CHANGES IN LEUCOCYTES FOLLOWING STIMULATION OF MITOSIS

by

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"....   You don't know what blood is."

"We do....It is red stuff that soaks into the ground and smells."

Kipling (1900)

Frontispiece.

The grey kangaroo, Macropus giganteus Shaw, 1790.

Statement required by the University of Adelaide from candidates for the Degree of Doctor of Philosophy:

This thesis contains no material previously submitted for a degree in any University either by me or by any other person, except when due reference is made in the text of the thesis.

D. W. Burton.

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ABBREVIATIONS

Only the four abbreviations listed below are used throughout the text.

DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
PHA	Phytohaemagglutinin
DNP	Deoxyribonucleoprotein

SUMMARY

1. Light microscopy of the leucocytes of the peripheral blood of the kangaroo showed that the various cell types were very similar to those found in human peripheral blood. The only cells surviving after 24 hours of incubation were lymphocytes, blastoid cells derived from lymphocytes, and eosinophils.
2. Synthesis of DNA in cultured kangaroo leucocytes is first detectable 12 hours after the addition of phytohaemagglutinin. This is 12 hours earlier than in cultures of human leucocytes.
3. The syntheses of histone and DNA are asynchronous in kangaroo lymphocytes stimulated with PHA. The onset of histone synthesis lags behind that of DNA synthesis, and histone synthesis continues after the synthesis of DNA is complete.
4. Synthesis of chromosomal residual protein commences at about the same time as histone synthesis, and continues at least until early prophase. Synthesis of chromosomal residual protein possibly continues at a low rate through mitosis.
5. There is an increase in the rate of RNA synthesis only twelve minutes after the addition of PHA.

At first, this is indicated only by incorporation of cytidine-H^3 into the nucleus, but eighteen minutes after the addition of PHA some labelling is detectable in the cytoplasm. Cytoplasmic labelling has not been detected until twelve hours after the addition of PHA in cultures of human leucocytes.

6. Cytidine-H^3 incorporation is closely followed by incorporation of phenylalanine-H^3 into the small lymphocyte. Label is seen first in the cytoplasm, and later (four hours after the addition of PHA) it is incorporated into nuclear protein.
7. Nuclear and cytoplasmic volumes of PHA-treated kangaroo lymphocytes showed significant increases within three hours of the addition of PHA.
8. Nuclear dry masses of the small lymphocytes started to increase within four hours of the addition of PHA, although the dry mass of the nucleic acids remained constant.
9. Lymphocytes treated with PHA almost immediately showed a sharp decrease in stainability with alkaline fast green, indicating a corresponding decrease in the amount of histone.
10. There is an initial slight decrease in the dry masses of nuclei of lymphocytes exposed to PHA,

and a corresponding increase in the cytoplasmic dry mass. This can be accounted for by the transfer of histone or histone-derived material from the nucleus to the cytoplasm.

11. Hypotheses are advanced to explain why kangaroo lymphocytes exposed to PHA commence the synthesis of DNA 12 hours earlier than human lymphocytes which have been similarly treated, and to explain some aspects of the mitogenic activity of phytohaemagglutinin.

1. INTRODUCTION

In 1936, Rich said of the lymphocyte "The complete ignorance of the function of this cell is one of the most humiliating and disgraceful gaps in all medical knowledge." Trowell (1958), after a lengthy review of the lymphocyte problem, came to much the same conclusion. Since then, numerous experiments under controlled conditions have provided new information on this cell's function and potentiality. Nevertheless, the lymphocyte still has many puzzling features, and is still the subject of intensive study and debate.

The discovery by Nowell (1960b) that the small lymphocyte could be induced to grow and divide in vitro when cultured with phytohaemagglutinin (henceforth abbreviated PHA) opened up a new dimension in the study of the lymphocyte. This finding stimulated a search for other blastogenic factors which could stimulate the small lymphocyte, and this search succeeded when Pearmain, Lycette, and Fitzgerald (1963) and Schrek (1963) independently found that specific antigens induced growth and mitoses in cultures of leucocytes from donors who had previously been exposed to the same antigen. This significant discovery gave new insight

into the function of the lymphocyte.

However, there are marked differences between the response of a lymphocyte to PHA and the response to antigen of a lymphocyte from a presensitised donor. Furthermore, Sarkany and Caron (1965), Agrell (1966), and Ioachim (1966) have recently demonstrated the mitogenic effect of PHA on non-lymphoid as well as lymphoid tissue.

These findings raise some important questions.

1. Is the mechanism of PHA-stimulation of lymphocytes basically the same as the mechanism of antigenic stimulation of presensitised lymphocytes? (i.e., antigenic stimulation of lymphocytes from presensitised donors.)

2. If the two mechanisms are different, how do they differ?

3. Precisely how does PHA stimulate lymphocytes and other cells to grow and divide?

Before any attempt can be made to answer these questions, three aspects of the problem must be considered.

The first aspect concerns the nature of PHA itself, and its precise effect on the lymphocyte population.

The second aspect centres around the nature and function of the lymphocyte. As there is as yet no

information on the intracellular changes induced by PHA in non-lymphoid cells, all the available information derives from studies on the lymphocyte. This means that some knowledge of the function of the lymphocyte and of the nature of its response to antigen is necessary when studying its response to PHA. The lymphocyte's response to antigen implies antigen recognition; this means that such a response will be a very characteristic, definable one. As the lymphocyte is presumably predisposed to make this type of response, it is highly likely that at least some of the aspects of lymphocyte response to antigen will be present in the response of lymphocytes to PHA. If this is so, then the changes induced by PHA in non-lymphoid tissue will be less obvious than the changes induced in lymphoid tissue; in the former case, the element of antigen recognition will not be present to mask the basic mechanism of PHA-stimulation. In the case of the lymphocyte, however, the situation becomes more confused. Therefore a comparison of antigen- and PHA-stimulation of lymphocytes must be made.

It can be shown that PHA has a marked and immediate effect on the histones of lymphocytes exposed to it. The nature of histone and its function in the lymphocyte make up the third aspect of the problem of the activity

of PHA on peripheral lymphocytes and other cells.

1.2. Phytohaemagglutinin - its chemistry and properties.

The term "phytohaemagglutinin" has been applied to the haemagglutinating extract prepared by Rigas and Osgood (1955) from the red kidney bean, *Phaseolus vulgaris*. However, the first use of a haemagglutinating extract from this plant dates back to 1916, when it was used to obtain clear, sterile serum for use in the treatment of hog cholera (Dorset and Henley, 1916).

In low concentrations, PHA agglutinates only the erythrocytes (Rigas and Osgood, 1955), but in higher concentrations it agglutinates the leucocytes as well (Mellman, Klevit, and Moorhead, 1963; Hirschhorn, Kolodny, Hashem, and Bach, 1963; Nordman, de la Chapelle, and Grasbeck, 1964). Nevertheless, by far its most interesting aspect is its mitogenicity, which was discovered by Nowell in 1960. He found that leucocyte cultures prepared in serum which had been separated with PHA showed a much higher percentage of blastoid cells and mitoses than had been reported by other workers, and by a process of elimination he decided that phytohaemagglutinin was the blastogenic factor.

Other blastogenic preparations have been obtained from P. vulgaris, but some of these preparations showed no sign of any red cell agglutinating activity, and this indicated that the two factors involved were separable (Beckman, Fichtelius, Finley, Finley, and Lindahl-Kiessling, 1962; Genest, 1963). Conversely, some batches of PHA were found to have haemagglutinating, but not mitogenic, activity (Cooper, Barkhan, and Hale, 1961; de la Chapelle, 1961; Barkhan and Ballas, 1963). This also indicated that the factors involved were separable, and several workers succeeded in separating the blastogenic factor in PHA from the haemagglutinating factor by adsorbing the latter onto erythrocytes (Hirschhorn, Kolodny, Hashem, and Bach, 1963; Barkhan and Ballas, 1963; Nordman, de la Chapelle, and Grasbeck, 1964; Borjeson, Bouveng, Gardell, Norden, and Thunell, 1964; Robbins, 1964 a) or erythrocyte stroma (Rigas and Johnson, 1964; Tunis, 1964). However, the leucoagglutinating activity of PHA is not removed by this treatment, and no success has been achieved in attempts to separate the leucoagglutinating and the blastogenic factors (Hirschhorn, Kolodny, Hashem, and Bach, 1963; Kolodny and Hirschhorn, 1964; Nordman, de la Chapelle, and Grasbeck, 1964; Tunis, 1964). This does not necessarily mean that the two factors are

one and the same; de la Chapelle (1961) and Genest (1963) suggested that the two factors may be on different parts of the same molecule.

The available preparations of phytohaemagglutinin are found to possess several protein fractions when they are analysed electrophoretically (Beckman, Fichtelius, Finley, Finley, and Lindahl-Kiessling, 1962; Beckman, 1962; Barkhan and Ballas, 1963; Robbins and Wachtel, 1963; Nordman, de la Chapelle, and Grasbeck, 1964; Rigas and Johnson, 1964; Spitz, 1964). Furthermore, the mitogenic factor itself is non-dialysable (Punnet, Punnet, and Kaufman, 1962; Genest, 1963), is destroyed at 100° C. (Nowell, 1960b), and its activity is always found in the presence of protein (Beckman, Fichtelius, Finley, Finley, and Lindahl-Kiessling, 1962; Rigas and Johnson, 1964; Borjeson, Bouveng, Gardell, Norden, and Thunell, 1964). It therefore seems very likely that the factor is proteinaceous.

1.3. Blastogenic factors other than PHA.

The finding that phytohaemagglutinin could induce mitoses in cultures of peripheral leucocytes stimulated a search for other factors that could do the same thing. Several other factors were found, but their effects on

leucocyte cultures are, in general, rather different from those of PHA. These factors can be divided into several groups.

(a). Antigenic factors.

The first known blastogenic factor apart from PHA was tuberculin purified protein derivative (PPD), the activity of which was independently discovered by Pearmain, Lycette, and Fitzgerald (1963) and Schrek (1963). Unlike PHA, PPD produced mitoses only in cultures prepared from the blood of Mantoux-positive donors. These findings were soon confirmed (Marshall and Roberts, 1963a; Cowling, Quaglino, and Davidson, 1963), and soon after this action was reported, Elves, Roath, and Israels (1963) showed that tetanus toxoid and polio-virus vaccine had a similar effect on leucocyte cultures prepared from the blood of specifically pre-sensitised donors. Several more antigens capable of producing divisions in cultures from specifically presensitised donors have since been reported (Hashem and Barr, 1963; Hirschhorn, Bach, Kolodny, Firschein, and Hashem, 1963; Hirschhorn, Kolodny, Hashem, and Bach, 1963; Lycette and Pearmain, 1963; Ling and Husband, 1964; Matsianotis and Tsenghi, 1964; Holland and Mauer, 1964).

In general, antigen-treated cultures show only a

small percentage of blastoid cells. Ling and Husband (1964) reported that more blastoid cells were formed in a culture treated with two antigens than were formed in cultures exposed to only one of the antigens. However, Cowling and Quaglino (1964) published results showing exactly the opposite. No further work of this nature appears to have been done. Some substances have been reported to produce a very high percentage of blastoid cells; Matsianotis and Tsenghi (1964) reported that smallpox-vaccine lymph which had been inactivated by heat affected 65 % of the cells in culture, and Ling and Husband (1964) gave a figure of 71 % for staphylococcal filtrate. These results are comparable with those obtained from treatment with PHA. It is still not clear whether the responses of the leucocyte cultures to these last two antigenic factors, when they are compared with the results obtained from other antigens, reflect differences in technique, differences in cell sensitivity, or differences in the mode of action of the smallpox-vaccine lymph and staphylococcal filtrate.

(b). Homologous leucocytes.

Bain, Vas, and Lowenstein (1964) showed that when leucocytes from the peripheral blood of two homologous individuals were cultured together in vitro, blastoid

cells were formed. This reaction was similar to the reaction caused by PHA, although it was less intense. This finding was later confirmed by others (Hirschhorn, Bach, Kolodny, Firschein, and Hashem, 1963; Hashem and Rosen, 1964).

(c). Antiserum to leucocytes.

On the assumption that PHA might induce blastogenesis by attaching to some part of the leucocyte, Grasbeck, Nordman, and de la Chapelle (1963) attempted to prepare a substance likely to make such an attachment. They succeeded in preparing antiserums to human leucocytes from rabbits; these antiserums had very similar effects to that of PHA, in that they showed some tendency to agglutinate leucocytes, and possessed comparable mitogenic activity.

(d). RNA from stimulated lymphocytes.

Hashem (1965) reported that ribosomal RNA, from peripheral lymphocytes which had been recently stimulated by specific antigens to which the donor was presensitised, was capable of promoting transformation and mitosis when added to cultures of autologous unstimulated lymphocytes. This is a very interesting observation, but it probably reflects only one aspect of the process of antigenic stimulation of presensitised lymphocytes.

(e). Drug sensitivity.

Holland and Mauer (1964) reported that leucocyte

cultures prepared from a patient with a severe reaction to phenytoin sodium, and incubated with the drug, showed a mitotic index similar to that obtained with antigen stimulation. Normal blood did not react.

1.4. Suggested mechanisms of PHA activity.

Following the report by Nowell (1960b) on the mitogenic activity of PHA, various attempts have been made to explain its action. These attempts have, in general, fallen into three categories.

(a). Beckman (1962) suggested that PHA precipitated a protein from serum which normally inhibits lymphocyte growth, and supported this with results from starch-gel electrophoresis studies.

(b). Hastings, Freedman, Rendon, Cooper, and Hirschhorn (1961) suggested that the surface-active properties of PHA may be responsible for its mitogenic activity.

(c). The third explanation of the mitogenic activity of PHA derives from Pearmain, Lycette, and Fitzgerald's observations on tuberculin-stimulated lymphocyte cultures from Mantoux-positive donors. Hirschhorn, Kolodny, Hashem, and Bach (1963) proposed that PHA acted as a general stimulus on immunologically competent cells.

These three proposed modes of action are fully discussed in chapter 4.

In an attempt to find the site of PHA activity, Michalowski, Jasinska, Brzosko, and Nowoslawski (1964) labelled PHA with fluorescein isothyocyanate. They found that PHA treated in this way did not lose its mitogenic activity, and showed that PHA actually entered the cell. This finding appears to support the antigenic stimulus hypothesis rather than the surface action hypothesis. However, although the PHA used by Michalowski and his co-workers had been previously exposed to erythrocytes to remove the haemagglutinating factor, there was no evidence to show that the PHA fraction labelled with fluorescein isothyocyanate was the fraction responsible for the mitogenic activity of PHA.

A further finding of great interest is that very few or no blastoid cells develop when PHA is added to cultures of cells from some patients with chronic lymphocytic leukaemia (Schrek and Rabinowitz, 1963; Robbins, 1964 a; Bernard, Geraldes, and Boiron, 1964). These results suggest that most of the lymphocytes from some patients with chronic lymphocytic leukaemia may be inherently unable to respond to the blastogenic stimulus of PHA, and thus differ from the majority of lymphocytes

from normal persons (Robbins, 1964 b).

One further aspect of PHA activity remains to be discussed, and this is probably the most significant aspect of all. Killander and Rigler (1965) detected early changes in the DNP complex of leucocytes treated with PHA. These changes were reflected by an increased uptake of acridine orange by the leucocytes, which was probably due to a liberation of PO_4^- groups in the DNP of the PHA-stimulated cells. Pogo, Allfrey, and Mirsky (1966) detected an increase in histone acetylation in cells treated with PHA. Pogo and his co-workers regard these changes as representing gene activation. All the same, it is obvious that there is much to be learned about the precise mode of action of PHA, and the significance of this action is still obscure.

1.5. The lymphocyte: its potentialities and functions.

A number of functions have been suggested for the lymphocyte; these centre around known or proposed transformations of the lymphocyte to other cell types. Only two of the suggested transformations are well established. Others have been suggested, but there is, as yet, not a great deal of supporting evidence for these.

(a). Transformation into blastoid cells in vitro.

Since the discovery of the mitogenic activity of PHA (Nowell, 1960b), this transformation has become well documented. There is now a large body of evidence to show that the small lymphocyte is the cell which undergoes blastogenesis in the presence of PHA and other blastogenic substances (Berman and Stulberg, 1962; MacKinney, Stohlman, and Brecher, 1962; McIntyre and Ebaugh, 1962; Carstairs, 1962; Marshall and Roberts, 1963 a, b; Elves and Wilkinson, 1963). Numerical considerations demand that the blastoid cells in PHA-treated cultures should be derived from lymphocytes. In several of the experiments the initial inoculum consisted almost entirely of lymphocytes. It is not known whether the larger lymphocytes are affected by PHA, but the monocytes probably do not undergo blastogenesis - they either remain unchanged or become macrophages (Schrek and Rabinowitz, 1963).

(b). Transformation into macrophages.

In 1937 Taliaferro and Mulligan, while studying the histopathological changes caused by malaria, became convinced that the majority of new macrophages originated heteroplastically from lymphocytes. Kolouch (1939) studied experimental inflammation in subcutaneous tissue by serial biopsy, and found that the lymphocytes

developed into macrophages within the first 14 hours. Rebuck and Crowley (1955) found that lymphocytes developed directly into macrophages without going through a monocyte stage. Recently, Gough, Elves, and Israels (1965) observed the transformation of unstimulated lymphocytes into macrophages in culture. Trowell (1958) concluded that there was fairly good evidence that lymphocytes could turn into macrophages under certain conditions, but said "It would be unwise to assume that this is a major destination or function of the blood lymphocytes." This comment is still valid.

The blastoid cells produced by PHA and other agents in cultures of peripheral leucocytes differ greatly from macrophages (Schrek and Rabinowitz, 1963). The blastoid cells do not phagocytose cell debris (Tanaka, Epstein, Brecher, and Stohlman, 1963; Newsome, 1963), whereas the macrophages do. There seems to be little to connect the two, apart from their established derivation from the small lymphocyte.

(c). Transformation into other cell types.

There is no convincing evidence that the blastoid cell or the small lymphocyte can give rise in vitro to any of the cells found in normal peripheral blood. The blastoid cell does not have the structure or the cyto-chemical properties of the erythrocyte, monocyte, or

granulocyte (Robbins, 1964b).

The question then arises: do the blastoid cells or their progeny revert in vitro to small lymphocytes? Some workers have suggested that this might happen (Nowell, 1960 a; Carstairs, 1962; Cooper, 1962; Elves and Wilkinson, 1962), but no convincing evidence has been presented.

The PHA-induced blastoid cells have some morphological resemblance to normal primitive cells such as reticulum cells, lymphoblasts, and plasmablasts (Berman and Stulberg, 1962; Carstairs, 1962; Barkhan and Ballas, 1963; Newsome, 1963; Elves, Gough, Chapman, and Israels, 1964), and cells sometimes present in diseases such as acute leukaemia (Tanaka, Epstein, Brecher, and Stohlman, 1963). However, this morphological resemblance need not be very important, and does not necessarily imply that the cells are closely related, or that they have the same origin, functions, or potentialities.

Much of the specific antibody of mammals, including human gamma-globulin, is produced in cells of the plasma cell series (Fagraeus, 1948 a, b; Leduc, Coons, and Connolly, 1955; Ortega and Mellors, 1957; Fagraeus, 1958; Bernhard and Granboulan, 1960; Thiery, 1960; McMaster, 1961; Mellors and Korngold, 1963; Solomon,

Fahey, and Malmgren, 1963). There is very little similarity between the typical blastoid cell and the cells of the plasma cell series. While the cytoplasm of both is markedly pyroninophilic, mature plasma cells have large amounts of well developed endoplasmic reticulum (Braunsteiner, Fellinger, and Pakesch, 1953; Bernhard and Granboulan, 1960; Bessis, 1961; Binet and Mathe, 1962), and the blastoid cells have very little (Marshall and Roberts, 1963 b; Tanaka, Epstein, Brecher, and Stohlman, 1963; Inman and Cooper, 1963; Elves, Gough, Chapman, and Israels, 1964).

Much more revealing are the reactions in vivo, in which a large pyroninophilic cell is formed in response to substances which are blastogenic in vitro. A large pyroninophilic cell with immature morphology is formed in homograft and graft-versus-host reactions in mammals, and this cell can apparently be derived from the small lymphocyte (Scothorne, 1957; Porter and Cooper, 1962; Gowans, McGregor, Cowen, and Ford, 1962; Gowans, 1962; Binet and Mathe, 1962; Andre, Schwartz, Mitus, and Dameshek, 1962). This large pyroninophilic cell closely resembles the blastoid cell formed in vitro (Berman and Stulberg, 1962; Marshall and Roberts, 1963 a, b; Tanaka, Epstein, Brecher, and Stohlman, 1963; Inman and Cooper, 1963;

Bain, Vas, and Lowenstein, 1963, 1964; Elves, Gough, Chapman, and Israels, 1964). A morphologically similar cell is found in lymphoid tissue stimulated with antigens (Fagraeus, 1948 a, b; Leduc, Coons, and Connolly, 1955; Bain, Vas, and Lowenstein, 1963, 1964). Nevertheless, it is not at all clearly established that the lymphocyte is the precursor of these cells, and there is no clear evidence to show that the lymphocyte is a precursor of the plasma cell.

(d). The immunological competence of the lymphocyte.

Animals may develop a fatal wasting disease if they are injected with adult lymphoid cells which lack antigens present in the tissues of the host. The ability of a particular cell type to induce such a disease is an index of its "immunological competence", in the sense that it must be able to react with antigens in the tissues of the host and initiate an immune reaction (Gowans, McGregor, Cowen, and Ford, 1962). Studies on the graft-versus-host reactions which occur when thoracic duct lymphocytes from genetically appropriate rats or mice are injected into new-born rats (Billingham, Defendi, Silvers, and Steinmullar, 1962; Hildeman, Linscott, and Morlino, 1962), adult F1 hybrid rats (Gowans, Gesner, and McGregor, 1961), and lethally X-irradiated mice (Gesner and Gowans, 1962; Gowans,

McGregor, Cowen, and Ford, 1962), show that the small lymphocyte is an immunologically competent cell. Gowans and McGregor (1963) found that drainage of thoracic duct lymphocytes severely depressed the immune response, but if the lymphocytes were removed immediately before a second antigen dose, a normal secondary response occurred. They concluded that the cell which mediated the response could not be withdrawn by drainage from the thoracic duct. However, when thoracic duct lymphocytes from a primarily stimulated rat were injected into a non-immunised rat, the recipient responded to antigen with a brisk secondary response. They thereupon concluded that the memory of the primary immunisation, in addition to being invested in fixed cells, is also carried by a circulating lymphocyte. Gowans (1965) proposed that the interaction from primary immunisation caused some lymphocytes to develop into large pyroninophilic cells in regional lymph nodes. "Activated" lymphocytes released from the lymph nodes into blood could be the offspring of these large pyroninophilic cells, and could play a crucial role in the secondary response.

This implicates the lymphocyte as a memory cell. This interpretation is supported by findings on the long life span of the lymphocyte (Brecher, Little,

Bradley, and Rose, 1961; Little, Brecher, Bradley, and Rose, 1962; Caffrey, Rieke, and Everett, 1962; Buckton and Pike, 1964 a, b), and by studies on the circulation of lymphocytes throughout the body (Mann and Higgins, 1950; Gowans, 1957, 1959; Gowans and Knight, 1963). These attributes, among others, are exactly what would be expected of a cell whose function was to recognise and respond to antigen throughout the body.

(e). Antibody production by lymphocytes.

There is some evidence that peripheral blood lymphocytes are capable of synthesising antibody, but the evidence is far from being conclusive. Hollander and Danielsson (1962) reported antibody production in vitro by thoracic duct lymphocytes on the second, third, and fcurth days of a secondary response. Hulliger and Sorkin (1963) and Landy, Sanderson, Bernstein, and Jackson (1964) recorded antibody production by leucocytes in peripheral blood. It is probable that the lymphocyte is capable of producing a small amount of antibody, but it is a minimal amount compared with production of antibody from other sources. Antibody production is almost certainly not a primary function of the small lymphocyte.

In summary, then, the following transformations,

properties, and potentialities are known or inferred for the small lymphocyte.

1. It can give rise to blastoid cells in culture.

2. Transformation into macrophages is established.

3. PHA-induced blastoid cells have a morphological resemblance to normal "primitive" cells such as reticulum cells, lymphoblasts, and plasmablasts.

4. The lymphocyte apparently gives rise to a large pyroninophilic cell in homograft and graft-versus-host reactions in mammals.

5. Lymphocytes are immunologically competent, have a long life span, and circulate freely throughout the body.

6. They are probably capable of producing small amounts of antibody.

Thus the lymphocyte problem has a number of different facets, and this in itself has contributed greatly to the confusion over the status and function of the lymphocyte. Since the time of Ehrlich and Lazarus (1898) many workers have regarded it as a mature cell which could not produce anything else, while others have regarded it as the temporarily resting stage of a primitive pluripotential cell capable of active development under suitable conditions. This fundamental dichotomy of thought stemmed from a quarrel

between Ranvier and Ehrlich at the time of the Franco-Prussian war (Trowell, 1958), and has since become compounded and confused by workers who concentrated on one aspect of the lymphocyte only and ignored or denied all other aspects.

Probably the most important and the most basic attribute of the small lymphocyte is its ability to recognise antigens and respond to them. All the observed and projected transformations and potentialities hang on this ability. Whether the lymphocyte is regarded as a pluripotential end cell capable of directly producing all other haematologic cells (Yoffey, 1962), or a cell with limited stem cell potentialities (Gropp and Fischer, 1964), or a cell which mediates the transfer of information to antibody-producing cells of a different stem-line, the recognition of antigen by the lymphocyte is vital to its functioning. In any case, it is fairly clear that only the inductive phase of the immune response can be studied in lymphocyte cultures. The study of the basic mechanisms of lymphocyte stimulation by PHA, antigens, and other blastogenic substances can shed a great deal of light on the function of the lymphocyte as a memory cell and on the process of antigen recognition and gene activation.

1.6. The histones.

The histones are the most basic proteins known apart from the protamines, and they have isoelectric points at about pH 11 (Phillips, 1962). Cruft, Mauritzen, and Stedman (1957) have suggested that the term "histone" should be applied only to the basic proteins, other than protamines, which occur in the cell nucleus. Basic proteins similar to those found in the nucleus (that is, with similar amino acid analyses) have been found in the cytoplasm (Crampton and Petermann, 1959). These proteins may be more conveniently referred to as "cytoplasmic histone"; throughout the text of this thesis "histone" refers only to nuclear basic protein which is intimately associated with DNA at some stage in the cell cycle.

1.7. The function of histone.

Hypotheses on the functions of histones fall into several groups.

(a). Histone plays a purely structural or passive role; Butler (1956) considered that histone protected DNA from combination with other proteins, such as enzymes, or else joined DNA molecules into a chromosome. This view is supported by Zubay (1964).

(b). Stedman and Stedman (1950, 1951) put forward a gene inhibition hypothesis. They proposed that histones specifically blocked those parts of the DNA which were not required to produce a given type of cell. Only the "exposed" DNA in the cell is active, and it is this portion which determines the character of the cell. Many workers now subscribe to this view, although with some modifications.

(c). Danielli (1953) suggested that histones might combine with RNA, and thus slow down protein synthesis. This idea is supported to some extent by the finding of Benjamin, Levander, Gellhorn, and DeBellis (1966) that RNA and histone can become intimately associated in mammalian cells; however, the precise significance of this finding is not yet known.

(d). Leslie (1961) suggested that the histones stabilise both nuclear and ribosomal RNA by combining with specific RNA with a single polynucleotide strand ("template RNA").

(e). Butler (1959) suggested that the histones were one step in the transfer of information from DNA to the protein synthesis mechanism.

(f). Bloch and Godman (1955 a, b) postulated that the histones could act as templates for DNA

synthesis, and vice versa.

(g). Mauritzen and Stedman (1959) concluded that histone did not support RNA synthesis, and so concluded that histone was a suppressor of genetic activity. This conclusion was supported by Huang and Bonner (1962), who showed that DNA which was fully complexed with histone was inactive in the support of RNA synthesis; they also concluded that histone was a suppressor of genetic activity. Allfrey, Littau, and Mirsky (1963 a, b) showed that the addition of histones to isolated thymus nuclei inhibited RNA synthesis. Billen and Hnilica (1963) reported that when calf thymus histone was added to an in vitro system capable of synthesising DNA, there was an inhibition of substrate incorporation into DNA. Busch, Steele, Hnilica, Taylor, and Mavioglu (1963) agreed with Bloch (1962 a, b, 1963 a, b) that the nucleohistone complex provided a basis for the existence of alternative hereditary gene states. Further evidence on the inhibition of protein synthesis by histone has been put forward by Irvin, Holbrook, Evans, McAllister, and Stiles (1963), Peck, (1963), and Allfrey, Faulkner, and Mirsky (1964). Some evidence has also been presented to show that histone can form a complex with RNA (Huang and Bonner, 1965; Benjamin, Levander,

Gellhorn, and DeBellis, 1966). However, some conflicting evidence has also been presented. Barr and Butler (1963) and Bonner, Huang, and Murray (1963) claimed that histone was active in the support of DNA-dependent RNA synthesis.

