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EFFECTS OF 5-AZACYTIDINE ON GROWTH CONTROL MECHANISMS IN PHA STIMULATED EQUINE LYMPHOCYTES

By

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CONTENTS

ABBREVIATIONS

These are in accordance with those published in the Biochemical journal in the section "Instruction to authors" revised (1971) with the following additions.

PHA	Phytohaemagglutinin
PWM	Pokeweed mitogen
5-azaCyd	5-azaCytidine
5-FUra	5-FluoroUracil
DC AMP	Dibutyryl cyclic AMP
WBC	White blood cells
RBC	Red blood cells
PPD	Tuberculin purified protein derivative
HPEHM	Modified Eagle's Minimum Essential medium Supplemented with 10% autologous horse plasma
BSS	Balanced solt solution
BSA	Bovine serum albumin
TCA	Trichoroacetic acid
SDS	Sodium dodecyl sulphate
HSS	High speed supernatant fraction
PPo	2, 5-diphenyloxazole
tRNA	transfer RNA
rRNA	ribosomal RNA
mRNA	messenger RNA

FITC	Fluorscein isothioCyanate
LDH	Lactic dehydrogenase
G-6-PDH	Glucose-6-phosphate dehydrogenase
ACp	Acid phosphatase
B-GUR	B. GlucoUronidase
DNA polymerase	DNA nucleotidyl transferase EC 2.7.7.7.
Thymidine kinase	ATP: thymidine 5-phospho-transferase. EC 2.7.1.21

INTRODUCTION

CHAPTER 1 - INTRODUCTION

1. GROWTH CONTROL MECHANISMS

One of the challenging problems today is to investigate the mechanisms of growth, cell division and cell differentiation in normal and malignant tissue. Cancer is the term applied to an abnormal and usually uncontrolled growth of a living tissue and hence for the cure of this disease an understanding of the basic mode of growth of a living tissue, and the regulation of its growth is essential.

The basic requirement for this type of research is a suitable system of rapidly growing cells. A number of systems meet this criteria of rapid growth i. e. growing tumours, regenerating tissue (liver, kidney and the granulation tissue of healing wounds) and cultured cells.

The work involving intact animals, such as the use of regenerating rat liver, is subject to variation between animals. In addition, in such complex systems, a number of parameters are to be considered. Bucher and Swaffield (1965) have shown that after partial hepatectomy the UTP pool increases significantly when starved rats are used.

On the other hand Ove, Adam, Abram and Lieberman (1966) have shown that when rats are fed ad libitum, the doubling in the rate of RNA synthesis is accompanied by only a 20-30% increase in the specific activity of the uridylate pool.

Thus difficult manipulations are required to make accurate quantitative analyses. In addition, such complex systems contain a mixture of different types of tissues and cells. It is easier to work with a tissue instead of working with an intact animal

and even easier to work with single cells, than with a tissue containing different types of cells.

Phytohaemagglutinin (PHA) stimulated lymphocyte cultures (invitro) seemed more promising to us, as a system for studies of growth control mechanisms. The reason being that lymphocytes, once considered to be inert and inactive, an endline of blood cell transformation, are today considered pluripotential cells. These cells are very important as they are involved in malignant, inflammatory and immunological processes. The involvement of the lymphatic organ, thymus, and its role in malignant diseases is well known. The use of lymphocytes in modern medicine e. g. in transplant surgery, is quite well known (Billingham - 1969). The part played by lymphocytes in immunological reactions is also worth mentioning. Inspite of these cells being present both in normal and in diseased states very little is known about them.

In recent years a lot of work has been done in this field, in order to understand the basic metabolic patterns of lymphocyte growth.

Lymphocytes from blood normally do not divide invitro, but they are made capable of undergoing mitosis by treating with phytohaemagglutinin, an extract of the kidney bean, phaseolus vulgaris. Comparison shows that similarities exist metabolically, morphologically and immunologically, between leukemic lymphocytes and PHA stimulated lymphocytes.

Hence we selected this system for our studies, since it seems to offer a possible tool to help us understand the mechanism of malignancy, cell division and cell differentiation. Compared to leukemic lymphocytes, PHA stimulated lymphocytes are easily available. For certain experiments it is difficult to

get sufficient quantities of human blood, so we started working with the cultures of horse lymphocytes invitro, stimulated by PHA.

The aim of our studies has been manifold.

(i) To investigate the basic metabolic patterns of lymphocyte growth.

(ii) To investigate the metabolic changes taking place as a result of PHA stimulation, in order to understand what actually happens when lymphocytes are stimulated by PHA. We are interested to know what PHA actually does to convert the metabolically stable and static cells into metabolically active dynamic cells, manifesting mitosis and growth.

The approach adopted to tackle this problem was to activate the cells with PHA; once the cells start growing, to inhibit the growth using certain growth inhibitors. From the knowledge of the mechanism of action of the drug we hoped to work out the mechanism of stimulation of the cells.

5-Azacytidine, an analogue of the normal nucleic acid base cytidine was selected as the growth inhibitor in our studies. The reason for choosing 5- azacytidine as the growth inhibitor in our studies is that Vesely, Cihak, Piskala and Sorm (1964) showed that this drug selectively inhibits the growth of lymphopoietic tissue. Thus, on injecting the drug into mice, it is concentrated into lymphatic organs and retained there.

It is hoped that the use of 5-Azacytidine might demonstrate a process unique to the growth of PHA stimulated lymphocytes thus, working out the mechanism of action of 5-azacytidine also became a part of our investigation.

2. LYMPHOCYTES invivo

Lymphocytes can be defined as a species of WBC which arise in the reticular tissue of the lymph glands and are prominently involved in certain inflammatory, malignant and immunological processes.

2.1 Morphology

In stained smears of blood, the lymphocyte can be distinguished from the rest of the WBC, by its dark rounded nucleus, surrounded by the so called nongranular cytoplasm. The mature cell has a deeply basophilic nucleus, with densely clumped chromatin. The population is extremely heterogeneous and cells of different sizes are found.

(a) Small Lymphocytes are the key cells of the immunological system. They are most numerous, with a diameter of 7-10 um and with densely packed chromatin. They have very little pale cytoplasm and a slightly eccentric nucleus (Maximow and Bloom 1957).

(b) Medium Lymphocytes have more cytoplasm and the size ranges between small and large cells.

(c) Large Lymphocytes with a diameter of 10-18 um are abundant in lymphnodes and spleen (Rebuck, Coffman, Bluhm and Bark - 1964). These cells divide in the lymphoid tissue to give rise to the peripheral blood lymphocytes. Lymphocytes do not stain with peroxidase stain.

2.2 Lymphopoiesis and Origin of lymphocytes:-

Lymphocytes are produced in the central lymphoid orga thymus or the peripheral lymphoid organs like lymphnodes, tonsils, periarterial pulp of the spleen, in bonemarrow etc. There is no unanimity regarding the genesis of blood cells and it is not known whether each cell has a common parent cell or arise from the separate progenitor (for further detail cf. Fletcher - 1968).

2.3 Function of lymphocytes and Lymphoid tissue:-

The lymphoreticular system is responsible for the immunological processes taking place in an animal. Lymph- nodes and spleen are the production centres of antigens and antibodies. Lymphocytes acquire an immune behaviour in th so called "cellular response", they recognise and migrate towards the antigens and mount a cellular attack. (Nossal & Makella - 1962). The lymphocyte recognises the antigens ar retains this ability for lengthy periods. (Immunological mem Proof that the small lymphocyte is the carrier of immunologi memory comes from the work of Gowan & Uhr (1966). Lymphocyte is the mediator of antibody formation. Within 48 of exposure to certain antigens, it transforms itself into large pyroninophilic cells which is virtually a lymphoblast wi antibody producing cytoplasm (Gowan & McGregor - 1962).

3. PHYTOHAEMAGGLUTININ or PHA:-

3.1 Historical:-

The existence of haemagglutinins in various plants has been known for a long time (Bendor & Prescot - 1962). The first use of PHA from Phaseolus vulgaris in the separation of leukocytes was made by Li & Osgood in 1949 when they accidentally rediscovered it. Later,

this PHA was isolated in 2 electrophoretically homogeneous forms as a mucoprotein PHA-M and as a Protein PHA-P (Coons, Ledu and Connolly - 1955). Nowell (1960), while studying differentiation in short term cultures of normal human leukocytes, which he obtained from peripheral blood by the use of Commercial PHA-M, observed that these preparations contained a substance capable of initiating mitosis in such cultures.

3.2 Commercial preparations:-

Studies have shown that not all batches of PHA are mitogenically active (Nowell - 1961) and Difco now market two preparations.

(a) Bacto PHA-P is a purified protein PHA, from which the polysaccharide molecules have been removed. It is said to have a powerful haemagglutinating property, but to be poor as a mitogenic stimulant.

(b) Bacto PHA-M the less purified product is recommended for mitotic studies (Difco Labs. Commercial Literature - 1961). This substance initiates mitosis in such a great number of cells that it has attracted a lot of people working in the field of Cytogenetics and Genetics. It has been used for karyotyping experiments, and also for investigating the effects of PHA on the kinetics of cellular proliferation.

3.3 Purification and Identification of PHA:-

After the first report about the mitogenic action of PHA on lymphocytes (Nowell - 1964) a number of attempts were made to separate the various active constituents of

PHA and to characterise their chemical structure. Punnet and Punnet (1962) after extensive fractionation isolated a blastogenic factor of macromolecular dimension, free of haemagglutinating activity and from their findings suggested that haemagglutinating and blastogenic factors are separate molecules. Robins (1964) confirmed the above mentioned findings by incubating the PHA with packed RBC at $4^{o}C$ for 25 minutes He centrifuged the cell suspension and repeated the absorption a second time. He found that the supernatant solution did not possess RBC agglutinating activity, but retained essentially all of the blastogenic and mitogenic activity of the original preparation. Hastings, Freedman, Cooper, Rendon and Hirschhorn et al (1961) showed the inter-relationship between mitotic activity and leukoagglutinating activity of PHA, which was further confirmed by Kolodny and Hirschhorn (1964).

Rigas and Johnson (1964) obtained a PHA in the protein form which was homogeneous by ultracentrifugation, continuous flow curtain electrophoresis at pH 8.0, starch gel electrophoresis at pH 8.0, polyacrylamide gel electrophoresis and chromatography on Sephadex G-200 and on DEAE cellulose. The material had a mol. wt. of the order of 128,000 and possessed both mitogenic and agglutinating activities. The results of mitogenicity assays of fractions obtained by chromatography suggested the existence of more than one mitogenic substance. Rivera and Mueller (1966) presented evidence for the existence of at least 3 separate and distinct activities of PHA.

a) A leukoagglutinin

b) A factor inducing RNA synthesis

c) A mitogenic factor inducing DNA synthesis.

Goldberg, Rosenau and Burke (1969) have recently fractionated PHA and purified the RNA and DNA synthesising stimulatory substances. They present evidence that these substances are not proteins. They suggest that the four activities found in PHA -

a) Erythroagglutination

b) Leukoagglutination

c) RNA synthesis stimulating (RSS)

d) DNA synthesis stimulating (DSS) are due to four separate substances. From their results Goldberg, Rosenau and Burke suggest that the mitogenic stimulant in PHA is not a macromolecule, since it is neither a protein, nor carbohydrate nor nucleic acid and they confirm Rivera and Mueller's finding that the RNA synthesis stimulating and DNA synthesis stimulating activities are probably associated with two separate molecules, since they are destroyed by periodate at different rates.

In the same year Breitner (1969) published his work on enzymic and chemical studies on the mitogen of PHA. Proteases, nucleases, periodate, phenol extraction and extraction with lipid solvents were tested for their effect on the mitogenicity of PHA. Breitner's results with protease digestion suggest that the mitogenic activity of PHA resides in a protein or is at least protein dependant.

Goldberg et al, suggest that the RSS and DSS activities of PHA are mediated by small mol. wt. substances, which become absorbed to proteins thus explaining the presence of activity in protein fractions. Thus inspite of a large amount of work done over a decade little is known of the chemical characteristics of the mitogenic principle in phytohaemagglutinin.

3.4 Properties of PHA:-

PHA, a crude extract of the kidney bean Phaseolous vulgaris, has the property of converting invitro the inert lymphocytes into actively growing cells. This phenomenon is called "blastogenesis" and will be discussed in detail later. Mitogenic activity of PHA i.e. the capacity to induce DNA and RNA synthesis in lymphocytes is the most important property of PHA. In addition PHA causes the clumping of WBC (leukoagglutination) and clumping of RBC (Erythro-agglutination) when added to these cell suspensions.

4. ACTION OF PHA invivo:-

4.1 Toxicity

The toxicity of PHA invivo was tested by injecting the preparation into different animals and in different doses. The results are controversial, some showing the substance to be toxic, the others proving it harmless. Thus Goddard and Mendel (1929) injected PHA-P (their own preparation) intraperitoneally into mice and rabbits and showed that it is nontoxic. On the other hand Marshall and Norins (1964) showed that

intracardiac injection of PHA-P at a dose of 100-800 mg into rabbits, rats and guinea pigs resulted in 100% mortality. Low doses in these animals caused an illness marked by muscular weakness, inactivity, hunched posture and some degree of paralysis.

4.2 Histological Changes:-

On administration of PHA-P intravenously into rabbits striking changes occur in the spleen and lymphnode (The thymus dependant areas). Within 24h after PHA-P injection a depletion of small lymphocytes is observed accompanied by a blastogenic response. By the 6th h after PHA administration an increase in the normoblasts in the spleenic red pulp takes place, which returns to normal by 48h, followed by enlargement of spleenic germinal centres and myelopoiesis (Naspitz - 1968).

4.3 Antibody formation:-

PHA inhibits the antibody formation invivo. The intraperitoneal injection of PHA-P simultaneously with or prior to injection of sheep RBC into mice depresses both the primary and the secondary immune responses as measured by hemolytic plaque technique. (Spraefico and Lerner - 1967).

4.4 Role in immunosuppressant Therapy:-

In rabbits PHA is capable of neutralizing the lethal effects of 6-mercapto-purine (Richter and Mendel 1967).

PHA has been used in combined immunosuppressive therapy in dogs with renal homotransplant (Calne,

Wheeler and Hurn - 1967). If PHA is administered together with azathioprene, it potentiates the immunosuppressive activity of the latter. (Israel et al, 1965).

Intravenous injection of PHA, into 27 Cancer patients on chaemotherapy permitted the administration of greater doses of antimetabolite drugs without the accompanying leucopenia. (Israel, Delobel and Bernard 1965).

4.5 Effect of PHA on tumour survival:-

PHA does not affect the rejection of allogenic tumour cells. On injecting Ehrlich's ascites cells into PHA treated CBA mice, the tumours survive in 95% of the animals (Rubio and Unsgaard - 1966).

4.6 PHA and aplastic anaemia:-

Human patients with aplastic anaemia, on treatment with PHA, gain recovery of haemopoietic function. (Humble - 1966). The lymphocytes from patients with aplastic anaemia respond to RNA invitro. On treatment with PHA invivo, the lymphocytes from the same patients and under the same conditions fail to respond to PHA. Moreover, the plasma obtained from these patients, at this time when added to cultures of normal homologous lymphocytes, inhibits their invitro response to PHA.

5. ACTION OF PHA invitro

5.1 Effect of PHA on cells other than lymphocytes:-

PHA-P inhibits the growth of mouse fibroblasts invitro, and alters their cellular morphology.

(Caso - 1968). Argell (1966) reports that PHA is a mitotic stimulator of free living amoebae. Iochaim (1966) observed that PHA can stimulate the cells from non-lymphoid cell lines invitro. It can also stimulate the epithetial cells in the skin.

5.2 Effect of PHA on lymphocytes invitro and Blastogenesis:-

When leukocytes of the normal human peripheral blood are cultured invitro, few if any of the cells enlarge and undergo mitosis. Nowell (1960) showed that, invitro, in the presence of PHA, lymphocytes from human blood undergo a transformation into large morphologically primitive blast like cells, capable of undergoing mitosis. This transformation which is called "Blastogenesis" has been extensively studied during the last decade.

The subject has been reviewed by Robbins (1964) Ling (1967) and Naspitz and Richter (1968).

This transformation process occurs in small lymphocytes which are metabolically inactive at the onset of the culture (MacKinney, Stohlman and Brecher 1962) and has now been demonstrated in lymphocytes from various other species.

Blastogenesis is defined as the acquisition by such cells of morphology which resembles that of the immature blood cells known as "blasts". Substances causing blastogenesis are referred to as "blastogenic" and the resulting cells are "blastoid". (Robins - 1964). Many of the findings obtained from blastogenesis have direct important medical applications, and also provide fundamental information about the lymphocyte and

blastogenesis. The uniqueness of this phenomenon is not so much in the transformation, but in the mitogenesis, and seems to offer a possible tool and model to investigate the mechanisms of malignancy, cell division and cell differentiation.

Our aim has been to gain insight into the mechanisms of growth control in lymphocytes and also to know what happens when lymphocytes are stimulated by PHA.

5.3 Cellular localization and metabolism of PHA:-

Kay (1967) made attempts to investigate the mechanism of action of PHA, in leukocyte cultures, with respect to the fate of the mitogen during the 3 day blastogenic period. Kay incubated the lymphocytes with PHA for short periods followed by 48 and 72h incubation without PHA, and assayed the growth by measuring the uptake of (^{3}H)-dThd into DNA. He showed that when the time of incubation with PHA was 1h growth was 50% of the value obtained when the PHA was not removed. After 3h incubation with PHA followed by removal of PHA from the culture the growth was 70-90% of the control.

Naspitz and Richter (1969) found that incubation with PHA for a time period as short as 5 min followed by incubation without PHA for 72h resulted in blastoid transformation. However, the minimum incubation time required to produce maximum induction was 6h. They demonstrated that the mitogen is bound to the cells in culture, and the cell bound mitogen is not degraded during the 3 day incubation period.

Attempts to determine the localization of PHA inside the cells were made by different people, but conflicting results have been reported. Most of the work has been done using commercial samples of PHA. Michalowski, Tasinka, Brzosko and Nowolowski (1964) labelled PHA-P (Difco) with Fluorescein isothiocyanate (FITC). During the early period of culture (4-9h), a distinct cellular localization of the fluorescent material was observed in the nuclei of lymphocytes, but no localisation occured in the cytoplasm of lymphocytes nor in granulocytes. This localization of fluorscein conjugated PHA did not change during the entire 3 days of culture.

However, Rizavi also using FITC conjugated PHA observed fluorescence in all the species of WBC in the early culture period and the fluorescein was localised in the cytoplasm of blast cells and was not at all associated with the nucleus.

Autoradiographic studies using (^{3}H)-PHA obtained by growing Phaseolus vulgaris plants in nutrient containing tritiated water by Conard (1967) showed the label to be present initially in the cytoplasm, but after 2-3 days it was mainly localized in the nucleus. Stanley, Frenster and Rigas (1969) exposed highly purified PHA to (^{3}H) gas for 2 weeks and used the product for autoradiographic studies. Their results reveal that grains were localized both in the nucleus and cytoplasm. The nuclear grains were localized exclusively over condensed masses of repressed heterochromatin.

5.4 Morphological and Histochemical Changes induced by PHA in lymphocytes invitro

On the third day of culture, in the absence of PHA, the small lymphocytes are essentially unchanged. (Robbins - 1964 b). In cultures containing PHA, more than 70% of the small lymphocytes may be transformed into blastoid cells. These cells are large in size and have deeply basophilic nongranular cytoplasm, surrounding a large rounded nucleus, which contains homogeneously staining chromatin and prominent nucleoli. In the cytoplasm there are often several small unstained cytoplasmic vacuoles. The diameter of the largest cell may be three times that of a small lymphocyte. Smaller blastoid cells varying in size between these very large cells and small lymphocytes are also present. Many of these cells are found aggregated in clumps. Some blastoid cells can be seen in mitotic division after the 48th h of culture (Robbins - 1964 a).

The cytoplasm of the blastoid cells stain intensively with pyronin and this pyroninophilia indicates the presence of RNA, for the staining does not occur after exposure of the cells to ribonuclease (Marshall and Roberts - 1963). Microdensitometric measurements of Feulgen stained preparations indicate that the DNA doubles in amount as the nucleus enlarges during blastogenesis in preparation for visible mitotic division (Barkhan and Hale - 1963). The blastoid cell does not stain in the peroxidase reaction. (Quaglino, Hayhoe and Flemans - 1962).

The electronmicroscopic studies reveal that the cytoplasm of blastoid cell, on the third day of culture, contains a well formed Golgi body, Sparse endoplasmic reticulum and many free ribosomes (Marshall and Roberts - 1963, Cooper and Barkhan - 1961). Inman and Cooper (1965) noted that cells synthesizing DNA possess more nucleoli mitochondria and elaborate Golgi zones compared to those not synthesizing DNA. In PHA stimulated lymphocytes there is a restricted development of endoplasmic reticulum.

5.5 Metabolic Changes induced by PHA in lymphocytes invitro:-

5.5.1 Changes in the deoxyribonucleoprotein Complexes:-

Stimulation of human lymphocytes by PHA invitro induces marked changes in the properties of the nuclear deoxyribonucleoprotein complexes. These changes manifest themselves in an increased capacity to bind acridine orange dye (Killander and Rigler 1965), acetylation of histones (Pogo, Allfrey and Mirsky - 1966) an altered affinity for Actinomycin-D (Darzynkiewicz, Blound and Ringertz - 1969) and can be observed before the increase in the rate of RNA synthesis is initiated.

An increased capacity to bind acridine orange is observed in all cells, but only part of the lymphocyte population appears to acetylate histones, and to show an enhanced actinomycin binding. Double emulsion autoradiography after labelling with ^{14}C-acetate and (^{3}H)-uridine suggests that only those cells which acetylate histones, also initiate RNA synthesis. (Darzynkiewicz, Blound

and Ringertz 1969). Darzynkiewicz et al, suggest that this phenomenon mainly reflects different stages of dissociation of nucleoprotein complexes and is a part of a multistep preparatory mechanism for initiation of transcription.

Soren (1970) reported the nuclear and cytoplasmic accumulation of protein in PHA stimulated lymphocytes measured by microinterferometry. Both the nuclear and cytoplasmic mass increases but the increase in cytoplasmic mass is relatively greater than in nuclear mass. A considerable part of nuclear mass accumulation occurs before the initiation of DNA synthesis. The present knowledge about the cellular mass is that for cells in a log-phase of growth the nuclear mass remains constant during G_1 phase, but it increases by a factor of two during S-phase. From this data Soren concludes that the growth of PHA stimulated lymphocytes is biphasic.

a) Growth connected with transformation of resting cells into "active cells".

b) The interphase growth of the transformed cells.

5.5.2 Effects on DNA metabolism:-

Studies involving incorporation of (^{3}H)-dTh
or ($^{32}P_i$)-Orthophosphate show that DNA synthesis begins 24-36h after the addition of PHA

(Bender and Prescot - 1962, Ling and Husband - 1964 and our own results). This is further supported by autoradiographic studies done by Yoffey, Winter, Osmond and Meek (1965).

5.5.3 Effect on RNA metabolism:-

Radioactive nucleic acid precursors have been used to study the synthesis of nucleic acid during blastogenesis (Cooper - 1962, Barkhan - 1963). The results of these studies are in accordance with the histochemical and morphological studies and can be presented as follows.

On treatment of lymphocyte cultures with PHA, total RNA decreases during the first 30 min of exposure, followed by an accelerated synthesis of RNA within an hour after PHA addition (Cooper and Rubin - 1966). Mueller and Mahieu (1966) have shown that RNA production increases exponentially as soon as PHA is added to cells. The analysis by centrifugation on sucrose gradients of the newly synthesized RNA reveals that the synthesis of all classes of RNA is stimulated by PHA. A lot of work has been done in this field by Cooper's, Rubin's, and Torelli's groups. Based on his findings Cooper has recently advanced a hypothesis regarding some aspects of the mechanisms of growth control in lymphocytes. He suggests that regulation of rRNA metabolism is the controlling factor in lymphocyte growth (Cooper - 1969a, 1969b and 1970).

In resting lymphocytes, motility is manifested and synthesis of small amounts of RNA and protein takes place, but these cells do not enlarge, replicate their DNA nor undergo mitosis (Epstein and Stohlman - 1964). They produce rRNA at a rate which is 2-4% that of overall RNA synthesis as part of a basal turnover of ribosomes. (Cooper and Rubin - 1965). Within 24h after addition of PHA, the absolute rate of rRNA production increases 50 fold over the resting levels. The growth of small lymphocytes into large cells which undergo mitosis is accompanied by a marked accumulation of new ribosomes. (Inman and Cooper - 1963). Thus it seems likely that early stimulation of rRNA synthesis is an important step in the induction of normal lymphocyte growth (Cooper - 1969 a).

In those cells in which rRNA synthesis is abolished, protein synthesis and cellular enlargement occur to a certain extent, but the cells do not divide (Kay, Leventhal and Cooper - 1970).

The currently accepted model for rRNA synthesis in animal cells is that it is synthesised in the nucleus as a 45S precursor, which is cleaved to form a 32S molecule and a molecule of 18s rRNA. The 32S molecule is further cleaved to give 28S rRNA (maden - 1968) from this it

follows that both 28S and 18S rRNA molecules are produced in equimolar proportions.

When newly synthesized RNA in growing human lymphocytes was labelled with (^{3}H)-Uridine, the ratio of the radioactive 18S:32S rRNA was consistent with equimolar production of the two rRNA classes as expected. However, resting lymphocytes showed an apparent deficiency of new 18S rRNA relative to the amount of 32-28S rRNA produced. The extent of 18S deficiency was estimated from methylation studies, and was found to be about 50%. That is half of the originally synthesized 18S rRNA molecules were being lost.

On addition of PHA, the degree of 18S rRNA degradation diminished immediately thereafter, and was minimum, 6-7h after PHA addition. After 20h of stimulation by PH^., the wastage was again significantly higher than at 6-7h and by 40h of stimulation this wastage was as high as in the resting cells. (Cooper - 1970). Addition of excess of PHA, during the maximal growth did not reverse the wastage of rRNA. Only after 9 days of culture, when the cells were reduced in growth activity to nearly resting levels, did the addition of PHA reduce the wastage of rRNA. Cooper suggests that PHA may have two effects.

i) An early event leading to the minimisation of 18S rRNA wastage.

ii) An event providing the lymphocytes with a means of increasing rRNA production.

It seems that PHA can act very promptly to prevent degradation of a large portion of those 18S molecules synthesized in resting lymphocytes which would normally have been wasted. This action requires continuous protein synthesis; thus both the normal survival of 18S rRNA seen in resting cells and the increased survival of 18S rRNA produced as a consequence of PHA stimulation are dependant on protein synthesis. The action of PHA in reducing 18S rRNA wastage is independant of the PHA stimulated rise in 45S RNA production, since the former effect was observed in the presence of actinomycin-D. The decrease of rRNA wastage requires continuous protein synthesis, whereas the production of 45S RNA does not.

Recently, Monjardino and MacGillivray (1970) have reported their findings about the RNA metabolism in PHA stimulated lymphocytes. They report that both rRNA and 4S RNA are synthesised as a result of PHA stimulation. The results confirm Cooper's findings that synthesis of rRNA follows the new accepted pathway (Maden - 1968).

Rapidly labelled 4S RNA appears after the first hour of incubation and persists and predominates with longer incubation periods. The nature of this RNA is not fully understood but it comprises a labile component and is actively methylated. Monjardino and MacGillivray did not find any RNA species with characteristics attributed to mRNA under the experimental conditions they used.

5.5.4 Effect on Protein Metabolism:-

i) Proteins and immunoglobulins:-

Kay (1966) showed that PHA causes an increase in the rate of protein synthesis measured by incorporation of 14C-leucine. This increase follows an early stimulation of (^{3}H)-Uridine incorporation into the acid insoluble fraction, but preceeds the stimulation of rRNA synthesis. Bach and Hirschhorn (1963) demonstrated protein production in lymphocytes stimulated with PHA by adding (^{14}C)labelled aminoacids to the culture and measuring the incorporation of radioactivity into proteins. They found that in the presence of PHA 5 X 10^{6} lymphocytes produced 10 ugm of protein at the end of 24h incubation. When the medium of these cultures was analysed by immunoelectrophoresis and autoradiography, precipitation bands were noted in the γ_1 A and γ_2 globulin regions.

Bach and Hirschhorn also showed by immunofluorescence studies that virtually all the cells stimulated by PHA fluoresced with fluorescein-conjugated antihuman 7S γ-Globulin. Lymphocytes taken from an individual with multiple myeloma when stimulated with PHA produced the specific myeloma protein invitro. Forbes and Henderson (1966) confirmed Bach and Hirschhorn's findings. Turner and Forbes (1966) studied the synthesis of proteins by PHA stimulated and nonstimulated human lymphocytes invitro, by measuring the incorporation of (^{14}C) aminoacids into proteins. They analysed the culture supernatant fluids by DEAE column chromatography and starch gel electrophoresis and showed that radioactivity was present in a broad range of serum proteins. By radioimmuno-electrophoretic analysis the IgG and IgM fractions were shown to be labelled, but not as strongly as some of the other fractions such as α_2-M and haptoglobins. Patterns from cultures not treated with PHA gave only a single faint band in the region of haptoglobins. They calculated that 10^8 lymphocytes synthesized 30μg IgG globulin in 48h.

However, neither Balfour, Cooper and Alpen (1965) nor Coulson and Chalmers (1967) were able to detect immunoglobulin synthesis in PHA stimulated lymphocytes.

Recently Scheurlen (1968) reported that 3-5 proteins are synthesized in lymphocytes stimulated by PHA. On the basis of immunoelectrophoretic criteria the proteins are identical with serum proteins.

ii) Enzymes:-

Nadler (1968) studied various enzymes in cells fractionated by nitrogen cavitation. By this method the cells rupture while the intracellular organelles remain intact. Following differential centrifugation this method is claimed to yield clearer separation of fractions and greater enzyme recoveries compared with other methods.

Lactic Dehydrogenase (LDH)and Glucose-6-Phosphate-dehydrogenase (G-6-PDH) appeared almost entirely in the highspeed supernatant fraction.

Acid Phosphatase (ACP) was confined to the lysosomal fraction.

B glucouronidase (B-GUR) and
α-glucosidase (-Glu) were distributed in all cell fractions. On comparing the PHA stimulated cells with unstimulated control cells LDH, G-6-PDH, ACP, Glu and protein decreased slightly during the first 6h and subsequently linearly increased to maxima at 65h. The changes in B-GUR were not consistent. ACP was not seggregated to the lysosomal fraction as in controls but remained widely distributed in all cell fractions in PHA stimulated cells.

This redistribution of enzymes suggests a direct effect of PHA on the lysosomal membrane. On stimulation with PHA, the proportion of M-type LDH increases (Rabinowitz-1968, Hellung - 1968).

Various cytochemical studies have been carried out in PHA - stimulated lymphocytes. Hirschhorn and Weissman (1967) observed the development of acid hydrolase rich granules between 24-48h after stimulation by PHA; prior to mitosis. This increase was measured biochemically as a net increase in the total activity of the lysosomal enzymes, acid B glycerophosphatase, acid-phenolphthalene phosphatase and

arylsulfatase. The enzymes behave as if they are membrane bound, i.e. their activity can be released by lysolecithin.

Hausen, Stein and Peter (1969) reported that RNA polymerase begins to rise at the same time as the cells begin to accumulate stable RNA. At 30h after PHA addition both bound and soluble enzyme activities are enhanced by a factor of two over the controls. Hausen et al suggest that these changes in RNA polymerase activity in lymphocytes are comparable to the changes found in other biological systems such as regenerating liver cells. Kay and Handmaker (1970) report that uridine kinase activity assayed invitro, increases in PHA stimulated lymphocytes, but this increase is not necessary for at least the initial increase in the rate of uridine incorporation.

Loeb, Agarwal and Woodside (1968) and Loeb, Argarwal and Ewald (1970) studied the DNA polymerase and DNA replication in PHA stimulated lymphocytes. The increase in DNA polymerase activity parallels in time and magnitude the ability of the cells to synthesize DNA. The activities of thymidine kinase and thymidine monophosphate kinase multiplies about 2-10 fold.

The stimulation of DNA synthesis is rigidly dependant on the induction of DNA polymerase.

5.5.5 Effect of PHA on Carbohydrate Metabolism:-

Barkhan and Ballas (1963) reported that during blastogenesis glucose is utilized and lactic acid is produced. These facts suggest that glycolysis may be the main energy source for blastogenesis. This suggestion is further supported by the finding that blastogenesis can occur in the complete absence of oxygen. (Nowell - 1960). Glucose metabolism in intact lymphocytes was found to be altered in the presence of PHA, when the operation of pentose phosphate pathway is found to be significantly enhanced. This finding is interpreted as reflecting an additional need of biosynthetic intermediates and NADPH by the lymphocytes to accommodate mitotic activity induced by PHA (Macchafi-1967).

5.5.6 Effect of PHA on lipid metabolism:-

Kay (1968) has shown that PHA causes a rapid increase in the rate of incorporation of precursors into the lipid fractions of lymphocytes, and the increase is independant of the effects of PHA on RNA and protein metabolism. It is yet to be established whether the effect is casually related to lymphocyte activation or a prerequisite for further events to occur. Fischer (1968)

reported that after 10 min exposure of lymphocytes to PHA, the rate of incorporation of ($^{32}P_i$) into phosphatidyl inositol (PI) is ten times accelerated. Incorporation into phosphatidyl choline, phosphatidyl ethamolamine, and phosphatidyl serine is stimulated by 8-60% Huber (1968) reported that with a given initial cell number, the incorporation of($^{32}P_i$) radioactivity into the phospholipid fractions of PHA stimulated lymphocytes increases by the following factors. Lysolecithin 3-9, Sphingomyelin 7.0, Lecithin 8.0, phosphotidyl-serine and Phosphatidyl inositol 8.8, and phosphatidylethanol-amine 7.3.

