of variations in rate between replicate cultures. However, the latter technique does not permit accurate measurements of changes occurring during relatively brief periods of time. In this study, 24 hour incubations of H^3-thymidine were used in some experiments, as described in section 3.4.12.

5.6.3. Autoradiography

Autoradiographic studies demonstrated that most of the H^3-uridine label taken up into the cells was initially nucleolar (i.e. when the cells have been incubated with the label for a period of 40 minutes). It was approximately one hour from the time that the H^3-uridine was first given that label appeared in the cytoplasm. These results suggest that there was a movement of RNA from the nucleus to the cytoplasm. However, the possibility that some direct incorporation of H^3-uridine might take place in the cytoplasm is not ruled out by these experiments.

It is interesting to compare these results with two papers by Hayhoe and Quaglino (1965) and by Winter and Yoffey (1965). Hayhoe and Quaglino gave pulse doses of H^3-uridine for one hour and measured uptakes into the nucleus and cytoplasm by grain counts during the first 1 to 5 hours.

They comment on the nuclear positivity with local accentuations in the nucleolar areas.

Winter and Yoffey also studied these cells following incubation with one hour of H^3-uridine. These authors also observed uptake in the cytoplasm. It is to be noted that these authors only used one hour of incubation with uridine and for this reason, the initial uptake of H^3-uridine may actually have been nuclear. The implication of their results was that there may be some cytoplasmic RNA synthesis which had been proposed previously by H. Harris. The present results, using shorter incubation periods, indicate that it takes about one hour before label begins to appear in the cytoplasm,in confirmation of the results of Hayhoe and Quaglino. Winter and Yoffey state that "whether the cytoplasmic labelling represent a relatively rapid transfer of more nuclear RNA to the cytoplasm once the process of cell growth initiated by PHA is under way, or whether it represents diffusion of acid soluble end products outward from nucleus remains uncertain".

5.6.4. Binding of PHA to Lymphocytes

This work was carried out as the first stage in the presently reported investigation of the nature of the surface action of phytohemagglutinin on lymphocytes. About the same time that these results were obtained, it became apparent that

Michalowski and Razavi had published similar data. We therefore did not pursue further these studies on the fluorescein-labelling of PHA, since it was desired not to repeat the work of others, and also because more original findings were obtained, as described in section 3.2 et seq.

Some discussion of the fluorescein-PHA results will, however, be useful. The mitogen, when labelled with fluorescein isothiocyanate, fluoresced at the surface of lymphocytes during the first two hours of exposure. Because there is relatively little cytoplasm in the lymphocyte (the nucleus: cytoplasm ratio is high), it is difficult to differentiate between intracellular label and label adhering only to the cell surface. Some cells, particularly those with considerable intracytoplasmic vacuolization, had uptake which was definitely cytoplasmic. Similar results were obtained by Michalowski, who found that the labelling of dead and dying cells was particularly noticeable.

Label also was seen within cells treated for two or more days with fluorescein-labelled PHA. The distribution of label, while definitely not nuclear, appeared in areas corresponding to the cell membrane, but also as focal conglomerations within cells. This work confirms findings of Michalowski who found label to appear in the cytoplasm of blast cells.

From our own results, it would appear that the labelled PHA first appears on the cell surface, but it cannot be said that it does not also enter the cell. After one or more days in the presence of PHA, cytoplasmic labelling seems to be present in many blast cells.

The presence of PHA within the cell does not necessarily imply that the internal mitogen caused the transformation. The present study and those of others suggest that at least for the first few hours in the presence of PHA, surface binding to the cell membrane occurs. Some of the most dramatic changes associated with early lymphocyte transformation occur during the first few hours; some of these involve membrane responses and others involve changes in nuclear protein and DNA. However, these results do not prove that the site of action of PHA is at the cell membrane.

5.6.5. Methotrexate

Methotrexate (amethopterin), an analogue of folic acid, is antagonistic to the metabolism of folic acid (Osborn et al, 1958), and probably acts by preventing the reduction of dihydrofolate to tetrahydrofolate by inhibition of the enzyme dihydrofolate reductase (Werkeiser, 1963). Tetrahydrofolate is an important cofactor required for the methylation of deoxyuridylic acid (Friedkin, 1963) by the enzyme thymidylate synthetase.