In summary, it can be said that the precise function of histone is still uncertain, but there is abundant circumstantial evidence to indicate that histone plays an important part in the control of the activity of genes. If this is so, then newly synthesised DNA should promptly form a complex with histone, so that any genetic information not required by the cell is not made available during the course of DNA replication. This requires either that DNA and histone syntheses should be synchronous, or else that histone should be synthesised prior to DNA. A number of workers in the past have shown that DNA/histone ratios in various tissues are constant throughout the mitotic cycle, and have inferred that DNA and histone were being synthesised concurrently. Alfert, Bern, and Kahn (1955) demonstrated a constant DNA/histone ratio in rat tissue in various functional states; Bloch and Godman (1955b) obtained the same results in studies on rat liver; Ansley (1957) found that DNA and histone syntheses were synchronous in normal cells of <u>Loxa</u>

flavicolor. McLeish (1959) recorded synchronous syntheses in plant nuclei, and De (1961) obtained the same result from studies on the microsporocytes of *Tradescantia*. Evans, Holbrook, and Irvin (1962), and Niehaus and Barnum (1965) recorded synchronous syntheses of DNA and histone in regenerating liver; Meek (1964) obtained the same result from work on mouse testicular cells. However, Umana, Updike, and Dounce (1962) concluded, from studies on dividing and interphase cells in liver, kidney, and pancreas, that histone doubled in quantity shortly after mitosis and not concomitantly with DNA. Nevertheless, the general consensus of opinion is that the synchrony of DNA synthesis and histone synthesis is established for a wide variety of normal, differentiated tissues, and it seems clear that the syntheses of histone and DNA in normal differentiated cells are closely linked.

Bloch (1962 a, b, 1963 a, b) decided that if histone synthesis was controlled by specific genes, the DNA-histone association would provide a means by which histone-producing genes could variably influence histone associating genes. During the life cycle of organisms, cells are found in which the main complement of histone has been replaced; this indicates that variation in histone association may occur among

homologous genes at different times during development. DNA replication must occur within the spectrum of histones found within the cell, and unless DNA-histone association is random, the newly-replicated DNA would be expected to associate with the same histone after replication as before replication. Bloch thought that it was unlikely that the associations would be restricted by inherent differences in the affinities of DNA for histone, and inferred that the factor which determined the specificity of association could be traced back to the association which existed before replication. Thus the nucleohistone complex would replicate as a unit: the DNA would act as its own template, and the pre-existing histone would determine the association of the newly-replicated DNA with the proper previously-synthesised histone. Bloch concluded that the DNA-histone association could provide a basis for alternative hereditary states of the gene which are moderately stable and potentially reversible, and so would correspond with the parachromatin postulated by Brink (1960).

From this point of view, the DNA-histone complex in lymphoid tissue becomes particularly interesting. If Bloch's conclusions are correct, then presensitised lymphocytes exposed to specific antigens would show some change in their DNA-histone complexes, and these

changes would reflect gene activation in the lymphocyte. The antigen-induced changes in lymphoid tissue recorded by Black and Speer (1958), Black, Ansley, and Mandl (1964), and Black and Ansley (1965, 1966) then become understandable. Similar changes have also been noted in PHA-stimulated lymphocytes by Killander and Rigler (1965), and Pogo, Allfrey, and Mirsky (1966). Pogo and his co-workers regard these changes as representing gene activation.

The undisturbed lymphocyte apparently has a very long life span between divisions (Brecher, Little, Bradley, and Rose, 1961; Little, Brecher, Bradley, and Rose, 1962; Caffrey, Rieke, and Everett, 1962; Buckton and Pike, 1964 a, b), but when presensitised lymphocytes are exposed to antigen, they grow and divide. This argues that a considerable amount of information which was previously unavailable is made available to the cell during the process of antigen recognition. As the first detectable changes in antigen-treated or PHA-treated cells occur in the nucleohistone complex, it is logical to suppose that these changes represent gene activation.

1.8. Selection of study material.

Leucocytes from the grey kangaroo *Macropus giganteus* were chosen as the study material for three main reasons.

(a). Blood was readily available at all times, and was relatively easily collected.

(b). It was hoped that marsupial leucocyte cultures would show some illuminating differences in response to PHA when compared with cultures from eutherian mammals.

(c). The grey kangaroo has about 14 % more DNA per cell than man (Martin, pers. comm.) and it is divided among only 16 chromosomes instead of 46. Thus the kangaroo's chromosomes are, on the average, about three times as big as those of man. It was hoped that this feature would be helpful in some studies but, in practice, it proved to be of only minor importance.

2. MATERIALS AND METHODS

2.1. Experimental animals.

Blood taken from three adult male and four adult female grey kangaroos (Macropus giganteus Shaw, 1790; synonym M. major Shaw, 1800) was used during the course of the investigation. Unless otherwise stated in the text, the sex of an animal was not taken into account when planning an experiment.

2.2. Radioactive materials.

The tritium-labelled amino acids and nucleic acid precursors used are shown in table 1.

The arginine was a product of the Radiochemical Centre, Amersham. All other radioactive materials were products of Schwarz BioResearch Inc., New York.

Thymidine was used in the study of DNA synthesis, as is is known to label DNA almost exclusively (Friedkin, Tilson, and Roberts, 1956).

Phenylalanine was used to study synthesis of protein other than histone. It was used by Bach and Hirschhorn (1963) to study gamma globulin production

TABLE 1

Tritium-labelled amino acids and nucleic acid precursors used.

Tritiated material	Specific activity
L-Arginine-T (G) monohydrochloride, aqueous solution.	238 mc/mM.
L-Phenylalanine, 50 % ethanol solution.	1.5 c/mM.
L-Lysine, 50 % ethanol solution.	171 mc/mM.
Cytidine 5'- monophosphate, diammonium, 50 % ethanol soln.	1.0 c/mM.
Thymidine (methyl labelled), aqueous solution.	1.9 c/mM.

in small lymphocytes treated with PHA, but it appears to label structural protein as well.

Arginine and lysine were used to study the syntheses of arginine-rich and lysine-rich histones respectively. Unfortunately, these amino acids can be incorporated into non-histone protein. This was demonstrated by Cave (1966) for lysine, and it is highly probable that arginine behaves the same way. Therefore some care is needed in interpreting results obtained through the use of these two amino acids.

Cytidine was used in the study of RNA synthesis. Cytidine 5'- monophosphate was used in preference to the di- or triphosphate because of its stability in comparison with the latter two preparations. Some cytidine is incorporated into DNA during DNA synthesis (Feinendegen, Bond, Shreeve, and Painter, 1960), but as cytidine incorporation was studied during the G1 period only, this drawback is not relevant.

2.3. Collection of blood.

Blood was collected by venipuncture, from a superficial vein on the side of the tail of the kangaroo. Prior to collection of blood, the selected animal was placed in a sack so that its tail protruded. The

kangaroo was placed on its side, the area around the vein was clipped and swabbed with 70 % alcohol, and a tourniquet was applied at the base of the tail. Blood was drawn through a sterile 19 g. hypodermic needle into a sterile 20 ml. "Luer-lok" glass syringe, the barrel and plunger of which had previously been coated with liquid paraffin to prevent clotting of blood following its contact with glass. The blood was promptly transferred to a sterile, siliconed 1 oz. MacCartney bottle, containing 0.8 ml. of dextran and two drops of 5000 I.U./ml. heparin (Boots). The bottles were siliconed with a 0.5 % (V/V) solution of Dow-Corning 1107 resin in ether. After the blood was transferred from the syringe, the bottle was gently inverted several times to mix the contents. Blood intended for serum extraction was placed in a sterile non-siliconed MacCartney bottle and allowed to clot.

2.4. Separation of leucocytes.

After the heparinised blood had been thoroughly mixed with the dextran, it was allowed to stand at room temperature. After some time, varying from 20 minutes to five hours, the erythrocytes agglutinated and settled, leaving a turbid, straw-coloured supernatant containing large numbers of leucocytes in the top 1/3 of the bottle.

This was pipetted off and transferred to a sterile, siliconed bottle. No attempt was made to separate lymphocytes from the rest of the leucocyte population.

2.5. Preparation of defibrinated serum.

Kangaroo blood clots very readily and quickly, and appears to contain a large amount of fibrin, so the addition of heparin could not be relied upon to prevent the formation of fibrin clots in culture. Three different methods were therefore devised so that defibrinated serum could be obtained.

(a). The leucocyte-rich plasma obtained after separation with dextran was centrifuged for three minutes at 200 g, the supernatant transferred to a sterile, non-siliconed MacCartney bottle, and the leucocyte pellet resuspended in Commonwealth Serum Laboratories medium 199. The leucocyte-free plasma thus obtained was stirred with a glass rod until no more fibrin was precipitated, then centrifuged for five minutes at 1000 g, and the supernatant pipetted off. This method reduced the tendency to clot, but did not eliminate it, as not all of the fibrin was precipitated.

(b). After the separation of leucocytes and plasma as in (a), the plasma was frozen at - 20° C.

for one hour. At the end of this time a considerable amount of fibrin was normally precipitated; this was separated from the serum by centrifugation at 1000 g for five minutes. This method also was only partially successful.

(c). Following the relative failure of the above methods, a third method was devised. For every 20 ml. of heparinised blood collected, a further volume of 20 ml. of blood was collected at the same time. This was placed in a sterile non-siliconed MacCartney bottle and allowed to clot. The clot was shaken to detach it from the sides of the bottle and allowed to stand for one hour, during which time defibrinated serum and haemolysed erythrocytes oozed out of it. This mixture was removed and centrifuged at 1000 g for five minutes, leaving a clear, straw-coloured supernatant above the erythrocyte pellet. This method yielded about 7 ml. of fibrin-free serum for every 20 ml. of whole blood collected.

This method of defibrination was used for long or medium term cultures (more than twelve hours) unless otherwise stated in the text. For short term cultures, the addition of one drop of 5000 I.U./ml. heparin to every five ml. of culture gave satisfactory results.

In some later cultures, Commonwealth Serum Laboratories foetal calf serum was substituted for

defibrinated autologous serum in long-term cultures.

2.6. Culturing technique.

Leucocytes stored in medium 199, as above, were centrifuged for two minutes at 200 g, and resuspended in a mixture of three parts 199 to one part serum. This serum was either autologous serum or foetal calf serum as stated in the text. Two drops of heparin (5000 I.U./ml.) and 0.1 ml. Difco Phytohaemagglutinin P were added to every 10 ml. of culture. The culture was then thoroughly shaken and divided into 4 ml. aliquots, which were placed in sterile, siliconed ½ oz. MacCartney bottles. To maintain the culture at an acceptable pH, the air above the culture in each bottle was replaced with a mixture of 95 % air and 5 % carbon dioxide. Cultures were incubated in a water bath at 37° C., and gently shaken once daily until they were harvested.

The cultures were normally uncontaminated, but at one stage they frequently became heavily contaminated with yeast. At this time, a dust-free hood, sprayed inside with a mixture of equal parts of ethylene glycol and absolute alcohol, and liberally swabbed with 70 % ethanol, was used when setting up cultures. No further contamination occurred.

2.7. Quantitative DNA estimation.

Feulgen staining was used to estimate the relative amounts of DNA in lymphocyte material, and the density of stain was measured with a Barr and Stroud Type GN2 Integrating Microdensitometer. Leucocyte pellets obtained from culture by centrifugation at 200 g for two minutes were resuspended in fresh Commonwealth Serum Laboratories medium 199, centrifuged again, fixed in two changes of 3:1 ethanol:acetic acid, and stored overnight at 4° C. They were then resuspended in 45 % acetic acid and squashed onto a clean marked slide. The slide, with coverslip, was inverted onto dry ice for one minute, the coverslip flicked off with a scalpel, and the slide immersed in absolute alcohol. The time schedule of the subsequent staining procedure is shown in table 2.

The densities of stain in thirty cells in each treatment were measured in comparison with staining densities in thirty cells of a control culture mounted at the other end of the same slide. The wavelength used was 5480 Å. The use of a control culture on every slide was an important feature of the method, as it ensured that the control culture and the experimental treatment were subjected to exactly the same staining conditions. This then allowed direct comparison between

TABLE 2

Time schedule of Feulgen-staining procedure

Bath	Time in minutes
Absolute alcohol	2
70 % alcohol	2
50 % alcohol	2
30 % alcohol	2
Distilled water 1	2
Distilled water 2	2
1 N hydrochloric acid (60° C.)	12
Leuco-basic fuchsin (Gurr)	120
SO_2 water 1	10
SO_2 water 2	10
50 % alcohol	2
70 % alcohol	2
90 % alcohol	2
Absolute alcohol	3
Xylol	3

Mounted in DePeX (Gurr).

the two. Cells were fixed in 3:1 ethanol:acetic acid rather than formalin, as formalin-fixed cells tended to float off the slide during subsequent treatment. Rinsing the slides with water after immersion in SO_2 water was omitted, as this step renders stain intensity less dependable (Darlington and La Cour, 1962).

2.8. Quantitative histone estimation.

Staining with alkaline fast green according to the method of Alfert and Geschwind (1953) was used to estimate relative quantities of histone in lymphocyte material. Leucocytes in culture were fixed in the same manner as those used in DNA estimation, except that 10 % neutral formalin was used as a fixative. Cells were transferred to slides by inversion over dry ice as before, but showed a marked tendency to float off the slides during subsequent treatment. The following method was developed to prevent this, as fixatives containing acetic acid could not be used because they tend to dissolve out histone (De, 1961).

Leucocyte suspensions in 10 % neutral formalin were placed in disposable 0.4 ml. Beckman polythene centrifuge tubes, and spun for 15 seconds in a Beckman "Spinco" Microfuge. The leucocyte pellet was resuspended in distilled water, spun down again,

and resuspended in 0.3 ml. of 5 % (W/V) trichloracetic acid to remove nucleic acids. All centrifuge tubes were placed in a 90° C. water bath for 90 minutes, at the end of which time the cells were spun down and resuspended in distilled water. The cell suspension was squashed onto a slide and the coverslip removed after inversion over dry ice as before; the slide was then allowed to dry. When dry, it was dipped briefly in a 0.75 % (W/V) solution of formvar in chloroform, drained, and allowed to dry once more. The formvar film thus applied was thick enough to hold the cells on the slide during staining, and yet porous enough to allow the stain to reach the cells readily.

The stain was adjusted to pH 8.2 with dilute sodium hydroxide, and the pH of the stain was checked immediately before and after staining with a model C pH electrometer, made by N. L. Jones of Melbourne.

The time schedule of the staining procedure for estimation of histone is shown in table 3.

The first bath in dilute sodium hydroxide at pH 10 was introduced to counteract the effects of any residual acidity due to the prior immersion of the slides in trichloracetic acid.

TABLE 3

Time schedule of alkaline fast green staining procedure

Bath	Time in minutes
Distilled water	2
Dilute NaOH, pH 10	2
Dilute NaOH, pH 8	2
0.1 % (W/V) Fast Green, pH 8.2, @ 25° C.	30
Distilled water	2
Alcohol series, as in table 2	

Mounted in DePeX (Gurr)

2.9. Autoradiography.

Cells labelled with radioactive materials were fixed in fresh 3:1 ethanol:acetic acid in the usual way, and then transferred to 45 % acetic acid in water. The cell suspension was squashed onto a subbed slide, and the slide inverted over dry ice. After one minute, the coverslip was flicked off in the usual way, and the slide transferred to 95 % ethanol. It was then taken slowly down through an alcohol series, washed twice in distilled water, and left in distilled water until the autoradiographic stripping film was applied. Kodak AR 10 autoradiographic stripping plates were used throughout the investigation; the film was applied to the slides in the usual way (Pelc, 1956). During application of the film and during development a Kodak "Wratten" Series 1 safelight was used, but as overexposure to the safelight caused fogging on the film, the slides were allowed to dry in darkness. Autoradiographic slides were racked in polythene slide boxes which were then wrapped in black paper and stored in light-tight cardboard boxes at 4° C. Film was exposed for periods ranging from 48 hours to 6 months.

After a suitable period of exposure, autoradiographs were developed for five minutes in Kodak

formula D - 19b developer, washed in distilled water, fixed for ten minutes, washed twice in distilled water, and stained for forty seconds in a 0.5 % (W/V) solution of toluidine blue in distilled water. Lymphocytes labelled with tritiated thymidine or tritiated phenylalanine were normally hydrolysed in 1 N hydrochloric acid for six minutes at 60° C. prior to application of the film, but this step was omitted in the case of arginine-labelled, lysine-labelled, or cytidine-labelled cultures, as some label would be lost during hydrolysis in each case. After staining, the slides were washed twice more in distilled water, the excess film cut away, and the slides allowed to dry under a fan.

In experiments involving labelling of histone, acetic alcohol still had to be used as a fixative, even though acetic acid does tend to dissolve histone out of the cell (De, 1961). It was important to ensure that the cells were well spread on the slide and that the various stages of mitosis were easily distinguishable, and acetic alcohol was the most suitable fixative for this. However, its histone-dissolving effects were largely minimised by fixing the cells in ice-cold fixative for two minutes only, and then transferring them to distilled water rather

than 45 % acetic acid. Autoradiographs prepared in this way were quite satisfactory, and microspectrophotometry with alkaline fast green showed that an appreciable quantity of histone remained in the cell.

2.10. Estimation of dry mass.

Estimations of nuclear and cytoplasmic dry masses of individual lymphocytes were carried out with a Watson interference microscope equipped with a half-shade eyepiece. A Wild micrometer eyepiece was used in conjunction with this equipment to determine the projected area of the cell being measured. Two experiments involving the estimation of dry mass were performed; the first involved measurement of the nucleus only, and the second involved measurement of both nuclear and cytoplasmic dry masses.

In the first experiment, the cells to be used for dry mass estimation were fixed in two changes of fresh form-acetic-alcohol, washed in distilled water, and squashed onto a clean slide. The coverslip was removed after inversion over dry ice in the usual way, and the slides stored in 70 % alcohol until required. Photomicrographs were taken of suitable areas of the slide (those areas containing large numbers of cells, and with some readily recognisable clumps present), and

these photographs were subsequently used to identify particular cells, the code numbers of which were written directly onto the relevant photograph.

Immediately prior to examination, the slides were rinsed in distilled water, a No. 1 coverslip placed over the selected area, the rest of the slide blotted dry, and the coverslip ringed with Dunlop rubber solution. Phase changes introduced by the lymphocyte nuclei were recorded, and two nuclear diameters at right angles were measured. After all measurements were completed, the rubber solution was peeled off and the slide replaced in 70 % alcohol. Nucleic acids were extracted by immersion in 5 % trichloracetic acid at 90° C. for 90 minutes. At the end of this treatment the slides were washed twice in distilled water, and stored in 70 % alcohol until the nuclear dry masses could be measured again.

During the course of this experiment, only the 40 X objective was available, but a 100 X water-immersion objective became available for the next experiment.

In the second experiment, cells were fixed in 10 % neutral formalin, washed in distilled water, and placed on the slides just before examination. The coverslip was then applied in the manner described

above. No examination of the cellular content of nucleic acids was attempted, and as each cell was measured only once, no reference photographs were required.

2.11. Photomicrography.

The photomicrographs were all taken with a Wild M-20 microscope equipped with a Wild camera attachment, on the top of which was attached a Wild 35 mm. casette holder. Ilford Pan F film was used throughout. The films were developed in Agfa "Rodinal" compensating developer, and prints were made on Ilford "hard" or "extra hard" single weight glossy paper according to the degree of contrast required.

3. RESULTS

Section 1: General characteristics of culture.

3.1.1. Leucocytes of kangaroo peripheral blood.

The leucocytes found in the peripheral blood of *Macropus giganteus* were, in general, similar to those found in eutherian peripheral blood, and the various cell types were easily identifiable. These are shown in figures 1 - 5. About 2 % of the lymphocytes possessed one to six well-defined cytoplasmic granules similar to those seen in the cytoplasm of the basophile leucocytes (see figures 4, 6, and 7). Ponder, Yeager, and Charipper (1929), in the course of a study on the blood of ten species of marsupials, noted similar granules in the cytoplasm of large lymphocytes of *Macropus robustus*, but *M. giganteus* was not studied by them, and they did not observe similar granules in the lymphocytes of other species.

In the early stages of the study it seemed advisable to find out how the various leucocyte populations were affected during incubation with PHA, so

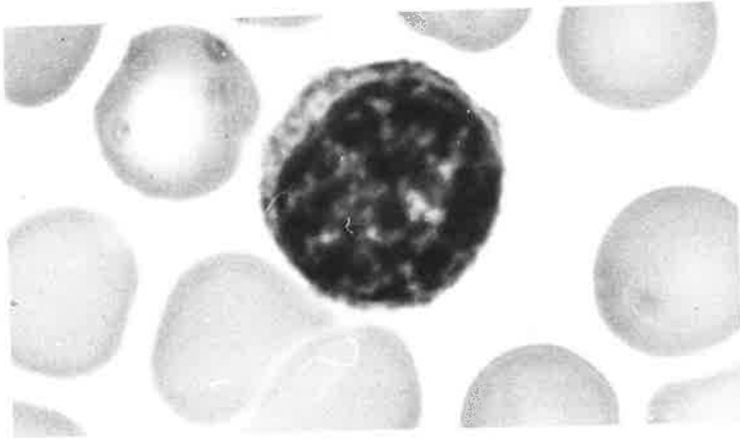

Figure 1. Typical small lymphocyte.

2900 X.

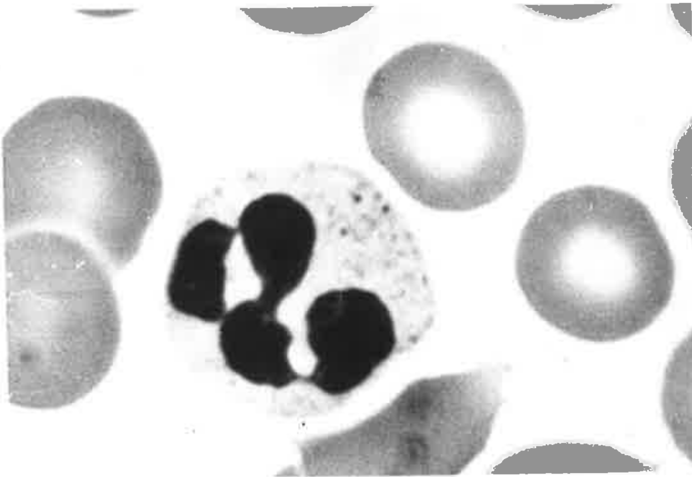

Figure 2. Neutrophile leucocyte. Differential counts showed that the proportion of neutrophils in the peripheral blood of the kangaroo was markedly lower than in human and other eutherian blood.

2900 X.

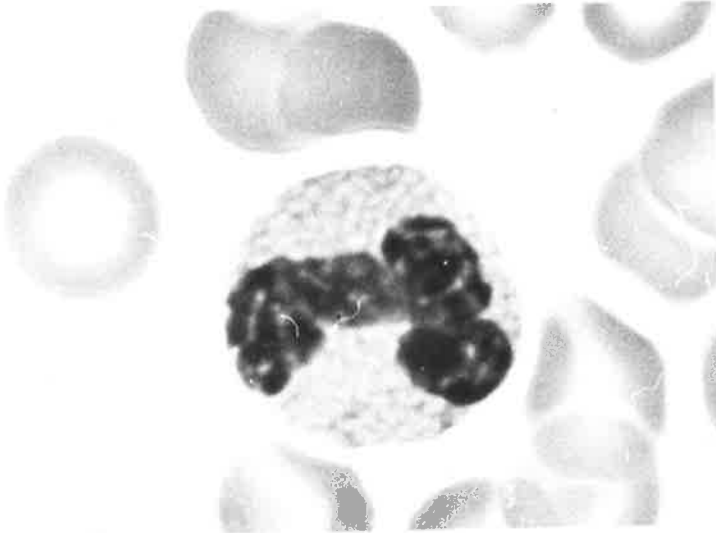

Figure 3. Eosinophile leucocyte. These made up about 6 % of the leucocytes of the peripheral blood. Apart from the lymphocytes, they were the only cell type able to survive prolonged culturing.

2900 X.

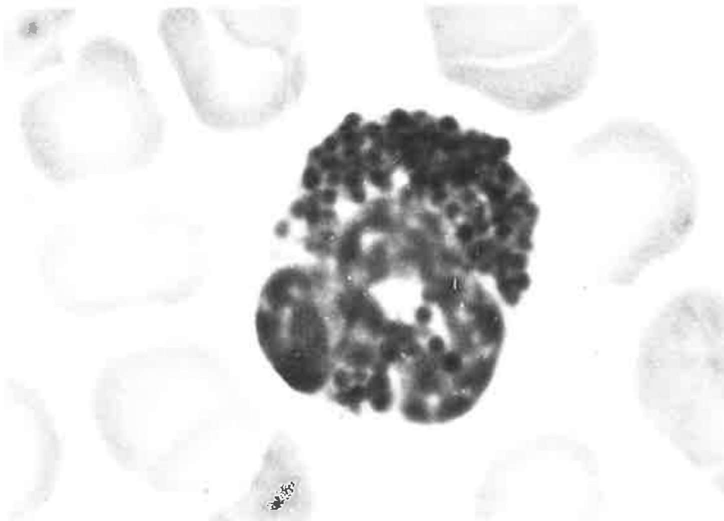

Figure 4. Basophile leucocyte. None were detected after the first hour of culture, although they were retained in separated plasma.

2900 X.

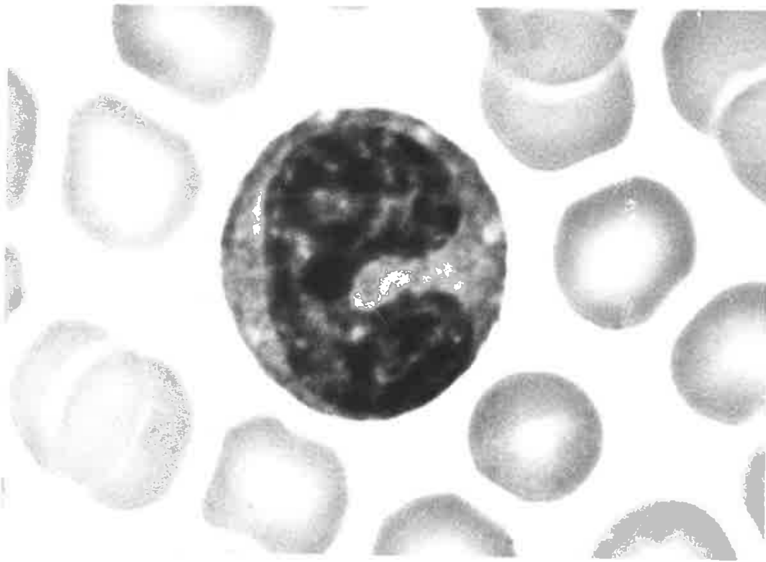

Figure 5. Monocyte. These appeared only in smears of whole blood, and apparently sedimented out during separation with dextran. They were difficult to distinguish from the larger lymphocytes, as apparently intermediate forms existed.

2900 X.

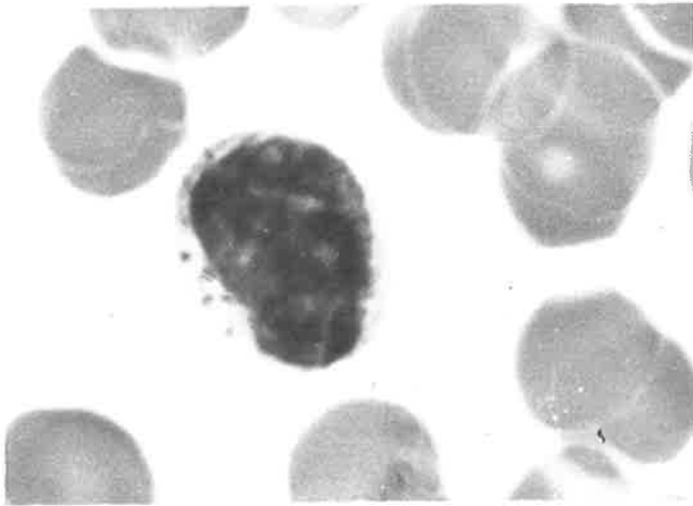

Figure 6. 2900 X.

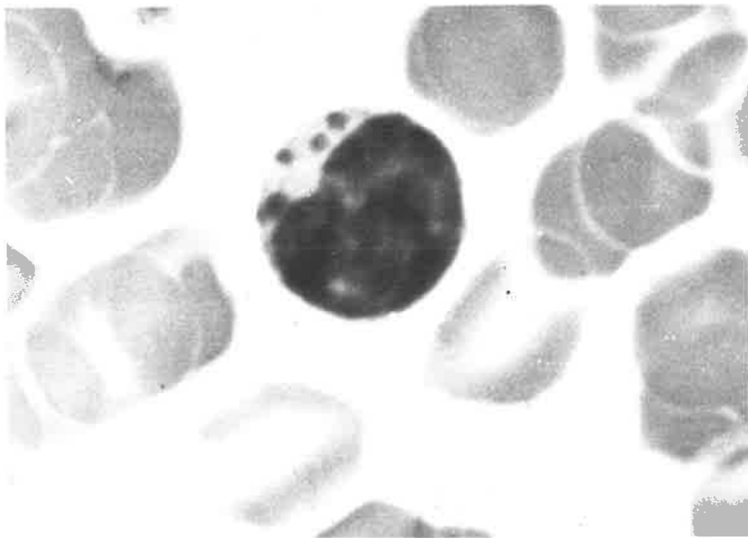

Figure 7. 2900 X.