5.6 Blastogenic factors other than PHA

A number of other factors apart from PHA have been reported to induce blastogenesis in lymphocytes invitro. These include:

i) Specific antigens and allergens:-

Marshall & Roberts (1963) reported that the addition of tuberculin purified protein derivative (PPD) to cultures of lymphocytes from individuals sensitive to tuberculin induces blastogenesis.

ii) Antileukocyte serum:-

Holt, Ling and Stanworth (1966) report that rabbit antihuman leukocyte antisera has a blastogenic effect on human lymphocytes invitro, and the activity is independant of the source of the cultured human leukocytes.

iii) Pokeweed mitogen:-

Farnes, Barker, Brownhill and Fanger (1964) reported that extracts of the plant phytolacca americana, pokeweed, induce transformation of human lymphocytes invitro. Barker and Farnes (1967) concluded from their studies that blast cells obtained from PHA stimulation and Pokeweed mitogen stimulation exhibit similar morphology and similar adaptations. Borjesson (1966) has described the biological properties of pokeweed mitogen and the physicochemical constituents in the pokeweed preparation.

5.7 Mechanism of action of PHA:-

Inspite of a number of attempts made over the past decade, the mechanism by which PHA induces blastogenesis and cell division in cultured lymphocytes is not clear. The problem is complicated as different authors have reported different and sometimes controversial results.

The following are the major proposed mechanisms.

i) Immunological basis for PHA action:-

As revealed by the light and electron microscope the morphology of the blast cells obtained by PHA stimulation is very similar to those cells obtained as a consequence of incubation with specific γ -globulins (Turner & Forbes 1966). in a similar way to cells stimulated by antigens invitro (Elves, Roath and Israels - 1963).

It has been shown conclusively that the small lymphocyte possesses all the attributes required for immunological competence (Gowans, Stohlman and Brecher et al, 1965). and it is the small lymphocyte which is transformed by PHA (MacKinney Stochlman and Brecher 1962). The small lymphocyte is capable of undergoing blast transformation following a variety of antigenic stimuli, both invivo and invitro i. e. invivo in hypersensitivity reactions (Parrot and Sousa - 1966), Graft versus host reaction (Gowans - 1965) and skin homograft rejection reactions. (Scothorne - 1957). The morphology of the blast cells arising as a result of the above mentioned reactions closely resembles that of the PHA induced blast cells invitro. (Gowans et al, 1965)

Blastogenesis has been observed when lymphocytes are cultured with antilymphocyte antisera and homologous mixed cell cultures. Thus, the presence of blast cells in all these cultures strongly suggests that this cell plays an important role in immunological reactions.

Simmons et al, - 1968 immunised mice against PHA and demonstrated the formation of two antibodies. To establish the relationship between antigenic determinants (responsible for the production of antibodies) and mitogenecity, they blocked the antigenic determinants with antisera and showed the disappearance of mitogenic activity, suggesting that the mitogenic moiety of PHA molecule is closely related to or

identical with the antigenic determinant sites.

Goldberg et al, (1969) have suggested that the mitogenic principle of PHA is probably absorbed to proteins. As PHA is not a pure substance, the antigen-antibody reaction is expected.

ii) Non-immunological basis of PHA action:-

Three days after stimulation of lymphocytes with PHA, the percentage of blast cells is 70-90% whereas in antigen stimulated cultures only 5-30% of the cells are transformed after 7-10 days of stimulation and with specific antigens no transformation is observed after 3 days. (Bach and Hirschhorn - 1965). The invitro transformation with PHA does not require the presence of macrophages, whereas macrophages are essential for the antigen-induced blastogenic response (Jones - 1966). The fact that PHA stimulates the epithelial cells in skin (Caron - 1966), non lymphoid cell lines (Iochaim - 1966) and free living amoebae further supports the non immunologic mechanism of PHA transformation.

iii) An alternate hypothesis proposed by Hirschhorn et al, (1963) is that PHA exerts its action by attaching to some immunologically nonspecific surface structure on the lymphocytes. Hirschhorn et al, proposed this hypothesis based on the observation of the leukagglutinating

property of PHA and its relationship to mitogenecity. On neutralizing PHA by absorption on lymphocytes both leukagglutinating and blastogenic activities are lost. This hypothesis is untenable because (a) blastogenic activity is different from leukagglutinating activity and (b) when invitro blastogenesis is induced by antigens leukagglutination does not occur.

Lindahl-Kiesling and Paterson (1969) reported that pretreatment of mice with PHA during the neonatal period does not alter the subsequent response to PHA. This finding strongly supports the view that antigenic properties of PHA are not the foundation of its capability to induce mitosis, had this been the case, induction of tolerance to PHA by neonatal injections would have abolished the invitro response.

It has been reported that in the tissue cultures PHA precipitates certain proteins of cell homogenates and plasma in the medium. Beckman (1962) suggests that probably PHA precipitates some serum protein which is an inhibitor of mitosis and growth. But Holland and Holland (1965) has shown that removal of the protein precipitating activity from PHA does not alter its ability to induce blastogenesis.

Nowell (1960) thinks that PHA might alter the cell membrane of the lymphocytes, so that some substance, either serum component or a cellular product could initiate the mitotic process.

Fischer (1968) while studying lipid metabolism in PHA stimulated lymphocytes, observed a stimulation of phosphatidyl inositol metabolism and suggest an activation of the lymphocyte secretary apparatus, promoting removal of an intracellular growth inhibitor (possibly γ-globulin).

Allison and Malluci (1964) noted an increased lysosomal content in blast cells (a product of PHA stimulation, and suggested that PHA probably destabilises the lysosomal membrane. They consider that release of the enzymes, due to changes in permeability could be responsible for the initiation of cell division.

Nadler (1968) studied the distribution of various enzymes in PHA stimulated and unstimulated lymphocytes and suggested that the redistribution of enzymes is a direct effect of PHA on lysosomal membrane.

"Staphylococcal filtrate" another blastogenic factor has been shown to release enzymes from lysosomes. It has also been shown (Hirschhorn et al, 1965) that the blastogenic action of PHA is inhibited by substances like chloroquine and prednisolone which stabilise the lysosomal membranes. They suggest that small lymphocytes under normal conditions are 'repressed' when PHA is added to the lymphocyte culture a 'derepression' occurs, associated with controlled release of lysosomal enzymes which breakdown the existing RNA.

The above mentioned finding is in agreement with that of Cooper and Rubin (1966) who demonstrated that within 30 min of the addition of PHA to the cell culture, there is a

rapid breakdown of the existing cellular RNA, followed by synthesis of increased amounts of new cellular RNA. This new cellular RNA has been shown to be nonribosomal.

In another communication Kay and Cooper (1969) describe rapidly labelled cytoplasmic RNA which is stimulated within 30 min of the addition of PHA to lymphocytes. Analysis on Sucrose gradients showed that 4S RNA is the predominant species. Gelfiltration on Sephadex G-100 revealed that this labelled RNA contained several components and very little tRNA. The properties of this RNA were compatible with it being an unmethylated precursor of tRNA.

Monjardino and MacGillivray (1970) also noted the presence of an RNA species in the early period of stimulation, whose nature is not fully understood. This RNA comprises a labile component, is actively methylated and does not have any characteristics attributable to mRNA.

An early non ribosomal RNA was also observed by Darzynkiewicz and Pienkowski (1969). It is synthesized within the first few hours following PHA stimulation and sediments in a polydisperse manner. By autoradiographic studies they detected two fractions of RNA, one with a turnover time of 60 min and the other with a turnover time of 300 min. The former sedimented in a polydisperse fashion and was degraded during a chase with unlabelled Uridine. Hirschhorn (1965) suggests this newly synthesized RNA may regulate the transition of the lymphocyte from a resting state to an active growth state. PHA may act in two ways.

It may act first on the cell membrane to change the permeability and then it may activate some enzymes. This would cause a transition of the inactive cell to an active state. The activation is probably followed by a normal growth phase. Support for this view comes from the work of Sören (1970) who measured the nuclear and cytoplasmic protein accumulation and mass increase by microinterferometery. From his observations he suggests that the growth of lymphocytes is biphasic (i) Connected with transformation of resting cells to active cells (ii) The interphase growth of transformed cells. Thus the early mechanism of PHA action is still obscure.

Cooper (1970) has recently described the effects of PHA on rRNA in lymphocytes and its connection with the regulation of growth in these cells, but the effect of PHA on rRNA can only be a secondary effect since an increased rate of rRNA synthesis is only detected 3-6h after PHA stimulation. PHA causes an increase in the rate of synthesis of rRNA which is disproportionately large relative to overall rate of RNA synthesis. In the resting lymphocytes half of the newly synthesized rRNA molecules fail to enter the cytoplasm. (Cooper 1969 (a) and 1969 (b)). The excess of the new 28S rRNA molecules remaining after the degradation of 18S rRNA are also degraded, albeit only after a lag period. Thus stimulation seems to act in two different ways to provide the lymphocytes with the means of increasing rRNA production (a) by increasing the rate of synthesis of rRNA (b) by decreasing the wastage of the rRNA. These two effects are independant of each other and show different biochemical characteristics (Cooper - 1970).

"The gene-activation theory" was put forward by Allfrey, Mirsky and Pogo (1967) during their experiments on acetylation of histones, they noted a remarkable difference in the behaviour of lymphocytes (which synthesize RNA on PHA stimulation) and granulocytes (which show a decreased capacity in RNA synthesis on PHA stimulation). They correlated these with the inhibitory or stimulatory response on RNA synthesis. On studying the turnover of acetyl group in lymphocytes and granulocytes it appeared that when the cells lost the capability to synthesize RNA the acetyl groups were lost from the histones, an observation they regarded as evidence of gene activation, where acetylation of histones plays a primary role. Mukherjee and Cohen (1969) by their cytological and autoradiographic studies confirmed Allfrey and coworker's biochemical studies describing the acetylation of previously existing histones in PHA stimulated lymphocytes in relation to RNA synthesis. Thus Allfrey's and Mukherjee's group suggests that histones may be involved in the regulation of gene expression at the transcription level and that changes in the binding of histones to template would make new segments of DNA available for transcription.

Support for this hypothesis also comes from various other sources. Darzynkiewicz (1969) and Zitterberg and Aver (1968) describe the changes induced by PHA in the deoxyribonucleoprotein complexes. Thus all these changes i. e. an increased capacity to bind acridine-orange, actinomycin-D, picric acid and bromophenol blue, might represent degrees of nuclear activation.

Monjardino and MacGillivray (1970) have recently put forward an "Operation-regulation theory" to explain the mechanism of PHA action. From their data they suggest that a programmed cell like the lymphocyte there could be a system of storing immunological information, so as to allow rapid retrieval in the presence of the previously met antigens. Thus, possibly long lived mRNA exist in such cells, and contact with both specific antigen and nonspecific mitogen like PHA might bring about the regulation of cellular function. PHA might act by removing a repressor or, somehow interfering with the mechanisms whereby the messenger is kept inactive in the absence of specific antigens. The regulation of mRNA function could be controlled by the synthesis of tRNA.

Contrary to Pogo et al, (1966), Monjardino and MacGillivray found that Purified PHA is associated with a decrease rather than an increase in the incorporation of (^{14}C)-acetate into histones.

Thus unless PHA is purified, and chemically characterized, at the moment, it is very difficult to decide what is the exact mechanism of PHA action.

6. ANTIMETABOLITIES:-

The pharmacologists have long recognised that substances having related structures may compete with the physiologically active substances. These substances alter the metabolic processes by virtue of their action on enzymes. In most of the cases the nature of the essential enzymic step is not known, such metabolic inhibitors or antimetabolites are potent inhibitors of the growth of pathogenic microorganisms as well as neoplasms.

Based on this concept of antimetabolites, artificially produced nucleic acid base analogues have been introduced. These substances are incorporated into the DNA and/or RNA and may have powerful mutagenic effects (Freese - 1963).

Many of these nucleic acid base analogues have been and are being tested for their effects on growth, particularly in relation to cancer therapy.

The action of these unnatural bases seems at least in some cases to be twofold.

a) They generally block some stage in the biosynthesis of the normal purine and pyrimidine nucleotides. In general the inhibitory effect takes place only after the conversion of the inhibitor into a nucleotide. e.g. 5-azaUracil is first converted to a nucleoside and then a nucleotide. The latter inhibits the action of the enzyme Orotidine-5-phosphate decarboxylase. Thus inhibiting the denovo synthesis of pyrimidines. (Vesely, Cihak and Sorm - 1968).

b) After conversion to nucleotides, these substances themselves get incorporated into RNA and/or DNA. The incorporation into the functional units of RNA especially mRNA leads to disturbance in the normal metabolism of the organism.

In addition to the synthetic base analogues, some antimetabolites are obtained from living organisms, such as bacteria or fungi and are named "Antibiotics".

Thus, in addition to their therapeutic and industrial uses, antimetabolites and antibiotics are used by research workers as tools to understand the mechanisms of

growth in various organisms. Synthetic antimetabolites are too numerous to be described, but a brief account follows of those which exert biological effects like cancerostasis, growth inhibition etc. Attention will be focused on the pyrimidine analogues which are directly connected with this project.

6.1 5-azacytidine

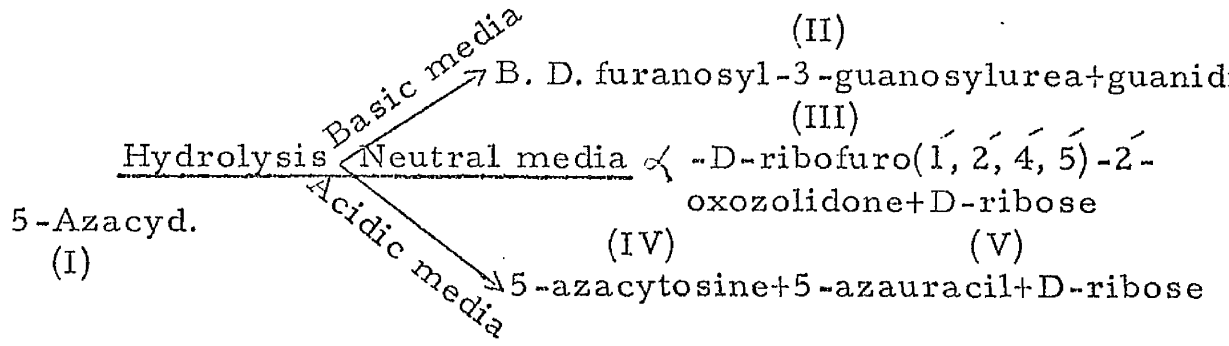
General Introduction:-

5-azacytidine is an analogue of the pyrimidine base cytidine. Azapyrimidines are a group of antimetabolites formed by the replacement of a ≻CH-group in the pyrimidine ring by a -N- atom. The mol. wt. of these bases is not altered significantly, but there is a considerable decrease in the pK value. A number of aza-analogues of nucleic acid bases like 5-azauracil, 5-azacytosine, 6-azauracil etc. have been prepared, and tested for their biological activity. The subject is reviewed by Skoda (1963).

5-azacytidine has been utilized in this project as the growth inhibitor for PHA-stimulated horse lymphocytes and will be discussed in the following section. It was first reported in 1964 by Piskala and Sorm.

It is a crystalline white solid (m. p. 228-230^{o}C), with an absorption maxima of 246 mu at pH 5.0. The substance is chromatographically homogeneous, and degrades at higher pH and temperature. It is soluble in water, but readily hydrolyses in solution, to give rise to other biologically active products. Pithova, Piskala

Pitha and Sorm (1965) suggested that the hydrolysis of the compound may be connected with the biological activity.



B. D. -furanosyl-3-guanosylurea (Cihak, Skoda & Sorm, 1963) and Compounds III, IV and V (Cihak and Sorm - 1965) have been shown to be biologically active. However, in E. coli, products of hydrolysis of 5-azacytidine itself are inactive. (Vesely, Piskala, Cihak and Sorm - 1964).

6.2 Biological Properties of 5-azacytidine:-

5-azacytidine has been shown to be a cancerostatic, bacteriostatic and mutagenic substance.

6.2.1 Cancerostatic Properties:-

Vesely et al, (1964) reported the antileukemic effects of 5-azacytidine in AKR-mice. They studied the cytological and histological effects of 5-azacyd, on the bone marrow blood and lymphoid tissue. Its mode of action appeared to differ from that of 6-azacytidine, because no accumulation of Oroticacid or Orotidine was observed in the Urine of treated mice. A single dose of 100mg/kg, though well below the lethal dose, produced complete or considerable sterilization of the leukemic cells. The

difference in sensitivity between leukemic tissue, intestinal epithelium and lymphopoietic tissue, led them to suggest that the drug is relatively selective in inhibiting proliferative leukemic cells.

Vadlamudi et al, (1970), recently confirmed the antileukemic effect of 5-azacyd in leukemic L1210 mice. These authors have shown that optimum doses (2.6 - 4.3mg/kg) of 5-azacyd were more active against the rapidly proliferating leukemic cells than the nonleukemic cells. Treatment with optimal doses for 3-11 days increased the survival time of the leukemic mice by 85-100% over the untreated mice.

6.2.2 Bacteriostatic properties:-

To quote Cihak and Sorm (1965) "the substance is the most powerful synthetic anomolous pyrimidine ribonucleoside inhibitor". At a concentration of 1.2×10^{-6} M, 5-azacyd causes complete inhibition of the growth of E. coli. The biological activity of the drug was shown to be temperature dependant, the drug decomposing at higher temperature.

6.2.3 Mutagenic Properties:-

Fucik et al, (1965) studied the effect of 5-azacyd on the primary roots of vicia faba seedlings. They planted the young seedlings in a solution of 4×10^{-5}M 5-azacyd for 24h and subsequently cultivated them in the absence of the

drug, when further growth was blocked. Inhibition of growth was accompanied by a high incidence of chromosomal aberrations and chromosomal stickiness.

At a concentration of 0.4 - 4.0 ug/ml of the growth medium 5-azacyd. caused mutagenic effects in E. coli. (wp 14 Pro⁻ strain), as revealed by an increase in the number of spontaneous mutations. On application of cytidine at a 20 fold higher concentration the mutagenic effect is neutralized. The decomposition products of 5-azacyd. (guanidyl urea riboside and guanidine) lacked mutagenic activity when tested similarly (Fucik et al, 1965 b).

6.3 Distribution of 5-azacytidine in mammalian tissue:-

Raska, Jurovcik, Sormova and Sorm (1965) studied the distribution of 5-azacyd in the organs of AKR mice. Both 5-azacyd, and 5-aza-2-deoxycyd, are excreted from the mice within few hours of application, but substantial portions of the two antimetabolites are retained in the tissue. The urine contains unaltered antimetabolites as well as products of deamination and degradation.

On injecting the radioactive drugs, the level of radioactivity in all organs decreases slowly, but, the concentration in the spleen remains higher than in the liver amd kidneys. The concentrations of both substances were significantly higher, and the retention longer, in lymphatic organs in agreement with the pronouned

morphological changes noted in the thymus, spleen and bone marrow. Liver and kidney show no such change.

5-azaCyd is metabolised very much more slowly in Ehrlich-ascites tumour cells, as compared with bacteria.

6.4 Phosphorylation of 5-azacytidine:-

6.4.1 In Mammals:-

In Ehrlich ascites tumour cells invivo, 5-azacyd is phosphorylated to mono, di and triphosphates. (Jurovcik et al, 1965). In cell-free extracts of mouse tissue it is phosphosylated to the monophosphate, but higher phosphates could not be detected. It is worth noting that phosphorylation invitro is somewhat higher in extracts from lymphatic organs than from liver or kidney, but, there was no significant difference in the activity of the extracts from normal and leukemic tissue. Phosphorylation of 5-azaCyd, by mousebrain is higher than by liver or ascites tumour cells. (Raska et al - 1966). In the cell free extracts of rat liver, the phosphorylation of cytidine is not significantly inhibited by 5-azaCyd, whereas at the same ratio of substrate to inhibitor, the phosphorylation of 5-azaCyd is inhibited by cytidine.

6.4.2 In Bacteria:-

Cihak and Sorm (1965) noted that within 5 min after addition to an E. coli culture,

5-azaCyd undergoes metabolic changes. (Showed by the use of (4-^{14}C)-5azaCyd). Radioactive 5-azauracil, biuret and ribosylbiuret were found in the medium and within 30 min 5-azaCyd disappeared completely from the culture medium. Examination of the intracellular radioactivity in the acid soluble fraction an hour after radioactive 5-azaCyd treatment revealed that the radioactivity was present in 5-azaCyd-5-phosphate.

On infection with T_4 phage, E. coli cells almost completely lose the ability to phosphorylate and incorporate 5-azacytidine, and the pool size of the intracellular acid soluble phosphorylated intermediates of 5-azaCyd is negligible compared with uninfected cells. (Doskocil and Sorm 1969).

6.5 Effect of 5-azacytidine on nucleic acid metabolism:-

6.5.1 Mammals:-

In mammalian tissue 5-azaCyd is phosphorylated and incorporated into RNA. Raska et al (1966 a) showed that the drug is incorporated invivo into mousebrain and liver and into isolated nuclei of calf thymus. Cihak et al (1966) injected radioactive 5-azaCyd intraperitoneally, into mice bearing Ehrlich ascites tumour. They isolated the radioactive RNA from the tumour tissue and proved incorporation of 5-azaCyd by enzymatic hydrolysis of the tRNA and isolation of radioactive

5-azaCyd by paper chromatography. The incorporation of (^{14}C)-5-azaCyd into the RNA of the liver of leukemic mice was established by autoradiography of smears of liver cells (Zadrazil et al, 1965). Kalousek et al (1966) injected radioactive 5-azaCyd intraperitoneally into mice. The animals were killed 4h later and the tRNA extracted. The tRNA isolated was tested in an invitro assay for its acceptor activity for different aminoacids. They concluded that after incorporation into tRNA 5-azaCyd inhibited the acceptor activity of all the aminoacids with the exception of leucine. The physicochemical properties of tRNA did not change after 5-azaCyd incorporation. Raska et al (1966 b) showed inhibition of RNA synthesis in the isolated nuclei of calf thymus treated with 5-azaCyd.

6.5.2 Plants:-

Pithova et al, (1965 b) showed that 5-azaCyd inhibits the growth of young seedlings of vicia-faba and causes chromosomal stickiness and abberrations. They demonstrated the incorporation of 5-azaCyd into DNA. They isolated the radioactive DNA, hydrolyzed it enzymatically, and chromatographed the nucleoside mixture. The radioactive spots containing the guanidino-groups were detected and radioactivity measured, proving the incorporation of the triazine ring.

6.5.3 Sea Urchin:-

5-azaCyd is a powerful inhibitor of nucleic acid synthesis during the early stages of sea urchin development. (Kuen jakof, Bajkovio and Glisin 1970).

6.5.4 Bacteria:-

General inhibition of RNA synthesis is not observed in E. coli. Synthesis of RNA, as measured by incorporation of radioactive adenosine and uridine is slightly inhibited. (Doskocil et al - 1967). Since overall RNA synthesis is normal in E. coli, the authors started investigations to establish whether the quality and function of RNA was altered in the presence of 5-azaCyd. Paeces et al (1968) showed that 5-azaCyd is incorporated both into DNA and RNA of E. coli, and studied the kinetics of (4-^{14}C)-5-azaCyd incorporation into various species of RNA.

When the incorporation of 5-azaCyd was terminated by the addition of excess Cyd or dThd, the rapidly labelled RNA was transformed and the radioactivity appeared in rRNA and tRNA. About 20-30% of Cyd was replaced by 5-azaCyd in both the RNA and the DNA. All the species of RNA containing 5-azaCyd showed normal sedimentation constants. The RNA pulse labelled in the presence of 5-azaCyd was capable of forming hybrids with homologous DNA.

However, when 5-azaCyd was present during the synthesis of mRNA, the synthesis of B. galactosidase was completely inhibited; on the other hand mRNA formed before addition of 5-azaCyd was utilized normally.

5-azaCyd did not inhibit the synthesis of DNA in E. coli - as measured by radioactive dThd incorporation. In E. coli cultures containing 5-azaCyd unbalanced growth was observed. As the protein synthesis was inhibited cell division ceased, while DNA was continuously synthesized.

The fact that 5-azaCyd produced chromosomal and gene mutations in E. coli led Zodrazil et al, (1965) to isolate DNA from E. coli cultured in the presence of 5-azaCyd, and compare it with normal DNA, on the basis of sedimentation properties, thermal denaturation, and electron microscopic examination. Both the sedimentation diagrams and electron micrographs revealed great heterogenity in molecular - dimensions in 5-azaCyd treated cells. The Tm values of the DNA isolated from 5-azaCyd treated cells were lower compared with the control.

In E. coli cells infected with T_4phage, the production of viable phage is strongly depressed as a result of direct specific inhibition of the synthesis of phage DNA (Doskocil et al, 1969).

6.6 Effect on protein metabolism:-

6.6.1 In Mammals:-

In isolated nuclei of calf thymus, protein synthesis (as measured by incorporation of radioactive aminoacids into proteins) is inhibited by 5-azaCyd, but only after a lag of ninety minutes. (Raska et al, - 1966 b). Administration of 5-azaCyd to rats within 12-14h after partial hepatectomy inhibits the normal increase in thymidine kinase activity, and is associated with a loss of absorbancy in the heavy polyribosome region. The drug causes the breakdown of hepatic polyribosomes to inactive monomers and dimers, and inhibits the incorporation of aminoacids into soluble liver proteins (Cihak et al, 1968).

The hormonally induced rises in the activity of tryptophan pyrrolase but not of tyrosine transaminase is inhibited by 5-azaCyd (Webb and Levitan 1969).

6.6.2 In Bacteria:-

The presence of 5-azaCyd in the cell free extract of E. coli resulted in the inhibition of uridine phosphorylase and cytidine deaminase activity (Cihak and Sorm 1965). On the basis of their results the authors suggest that 5-azacytosine as a substrate analogue and 5-azauracil as a product analogue represent highly effective inhibitors of cytosine deaminase activity. But the

major inhibition of growth is caused by the highly bacteriostatic 5-azaCyd.

Doskocil and Paeces (1967) showed that, when added to a culture induced to synthesize B. galactosidase, 5-azaCyd completely inhibits the synthesis of the enzyme within 12minute. Total protein synthesis is inhibited to about 6% of the control.

The inhibition of B. galactosidase synthesis was reversed by the addition of Urd or Cyd and the extent of inhibition depended on the time of addition of the bases. Doskocil and Paeces thus conclude that inhibition of protein synthesis is due to the selective effect of 5-azaCyd on the function of some species or portions of RNA, which is vital for protein synthesis. They provide evidence of 5-azaCyd incorporation into bacterial RNA and suggest that the incorporation of the drug into RNA results in concurrent inhibition of B. galactosidase synthesis.

6.7 Proposed mechanism of 5-azaCyd action:-

By virtue of its spontaneous and enzymatic decomposition into biologically active compounds 5-azaCyd exhibits a polyvalent mechanism of action. Its pronounced effects seem to start only after its conversion into 5-azaCyd-5-phosphate. This follows from the observation that during studies on leukemic mice, some animals developed a resistance to 5-azaCyd associated with decreased uridine kinase activity, and impaired anabolic transformation of the

drug (Vesely and Cihak - 1967).

So far, the following mechanisms of interference with nucleic acid synthesis have been reported.

a) Competition by 5-azaCyd with uridine and cytidine for the kinases (Raska et al - 1966).

b) Inhibition of orotidylic acid decarboxylase by 5-azaCyd-5-phosphate. (Vesely, Cihak et al - 1968).

c) Incorporation into RNA (Raska et al - 1965, 1966 a, 1966 b).

d) Incorporation into DNA (Zodrazil et al - 1965).

It has been considered that the major inhibitory effect of the drug is connected with its incorporation into the nucleic acids.

Sorm's group suggested that following incorporation into nucleic acids the triazine ring of 5-azaCyd breaks open and causes mutations, but this suggestion was later on ruled out, because Paeces et al, (1968) were able to show that almost all the radioactivity of 5-azaCyd, initially incorporated into rapidly labelled RNA was present in rRNA and tRNA in the form of 5-azaCyd.

At present the most widely held view is that 5-azaCyd exerts its action following incorporation into mRNA by the production of faulty and fraudulent messengers, which lead to the abnormalities in the basic metabolism of the organism.

6.8 5-FluoroUracil:-

6.8.1 General Description:-

5-FUra is a derivative of uracil in which a fluorine atom is substituted for a hydrogen atom at the 5th carbon in the pyrimidine ring. In general the physicochemical properties of 5-FUra resemble those of Uracil except that the former has a greater solubility in water. The subject fluorinated pyrimidines has been reviewed by C. Heidelberger (1965). It has considerable activity against bacteria and inhibits the growth of several transplantable tumours in mice and rats. The effects of 5-FUra and its derivatives on different cell lines have been studied by Umeda and Heidelberger (1968).

In the animal system it is first converted to the ribonucleoside which in turn gets phosphorylated to the mono, di and tri phosphate prior to incorporation into RNA. It is further converted into F-dUMP which inhibits the enzyme thymidylate synthetase, which catalyzes the methylation of deoxyuridylate to deoxythymidylate.

6.8.2 Metabolic effects:-

i) Nucleic acid metabolism:-

5-FUra is incorporated into the total cellular RNA of mice (Chaudhari and Heidelberger 1958). In Ehrlich ascites tumour cells it may replace uracil by 6%.

5-FUMP, 5-FUDP and 5-FUTP are detected on ion exchange chromatography of the acid soluble fraction of Ehrlich ascites tumour cells treated with 5-FUra. It is readily incorporated into tRNAs of E. coli. and tRNA prepared in the presence of 5-FUra may have up to 100% replacement of uracil by 5-FUra. The tRNA containing 5-FUra has been separated from contaminating unsubstituted tRNA by chromatography on columns of methylated albumin Kieselguhr and shown to posses an altered secondary structure. (Lowrie and Burgquist - 1968). Incorporation of 5-FUra into DNA has never been detected.

In mice bearing Ehrlich ascites tumour 5-FUra inhibits the incorporation of (^{14}C)-formate into DNA thymine. The incorporation of thymidine is stimulated. These results indicate that a metabolite closely resembling deoxyUridine inhibits the enzyme thymidylate synthetase (Dannberg et al - 1958). At high doses 5-FUra inhibits the incorporation of (^{14}C)-uracil and ($^{32}P_i$) -orthophosphate into RNA, but chaemotherapeutic doses of 5-FUra do not exhibit any inhibitory effect on the incorporation of uracil into RNA hence the chaemotherapeutic action of 5-FUra is not a result of inhibition of RNA synthesis.

A lot of work has been done on RNA metabolism in bacteria and Fungi. In Saccharomyces carlsbergensis Mayo et al, (1968) have shown that 5-FUra interferes severely with the formation of RNA. In its presence high mol. wt. RNA accumulates which is a mixture of small amounts of rRNA and RNA with a DNA like base composition, possibly mRNA. 5-FUra inhibits the rRNA synthesis. Gressel and Galun (1966) report the suppressed photoinduced sporulation of Trichoderma viriae by 5-FUra.

ii) Protein metabolism :-

Polymers of 5-FUra code exactly like polymers of uridine and with even less ambiguity. Soffer (1964) has shown that RNA extracted from 5-FUra treated E. coli stimulates incorporation of a numb-r of aminoacids into proteins. 5-FUra neither alters the amino acid incorporation nor the composition of proteins synthesized in its presence. There is no major inhibition of total protein biosynthesis (Dekloet - 1968). 5-FUra inhibits the induction of enzymes like B. galactosidase, serine dehydrase, alkaline phosphatase etc.

Horowitz and Kohlmeier (1967) showed that 5-FUra is incorporated into mRNA in E. coli and functional mRNA specific for

B. galactosidase is synthesized in its presence.

6.8.3 Mode of 5-FUra action:-

The exact mechanism of 5-FUra action is not clearly understood. The modes of action reported in the literature include:

a) 5-FUra interferes with pyrimidine nucleotide biosynthesis (Anderson and Brockman - 1964).

b) 5-FUra is incorporated into mRNA, rRNA and tRNA but the synthesis of proteins is not inhibited. (Lowrie and Burgquist - 1968).

c) Evidence indicates that the incorporation of 5-FUra into RNA increases the frequency of base pairing errors leading to misreading of 5-FUra containing codons or mutations (Cooper - 1964).

d) The major chaemotherapeutic and toxic effect of the drug is produced as a result of inhibition of DNA synthesis (by conversion to Fluro-deoxyuridine phosphate which inhibits the enzyme thymidylate synthetase). (Hartman and Heidelberger - 1961).

Before closing the chapter a brief account will be given about the aim of the present studies, the problem undertaken for research the approach adopted to tackle the problem, and how far our approach has been successful.

It has already been mentioned that the aim of our present work has been to study the growth control mechanisms of PHA stimulated lymphocytes, and to understand the mechanism of PHA action, as well as the basic mode of growth of these cells.

In an attempt to understand the action of PHA, we considered the possibility of PHA altering the permeability of the cell membrane. If this is true, then Cyclic-AMP being capable of such an action (Rasmussen et al 1968) may also induce mitotic activity in PHA stimulated lymphocytes. However, in contrast to (Cross and Ord - -1970) we did not get results to agree with the above idea, inspite of testing a wide range of Cyclic-AMP concentrations. Similar work with Adams (unpublished observations) has shown that theophylline, an inhibitor of the enzyme degrading Cyclic-AMP (Sutherland and Rall - -1958) also has no blastogenic action.

Our next approach was to make use of some growth inhibitors, and we selected 5-AzaCyd, as it was reported to be a potent inhibitor of lymphocyte growth, and especially the growth of leukemic lymphocytes (Piskala and Sorm - 1964).

Since PHA stimulated lymphocytes resemble leukemic lymphocytes in many ways, it was hoped that the information obtained from these studies might prove beneficial in basic biochemistry, and might have some application in medicine. Thus, we developed an interest in the mechanism of action of 5-azaCyd in lymphocytes.

The cells were treated with the inhibitor at 2 different time periods.

To gain understanding into the mechanism of PHA action, studies were made during the early phases of growth, and the cells were treated with the drug simultaneously with PHA.