Svet-Moldavsky et al (1968a) have proposed that methotrexate induces non-immune lymphocytes to destroy target cells in a way similar to PHA-activation of cytotoxicity (Holm et al, 1964). They have also suggested (1968b) that the therapeutic effect of methotrexate on neoplasms such as malignant chorion-epithelioma is brought about by an anti-tumor activation of lymphocytes. Evidence supporting this was given by Stewart (1969) who found that tumors with marked stromal infiltrate by mononuclear cells (primarily lymphocytes) were most responsive to methotrexate therapy.

The prediction was therefore made, together with Stewart, that methotrexate should enhance the incorporation of H^3-thymidine by transforming lymphocytes in vitro, since this parameter has been shown by Möller et al (1966) and Holm and Perlman (1967) to be associated with the production of optimal cytotoxic effects of lymphocytes stimulated by PHA. It would also be expected that lymphocytes would show some degree of 'resistance' to effects of methotrexate.

The results obtained supported the above predictions. Methotrexate enhanced the incorporation of H^3-thymidine into PHA-treated leucocyte cultures. In addition, substantial incorporation of this precursor into the culture was observed after a week of continuous culture with methotrexate, even at

concentrations as high as 10^{-4}M, although monkey kidney BSC-1 cells showed a pronounced inhibition of incorporation after the same period of time.

Nevertheless, explanation of the observed phenomena is not obvious and requires further study. Possible reasons for the rate of incorporation of H^3-thymidine include;

a) Enhanced incorporation of tracer into DNA may occur, based on an increase of thymidine kinase activity and/or reduced degradation of H^3-thymidine (Caron, 1969; Rubini, 1966);

b) H^3-thymidine specific activity in the methotrexate-treated cells may be increased, possibly due to a reduced level of endogenous precursors;

c) Reversal by carrier thymidine of a methotrexate-induced block of DNA synthesis, such as that suggest by Tormey and Mueller (1965). It might be noted, however, that the latter workers used 100 times as much thymidine as in these experiments;

d) An enhancement of the rate of DNA synthesis may occur, possibly 'unscheduled', such as that observed after irradiation (Mitchell, J.S., personal communication).

For the apparent methotrexate 'resistance' observed in the PHA-treated leucocyte cultures, one must consider;

e) An induction of dihydrofolate reductase activity by methotrexate: (Bertino et al, 1963; Roberts and Hall, 1967; Hakala et al, 1961);

f) Reduced transport of the drug into the cell (Sirotnak et al, 1968; Kessel et al, 1968);

g) Enhancement of thymidine kinase activity as in (a), which might also lead to some degree of methotrexate 'resistance' by rendering DNA synthesis less dependent on the methotrexate-sensitive thymidylate synthetase pathway.

The results reported then do not permit a choice between these alternatives. The observations are, however, intriguing since they suggest a relative resistance of the transforming lymphocyte to methotrexate and appear to confirm the prediction of increased H^3-thymidine incorporation induced by methotrexate.

5.6.6. Effect of ouabain on homografts

The results herein described were preliminary and represented 'pilot' studies.

The inhibitory effect of ouabain on lymphocyte transformation suggested that the glycoside might have application to the problem of homograft rejection, provided that the compound caused a similar effect on lymphocyte transformation in vivo. Pilot studies, first with rats and then with pigs, were set up with the object of determining the most appropriate experimental animal and whether any immediate effect of the glycoside on homograft rejection could be observed by direct injection into skin transplanted animals.

As described in section 3.5.6.1, rats proved unsatisfactory because of the high resistance of their transforming lymphocytes to ouabain *in vitro*. The cause for this is not known, but is probably related to the characteristic resistance of the rat to ouabain. Pigs were more satisfactory as far as lymphocyte transformation was concerned, but major problems involving dosage and extracellular fluid concentrations of the glycoside remained. Preliminary results, however, indicated that ouabain, in the doses used, seemed to accelerate rather than retard the rejection of the graft. Whether this may be due to a stimulatory effect of ouabain on cation transport at very low concentration (Repke, 1963) or to some other cause, is not clear.