These photographs show granules in the cytoplasm of lymphocytes. These granules were found only in the larger lymphocytes, and always opposite an indentation of the nucleus. They appeared similar in size and in staining characteristics to the granules found in the cytoplasm of basophile leucocytes.

that an estimate could be made of the proportions of lymphocytes and lymphocyte-derived cells at various times after the start of growth. Differential leucocyte counts were carried out for smears of whole blood, and for smears of leucocyte suspensions after separation with dextran. 1000 cells were counted in each case, and the results are given in table 4.

The high differential lymphocyte count is striking. In human blood the lymphocytes normally account for 20 - 25 % of the leucocyte population. Similarly high lymphocyte counts were obtained for a number of kangaroos throughout the study. The relatively high eosinophil count may reflect exposure to an otherwise undetected infection; however, Ponder and his co-workers recorded eosinophil counts ranging between 3 and 6 % in most cases.

The increase in the proportion of lymphocytes after separation with dextran is probably due to a low lymphocyte sedimentation rate. No buffy coat cells were taken. As all other leucocytes are larger than lymphocytes they would probably sediment more rapidly and thus remain in suspension for a shorter time than lymphocytes. This would result in a higher proportion of lymphocytes in the separated plasma.

TABLE 4

Differential counts of whole blood and separated leucocytes.

Cell type	Whole blood	Separated plasma
Lymphocytes	64.9 %	78.3 %
Neutrophile leucocytes	23.4 %	15.5 %
Eosinophile leucocytes	7.9 %	5.4 %
Basophile leucocytes	0.8 %	0.8 %
Monocytes	3.0 %	0.0 %

3.1.2. Changes in differential leucocyte counts in culture.

A culture prepared in autologous serum was divided into two equal lots; one lot was treated with 0.01 ml./ml. PHA immediately before incubation, and the other lot was incubated untreated. Each culture was sampled at intervals up to 48 hours after the commencement of incubation; the samples were smeared onto slides and air dried, dipped briefly into a 0.75 % solution (W/V) of formvar in chloroform so that cells would not float off differentially during staining, and stained in the usual way with Leishmann's stain. Differential leucocyte counts were made for 1000 cells in each sample, and the results are shown in figures 8 and 9.

Apart from the appearance of blastoid cells in the cultures treated with PHA, two other factors require comment.

(a). Neutrophile leucocytes degenerated more rapidly in PHA-treated cultures than in untreated cultures. This finding agrees with the results of Elves and Wilkinson (1962, 1963).

(b). Eosinophile leucocytes remained intact for most of the culture period, although numbers of degenerating eosinophils could be seen in the later

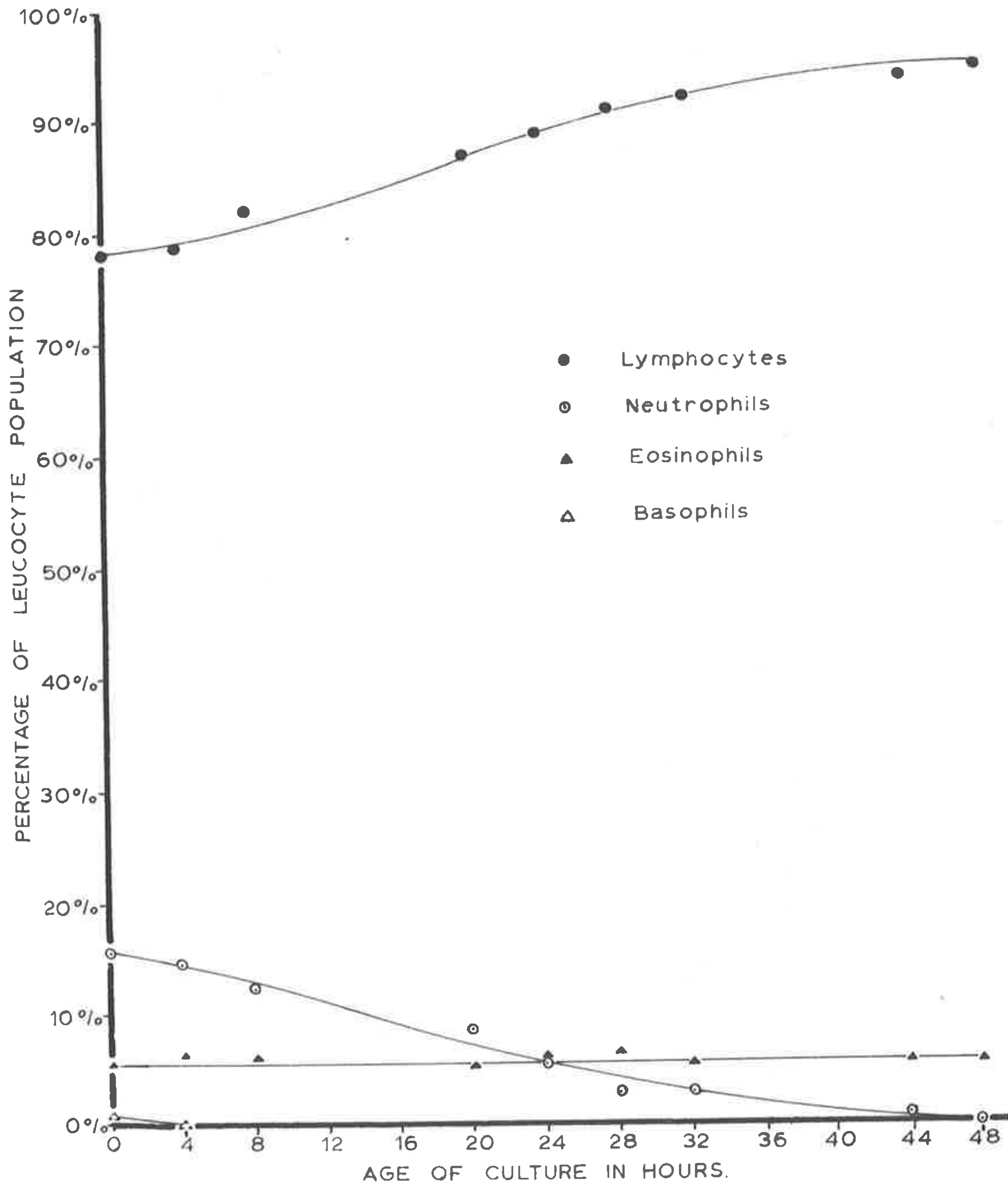

Figure 8.

Differential leucocyte count - untreated culture.

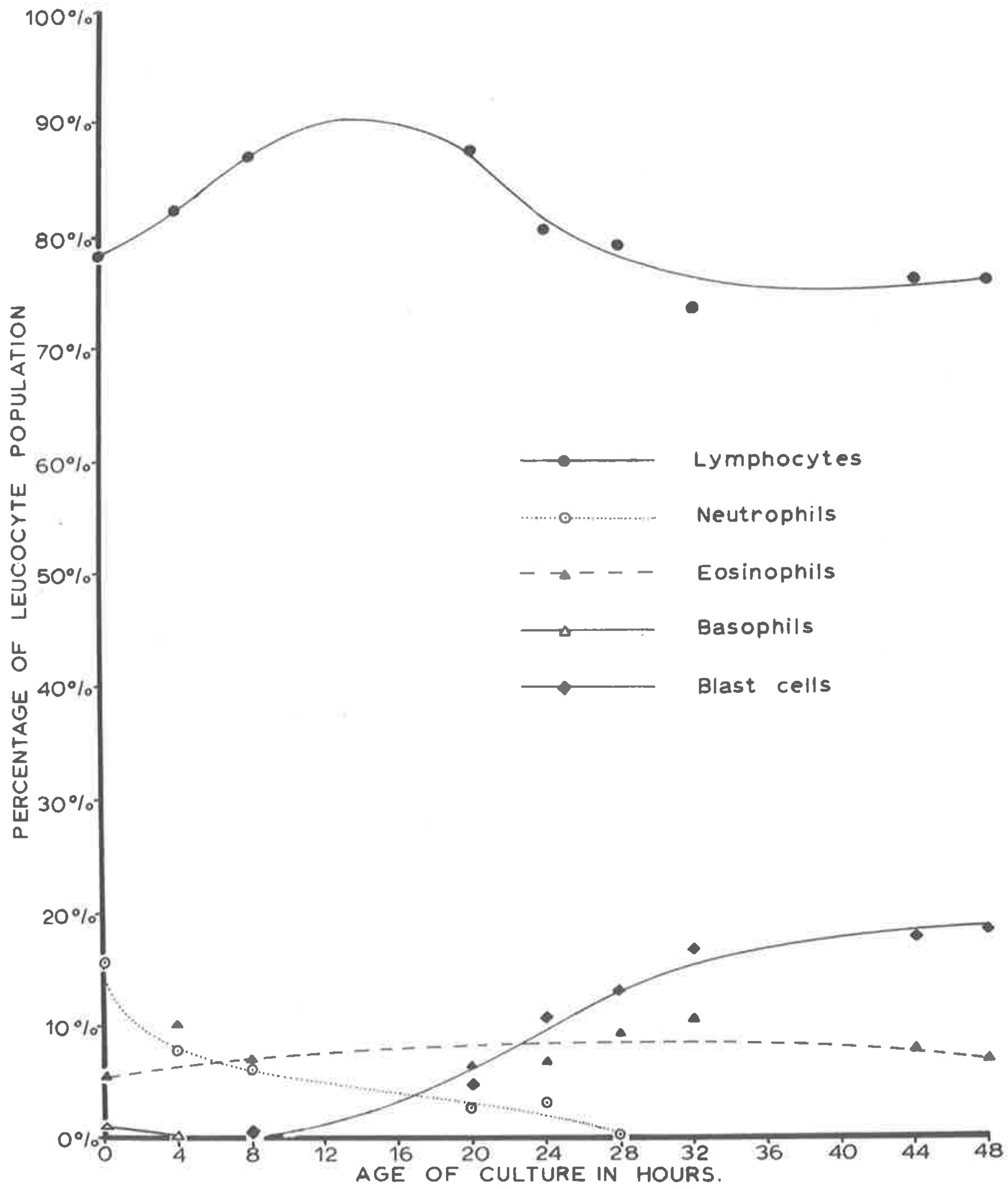

Figure 9.

Differential leucocyte count - PHA-treated cultures.

cultures. MacKinney, Stohlman, and Brecher (1962) showed that in human blood all neutrophils and eosinophils had disappeared after 24 hours of incubation. In cultures of kangaroo leucocytes treated with PHA, the eosinophils were invariably found only in the leucocyte clumps, and sometimes accounted for 30 % of the cells in a clump. This helps to explain the fluctuations in the eosinophil counts recorded in the PHA-treated cultures, as the distribution of eosinophils in these cultures was not random. The significance of these two characteristics of the eosinophil population is not known.

3.1.3. Commencement of DNA synthesis.

A series of four experiments was prepared to find the timing of the commencement of DNA synthesis. Each experiment was essentially similar, and involved the use of tritiated thymidine as a precursor for DNA. In each experiment, samples from a pooled culture were incubated with 0.1 μc./ml. thymidine-H^3 for periods of one hour, at intervals ranging from 9 - 10 hours to 24 - 25 hours after the addition of PHA. Autoradiographs were prepared in the usual way, and developed after exposure times ranging up to four weeks. The results of the four experiments, represented in figure 10

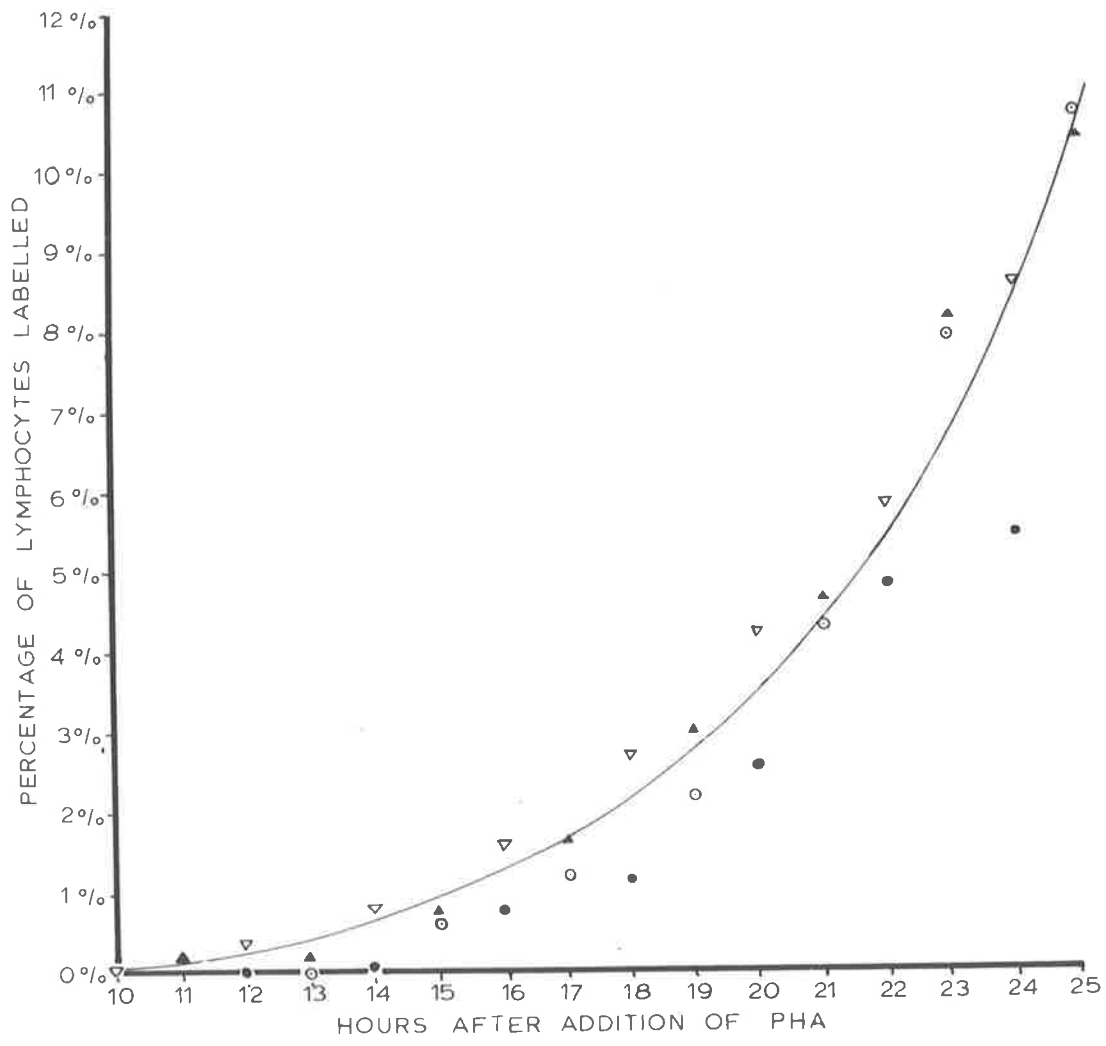

Figure 10.

Uptake of ^{3}H - Thymidine.

by four different symbols, were similar. Uptake of thymidine first occurs at about 12 hours and the proportion of labelled cells increases thereafter.

Various estimates of the timing of commencement of DNA synthesis in human lymphocyte cultures have been made. Cooper, Barkhan, and Hale (1961, 1963) gave 24 hours as the timing of the first significant increase in the rate of incorporation of thymidine-H^3; MacKinney, Stohlman, and Brecher (1962), Bender and Prescott (1962), and Killander and Rigler (1965) all confirm this figure. Cooper (1962) estimated that the onset of DNA synthesis occurred at 20 hours, whereas Lima-de-Faria, Reitalu, and Bergman (1961), and Michalowski (1963) place the event at thirty hours after the addition of PHA.

It is quite clear that DNA synthesis in the lymphocytes of the grey kangaroo commences much earlier than it does in human lymphocytes. Similarly, the first wave of mitoses appears to commence much earlier in PHA-treated cultures of kangaroo lymphocytes than it does in similar cultures of human lymphocytes. Bender and Prescott (1962) place this event at 42 - 48 hours for human lymphocytes, and Cooper, Barkhan, and Hale (1963) place it at 48 hours after the addition of PHA. During the present study, mitoses have been

seen as early as 28 hours after the addition of PHA, but significant numbers of mitoses were not normally seen before the 36th hour of culture. It is clear that both DNA synthesis and mitosis occur much earlier in cultures of kangaroo leucocytes than they do in similar cultures of human leucocytes, and, as will be shown, the cause can be traced to a more rapid response by the kangaroo lymphocytes to PHA in the early part of the G1 period, resulting in a marked reduction of the duration of this stage.

Section 2: Syntheses of DNA, histone, and chromosomal residual protein.

Bloch and Godman (1955 a, b) showed that histone and DNA levels ran parallel during the course of the cell cycle in rat liver, and suggested that, in effect, the material was being synthesised as nucleoprotein. However, Flamm and Birnstiel (1964) showed that when DNA replication was inhibited with 5-fluorodeoxyuridine, histone synthesis still occurred; this appears to indicate that the syntheses of DNA and histone are not closely linked.

There is a large amount of circumstantial evidence indicating that histones are involved, through the inhibition of RNA synthesis, in the regulation of protein synthesis (Huang and Bonner, 1962; Allfrey, Littau, and Mirsky, 1963a, b; Billen and Hnilica, 1963; von Hahn, 1965).

These two aspects assume particular importance in the case of lymphoid tissue, the function of which is to produce a wide variety of antibody molecules in response to antigenic stimuli. In view of these considerations, a series of experiments was designed to determine the relative timing of DNA and histone syntheses. Five of the experiments used autoradio-

graphic techniques; the sixth made use of micro-spectrophotometry.

The first experiment involved the use of tritiated thymidine and tritiated lysine to determine the timing of the start of DNA synthesis and the start of histone synthesis respectively. A culture was prepared in autologous serum, treated with 0.01 ml./ml. PHA, and incubated at 37° C. for twelve hours. At the end of this time, two four ml. samples were incubated with 0.1 μc./ml. thymidine-H^3 and 0.125 μc./ml. lysine-H^3 respectively for one hour; they were then spun down and fixed in the usual way. At two-hour intervals from 12 hours up to 24 hours after the addition of PHA, further samples were treated in the same way. Autoradiographs of each treatment were prepared and exposed for up to four weeks before development, and the percentage of cells labelled was estimated from counts of 500 cells on each slide. The results of this experiment are shown in figure 11.

A further experiment, with slightly different timing, was carried out to confirm these results. The first samples in this case were labelled 9 hours after the addition of PHA, and exposed to label for one hour before being fixed in the usual way.

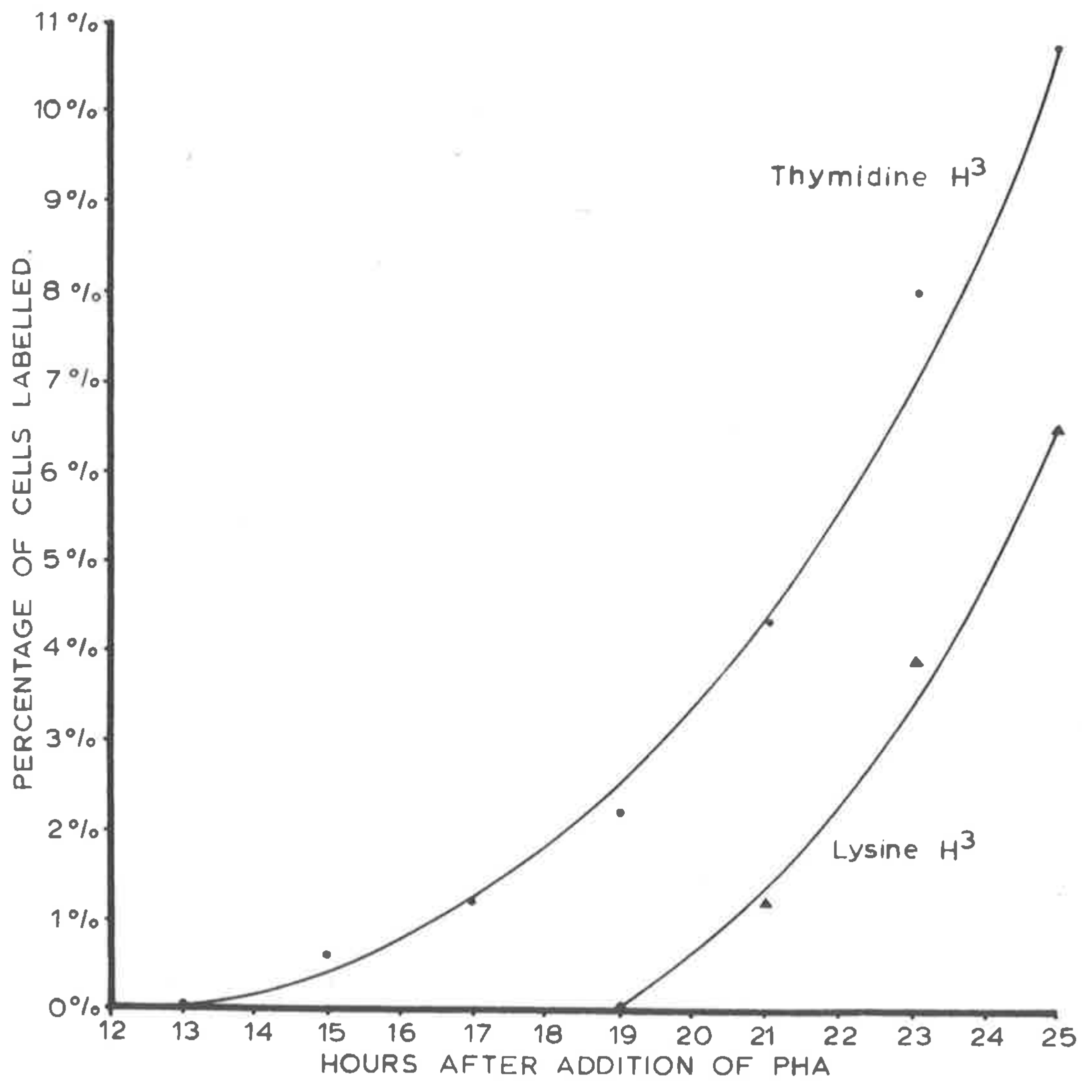

Figure 11.

Relative timing of commencements of DNA and histone syntheses.

Further samples were treated in the same way at two-hour intervals up to 24 hours after treatment with PHA. Autoradiographs were prepared and exposed for periods ranging up to four weeks before development, and the percentage of cells labelled was estimated from counts of 500 cells in each treatment. The results of this experiment are shown in figure 12.

Superficially, it seemed fairly clear from both these experiments that the onset of histone synthesis lagged behind the onset of DNA synthesis by some three to four hours. However, it is possible to interpret these figures in other ways.

(a). Lysine-rich histone may well be dissolved out of the cell during fixation, and the label seen in the lysine-labelled cultures may be due to the incorporation of lysine-H^3 into non-histone protein. Cave (1966) has shown that tritiated lysine is incorporated into chromosomal residual protein; the implications of this finding are discussed fully on page 86.

(b). Even though only a portion of the lysine-rich histone may have dissolved out during fixation, this may have been sufficient in the early stages of lysine uptake to render a lightly labelled cell effectively unlabelled, so that the apparent lag

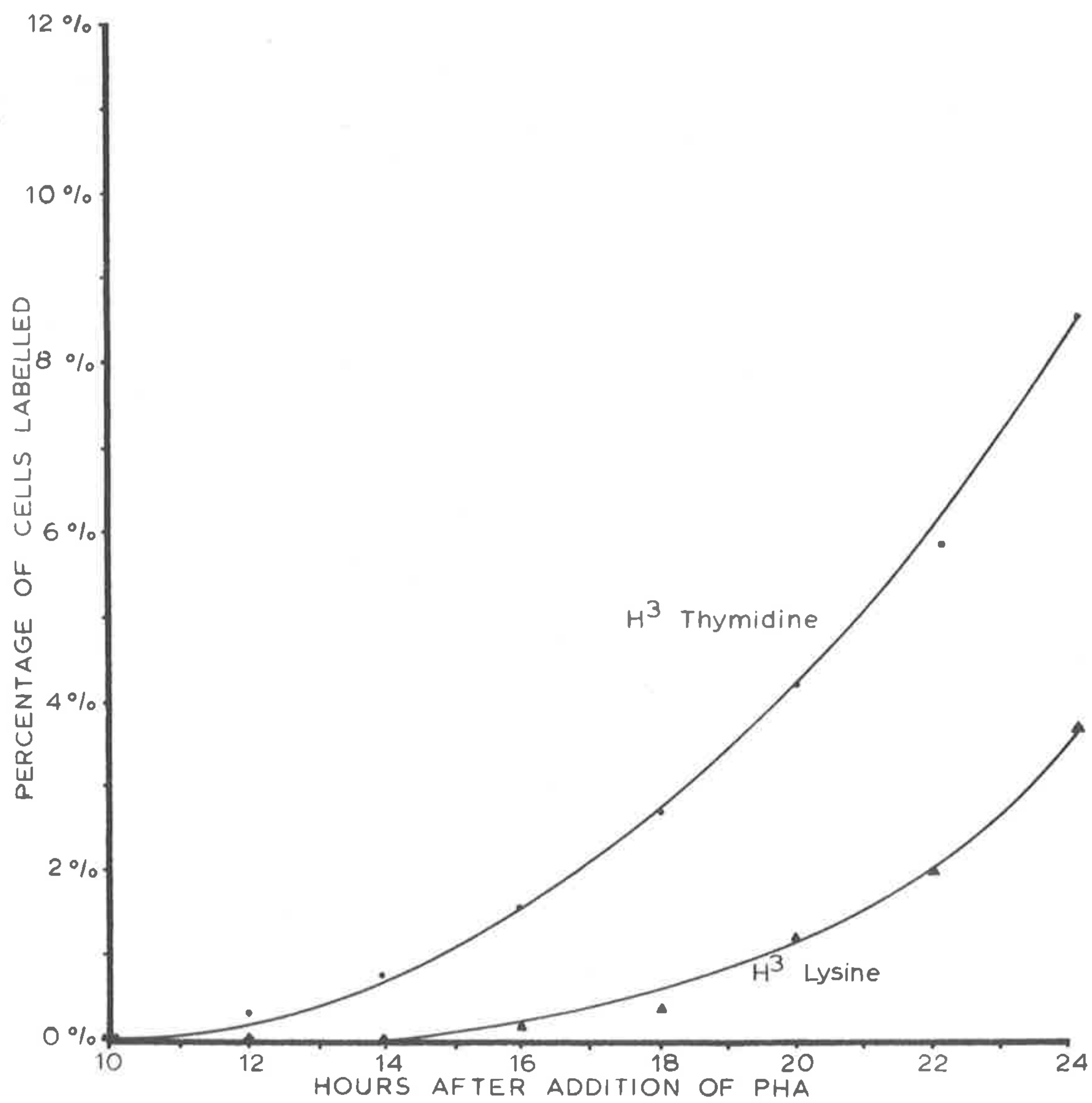

Figure 12.

Relative timing of commencements of DNA & histone syntheses.

shown in figures 11 and 12 could be an artifact.

In the light of these possibilities, a different approach was thought necessary, and an attempt was made to estimate the relative quantities of histone and DNA by microspectrophotometry, so that the results obtained from the autoradiographs could be verified.

A culture was prepared in autologous serum, and two hours after the addition of PHA a large sample was fixed in 10 % neutral formalin. This sample was used as a standard. Starting 13.5 hours after the addition of PHA, samples were fixed in 10 % neutral formalin every two hours up to 25.5 hours after the initiation of cell growth. Cells from each sample were squashed onto one end of an appropriately marked slide, cells from the standard sample were squashed onto the other end, and the cells were Feulgen-stained or stained with alkaline fast green according to the staining schedules on pages 38 and 41. Cells in each treatment were measured for stain density in comparison with the cells from the standard sample on the same slide. The results of this experiment are given in table 5.

Several aspects of this table need discussion.

(a). The variation in stain-density values of the cells in the standard sample is due to variation

TABLE 5.

1. DNA estimation.

Age of culture (hours)		Mean (Arbitrary units)	Standard error.	Difference in means.	t.
13.5	Expt.	14.10	2.98	0.20	0.29 ns.
	Control	13.90	2.12		
15.5	Expt.	31.83	3.50	1.47	1.75 ns.
	Control	30.36	2.85		
17.5	Expt.	34.03	4.02	4.33	4.10 xxx
	Control	29.70	4.03		
19.5	Expt.	36.20	3.85	5.37	5.73 xxx
	Control	30.83	3.26		
21.5	Expt.	37.40	4.83	9.37	8.96 xxx
	Control	28.03	2.90		
23.5	Expt.	31.80	5.11	9.24	8.25 xxx
	Control	22.56	3.21		
25.5	Expt.	42.40	3.56	11.83	14.45 xxx
	Control	30.57	2.60		

2. Histone estimation.

Age of culture (hours)		Mean (Arbitrary units)	Standard error.	Difference in means.	t.
13.5	Expt.	15.67	3.35	0.27	0.25 ns.
	Control	15.40	4.64		
15.5	Expt.	14.93	2.53	1.34	1.35 ns.
	Control	16.27	4.69		
17.5	Expt.	15.26	3.48	0.07	0.06 ns.
	Control	15.33	4.65		
19.5	Expt.	21.20	5.34	0.27	0.21 ns.
	Control	20.93	4.52		
21.5	Expt.	19.97	4.80	2.00	1.79 ns.
	Control	17.97	3.64		
23.5	Expt.	19.50	4.41	3.30	3.38 xxx
	Control	16.20	2.87		
25.5	Expt.	26.90	6.22	6.90	5.34 xxx
	Control	20.00	3.11		

in the staining procedure. However, as each experimental sample was compared only with the standard sample on the same slide, and as the cells were stained on the slide, these variations are irrelevant; both the experimental sample and the standard sample on a slide were subjected to exactly the same staining conditions.