To help elucidate the basic biochemical changes taking place as a consequence of PHA stimulation studies were made in well stimulated cultures.

The approaches gave us much information about the basic metabolic alterations taking place in PHA stimulated cells compared to the unstimulated controls. In addition, some understanding of the mode of 5-azaCyd action has been achieved.

MATERIALS AND METHODS

CHAPTER II - MATERIALS AND METHODS

1. MATERIALS:-

1.1 Lymphocytes and Materials required for their collection, isolation and Purification:-

a) Lymphocytes: Horse blood used in our experiments was kindly provided by Dr. T. A. Douglas, from the Verterinary School, Glasgow University.

b) Heparin: "Pularin" 25,000 i.u/ml, (5ml bottles with the preservative) were obtained from Evans Medical Company, Liverpool.

200ml of the blood was collected, aseptically, into a 14oz medical flat bottle containing 5000 i.u. of heparin.

c) Glass beads: Type 070-5005, Superbrite brand, were purchased from reflective products division 3M Company, St. Paul, Minnesota, 55119.

The glass beads were soaked overnight, in conc. HNO_3, washed exhaustively with tap water, rinsed thoroughly with distilled water. They were dried in the oven, at 110°C and sterilized in small aliquots at 360°C for 3h.

d) Triosil: (Na-metrizoate or Na-N methyl, 3:5 diacetamido, 2:4:6 triodobenzoate). Triosil was obtained from Glaxo Laboratories Ltd., Greenford, Middlesex, England. It was provided as a radiographic contrast medium; a 60% ($^{w}/v$) solution containing 55.2% ($^{w}/v$) Na-metrizoate, 2.8% ($^{w}/v$)

Ca-metrizoate and 2% ($^{w}/v$) Mg-metrizoate. The density of the solution measured by weighting known quantities of the substance, was found to be 1.39 gm/ml. Hence, for our experimental purposes, it was diluted with water (10 parts triosil + 8.5 parts of water) to get a density of 1.2 gm/ml.

e) Ficoll: was obtained from the Pharmacia Chemicals, Uppsala, Sweden. A 9% ($^{w}/v$) solution in water was prepared. It was sterilized in small aliquots, in an autoclave, at 120°C, at a pressure of 15 pounds per square inch for 20 minutes.

f) Separation Fluid: Just before use, 10 parts of triosil (density 1.2 gm/ml) was mixed with 24 parts of 9% ($^{w}/v$) ficoll.

1.2 Materials required for the culture and growth of lymphocytes invitro:-

a) Culture tubes: Round bottomed screw capped glass tubes of two sizes; small 12 Cm X 13 mm and large 14 Cm X 30 mm were used. The tubes were purchased from Flow Laboratories, Irvine, Scotland.

b) Growth Media: TC-199, NCTC 109 and Eagle's MEM media were obtained from Flow Laboratories, Irvine, Scotland.

Eagle's MEM was modified by adding a mixture of Serine and Glycine (10ug Serine, 7.5ug Glycine/ml of the medium) and 50 i.u./ml of Penicillin and Streptomycin. It was supplemented with 10% ($^{v}/v$) autologous horse plasma.

c) Earle's balanced salt solution (BSS): devoid of glucose, was provided by the tissue culture unit of our deparment.

d) Autologous horse plasma: This was prepared from the horse blood as follows:- 200 ml of horse blood containing 20 i.u. of heparin/ml, was allowed to stand at room temperature, for 40 min. The leukocyte rich plasma was withdrawn, carefully and aseptically, into sterile, 14cm X 30 mm glass tubes. The plasma was centrifuged at 600 xg for 15 min at room temperature in the MSE centrifuge. The supernatant plasma was carefully transferred to another set of tubes. It was recentrifuged at 1000 xg for 10 min at room temperature, to remove the traces of cellular debris. The plasma was decanted into a bottle and preserved at 0^{o}C till further use. Generally it was used the same day of isolation, if required for media change, it was preserved at -20^{o}C.

e) Bacto PHA-M: A product of Difco Laboratories, Detroit, Michigan U. S. A. Each bottle provided contained 100 mg of dehydrated powder. For use it was rehydrated with 5ml of sterile distilled water. 1ml of this solution was designated as 1 unit of activity (arbitrary). The stock solution was further diluted, in 1:20 ratio with the growth medium and 0.1ml of this diluted solution was used/2ml of culture. medium.

f) Pokeweed mitogen: 5ml vials (lyophilized) were obtained from Green Island Biological Co. New York, 14072. (1ml of the solution was designed as 1 unit

of activity).

g) Amethopterin: Na-methotrexoate was obtained from Lederle Laboratories American Cyanamid Company - Pearl River New York.

The solution was prepared by dissolving 50mg in 110ml of distilled water, 0.4ml of 1N NaoH was added. The solution was sterilized through 0.2mu halogene disposable filter.

h) Human and Horse Transferrin: Horse transferrin used in our studies was a product of Pentex Biochemicals U.S.A. The human transferrin was purchased from Kochlight Laboratories.

1.3 Dyes and Stains:

a) Leishman Stain: was obtained from British Drug Houses Ltd., Poole, England. 0.15g of the powder was dissolved in 100ml of methanol.

b) Acridine Orange: was obtained from George T Gurr Ltd., London.

1.4 Inhibitors of lymphocyte growth:-

a) 5-Fluorouracil (5-FUra) was purchased from Calbiochem., Los Angeles, California. To prepare a stock solution, it was dissolved in 0.005N Hcl, sterilized by passing through a 0.22um millipore filter and the absorption at 265.5mu determined.

$$\text{Concentration of the solution} = \frac{\text{O.D.} \times 200}{70.7} \times 10^{-2} M$$

b) 5-azaCytidine (5-azaCyd) was a product of Mann Research Laboratories, Orangeburg, New York.

Freshly made aqueous solution (sterlized by passing through 0.22um millipore filter) was used.

1.5 Materials for Autoradiography:-

a) Nuclear track emulsion type L_4 and

b) 1D-19 developer were purchased from Ilford Ltd., Essex.

c) Amfix was obtained from May & Baker Ltd., Dagenham. It was diluted in 1:5 ratio with water, before use.

1.6 Inorganic Chemicals:- All the inorganic Chemicals used were of high purity, and whenever possible ANALAR grade reagents were used. The Chemicals were normally obtained from B.D.H. Biochemicals, Poole, Dorset, England.

1.6.1 Solvents and Buffers:

a) Borate Buffer used in the Chromatographic Separation of deoxyribonucleosides and their mono, di and tri phosphates was prepared as follows:

A solution of 1M Ammoniumacetate containing 0.01M EDTA was prepared. The pH was adjusted to 9.0 with liquor ammonia and saturated with sodium tetraborate. To 3 volumes of the above solution 6.5 volumes of absolute ethanol was added, and allowed to stand for one hour, before use.

1.6.2 Buffers used in the Sucrose density gradient analysis of RNA:-

1.6.2 Buffers used in the Sucrose density gradients analysis of RNA:

a) Buffer for the preparation of rat liver Post mitochondrial supernatant

Sucrose	0.25M	
Tris	0.02M	pH 7.4
Mg^{++}	0.003M	

b) Buffer employed for dissolving RNA and preparing sucrose solutions:

Sodium acetate	0.01M	
Mg^{++}	0.001M	pH 5.2
Nacl	0.1M	
Sodium-dodecyl sulphate	0.01% ($^{w}/v$)	

1.6.3 For disruption of cells and fractionation of nucleir

Sucrose	0.25M	
Tris	0.02M	pH 7.5

1.6.4 Buffer used in RNA polymerase Assays

Tris/Hcl	0.02M	pH 7.4
Sodium deoxy-cholate	1% ($^{w}/v$)	

1.7 DNA and RNA precursors:

Non radioactive deoxyribonucleotide triphosphates were purchased from Calbiochem, Los Angeles, California.

The ribonucleosides were the product of Kochlight laboratories.

1.8 Radioactive Isotopes:

All the radioactive isotopes used (except those mentioned otherwise) were purchased from Radiochemical Centre, Amersham, Bucks, England.

($6\text{-}^{3}H$) - thymidine was diluted with cold thymidine according to need

($6\text{-}^{3}H$) & ($5\text{-}^{3}H$) - Uridine with sp. activities of 22Ci/m mol. and 31Ci/m mol was employed

(2-^{14}C) - thymidine used had a sp. activity of 3.66mc/m mol.

(8-^{3}H) - Guanosine with sp. activity of 1.8 Ci/m mol was used.

(4, 5, -^{3}H)-L-Leucine, sp. activity 22 Ci/m mol.

(Me-^{3}H) -dTTP was a product of Schwarz Bioresearch Incorporation, Orangeburg, New York.

CTP - CTP with sp. activity 4.39 Ci/m mol.

($^{32}P_i$) - orthophosphate with sp. activities of 92 Ci/mg Pi and 51 Ci/mg Pi.

1.9 Materials for Liquid Scintillation Counting:-

2.5 diphenyloxazole (PPO) was purchased from Kochlight Laboratories Colnbrook, Bucks. Hyamine hydroxide (1M in methanol) was a product of Nuclear Enterprises Limited, Edinburgh, Scotland. Scintillation grade naphthalene was purchased from Kochlight Laboratories, Colnbrook, Bucks.

Scintillation Fluids:-

a) Toluene Scintillation fluid contained a 0.5% (^{w}/v) solution of PPO in ANALAR grade toluene.

b) Dioxan Scintillation Fluid contained 0.7% (^{w}/v) PPO and 10% (^{w}/v) naphthalene in scintillation grade dioxan.

c) Methoxyethanol and Toluene Scintillation Fluid:

A solution of 0.5% (^{w}/v) PPO in methoxyethanol, and 0.5% (^{w}/v) PPO in toluene, were mixed in a 1:2 ratio.

2. METHODS:-

2.1 Isolation and purification of lymphocytes:-

Two different methods were employed for the purification of horse lymphocytes.

a) Glass bead column method (Rabinowitz, 1964) or Triosil-Ficoll (Boyum 1968). In both cases the heparinised blood was allowed to stand at room temperature for 40 min. The leukocyte rich plasma was withdrawn, aseptically, into sterile, screw capped, 50ml capacity glass tubes.

The original cell number (number of leukocytes/ ml of the plasma) was counted by diluting an aliquot of plasma with 2% acetic acid in a 1:20 ratio in a WBC diluting pippette. A drop was counted on the Neubauer counting chamber.

The plasma was centrifuged at 400xg for 15 min at room temperature in the MSE centrifuge, and the supernatant plasma, transferred to another set of tubes for preparation of autologous horse plasma (See Materials, Section 1.2 d). The cell pellet was resuspended into 20ml plasma and treated as follows:

2.1.1 Glass bead column method:-

A sterile glass condenser (40Cm X 2Cm) covered with tinfoil on both the ends, was used as a column. The column was clamped in a vertical position, with the water circulating through it at 37°C. The sterile glass beads were poured in carefully, to $\frac{2}{3}$ of the column height.

In order to saturate the glass beads with the medium the column was washed once with 20ml of Eagle's MEM gassed with 5% Co_2 in air.

The leukocyte suspension was applied on the top of the column, and allowed to diffuse on to the glass beads. The cells were left on the column, at 37°C for 30 min, and then eluted with 40ml of Eagle's MEM supplemented with 50% autologous horse plasma. The volume of the eluate was recorded, and a small aliquot counted as mentioned before.

2.1.2 Triosil-Ficoll gradient centrifugation method:-

a) Preparation of the gradients:-

20ml of leukocyte suspension (2.1) was diluted with 40ml of sterile 0.9% NaCl.

28ml of this suspension was layered carefully over 10.5ml of the separation fluid (1.1f) contained in a screw capped tube (14cm X 30min). The tubes were centrifuged at 400xg for 25 min at 20°C in the MSE centrifuge.

b) Separation and Washing of lymphocytes:-

At the end of centrifugation, the lymphocytes separate in the form of a white band at the junction of the separation fluid and saline layers. The upper layer was carefully sucked out and discarded, and the cell pellet resuspended in 40ml of Eagle's

MEM (modified) devoid of plasma, and centrifuged at 800xg for 10 min at room temperature. The washing was repeated once more. The pellet was resuspended in a known quantity of 10% HPEHM, gassed with 5% Co_2 in air and left at room temperature till further use. A small aliquot was counted as described. (2.1(a)).

2.2 Standard Method of culturing lymphocytes:-

The cells were cultured as follows:-

i) Two ml cultures: lymphocytes were suspended in Eagle's MEM supplemented with autologous horse plasma 10% (v/v), at a concentration of 0.5 - 1.0 X 10^6 cells/ml of HPEHM. Two ml suspension was pippetted out into 12Cm X 13mm culture tubes; 0.005 unit of PHA-M was added to each culture and the tubes incubated at 37°C, in an atmosphere of 5% Co_2 in air, for 1-4 days. The tubes were loosely capped and were held in a vertical position. (More satisfactory results were obtained when the cells were suspended into half the volume of medium, and PHA-M suspended in half the volume, and the two solution pippetted out separately).

ii) 5-10ml cultures: For culturing 10-20 X 10^6 cells 14cm X 30mm tubes were employed. The rest of the conditions were same as above.

iii) 50 ml cultures: For culturing 100 X 10^6 cells or more, Winchester bottles were used. The cell suspension (2 X 10^6 cells/ml) was pippetted into the burrlars, followed by PHA-M. The Winchester

bottles were gassed with 5% Co_2 in air and sealed tightly, and were incubated in a warm room at 37°C, on burling machines.

2.3 Method of radioactive labelling:-

2.3.1 Labelling of DNA with thymidine:-

a) (2-^{14}C)-Thymidine: (2-^{14}C)-dThd, 0.1ml of a solution (30uc/ml 3.66mc/m mol) was added per 2ml culture medium 5h before harvesting. After pulse labelling for 6h the cells were harvested by cooling to 0°C, centrifuged at 1000xg for 10min, at 0.4°C, in the MSE centrifuge. The supernatant medium was discarded.

b) (3-^{3}H)-Thymidine: The cultures were pulse labelled for 2h immediately before harvest with 5uc of (^{3}H)-dThd per two ml of culture medium, at a final concentration in the medium of 4 X 10^{-6}M. The rest of the procedure was same as mentioned above, except that after discarding the supernatant the cells were washed thrice with 15ml of cold Earle's BSS devoid of glucose.

2.3.2 Labelling of RNA with Uridine or Cytidine:-

The cells were pulse labelled for 2h immediately before harvesting with 2-5uc of (5-^{3}H) or (6-^{3}H)-Uridine per two ml of the medium. The rest of the procedure is same as shown in Section 2.3.1 (b).

2.3.3 Labelling with ($^{32}P_i$) - orthophosphate (RNA and DNA)

i) Labelling in a low phosphate medium:-

The cells 1-2 X 10^6/2ml of the growth medium, were pulse labelled immediately before harvesting for 2h with 50uc($^{32}P_i$)-orthophosphate (sp. activity 92 or 51 Ci/mg of P_i). Just before labelling, the medium was changed. The cells were centrifuged at 800xg for 10min, at 37°C, the medium was discarded and replaced by 2ml of warm 10% HPEHM with 1/10th of the normal phosphate content, containing 50uc $^{32}P_i$ - orthophosphate. (In case of larger volumes and higher cell concentrations, the proportion of the label was the same as shown above).

The cells were harvested as described in Section 2.3.1(b).

ii) Labelling in normal medium:-

The cultures were labelled without any prior change in the medium. The cultures were labelled for varying time periods and processed as mentioned above.

2.3.4 Labelling of proteins with (^3H)-Leucine or (^3H)-methionine:-

The 2ml cultures were pulse labelled with 5-10uc of (^3H)-leucine or (^3H)-methionine with

different sp. activities for 2-4h before harvesting. The rest of the operations were as described in Section 2.3.1.

2.4 Preparation of the Samples for measurement of radioactivity:-

2.4.1 a) Measurement of (^{3}H) or (^{14}C) incorporated into DNA or RNA:-

The harvested and washed cells were extracted thrice with 15ml of 5% ($^{w}/v$) triochloroacetic acid (referred to as TCA), at 0-4^{o}C. The TCA-insoluble material was centrifuged at 1000xg, for 10 min at 0-4^{o}C in a MSE centrifuge. The precipitate was resuspended in TCAand the procedure repeated twice. The TCA extraction was followed by extraction with 10ml of absolute ethanol, which was followed by an extraction with diethylether. The precipitate was dissolved in 0.5ml of hyamine hydroxide (1M in methanol), by incubating at 60^{o}C for 20 min. The contents of the tubes were washed into scintillation vials with 10ml of toluene scintillation fluid and counted in Phillips scintillation counter with a counting efficiency of 18-20% for (^{3}H) and 60-80% for (^{14}C).

b) Measurement of (^{3}H) or (^{14}C) radioactivity incorporated into the acid soluble fraction:-

In certain experiments where acid soluble radioactivity was also measured the harvested

and washed cells were extracted first with 1ml of 5% (^{w}/v) TCA and centrifuged at 1000xg for 10min at 0-4^{o}C. A 0.5ml aliquot was counted either in 10ml of dioxanscintillator or methoxyethanol toluene scintillation fluid.

c) Measurement of (^{3}H) or (^{14}C) radioactivity incorporated into Proteins:-

The cells were treated as described in section 2.4.1 (a) except that the cells were not extracted with ethanol but with 1:1 ethanol: ether. After extractions with 25ml of TCA, (five times), the cells were solubilised in 0.5ml hyamine hydroxide and counted with toluene scintillator, or dried at 37^{o}C overnight and solubilised in hyamine-hydroxide and treated as mentioned in section 2.4.1(a).

2.4.2 Measurement of ($^{32}P_i$)-orthophosphate radioactivity into DNA and RNA:-

a) ($^{32}P_i$) incorporation into RNA:- The cells were harvested and washed with BSS and extracted 5 times with 25ml of 5% (^{w}/v) TCA containing 0.005M Na-orthophosphate (TCA/P_i) as described in section 2.4.1. The acid insoluble precipitate was then incubated with 1ml of 0.3N NaoH, at 37^{o}C for an hour. To each tube 1mg of bovine serum albumin (BSA) was added and the acid-insoluble material precipitated with 0.2ml of 50% (^{w}/v) TCA. The tubes were centrifuged at 800xg, for 10min at 0-4^{o}C. 0.5ml of the supernatant fluid was counted, using

5ml of dioxan scintillator, either in the Nuclear Chicago or Phillips liquid scintillation spectrometer with a counting efficiency of 100%.

b) ($^{32}P_i$) - orthophosphate incorporated into DNA:

The acid insoluble material obtained after alkalidigestion (Section 2. 4. 2(a)) was washed twice with 10ml of TCA/P_i and suspended into 1ml of 0. 5N Perchloric acid. The suspension was incubated at 70°C for 30min. After cooling, the tubes were centrifuged at 800xg for 10min, at 0-4°C in MSE centrifuge. 0. 5ml of the hot perchloric acid soluble supernatant solution was counted in 5ml of dioxan scintillation fluid.

2. 5 Technique of Changing the Culture Medium:-

The cells, 1-2 X 10^6 per 2ml of 10% HPEHM in 12Cm X 13mm culture tubes were centrifuged at 800xg, for 10min, at 0-4°C. The medium was discarded and replaced by 2ml of Eagle's MEM (modified) supplemented with 10% homologous horse serum. After further centrifugation, the cells were resuspended in 2ml of warm HPEHM (10%) containing PHA-M (0. 0025 unit/ml) and recincubated under standard conditions.

2. 6 Standard Assay of lymphocyte growth:-

The growth of lymphocytes was measured as an extent of incorporation of radioactive thymidine into DNA, pulse labelled for a fixed time. Attempts to correlate the growth of lymphocytes in terms of changes in cell number were unsuccessul, since the majority of

the cells were present in big clumps due to the leukoagglutinating property of PHA making it difficult to count the cell number accurately. Thus the former assay method was considered more reliable than the latter.

Cultures of lymphocytes were established at a concentration of 0.5×10^6 cells per ml of the growth medium (Eagle's MEM containing 1mg serine and 0.75mg of glycine/100ml of the media, supplemented with 10% v/v autologous horse plasma (hereinafter referred to as 10% HPEHM).

Two ml cultures in 12Cm X 13mm, screw capped round bottomed tubes containing 1×10^6 cells were set. 0.005 unit of PHA-M was added to the respective cultures, and incubated at 37°C in an atmosphere of 5% Co_2 in air. The time of PHA addition in all the cases is counted as 0h of culture. The cells were labelled by adding 3uc of (2-^{14}C) dThd (3.66mc/m mol) 6h before harvesting. However, if the label employed was (^{3}H) - dThd usedconcentration of 4×10^{-6}M for two hours immediately before harvesting. The cultures were harvested by cooling to 0°C, centrifuged at 1000xg for 10min at 0-4°C. The medium was discarded and the radioactivity incorporated into DNA was measured as described in Section 2.4.1. The counts of radioactivity incorporated into DNA were taken as a measure of growth.

2.7 Morphological studies:-

For morphological studies the standard 2ml cultures were harvested without labelling at the end of each day

and washed with cold BSS as described in Section 2. 3. 1(b)

The BSS was drained off, and the cell pellet was resuspended into the drop of BSS remaining in the tube. A tiny drop of the cell suspension was placed on a slide and covered with a glass coverslip. A small drop of aqueous solution of acridine orange, was added and the cells examined using a fluorescent microscope.

2. 8 Techniques employed in Autoradiographic studies:-

2ml standard cultures containing 2 X 10^6 lymphocytes, were grown under standard conditions. The cells were labelled continuously with (^{3}H)-dThd (10uc, 10^{-6}M) from 0h of culture till harvest. At the end of each day the cells were harvested (as described in Section 2. 3. 1). The cell pellet was suspended in a drop of Saponin to break the clumps, and smears of cell suspension on glass slides were made. The smears were airdried, and washed thoroughly with 5% TCA. The slides were airdried and coated with the nuclear track emulsion in a dark room and exposed for 3-4 weeks in the dark. The slides were developed with Id-19 developer and fixed with Amfix (diluted 1:5 with H_2o). The cells were stained with haematoxylin and the number of labelled cells was counted.

2. 9 Disruption of cells and preparation of nuclei and highspeed supernatant (HSS) fraction:-

2. 9. 1 High speed supernatant fraction - (HSS):-

The cells were harvested by cooling to 0^{o}C centrifuged and washed as described in Section 2. 3. 1(b). After washing with Earle's

BSS, the cells were washed once with 10ml of cold buffer (Section 1.6.3). The washings were discarded and the cells were suspended in the same buffer at a concentration of 1×10^8 - $1\text{-}10^9$ cells/ml. The suspension was homogenised in a Potter Elvehjem homogeniser (Teflon pestle of 0.308 inch diameter; clearance between tube and pestle 0.004 - 0.006 inch, tube with 2ml capacity). The homogenate was centrifuged at 800xg, for 10min at 0-4°C, to sediment the nuclei and cell debris. The supernatant fraction was further centrifuged, at 50K r.p.m. for 60min, at 0°C, in Spinco Model L-ultracentrifuge, using the AL-40 or Ti-50 type rotor. The resultant supernatant was either immediately used or preserved at -20°C for a day.

2.9.2 Preparation of nuclei:-

The nuclear pellet (2.9.1.) was washed twice more with the buffer (1.6.3.) by resuspension and recentrifugation of the nuclei at 800xg for 10min at 0-4°C in the MSE centrifuge. The purity of the nuclei was checked by fluorescent microscopy, after staining with an aqueous solution of acridine orange.

a) For DNA polymerase assays the nuclei were suspended in the same volume of the buffer as used for disrupting the cells, to use as a source of nuclear enzyme.

b) To assay the RNA polymerase the nuclei were suspended in Tris/Hcl buffer containing deoxycholate (c.f. section 1.6.4).

2.10 Studies connected with the uptake and phosphorylation of Thymidine and Uridine:-

a) The lymphocyte cultures were harvested, and washed as described in section 2.3.1 (b) after pulse labelling with ^{3}H-dThd. The cells were extracted once with 1ml of 5% TCA (^{w}/v), and were centrifuged at 1000xg for 10min at 0-2^{o}C, in the MSE centrifuge. The supernatant was decanted into a test tube, and extracted thrice with 5ml of diethylether.

Deoxyribonucleosides and their mono, di and tri phosphates were separated by chromatography of 100u litre of the supernatant fraction on Whatman No. 1 paper, using the conditions described by Keir and Smellie (1959).

Isobutyric acid: NH_4 (sp. gravity 0.88) EDTA (0.1M): H_2O (100:4.2: 1.6: 55.8 by volume) was used as a solvent and ascending chromatograms were developed overnight. Samples of thymidine, dTMP, dTDP and dTTP were used as the markers. The chromatograms were dried at 100^{o}C for an hour and spots detected under U.V. The spots were cut into small pieces, incubated with 0.5ml of hyaminehydroxide (1M in methanol) and counted with 10ml of toluene scintillation fluid.

b) Uptake and Phosphorylation of Uridine:-

Cultures of lymphocytes were labelled with (6-^{3}H)-Uridine for 2h. The cells were harvested and washed as described in Section 2.3.1(b) and the acid soluble fraction collected (as described in

Section 2. 10). The acid soluble material was boiled for 10min to convert all the nucleotides to monophosphate level, and was extracted thrice with diethyl ether.

100u litre of the solution was chromatographed on Whatman No. 1 paper using the conditions described by Klenov and Lichtler (1957). The chromatograms were developed for 66h in borate buffer (Section 1. 6. 1a). The chromatograms were dried at 100°C for an hour, and the spots detected under U. V. light.

The spots were cut into 1cm strips, the strips were incubated with 0. 5ml of hyamine hydroxide at 60°C, for 30min, and counted in 10ml of toluene scintillation fluid.

2. 11 Enzyme Assay Procedures:-

i) DNA polymerase or DNA nucleotidyl transferase E. C. 2. 7. 77:-

The basic assay procedure was that of Shepherd and Keir (1966) for comparison Loeb's procedure was also used (Loeb and Agarwall - 1968).

The enzyme activity was measured by incorporation of (^{3}H)-dTTP into the acid insoluble material, over a 60min period at 37°C. The added primer was either native or heat denatured Salmon tested DNA. The enzyme activity was measured, either in the crude cell homogenate or the high speed supernatant fraction (Section 2. 9. 1) or in the whole nuclei suspended in the buffer (Section 2. 9. 2).

Denaturation of DNA: The commercially

obtained Salmon tests DNA was dissolved in 0.05M KCl, at a concentration of 2mg/ml, heated at 100°C for 10min, and cooled rapidly to 0°C by placing the tube in ice water.

The crude cell homogenate, nuclear or HSS fraction, used as a source of DNA polymerase activity, was incubated at 37°C for 60min, in a total volume of 0.25ml with either Keir's or Loeb's enzyme assay mixture. The reaction was terminated by the addition of 0.05ml of 2N-NaoH and samples were incubated overnight at 37°C before being prepared for radioactivity counting (c.f. 2.4.1).

Enzyme Assay Mixtures:-

a) Loeb's Cocktail: 0.25ml of the assay mixture contained 0.1ml of enzyme preparation, 25µ mole of trismaleate buffer pH 8.0, 3u mole of $MgCl_2$, 1u mole of KCl 0.3µ mole of 2-mercaptoethanol, 25µ mole each of dATP, dGTP and dCTP and (Me-^{3}H) dTTP, (1µc), 100µg of Salmon testes DNA.

b) Keir and Shepherd's Cocktail: 0.25ml of the assay mixture contained 0.1ml of enzyme preparation. 5µ mole of tris/Hcl buffer pH 7.5, 1µ mole of $MgCl_2$, 15µ mole of KCl 0.1µ mole EDTA, 1.5µ mole 2-mercaptoethanol, 100µg of DNA, 50mµ mole each of dATP, dGTP, dCTP and (Me-^{3}H) dTTP (1µc).

ii) RNA polymerase or (DNA dependant RNA polymerase E.C. 2.7.7.6)

The basic assay system was that of Lieberman et al, The nuclei were prepared (as described in Section 2.9.2),

and were suspended in 0.02M tris/Hcl pH 7.4 buffer containing 1% Nadeoxycholate. The suspension was vortexed, and the resultant viscous solution was not sonicated, as this resulted in the loss of enzyme activity. The reaction mixture was incubated at 37°C, for 15min, and the reaction terminated by the addition of 5ml of 5% TCA (^{w}/v). The acid insoluble precipitate collected by centrifugation at 1200xg for 4min at 0°C in MSE centrifuge. The supernatant fluid was discarded, and 1 drop of bromothymol blue indicator was added to the pellet, followed by 0.15ml of 1M KOH. The tubes were vortexed immediately and 8ml of 5% (^{w}/v) TCA was immediately added. The procedure of extraction was repeated 4 times by centrifugation and resuspension of the acid insoluble material. This was followed by washing the precipitate thrice with 15ml of 5% (^{w}/v) TCA and the samples prepared for radioactivity measurement (Section 2.4.1).

2.12 Extraction and Fractionating of lymphocyte RNA:-

The method is based on that of Kay and Korner (1966) with a few modifications. Rat liver postmitochondrial supernatant was used as the source of carrier RNA.

i) Preparation of the rat liver postmitochondrial supernatant:-

Albino rats of either sex, weighing 110-150g, were killed by decapitation, and the livers removed quickly. The livers were cut into small pieces,

rinsed thoroughly with cold buffer (c. f. 1. 6. 2 (a)) and homogenised in 3 volumes of the same buffer, in a Potter Elvehjem homogeniser, fitted with a teflon pestle with 3 strokes at full speed. The homogenate was centrifuged for 15min, at 12, 000 r. p. m. at 0^{o}C in MSE-18 centrifuge. The supernatant was decanted, and the volume adjusted to 35ml per liver with the above mentioned buffer.

ii) Extraction of RNA:-

a) Cytoplasmic RNA extraction by Cold phenol:-

5ml of the rat liver supernatant was mixed with 20 X 10^{6} radioactive lymphocytes. An equal volume of 80% (^{w}/v) phenol, made 0. 01% (^{w}/v) with respect to 8-0H quinoline was added. The mixture was extracted at room temperature for 10min, and the aqueous layer separated by centrifugation at 6, 000xg for 10min, at 0-4^{o}C in the MSE-18 centrifuge. The top aqueous layer was removed carefully. An equal volume of buffer was added to the phenol layer and the extraction repeated. The aqueous layers from both the extractions were pooled and made 0. 2% (^{w}/v) with respect to sodium dodecylsulphate (S. D. S).

b) Nuclear RNA extraction by hot phenol:-

To the phenol layer (2. 12. 2(a)) was added an equal volume of the buffer (c. f. 1. 6. 2(a)) made

0.5% with respect to S.D.S. and the mixture heated at 63°C for 3min. The extraction was then carried out at room temperature for 10min The two layers were separated by centrifugation as described in the previous section. The phenol layer was extracted once more with an equal volume of buffer, and the two aqueous layers were pooled.

The aqueous layers obtained from cold and hot phenol extractions, were extracted separately with an equal volume of fresh 80% ($^{w}/v$) phenol made 0.01% with respect to 8-hydroxyquinoline) and 2% with respect to sodium acetate pH 5.2.

The RNA was precipitated, at -20°C, with 2.5 volumes of ethanol (previously chilled to -20°C) for a length of time exceeding 2h. The RNA was collected by centrifugation at 12,000 r.p.m. for 5min, at -20°C, and washed once with cold ethanol containing 2% Na-acetate. The supernatant fluid was poured off, and the precipitated RNA dissolved in 0.5ml of buffer pH 7.4 (c.f. Section 1.6.2(b)). The solution was centrifuged once at 2,000xg, at 0°C to sediment the insoluble impurities, and extracted thrice with diethylether. The last traces of ether evaporated under Nitrogen and the sample layered on the gradient as described below.

iii) Sucrose density gradient analysis of RNA:-

12ml, 15-30% ($^{w}/v$) linear sucrose gradients were made on the top of a 2ml cushion of 2M sucrose (The sucrose solution was prepared in pH 7.4 buffer described in Section 1.6.2 (b)). 0.2ml of the RNA was layered on the top of the gradient and centrifuged at 24,000 r.p.m. at $4^{\circ}C$, for 12h, in a Beckman - L_2-Ultracentrifuge using SW_{40} rotor.

At the end of the run, the gradients were analysed by passing through a flowcell, fitted to Gilford Model 240 spectrophotometer and the absorption at 260mu was measured. 0.5ml fractions were collected, and the acid insoluble radioactivity was measured as described in Section 2.3.2. or 2.4.2. depending on the type of the label used.

RESULTS

CHAPTER III - RESULTS

1. STUDIES ON ESTABLISHING THE OPTIMUM CULTURE CONDITIONS FOR THE GROWTH OF EQUINE LYMPHOCYTES:-

1.1 Introduction:-

Studies involving assessments of biochemical changes require reasonable quantities of tissue and cells. As it is very difficult to get sufficient quantities of human blood for certain experiments, we started looking for some other source of lymphocytes. Preliminary experiments with sheep blood were not encouraging. Sheep lymphocytes responded, albeit poorly, to the action of PHA, and it was very difficult to separate the lymphocytes from erythrocytes because of the small size of the latter.

Horse lymphocytes gave us promising results, responded well to the action of PHA, and it was easier to separate them from erythrocytes. The degree of transformation of lymphocytes may be assessed using any of the following three basic techniques.

a. Morphological assessment of the blastoid cells
b. Labelling the newly synthesized DNA with a radioactive DNA precursor and counting the labelled cells autoradiographically.
c. Estimating the total radioactive uptake of radioactive thymidine by liquid scintillation counting.

Theoretically the above methods should give precise results and reproducible measures, but, in practice a number of variable and controllable factors operate, starting from the purification, culturing and growth of

the cells to estimation of radioactivity.

Thus, in order to get reproducible results and maximum growth, we had first to develop a standard method for the growth of equine lymphocytes.