Further experimentation is planned, but with a more gradual progression from *in vitro* to *in vivo*, i.e., by testing immune responses of progressively increasing complexity *in vitro*, before progressing to the *in vivo* situation.

5.7. APPLICATION OF MEASURED PARAMETERS TO THEORY

5.7.1. Flux data

The potassium flux values were within the same range of magnitude though somewhat smaller than those obtained by Hodgkin and Horowicz (1959) using frog muscle fibers.

For example, the influx for lymphocytes was about 0.5×10^{-12} moles/cm^2/sec; for frog muscle fibers, 5.4×10^{-12} moles/cm^2/sec. Outflux for lymphocytes was 1.5×10^{-12} moles/cm^2/sec, and for muscle fibers, 8.8×10^{-12} moles/cm^2/sec. The estimated permeability of lymphocytes to potassium, 4.2×10^{-8}/cm^2/sec, was lower by a factor of about 13 below that of muscle fibers (6×10^{-7} cm/sec).

It is encouraging that the V_{max} could be predicted from flux data so closely, although the accuracy obtained was surprising. In Table 4.5.1, the V_{max} for stimulated and unstimulated cells was obtained in 2 different ways. The close correspondence of the calculated and directly measured values for both unstimulated and stimulated cells is evidence that (a) the permeability of flux value were within the right magnitude, and (b) that PHA induced an increase in the V_{max}.

5.7.2. Models

The models (Section 4.4) were developed to determine whether ouabain effects on the synthesis of DNA could be predicted, on the theory that synthesis was determined by potassium levels and/or potassium transport across the cell membrane. Both non-threshold and threshold models were examined.

It was found that the threshold models would fit the observed data, but the threshold values were rather high. For example, a potassium threshold as high as 120 mM was predicted, and a transport threshold at the level found in stimulated cells was also predicted. These values depended on the K_i for ouabain. If the K_i was over estimated, and was actually somewhat smaller than that used (1.45×10^{-7}M) these thresholds would be somewhat lower in magnitude.

These predictions imply that the lymphocyte must build its internal potassium to relatively high levels before synthesis can occur, suggesting an explanation for the sensitivity of the cell to ouabain.

The K_i (1.45×10^{-7}M) found for ouabain was relatively close to that observed by Matsui and Schwartz (1966) for the inhibition of heart muscle ATPase by this glycoside. They obtained a value of 2.2×10^{-7}M. Half-maximal inhibition of the

enzyme was obtained by Post and Albright (1961) at a concentration of 3-7 x 10^{-8}M; these concentrations gave 1/2 maximal synthetic in the present study at extracellular potassium levels of 5.8 mM.

The Km found for potassium (1.5 mM) is relatively close to that obtained for red cells by Glynn (1956), who reported a value of 2.0 mM. Post and Jolly (1957) obtained a value of 2.2 mM.

5.8. MODE OF ACTION OF PHA

5.8.1. Dose PHA Enter the Cell to Cause its effect?

Coulson (1969) has adduced some indirect evidence in favour of his view that the site at which PHA acts is different from and influences a step later in the sequence of events than that at which the specific antigens cause lymphocyte activation: that is, he supposes that PHA acts to short-circuit certain of the initial steps following activation by specific antigens. In this way, he explains the fact that the latter activate only a relatively small fraction of the lymphocytes, whereas PHA affects most of the lymphocytes in the culture. He speculates further that the PHA site is not at the cell surface but somewhere within the cell. However, the evidence in favour of this view is not compelling; no hard data exclude the possibility that (a) the PHA sites are at the cell surface, and (b) the sites are identical to, or include the specific antigenic sites. The latter hypothesis requires only that there be a non-specific component common to all the specific antigenic recognition sites present at the surface of the lymphocytes, and that this component recognize and bind PHA. Indeed, the recent data of Kay (1970) and Hausen and Stein (1968) on the action of anti-phytohemagglutinin serum suggest that PHA does not enter the cell to cause its stimulatory effect.