(b). The standard errors in the DNA control series are consistently lower than the standard errors in the corresponding histone series. This does not necessarily reflect a greater variation in cellular histone values than there is in cellular DNA values; the greater variation could be due to a lower specificity of the alkaline fast green reaction in comparison with the highly specific Feulgen reaction.

(c). Even if DNA and histone syntheses were synchronous, a significant difference could still be expected earlier in the Feulgen-stained cultures than in those stained with alkaline fast green. This is due to the higher mean values and lower standard errors recorded with Feulgen-stained cells, and can be illustrated in the following way. The mean control value overall for the Feulgen-stained cells is approximately 27 arbitrary units, and the overall mean standard error is approximately 3.0 units. The

corresponding figures for the cells stained with fast green are 17.4 and 4.0 respectively. If it is assumed that the S period lasts 10 hours, then the mean histone values will increase by 1.74 units per hour, whereas DNA values will increase by 2.7 units per hour. Thus, if DNA and histone syntheses are synchronous, and each commences at 0 hours, a significant difference will be recorded between experimental and control DNA values after one hour, and between the corresponding histone values after two hours, thus implying a non-existent one hour lag between the syntheses of DNA and histone. However, it is impossible to account for the figures in table 5 in this way. If it is assumed that the quantities of DNA and histone must both double during the cell cycle, then it is clear that at 19.5 hours after the addition of PHA, over 17 % of DNA synthesis is complete, whereas histone synthesis at this stage is barely detectable. Similarly, at 21.5 hours after the addition of PHA 33.4 % of DNA synthesis is complete, against 11 % of the synthesis of histone. It seems quite clear that DNA and histone syntheses are not synchronous; this finding is reinforced to some extent by the results obtained from the two preceding experiments.

Nevertheless, these three experiments gave a rather

incomplete picture of the relationship between the synthesis of DNA and the synthesis of histone, and so two further experiments were designed. In the first of these experiments, a culture of leucocytes drawn from a female kangaroo was prepared with foetal calf serum, treated with PHA, and incubated at 37° C. for 60 hours. The culture was then divided into two equal lots, one of which was labelled with tritiated thymidine at a concentration of 0.1 μc./ml., and the other with tritiated lysine at the same concentration. After one hour, a sample was taken from each treatment, and treated with 0.1 ml./ml. 0.002 % colchicine. Samples were exposed to colchicine for two hours prior to fixation, and were extracted sequentially from the parent cultures at hourly intervals ranging from one to five hours after the addition of isotope; thus cells were fixed, after two hours' exposure to colchicine, at hourly intervals ranging from three to seven hours after the addition of isotope. The leucocyte suspensions were fixed in the usual way. Autoradiographs were prepared, and developed after exposure times ranging from three days, in the case of the late-harvested thymidine-treated cultures, to eight months, in the case of the early lysine-treated cultures. The percentage of labelled mitotic cells in each treatment

was estimated, and the results of this experiment are shown in figure 13. (This experiment will be discussed in conjunction with the one immediately following).
The next experiment, using arginine-H^3 as the label for histone, was designed to give a more comprehensive picture of histone synthesis immediately prior to mitosis. A culture of leucocytes drawn from a female kangaroo was prepared with foetal calf serum, treated with PHA, and incubated for 60 hours at 37° C. It was then divided into two equal lots, one of which was labelled with arginine-H^3 at a concentration of 0.5 µc./ml., and the other with thymidine-H^3 at a concentration of 0.1 µc./ml. Each culture was sampled sequentially, at hourly intervals, up to seven hours after addition of the isotopes. The samples were fixed and autoradiographs prepared in the usual way, and the autoradiographs exposed for periods ranging up to seven days. Results are shown in figure 14.

Upon examination of the autoradiographs from both experiments, it was found that all the dividing cells in the arginine- and lysine-labelled cultures were labelled, even in the arginine-treated culture fixed after only one hour of exposure to label. (In the corresponding thymidine-treated culture, only 3 % of the dividing cells were labelled.) This clearly indicates that cells in the G2 period, which are no longer synthesising DNA, still continue to incorporate lysine and arginine. The

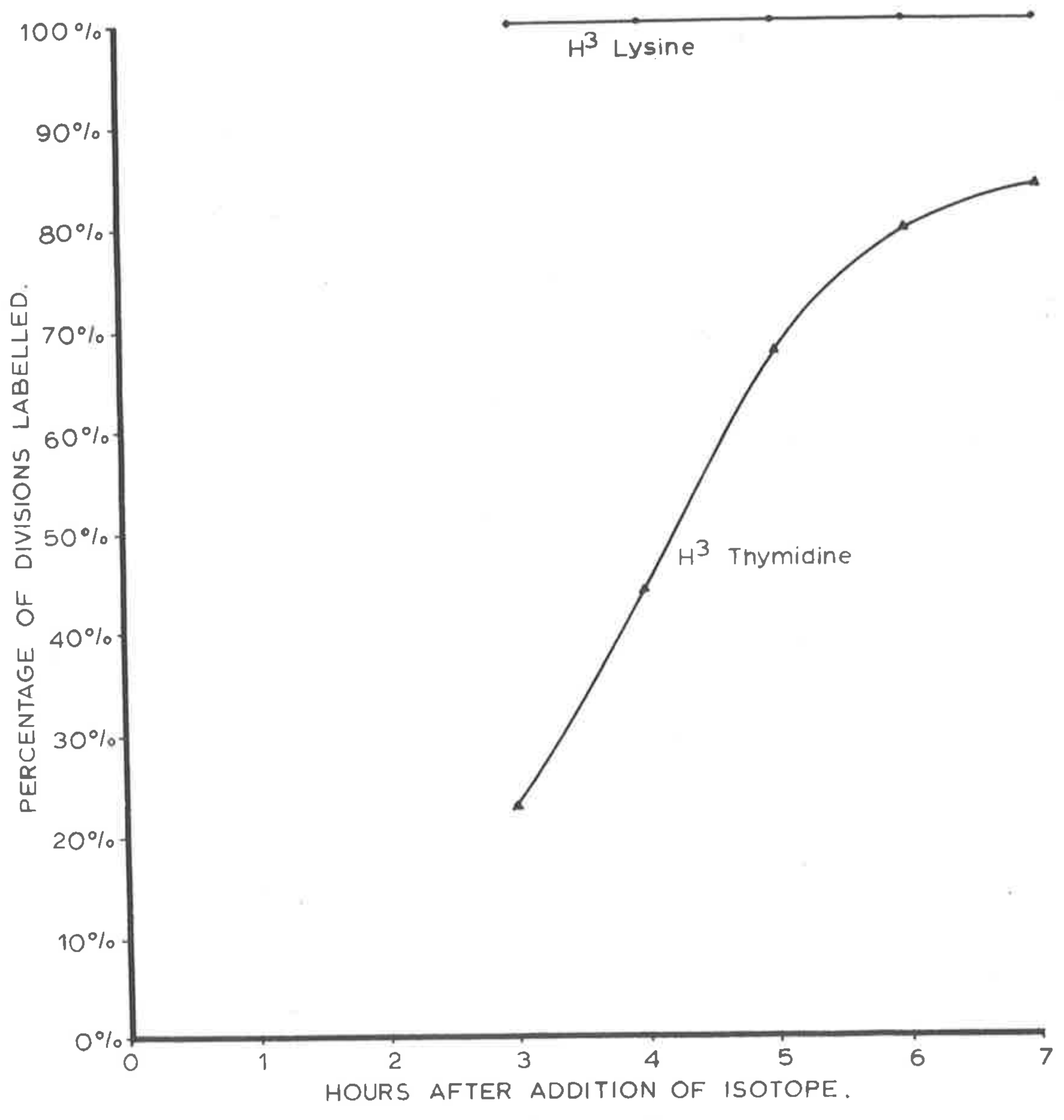

Figure 13

DNA and histone synthesis at end of interphase.

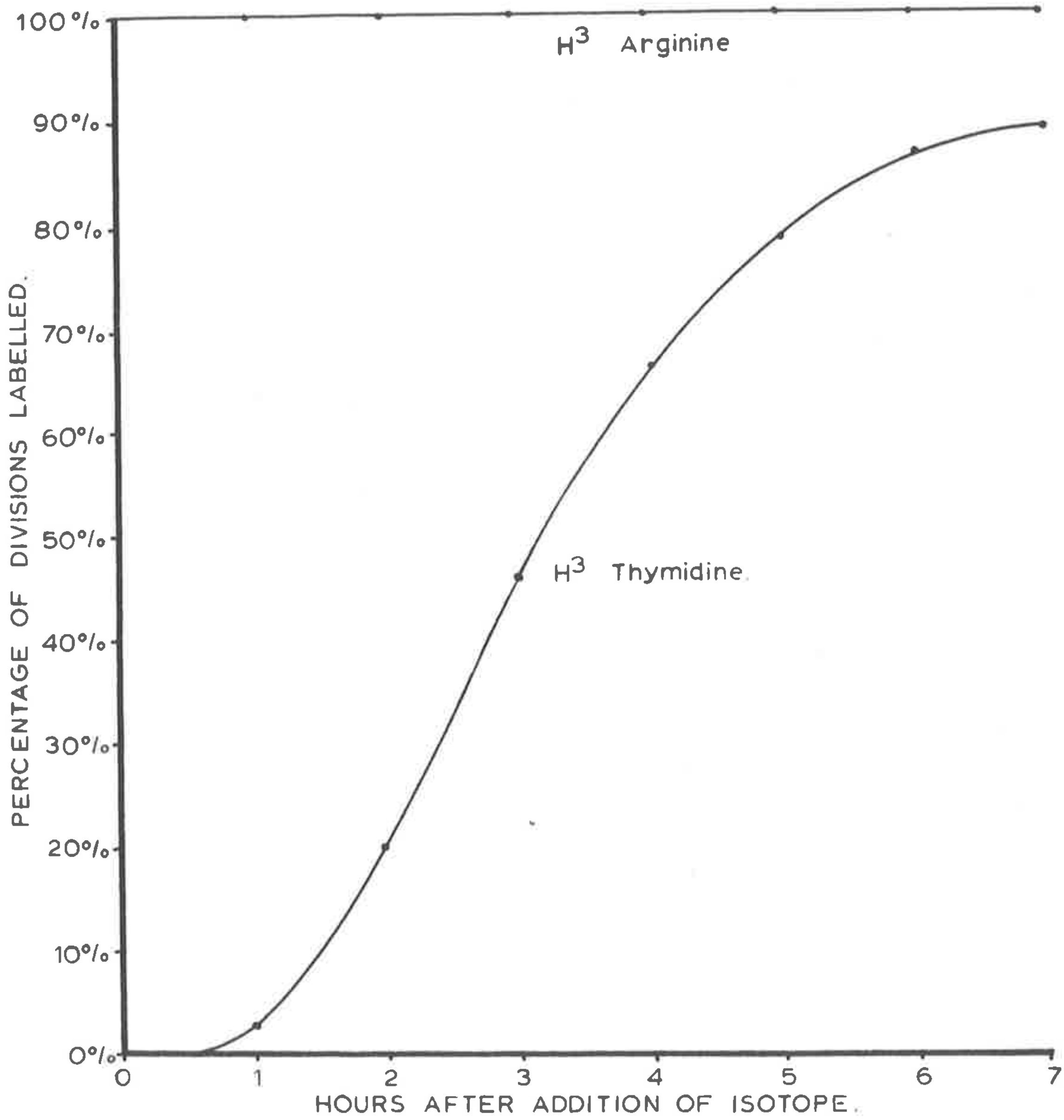

Figure 14.

DNA and histone synthesis at end of interphase.

problem of accounting for the nature of the protein into which these two amino acids are incorporated is fully discussed later, on page 82. In the meantime, some analysis of the results of these two experiments is necessary.

The duration of the G2 period in the thymidine-labelled cells is interesting. Bender and Prescott (1962) recorded a minimum of 2 hours and a maximum of 6 hours for the G2 period in human lymphocytes, and these results were verified by others. Cooper, Barkhan, and Hale (1963) recorded a minimum G2 period of 2 hours; Hayhoe and Quaglino (1965) gave a figure of three hours for G2. However, the minimum duration of the G2 period in cultures of kangaroo lymphocytes is about one hour (figure 15) and the maximum duration must be more than 7 hours (figure 16).

In the case of the colchicine-treated cells in the first of the two experiments under discussion, the addition of colchicine extended the actual G2 period by one hour. The presence of colchicine must tend to give a higher proportion of unlabelled metaphases, as any unlabelled cells caught in G2 by colchicine would be arrested at metaphase. If a constant rate of entry of cells into mitosis is assumed, then the mean age of the metaphases seen would be one hour, as the colchicine was added two hours before harvesting. This applies equally to

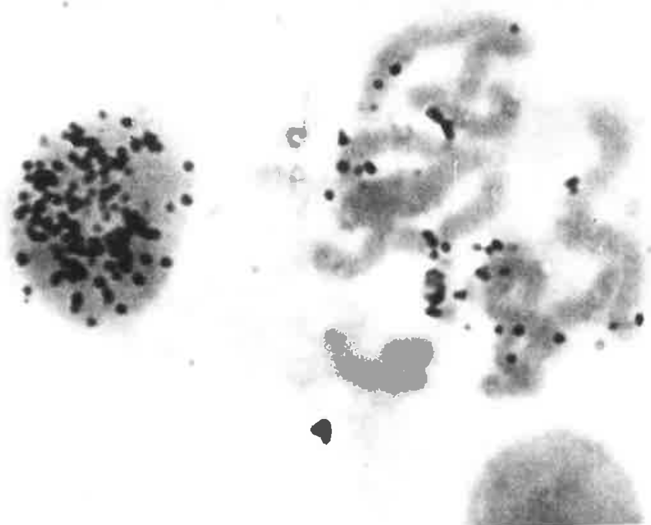

Figure 15. Thymidine-H^3 -labelled metaphase, one hour after addition of isotope. This indicates that the minimum G2 period must be less than one hour. The localisation of label probably represents a late-replicating X chromosome.

2700 X.

Figure 16. Unlabelled metaphase, after 7 hours' exposure to thymidine-H^3.

2700 X.

cultures with long or short term labelling. The increase in the incidence of unlabelled metaphases accounts for the difference in the estimated durations of the G2 period derived from this and the subsequent experiments. (See figures 13 and 14, pages 72 and 73).

The cultures used in both the experiments under discussion were obtained from female kangaroos, and some divisions with late-replicating X chromosomes were seen in cultures labelled with thymidine-H^3. (See figure 17). However, despite a careful search, no corresponding late-replicating X chromosomes were seen in the lysine-labelled or arginine-labelled divisions.

Upon further examination of the arginine-labelled culture fixed after one hour's exposure to the isotope, it was found that the later stages of mitosis had, in general, less label than the earlier stages, which themselves showed less label than did the S or G2 cells in the culture. (See figures 18, 18a, and 19). Dot counts were taken over 60 cells for each stage of mitosis, and the results of this aspect of the experiment are shown in table 6. Despite the low mean and relatively high standard deviation of the dot counts over the telophase cells, no unlabelled telophases were found. There may be two different explanations for this.

(a). There may be little variation in the

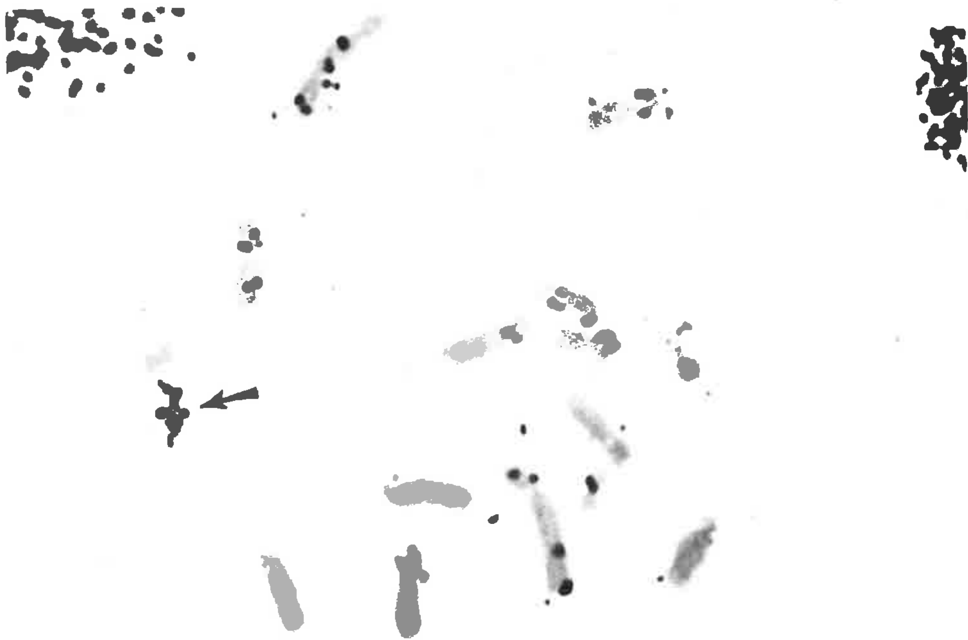

Figure 17. Metaphase spread labelled with tritiated thymidine, with a late-replicating X chromosome (arrowed). This pattern of chromosome replication was seen only in thymidine-labelled cultures.

2700 X.

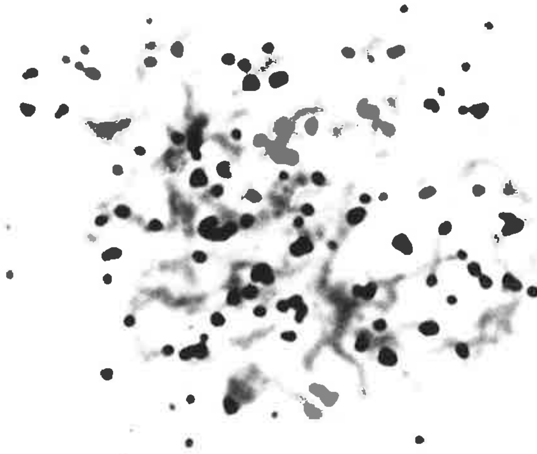

Figure 18. Arginine-H^3-labelled prophase, one hour after addition of isotope. Labelling is nearly as dense as that in late interphase cells, and the greater part of it lies over the chromosomes.

2700 X.

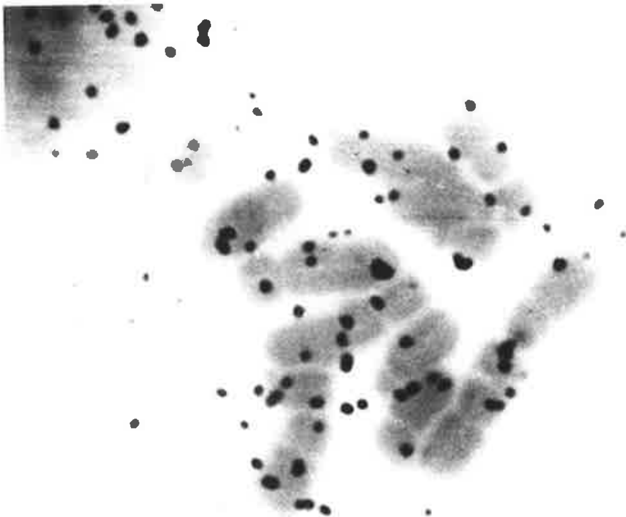

Figure 18a. Arginine-H^3-labelled metaphase, one hour after addition of isotope. Labelling is less dense than in fig. 18, indicating that the cell has been incorporating arginine for a shorter time.

2700 X.

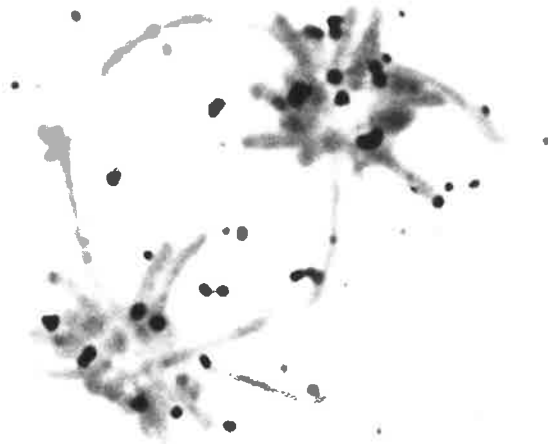

Figure 19. Arginine-H^3-labelled anaphase, one hour after addition of isotope. Some non-chromosomal label is present.

2700 X.

TABLE 6.

Dot counts over mitotic stages in 1-hour arginine-labelled culture.

Stage	Late S & G2	Prophase	Metaphase	Anaphase	Telo-phase
Mean dot count	100.1	80.0	50.4	32.0	10.7
Standard error	13.0	12.8	11.2	10.3	6.1

duration of mitosis in individual cells, and the interval between the cessation of incorporation of arginine and the beginning of telophase must be less than one hour.

(b). Although most of the uptake of arginine may cease at or near the beginning of prophase, label incorporation may continue at a very slow rate through mitosis. Prescott and Bender (1962) found that the incorporation of histidine-H^3 into HeLa cells was severely curtailed, but not stopped, during mitosis. This explanation seemed more likely than the preceding one, but it raised important problems. It seemed very unlikely that histone synthesis would continue throughout mitosis; it is much more likely that the label seen over telophase cells after one hour's exposure to tritiated arginine reflected the synthesis of chromosomal residual protein rather than the synthesis of histone. Thus a critical examination of the destination of labelled arginine and labelled lysine incorporated into the cell became vitally important.

During inspection of the lysine-labelled and arginine-labelled divisions obtained from the last two experiments, it could be seen that labelling lay largely over the chromosomes, with some label being distributed over other parts of the cell. Radioactive

emissions from cells labelled with tritiated lysine or tritiated arginine may emanate from one or more of the following sources.

(a). Cytoplasmic or nuclear protein, not directly associated with the chromosomes, synthesised by the cell during the labelling period. This certainly accounts for some of the label seen, but does not account for the greater part of it. Typically, only a small part of the label over an interphase cell lies over the cytoplasm; the greater part of it lies over the nucleus. (See figures 20 and 21). Nuclear protein labelled with tritiated arginine or tritiated lysine need not necessarily be directly associated with the chromosomes, but may fulfil other functions instead, such as forming part of the mitotic apparatus. However, the greater part of the label in dividing cells lies directly over the chromosomes. (See figures 18 and 18a, page 78; figure 19, page 79; figure 22, page 85).

(b). The labelled lysine and arginine may be incorporated into basic protein associated with nucleolar RNA, which is distributed over the chromosomes following the dissolution of the nucleolus during prophase (Kusanagi, 1964 a, b). If this was the case, a concentration of label over the nucleoli of interphase

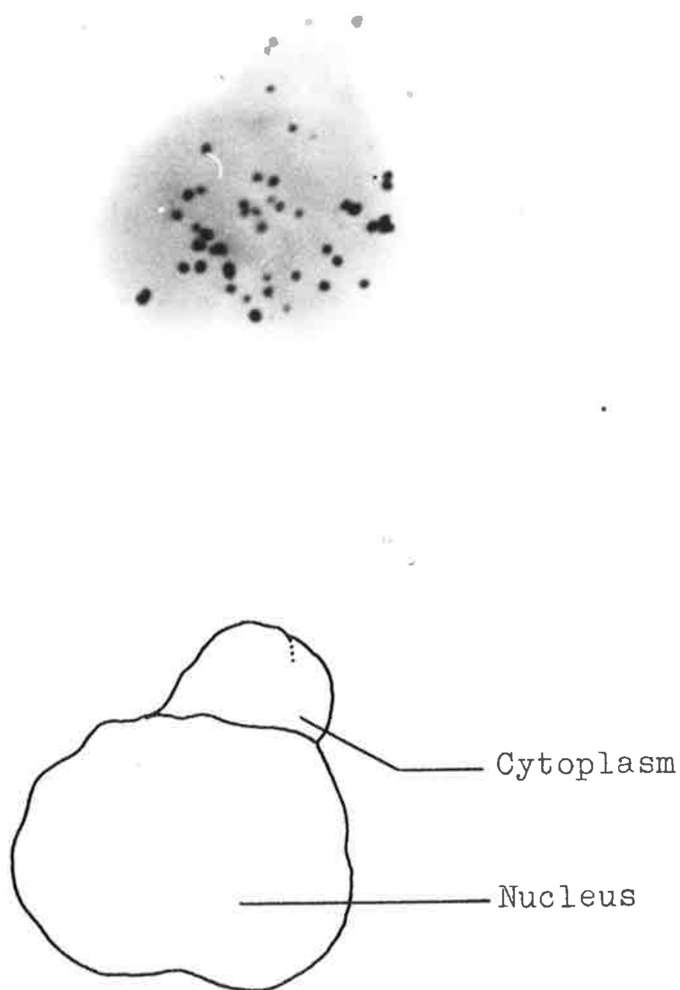

Figure 20. Arginine-H^3-labelled interphase cell. The label is largely nuclear and is not localised.

2000 X.

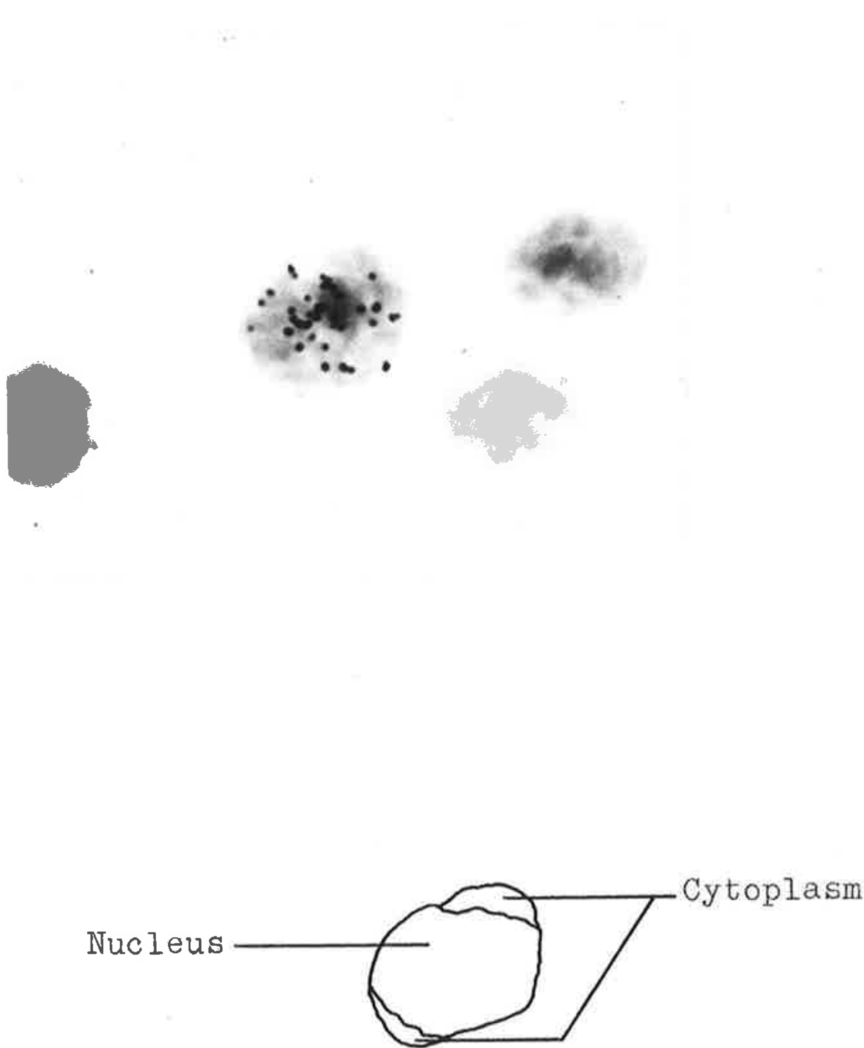

Figure 21. Lysine-H^3-labelled interphase cell, 20 hours after the addition of PHA to the culture. The cell is rather larger than the unlabelled lymphocytes in the photograph. No cytoplasmic label is present.

2000 X.

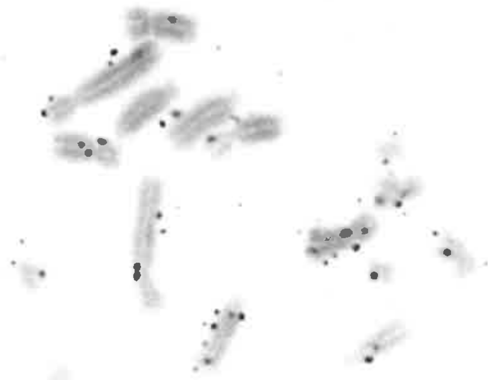

Figure 22. Lysine-H^3-labelled metaphase, three hours after addition of isotope. The greater part of the label lies over the chromosomes.

2000 X.

cells could be expected. No such concentration was seen in any cell.