Several reports appeared in literature regarding lymphocyte-culture transformation techniques, (Coulson and Chalmers, 1966: Tormey & Mueller, 1965: Schelleken and Eijsvoogel, 1968: Ling, 1967) but most of these studies involved the use of human lymphocytes. Pogo et al (1966) reported the use of equine lymphocytes, but the controlling factors in tissue culture have not been properly studied and there is no clue to say that the conditions used are optimal.

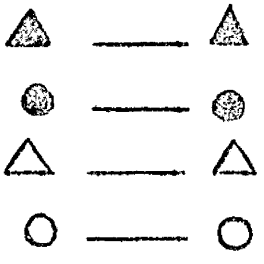
In addition to human lymphocytes, peripheral blood lymphocytes from rats (Metcalf and Osmond, 1966) Mice (Hybertson and Byran, 1967) Pig (Forsdyke, 1968), Guinea pig, rabbits, monkey and hamster (Knight, Ling, Oxnard and Normansell, 1965) have been reported to be stimulated by PHA.

One of the basic essentials for successful culture is to maintain the cells in an environment as closely as possible resembling that of the parent organism. The pH of the culture medium, the oxygen tension, depth of the medium, size and shape of the culture vessels, the nutrients and stimulants in the medium (i. e. inorganic ions, body fluids like serum or plasma, aminoacids, nucleotides, vitamins and coenzymes) and gases have a remarkable effect on the growth of the cells.

FIG III - 1

Effect of variation in the growth media on lymphocyte growth

Cultures of lymphocytes were established, using 1 X 10^6 cells (purified by glass bead column method) in two ml. of the respective growth medium supplemented with 5 or 10% (^{v}/v) autologous horse plasma. 0.005 units of PHA-M was added to each culture and incubated under standard conditions (Materials & Methods Section 2.2.1). The time of PHA addition is always counted as 0h of culture. The cells were pulse labelled for 6h (66-72h) with 0.1ml of a solution of (2-^{14}C)-dThd (3.66 mc/mM, 30 uc/ml). The radioactivity incorporated into DNA was determined as in (Methods Section 2.4.1). The results are expressed as c.p.m. X 10^{-2} dThd-2-^{14}C incorporated into the acid insoluble material/culture.



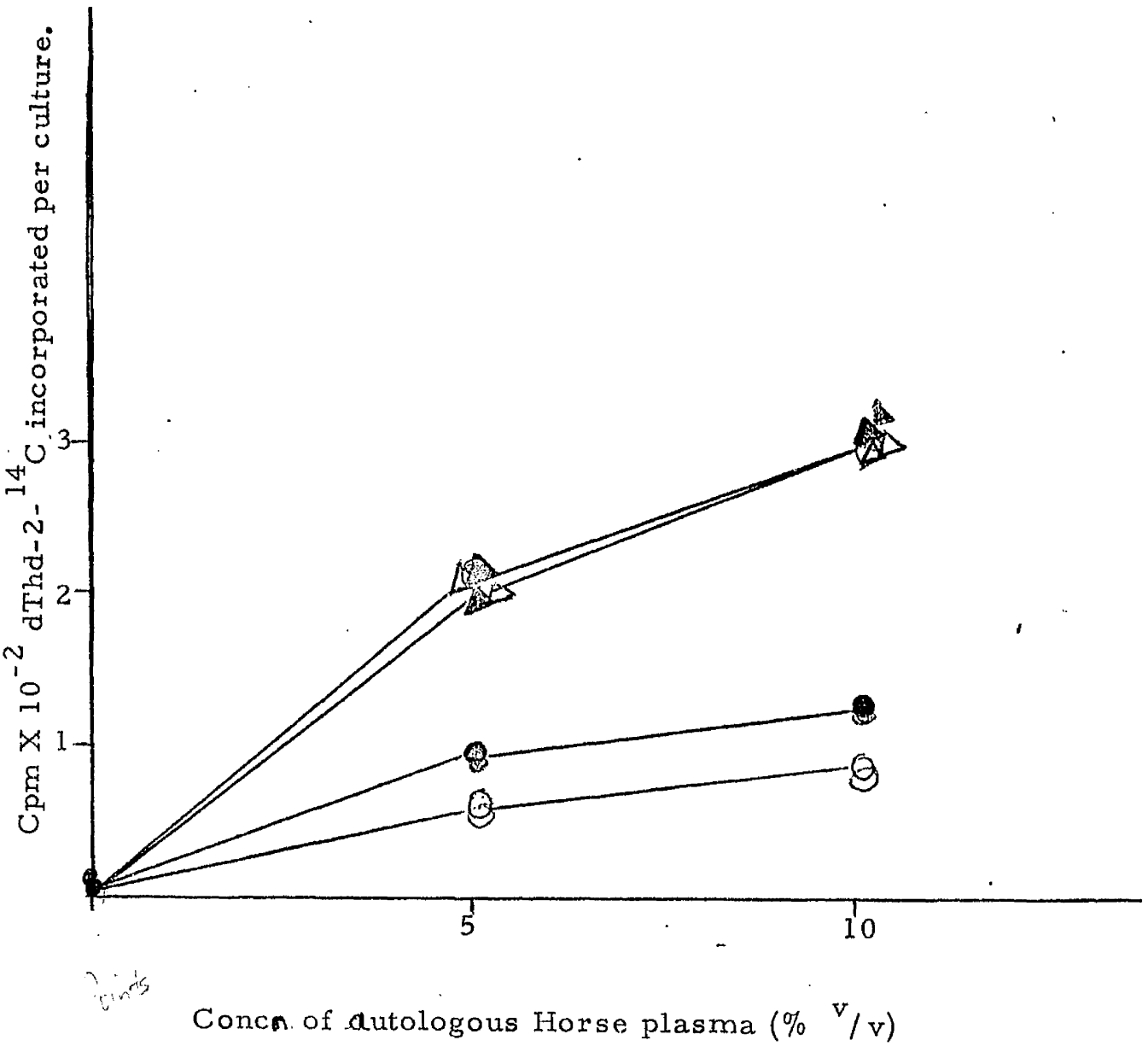
Medium NCTC 109

Medium TC - 199

Modified Eagle's MEM (EHM)

EHM containing 10mg/litre dThd

Fig III - 1


Cpm X 10^{-2} dThd-2-^{14}C incorporated per culture.
1
2
3
5
10
Concn of autologous Horse plasma (% v/v)

Thus, in order to develop a suitable and reproducible method for the growth of horse lymphocytes invitro, we studied various conditions for growth.

We do not claim that we have covered all the factors operating but we did study some of the factors which considerably affect the cell growth and which, if not carefully controlled, give either significant variations or suboptimal uptake of the precursors.

1.2 Effect of the type of culture tubes on the growth of the cells:-

The shape of the culture tube produces variation in the uptake of (^{3}H)-dThd as it determines the depth of the medium and consequently the oxygen tension at the bottom of the tube. Hughes and Caspray (1970) have shown that wide variations in the bottom of the tubes could give up to 30% discrepancies in the uptake of thymidine.

Since the conical tubes with the restricted bottom are not completely uniform in shape, some having narrow and some wider bottoms, we selected the standard Flow Laboratories, screw capped, round bottomed tubes. Depending on the size of the culture, tubes of two different sizes were used as described in Materials and Methods (Section 1.2 a).

1.3 Effect of variation in the growth medium on the growth of lymphocytes:-

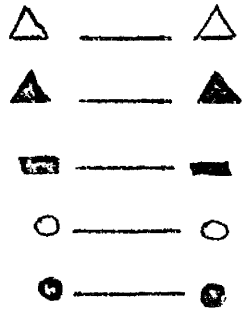
Since the concentration of nutrients and stimulants in the growth medium effects the growth of the cells and the uptake of radioactive thymidine (Woodliff H.J.

FIG III - 2

Effect of variation in the type and concentration of the sera

Similar cultures as mentioned in Fig III-1 were established using EHM and the rest of the procedure was identical to that described in Fig III-1 Concentrations of different types of sera were used.

The results are expressed as the d. p. m. $X\ 10^{-2}$ dThd-2-^{14}C incorporated into the acid insoluble fraction per culture.



Horse Serum (autologous)

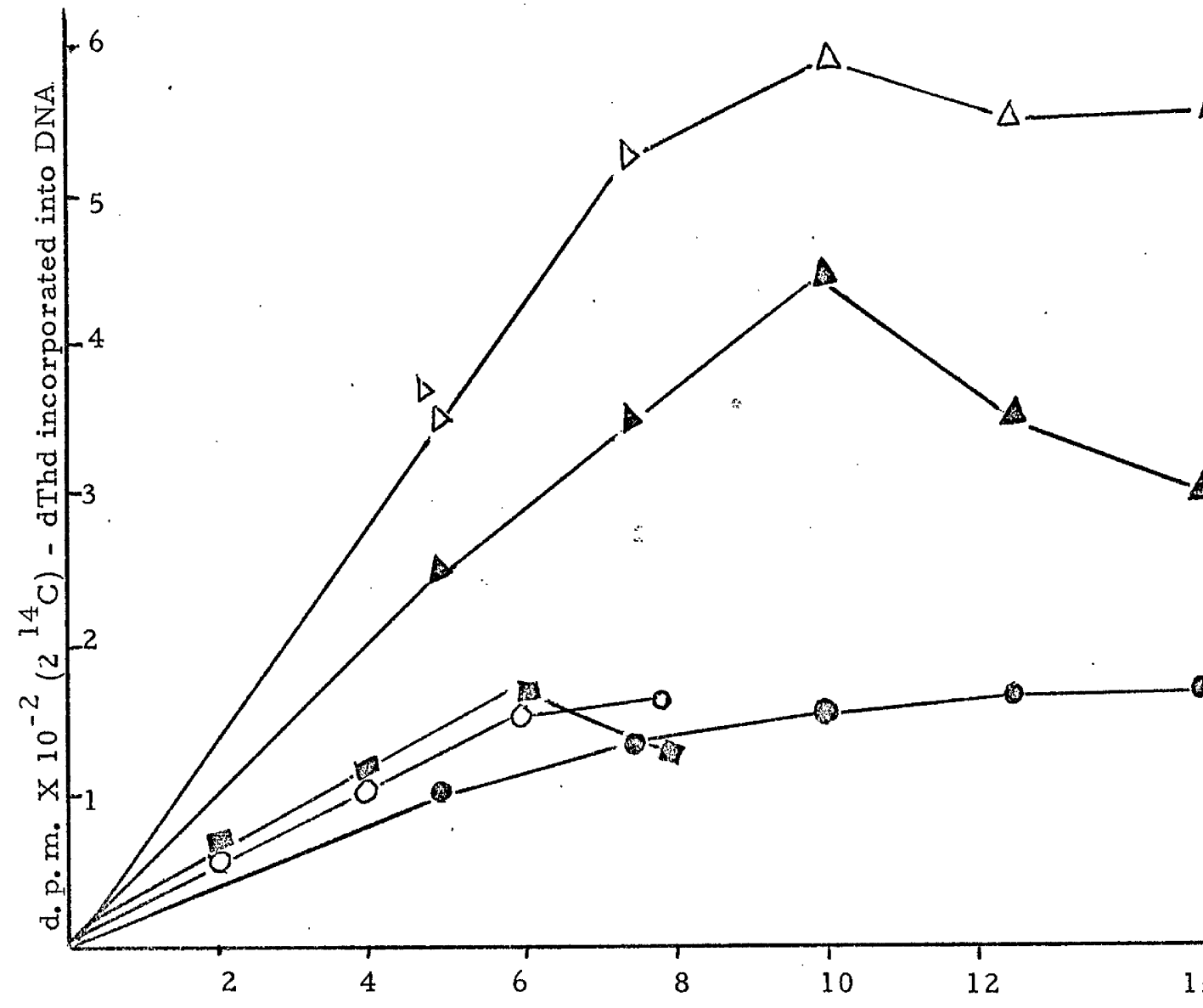
Horse plasma (autologous)

Foetal calf serum

Calf serum

Horse serum homologous

Fig III - 2


d. p. m. X 10^{-2} (2^{14}C) - dThd incorporated into DNA
6
5
4
3
2
1
2
4
6
8
10
12
Concn. of plasma or sera

1964) the effect of variation in the growth media on the growth of lymphocytes was assessed.

Comparison was made between Medium NCTC 109, TC-199 and Eagle's MEM (modified as shown in Materials Section 1.2 (b)). Since thymidine is not a component of Eagle's MEM, the same concentration of thymidine as is present in NCTC-109 was added to Eagle's MEM to check the efficiency of this altered medium.

Results from Fig III-1 show that, Eagle's MEM and NCTC-109 give the best response while TC-199 proves to be inferior under our experimental conditions.

Addition of thymidine to Eagle's MEM reduces the incorporation of radioactive dThd into DNA. The experiment was performed under identical conditions using cells from the same animal.

1.4 Effect of variation in the type and concentration of the sera:-

Living cells cannot be maintained alive for long time periods by culturing in simple salt solutions. They do survive for a short period but the growth of the cells either does not occur or is very poor.

On addition of serum or plasma an immediate improvement ensures, (Woodliff, 1958). Because of the complex nature of the plasma and serum, it is very difficult to decide which of the factors in it is essential for cell survival and growth.

There is some evidence that the macromolecules present in the serum promote the health of the cells

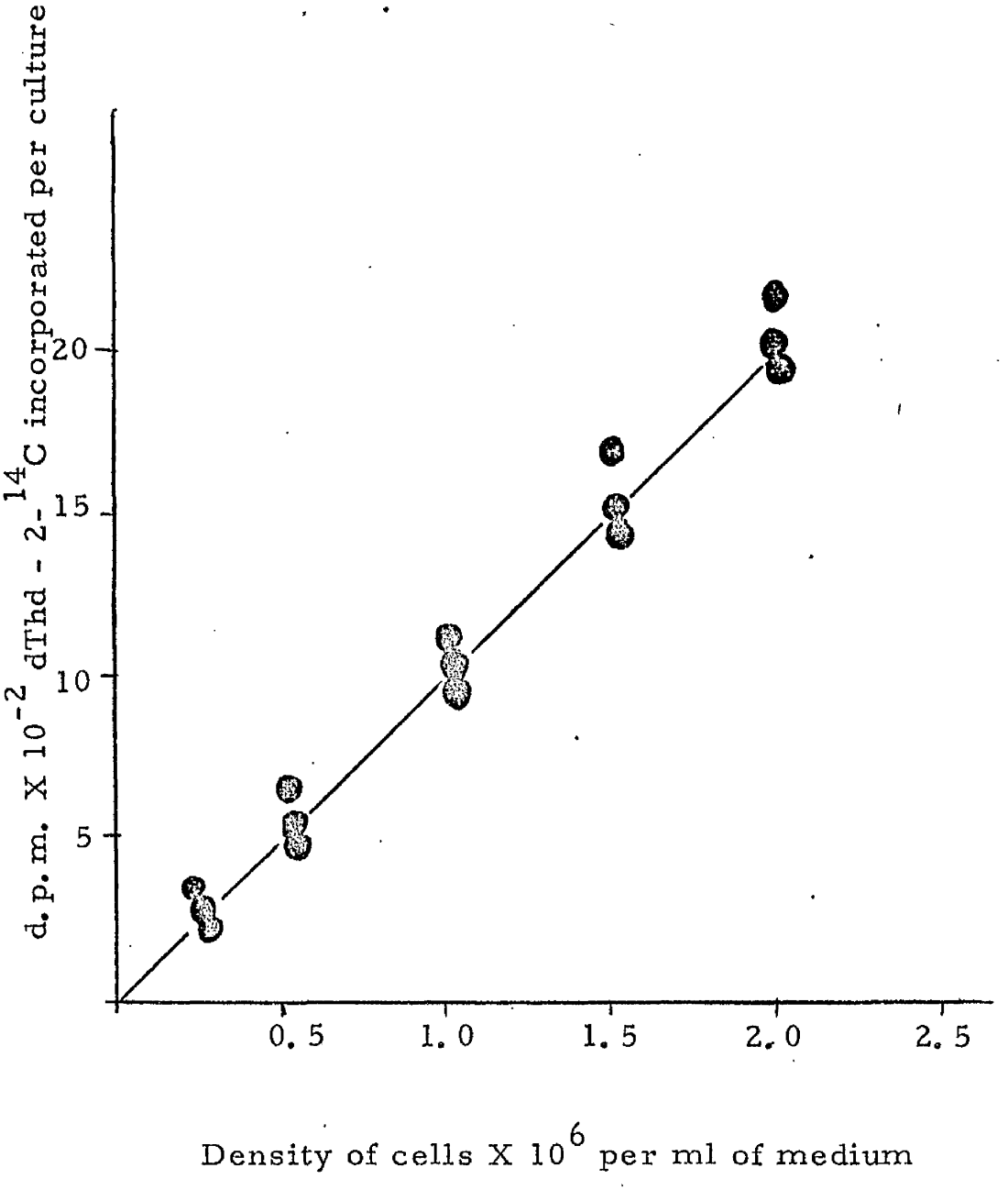
FIG III - 3

Effect of variation in the concentration of the cells

Cultures of lymphocytes containing varying concentrations of the cells (purified by Rabinowitz method) suspended in 3ml of EHM supplemented with 10% autologous horse plasma were used. The rest of the operations were similar to those described in Fig III-1

The results are expressed as the d. p. m. X 10^{-2} of 2-^{14}C-dThd incorporated into the acid insoluble fraction per culture.

Fig III - 3


d.p.m. X 10^{-2} dThd - 2-^{14}C incorporated per culture
20
15
10
5
0.5
1.0
1.5
2.0
2.5
Density of cells X 10^{6} per ml of medium

(Woodliff, 1964).

In addition it has been shown (Johnson & Russel, 1965), that foetal calf serum has stimulatory effect on human lymphocyte growth. It has been shown by a number of workers that serum from different sources contains different stimulatory or inhibitory factors. Thus in order to achieve optimum conditions for PHA stimulated horse lymphocytes growth in invitro cultures, different concentration of sera or plasma from varying sources were tested.

Fig III-2 shows that Horse plasma (autologous), Horse serum (autologous or homologous), foetal calf serum and calf serum at a concentration ranging from 2-15% (^{v}/v) was tested. The best response was given by autologous horse serum at a concentration of 10% (^{v}/v). The next best response was obtained by autologous Horse plasma 10% (^{v}/v).

Shellekens & Eijsvoogel (1968) have shown that 20% foetal calf serum in human lymphocyte cultures invitro stimulates the growth, but in our experiments with horse lymphocyte at the serum concentrations tested, foetal calf serum seems to be inhibitory.

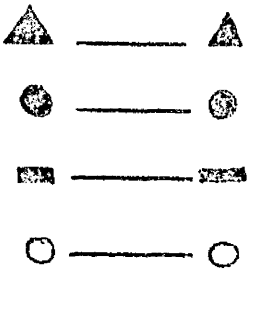
We selected autologous horse plasma (10% ^{v}/v) in our experiments, as the plasma is derived from the same blood specimen from which the cells are obtained thus minimising the wastage of blood. In addition there are the possibilities of the metabolic rates of lymphocytes being changed and the presence of components of the foreign sera might induce or inhibit the blastogenesis in lymphocytes, thus producing variations.

FIG III - 4

Effect of variation in the PHA-M Concentration on lymphocyte growth

The procedure was similar to that described in Fig III-1 except that PHA-M was added at different concentrations, and the response of PHA to cells cultured in different types of media was tested.

The results are expressed as c.p.m. X 10^{-2} dThd - $2^{14}C$ incorporated into the acid insoluble fraction per culture.



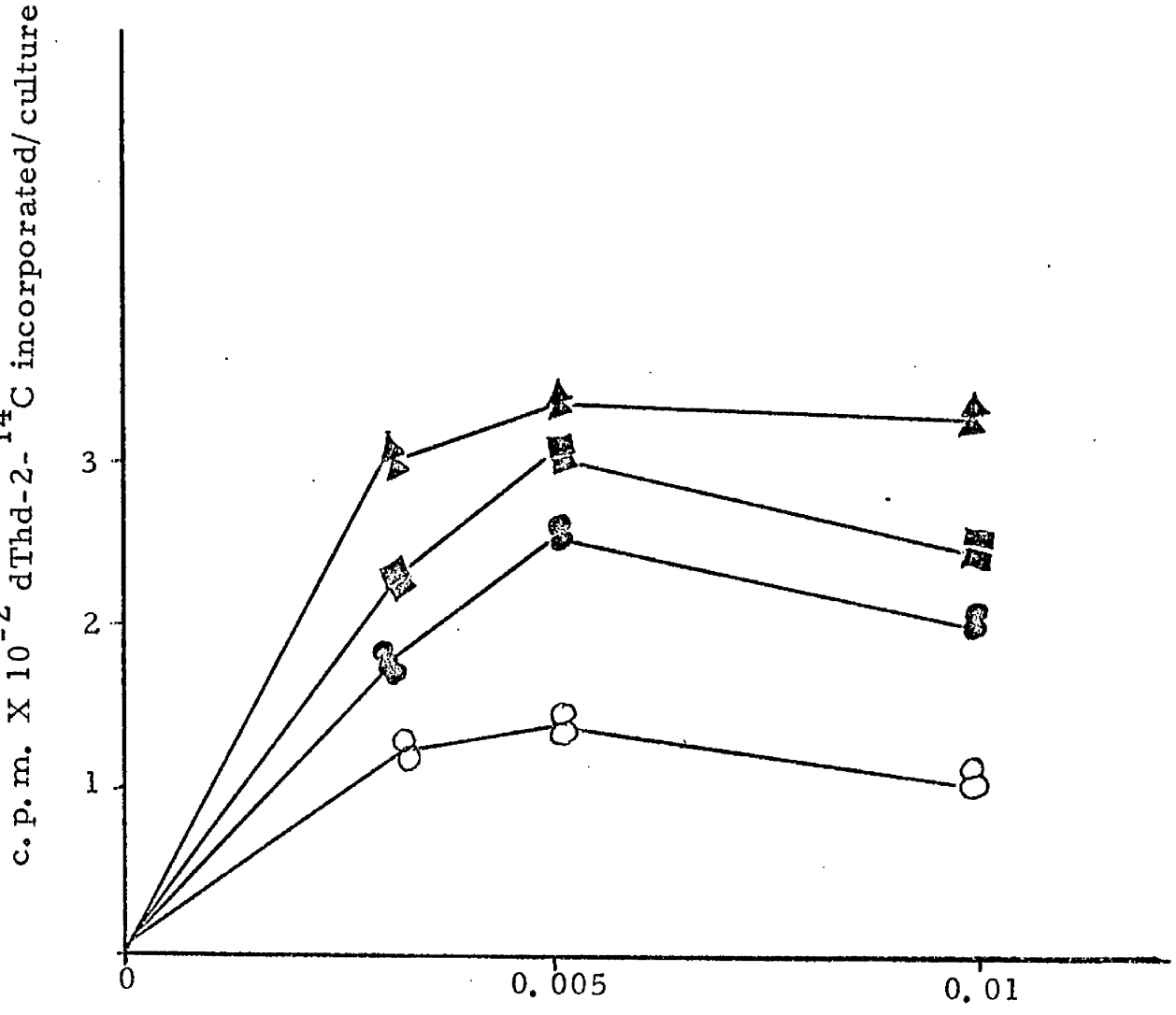
△ ——— △ Media NCTC 109

● ——— ● TC 199

▬ ——— ▬ EHM

○ ——— ○ EHM supplemented with dThd (10mg/litre)

Fig III - 4


c. p. m. X 10^{-2} dThd-2-^{14}C incorporated/culture
3
2
1
0
0. 005
0. 01
Conc. of PHA-M (units/culture)

1.5 Effect of variation in the cell concentration:-

In order to study the effect of number of lymphocytes on the uptake of radioactive thymidine, this experiment was performed. Fig III-3 reveals that the uptake of radioactive thymidine and the number of lymphocytes show a linear relationship in the range 0.5 to 4 X 10^6 lymphocytes per culture.

1.6 Effect of PHA-M Concentration

Studies have shown that all batches of PHA are not active to the same extent mitogenically. (P. C. Nowell 1961). Moreover, some authors have used PHA-P, some PHA-M and others PHA from Burroughs-Wellcome. Bacto PHA-M, the less purified product is recommended for mitotic studies (Difco Labs. Commercial literature 1961). De Lachapelle (1961) reported PHA-M to be inactive. Thus we studied the effect of PHA-M to assess its mitogenic activity and also to know the concentration required for optimum response. Tormey & Mueller (1965) have devised an assay for the mitogenic activity of PHA preparations.

The concentration of added PHA in the culture tubes plays an important role in inducing growth of lymphocytes invitro, suboptimal concentrations slowing down the growth (Robins & Levis 1971).

Schellekens & Eijsvoogel (1968) using PHA from Burroughs-Wellcome have shown that 0.05ml of PHA in a 4ml culture gives an optimum response in the uptake of (2-^{14}C) dThd.

FIG III - 5

Testing the action of Pokeweed mitogen and PHA - M

Standard lymphocyte cultures (c. f. Methods Section 2. 2. 1) containing 2×10^6 lymphocytes per 2ml of the growth medium were employed. The cells were stimulated either by PHA-M or by Pokeweed mitogen. A range of doses shown in Fig III-5 were tested. The growth of the cells was measured by incorporating ($2\text{-}^{14}C$) dThd into DNA as described in Fig III-1

The results are expressed as d. p. m. $2\text{-}^{14}C$ dThd $\times 10^{-2}$ incorporated into DNA per culture for 6h (66-72h of culture).

● ——— ● PHA-M

○ ——— ○ Pokeweed mitogen

Fig III - 5

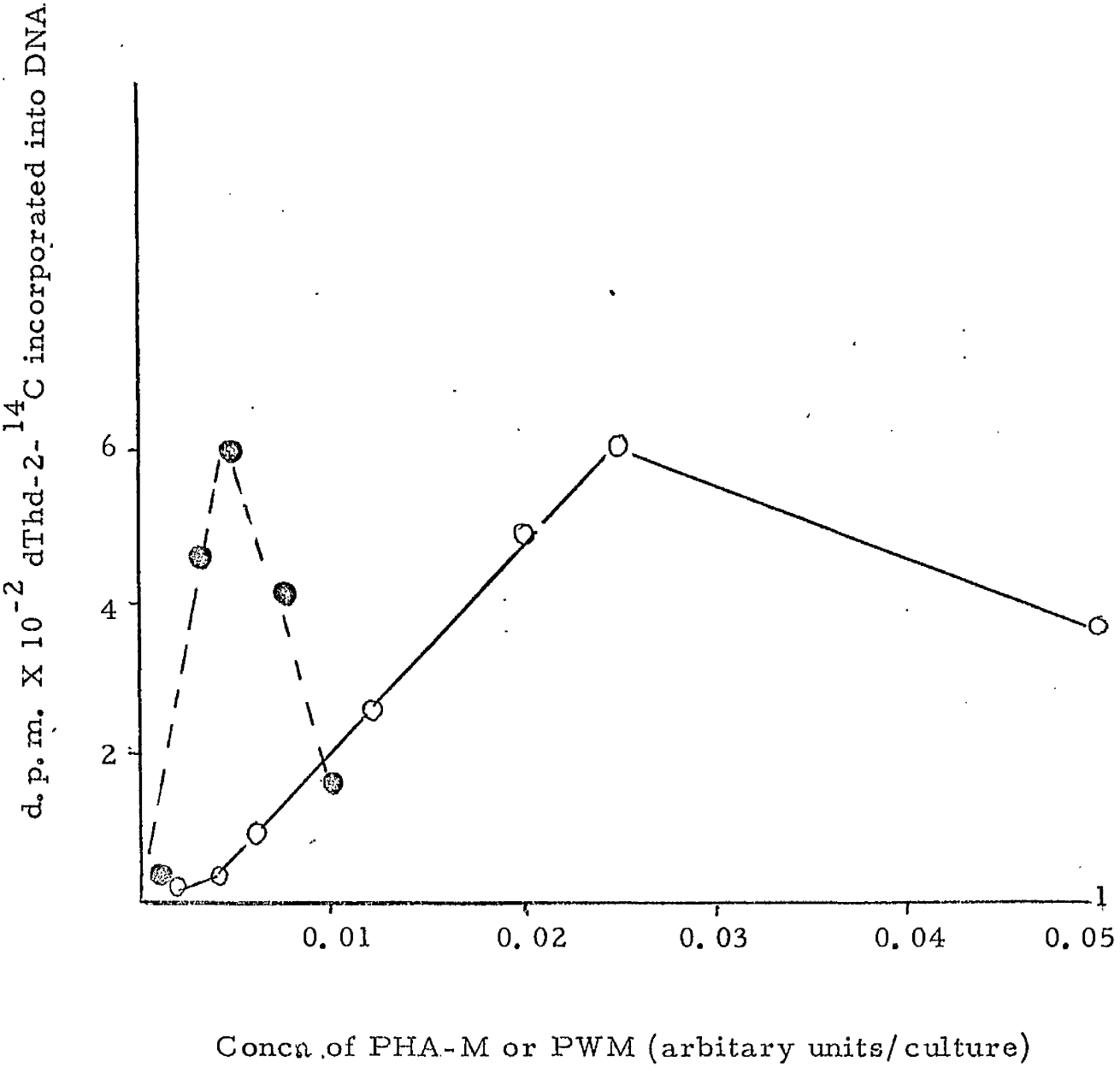

d.p.m. X 10^{-2} dThd-2-^{14}C incorporated into DNA
6
4
2
1
0.01
0.02
0.03
0.04
0.05
Concn of PHA-M or PWM (arbitary units/culture)

Fig III-4 reveals that under our experimental conditions 0.005 unit of PHA-M per culture gave the optimum response. The response of PHA-M in lymphocytes grown in different types of growth medium was studied.

Irrespective of the type of medium employed, optimum growth response is given by cultures containing 0.005 unit of PHA-M.

The same dose response was obtained in human lymphocyte cultures by Tormey & Mueller (1965) when PHA-M was used.

1.7 Action of Pokeweed mitogen on lymphocyte stimulation:-

One of the disadvantages with PHA stimulated lymphocyte cultures is that it is very difficult to count the cell number in the cultures once PHA has been added to the cultures. The clumping is so rapid that it is risky to mix up the cell suspension with PHA before pipetting into the culture tubes, and as a precautionary measure the PHA-M was to be added individually to each culture tube.

Thus we tested the blastogenic action of pokeweed mitogen on equine lymphocytes. A comparison was made between PHA and PWM, by assigning arbitrary units of activity to equal volumes of the standard available samples. The vials are provided as 5ml solutions and we assigned one unit of activity to one ml of this standard solution.

Fig III-5 reveals that Pokeweed mitogen does induce blastogenesis in equine lymphocytes, under our

FIG III - 6(a)

Dose response curve of dibutyryl cyclic AMP

Two ml cultures containing 2 X 10^6 lymphocytes per culture (purified by Boyum's method) were established according to the standard method. Two cultures were stimulated by PHA at 0h of culture the rest of the cultures received dibutyryl cyclic-AMP at the concentrations shown in Fig III-6(a). The cultures were incubated under standard conditions for 72h and pulse labelled with (6-^{3}H) dThd (5uc, 4 X 10^{-6}M) for 2h (70-72h of culture).

The growth was assayed as described in Methods (Section 2. 6). The results are expressed as d. p. m. of (^{3}H)-dThd X 10^{-3} incorporated into DNA per culture.

FIG III - 6 (a)

Concentration of PHA-M	Concentration of dibutyry-cyclic AMP	d. p. m. (^{3}H)-dThd X 10^{-3} incorporated/ culture	
-	-	0.73	0.8
+	-	20.0	18.0
-	10^{-2}	0.8	0.72
-	10^{-3}	0.75	0.79
-	10^{-4}	0.76	0.78
-	5×10^{-5}	0.8	0.82
-	10^{-5}	0.8	0.76
-	5×10^{-6}	0.79	0.82
-	10^{-6}	1.0	0.96
-	5×10^{-7}	1.5	2.0
-	10^{-7}	0.9	0.85
-	5×10^{-8}	0.9	0.95
-	10^{-8}	0.75	0.55

experimental conditions and a greater volume of the PWM solution is required compared to PHA-M, in order to induce the same extent of stimulation in DNA synthesis. Compared to 0.005 unit of PHA-M, the PWM optima is 0.025. Eridani et al (1969) report that in human lymphocyte cultures 0.2 ml of PHA-M (equivalent to 0.02 unit/ml medium induces blastogenesis in 72% cells while 0.1 ml of or 0.01 unit of PWM induces blastogenesis in 60% of the cells under their experimental conditions measured by autoradiographic studies. Douglas et al, (1967) reported that some of the blast cells obtained by stimulation with PWM differ from those obtained by PHA stimulation.

Though Fig III-5 reveals that to induce blastogenesis in lymphocyte cultures PWM can be substituted by PHA, we did not use PWM, because it is not clear whether PHA and PWM do have the same mechanism of action.

1.8 Action of dibutyryl 3' - 5' cyclic-AMP on horse lymphocytes invitro:-

Nowell (1960) and Hastings et al, (1961) suggested that blastogenesis invitro might be related to the activity of PHA at the cell membrane by effecting the permeability. Some workers also suggested that the action of PHA is similar to the action of hormones. Thus in order to test these possibilities in an attempt to understand the mechanism of PHA action on lymphocytes, we checked the effect of cyclic AMP on horse lymphocytes invitro.

Rasmussen et al, (1968) reported cyclic AMP to be the important regulator of the permeability of cellular membranes. It has been suggested that an important

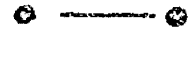
FIG III - 6(b)

Comparison of the action of PHA-M and dibutyryl Cyclic AMP on the growth of lymphocytes

Standard cultures of lymphocytes containing 1 X 10^6 cells (purified by Boyum's method) per two ml of the culture medium were used. To half the cultures the standard dose of PHA-M (c. f. Methods Section 2. 2. 1) was given and the other half were treated with dibutyryl cyclic AMP (10^{-5} M).

The cells were incubated under standard conditions (c. f. Methods Section 2. 2. 1). They were pulse labelled with CH_3 (^{3}H)-dThd 10uc (4 X 10^{-6} M) for two hours at the end of each day and harvested. The amount of radioactivity incorporated into the acid insoluble fraction was measured as described in Methods Section (2. 4. 1).