We thus prefer our model, involving stimulation of the membrane-bound Na-K-ATPase, as being more compatible with all the available data.

5.8.2. A Speculative Model for the Action of PHA on Lymphocytes

5.8.2.1. Sequence of events

Table 5.8.1 shows some of the initial events which have been observed to occur in lymphocytes following the administration of PHA. This table summarizes much of the data discussed at length in section 1.5 of the introduction. In Figure 5.8.1, an attempt has been made to arrange these events of transformation into a sequential order.

5.8.2.2. Possible mechanism of action of PHA at the membrane

The following observations are to be explained;

(a) An action of PHA at the cell surface;

(b) The activation of the Na-K-ATPase of the plasma membrane, characterized by a conformational change without requiring extra protein synthesis;

(c) The possibility that other enzyme systems associated with the plasma membrane may also be activated;

(d) The 'non-specific' nature of PHA action;

TABLE 5.8.1.

Some early cellular events associated with lymphocyte transformation

location	first hour	second hour	fourth hour
membrane	phosphorylation of membrane lipids; (phosphatidyl inositol)	increased potassium transport; increased 'binding' of K-42 to cell surface; increased amino-acid transport ? increased adenyl-cyclase activity	increased synthesis of phosphatidyl choline
cytoplasm	? increased breakdown of RNA	increased endocytosis; increased protein synthesis;	increased glycolysis; increased LDH formation; increased lysosomal size;
nucleus	acetylation and phosphorylation of nucleoproteins; increased binding of actinomycin; altered acridine orange staining;	increased RNA synthesis and alteration in the kind of RNA formed; increased uridine kinase activity;	

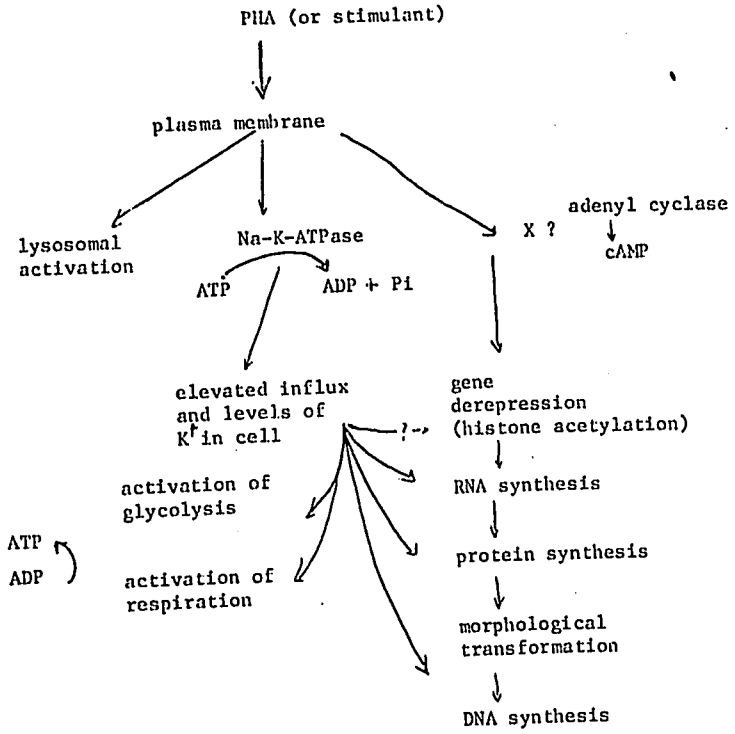


Fig. 5.8.1.

Simplified scheme showing sequential events during lymphocyte transformation and proposed rate of Na-K-ATPase. The arrows leading from the statement; 'elevated influx and levels of K^+ in cell', point to cellular activites which are presumably affected by the level of potassium in the cell.

(e) The observation that K^{42} 'bound' to the cell surface may be increased, as well as the amount of K^{42} actually transported into the cell:

(f) The observation (by Vassar and Culling, 1964) that PHA reduces the cell charge:

The following model is proposed which, though highly speculative, does lend itself to experimental testing.