(c). The label may be incorporated into histone in the nucleohistone complex. Even though histone does tend to dissolve out of the cell during fixation, there is still an appreciable amount left in the nucleus, as can be shown by microspectrophotometry of cells stained with alkaline fast green. If the label is incorporated into histone, then the label should be evenly distributed over the whole of the nucleus of a cell in the S or G2 stages, confined to the chromosomes of a cell at any stage of mitosis (disregarding any portion incorporated into non-histone protein), and absent over cells still in the G1 period. This is in fact the case, except that the label over the chromosomes is not quite so specific as it is in cultures labelled with tritiated thymidine. This is probably due to the limitations imposed by fixation in acetic/alcohol.

(d). After these experiments were completed, Cave (1966) showed that tritiated lysine was incorporated into chromosomal residual protein. This meant that information gained from autoradiographic studies of histone metabolism could not be relied on, as it was impossible to tell whether the radioactive emissions registered in the autoradiographs had emanated from

histone or chromosomal residual protein - the labelling patterns of each are identical. At this point, the only experiment clearly demonstrating asynchrony in DNA and histone syntheses was the one involving microspectrophotometry. To clear up this point, one further experiment was carried out.

A leucocyte culture was prepared with foetal calf serum, and incubated with PHA for 60 hours. It was then divided into two lots. 0.1 μc./ml. thymidine-H^3 was added to the first lot, and 1.0 μc./ml. arginine-H^3 was added to the second lot. At the end of one hour, the thymidine-labelled culture was fixed in the usual way in acetic/alcohol, and the arginine-labelled culture was divided into two equal parts. One part was fixed in acetic/alcohol, and the other was fixed in form-acetic-alcohol, which fixes histone satisfactorily (De, 1961) and is suitable for autoradiography (Darlington and La Cour, 1962). The arginine-labelled acetic/alcohol-fixed cells were then subjected to four changes of fixative, and all cultures were left overnight. This ensured that all histone was dissolved out of the acetic/alcohol-fixed cells. Autoradiographs were prepared of each culture in the usual way, and developed after a standard exposure time of 45 hours. This ensured that dot counts over labelled

cells could be regarded as a measure of the amount of label in the cell.

The cells labelled with tritiated thymidine were examined first. Nuclear diameters in two directions at right angles were measured for fifty labelled cells, and the dot count over each labelled cell was recorded. The dot counts were then plotted against the mean nuclear diameters, and the results are shown in figure 23.

No labelled cells with a mean nuclear diameter of less than 14 μ were seen. For cells with mean nuclear diameters between 14 μ and 20 μ, the amount of label present was, in general, directly proportional to the diameter of the cell - that is, the larger the cell, the higher the rate of incorporation of tritiated thymidine, and hence the higher the rate of DNA synthesis. The rather broad scatter of readings over this range probably reflects the complexity of the labelling pattern over the chromosomes throughout the S period. When the mean nuclear diameter of the cell becomes greater than 20 μ, the rate of thymidine incorporation drops markedly, and it appears that cells with a nuclear diameter of 20 μ or greater are either on the verge of, or else actually in, the G2 period. Thus cells with a nuclear diameter of less than 14 μ

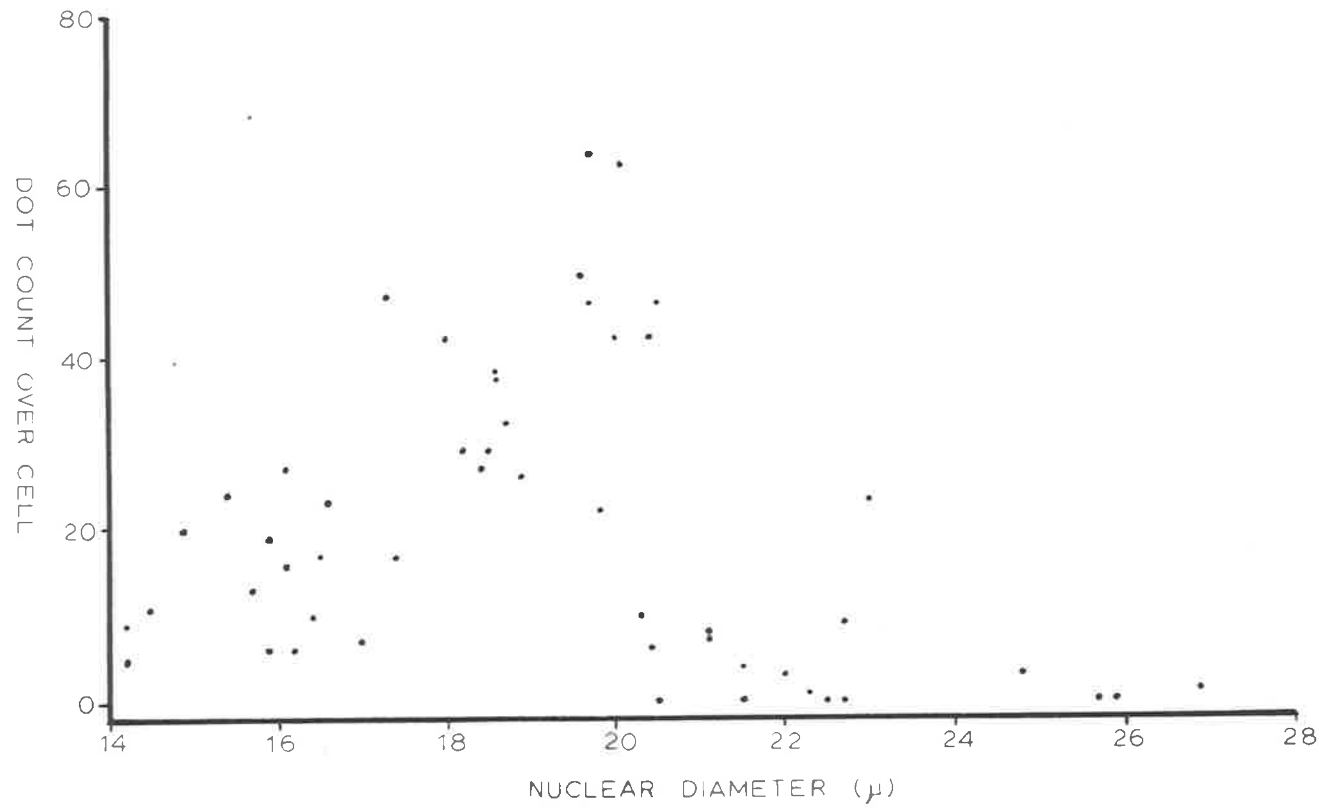

Figure 23

Rate of synthesis of DNA plotted against nuclear diameter

will be in the G1 period, cells with nuclear diameters between 14 μ and 20 μ will be in the S period, and cells with nuclear diameters greater than 20 μ will be in the G2 period of the cell cycle.

Autoradiographs of cells labelled with arginine-H^3 and fixed in acetic alcohol were examined next. As the cells are squashed during the preparation of autoradiographs, the size-distributions of cells in the thymidine-labelled and lysine-labelled cultures were compared. There was no significant difference in cell size between the two samples, each of 100 cells, taken from the two treatments. Once again, mean nuclear diameters were measured in conjunction with dot counts over each cell. The results are shown in figure 24, and statistical analyses of the data obtained from this and the subsequent treatment are given on page 169.

No labelled cells with a mean nuclear diameter of less than 14.4 μ were seen. For cells with nuclear diameters greater than this, the rate of labelling increased in direct proportion to the nuclear diameter of the cell, and this still held true for cells with a nuclear diameter greater than 20 μ (that is, cells in G2.) This labelling represents synthesis of chromosomal residual protein only (Cave, 1966), and no labelled histone would be present in the cell. Thus it appears that the synthesis of chromosomal residual protein

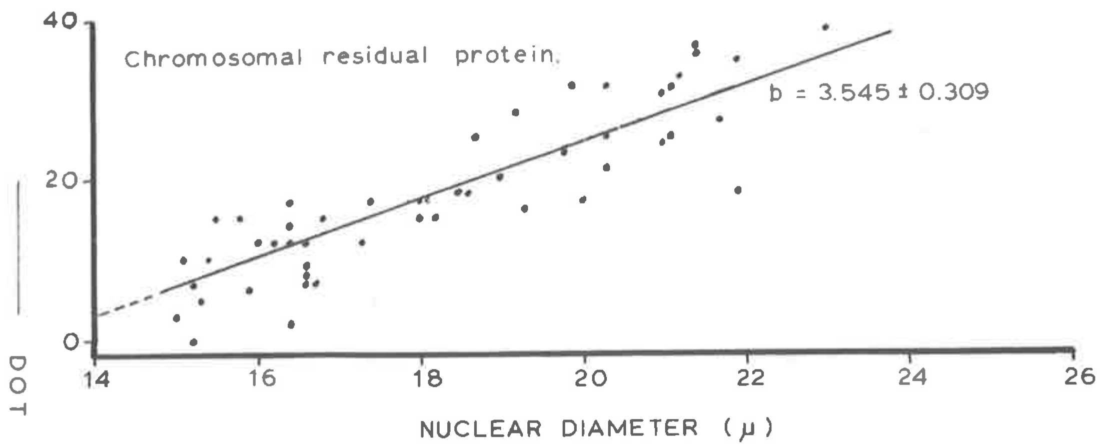

Figure 24

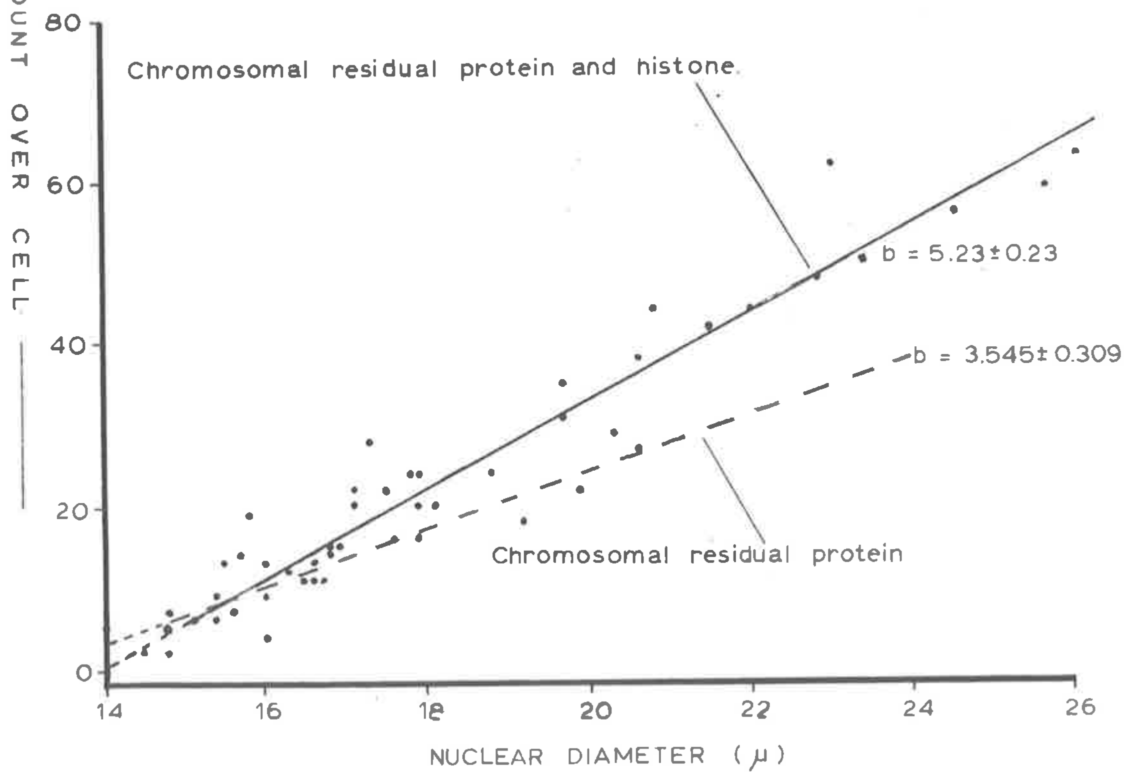

Figure 25

Rates of arginine incorporation in relation to nuclear diameter

commences after the start of the S period, and does not continue right through the G1 period as well. This conflicts with Cave's results; he concluded that chromosomal residual protein was synthesised all through interphase in cultures of human leucocytes, with an increase in the rate of labelling occurring through the S and G2 periods.

The autoradiographs of the cells labelled with tritiated arginine and fixed in form-acetic-alcohol were examined last. There was no significant difference in size between the cells in this culture and the cells in the two preceding treatments. Once again, the dot counts over individual labelled cells were estimated in conjunction with their mean nuclear diameters. The results are shown in figure 25, with the trend line obtained from figure 24 superimposed as a dotted line. No labelled cell was seen with a nuclear diameter less than 14.2 μ. Cells with nuclear diameters between 14.2 μ and 20 μ showed labelling rates rather higher than those found in the corresponding cells fixed in acetic/alcohol, thus indicating that some label was being incorporated into histone. The incorporation rate diminished slightly after the cells reached a nuclear diameter greater than 20 μ, but it is clear that synthesis of histone

was still proceeding throughout the G2 period, although at a reduced rate. If synthesis of histone ceased as the cell entered the G2 period, then a sharp drop in the labelling rate could be expected, as the label would be incorporated only into the chromosomal residual protein. Such a drop does not occur, and it seems clear that synthesis of histone proceeds at a reduced rate after DNA synthesis stops. It is possible that cells which had been in the S period during label incorporation and entered the G2 period during the course of exposure to label would have an artificially high dot count if the synthesis of histone stopped at the end of the S period. However, this is not enough to explain the high dot counts over cells with nuclear diameters greater than 22 μ, as these cells must have been in G2 for a longer time than they had been exposed to label. The average G2 period in the lymphocytes of the kangaroo is about three hours (see figure 14, page 73), whereas the cells in the present experiment were exposed to label for one hour only.

One further check was made, using the autoradiographs obtained from the last experiment. The percentages of labelled cells in random samples of 500 cells were estimated for all three treatments. The results are given in table 7.

TABLE 7.

Percentages of cells labelled in thymidine-labelled and arginine-labelled autoradiographs.

Cell constituent labelled.	Labelled cells.	Unlabelled cells.
DNA	27.3 %	72.7 %
Chromosomal residual protein.	33.9 %	66.1 %
Chromosomal residual protein and histone.	34.5 %	65.5 %

11.7 % of the cells in the culture treated with thymidine were in G2, and were thus unlabelled.

Contingency tables derived from this table are given on page 169.

The G2 cells referred to in the DNA-labelled cells were determined on the basis of size alone, as these cells were not labelled. There is no significant difference between the two arginine-labelled treatments. This means either that histone and chromosomal residual protein commence synthesis at the same time, or else that the synthesis of histone starts later than the synthesis of chromosomal residual protein. The higher proportion of labelled and G2 cells in the thymidine-treated culture presumably indicates that the synthesis of DNA starts earlier than either the synthesis of histone or the synthesis of chromosomal residual protein. If the commencements of the two latter labelling patterns are synchronous, then the earlier lysine-labelling and arginine-labelling experiments (see figure 11, page 63; figure 12, page 65; figure 13, page 72; figure 14, page 73) can be regarded as displaying a definite lag in histone synthesis after the synthesis of DNA commences. However, no such lag can be demonstrated from those experiments at the <u>end</u> of DNA synthesis.

In summary, then, the following conclusions can be arrived at in this section.

(a). Histone synthesis starts later and finishes later than DNA synthesis, and some degree of asynchrony

seems clear.

(b). Chromosomal residual protein is synthesised in significant amounts throughout the S and G2 periods, and this activity masks the labelling of histone in autoradiographs.

(c). Because of the difficulties raised by the synthesis of chromosomal residual protein, it is very difficult to get a precise idea of the relative timing of the synthesis of histone. Nevertheless, for the purposes of discussion a demonstration of asynchrony in itself is sufficient, and some degree of asynchrony of DNA and histone syntheses seems clear from the results obtained in this section.

Section 3: Initiation of lymphocyte growth by PHA.

It is now well known that lymphocytes stimulated with PHA increase their rate of RNA turnover and start to synthesise protein (Bach and Hirschhorn, 1963; Hirschhorn, Kolodny, Hashem, and Bach, 1963; Hirschhorn, Bach, Kolodny, Firschein, and Hashem, 1963; Epstein and Stohlman, 1964; Killander and Rigler, 1965; Winter and Yoffey, 1965; Mueller and le Mahieu, 1966). However, most workers have concentrated on the question of RNA synthesis and gamma-globulin production by lymphocytes, and little light has been shed on the synthesis of protein more directly concerned with the cell cycle. Furthermore, as the present study was carried out on kangaroo lymphocytes, it seemed advisable to investigate the immediate response of the lymphocytes to PHA, and to find out if there were any differences in response in comparison with human cells.

Experiments were therefore designed to compare the rates of RNA and protein synthesis in kangaroo lymphocytes after exposure to PHA, to measure the rate of accumulation of nuclear protein, to check on the function of protein synthesised by the cell as a response to PHA treatment, and, finally, to attempt to account for the action of PHA on the cell.

3.3.1. Experiments on RNA and protein synthesis.

The first experiment in this section was designed to compare the relative rates of incorporation of tritiated cytidine and tritiated phenylalanine. It was hoped that this would provide data which could be used as a basis for the design of further experiments.

A leucocyte culture was prepared in autologous serum and allowed to stand at 37° C. for twelve hours before the addition of PHA, so that changes in the rate of synthesis of protein recorded by Bach and Hirschhorn (1963) in fresh, unstimulated cultures would not affect the control cells. At the end of twelve hours the culture was equally divided into two parts, one part being treated with 0.1 μc./ml. tritiated phenylalanine, and the other with 0.1 μc./ml. tritiated cytidine. Each treatment was thereupon divided equally again, and one half from either treatment was stimulated with 0.01 ml./ml. PHA. Each of the four treatments was then sampled sequentially at 30 minute intervals up to 150 minutes after the addition of PHA, the samples were fixed in fresh 3:1 ethanol:acetic acid, and autoradiographs were prepared in the usual way. The autoradiographs were developed after an exposure time of 9 weeks, and the slides were examined for labelled lymphocytes. 500 cells were counted on each

slide. The results of this experiment are shown in figure 26.

RNA labelling in the control cultures was exclusively nuclear, but a small amount of cytoplasmic labelling was found in the cells treated with PHA. This cytoplasmic labelling became more noticeable in the samples collected towards the end of the experiment. An abrupt rise in the proportion of cytidine-labelled cells in the control culture after 120 minutes remains unexplained; some slight clumping, absent in the other control cultures, was also apparent.

It was clear from this experiment that the response of kangaroo lymphocytes to stimulation by PHA was very much more rapid than had been anticipated, especially where RNA synthesis was concerned. It was also clear that RNA synthesis preceded protein synthesis by a considerable period. The relatively high rate of cytidine incorporation by the unstimulated cells may be due to the synthesis of short-lived RNA in the nucleus (Harris, 1964), and the commencement of protein synthesis may depend on the formation of more stable template RNA.

A further experiment was designed to determine the rate of increase of RNA turnover after exposure of the lymphocytes to PHA, and to investigate the appearance of

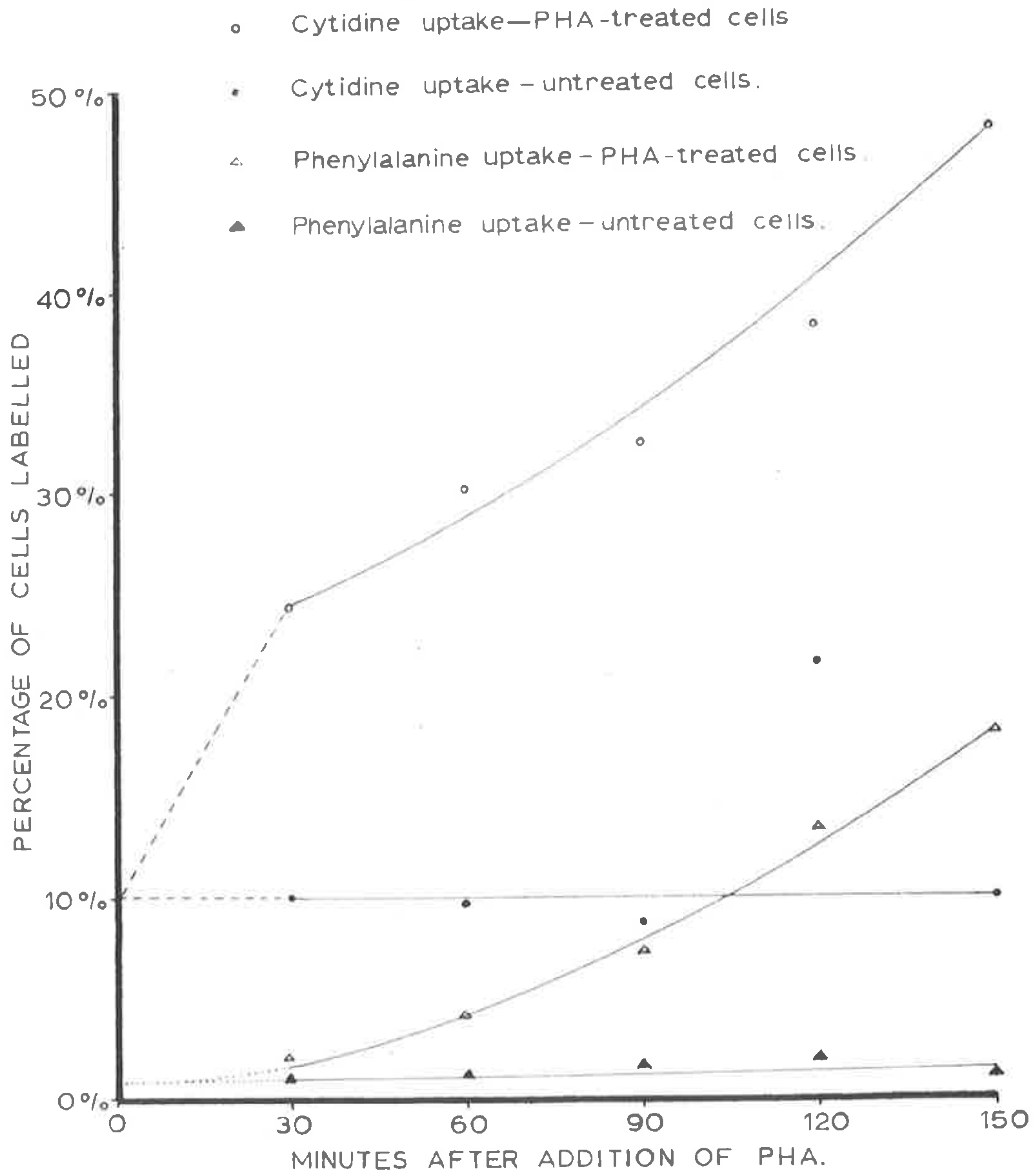

Figure 26.

Commencement of RNA and protein synthesis.

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label in the lymphocyte cytoplasm after exposure to tritiated cytidine.

A leucocyte culture was prepared in autologous serum, and 0.1 μc./ml. cytidine-H^3 was added. The culture was immediately divided into two equal lots, one of which was treated with 0.01 ml./ml. PHA. Sequential samples were taken from each treatment every six minutes up to thirty minutes after the addition of PHA. The cells were fixed in fresh 3:1 ethanol: acetic acid, and autoradiographs were prepared in the usual way. These were developed after an exposure period of six months, and the slides examined for labelled cells. 500 cells were counted on each slide, and the results of this experiment are shown in figure 27.

These cultures were also examined for cytoplasmic label, and the percentages of <u>labelled</u> lymphocytes showing cytoplasmic labelling are given in table 8.

No cells in the control culture showed any sign of cytoplasmic labelling (see figure 28, page 104), and those cells with labelled nuclei were generally rather larger than the unlabelled cells. In the cultures treated with PHA, any label found in the cytoplasm was light compared with that over the nucleus. (See figure 29, page 105; figure 30, page 106).

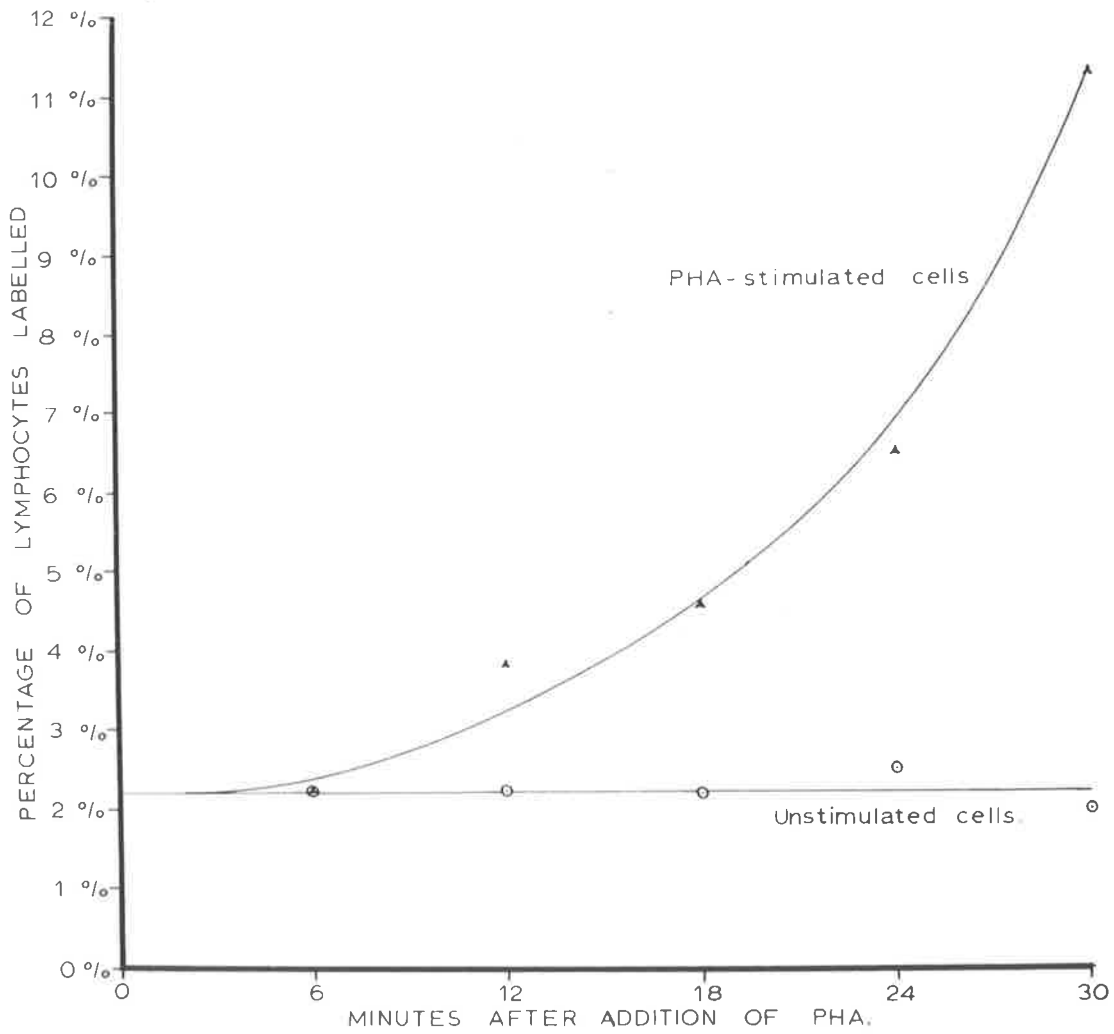


Figure 27.

^{3}H-cytidine labelling after exposure to PHA.

TABLE 8.

Percentages of labelled cells with cytoplasmic cytidine-H^3 labelling.

Age of culture (minutes)	6	12	18	24	30
% labelled cells with cytoplasmic labelling.	0 %	0 %	3.1 %	5.5 %	16.2 %

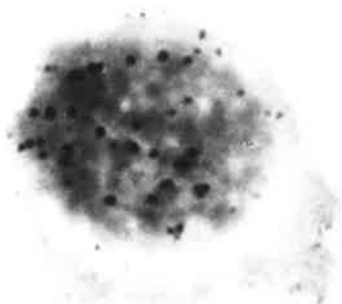
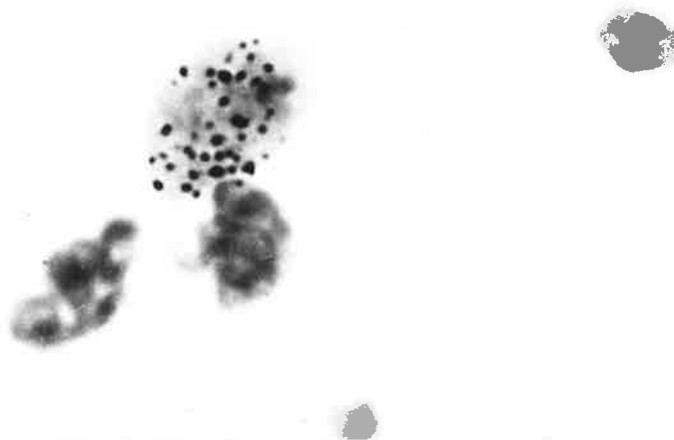


Figure 28. Cytidine-H^3 -labelled cell from an unstimulated culture. The label is exclusively nuclear, and the cell is rather larger than other cells in the culture.

2000 X.



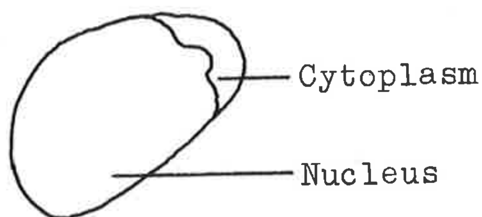


Figure 29. Cytidine-H^3-labelled cell, twelve minutes after the addition of PHA. The label is exclusively nuclear.