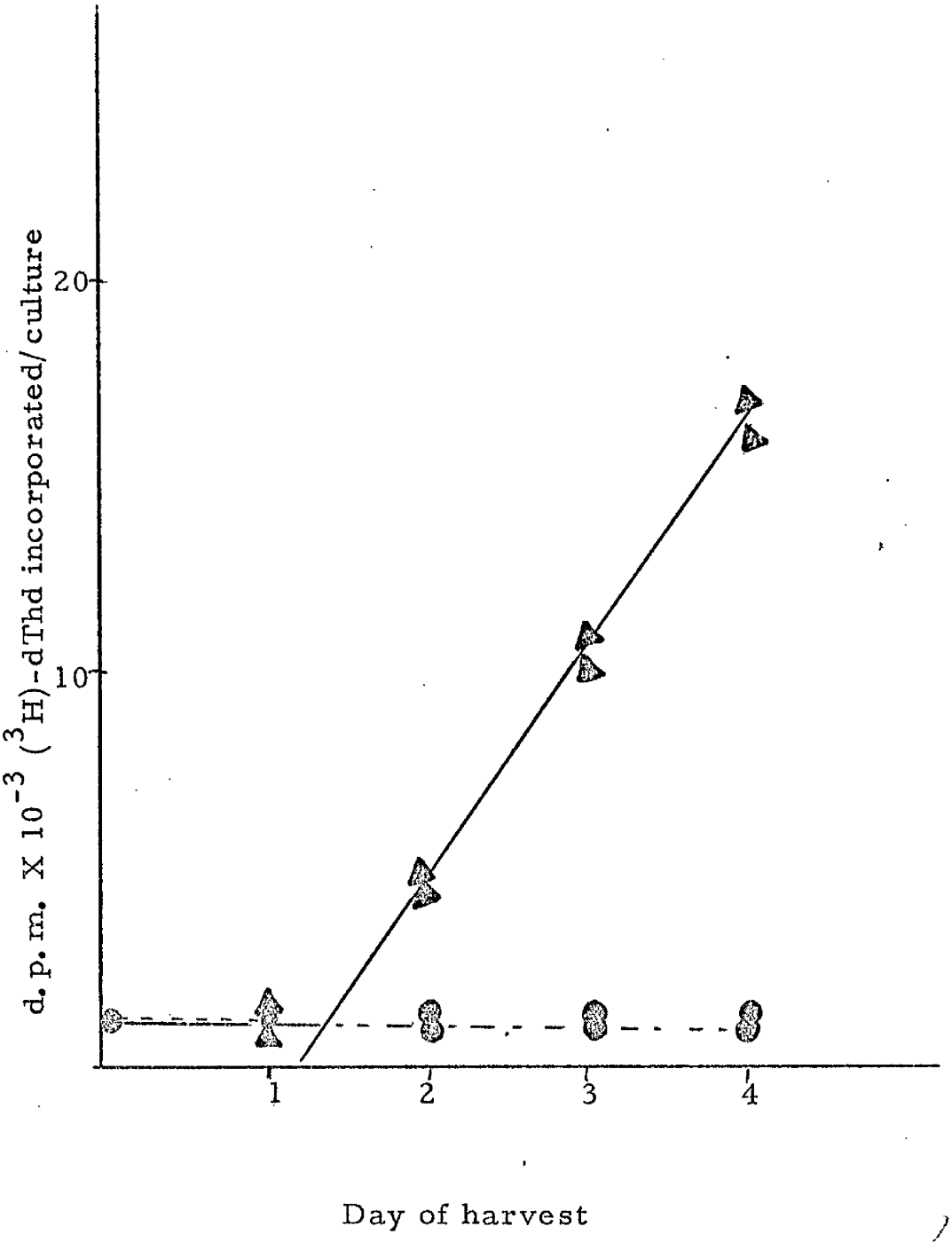
The results are expressed as the d. p. m. X 10^{-3} (^{3}H)-dThd incorporated into DNA per culture.



Cells treated with dibutyryl Cyclic AMP

Cells treated with PHA-M

Fig III - 6 (b)


d. p. m. X 10^{-3} (^{3}H)-dThd incorporated/culture
20
10
1
2
3
4
Day of harvest

cellular function of cyclic AMP is that of regulating secretary activity whether it be a secretion from exocrine gland or endocrine gland or from nerve ending. Evidence suggests that cyclic AMP is the common intracellular mediator of the diverse physiological actions of a variety of hormones. (Pastan & Macchia 1967, Fain and Caldwell 1969).

If the mechanism of action of PHA is by an initial attack on the permeability of cell membrane, cyclic AMP should also induce blastogenesis in lymphocytes.

Fig III-6(a), b and c reveal that dibutyryl cyclic AMP does not induce blastogenesis in equine lymphocytes invitro. The concentration range studied was 10^{-8} to 10^{-2}M (Fig III-6(a)). In contrast to our results Macmanus & Whitfield (1970) report the stimulation of human lymphocytes at a concentration of 10^{-8} to 10^{-6}M cyclic AMP, and Cross & Ord (1970) report the stimulation of Pig lymphocytes.

The effect of the substance on lymphocyte stimulation was studied everyday for four subsequent days. Fig III-6(b) reveals that compared with PHA there is no effect whatsoever on the stimulation of lymphocytes at any time during the four days.

Studies of the effect of 10^{-5}M dibutyryl cyclic AMP on PHA stimulated lymphocytes, added at 0, 24, 48 and 64 hours and studied at 72nd h of culture reveals that the substance is inhibitory to the growth of PHA stimulated lymphocytes, if added at a time period later than 24h of culture. The inhibition is more marked if added at the later hours of culture (Fig III-6(c))ie.addition made

FIG III - 6(c)

Mixed Action of PHA and dibutyryl Cyclic-AMP on horse lymphocytes invitro

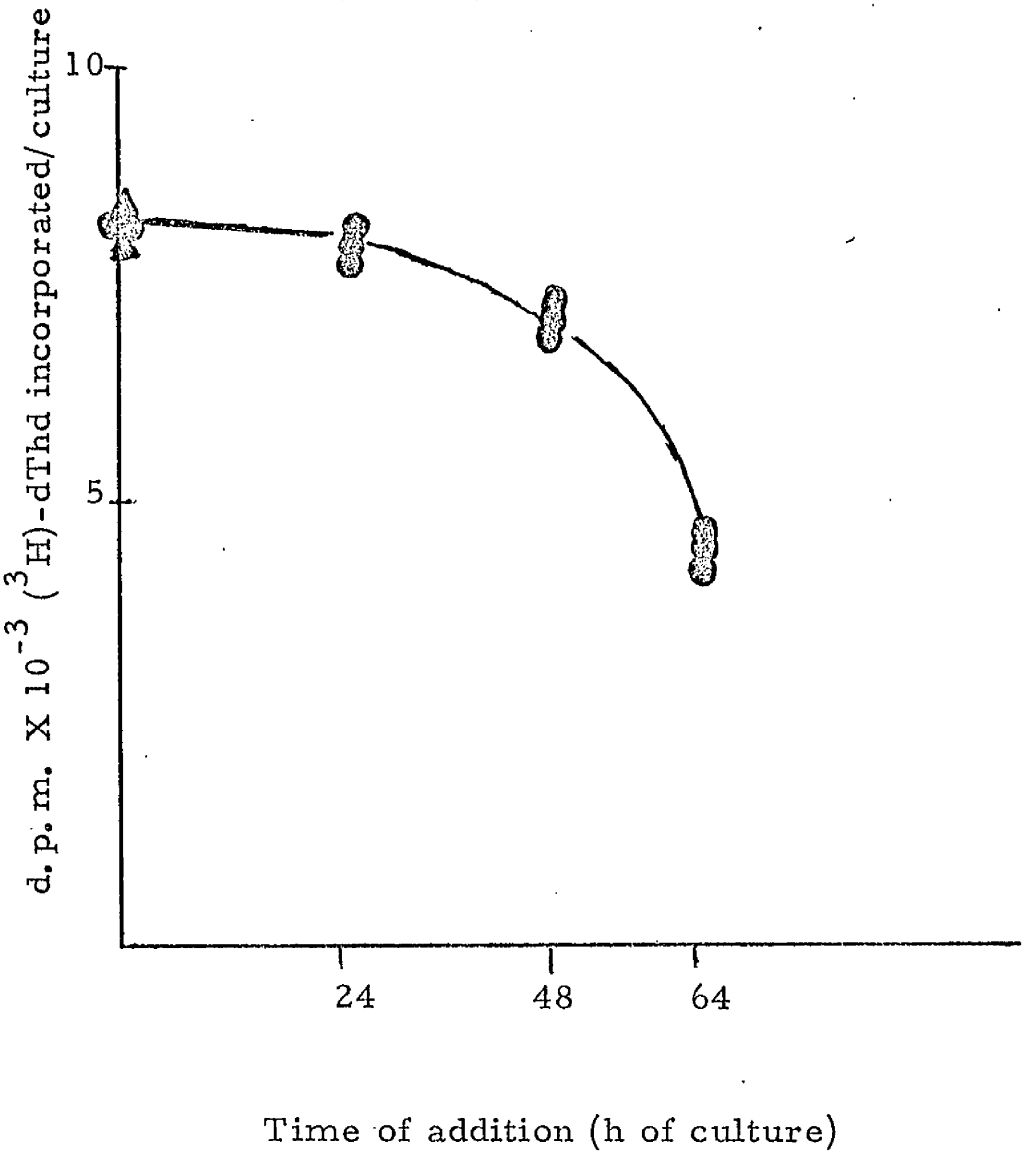
Two ml cultures containing 1 X 10^6 lymphocytes (purified by Boyum's method) were used. The cells were incubated after PHA addition as per standard method, and cyclic AMP added at 0, 24, 48 and 64th h of culture. The cells were pulse labelled with (6^3H) dThd (5uc, 4 X 10^{-6}M) for 2h, immediately before harvesting at 72h. The growth was assayed according to the standard method described in Methods section 2.6).

The results are expressed as d.p.m. dThd (6-^{3}H) incorporated into the DNA of cells present per culture.

▲ +PHA without cyclic AMP

● +PHA and + 1 X 10^{-5}M dibutyryl cyclic AMP

Fig III-6 (c)


d.p.m. X 10^{-3} (^{3}H)-dThd incorporated/culture
10
5
24
48
64
Time of addition (h of culture)

at 64th h of culture).

Macmanus & Whitfield (1970) also observed an inhibitory effect of the substance on rat thymocytes at concentrations above 10^{-4}M. Smith, Steiner, Newberry and Parker (1969) reported that 10^{-4}M dibutyryl cyclic AMP inhibited the uptake and incorporation of (^{3}H)-dThd by more than 60% and the inhibition was more marked during the first hour of a 72h incubation.

1.9 Time of onset of DNA synthesis and the effect of amethopterin: -

In order to see how many hours after PHA treatment horse lymphocytes start DNA synthesis, and how long it is continued, PHA was added to the cultures, and the rate of DNA synthesis was assayed every day for four days. Fig III-7 reveals that the cells start synthesizing DNA somewhere between 24-30h after PHA addition. The rate of DNA synthesis seems to be linear for the following four days after PHA treatment.

Amethopterin is an analogue of folic acid. It has been shown to be an inhibitor of DNA synthesis as it blocks the conversion of dUMP to dTMP. Amethopterin also changes the activities of a number of enzymes involved in the DNA synthetic pathway (Bertino - 1963).

Rueckert & Miller (1960) working with Hela cells showed the endogeneous synthesis of dThd and concomitant DNA synthesis can be blocked with amethopterin, under culture conditions which permit the continued RNA and protein synthesis.

FIG III - 7

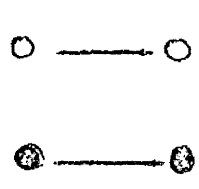
Extent of DNA synthesis and the effect of amethopterin

Cultures of lymphocytes (purified by Rabinowitz' method) containing 3 X 10^6 cells/3ml of the culture medium were established. The cells were labelled at the end of 1, 2, 3 and 4 days of culture by addition of 0.1ml of solution of (2-^{14}C)-dThd (3.66mc/mM, 30uc/ml) 6h immediately before harvesting.

The radioactivity incorporated into DNA was determined (c.f. Methods section 2.4.1).

A similar experiment was conducted as shown above, except that amethopterin (1 X 10^{-6}M) was added 16h before labelling the cells with thymidine.

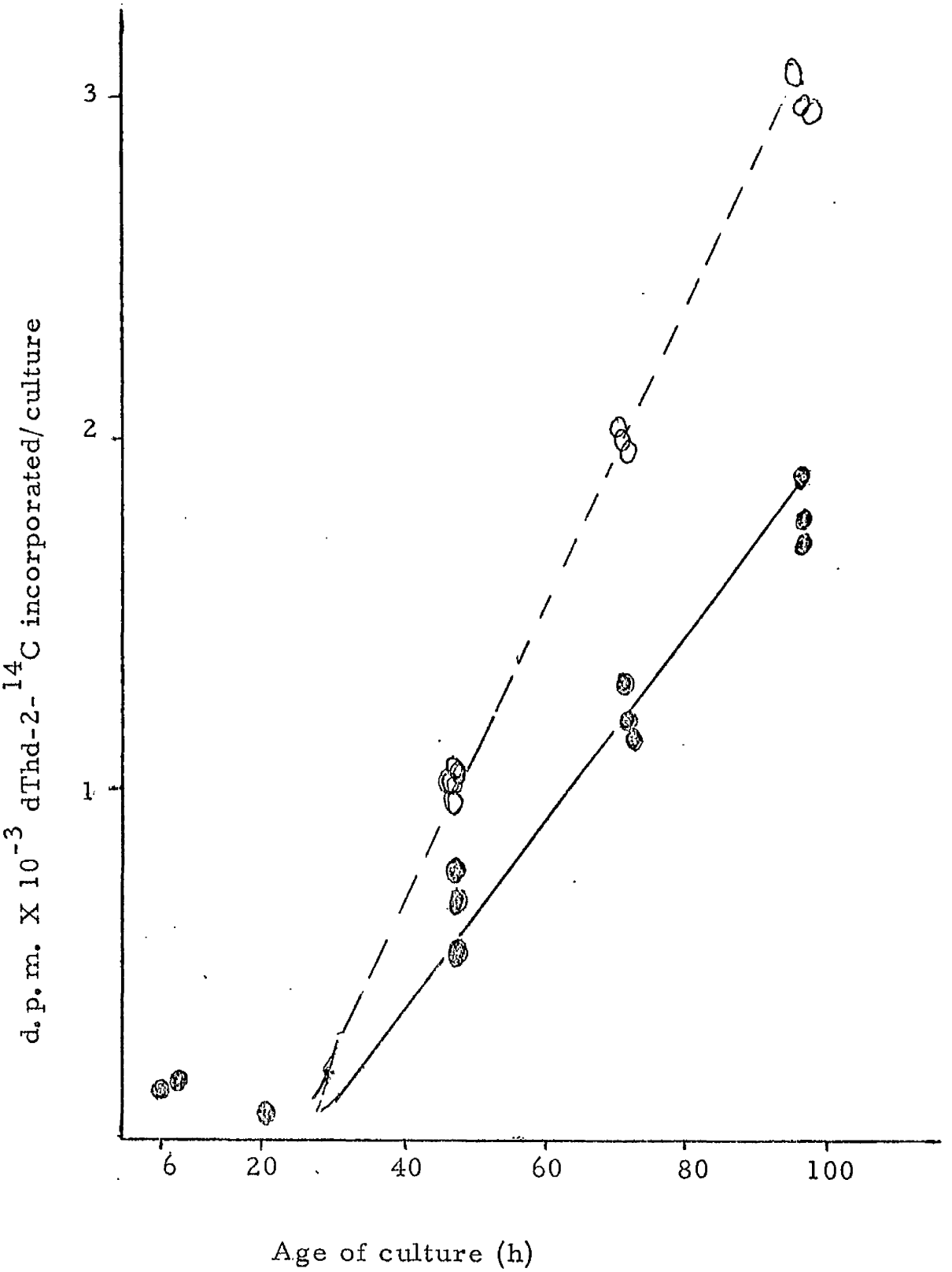
The results are expressed as d.p.m. (2-^{14}C) dThd incorporated into DNA per culture.



Amethopterin treated PHA stimulated cells

PHA stimulated lymphocytes

Fig III - 7


d.p.m. X 10^{-3} dThd-2-^{14}C incorporated/culture
3
2
1
6
20
40
60
80
100
Age of culture (h)

If however 16h after amethopterin treatment the inhibition is reversed by the exogeneous supply of dThd, a synchronous wave of DNA synthesis ensues in almost all cells in the population. Later on Tormey & Mueller (1965) tested this action of amethopterin in leukocyte cultures during the period of rise in DNA synthesis and showed that the concept applies to leukocyte cultures. It has been shown to reversibly block cells in S phase. (Steffen and Stolzman - 1969).

In order to study the sensitivity of our assays, we checked the effect of amethopterin on DNA synthesis in horse lymphocyte cultures. The results Fig III-7 reveal that amethopterin treated cultures incorporate significantly higher amount of 2-^{14}C dThd compared to the untreated controls.

Pegoraro and Benzio (1971) have shown that 5ug amethopterin per ml of culture increases the incorporation of (^{3}H)-dThd threefold compared to the untreated cells. Fig III-7 reveals that 1 X 10^{-6}M amethopterin increases the incorporation of exogeneous (2-^{14}C)-dThd in horse lymphocytes by a factor of 1.8 compared to untreated cells.

2. <u>ISOLATION, PURIFICATION AND STORAGE OF LYMPHOCYTES FROM HORSE BLOOD</u>:-

Lymphocytes employed in our studies, have been isolated from the blood and purified by two different techniques. To start with the glassbead column method of Rabinowitz was employed (Rabinowitz Y. 1964).

2.1 Glass bead column method:-

In this method the granulocytes are allowed to adhere to the glass beads, under proper conditions (c. f. Methods Section 2. 1. 1(a)) while lymphocytes and erythrocytes are eluted out of the column. Thus there are no means of separating RBC from lymphocytes.

Initially the presence of RBC in the cultures was not a problem since, the RBC do not synthesize DNA and hence did not interfere with our assays. The trouble started when we came to study protein-metabolism, i. e. to isolate and characterize the new proteins synthesized by lymphocytes as a result of stimulation with PHA. Preliminary attempts to isolate the lymphocyte proteins by electrophetic fractionation on polyacrylamide gels were not successful. The major problem was the presence of unwanted RBC proteins and the latter were present in such a high concentration that they masked the lymphocyte proteins.

Attempts to destroy the RBC using differential lysing procedures with hypotonic saline (Dane & Hall - 1967) resulted in the destruction of lymphocytes leading to loss of lymphocyte proteins. Thus we started looking for some other technique of lymphocyte purification . Application of the triosil-ficoll grandient centrifugation method of Boyum (1968) for the isolation and purification of horse blood lymphocytes, for invitro cell culture studies proved satisfactory.

2.2 Triosil-Ficoll method:-

When anticoagulated blood is layered on the top of a

layer of ficoll and triosil, the red cells are clumped by ficoll and fall down to the bottom of the tube, due to mixing and diffusion in the top and bottom layer a gradient layer is formed. The density and viscosity increases in the downward direction. ~~On centrifugation~~ even separation is accelerated, even on standing at 1g gravity field the driving force remains constant and the cellular elements are divided into 2 main fractions; granulocytes and red blood cells sediment to the bottom, while mononuclear cells together with platelets remain at the interphase.

2.3 Characteristics of purified lymphocytes:-

2.3.1 Yield and purity of lymphocytes separated by Boyum's method:-

Purity:-

The cell pellet obtained after purification (Section 2.2.1) of lymphocytes on triosil-ficoll gradients was suspended into 10ml of growth medium. A small aliquot was transferred to a tube & centrifuged at 1000g for 10 min at room temperature. The supernatant was discarded, and the cell pellet was suspended in a drop of autologous horse plasma. Smears of the cell suspension were made on glass slides and stained with Leishman Stain. A differential WBC count was done (Hunter and Bomford, 1968). The differential white blood cells count reveals that about 80-90% of the total cells present are lymphocytes.

FIG III - 8

Effect of centrifugation on Column Purified Cells

Cells were purified by Rabinowitz' method and 2ml cultures containing 1 X 10^6 cells/culture were set up according to the standard method (Section 2. 2. 1 Methods). Before the onset of incubation a set of cultures were centrifuged at 1000g for ten minutes at room temperature in MSE centrifuge, tubes were shaken thoroughly and the cells resuspended in the same medium. The procedure of centrifugation and resuspension was repeated twice. A similar set of tubes which were not centrifuged served as a control. The cultures were incubated under standard conditions, and the cell growth was assayed (c. f. Section 2. 6). at the end of 72h incubation, after labelling the cells with 0. 1ml of a solution of 2-^{14}C-dThd (3. 66mc/mM, 30uc/ml).

The results are expressed as c. p. m. of (2-^{14}C)-dThd incorporated into DNA per culture.

FIG III - 8

DNA synthesis (c. p. m.)

Control	Test
200	170
190	200
180	180

The contamination with granulocytes is very low, being only 0-1%. Erythrocyte contamination is decreased manifold and the number of red cells present is only 3-5% of the total cells.

Yield:-

The number of WBC present in the leukocyte rich plasma (Section 2.1 Methods) was counted on a haemocytometer (Hunter & Bomford, 1968). The initial WBC count varied from animal to animal. The range was 6-20 X 10^6 cells/ml of leukocyte rich plasma. The lower the initial peripheral blood count, the greater was the stimulation achieved.

The total yield of lymphocytes separated by Rabinowitz' method was only 15-20% calculated from fifteen individual experiments. The yield of lymphocytes separated by Boyum's method was about 90-95%.

Loss of cells in Rabinowitz' method occurred while transferring the concentrated cell suspension onto the top of the column. Cells remained sticking on to the sides of the tubes, in the pippette and on the column. In Boyum's method, the cells can be rinsed with saline and transferred completely to the top of the gradient.

2.3.2 Effect of Centrifugation on Lymphocyte growth:-

Rabinowitz (1964) demonstrated damage in lymphocytes that had been centrifuged at only 150g and Elves (1965) showed reduced viability.

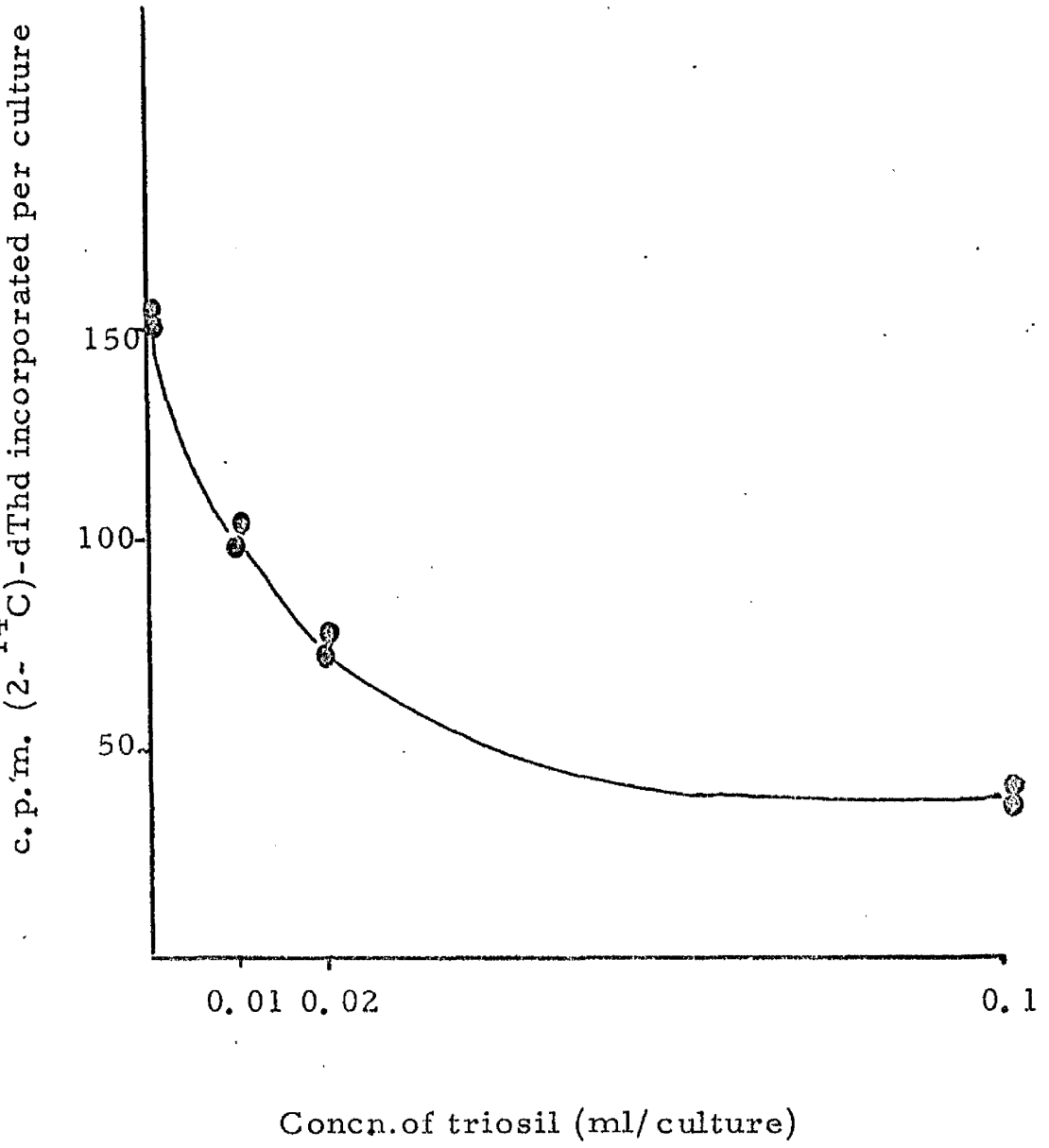
FIG III - 9

Effect of Triosil on column purified cells

Cells were purified by Rabinowitz' method and cultures similar to those described in the previous section were set up. After the addition of PHA-M (0.005 units/culture) different volumes of stock triosil solution (density = 1.200g/ml) were added to sets of cultures. The growth of the cells was assayed at the end of 72h as described in the previous section.

The results are expressed as c.p.m. of (2-^{14}C)-dThd incorporated into the DNA per culture.

Fig III - 9


c.p.m. (2-^{14}C)-dThd incorporated per culture
150
100
50
0.01 0.02
0.1
Concn.of triosil (ml/culture)

In contrast to the above mentioned effects, Fig III-8 shows that centrifugation of equine lymphocytes at 1000g, thrice for 10 min intervals, at room temperature, does not damage the cells and the extent of growth is equivalent to the cells not centrifuged.

2.3.3 Effect of Triosil on the growth of lymphocytes:-

Fig III-9 reveals that the presence of 0.01ml (600ug) triosil solution (Stock, 60% $^{w}/v$) inhibits the lymphocyte growth by 66% of the control.

However triosil is present in the separation fluid and this may have an inhibitory effect. If it does, then it can be removed by washing the cells as shown in Fig III-10.

2.3.4 Effect of washing and the presence of separation fluid in the culture:-

Caron (1967) showed reduced transformation of lymphocytes after repeated washing and centrifugation. Fig III-8 reveals that washing and centrifugation twice under our experimental conditions does not effect the growth of equine lymphocytes.

Fig III-10 however, shows that the presence of separation fluid 5% $^{v}/v$ (c.f. Materials 1.1.f) inhibits the growth of the cells by 63% of control but this effect can be completely removed by washing the cells twice with the growth medium. (Fig III-10).

FIG III - 10

Washing away the separation fluid on lymphocyte growth

A suspension of 50 X 10^6 lymphocytes (purified by Boyum's method) in 5ml of the separation fluid (section 2.1.2 Methods) was diluted with 95ml of 10% HPEHM and used for setting up the cultures.

2ml cultures containing 1 X 10^6 lymphocytes were set up according to the standard method (c.f. Methods section 2.2.1) and divided into 3 sets. Before the addition of PHA, all 3 sets of cultures were centrifuged at 800xg, for 10min, at room temperature and the media was discarded. The cells in set 2 and 3 were suspended in 2ml of EHM devoid of Plasma and washed by recentrifugation and resuspension once and twice respectively. Finally the cells, in all the sets were suspended into 2ml of 10% HPEHM. PHA-M at a concentration of 0.005 unit per culture was added, and the cells incubated under standard conditions. The growth was assayed in 72h old cultures, by labelling the cells for 6h (immediately before harvest) with 0.1ml of a solution of (2-^{14}C)-dThd 3 uc, 3.66mc/mM. The control was a set of cultures, similar to those described above, containing the same number of lymphocytes purified by Rabinowitz method which had not undergone any washing operations. The results are expressed as c.p.m. of (2-^{14}C)-dThd incorporated into DNA per culture.

CP Cells = Column Purified Cells

TP Cells = Triosil Purified Cells

FIG III - 10

c. p. m. (2-^{14}C)-dThd incorporated per culture			
CP Cells	T. P. Cells		
	No. of washings		
	0	1	2
180	120	150	190
190	125	160	200
180	115	150	170

2.4 Conditions for the storage of lymphocytes:-

In certain experiments, especially in those where time course studies were made, it was inconvenient to set up the cultures and start the experiment the same day. Thus we sought for the optimum storage conditions of purified lymphocytes.

Fig III-11 shows that preservation of the cells at $0^{o}C$ overnight, with or without PHA, delays or inhibits the growth response studied after 72h incubation with PHA under standard conditions (Methods, Section 2.2.1). Tullis (1953) has shown that preservation at $0^{o}C$ is harmful for the cells, as both the cell number and the viability of the cells is decreased.

Preservation of the cells with PHA at room temperature probably induces growth in the cells. When growth of the cells is measured at 72h of incubation under standard conditions, following preservation overnight with PHA at room temperature ($22^{o}C$), the extent of growth is higher in the test cultures, compared to the 72h controls which were not preserved.

The change in the medium has a marked effect in reducing the growth of the cells, in cultures which are preserved either at $22^{o}C$ or $37^{o}C$ in the presence of PHA, compared with the cells to which PHA was added next morning.

There is no significant difference in growth of cultures which are preserved without PHA, whether the growth medium is changed next morning or not, and the extent of growth is almost comparable to the controls.

FIG III - 11

Conditions for the storage of lymphocytes

Cultures containing 1 X 10^6 cells in 2ml of 10% HPEHM were set up. To half the cultures PHA-M, at a concentration of 0.005 unit/culture was added. The cultures with and without PHA were divided into 3 groups and preserved at $0^{\circ}C$, $37^{\circ}C$, and at room temperature ($22^{\circ}C$), after gassing with 5% Co_2 in air.

Next morning the following treatment was done.

All the cultures preserved at different temperatures were divided into 2 groups. To the first group PHA-M (0.005 unit/culture) was added (to those cultures devoid of it) and incubation was carried out under standard conditions without a change in the medium.

In the case of the other set, media was changed (c.f. Methods section 2.5) and all the cultures received 0.005 unit of PHA-M before incubation at $37^{\circ}C$. The cells were incubated for 72h, labelled with 0.1ml of a solution of (2-^{14}C)-dThd 3uc (3.66mc/mM) for 6h immediately before harvesting, and the growth assayed (c.f. Methods, Section 2.6).

The results are expressed as d.p.m. of (2-^{14}C)-dThd incorporated into DNA per culture.

(*the time of incubation with PHA at $37^{\circ}C$ in these cultures has been 86h).

Control was a set of cultures containing cells which were not preserved. Cultures of freshly isolated cells were incubated with PHA and grown for 72h under standard conditions.

DNA synthesis in control cultures
expressed as d.p.m. = 350 and 320

FIG III - 11

Storage Temp.	Cells preserved with PHA		Cells preserved without PHA	
	Medium changed	Medium not changed	Medium changed	Medium not changed
0°C	200	201	121	133
	156	172	125	138
22°C	287	418	323	323
	183	384	320	311
37°C	280*	450*	320	325
	220*	430*	330	308

at 37°C +PHA

Thus we selected the following conditions as the standard conditions for the preservation of purified lymphocytes.

After purification, the standard cultures were set up according to the experimental need, and left overnight at 37°C either in Co_2: air (5% Co_2 in air) incubator or, in the case of burrlars, after gassing the cultures with 5% Co_2 in air, without the addition of PHA. The following morning, PHA-M was added, and the time of addition of PHA-M counted as 0h of culture.

3. BLASTOGENESIS IN EQUINE LYMPHOCYTES:-

3.1 Morphological studies:-

At the beginning of culture, small lymphocytes are the predominant cells. These cells are characterised by having a small size (7-10um) containing a large nucleus with only a small ring of cytoplasm. Within minutes of PHA-M addition the cells start clumping, (Fig III-12(b)) and the size of the clump increases with the time of incubation.

When 1×10^8 cells were grown in burrlars, on the third day of culture we observed that the size of the clump was so big that it looked like a sheet of cells, and very few single cells were found. Killander and Rigler (1965) reported that on stimulation with PHA the cells acquire an increased capacity to bind the dye acridine orange.

The photographs (Fig III-12b) reveal that in PHA treated cultures though the cells are present in small clumps on the first day of culture neither their size nor

FIG III - 12

Morphological studies:-

The procedure was the same as described in Methods section 2.7.

Fig III-12 (a) Lymphocytes in a freshly prepared culture soon after the addition of PHA. (X 10 magnified).

Fig III-12 (b) Lymphocytes, 24h after PHA addition (X 10 magnified).

Fig III-12 (c) Lymphocytes, 48h after PHA treatment (X 40 magnified).

Fig III-12 (d) Lymphocytes 72h after PHA treatment (X 40 magnified).

Fig III-12 (e) Unstimulated lymphocytes (fresh) (X 10 magnified).