It is suggested that a repression mechanism is operative at the plasma membrane of resting lymphocytes, and that PHA affects this repression by causing the exposure of active enzyme sites to substances in the medium, such as potassium.

In Figure 5.8.2, it is suggested that the glycoprotein associated with the lymphocyte surface (see section 1.10) covers enzyme sites. It is proposed that PHA binds to this glycoprotein and in doing so, lifts it up away from the active enzyme sites, which are exposed to the medium. The increased number of negative changes exposed to the medium causes an increased binding of cation (and a resultant lowering of cell change) and the binding of potassium to Na-K-ATPase in the membrane leads to enzyme activation.

While this model is admittedly very speculative, and overly simple, it lends itself to certain experimental predictions. For example, it would be predicted that after PHA

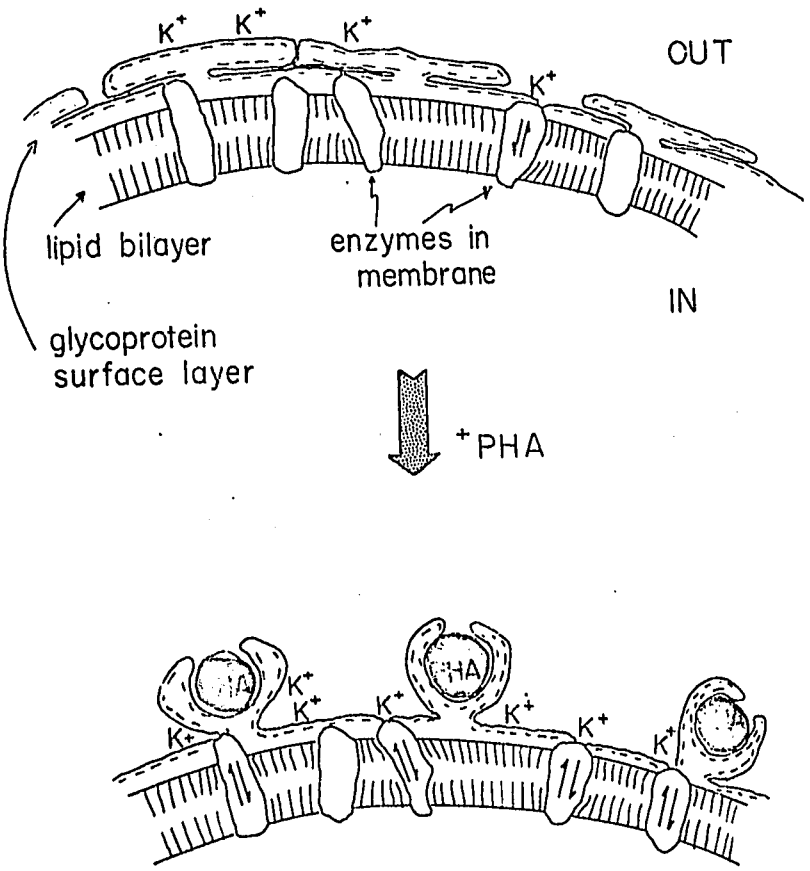


Fig. 5.8.2.

Speculative model of the action of PHA on the cell surface.
Upper figure: Lipid bilayer of the plasma membrane contains various surface enzymes including Na-K-ATPase. An active transport enzyme complex is shown with the double arrow. The plasma membrane covered by a negatively charged glycoprotein layer containing pores or cracks. This layer partially prevents access of potassium and other substances from the medium.
Lower figure: Same portion of membrane after interaction with PHA molecules. Because of its affinity to the sugar molecules, the PHA molecules cause a lifting away, or a dissociation of the glycoprotein layer from the membrane, thus exposing enzyme sites to the external medium. More transport enzyme complexes are activated, as shown by the double arrows.

treatment, the binding of H^3-ouabain would be increased. It is also predicted that should an irreversible inhibitor of the Na-K-ATPase be added before PHA treatment, PHA should have a stimulating effect after washing the cells, since the exposed enzyme sites would not be occupied by the inhibitor. However, stimulation would not occur if the irreversible Na-K-ATPase inhibitor were given after the administration of PHA, since all the enzyme sites would then be occupied.

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