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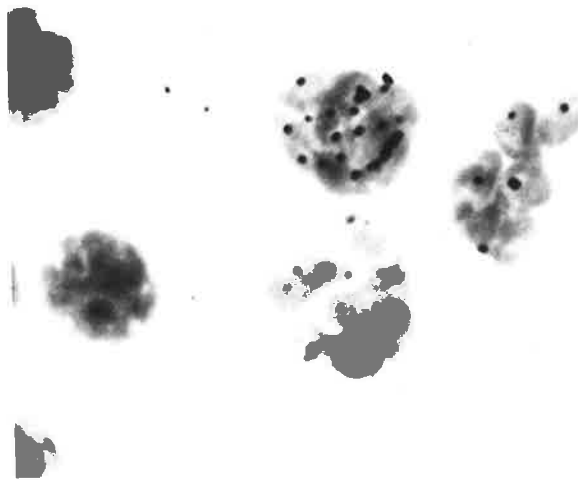

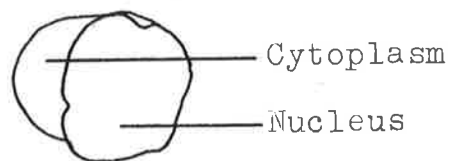

Figure 30. Cytidine-H^3-labelled cell, eighteen minutes after the addition of PHA. Some clear cytoplasmic labelling is present, although it is light in comparison with the labelling over the nucleus.

2000 X.

The appearance of label in the cytoplasm almost certainly represents an important step in the cell cycle, and may reflect the formation of the stable template RNA postulated by Harris (1964).

Clumping, absent in the unstimulated cultures, was evident in the PHA-treated cultures as early as six minutes after the addition of PHA, with aggregations of six to ten cells being fairly common at this time.

The very considerable difference (between 2 % and 10 %) in the cytidine-H^3 labelling rates in the control cultures of the two experiments is probably due to two different factors; first, one experiment was incubated for twelve hours before stimulation with PHA, while the other was not, and second, slight contamination was seen in the control cultures of the first experiment. Nevertheless, the increased labelling rate induced by PHA can be clearly seen in both experiments.

From these two experiments, the following points are clear:-

(a). There is a significant increase in nuclear RNA labelling after only twelve minutes' exposure to PHA.

(b). The appearance of labelled RNA in the cytoplasm occurs very early in comparison with PHA-treated human lymphocytes. Winter and Yoffey (1965)

place the advent of cytoplasmic RNA labelling of human lymphocytes at twelve hours after the addition of PHA.

(c). The pattern of phenylalanine uptake is similar to that recorded by Bach and Hirschhorn (1963) in human lymphocytes treated with PHA. However, they found that protein synthesised at this stage travelled electrophoretically as gamma-globulin. Certainly most of the label seen in cells labelled at this time with phenylalanine-H^3 is cytoplasmic. These experiments on kangaroo lymphocytes show no evidence that nuclear protein is being synthesised at this stage.

It became necessary, following these experiments, to determine the fate of the phenylalanine incorporated into the cells.

A culture was prepared in foetal calf serum, and incubated with 0.01 ml./ml. PHA and 1.0 μc./ml. phenylalanine-H^3 for four hours. At the end of this time the culture was centrifuged, and the leucocyte pellet washed three times with medium 199 before being resuspended in fresh culture medium. PHA was added to the fresh culture medium, and samples were taken at intervals from four hours to forty eight hours after the first exposure of the cells to PHA. Autoradiographs were prepared in the usual way, and developed after an exposure period of fifteen weeks.

The timing of this experiment was arrived at after consideration of the following points:-

(a). If the protein synthesised by the cell in the early stages is exclusively gamma-globulin, this will be reflected by a preponderance of cytoplasmic label in the autoradiographed cells. However, studies on dry mass and nuclear volume indicate that there is some synthesis of nuclear protein by the third hour of cellular response to PHA. The four hour period of exposure to phenylalanine-H^3 was chosen in the hope that some nuclear label would be detected.

(b). Samples were taken at intervals up to 48 hours after initiation of cell growth so that any changes in the distribution of label could be detected. It was also hoped that the position of the label, if still present, in the S, G2, and mitotic cells would help to show the function of protein synthesised in the early stages of the cell cycle.

In the early (4 hour) culture, two main labelling patterns were apparent. The first pattern showed a marked preponderance of label in the cytoplasm (see figure 31). The second pattern, while still showing a considerable amount of cytoplasmic label, showed heavy nuclear labelling as well (see figure 32).

The second pattern became increasingly common as

Figure 31. Phenylalanine-H^3-labelled small lymphocyte, four hours after addition of PHA. Most of the label is cytoplasmic.

2000 X.

Figure 32. Phenylalanine-H^3-labelled cell four hours after the addition of PHA. A considerable amount of nuclear label is present.

2000 X.

the culture progressed; after 48 hours most cells showed both nuclear and cytoplasmic labelling. Cells in mitosis showed label scattered around the chromosomes, but little label over the chromosomes themselves (see figure 33).

It seems likely that phenylalanine is incorporated first into gamma-globulin, as Bach and Hirschhorn (1963) reported. This is probably reflected by the high incidence of cytoplasmic labelling in the early cultures. However, the onset of nuclear labelling probably reflects the initiation of the synthesis of nuclear structural protein. Synthesis of nuclear protein at this stage is also indicated by changes in nuclear volume and nuclear dry mass (see pages 115 and 118).

Unfortunately, the distribution pattern of label in the cells of the later cultures is not a reliable guide to the function of the protein into which the phenylalanine had first been incorporated. First, nuclear and cytoplasmic protein, if formed, may be degraded and the label lost to other parts of the cell. Second, the death of labelled lymphocytes during the culture period would liberate labelled protein which could later be taken up by other cells. Third, any gamma-globulin released by the cells after

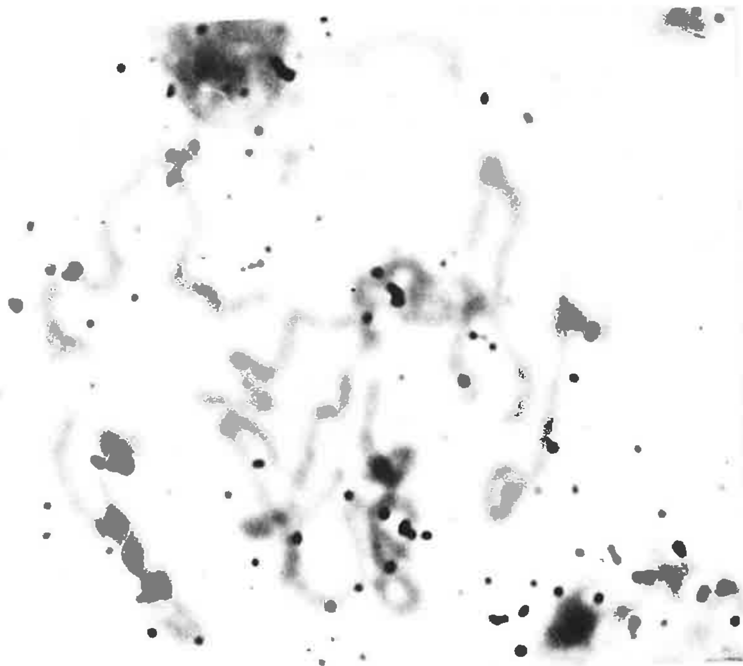

Figure 33. Phenylalanine-H^3-labelled **mitotic** cell. The cell was pulse labelled over the first four hours of culture. There is a preponderance of non-chromosomal label, in marked contrast to the labelling patterns obtained when the cell is exposed to arginine or lysine just before harvesting.

2000 X.

resuspension in fresh culture could be a source of label during a later stage of the culture. Thus the only sample from this experiment which can be regarded as valid is the first sample, taken four hours after the addition of isotope and PHA.

However, it is useful to compare the labelling pattern over mitotic cells with that obtained with tritiated lysine or arginine. While much of the label is nuclear in the phenylalanine-treated cells, very little of it lies directly over the chromosomes; this is in direct contrast to the situation in cells labelled with arginine or lysine.

3.3.2. Size and dry mass changes after PHA treatment.

It has been shown that some at least of the protein produced in the early stages of PHA-induced cell growth is gamma-globulin (Bach and Hirschhorn, 1963; Hirschhorn, Bach, Kolodny, Firschein, and Hashem, 1963; Hirschhorn, Kolodny, Hashem, and Bach, 1963). In view of these results, it seemed advisable to measure increases in the sizes and dry masses of lymphocytes following their stimulation with PHA, and to find out if nuclear protein was synthesised at the same time as gamma-globulin.

Nuclear and whole-cell diameters of formalin-fixed

cells, sampled at intervals up to five hours after the addition of PHA, were measured (in suspension) for 75 lymphocytes in each sample. Nuclear, whole cell, and cytoplasmic volumes were calculated from these data. The diameters, both of the nucleus and of the whole cell, were measured in two directions at right angles, and the volume in each case calculated from the formula

$$\text{volume} = \frac{4}{3}\pi\, r_1 r_2^{\,2}$$

where r_2 is the lesser of the two radii measured. Cytoplasmic volume was obtained by subtraction of the nuclear volume from the whole cell volume. The results are given in figures 34 and 35.

Both the nuclear and cytoplasmic volumes of lymphocytes exposed to PHA commenced a steady increase about three hours after stimulation, indicating that both cytoplasmic and nuclear protein synthesis was proceeding. However, this did not necessarily mean that the dry mass of the nucleus and the dry mass of the cytoplasm had increased by the same relative amount, as the experiment shed no light on the concentration of dissolved solids in the cell.

The next experiment was designed to give more

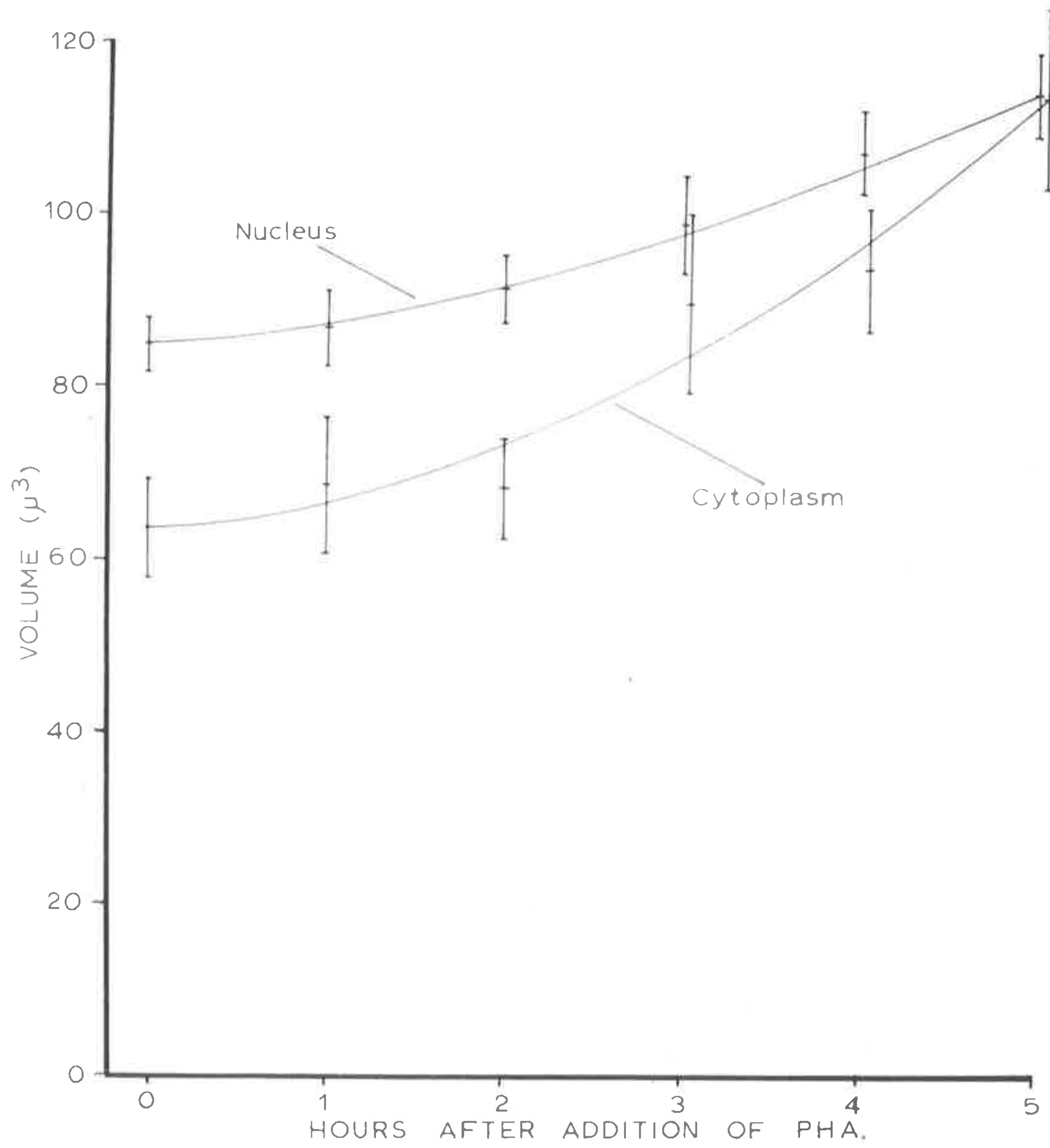

Figure 34

Nuclear and cytoplasmic volumes after PHA treatment.

95% confidence intervals of means shown.

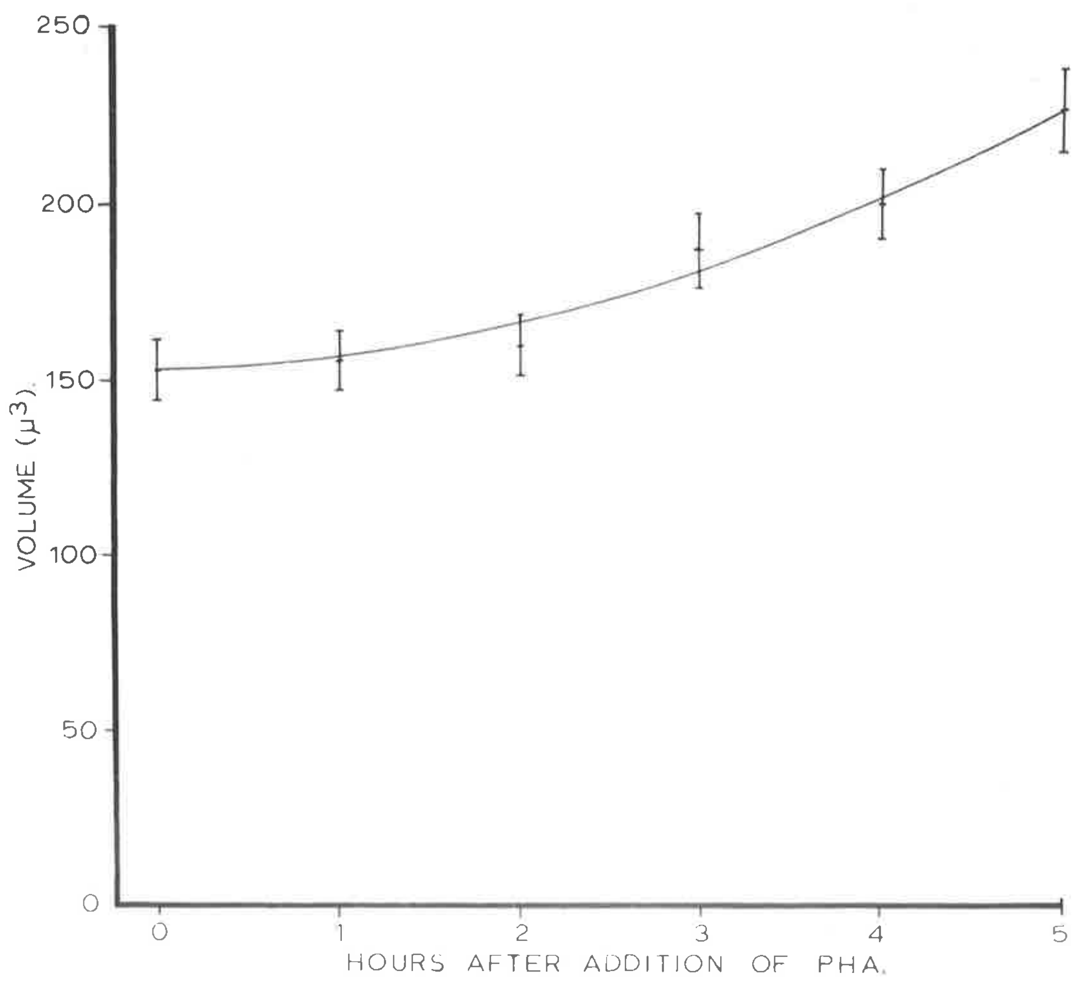

Figure 35.

Whole cell volumes after PHA treatment.

95% confidence intervals of means shown.

information on the accumulation of nuclear protein prior to DNA synthesis. A culture was sampled at two-hour intervals up to ten hours after the addition of PHA, and the samples were fixed in form-acetic-alcohol. This fixative was chosen for two main reasons; first, it fixes histone adequately, (De, 1961), and second, cells fixed in form-acetic-alcohol did not show any tendency to fall off the slide during subsequent treatment. The cells were then treated according to the method described on page 44. Dry masses of nuclei were measured both before and after the extraction of nucleic acids. The results from this experiment are given in figures 36 and 37.

The following points arise from this experiment:-

(a). A significant increase in the mean nuclear dry mass occurs after four hours of incubation. From this time on, active synthesis of nuclear protein proceeds, at least up to ten hours after the addition of PHA to the culture.

(b). There is no significant change in the amount of nucleic acid present at this time. The very slight rise seen after two hours of culture may be due to synthesis of nuclear RNA, but the increase is not significant.

(c). The fall of 1.8×10^{-12} gms. in the mean

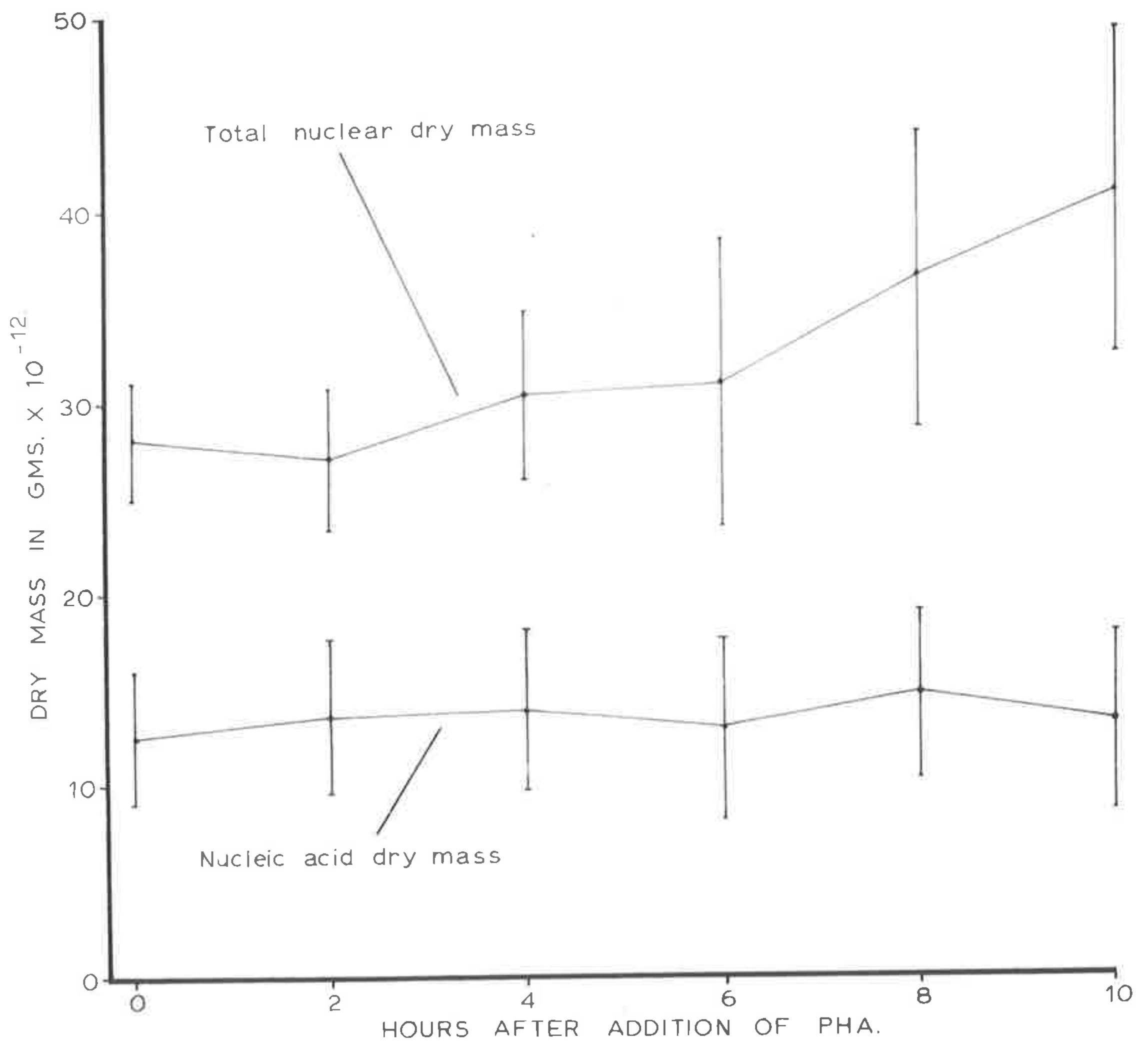

Figure 36

Nuclear and nucleic acid dry mass after exposure to PHA.
Means and standard deviations shown.

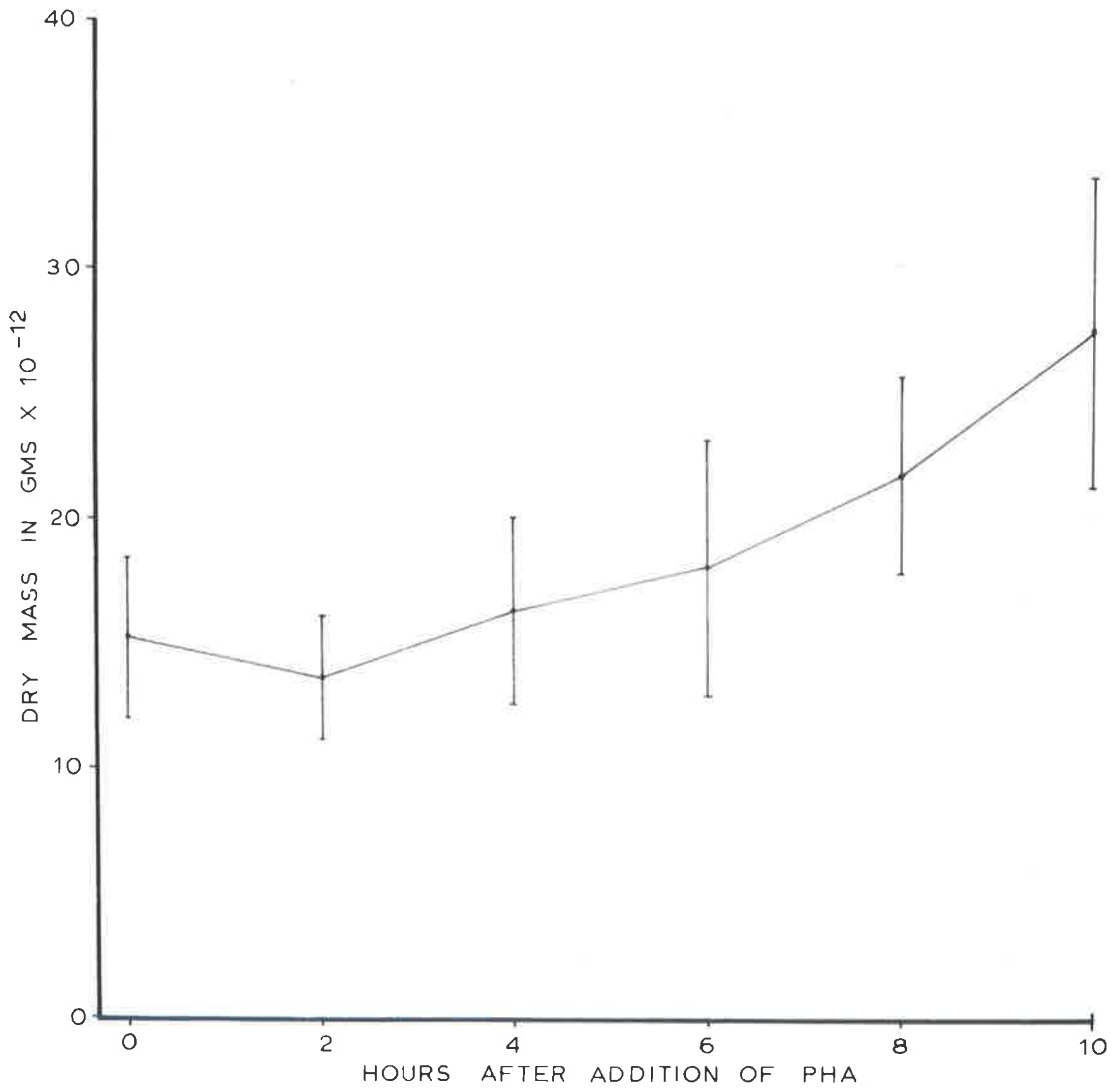

Figure 37

Nuclear dry mass after extraction of nucleic acids.
Means and standard deviations shown.

dry mass of nuclear protein after two hours of incubation with PHA is significant at the 5 % level. This fall may possibly be accounted for by PHA-induced loss of histone from the nucleus. (See page 144.)

3.3.3. Changes in histone at the commencement of cell growth.

Black and Ansley (1965) described changes in mouse thymocyte histones induced by an antigenic stimulus, and interpreted a decrease in stainability with fast green and ammoniacal silver as representing a probable loss of histone from the thymocyte nuclei. As some workers have proposed that the action of PHA on lymphocytes is essentially similar to that of an antigen (Pearmain, Lycette, and Fitzgerald, 1963; Hirschhorn, Kolodny, Hashem, and Bach, 1963; Lindahl-Kiessling and Böök, 1964; Tao, 1964), an experiment was designed to find out if a similar loss of histone stainability could be induced in lymphocytes by PHA.

Lymphocytes were harvested at intervals up to two hours after exposure to PHA, and fixed in 10 % neutral formalin. Nucleic acids were extracted from all cells by immersion in 5 % trichloracetic acid at 90° C. for 90 minutes. Cells were then squashed onto one end of an appropriately marked slide, and

control cells taken from the culture immediately prior to the addition of PHA were squashed onto the other end of the slide. The slides were then treated and stained with alkaline fast green according to the method described on pages 40 and 41. They were then examined microspectrophotometrically. The results of this experiment are shown in figure 38.

These data may be interpreted in several ways.

(a). The decrease in histone staining may reflect the loss of histone from the cell as a whole.

(b). Some histone may be degraded by PHA so that it no longer stains with fast green and yet remains in the nucleus (Killander, 1966).

(c). Busch, Steele, Hnilica, Taylor, and Mavioglu (1963) proposed that specific acidic proteins may be capable of forming a complex with the same affinity points in histones that complex with DNA. PHA may compete with DNA for these histone affinity points, and the histone not affected by PHA would be available for staining after the removal of nucleic acids. The histone/PHA complex, meanwhile, could remain in the nucleus, migrate to the cytoplasm, or be lost from the cell altogether.