Fig III-12 (f) 72h old PHA stimulated lymphocytes (X 100 magnified)

FIG III - 12 (a)

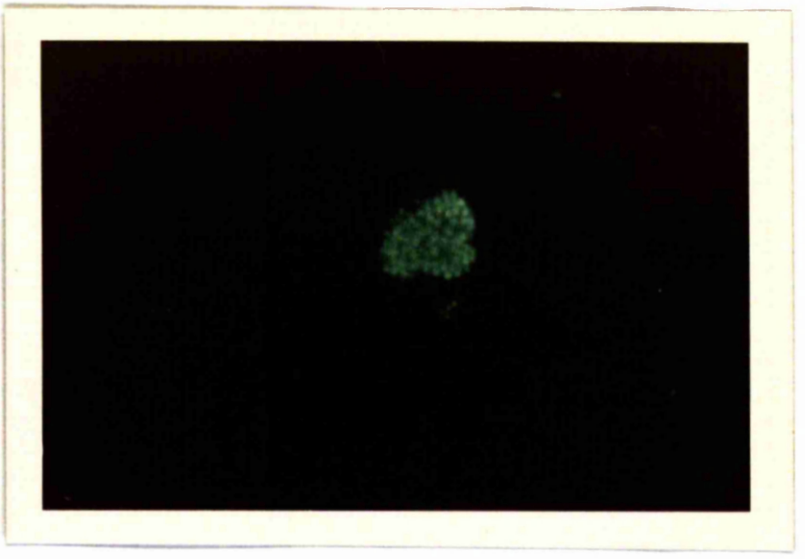


FIG III - 12 (b)

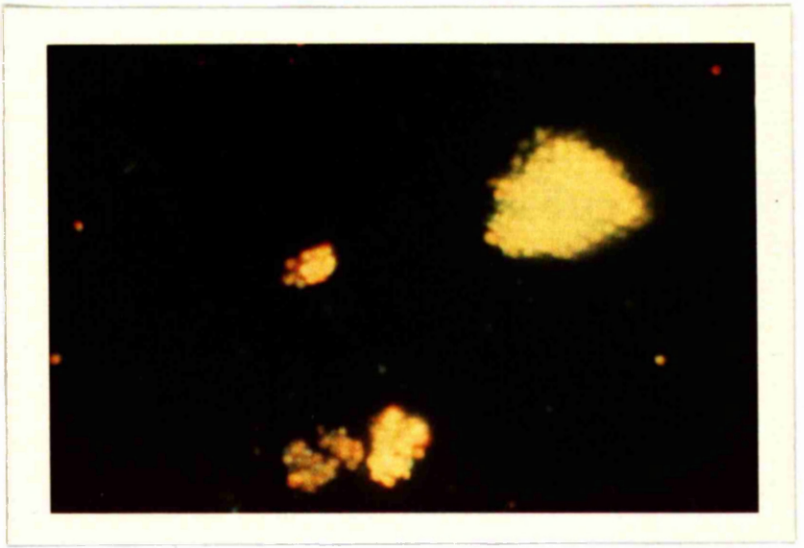


FIG III - 12 (c)

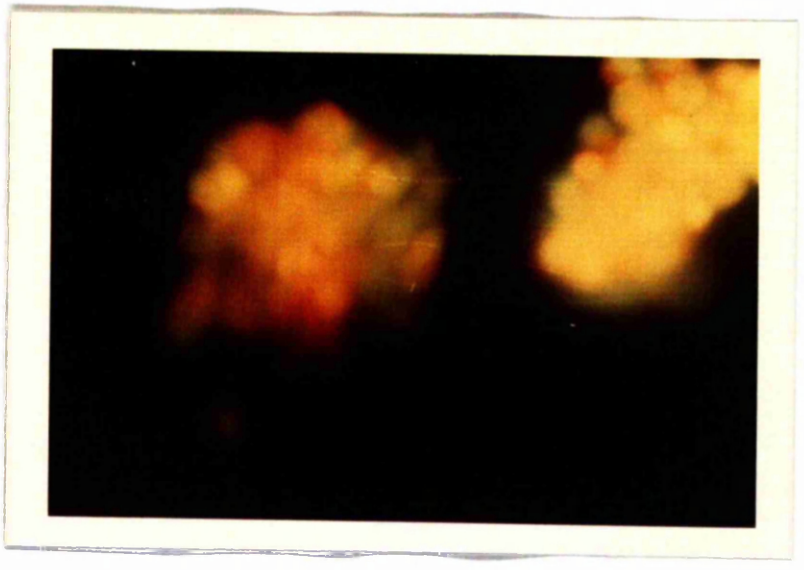


FIG III - 12 (d)

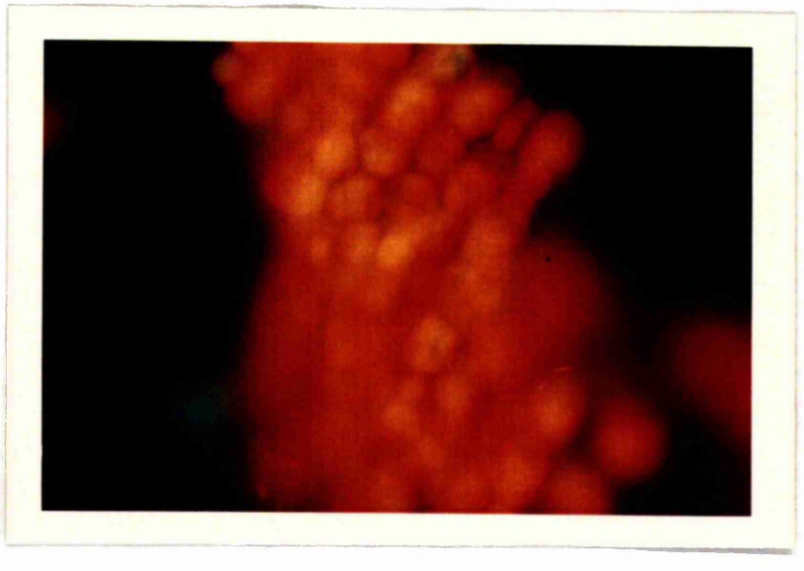


FIG III - 12 (e)

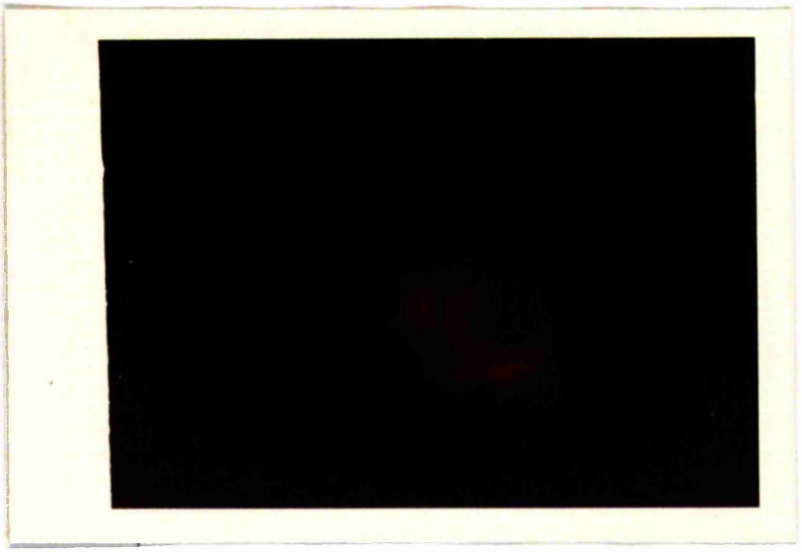


FIG III - 12 (f)

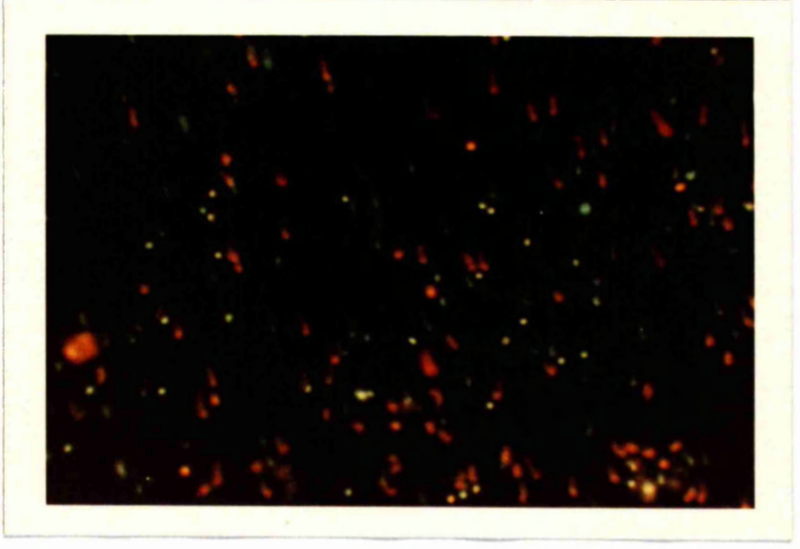


FIG III - 13

Autoradiographic Studies:-

The autoradiographs were prepared as described in Methods section 2. 8.

After staining with haematoxylin the number of labelled cells were counted. Cells having more than 5 grains were counted as labelled cells.

Results are expressed as % of labelled cells on different days of culture.

FIG III- 13

Day	No. of labelled cells	No. of unlabelled cells	% of labelled cells
I	7	100	7%
II	20	105	19%
III	110	5	95%
IV	107	6	100%

the acridine organge binding capacity is significant different from that of the cells not treated with PHA (Fig III-12c).

Deoxyribonucleoprotein (DNP) and ribonucleoprotein (RNP) in fixed cells bind acridine orange and other basic dyes because of the presence of the negative charges on the free phosphate groups in the nucleic acid components. The extent of dye binding has shown to be dependent on the functional state of the nuclear chromatin, a high dye binding being associated with active RNA synthesizing cell nuclei and low in the case of condensed and inactive nuclei (Gledhil, Gledhil, Rigler and Ringertz (1966)).

The results in Fig III-12 show that in unstimulated lymphocytes and in the PHA stimulated lymphocytes on the first day of PHA addition the fluorescence is weak and greenish. As the incubation with PHA is prolonged the fluorescence becomes red. Fig III-12c reveals that by 48h of stimulation, the binding of the dye is stronger and the green fluorescence is being replaced by the orange fluorescence.

Fig III-12d reveals that by the third day of culture in PHA stimulated lymphocytes, almost all the cells present in clumps bind the dye to a very

great extent and the size of majority of the cells in the clumps is increased.

Both the size of the nucleus and bulk of the cytoplasm increases significantly, but it is to be noted that it is the cytoplasm which is increased considerably (Fig III-12e).

3.2 Autoradiographic studies:-

The measurement of (^{14}C) or (^{3}H)-dThd radioactivity incorporated into the DNA gives an indication of the rate of DNA synthesis, but it does not give the information as to how many cells are synthesizing DNA since the population of lymphocytes separated from the blood is heterogeneous. We were interested to know how many cell in the culture are synthesizing DNA at different time periods during a 4 day incubation period.

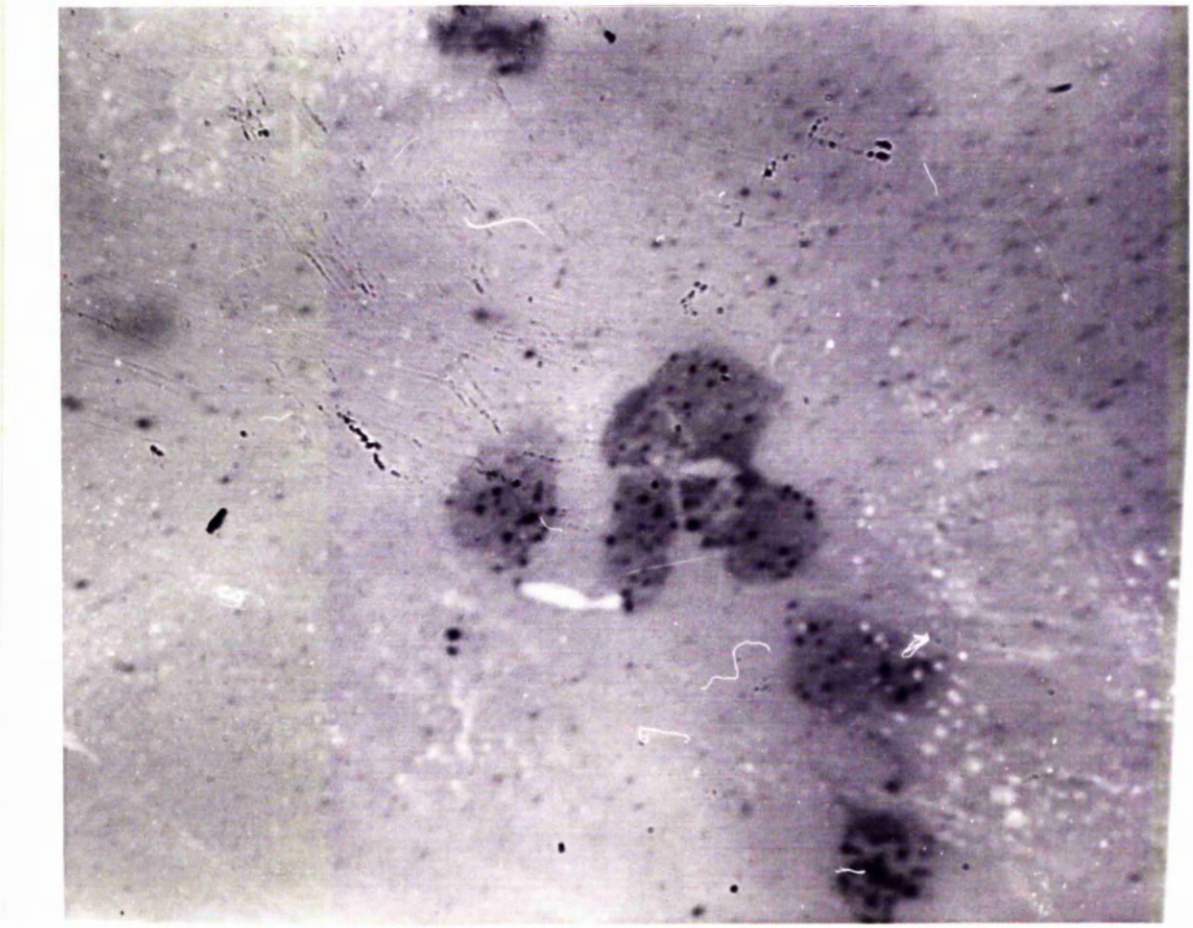
Fig III-13 reveals that on continuous label from 0h 90% of cells are labelled at 72h of cultur and on the 4th day all the cells were labelled. However, the extent of labelling varied considerab and some of the cells were heavily labelled and others not very heavily. (Fig III-14).

Similar type of studies in human lymphocytes have been done by a number of people. Yoffey et a (1965),

FIG III - 14

The experiment is the same described in Fig III-13. Photographs are shown.

FIG III - 14



Darzynkiewicz (1969), Eridani, et al (1969). Our results confirm their results.

3.3 Biochemical Studies:-

Stimulation of lymphocytes with PHA leads to a number of morphological, histological and biochemical changes. Cooper and Rubin (1966) have shown that within a few hours of PHA addition, there is a significant increase in the rate of RNA and protein synthesis, and by 24h of culture, the cells start synthesizing DNA and mitosis sets in.

Since most of these studies were made using human lymphocytes, we studied these changes to see the effect of PHA in horse lymphocytes. Pogo, Allfrey and Mirsky (1959) have made studies using horse lymphocytes, but most of their work is connected with changes in the histone acetylation properties and the deoxyribonucleo-protein complexes in these cells.

We studied a few important biochemical changes in horse lymphocytes to establish that these cells behave like human lymphocytes. Secondly we studied the effects of 5-azaCyd (andin some cases 5-FUra) on these biochemical alterations.

The studies were made during one of two different phases of growth depending on whether we wished to follow:-

a) The effect of the drugs on the induction of growth stimulation and blastogenesis in lymphocytes, when 5-azaCyd was added to the culture simultaneously with the inducer, PHA, or

FIG III - 15 (a)

Rate of DNA synthesis in PHA stimulated and unstimulated horse lymphocytes

Standard 2ml cultures containing 1 X 10^6 lymphocytes (gradient purified) per culture were set up as described in Methods section 2. 1. 1. To one set PHA-M (0. 005 units/culture) was added while the other served as an unstimulated control. The cells were labelled with 5uc (^{3}H)-dThd (4 X 10^{-6}M) for two hours immediately before harvesting and harvested at the end of each day for four days. The radioactivity incorporated into DNA was measured as described in Methods section 2. 4. 1 (a). The results are expressed as the c. p. m. X 10^{-3} (^{3}H)-dThd incorporated into DNA per culture.

O =-PHA cultures ● =+PHA cultures

FIG III - 15 (b)

DNA content of lymphocytes on different days of culture (± PHA)

The cultures were identical to those described above. The cultures were harvested without labelling at the end of each day and washed with 15ml of BSS (cf. Methods, section 2. 3. 1).

The DNA content of the cells present in each culture was measured using Burton's Method (Burton, 1956).

The results are expressed as ug of DNA per culture.

= Cultures - PHA = Cultures +PHA

FIG III - 15 (c)

Protein Content of lymphocytes on different days of culture (+ PHA)

The cultures were identical to those described in Fig 15-b. The protein content of cells present in each culture was determined by the method of Lowry et al, (1951). A solution of BSA (1mg/ml) was used as a standard.

The results are expressed as ug of protein per culture

○ = Cultures -PHA ● = Cultures +PHA

FIG III - 15 (a)

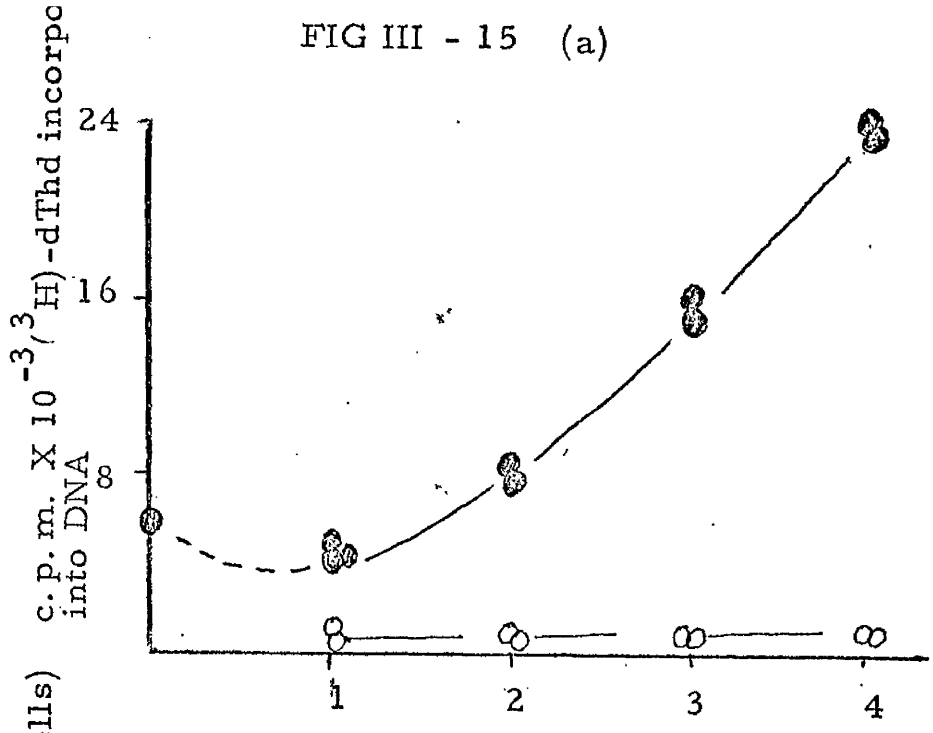


FIG III - 15 (b)

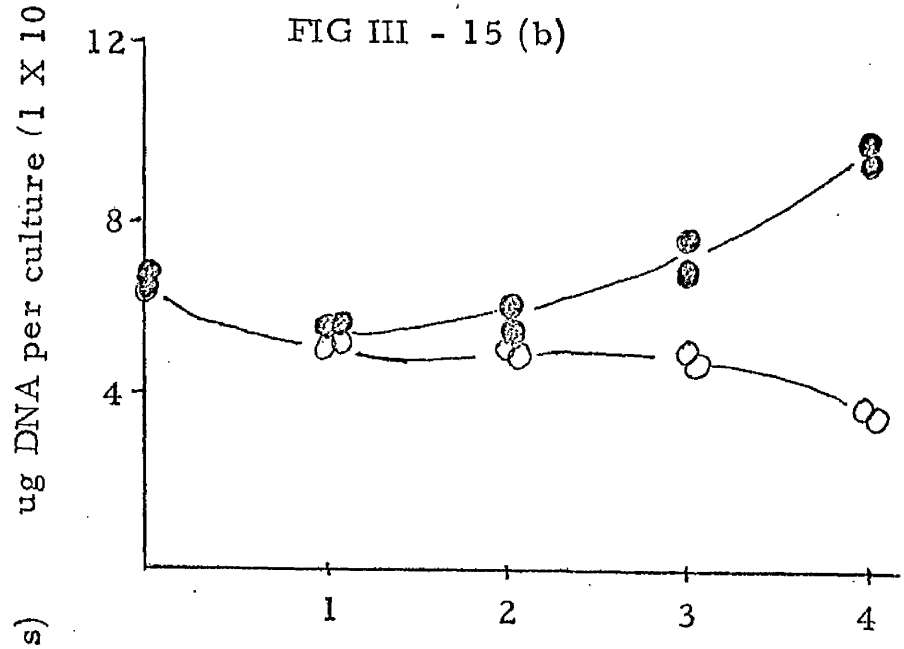
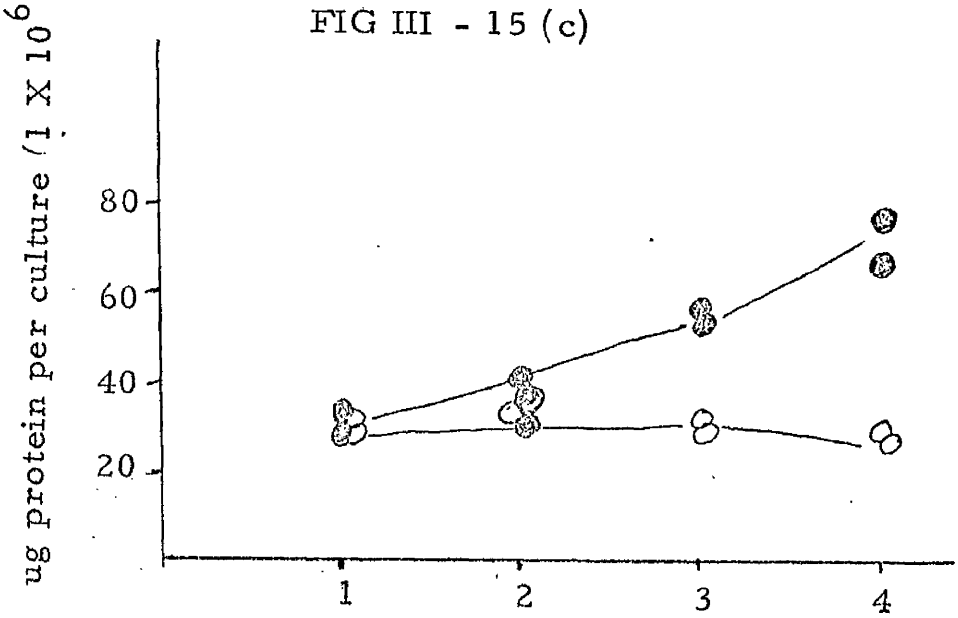


FIG III - 15 (c)



b) The effect of the drug on the events resultant of blastogenesis when the action of the drug in the well stimulated cultures was studied.

3.3.1 Rate of DNA synthesis in un-stimulated and PHA stimulated equine lymphocytes:-

Fig III-15(a) reveals that like human lymphocytes (Presscot & Bender 1962: Ling and Husband, 1964) equine lymphocytes also start DNA synthesis sometime between 24 and 48h, and the rate of incorporation of (^{3}H)-dThd continues to increase over the four days period studied.

3.3.2 DNA content of equine lymphocytes on different days of culture (+PHA):-

In order to further confirm the above mentioned results and also to know the increase in actual amount of DNA as a result of PHA stimulation, we determined the total DNA content of cultures treated with and without PHA, every day for four days of culture. Shellekens and Eijsvoogel (1968) also determined the DNA content in cultures of human lymphocytes. The figures quoted by them are very high compared to ours. To start with at 0h of culture their value is 11.4g DNA per 1 X 10^{6} lymphocytes. While Fig III-15(b) reveals that 6.7ug of DNA is present per 10^{6} equine lymphocytes at the beginning of culture. The mean DNA content per nucleus in leukocytes quoted by Davidson (1969) is 6.6 X 10^{-12}g i.e. 6.6ug/1 X 10^{6} cells.

Fig II - 15 (b) also shows that the DNA content falls slightly on the first day of culture. On 2nd day of culture it comes to the initial level but on the 3rd day in PHA stimulated cells it is equivalent to the original amount present before culturing the cells. On the 4th day the content of DNA in PHA stimulated cultures is significantly higher than the original amount present before the onset of culture.

In unstimulated cells the DNA content falls down on the 3rd day of culture and it is quite low on the 4th day compared to the original DNA content of equine lymphocytes at 0h. These observations are similar to those reported by Shellekens and Eijsvoogel (1968).

3.3.3 Protein content of equine lymphocytes on different day of culture ± PHA:-

Bach & Hirschhon (1963) reported protein production in lymphocytes incubated with PHA. They report that in the presence of PHA 5×10^6 human lymphocytes produced 10ug of protein at the end of 24h of culture.

Turner and Forbes (1966) calculated from their results at 10^8 lymphocytes synthesized 30ug 1gG globulin in 48h. Fig III-15(c) reveals that on the first day of culture the protein content of 1×10^6 equine lymphocytes is about 30ug per culture (1×10^6 cells), by the end of 4th day it almost doubles.

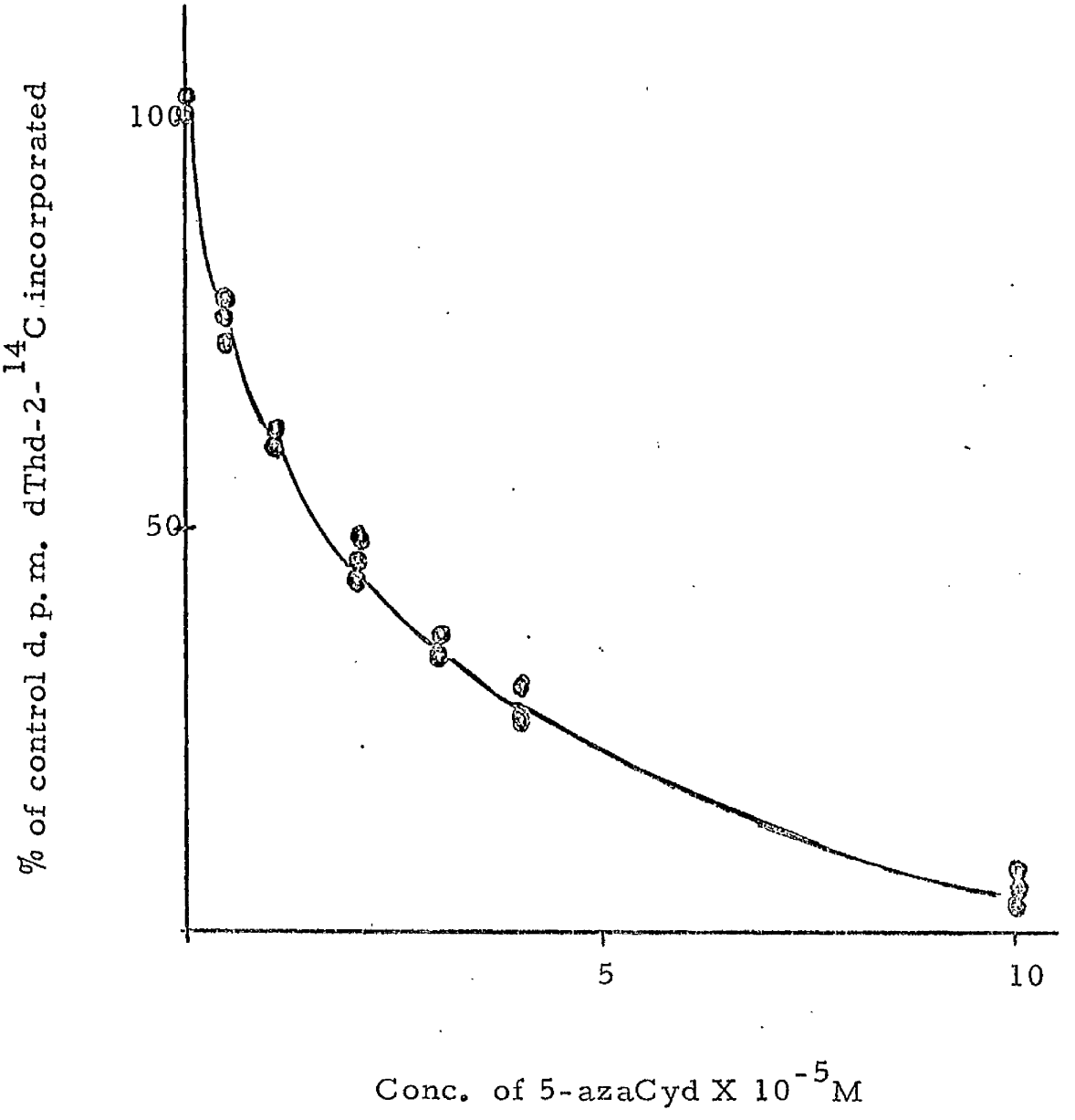
FIG III - 16

Dose response Curve of 5-azaCyd

Lymphocyte cultures containing 1 X 10^6 cells (purified by Rabinowitz method) in 2ml of 10% HPEHM were set according to the standard method (Methods 2. 2. 1.). 5-azaCyd at the concentration shown in Fig III-16 and PHA-M (0. 005 unit/culture) were added at zero time. Incubation was carried out under standard conditions for 2h, and then the drug was removed by washing and changing the medium (c. f. Methods section 2. 5), the cells were suspended in 2ml of fresh medium containing 0. 005 unit of PHA-M and reincubated under standard conditions. The cells were labelled for 6h immediately before harvest with (2-^{14}C)-dThd (0. 1ml, 3. 66mc/mM, 30uc/ml) and the growth assayed as described in Methods section 2. 6.

The results are expressed as d. p. m. (2-^{14}C) dThd incorporated into DNA per culture.

Fig III - 16


% of control d. p. m. dThd-2-^{14}C incorporated
100
50
5
10
Conc. of 5-azaCyd X 10^{-5}M

4. METABOLIC STUDIES USING THE ANTIMETABOLITES 5-AZACYTIDINE AND 5-FUra:-

The basis of undertaking these studies was to ascertain the metabolic changes taking place in stimulated and unstimulated cells, and by using 5-azaCyd and 5-FUra as a tool in understanding the mechanisms of metabolic alterations, we attempted to understand the metabolic patterns and growth control mechanisms in these cells. Since proteins and nucleic acids are the controlling factors of the cell survival, growth and division, we studied the DNA, RNA and protein metabolism.

4.1 Dose response curve of 5-azaCyd:-

5-azaCyd has been reported to be a potent inhibitor of the growth of bacteria (Cihak and Sorm, 1965) an inhibitor of Ehrlich ascites tumour survival and inhibitor in the development of sea urchin (Kvenjakof et al, 1970) (rats, Jurovcik et al, 1965
It has also been reported to be a specific inhibitor of leukemic lymphocytes in AKR mice (Vesely, Cihak, Piskala and Sorm, 1964). Since PHA stimulated lymphocytes in many ways resemble leukemic lymphocytes, we tested the effect of this drug on the growth of equine lymphocytes invitro.