It is obvious that PHA affects histone in a radical manner, but whether histone or histone-derived

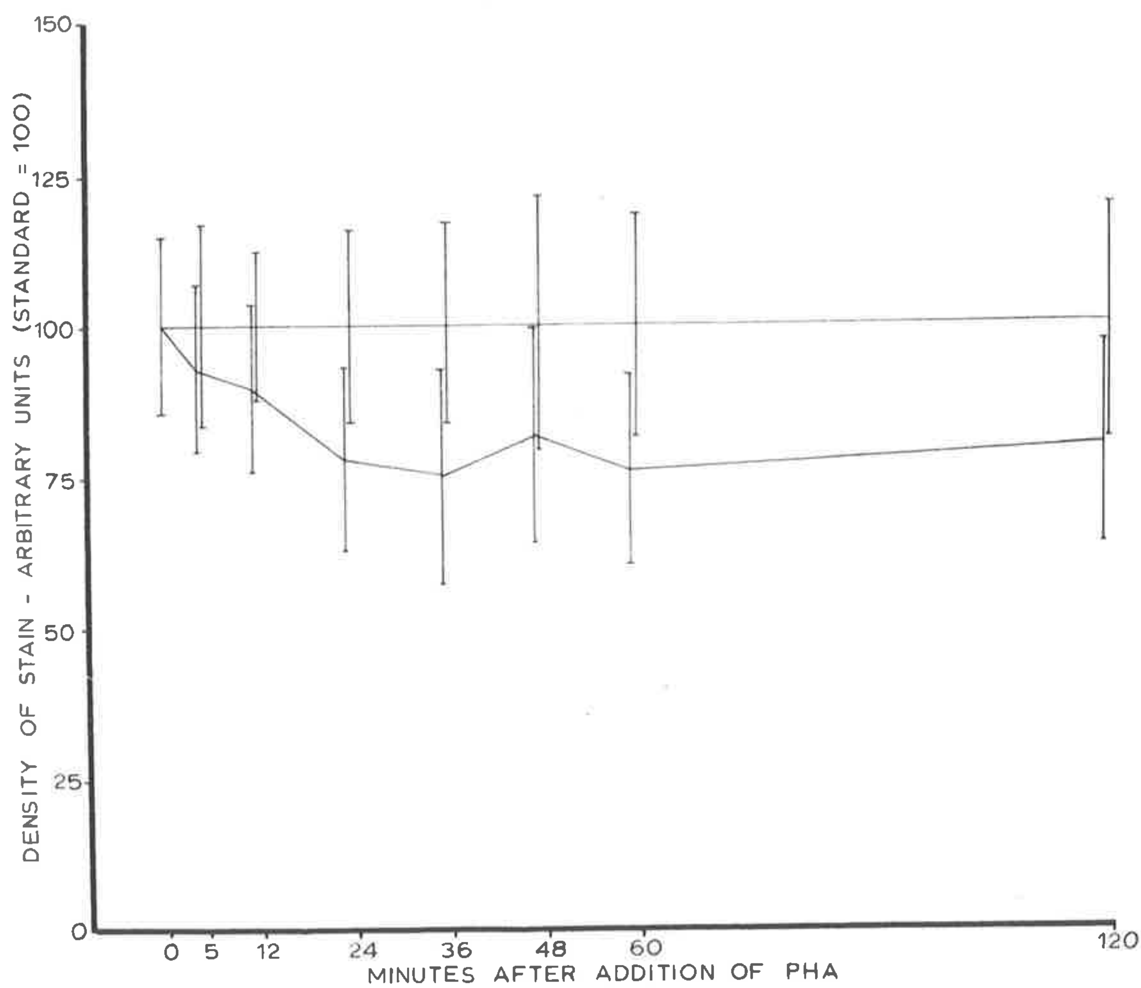

Figure 38

Changes in histone staining after exposure to PHA
(Alkaline fast green stain)
Means and standard deviations shown.

material is physically removed from the nucleus is a question that cannot be answered on microspectrophotometric grounds.

An experiment was therefore designed to see if there were any detectable changes in mass of the nucleus and cytoplasm of cells exposed to PHA. A culture was prepared in autologous serum, and a sample fixed in 10 % neutral formalin just before the addition of PHA to the rest of the culture. Further samples were fixed in 10 % neutral formalin at intervals ranging up to 120 minutes after the addition of PHA. Immediately prior to examination, the cell samples were washed in distilled water, placed on a slide, and covered with a coverslip which was subsequently ringed with rubber solution to prevent the cells from drying out. The optical path differences and dimensions of both nucleus and cytoplasm for each lymphocyte examined were estimated with an interference microscope equipped with a micrometer eyepiece. The results of this experiment are shown in figures 39, 40, and 41.

The mean decrease in nuclear dry mass of 1.9×10^{-12} gms. after 40 minutes' exposure to PHA can be accounted for by transfer of histone-derived material to the cytoplasm, and this conclusion is

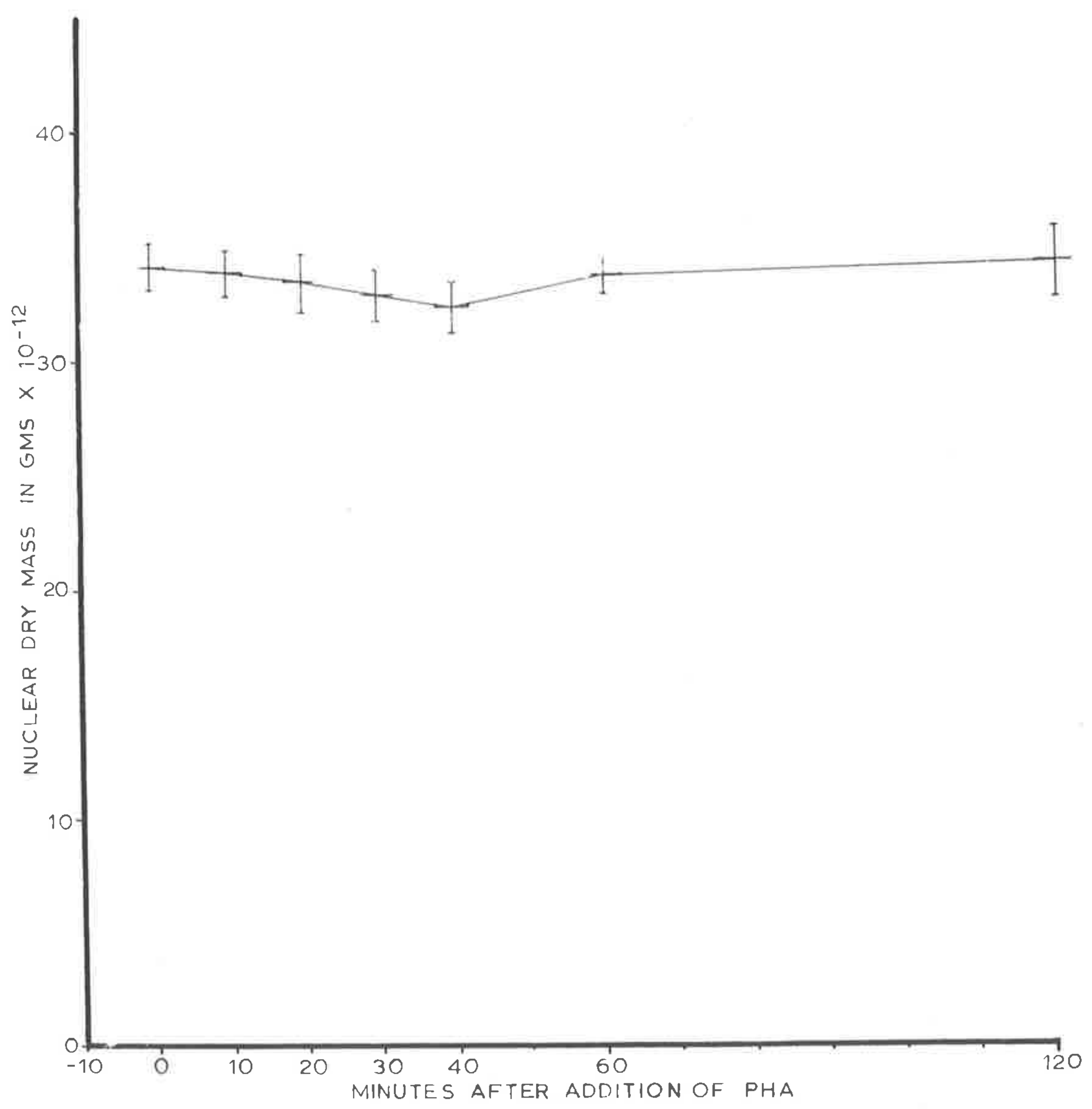

Figure 39.

Changes in nuclear dry mass after addition of PHA.

95 % confidence intervals of means shown.

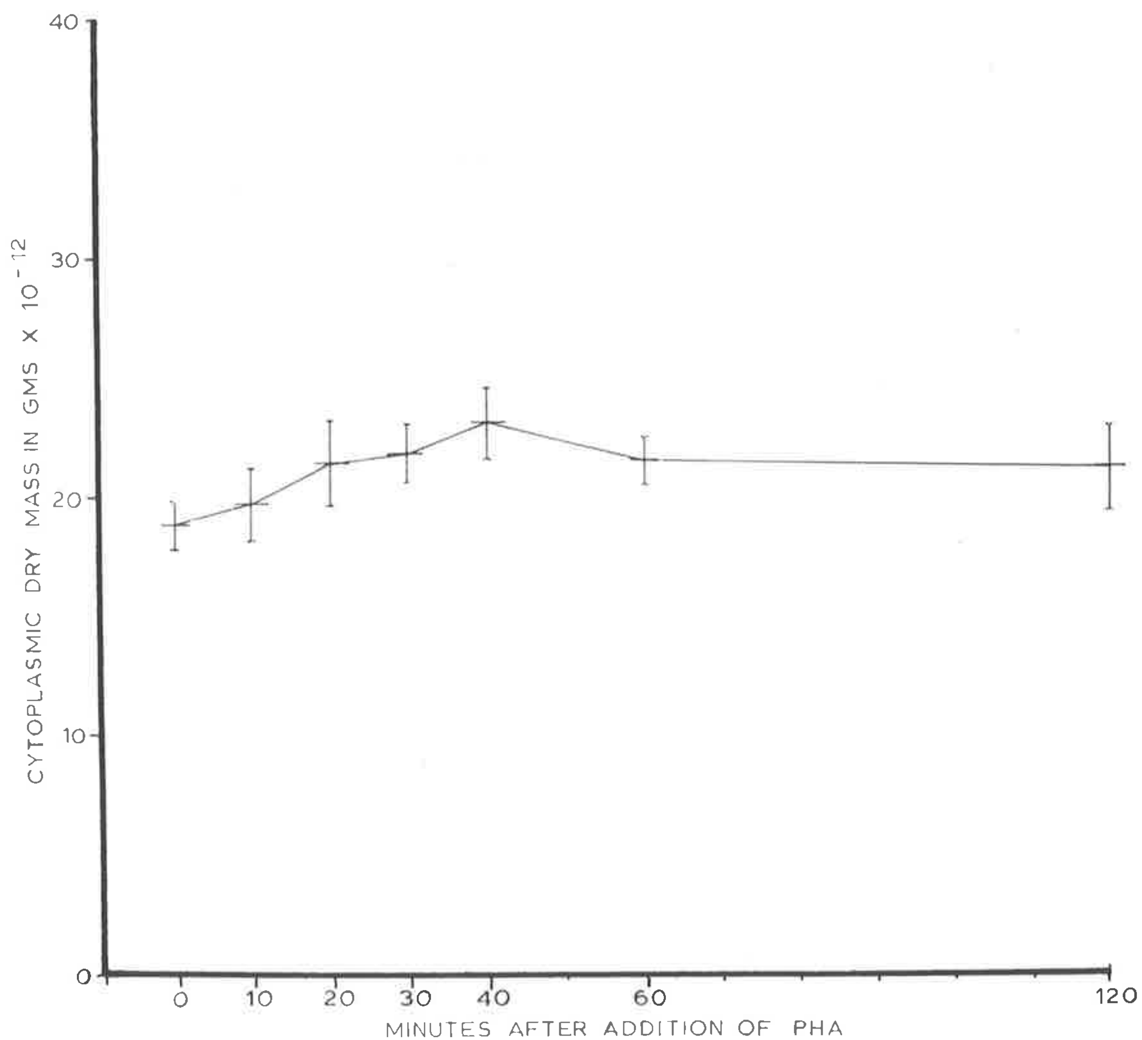

Figure 40.

Cytoplasmic changes in dry mass after addition of PHA.

95 % confidence intervals of means shown.

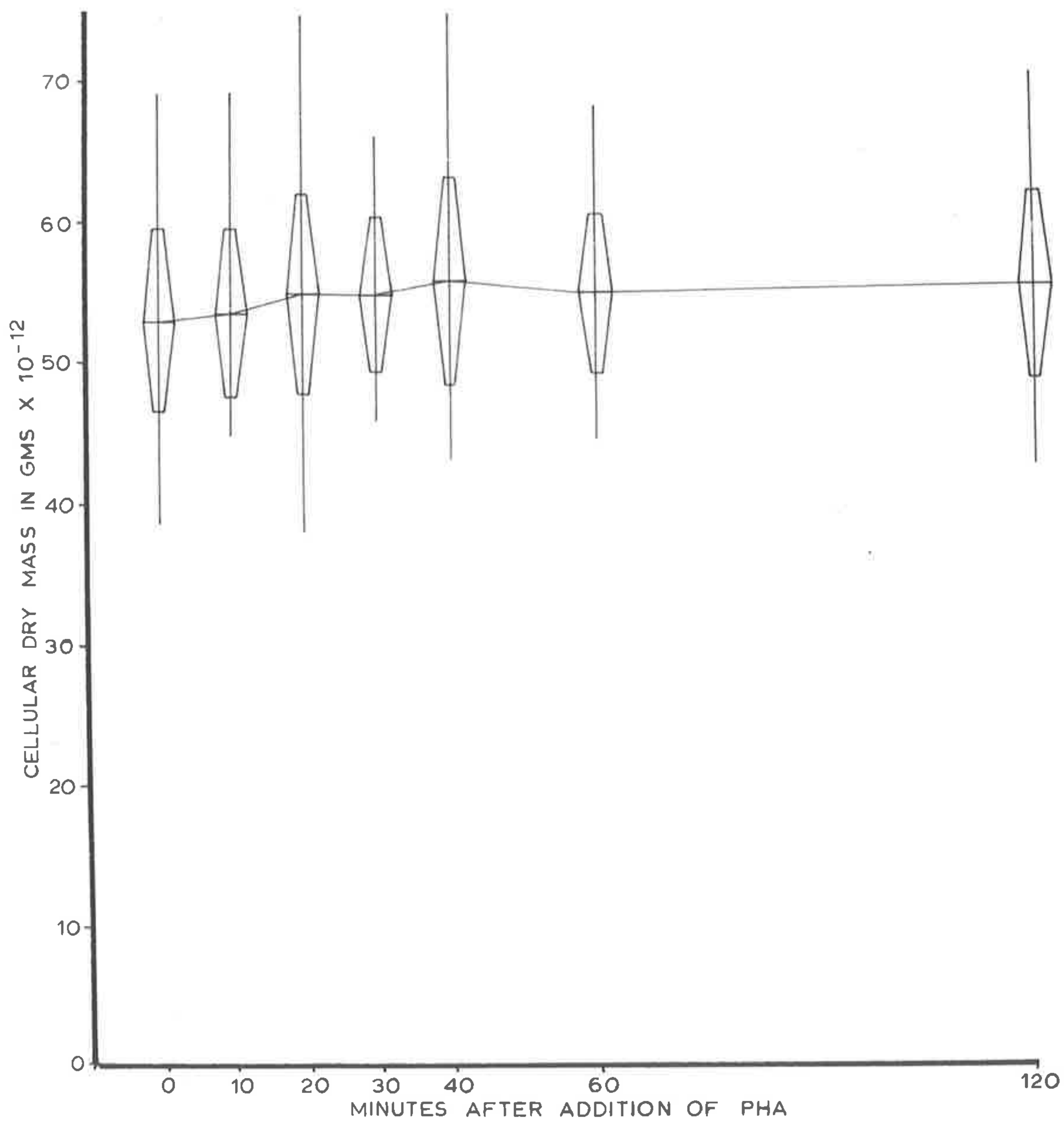

Figure 41.

Changes in cellular dry mass after addition of PHA.

Means, standard deviations, highest and lowest values shown.

supported, to some extent, by a corresponding increase in the mean dry mass of the cytoplasm; however, the evidence is circumstantial. The difference between the mean dry masses of the unstimulated and stimulated lymphocyte nuclei, quoted above, is significant at the 5 % level. This problem is fully discussed on page 144.

4. DISCUSSION.

In any study of the mitotic cycle of the lymphocyte, the nature and function of the lymphocyte must be taken into account. Typically, the circulating small lymphocyte appears to be relatively inactive until it is exposed to a mitogenic stimulus. When it is exposed to such a stimulus, the lymphocyte not only commences preparations for division, (such as synthesis of nuclear and cytoplasmic protein, synthesis of DNA, and synthesis of histone), but also commences the synthesis of gamma-globulin. It appears that the lymphocyte functions as a memory cell, and probably the most important attribute of the small lymphocyte is its ability to recognise antigens and respond to them. For this reason, in any study of antigen-stimulated lymphocyte response, both the mitotic cycle itself and the production of gamma globulin can be considered as resulting from the same process of antigen recognition.

However, there are quite marked differences between the responses of lymphocytes exposed to PHA and the responses of presensitised lymphocytes exposed to

specific antigens. The problems of differentiating the responses of a lymphocyte exposed to PHA from the responses of one exposed to antigen then arise, as well as the puzzle of sorting out the direct results of exposure to antigen, such as the production of antibody, from events more closely concerned with the mitotic cycle, such as the synthesis of nuclear protein.

4.2. Commencement of DNA synthesis.

At the start of the study it was expected that the timing of the commencement of DNA synthesis would be approximately the same as it is in cultures of human peripheral leucocytes treated with PHA; that is, between twenty and thirty hours after the addition of PHA (Cooper, Barkhan, and Hale, 1961, 1963; Lima-de-Faria, Reitalu, and Bergman, 1961; Beckman, Fichtelius, Finley, Finley, and Lindahl-Kiessling, 1962; MacKinney, Stohlman, and Brecher, 1962; Bender and Prescott, 1962; Michalowski, 1963; Killander and Rigler, 1965). However, with the first increase in the rate of incorporation of thymidine-H^3 occurring 12 hours after the addition of PHA, the G1 period in kangaroo lymphocytes is clearly much shorter than that found in human lymphocytes. The problem of accounting for this markedly shorter G1 period then arises, and several

possibilities must be considered.

(a). Events necessary for the initiation of DNA synthesis may occur at a faster rate in the lymphocytes of the kangaroo than they do in human lymphocytes.

(b). Events normally occurring in the G1 period and necessary for the initiation of DNA synthesis in human cells may not be necessary prior to the synthesis of DNA in the lymphocytes of the kangaroo, and thus need not necessarily occur in the G1 period at all. This could result in a shorter G1 period.

(c). If the initiation of DNA synthesis is dependent on the attainment of a certain critical cell mass, (Killander and Zetterberg, 1965a, b) this would then imply the completion of a certain amount of protein synthesis. This protein may either be synthesised at a faster rate, or else commence synthesis earlier in kangaroo lymphocytes than is the case in human lymphocytes treated with PHA.

Two lines of evidence throw some light on this problem. The first is the pattern of cytidine-H^3 labelling immediately after the exposure of the lymphocytes to PHA. Many workers have reported a rapid increase in the rate of turnover of RNA in lymphocytes treated with PHA (Epstein and Stohlman, 1964; Winter and Yoffey, 1965; Cooper and Rubin, 1966;

Mueller and le Mahieu, 1966; Pogo, Allfrey, and Mirsky, 1966). However, this rapidly-labelled RNA is almost exclusively nuclear, and its precise function is still not known. Cooper and Rubin (1966) believe that it may be messenger RNA being produced in large quantity, perhaps as a result of the abrogation of normal regulatory processes by PHA. They support this conclusion with evidence that the RNA molecules produced by lymphocytes treated with PHA differ in size distribution from those produced by lymphocytes stimulated with antigen. They have also pointed out that PHA-stimulated RNA is non-ribosomal (Cooper and Rubin, 1965). Further, Winter and Yoffey (1965) noted the appearance of labelled RNA in the cytoplasm of lymphocytes treated with PHA only after twelve hours of incubation. On the basis of their evidence they were unable to decide whether the cytoplasmic labelling represented a transfer of the stable template RNA postulated by Harris (1964) to the cytoplasm once the process of cell growth initiated by PHA was under way, or whether it merely represented diffusion of acid-soluble end products outwards from the nucleus, or both. They noted that cytoplasmic labelling was more marked from eighteen hours onwards. In the kangaroo lymphocytes studied, however, cytoplasmic labelling could be detected as early as eighteen <u>minutes</u>

after the addition of PHA (see table 8, page 103). It therefore seems very unlikely that this labelling reflected the diffusion of acid-soluble end products from the nucleus. Furthermore, if this cytoplasmic labelling does represent a transfer of stable RNA from the nucleus, it is clear that a radical short cut has been taken by the marsupial lymphocytes. This would naturally result in a correspondingly rapid initiation of nuclear and cytoplasmic protein synthesis, and a correspondingly shorter G1 period.

The second line of evidence involves the commencement of protein synthesis itself. Here it is important to separate the production of gamma-globulin from the synthesis of nuclear protein and the building up of cytoplasmic structural protein. The early incorporation of phenylalanine-C^{14} demonstrated by Bach and Hirschhorn (1963) was shown by them to reflect the synthesis of gamma-globulin. Cooper and Rubin (1965) also concluded that RNA synthesis was intimately bound up with the synthesis of gamma-globulin. As far as protein synthesis in the early stages of lymphocyte response is concerned, most workers have concentrated on the synthesis of gamma-globulin alone; the build-up of nuclear and cytoplasmic structural protein has been largely ignored, although work has been done on other

cell types. (Killander and Zetterberg, 1965a, b) used mouse fibroblasts). However, estimations of nuclear and cytoplasmic volumes and measurements of nuclear and cytoplasmic dry mass both before and after the extraction of nucleic acids can be used to give some indication of the timing of changes in the amount of protein in the cell. Using these methods, a significant increase in nuclear volume can be detected after only three hours in culture in the case of kangaroo lymphocytes. However, a significant increase in nuclear dry mass was not detected until the cells had been exposed to PHA for six hours. After this time, there is a steep rise in the mean nuclear dry mass during the rest of the G1 period. (See figures 34, page 116, and 36, page 119).

From this evidence it can be seen that there is a fairly prompt commencement of nuclear protein synthesis in kangaroo lymphocytes stimulated with PHA; this conclusion is backed up by the presence of nuclear labelling in phenylalanine-H^3-labelled cells after four hours exposure to PHA (see figure 32, page 111).

The following points can therefore be made:-

(a). The initial rapid increase in the rate of turnover of RNA in kangaroo lymphocytes stimulated with PHA parallels that found in similarly treated

human lymphocytes.

(b). Labelled RNA appears in the cytoplasm much sooner in kangaroo lymphocytes treated with PHA than is the case in similarly-treated human lymphocytes.

(c). Gamma-globulin production by both kangaroo and human lymphocytes exposed to PHA appears to be parallel in timing, and is a very rapid response. This form of protein synthesis appears to be separate from the synthesis of structural nuclear and cytoplasmic protein.

(d). The appearance of labelled cytidine in the cytoplasm probably reflects important changes in cellular RNA metabolism, which enable the cell to synthesise structural cytoplasmic and nuclear protein. This event has been recorded 12 hours prior to the increase in the rate of DNA synthesis in human cells (Winter and Yoffey, 1965) and 12 hours prior to the increase in the rate of DNA synthesis in kangaroo lymphocytes (see table 8, page 103; figure 10, page 58). This implies that the rates of protein synthesis are similar in both cases after synthesis has started, if it is assumed that approximately the same amount of protein must be synthesised in each case.

However, the difference in timing of the initiation of structural protein synthesis is puzzling. The

marsupial lymphocytes obviously respond to treatment with PHA more rapidly than do the human lymphocytes, at least as far as events directly associated with the cell cycle are concerned, but the significance of this and the precise mechanism involved are hard to assess. If the delay found in human lymphocytes allows time for increased synthesis of gamma-globulin, then this could be an advantage. However, the amount of gamma-globulin produced by the lymphocyte is probably so small that any advantage gained would be negligible. Furthermore, it is highly probable that gamma-globulin production by the lymphocyte is only a minor aspect of its function, and so the reason for the delayed onset of protein synthesis in the human lymphocyte must be looked for elsewhere.

It is not surprising that differences in response between the marsupials and the eutherians exist. Colbert (1955) concluded that the marsupials and the placental mammals both arose from one of the four early mammalian orders, the Pantotheria, during the Cretaceous; this long separation would allow ample time for the evolution of marked differences in lymphocyte response between the two groups.

The more rapid initiation of nuclear protein synthesis in kangaroo lymphocytes treated with PHA

(in comparison with similarly treated human lymphocytes) is probably responsible for the more rapid onset of DNA synthesis in the marsupial cells. Killander and Zetterberg (1965 a) showed that the intercellular mass variation among cells which had just initiated DNA synthesis (early S period cells) was significantly less than that among cells in the initial stage of interphase. It has also been shown that cells in populations with low initial masses spent a relatively long time in the G1 period and a relatively short time in the S and G2 phases of the cell cycle, while the opposite applied when the cells had high initial masses (Killander and Zetterberg, 1965 b). These findings support the hypothesis that the initiation of DNA synthesis is dependent on, or related to, the cell mass.

If the initiation of DNA synthesis is dependent on the attainment of a certain critical cell mass, then it is clear that the earlier that protein synthesis commences, the earlier will DNA synthesis commence. As protein synthesis (apart from gamma-globulin synthesis) commences in kangaroo lymphocyte cultures only three to six hours after the addition of PHA, the onset of DNA synthesis will be correspondingly advanced, as the early commencement of protein synthesis

enables the earlier attainment of the critical cell mass necessary for the commencement of DNA synthesis. However, this in turn depends on the relationship between two factors - the rate of protein synthesis, and the amount of protein synthesis required before the synthesis of DNA can commence. Nevertheless, it appears that once protein synthesis has commenced, the timing of the cell cycle is much the same in human and marsupial lymphocytes.

4.3. Asynchronous syntheses of DNA and histone.

Studies on various tissues in the past have shown that DNA/histone ratios during the mitotic cycle remained constant, indicating that DNA and histone were being synthesised concurrently (Bloch and Godman, 1955 a, b; Alfert, Bern, and Kahn, 1955; Ansley, 1957; De, 1961; Evans, Holbrook, and Irvin, 1962; Irvin, Holbrook, Evans, McAllister, and Stiles, 1963; Meek, 1964; Niehaus and Barnum, 1965). These observations led to the conclusion that DNA and histone were, in effect, being synthesised as a nucleoprotein complex (Bloch and Godman, 1955 a, b). However, the only observation on the synthesis of histone in cells of lymphoid tissue was made by Pogo, Allfrey, and Mirsky (1966), who showed that the incorporation of alanine-1-C^{14} into the

histones of human lymphocytes treated with PHA was negligible up to 24 hours after the addition of PHA, but increased after that; unfortunately, they made no attempt to relate this to the synthesis of DNA.

It appears that the primary target of PHA activity is part of the histone of the nucleohistone complex. This applies also to antigenic stimulation of presensitised lymphoid cells; however, PHA apparently has a more radical effect than antigen on the nucleohistone complex. Some differences in the synthesis of RNA between PHA-treated and antigen-stimulated lymphocytes have been reported. Cooper and Rubin (1965) showed that there was a higher percentage of non-ribosomal RNA synthesised in the early stages of lymphocyte response to PHA than there was in presensitised lymphocytes treated with antigen. Torelli, Quaglino, Artusi, Emilia, Ferrari, and Mauri (1966) have shown that there is a marked difference in the pattern of synthesis of RNA in PHA-treated cells in comparison with cells of the normal human bone marrow plasma cell series. In the cells treated with PHA they showed a striking and parallel increase in the rates of both RNA synthesis and protein synthesis, whereas in the plasma cell series an increase in the rate of protein synthesis was accompanied by a progressive decrease in the rate of RNA synthesis during

maturation. Unfortunately, no similar comparison has been made between PHA-treated and antigen-treated lymphocytes.

It seems likely, from the above evidence, that PHA damages mechanisms which normally regulate the synthesis of RNA. This damage, furthermore, persists throughout the cell cycle. If it is accepted that histone plays some part in these control mechanisms, then it is logical to expect that some differences in histone metabolism will occur throughout the cell cycle, as the original alteration in the nucleohistone complex is apparently non-reversible. In this context, it is interesting to find that the synthesis of DNA and the synthesis of histone are asynchronous in lymphocytes treated with PHA.

The asynchrony of the syntheses of histone and DNA in PHA-treated kangaroo lymphocytes was demonstrated independently by different methods, using different fixation techniques. The asynchrony observed was almost certainly due to the late synthesis of histone, rather than a delay in the complexing of existing histone with newly-synthesised DNA. The data obtained by using microspectrophotometry with alkaline fast green is slightly ambiguous in this respect, as only histone included in the nucleohistone complex will be stained.

However, delayed synthesis of histone is shown by the delayed incorporation of tritiated lysine, relative to the uptake of tritiated thymidine. Similarly, the finding that histone synthesis continues after the synthesis of DNA has stopped also reflects an important departure from the usual pattern of histone synthesis. It has been fairly conclusively established that DNA and histone are synthesised concurrently in normal tissues, and, although it has not been established, the same relative timing can be assumed for lymphoid tissue. If the asynchrony of the syntheses of histone and DNA in kangaroo lymphocytes treated with PHA reflects damage to the systems regulating the synthesis of histone in the lymphocyte nucleus, then it can be predicted that no such asynchrony will be found in presensitised lymphocytes stimulated with specific antigens. Alterations induced by PHA in the nucleo-histone complex could account for the differences in the patterns of synthesis of RNA detected by Torelli and his co-workers. Cooper and Rubin (1965, 1966) concluded that the abundant RNA produced at the commencement of growth of PHA-treated cells could be messenger RNA being produced in large quantities, "perhaps as a result of the abrogation of normal regulatory processes by PHA." This comment could

also apply to the later stages of the cell cycle.

4.4. Changes in histone after exposure to PHA.

Black and Ansley (1965) showed that when mouse thymocytes were exposed to antigen, they displayed a decrease of 20 % in fast green stainability within one hour of exposure to antigen. A 32 % decrease in stainability with ammoniacal silver was also recorded by them over this period. On the basis of this evidence they suggested that there was a real decrease in the content of histone, and a loss of histone from the nucleus, but they were unable to decide whether the decrease reflected discharge or degradation of the histones. On the other hand, Killander (1966) pointed out the possibility that the staining alterations might have been due to changes in the dye-binding groups of the histone, possibly brought about by a blocking effect of non-histone substances.

A precisely similar effect can be shown in lymphocytes exposed to PHA (see figure 38, page 123). Furthermore, Killander and Rigler (1965) showed that there was an immediate increase in the fluorescence of lymphocytes which had been exposed to PHA and stained with acridine orange. Pogo, Allfrey, and Mirsky (1966) showed that histone acetylation (but not histone

synthesis) was greatly increased in lymphocytes responding to PHA. These results certainly show that PHA detaches histone from the DNA-histone complex, but shed little light on the subsequent distribution of histone within the cell. There are several possibilities which can be considered.

(a). PHA may compete with DNA by forming a complex with the same affinity points in histone which complex with DNA. The histone fractions not affected by PHA would then be available for staining after the extraction of nucleic acids, while the histone in the PHA-histone complex would remain unstained.

(b). There may be degradation of histone by PHA; this would imply that only a certain fraction of the histone present would be susceptible to PHA-induced degradation. Black and Ansley (1965) conclude that the antigen-labile histone fraction in mouse thymocytes is lysine-rich. Possibly it is this fraction, or an essentially similar one, which is also affected by PHA.

(c). The histone or histone-derived material may remain in the nucleus.

(d). The histone or histone-derived material may migrate to the cytoplasm.

(e). Histone or histone-derived material may leave the cell altogether.

These alternatives cannot be resolved on microspectrophotometric grounds, and at this point the changes in nuclear and cytoplasmic dry mass must be considered. During the first forty minutes of culture after treatment with PHA the mean loss of dry mass recorded from the nuclei of small lymphocytes was 1.9×10^{-12} gms. (See figure 39, page 125). There is no direct evidence to show that this loss is due to the migration of histone or histone-derived materials. However, the mass lost correlates fairly closely with the theoretical mass of histone affected by PHA. This figure can be calculated on the basis of the proportion of histone in the nucleus (Stedman and Stedman, 1951) coupled with the decrease of histone stainability with alkaline fast green, and it amounts to 2.17×10^{-12} gms. This figure, however, must be treated with some reserve, as the proportion of histone in the nuclei of lymphoid cells of marsupials has not been estimated. Nevertheless, in the absence of further information the figure can be used as a general guide.

It seems possible that histone is removed from the nucleus through the action of PHA. If removal of histone is responsible for the decrease in mean nuclear dry mass, then this removal must occur in most, if not all, of the lymphocytes exposed to PHA; otherwise

the mean decrease in nuclear dry mass would not be so high.

Two complicating factors must be discussed here. First, Black and Ansley (1965) recorded a 32 % loss of ammoniacal-silver-stainable histone in thymocytes stimulated with antigen, as against a loss of only 20 % measurable by alkaline fast green staining in similarly treated thymocytes. This figure of 32 % would then suggest a much greater loss of histone than would the 20 % decrease in fast green stainability. However, Black and Ansley also found that ammoniacal silver stained lysine-rich histones more readily than it stained arginine-rich histones, whereas alkaline fast green was more selective. De (1961) concluded that the density of staining with alkaline fast green was directly proportional to the total amount of histone present. It would thus appear that the decrease in stainability with alkaline fast green does represent the true proportion of DNA-bound histone affected by PHA.

Second, Cooper and Rubin (1965) detected a decrease in the total amount of RNA in lymphocytes after 30 minutes' exposure to PHA. In a series of experiments, they found that varying amounts of RNA were lost, ranging from 5 % to 33 % of control totals. RNA levels

continued to fall for one to two hours after the addition of PHA. The problem thus arises: does the decrease in nuclear dry mass (and the corresponding increase in cytoplasmic dry mass) detected in kangaroo lymphocytes represent a loss of histone from the nucleus to the cytoplasm, or a loss of RNA, or both?

The evidence available to resolve this problem is circumstantial. The loss of RNA recorded by Cooper and Rubin was apparently a loss from the cell as a whole. However, they concluded that the observed decrease was due to the degradation of RNA by PHA, rather than a physical removal of RNA. They noted that the first detectable decrease in the amount of RNA occurred only after thirty minutes' exposure to PHA, with accelerated RNA synthesis occurring after one hour. This timing does not coincide with the timing of the decreases in nuclear dry mass found in kangaroo lymphocytes (see figure 39, page 125). The timing of dry mass losses in this case corresponds much more closely with the observed decreases in histone stainability. Furthermore, the rapid increase in the rate of synthesis of RNA in kangaroo lymphocytes could mask the decrease in total cellular RNA resulting from exposure to PHA. Two hours after treatment with PHA, a slight <u>increase</u> in the dry mass of nucleic acids in

kangaroo lymphocytes is detectable, (see figure 36, page 119), and this also argues against any earlier detectable decrease in the amount of RNA in the cell.

A further point arises here. If histone is transferred from the nucleus to the cytoplasm by the action of PHA, it would still be present in the cell; no decrease in ammoniacal silver stainability or fast green stainability should therefore be detected. However, if PHA forms a complex with the same affinity points in histone which form a complex with the stain or with DNA, then the subsequent removal of nucleic acids prior to staining would not liberate this histone from the PHA-histone complex formed, and a decrease in ammoniacal silver or fast green stainability would result. A similar decrease would result if PHA merely split the DNA-histone complex so that the freed histone could form a complex with the acidic proteins available in the nucleus.

Black, Ansley, and Mandl (1964) found that after an injection of antigen there were decreases in the sizes of spleen and thymus associated with a decreased histone content of these organs. This implies a loss of histone from the cell as a whole, and not merely a transfer of histone from the nucleus to the cytoplasm.

In summary, the following points can be made.

(a). The rapidity of the recorded dry mass changes in the nucleus and cytoplasm implies that the changes are due to the transfer of histone-derived, rather than RNA-derived, material.

(b). The magnitude of the mean nuclear dry mass decrease corresponds with the theoretical mass of histone affected by PHA.

(c). If the timing of the loss of RNA recorded by Cooper and Rubin applies also to kangaroo lymphocytes, then this loss would be masked to some extent by the more rapid increase in the rate of synthesis of RNA recorded in the marsupial cells.

(d). If histone or histone-derived material is lost from the cell, then this loss must occur after the commencement of uptake of extracellular material, or there would be an initial decrease in the mean cellular dry mass.

4.5. The mechanism of lymphocyte stimulation by PHA.

A number of different aspects of the action of PHA on blood cells have been recorded. Its agglutinating activity on erythrocytes has been known for many years (Dorset and Henley, 1916). However, this property can be removed from PHA without impairing its mitogenic qualities (Barkhan and Ballas, 1963), and so

this aspect does not need to be considered in any attempt to explain the mitogenic effects of PHA on lymphocytes. However, the removal of the haemagglutinating property of PHA does not affect its leucoagglutinating properties, and this has prompted several workers to conclude that agglutination of leucocytes plays an important part in, or is primarily responsible for, the mitogenic activity of PHA (Hastings, Freedman, Rendon, and Cooper, 1961; McIntyre and Ebaugh, 1962; Kolodny and Hirschhorn, 1964; Mueller and le Mahieu, 1966). Recently, however, other actions of PHA on the lymphocyte have been recorded, and these must be taken into account in any explanation of the mitogenicity of PHA. These actions are as follows:-

(a). Acetylation of histone was observed by Pogo, Allfrey, and Mirsky, (1966). It was found that histone acetylation (but not histone synthesis) was greatly increased in lymphocytes responding to PHA. This increase in acetylation of the basic proteins of the chromosomes appeared to precede the increase in the rate of synthesis of nuclear RNA.

(b). Killander and Rigler (1965) detected an increase in the fluorescence of acridine-orange-stained lymphocytes exposed to PHA. This represents an increase in the number of acridine-orange-binding PO_4^-

groups in the deoxyribonucleoprotein complexes of the stimulated lymphocytes.

(c). A decrease in the total amount of cellular RNA was detectable in human lymphocytes after an exposure of thirty minutes to PHA (Cooper and Rubin, 1965).

(d). A decrease in stainability with alkaline fast green was detectable in kangaroo lymphocytes after six minutes' exposure to PHA. This decrease levelled off after twenty-four minutes, and amounted to about 22 % of control total histone values. A similar decrease has been recorded in antigen-stimulated mouse thymocytes (Black and Ansley, 1965).

(e). A small decrease in nuclear dry mass and an increase in cytoplasmic dry mass was observed in kangaroo lymphocytes. (See figures 39 and 40).

(f). Increases in the rate of turnover of nuclear RNA have been detected by several workers (Winter and Yoffey, 1965; Cooper and Rubin, 1966; Mueller and le Mahieu, 1966; Pogo, Allfrey, and Mirsky, 1966). A correspondingly rapid increase was also detected in kangaroo lymphocytes.

These effects of PHA appear at first sight to be diverse and unrelated; however, they can be readily divided into two groups. The first of these groups

can be considered as primary effects, and are directly attributable to the action of PHA on the DNP of the nucleus. This group includes the acetylation of histone, the increase in the number of acridine-orange-binding PO_4^- groups in the DNP complex, and the reduction in the stainability of histone with alkaline fast green. The second group comprises the secondary effects, which arise as a result of the modifications seen in the first group. These secondary effects are the decrease in total RNA, the increase in the rate of turnover in RNA, and the changes in mean nuclear and cytoplasmic dry mass after treatment with PHA.

It can be argued that the primary effects are all aspects of the same phenomenon. Killander and Rigler showed in 1965 that mature human spermatozoa have six times less capacity for binding acridine orange than human lymphocytes unstimulated with PHA; the DNP complex of the spermatozoa is characterised by a higher percentage of basic amino acids having stronger positive charges. This implies that acridine orange fluorescence values in the lymphocytes in question are proportional to the extent of the dissociation of the nucleohistone complex. Thus an increase in acridine orange fluorescence values after exposure of lymphocytes to PHA implies a PHA-induced dissociation of histone from

DNA. Similarly, the increased acetylation of histone detected by Pogo and his co-workers reflects a higher availability of free histone in the nucleus. This is supported by the decrease in histone stainability with alkaline fast green. This staining reaction is carried out after the removal of nucleic acids; any histone removed from the DNP complex and bound elsewhere in the cell would no longer be available for staining after the removal of nucleic acids.

The secondary effects can largely be explained in the light of histone removal from the chromosomes. Since Stedman and Stedman (1951) proposed that histones were active as gene inhibitors, much circumstantial evidence has been put forward to support this view (Huang and Bonner, 1962; Allfrey, Littau, and Mirsky, 1963 a, b; Billen and Hnilica, 1963; Busch, Steele, Hnilica, Taylor, and Mavioglu, 1963; Allfrey, Faulkner, and Mirsky, 1964). Allfrey, Faulkner, and Mirsky showed that acetylation of histones lowered their effectiveness as inhibitors of the RNA-polymerase reaction. Histone removed from the DNP complex through the action of PHA would no longer inhibit RNA synthesis by those parts of the nucleoprotein complex that were affected. Thus the increase in RNA turnover recorded in PHA-treated lymphocytes could result directly from

removal and acetylation of DNA-bound histone. The decrease in nuclear dry mass in kangaroo lymphocytes treated with PHA may be due either to transfer of histone or histone-derived material from the nucleus, or else to the loss of RNA from the cell.

The precise significance of the decrease in total cellular RNA reported by Cooper and Rubin is hard to assess. They consider that the primary effect of PHA may be the degradation of pre-existing RNA, including stable messenger RNA concerned with the maintenance of the resting state, causing interruption of resting cell processes and allowing the onset of growth. RNA values were measured by ultraviolet absorption at 2600 Å, and the earliest change recorded was a decrease in total RNA after thirty minutes' exposure to PHA. As changes in histone binding are detectable much earlier than this, it appears that the degradation of pre-existing RNA is not one of the primary effects of PHA, and need not be due to the direct action of PHA at all. If PHA does actively degrade RNA, it would presumably continue to do so throughout interphase. There is, as yet, no evidence to support this; however, the absence of evidence does not rule out the possibility.

Other explanations of the mitogenic activity of

PHA besides that of Cooper and Rubin have been put forward. The first of these was advanced by Hastings, Freedman, Rendon, Cooper, and Hirschhorn in 1961; they suggested that the surface-active properties of PHA may be responsible for its mitogenicity. Other workers have supported this theory (McIntyre and Ebaugh, 1962; Kolodny and Hirschhorn, 1964; Mueller and le Mahieu, 1966). Vassar and Culling (1964) demonstrated that PHA was adsorbed onto the surface of lymphocytes, but said that the significance of this finding in relation to the subsequent blastic transformation of the cells was not known. In fact, no detailed explanation of any mechanism by which the surface active properties of PHA could be directly responsible for its mitogenicity has been put forward.

A second hypothesis was formulated by Beckman (1962), who suggested that PHA precipitated a protein which normally inhibited mitosis. He supported this hypothesis with evidence obtained from starch-gel electrophoresis studies, which showed that both serum and cell proteins were precipitated by PHA. However, Holland and Holland (1965) found that when PHA was heated for five minutes at 85° C., its haemagglutinating activity was reduced, and its serum-precipitating activity entirely removed, but its mitogenicity was not affected. They also

reported that the serum protein precipitated by PHA were not antibody, and concluded that PHA was not an indiscriminate antigen.

A third explanation is now more popular; this derives from the observation by Pearmain, Lycette, and Fitzgerald (1963) that tuberculin-stimulated leucocyte cultures from Mantoux positive donors showed mitotic activity, similar to that found in cultures stimulated with PHA. The logical outcome of this observation was the hypothesis that PHA acted as a general immune-like stimulus on immunologically competent cells (Hirschhorn, Kolodny, Hashem, and Bach, 1963; Lindahl-Kiessling and Böök, 1964; Tao, 1964; Bain, Vas, and Lowenstein, 1964).

There are numerous difficulties in the way of this interpretation. First of all, 95 % of the lymphocytes in culture are affected in some way by PHA (Hirschhorn, Bach, Kolodny, Firschein, and Hashem, 1963), whereas the proportions of presensitised lymphocytes affected by antigen are markedly lower, and range from 5 % to 33 % (Pearmain, Lycette, and Fitzgerald, 1963; Elves, Roath, and Israels, 1963; Bain, Vas, and Lowenstein, 1964). Second, the response to PHA is more rapid than it is to antigenic stimulus; mitosis in cultures treated with PHA starts 42 - 48

hours after the addition of PHA (Bender and Prescott, 1962; Cooper, Barkhan, and Hale, 1963; Michalowski, 1963), whereas antigen-stimulated presensitised lymphocytes only showed blastoid cells after 3 - 5 days of culture. Third, Cooper and Rubin (1966) showed that when lymphocytes were treated with PHA, the rapidly synthesised RNA produced as a result was mainly non-ribosomal, in contrast to the mainly ribosomal RNA produced by presensitised lymphocytes exposed to antigen. Fourth, the patterns of synthesis of RNA throughout interphase in PHA-treated lymphocytes and cells of the normal plasma cell series are not similar (Torelli, Quaglino, Artusi, Emilia, Ferrari, and Mauri, 1966). Fifth, PHA has been observed to induce mitoses in cultures of human epithelial cells of adult skin (Sarkany and Caron, 1965), free living amoebae (Agrell, 1966), and in various normal and neoplastic cells in vitro (Ioachim, 1966).

All this evidence argues against an immunologic mechanism of PHA activity. Certainly the products of lymphocyte stimulation by PHA and by antigens are morphologically very similar, and some of the initial effects of PHA parallel the initial effects of antigens very closely. The decrease in stainability with alkaline fast green in kangaroo lymphocytes exposed to

PHA closely parallels that in mouse thymocytes treated with antigen. However, Inman and Cooper (1963) concluded, after an electron microscope study of human lymphocytes stimulated with PHA, that the products of division were unlike a normal small or large lymphocyte, but had much in common with cancer cells. Lymphocytes treated with antigens exhibit a controlled response; this control of intracellular interphase processes is not so apparent in the case of cells stimulated with PHA, possibly because the splitting of the DNP complex is not so selective. This is reflected by the higher turnover of non-ribosomal RNA reported by Cooper and Rubin (1965), and possibly by the difference in the patterns of RNA synthesis throughout interphase reported by Torelli and his co-workers.

Roth (1965) put forward the view that tumour formation may result from interference with histone acetylation or with other characteristics of histone, resulting in malfunctioning of regulatory processes. This would result in a fairly random release of some histone repressions and the subsequent synthesis of some enzymes. In a few cases the pattern of random changes resulting might be such as to favour continued growth and cell division.

A similar sequence of events applies in the case

of phytohaemagglutinin; however, in this case only about 20 % of the DNA-bound histone can be regarded as the direct target of the action of phytohaemagglutinin. This histone fraction may possibly correspond with the antigen-labile, lysine-rich histone identified by Black and Ansley (1965). However, as PHA also induces mitoses in non-lymphoid tissue (Sarkany and Caron, 1965; Agrell, 1966; Ioachim, 1966), it presumably affects other histone fractions as well. Probably only a small change would be necessary. It would be very interesting to study the effect of PHA on the histones of the cells of non-lymphoid tissue known to respond to it.

4.6. Synthesis of chromosomal residual protein.

Cave (1966) studied the incorporation of lysine-H^3 into the chromosomal residual protein of human lymphocytes treated with PHA. His conclusions were reached on the basis of pulse labelling experiments, in which no isotope was added until the cells had been exposed to PHA for 60 hours. He concluded that lysine-H^3 was incorporated into chromosomal residual protein throughout the whole of interphase, with the highest rates of incorporation being found late in the S period and through the whole of the G2 period. He noted that an average of 73.5 % of the cells in culture after 60

hours of incubation with PHA had incorporated lysine-H^3 into nuclei, and suggested that the remainder were cells which were not influenced by PHA to undergo DNA synthesis and subsequent division. He further suggested that "Granulocyte leukocytes which do not show a mitotic response to PHA probably account for the non-duplicating population." MacKinney, Stohlman, and Brecher (1962) concluded that in human blood all neutrophils and eosinophils had disappeared after 24 hours in culture. Similar results apply to cultures of kangaroo leucocytes, except that some eosinophile leucocytes persist; these account for about 5 % of the cell population after 48 hours of incubation with PHA (see figure 9, page 56). Cave's conclusion that lysine-H^3 is incorporated into chromosomal residual protein throughout the <u>whole</u> of interphase is hard to support. It seems much more likely that this synthesis starts during the DNA-synthesis period. This is supported by two findings:-

(a). Kangaroo lymphocytes exposed to lysine-H^3 over the first 14 hours of culture showed no sign of label incorporation whatsoever (see figure 11, page 63; figure 12, page 65).

(b). After 60 hours of incubation with PHA, the percentage of the cell population incorporating arginine

into chromosomal residual protein is lower than the percentage of cells in S and G2 (see table 7, page 94).

The unlabelled cells seen by Cave could thus be cells in G1 prior to their second division in culture.

Nevertheless, the pattern of synthesis of chromosomal residual protein in marsupial lymphocytes could conceivably be quite different from the pattern found in human lymphocytes.

4.7. Does PHA-induced cell replication represent a normal cell cycle?

It was hoped at the commencement of the study that an investigation of the changes induced by PHA in leucocytes of peripheral blood would yield information on the relative timing and interdependence of various events in the cell cycle. However, as PHA appears to act by interfering with the normal regulatory processes of the cell, the cell cycle induced by PHA cannot be regarded as a "normal" one. This conclusion arises, in fact, from differences in the responses of PHA-treated and antigen-stimulated lymphocytes, and from differences in the mitotic cycles of PHA-treated lymphocytes and cells of the normal plasma cell series.

There are, of course, some similarities; the decrease in the stainability of histone which is induced

by PHA in kangaroo lymphocytes closely parallels that induced in mouse thymocytes by antigen (Black and Ansley, 1965). Nevertheless, there is a slight difference here also, in that the drop recorded in the kangaroo lymphocytes is slightly more than the drop in stainability recorded for the mouse cells.

Three problems must therefore be considered in this section:-

(a). How does antigen stimulate controlled growth and gamma-globulin synthesis in presensitised lymphocytes?

(b). How does the action of PHA differ from that of an antigen, and why does PHA induce growth in cells which are not immunologically competent?

(c). Why do the results of PHA stimulation differ from those induced by antigens?

To answer the first question, the function of the circulating small lymphocyte must be considered. Gowans (1959) concluded that there was a lymphocyte population whose cells had a life span of 180 to 200 days. This conclusion has been supported by the findings of other workers (Brecher, Little, Bradley, and Rose, 1961; Caffrey, Rieke, and Everett, 1962; Little, Brecher, Bradley, and Rose, 1962; Buckton and Pike, 1964 a, b). Lymphocytes circulating in

peripheral blood are in a resting state under normal conditions, and the only genetic information needed by them is that information which is required to maintain the resting state of the cell. Paul and Gilmour (1966) concluded that in differentiated cells only a part of the total genetic complement may be available for transcription, and they proposed the term "nome" for this part. Viewed in this context, the lymphocyte must be regarded as a special case. Stimulation of presensitised lymphocytes by specific antigens induces growth and division (Pearmain, Lycette, and Fitzgerald, 1963; Schrek, 1963; Marshall and Roberts, 1963a; Cowling, Quaglino, and Davidson, 1963; Elves, Roath, and Israels, 1963; Hashem and Barr, 1963; Hirschhorn, Kolodny, Hashem, and Bach, 1963; Hirschhorn, Bach, Kolodny, Firschein, and Hashem, 1963; Lycette and Pearmain, 1963; Ling and Husband, 1964; Matsianotis and Tsenghi, 1964; Holland and Mauer, 1964). This growth and division is under genetic control, and the resting cell must retain this information within the nucleohistone complex. However, this information is obviously not available for transcription during the resting stage of the lymphocyte life cycle. As a direct result of exposure to specific antigens, presensitised lymphocytes commence growth. This implies

that the information regulating growth is made available for transcription either as an integral part of the process of antigen recognition, or as a direct result of that process.

Antigen recognition and gene activation in lymphoid cells is marked by a striking loss of histone stainability (Black and Ansley, 1965). This implies a loss of histone from the nucleohistone complex. Huang and Bonner (1962) presented evidence to show that DNA which was fully complexed with histone was inactive in the support of RNA synthesis, and concluded that histone functioned as a suppressor of genetic activity. Thus a removal of histone from the DNP complex would allow an increase in the rate of turnover of RNA. The histone fraction affected by antigen would presumably be specific for lymphoid cells, and its removal would allow the cell to embark on a controlled pattern of response. Effectively, the removal of histone would represent a broadening of the "nome" of Paul and Gilmour.

It is now well established that PHA also affects a histone fraction in lymphocytes (Killander and Rigler, 1965; Pogo, Allfrey, and Mirsky, 1966; see also page 123). This fraction may very well correspond with the antigen-labile fraction mentioned above, but an

explanation of the activity of PHA based on the supposition that PHA is a universal antigen is clearly insufficient (see page 155). It is logical to conclude that the activity of PHA is mediated through its effect on histone, but it is also clear that this effect is not precisely similar to the effect of antigen on presensitised lymphocytes. It appears that the response of lymphocytes to PHA is not a precisely controlled one. PHA probably affects other histone fractions apart from the antigen-labile lysine-rich fraction reported by Black and Ansley (1965). If cellular synthetic processes are under the control of the nucleohistone complex, with histone functioning to control the availability of genetic information contained in the DNA, then any damage to the nucleo-histone based regulatory mechanism will lead to a loss of control of synthetic processes. Changes in the association between DNA and histone could be expected as a result of this damage, and these changes are reflected by the asynchrony of the syntheses of DNA and histone recorded in marsupial lymphocytes treated with PHA. Growth induced by PHA in other cells which are not immunologically competent could also be explained along these lines. Furthermore, the high death rates in lymphocyte populations, and in the

cells of the granulocyte series, when they are cultured with PHA are understandable if PHA interferes with the nucleohistone-based regulatory processes of the cell.

From this point of view also, any attempts to identify the end products of growth induced by PHA as normal leucocyte types become meaningless. Inman and Cooper (1963) concluded, from electron microscope studies, that the products of division of human lymphocytes stimulated with PHA had much in common with cancer cells.

Roth (1965), in a discussion of the role of histones in cancer, suggested that interference with histone acetylation or other characteristics of histone may result in a fairly random pattern of release of some histone repressions, with consequent uncontrolled growth. Seed (1966 a, b, c, d) observed differences in RNA and protein metabolism in cancer cells in comparison with results obtained from healthy embryo cells; the differences largely centred around a dissociation of the syntheses of RNA and protein from the synthesis of DNA. This dissociation can also be observed in PHA-treated cells, and may arise from a similar interference in cell regulatory mechanisms.

5. APPENDIX

5.1. Calculation of dry mass.

Dry mass calculations were based on the method developed by Barer (1953, 1956). The dry mass of a cell can be estimated by measuring phase changes introduced in polarised monochromatic light by the cell, as

$$\text{dry mass} = \frac{\theta \times \text{projected area of cell}}{100\alpha}$$

where α = 0.0018, which is the "specific refraction increment" for protein.

$$\text{projected area} = \frac{\pi}{4} \times \left(\frac{\text{diameter 1} + \text{diameter 2}}{2}\right)^2$$

$$\text{and } \theta = \frac{\lambda \times \text{"path difference"}}{180}$$

The path difference referred to could be measured directly by the microscope used, and was simply the difference between the reading over the cell and the reading over an adjacent clear space.

The wavelength used (λ) was 5460 Å.

5.2. Calculation of regression coefficients.

The regression coefficient, b, of y on x in figures 24 and 25 (page 91) was determined from the expression

$$b = \frac{\Sigma xy - \frac{\Sigma x \Sigma y}{n}}{S^2x}$$

where $S^2x = \Sigma x^2 - \frac{(\Sigma x)^2}{n}$

The variance about the regression line is given by

$$s^2 = \frac{\Sigma(y - \bar{y})^2 - b^2S^2x}{n - 2}$$

The estimate of b, with its attached standard error, may then be written as

$$b \pm \frac{s}{\sqrt{\Sigma(x - \bar{x})^2}}$$

The statistical significance of the difference between the trend lines shown on figures 24 and 25 is determined on page 169.

	Acetic/alcohol-fixed cells.	FAA-fixed cells.
n	50	50
Σx	913.2	906.1
Σy	902	1123
Σx^2	16938.24	16863.69
Σy^2	20750	38477
Σxy	17420.4	22671.7
$\Sigma \bar{y}$	18.04	22.46
$\Sigma \bar{x}$	18.26	18.12
$\Sigma (y - \bar{y})$	382.0	639.0
$\Sigma (y - \bar{y})^2$	4494.0	13254.5
$\Sigma (x - \bar{x})$	101.7	120.3
$\Sigma (x - \bar{x})^2$	269.99	442.84
S^2x	259.56	443.35
b	3.54 ± 0.31	5.23 ± 0.23
s^2	25.685	23.491
s	5.068	4.847

The two regression coefficients were then compared, using the expression

$$d = \frac{b_1 - b_2}{\sqrt{\frac{s_1^2}{\Sigma_1 (x_1 - \bar{x}_1)^2} + \frac{s_2^2}{\Sigma_2 (x_2 - \bar{x}_2)^2}}}$$

where the suffixes 1 and 2 refer to the two samples, and

d is normally distributed with zero mean and unit standard deviation.

$$d = 4.391$$

$$p < 0.001$$

The difference between the regression coefficients of the two populations is thus highly significant.

5.3. Contingency tables from table 7, page 94.

	Labelled	Unlabelled	Total
DNA	138	367	505
Protein	350	673	1023
Total	488	1040	1528

$\chi^2 = 7.376$ ** , with one degree of freedom. This indicates that labelling of protein continues after labelling of DNA has stopped.

			Total
DNA	Labelled & G2 197	G1 308	505
Protein	Labelled 350	Unlabelled 673	1023
Total	547	981	1528

$\chi^2 = 3.385$, which, with one degree of

freedom, is not significant.

5.4. Theoretical mass of histone affected by PHA.

The dry mass of histone affected by PHA can be calculated from the formula

$$m = a \times \frac{b}{100} \times \frac{c}{100}$$

where m = mass of histone affected

a = mean dry mass of control nuclei

b = percentage of nuclear dry mass which is accounted for by histone

and c = mean percentage loss of stainability with alkaline fast green.

Thus $m = 34.0 \times \frac{28}{100} \times \frac{22}{100} \times 10^{-12}$ grams,

$= 2.09 \times 10^{-12}$ grams after the cells had been exposed to PHA for 24 minutes.

"b" has not been estimated for kangaroo lymphoid cells, and the estimated figure for ox spleen and thymus (Stedman and Stedman, 1951) must be used instead. There is no reason to believe that the histone content of the lymphoid cells of the kangaroo will differ greatly from this figure; nevertheless, there is an element of

doubt. However, it is possible, using this approach, to directly compare the data given in figure 38, page 123, with that in figure 39, page 125. By inspection of the graphs, it can be seen that the percentage decrease in stainability with alkaline fast green stabilises 24 minutes after the addition of PHA. Similarly, the lowest mean value for nuclear dry mass is found 40 minutes after the addition of PHA. If it is assumed that the affected histone takes about 15 minutes to leave the nucleus, then the two figures can be compared directly, as follows:-

Minutes after addition of PHA.	Theoretical mass of histone affected (gms. x 10^{-12})	Actual time plus 15 minutes.	Actual mean mass decrease (gms. x 10^{-12})
5	0.67	20	0.7
12	1.01	27	1.2
24	2.09	39	1.9

$\chi^2 = 0.06$ with two degrees of freedom

$p > 0.99$, which indicates suspiciously good agreement.

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