Fig III-17 shows that 2 hour treatment at the beginning of the culture with 1×10^{-4} M 5-azaCyd completely inhibits the growth of horse lymphocytes which is induced as a result of PHA stimulation. 4×10^{-5} M 5-azaCyd inhibits the growth by 70-80%

4.2 Dose response curve by 5-Fluorouracil:-

In addition to 5-azaCyd, 5FUra a potent tumour

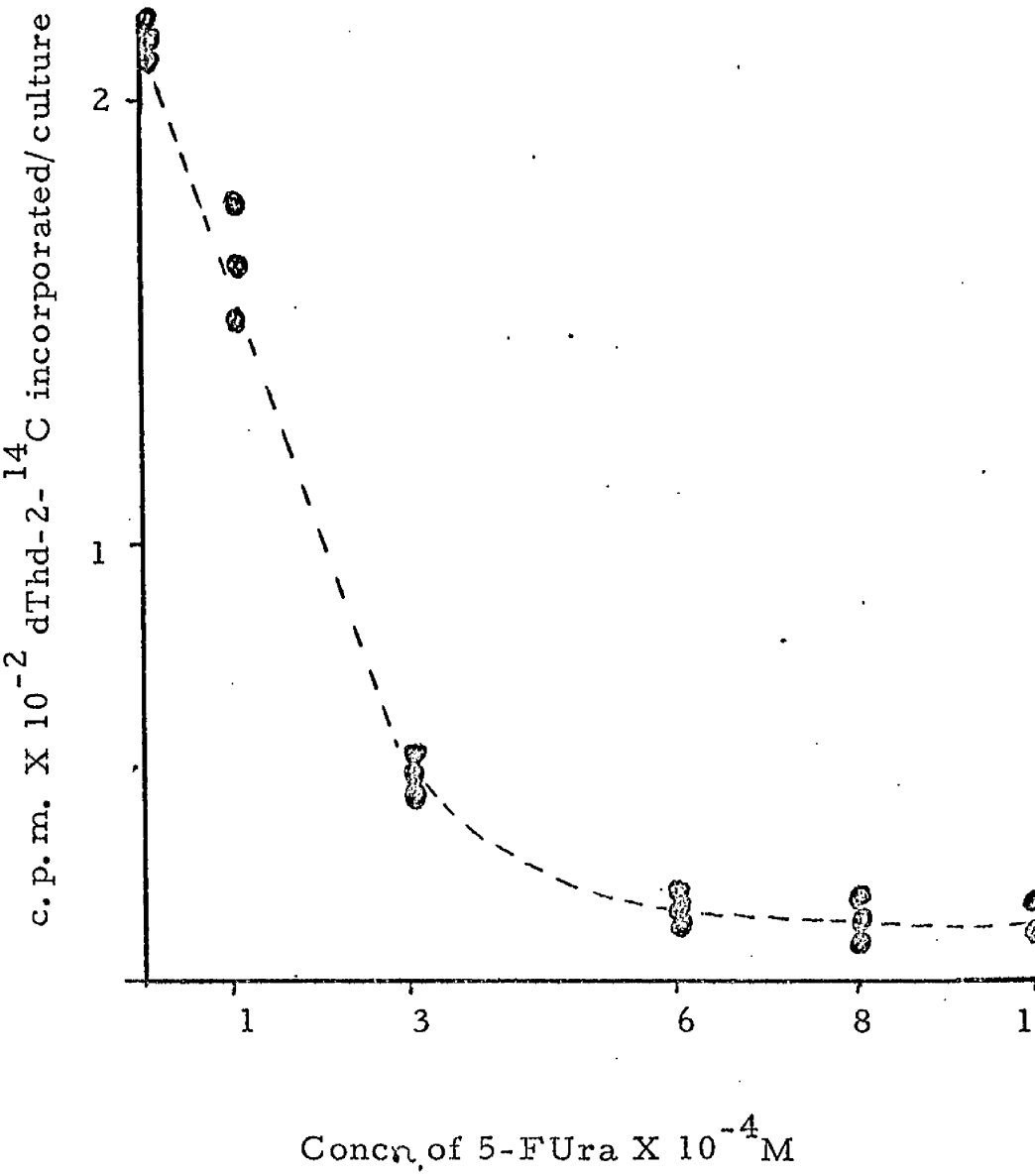
FIG III - 17

Dose Response of 5-FUra

The experiment was exactly the same as described in Fig III-16 except that instead of 5-azaCyd, 5-FUra was added to the cultures.

The results are expressed as c. p. m. (2-^{14}C)-dThd incorporated into DNA per culture.

Fig III-17


c.p.m. X 10^{-2} dThd-2-^{14}C incorporated/culture
2
1
1
3
6
8
1
Concn of 5-FUra X 10^{-4}M

inhibitory drug was also tested to see whether it inhibits the stimulation and growth of equine lymphocytes. Imrie and Robinson (1968) reported the inhibition of DNA synthesis and growth of PHA stimulated human lymphocytes by 5-FUra.

Fig III-16 reveals that 2h treatment at the beginning of culture with 6×10^{-4}M 5-FUra completely inhibits the growth of horse lymphocytes studied at 72h of culture. Growth inhibition to about 20-30% of control is obtained by 3×10^{-4}M 5-FUra.

4.3 5-azaCytidine, inhibitor of growth and stimulation of equine lymphocytes:-

5-azaCytidine has been reported to have a polyvalent mechanism of action (Vesely and Cihak 1967). The drug is unstable and undergoes spontaneous and enzymatic degradations to form some biologically active products. (Pithova et al, 1965). To avoid complications produced by the side products we selected a short time period of drug treatment, instead of allowing the substance to be present in the media throughout the culture period, the cells were treated with 5-azaCyd only for 2h, the medium was changed, cells were washed and fresh growth medium was supplemented.

The extent of growth inhibition was studied at different time periods during the culture. The treatment of 5-azaCyd along with the inducer (PHA) gives an indication of the effect of the drug on stimulation of lymphocytes. Treatment at later time periods when the cells have started DNA synthesis gives an indication of the effect on growth and proliferation of the cells.

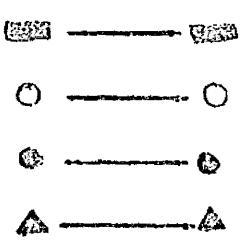
FIG III-18

The effect of 5-azaCyd (treated at varying time periods) on the growth of horse lymphocytes stimulated by PHA

on the growth of horse lymphocytes stimulated by PHA

The lymphocytes cultures were similar to those described in Fig III-16. All the operations were similar except that the cells were treated for two hour intervals from 0-2, 2-4, 26-28 and 48-50h of culture with different doses of the drug as shown in Dig III-23. All the control cultures, without 5-azaCyd were treated the same way during the washing procedure and changing the medium.

The results are expressed as d. p. m. (% control) (2-^{14}C)-dThd incorporated into DNA per culture over a 6h period.



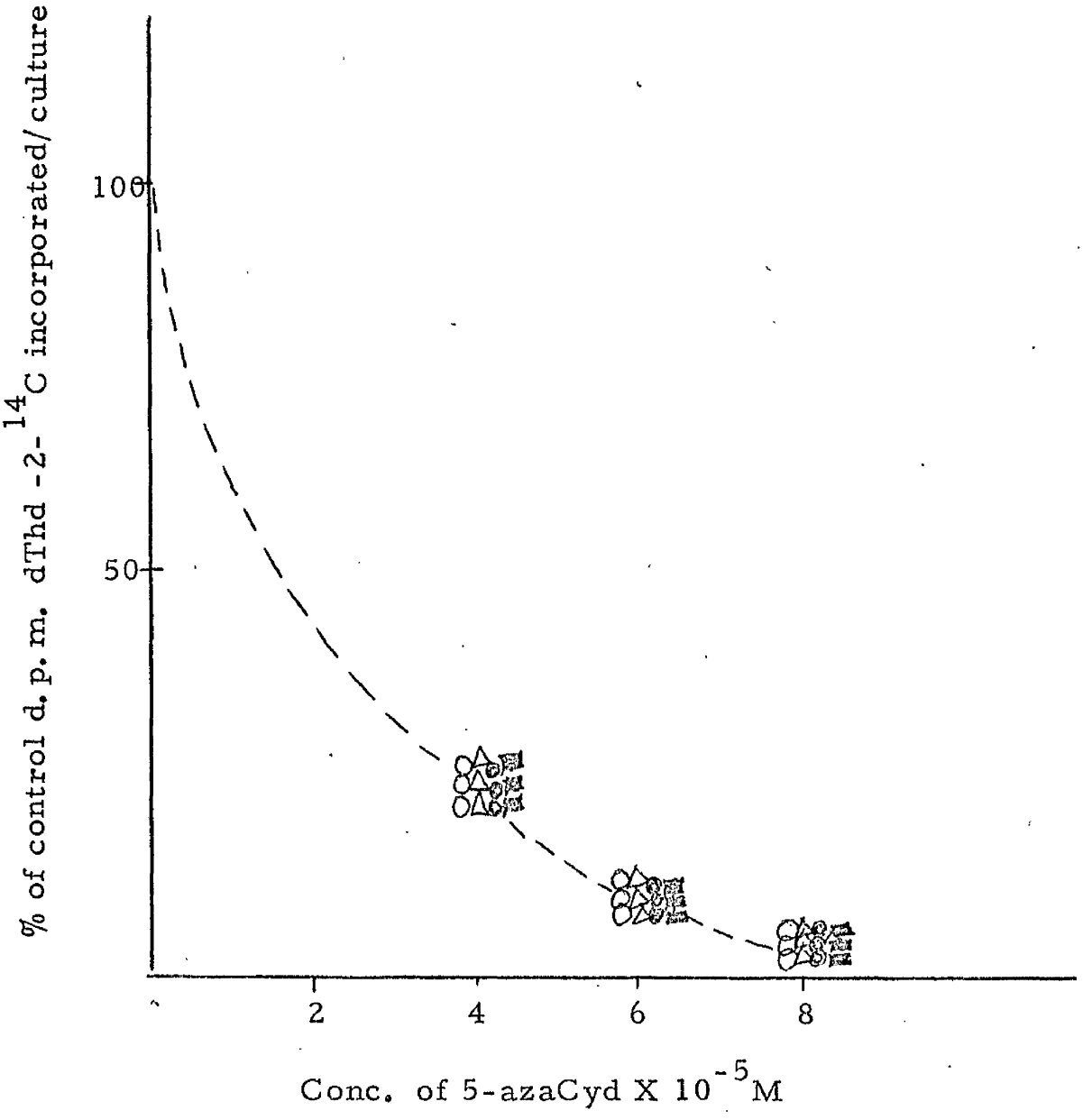
0-2h 5-azaCyd treatment

2-4 5-azaCyd treatment

26-28h 5-azaCyd treatment

48-50h 5-azaCyd treatment

Fig III - 18


% of control d.p.m. dThd -2- ^{14}C incorporated/culture
100
50
2
4
6
8
Conc. of 5-azaCyd X 10^{-5}M

In addition some knowledge is gained if the extent of inhibition is different if addition is made at different time periods. The inhibition may be more pronounced if the degradation products are also involved in inhibitory action. Treatment at early periods and growth assay at the late periods might give an indication, whether or not the cells recover. Thus bearing all these things in mind studies were made by treating the cultures with 5-azaCyd at different time periods during the culture.

Fig III-18 reveals that no matter at what time during the 50h of culture the cells are treated with the drug, the extent of inhibition measured at 72h is the same. This may be interpreted by assuming that the drug is effecting some substance, which is present throughout the culture period and is being continuously synthesized. This substance seems to be essential for the stimulation and growth of the cells.

4.4 Effect of 5-azaCyd on DNA synthesis:-

Doskocil and Sorm (1969) have shown that E. coli cells infected with T_4 bacteriophage, on treatment with 5-azaCyd stop the production of viable phage as a consequence of direct specific inhibition of synthesis of phage DNA.

One of the characteristics features of PHA is that it induces DNA synthesis in lymphocytes, Fig III - 18 and Fig III-19 (a) reveals that the incorporation of (^{3}H) or (^{14}C) dThd into the acid insoluble fraction is inhibited by 5-azaCyd. If the drug is treated at the beginning of the culture, the induction of DNA synthesis is inhibited and there is no DNA synthesis taking place at 28 h and 52 h

FIG III - 19 (a & b)

Effect of 5-azaCyd on the DNA synthesis in equine lymphocytes

Standard 2ml cultures (cf. Methods section 2. 2. 1) containing 2×10^6 lymphocytes (purified by Boyum's method) were used. PHA-M (0. 005 unit per culture) was added at 0h of culture and incubation carried out at $37^{\circ}C$ in a Co_2 in air incubator.

At the time periods shown in Fig III-19, 1×10^{-5} M 5-azaCyd was added to the respective cultures, and reincubated. 5-azaCyd was allowed to remain in the media and the cultures harvested subsequently at the time periods shown in Fig III-19.

2h before harvest the cells were labelled with 10uc of (^{3}H)-dThd (37 Ci/mM) and the amount of label incorporated into DNA was measured as described in Methods section 2. 4. 1.

The results are expressed as d. p. m. $\times 10^{-5}$ (^{3}H)-dThd incorporated into DNA per culture.

FIG III-19(b)

The acid soluble fraction from Fig III-19(a) was collected as described in Methods section 2. 4. 1, 0. 5ml was counted in dioxan scintillation fluid.

The results are expressed as d. p. m. (^{3}H)-dThd incorporated into the intracellular acid soluble fraction per culture.

FIG III - 19 (a)

d. p. m. X 10^{-5} (^{3}H)-dThd incorporated into DNA per culture

Time of harvest (h of culture	Type of treatment	Time of 5-azaCyd addition				
		0h	16h	25h	48h	72h
4h	Control Test	0.7, 0.8 0.7, 0.7				
16h	Control Test	0.0, 0.0 0.0, 0.0				
28h	Control Test	1.9, 2.4 0, 08	2.4, 1.9 0.09, 0.3			
52h	Control Test	7.9, 11.3 2.5, 5.4	7.9, 11.3 0.1, 0.1	7.9, 11.3 0.3, 0.6		
74h	Control Test	13.2, 19.0 5.0, 5.6	13.2, 19.0 3.6	13.2, 19.0 2.2, 2.7	13.2, 19.0 1.3, 1.4	13.2, 19.0 13.3, 12.9

FIG III - 19 (b)

d. p. m. X 10^{-3} (^{3}H)-dThd incorporated into Acid Soluble Fraction

Time of harvest (h of culture)	Type of treatment	Time of 5-azaCyd addition				
		0h	15h	25h	48h	72h
4h	Control Test	0.70, 0.71 0.67, 0.76				
16h	Control Test	0.94, 0.97 0.47, 0.47				
28h	Control Test	1.0, 1.1 0.37, 0.38	0.93, 1.0 0.45, 0.45			
52h	Control Test	1.5, 1.7 0.83, 0.89	1.5, 1.7 0.28, 0.34	1.5, 1.7 0.43, 0.47		
74h	Control Test	1.5, 1.7 1.2, 1.4	1.5, 1.7 0.61, 0.92	1.7, 1.5 0.62, 0.72	1.5, 1.7 0.49, 0.64	1.88, 1.77 1.76, 1.71

of culture. By 74 h of culture a slight increase in (^{3}H)-dThd incorporation takes place, which means either the inhibited cells are recovering or a few cells in the culture which are resistant to the action of 5-azaCyd have been proliferating.

The intracellular acid soluble radioactivity reflects a similar change as described for acid insoluble radioactivity.

As the extent of DNA synthesis was assayed by incorporation of (^{3}H) or (^{14}C)-dThd into DNA, abnormality in the phosphorylation of the dThd might show a depressed incorporation of the precursor, showing an apparent inhibition of DNA synthesis. To confirm this DNA inhibitory effect of 5-azaCyd, we studied the rate of DNA synthesis by the incorporation of ($^{32}P_i$)-orthophosphate in these cells.

Fig III-20 shows that treatment of lymphocytes with 5-azaCyd at the beginning of the culture completely inhibits the DNA synthesis studied at the end of 1st, 2nd 3rd and 4th day of culture.

4.4.1 <u>DNA synthesis in well stimulated cells and the effect of 5-azaCyd on it</u>:-

Previous experiments revealed that treatment of the drug for 2h intervals in between 0-50h of culture and measurement of DNA synthesis at 72h results in the inhibition of DNA synthesis. In an attempt to know whether the drug directly inhibits the DNA synthesis or whether this inhibition is a secondary effect on cell metabolism we conducted the

FIG III-20

Effect of 5-azaCyd on ($^{32}P_i$)-orthophosphate incorporation into DNA of PHA stimulated horse lymphocytes

Two ml lymphocyte cultures containing 1 X 10^6 cells were established, according to the standard method (cf. Methods, section 2.2.1). At 0h of culture 5-azaCyd (4 X 10^{-5}M) was added to the respective tubes, and incubated under standard conditions. At the end of 1st, 2nd, 3rd and 4th day, the culture medium was changed (cf. Methods, section 2.5) and was substituted by 2ml of warm low phosphate medium (1/10th of the normal phosphate concentration) containing 0.005 unit of PHA-M and 50uc ($^{32}P_i$)-orthophosphate (sp. activity 92 Ci/mg P_i).

After a 2h pulse in the presence of ($^{32}P_i$)-orthophosphate and in the absence of aza-Cyd the cells were harvested and the amount of ($^{32}P_i$) incorporated into DNA measured (Methods, section 2.4.2.(b)).

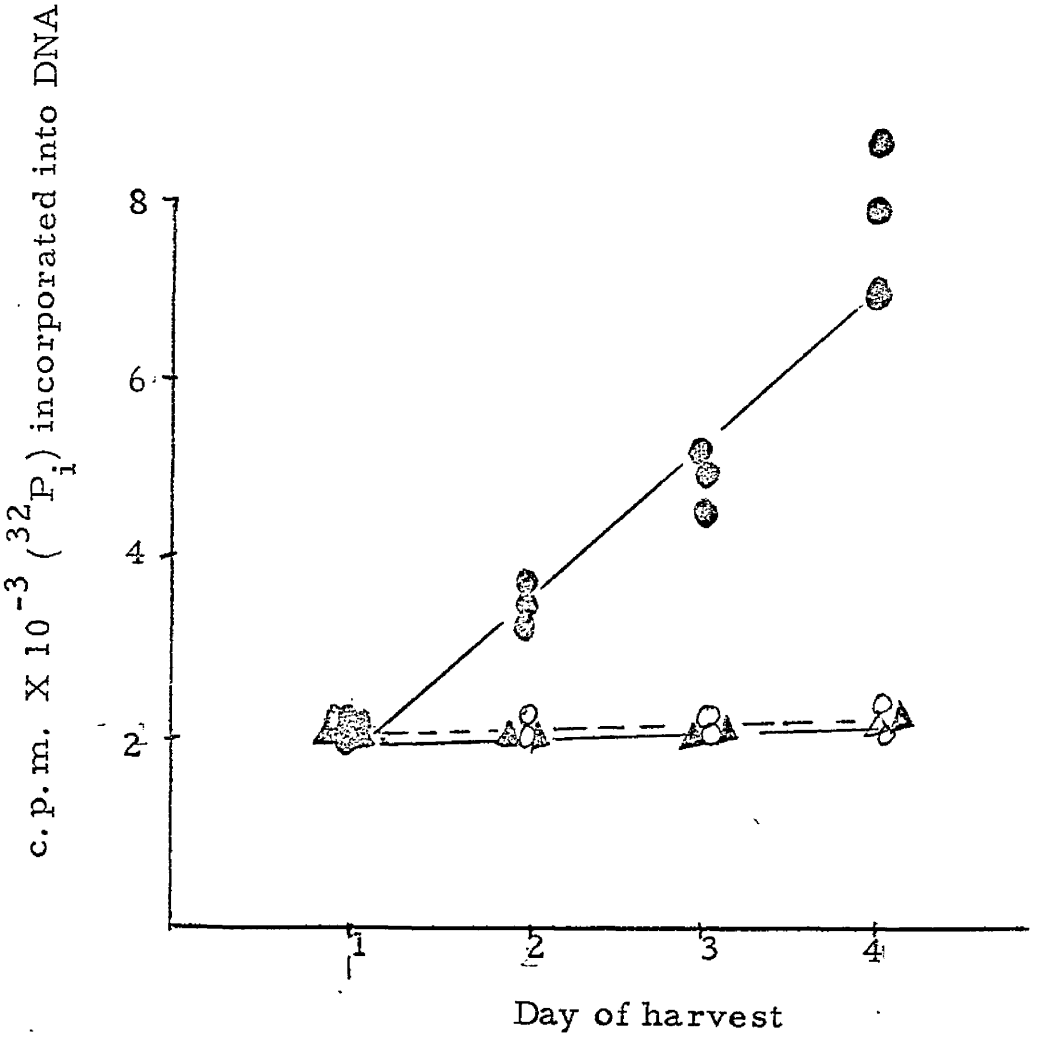
The results are expressed as the c.p.m. ($^{32}P_i$)-orthophosphate incorporated into DNA per culture.

●——● Control cells stimulated by PHA

○——○ Unstimulated cells

▲——▲ 5-azaCyd treated cells

Fig III - 20


c.p.m. X 10^{-3} ($^{32}P_i$) incorporated into DNA
8
6
4
2
1
2
3
4
Day of harvest

experiment described in Fig III-21

Fig III-21(a) reveals that 5-azaCyd does not inhibit the DNA synthesis directly, but it has an indirect effect. The inhibition is not immediate, but after a lag of one hour the depression in the (^{3}H) dThd incorporation becomes apparent, and it takes at least 8h to get 80% of growth inhibition.

4.4.2 Effect of 5-azaCyd on DNA synthesis in mouse fibroblasts (L929 cells):-

Released stationary cells of mouse fibroblasts L929 strain, are characterized by having a high rate of DNA synthesis. Lindsay and Adams (1968) have shown that release of L929 cells from stationary phase results in a synchronised burst of DNA synthesis after a lag period of 16h, and around 20h 70% of the population are in S phase.

Fig III-21(b) shows that a similar treatment as was done for PHA stimulated equine lymphocyte, with the same concentration of 5-azaCyd as described in Fig III-21(a) does not inhibit the rate of DNA synthesis in L929 cells.

Similar observations have been made by Adams (Personal communication).

4.5 Studies connected with the enzymes involved in DNA synthesis:-

Effect of PHA on unstimulated cells and effect of 5-azaCyd on PHA stimulated cells:-

As it has already been mentioned, the aim of our

FIG III -21(a)

Effect of 5-azaCyd (late hour treatment) on PHA stimulated horse lymphocytes

Standard 2ml cultures (cf. Methods section 2. 2. 1) containing 1 X 10^6 cells (purified by Boyum's method) were used. The cultures were incubated for 64h, under standard conditions. 5-azaCyd (4 X 10^{-5}M) was added at 65th h and every subsequent hour for the next 6h to separate cultures, and at 70th h (^{3}H)-dThd 5uc (4 X 10^{-6}M) was added. After pulse labelling for 2h the cells were harvested at 72nd h of culture and the growth assayed. (cf. Methods, section 2. 6). The results are expressed as c. p. m. (^{3}H)-dThd incorporated into DNA per culture.

FIG III-21(b)

Effect of 5-azaCyd on DNA synthesis in release stationary L929 cells

5cm plastic dishes were incubated under standard conditions with 3ml of a suspension of mouse fibroblast cells (L929 strain) at a concentration of 2 X 10^5 cells/ml of Eagle's MEM supplemented with 10% calf serum.

At 25th h of seeding 4 X 10^{-5}M 5-azaCytidine was added to a set of dishes, followed by addition at every subsequent hour for the next seven hours to seven separate set of dishes. Immediately before harvesting the cells were pulse labelled with 5uc (6-^{3}H)-dThd (4 X 10^{-6}M) for 2h and harvested as described in the Methods section. The cells were washed X 4 with Eagle's BSS (Cold), followed by four extractions with 20ml of 5% TCA. The acid insoluble material was solubilised

cont'd.

by incubating with 1ml of 0.3N NaoH at 37°C for 2h and the radioactivity incorporated into the acid insoluble alkali resistant fraction measured by counting the alkali solubilised acid precipitate material is dioxan scintillation fluid in Packard Tricarb spectrometer.

The results are expressed as the c.p.m. (^{3}H)-dThd incorporated into DNA per culture.

Fig III - 21 (a)

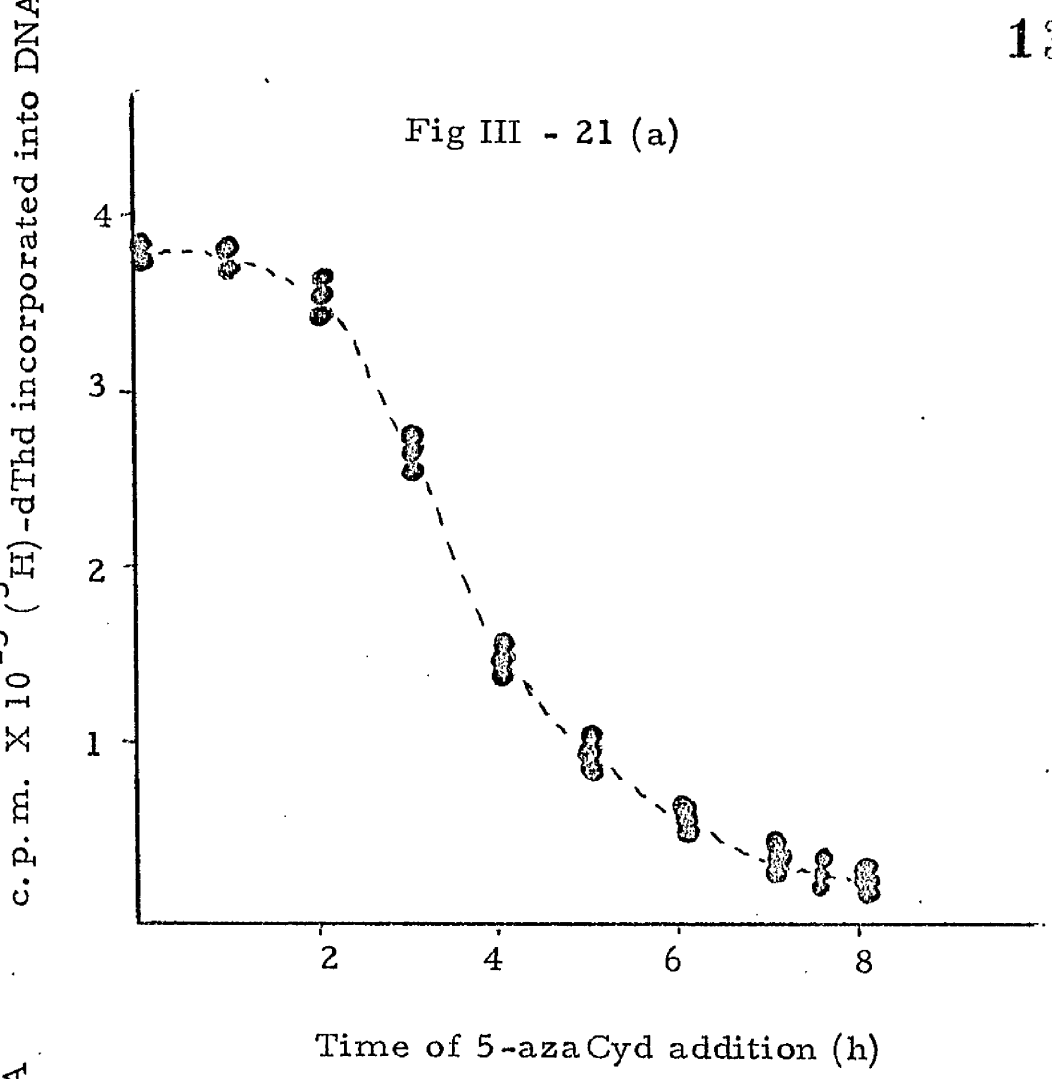
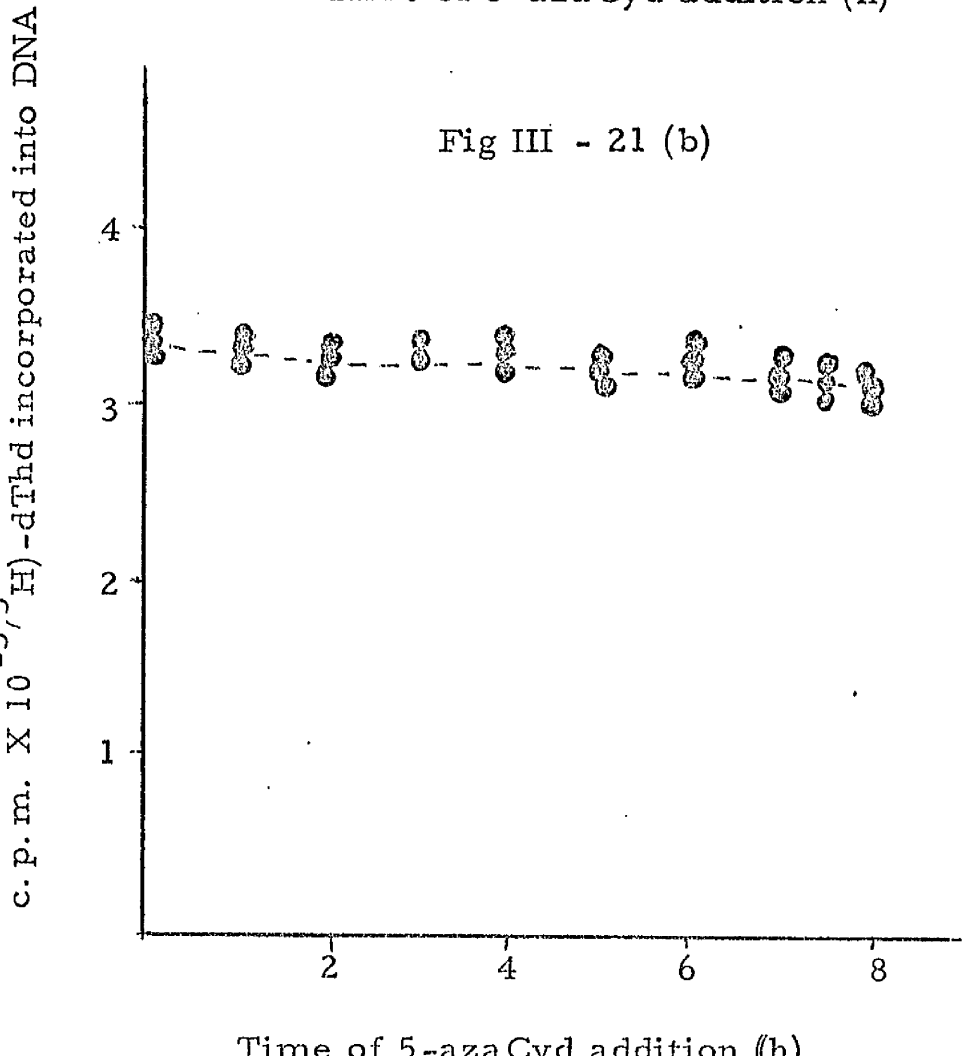


Fig III - 21 (b)



studies has been to learn the mechanism of PHA action. We have been trying to learn the control mechanism for the initiation of DNA synthesis during lymphocyte transformation, and in an attempt to understand these mechanisms we used the antimetabolites as the tool. The experiments described in this section were conducted to look at the major enzymes involved in DNA synthesis (DNA polymerase, thymidinkinase, dTMP kinase dTDP kinase and ribonucleotide reductase).

4.5.1 Thymidine kinase system:

Uptake and phosporylation of Thymidine:-

Since thymidine is the specific precursor of DNA biosynthesis, and a number of steps are involved by the time thymidine is taken up by the cells and is incorporated into DNA. We looked at the uptake and phosphorylation of exogeneous (^{3}H)-dThd in the unstimulated and PHA stimulated equine lymphocytes and also studied the effect of 5-azaCyd on the uptake and phosphorylation of (^{3}H)-dThd in well stimulated cells.

By stepwise phosphorylation the intracellular thymidine is converted to dTTP.

$$\text{dThd} \xrightarrow[\text{kinase}]{\text{Thymidine}} \text{dTMP} \xrightarrow[\text{kinase}]{\text{dTMP}} \text{dTDP} \xrightarrow[\text{kinase}]{\text{dTDP}} \text{dTTP}$$

Each of these steps is catalysed by separate enzyme. Adams (1969) presented evidence that cells in S phase and early G_2 phosphorylate the exogeneously supplied thymidine. Our results from autoradiographic experiments (cf Results Section 3.3.2) reveal that almost all the cells treated with

FIG III - 22

Phosphorylation and uptake of Thymidine

5ml lymphocyte cultures containing 10 X 10^6 cells purified by Boyum's method were set according to the standard method. The cultures were incubated under standard conditions. Four sets of cultures were used, and treated as follows:

i) Cultures of unstimulated cells without PHA, (72h old)

ii) Cultures of cells with 0.01 unit of PHA-M per culture (72h old)

iii) Cultures of cells with 0.01 unit PHA + 5-azaCyd (4×10^{-5} M) added at 69.5h of culture

iv) Cultures of lymphocytes with 0.01 unit PHA + 5-azaCyd (4×10^{-5} M) added at 64h of culture.

The cells were pulse labelled with 50uc (4×10^{-6} M) (^{3}H)-dThd for 2h (70-72h). The cells were harvested after cooling to 0°C, washed thrice with cold Earle's BSS, and the lymphocytes extracted with 1ml of 5% TCA ($^{w}/_{v}$) and the acid soluble fraction collected.

The acid soluble extract was extracted thrice with diethylether and 100u litre aliquots chromatographed as described in Methods section 2.10(a).

The radioactivity in the dThd, dTMP, dTDP and dTTP was counted as described in Methods, section 2.10 (b).

The results are expressed as (a) d.p.m. of (^{3}H)-dThd incorporated in the acid soluble fraction present in dThd, dTMP, dTDP and dTTP fractions giving an indication of extent of dThd uptake and phosphorylation.

FIG III - 22 (a)

Type of treatment	Uptake	Phosphorylation			DNA (Acid insoluble)
	dThd	dTMP	dTDP	dTTP	
-PHA	300	300	100	0	569
+PHA	3100	7300	3800	500	61048
+PHA + 5-azaCyd ($2\frac{1}{2}$h)	2500	4400	4000	200	41504
+PHA + 5-azaCyd (8h)	2000	2800	2600	400	3418

PHA are labelled on the 3rd day of culture whereas about 17-20% start synthesizing DNA by 48h. Thus almost all the cells on 3rd day of culture have been or still synthesizing DNA.

Bearing this in mind the uptake and phosphorylation studies on the 3rd day of culture.

Fig II-22 (a) shows the uptake and phosphorylation in unstimulated, PHA stimulated and 5-azaCyd treated cells. On stimulation with PHA, on the 3rd day of culture the uptake of (^{3}H)-dThd is increased by 10 fold and so is phosphorylation. $2\frac{1}{2}$h treatment with 5-azaCyd inhibits the uptake by 17% when the DNA synthesis is inhibited by 30%, the longer treatment (8h) effects it further. Both $2\frac{1}{2}$h and 8h treatment inhibits the phosphorylation of dThd. Loeb, Agarwal and Ewald (1970) have recently reported that the activities of thymidine kinase and thymidine monophosphate kinase multiply about 2-10 fold on stimulation of human lymphocytes by PHA.

Cihak, Vesely and Sorm (1968) reported that administration of 5-azaCyd 12-14 h after partial hepatectomy inhibits the normal increases in the thymidine kinase activity in regenerating rat liver.

4.5.2 <u>DNA polymerase or DNA nucleotidyl transferase EC 2.7.7.7.</u> :-

Resting cells are characterized by having low activities of enzymes required for the synthesis of DNA. Lindsay and Adams (1968) have shown that in stationary phase the DNA polymerase activity is increased and the cells start synthesizing DNA.

FIG III - 22 (b)

b) The experiment is the same as described in Fig III-22, but the results are expressed as the enzyme activity.

dThd kinase activity = d. p. m. X 10^{-3} (^{3}H) radioactivity in the intracellular dTMP + dTDP + dTTP + Acid insoluble fraction per 1 X 10^6 cells.

dTMP kinase activity = (^{3}H) radioactivity (d. p. m. X 10^{-3}) in intracellular dTDP + dTTP + Acid insoluble fraction per 1 X 10^6 cells.

dTDP kinase activity = (^{3}H) radioactivity (d. p. m. X 10^{-3}) in intracellular dTTP + Acid insoluble fraction per 1 X 10^6 cells

DNA polymerase activity = d. p. m. X 10^{-3} (^{3}H)-dThd incorporated into DNA per 1 X 10^6 cells.

FIG III - 22 (b)

Type of treatment	Thymidine Kinase dTMP+dTDP + dTTP + AI	dTMP Kinase dTDP + dTTP + AI	dTDP Kinase dTTP + AI	DNA Polymerase AI
-PHA	0.97	0.67	0.57	0.57
+PHA	17.7	10.4	6.6	6.1
+PHA (h) + 5-azaCyd $2\frac{1}{2}$h	12.7	8.3	4.3	4.1
+PHA +5-azaCyd for 8h	6.1	3.3	0.7	0.34

d. p. m. X 10^{-3} (^{3}H)-dThd incorporated into the acid soluble (AS) and acid insoluble (AI) fractions per 1 X 10^{6} cells

Unstimulated lymphocytes are quiscent cells and they do not synthesize DNA. On stimulation with PHA, however, the cells start DNA synthesis. Loeb et al, (1968) have shown that on stimulation with PHA, the activity of DNA polymerase increases in human lymphocytes. This finding is confirmed by Rabinowitz et al, (1969) who reported an increase in the DNA polymerase activity after treating the normal human lymphocytes with PHA.

Equine lymphocytes treated with PHA show a similar change (Fig III-23). The figure shows an increase of DNA polymerase activity by 50 fold in PHA stimulated cells on the 4th day of culture. In different experiments the increases ranged from 20-150 fold, the extent of enhancement depending on the initial total peripheral WBC counts in the blood. In one case with an initial WBC count of 4.5×10^6 cells/ml of the leukocyte containing plasma, a 150 fold increase in the DNA polymerase activity was obtained on the 4th day of culture, whereas with an initial WBC count of 11×10^6 cells/ml of plasma a 50 fold increase is shown in Fig III - 23.

1. Comparison of Loeb and Keir's method for DNA polymerase assay:

DNA polymerase activity was measured in the crude cell homogenate (Fig III-23). High speed supernatant fraction (Fig III-25) and pure nuclei (Fig III-25). The high speed supernatant fraction was more active when heat denatured

FIG III - 23

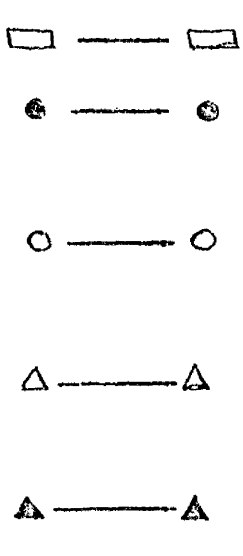
DNA polymerase in PHA stimulated and Unstimulated equine lymphocytes

Two burrlars containing a suspension of 100 X 10^6 cells (purified by Boyum's method, in 100ml of the growth medium were set up as described previously (Methods section 2. 2. 3). One burrlar was incubated without PHA whereas the other received 0. 25 unit of PHA-M at 0h of culture. The cells were grown under standard conditions for 4 days. The cells were harvested, and the cell homogenate used in the assays as a source of DNA polymerase activity was prepared as described in (Methods, section 2. 9. 1). The enzyme activity was assayed using two different types of assay mixture.

a) Loeb's assay mixture (cf. Methods section 2. 11. 1(a)

b) Keir's assay mixture (cf. Methods section 2. 11. 1(b)

The cocktails contained either the native DNA primer or the heat denatured DNA primer. A range of enzyme concentration was used 0. 1ml of homogenate respresents the enzyme activity from 5 X 10^6 cells. The enzyme activity is expressed as n mole or d. p. m. (^{3}H) dTTP (50u mole, 1 uc/assay) incorporated into DNA by the amount of enzyme shown in Fig III-23.



Unstimulated cells

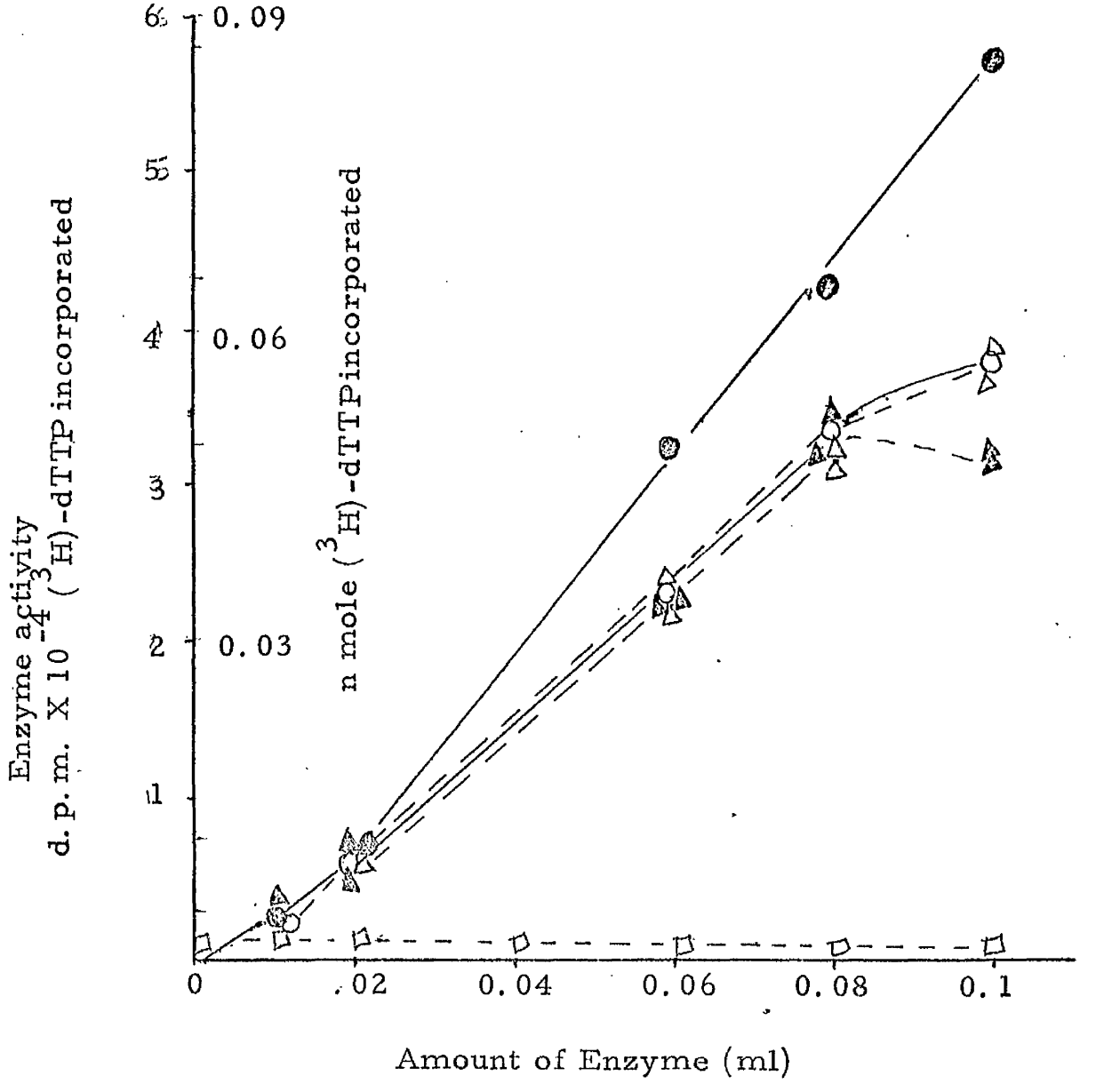
PHA stimulated cells, Keir's cocktail with heat denatured primer

PHA stimulated cells, Keir's cocktail with native primer

PHA stimulated cells Loeb's cocktail with heat denatured primer

PHA stimulated cells, Loeb's cocktail with native primer

FIG III - 23


Enzyme activity
d. p. m. X 10^{-4} (^{3}H)-dTTP incorporated
n mole (^{3}H)-dTTP incorporated
6
5
4
3
2
1
0.09
0.06
0.03
0
.02
0.04
0.06
0.08
0.1
Amount of Enzyme (ml)

primer and Shepherd and Keir's (1966) assay mixture (described in Methods Section 2. 2. 11). was used. Fig III-23 shows a typical assay using either the assay procedure described by Loeb and Agarwal (1968) or Shepherd and Keir (1966). With the assay system containing the assay mixture described by Shepherd and Keir heat denatured DNA primer and crude cell homogenate shows higher enzyme activity compared to that using the same assay mixture containing the native primer or the assay mixture described by Loeb containing either native or heat denatured DNA primer.

Fig III-25(a) shows that the supernatant enzyme is more active in the presence of denatured DNA.

The activity of DNA polymerase from a variety of mammalian tissue is greatly influenced by the physical state of the DNA primer. Yoneda and Bollum (1965) have shown that enzymes from calf thymus gland show little activity with native DNA and preferentially utilize single stranded DNA. The same result was reported by Shepherd and Keir (1966) using enzymes from Ehrlich ascites tumour cells.

Lindsay and Adams (1968) using exponentially growing mouse fibroblasts (L929 strain) report that the nuclei exhibit a 3-4 fold preference for native DNA as a primer, but the supernatant enzyme is 3-4 times more active in the presence

FIG III - 24

Effect of 5-azaCyd on the induction of DNA polymerase activity

10ml cultures of lymphocytes (purified by Boyum's method) containing 10 X 10^6 cells were set up by the standard method (cf. Methods section 2. 2. 2). Three sets were used, one set devoid of PHA and the other two containing 0. 05 unit of PHA-M per culture added at 0h. One of the PHA containing sets received 4 X 10^{-5}M 5-azaCyd added simultaneously with PHA. The cultures were incubated for 2h under standard conditions, the drug washed off and media changed as described (Methods section 2. 5). All the cultures, control and test were treated similarly while changing the medium. Finally the cells were reincubated under standard conditions and harvested at the end of each day for four subsequent days. The cell homogenate was prepared in 0. 5ml of the buffer (cf. Methods, section 2. 9. 1). DNA polymerase activity of unstimulated, PHA stimulated and 5-azaCyd treated cells was assayed using cell homogenate (equivalent to 2 X 10^6 cells/assay), and Loeb's assay mixture with heat denatured DNA primer.

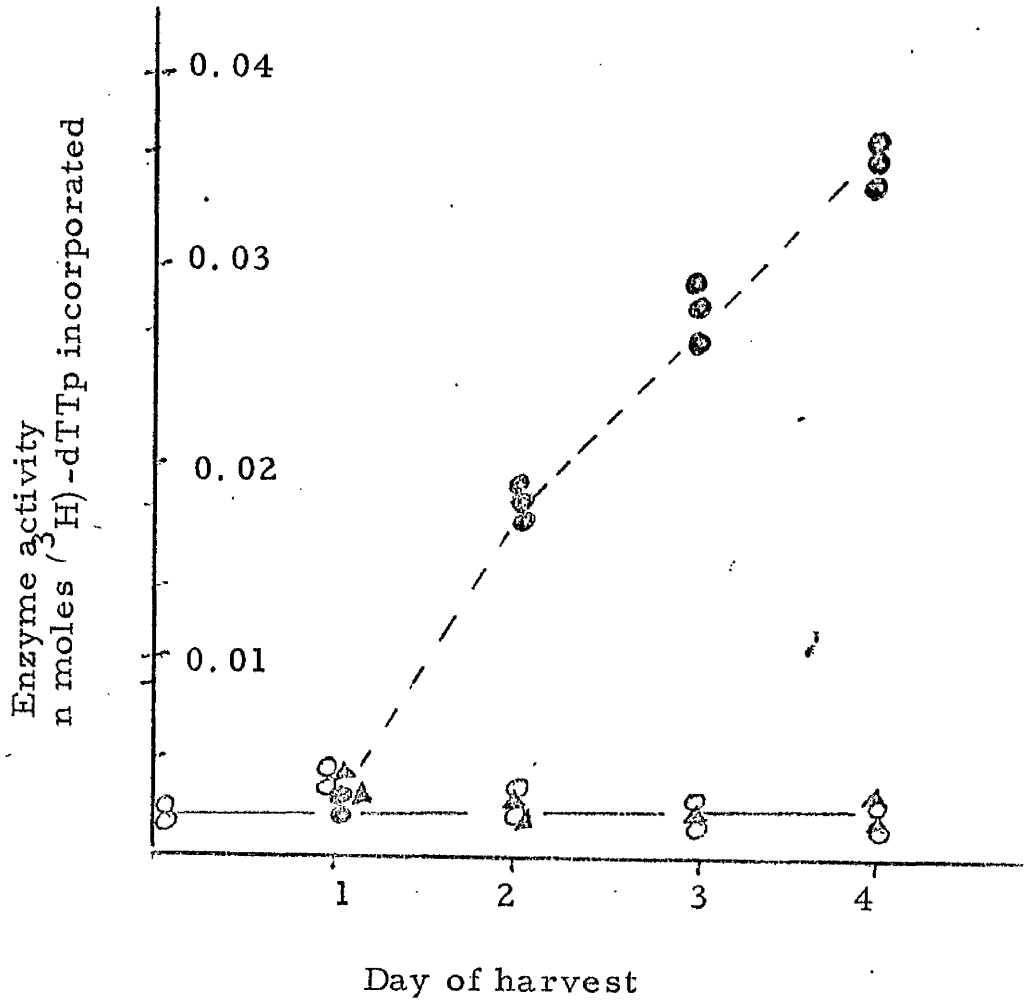
The results are expressed as the amount of (^{3}H)-dTTP (1uc, 50n mole/assay) incorporated for an hour, at 37^{o}C, into the acid insoluble, alkali resistant fraction by enzyme from 2 X 10^6 cells.

o——o Unstimulated cells

⊗——⊗ PHA stimulated cells

△——△ 5-azaCyd treated cells

Fig III - 24


Enzyme activity
n moles (^{3}H)-dTTp incorporated
0.04
0.03
0.02
0.01
1
2
3
4
Day of harvest

of denatured DNA.

ii. Induction of DNA polymerase activity and effect of 5-azaCyd on it:-

Fig III-24 shows that DNA polymerase activity is induced within 2 days after the treatment of cells with PHA. The activity steadily increases until four days, while in unstimulated cells the enzyme activity does not change. Treatment of the cells with 5-azaCyd for 2h at the beginning of culture inhibits the induction of this enzyme activity studied for the subsequent four days.

This induction in DNA polymerase is quite expected. The enzyme activity starts rising on the second day of culture, before the cells are actively engaged in DNA synthesis. A similar result has been reported by Loeb, Agarwal and Woodside (1968) working with human lymphocytes.

Cihak, Vesely and Sorm in 1967 reported that administration of 5-azaCyd along with the inducer inhibits completely the hormonal induction of tryptophan pyrrolase.

iii. Effect of 5-azaCyd on equine DNA polymerase in a cell free system:-

Results in Fig III-23 reveal that 5-azaCyd inhibits the induction of the DNA polymerase activity. To see whether the drug inhibits the enzyme we tested the effect of 5-azaCyd in a

FIG III - 25 (a)

Invitro Effect of 5-azaCyd on lymphocyte DNA polymerase activity

100×10^6 lymphocytes purified by Boyum's method were grown, harvested at 4th day of culture, and the homogenate prepared in exactly the same way as described in Fig III-23.

The homogenate (50×10^6 cells/ml of the homogenate) was used for the preparation of a high speed supernatant fraction (cf. Methods section 2.11) and the pure nuclei. The nuclei were suspended into a volume of buffer equivalent to the high speed supernatant fraction, and used to assay the nuclear DNA polymerase activity.

Using Keir's assay mixture (Methods 2.11.1) containing either native or denatured DNA primer, the DNA polymerase activity in either the supernatant fraction or in the pure nuclei was assayed. The effect of 5-azaCyd on high speed supernatant enzyme requiring native DNA primer, or the heat denatured DNA primer, and also on the nuclear enzyme requiring native primer or the heat denatured primer was determined. 5-azaCyd was added to the assay mixture before the onset of incubation at $87^{\circ}C$, and the assay was done using Keir's method described in Methods section 2.11.1.

FIG III-25(b)

Stability of DNA polymerase activity in high speed supernatant and nuclear fraction

The whole procedure was exactly the same as described above except that the high speed supernatant fraction (HSS) and pure nuclei were frozen and preserved at $-20^{\circ}C$ for 3 weeks, and the enzyme activity was assayed after 3 weeks using Keir's cocktail with either native or heat denatured DNA primer and using either the high speed

FIG III - 25(b)

supernatant or pure nuclei suspended in an equal volume of buffer as the HSS fraction.

Assay using HSS and heat denatured DNA primer.

Assay using HSS and native DNA primer

Assay using nuclei with heat denatured primer

Assay using nuclei with native DNA primer

Fig III - 25 (a)

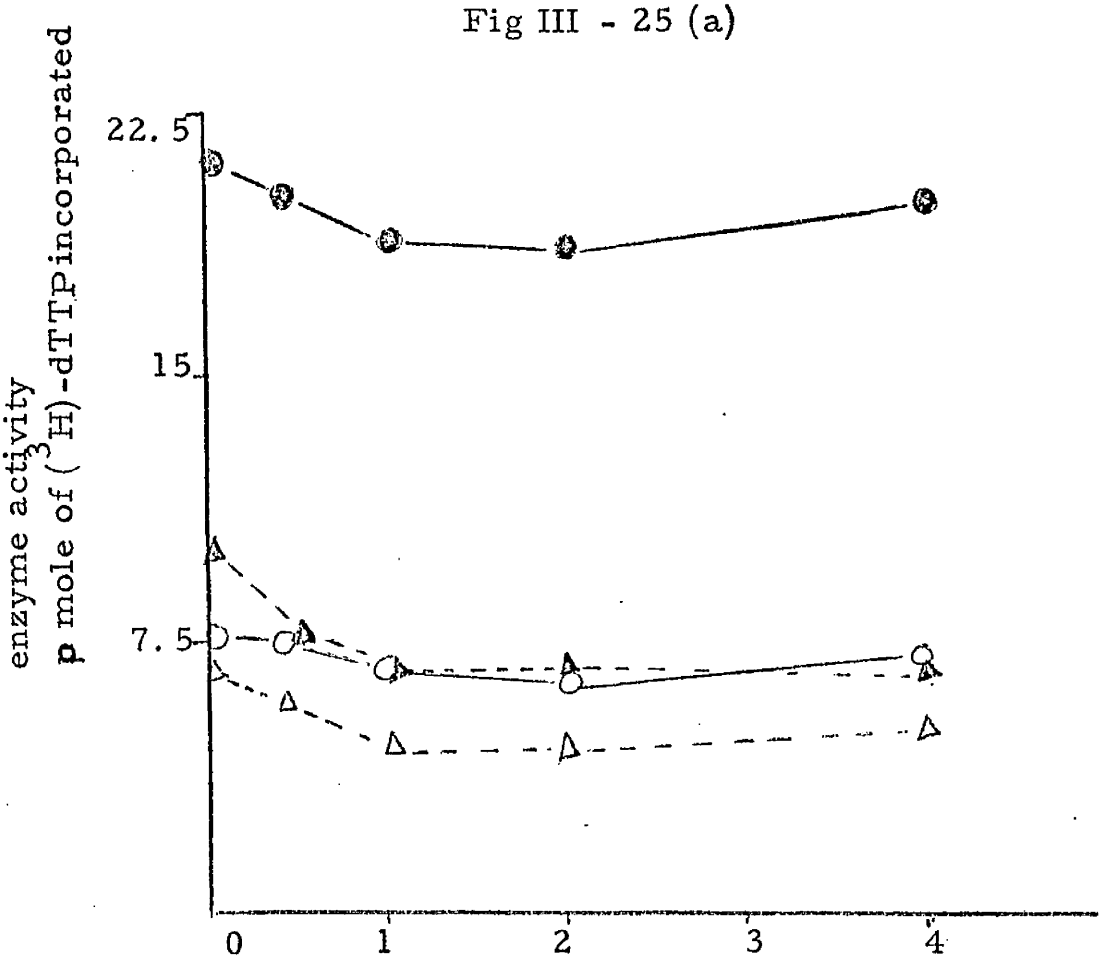
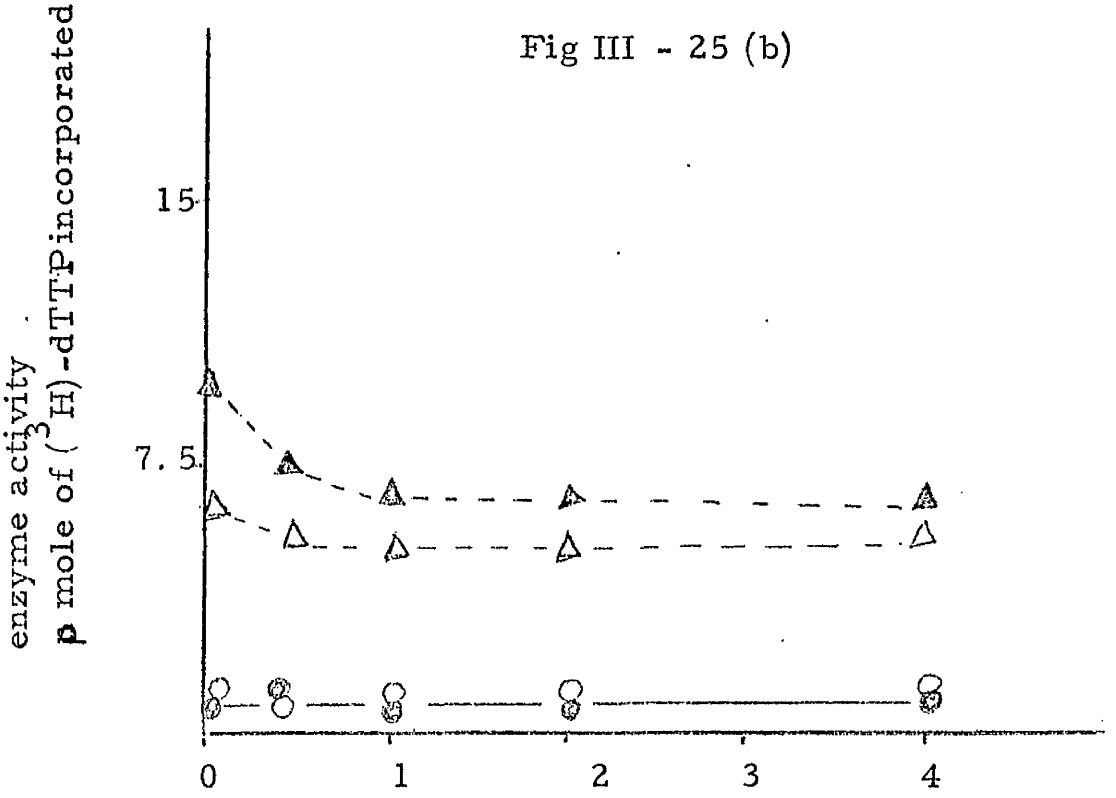


Fig III - 25 (b)



cell free system.

Fig III-25 (a) reveals that 5-azaCyd does not effectively inhibit the equine DNA polymerase activity in the cell free system. Enzyme activities from both supernatant and nuclear fraction give the same response towards 5-azaCyd.

Fig III-25(a) also reveals that with denatured primer the high speed supernatant fraction shows more than 3 fold increased activity compared to the use of native DNA primer. The nuclear enzyme activity is not effected by the physical state of the DNA primer.

Fig III-25 (b) reveals that the supernatant enzyme activity is lost if the enzyme is preserved for a fortnight at -20°C.

The nuclear enzyme activity is stable and preservation under similar conditions does not destroy the enzyme activity.

4.6 Effect of 5-FUra on the growth of horse lymphocytes:-

5-Fluorouracil, an antitumour drug has been studied widely and is employed as an anticancer drug.

One of the accepted mechanisms of action of this drug is that it inhibits the DNA synthesis in Ehrlich ascites tumour cells. The most widely accepted mechanism of action of fluorouracil series is that they are converted to the nucleotide, 5-fluorodeoxyuridine phosphate which blocks the DNA synthesis as a

FIG III - 26

Effect of 5-FUra on DNA synthesis in PHA-stimulated horse lymphocytes

2ml culture of lymphocytes, containing 1 X 10^6 cells (purified by Rabinowitz' method) were set up according to the standard method. After the addition of 0.005 unit/culture of PHA-M, the cells were treated with 3 X 10^{-4} M 5-FUra at varying time periods shown in Fig III-26. Following 2h treatment with the drug, the cells were washed and the media changed (c.f. Methods section 2.5). Finally the cells were resuspended in 2ml of the growth medium containing 0.005 unit of PHA-M and incubated under standard conditions (c.f. Methods section 2.2.1). The growth was assay (c.f. Methods Section 2.6) in 72h old cultures, after pulse labelling the cells for 6h with 0.1ml of a solution of (2-^{14}C)-dThd (3uc 3.66mc/mM,) at the end of the culture. The results are expressed as c.p.m. (2-^{14}C) dThd incorporated into DNA per culture.

FIG III - 26

Time of treatment	Culture	c. p. m. dThd-2-^{14}C incorporated/culture		
1-3h	Control	390	430	400
	5-FUra treated	56	65	60
18-20h	Control	689	690	740
	5-FUra treated	51	72	80
28-30h	Control	690	780	825
	5-FUra treated	51	45	42

consequence of inhibition of the enzyme thymidylate synthetase, thus leading to the inhibition of growth. Incubation of horse lymphocytes with 5-FUra for two hours at different time period during the culture, leads to inhibition of DNA synthesis measured by incorporation 2-^{14}C) dThd at 72h. (Fig III-26).

This inhibition is not a consequence of inhibition of thymidylate synthetase since exogeneous dThd was supplied. A similar result has been obtained by Imrie and Robinson (1968) in human lymphocytes and this demonstrates a similarity in the action of 5-azaCyd in 5-FUra.

4.7 Binding of 5-azaCyd in PHA stimulated equine lymphocytes:-

Results described in Section 4.5 reveal that 5-azaCyd does not inhibit any of the major enzymes involved in DNA synthesis, and that it is the induction of enzyme activity which is being inhibited. Thus a search started to find out the primary site of 5-azaCyd action and its mechanism of action.

Since only two hours treatment with the drug is sufficient to inhibit the stimulation and growth of lymphocytes for the rest of the 72h culture period, we considered the possibility of the drug getting bound somewhere in the cells.

The shape of the curve (Fig III-27) obtained on treatment of lymphocytes with 5-azaCyd for short periods suggests that the drug is getting bound at

FIG III - 27

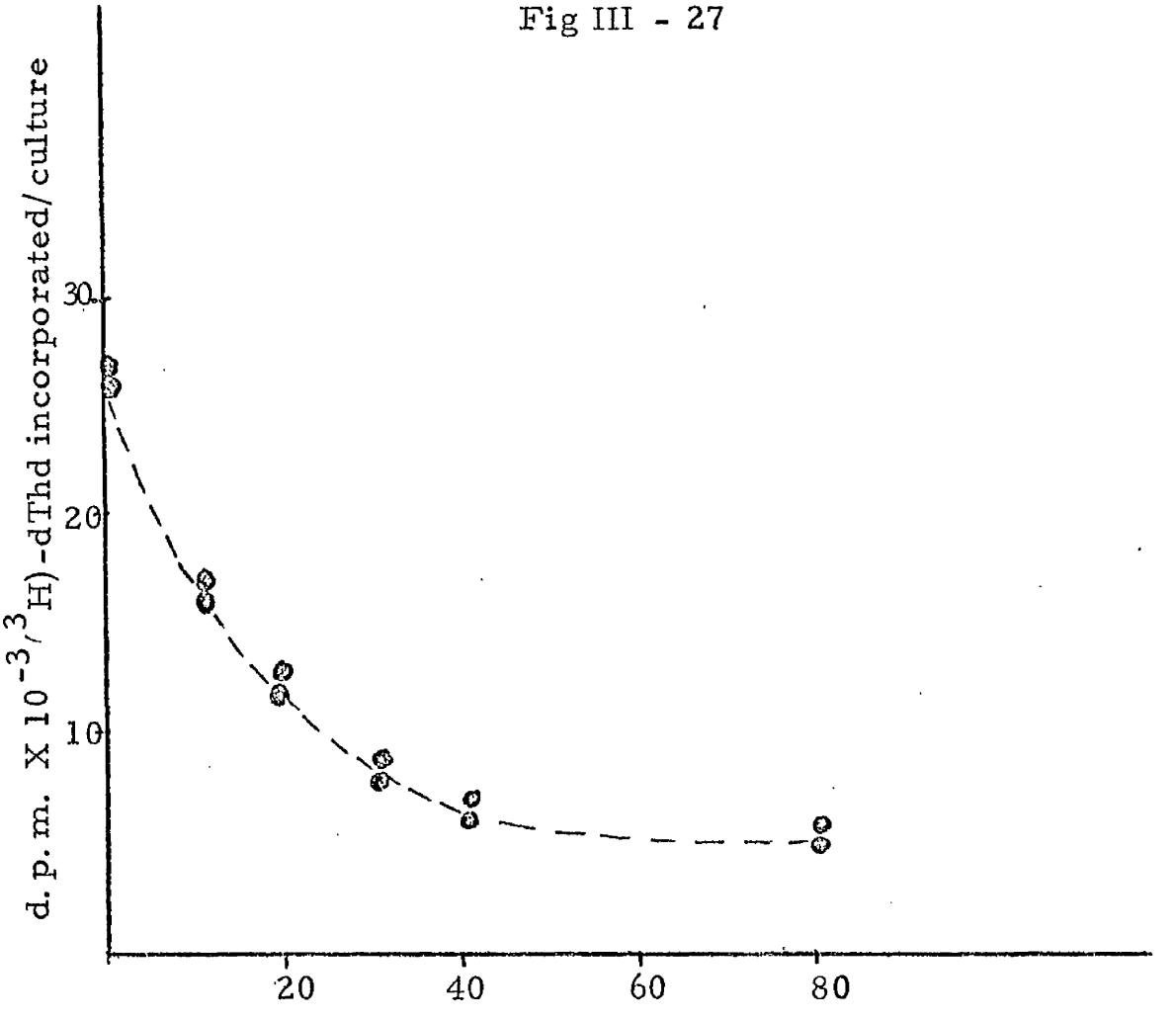
Binding of 5-azaCytidine

Effect of short treatment with 5-azaCyd on lymphocyte growth

Cultures of lymphocytes containing a 2ml suspension of lymphocytes (purified by Boyum's method) at a concentration of 1×10^6 cells/ml of 10% HPEHM were set up as described (methods, section 2.2.1) 5-azaCyd at a concentration of 4×10^{-5}M was added at the beginning of the culture, along with PHA. The cells were treated with the drug for the time periods shown in Fig III-27, and cooled to 0°C immediately after treatment. The cells were washed and the medium was changed as described (Methods, section 2.5). Finally, the cells were reincubated under standard conditions after suspending the cells in 2ml of fresh medium containing 0.005 unit of PHA-M. The cells were harvested at 72h after pulse labelling with (^{3}H)-dThd (5uc 4×10^{-6}M) for two hours, immediately before harvesting. The growth of the cells was assayed as described elsewhere (c.f. Methods section 2.6).

The results are expressed as d.p.m. (^{3}H)-dThd incorporated into DNA per culture.

Fig III - 27


d.p.m. X 10^{-3}/^{3}H)-dThd incorporated/culture
30
20
10
20
40
60
80
Time of 5-azaCyd treatment (min)

some site. It is quite straightforward and fruitful to use the radioactive drug and get direct evidence of its incorporation or binding at a particular site. Unfortunately the radioactive drug is not available in the market, and attempts to tritiate the drug resulted in failure because of its unstability. Sorm et al, (1964) synthesized the radioactive (^{14}C)-5-azaCyd and reported its incorporation into DNA and RNA of plants, bacterial and mammalian cells (Piskala and Sorm 1964; Paeces, Doskocil and Sorm 1968; Pithova et al, 1965, and Raska et al, 1966a and b; Cihak, Tykva and Sorm 1966, and Kalousek et al, 1966). Because of the lack of the radioactive drug we had to take an indirect approach to give evidence of its incorporation or binding.

Jurovcik, Raska, Sarmova and Sorm demonstrated in 1965 that 5-azaCyd is phosphorylated to mono, di and triphosphate in Ehrlich ascites tumour cells invivo. In cell free extracts of mouse tissue it is phosphorylated to its monophosphate. The phosphorylation of the drug has been shown to be greater in the lymphatic organs like thymus and spleen than in nonlymphoid tissues such as liver or kidneys. Results from Fig III-27 thus suggest that a pool of the phosphates of 5-azaCyd is built up in the cells and that equilibriation is attained within 40 min. (cf. equilibriation of radioactive thymidine with the acid soluble pool Adams - 1969). If this were the case then this pool should be diluted by simultaneous incubation with Cyd.

FIG III - 28(a)

Reversal of growth inhibition by Cytidine in 5-azaCyd inhibited cells

Standard 2ml cultures containing 1 X 10^6 lymphocytes purified by Rabinowitz' method were used. The cultures were incubated under standard conditions and treated simultaneously with 5-azaCyd (4 X 10^{-5}M) and Cyd (X 100) for 2h periods at the time periods shown in Fig III-28(d). After 2h treatment with the drug the cells were washed and the medium changed as described in Methods section 2.5. Three sets of controls were used:

i) Control without Cyd or 5-azaCyd
ii) Control with 4 X 10^{-5}M 5-azaCyd and
iii) Control with 4 X 10^{-5}M Cyd

The controls were treated in exactly the same way as the tests. The cultures were reincubated under standard conditions, labelled for six hours immediately before harvest with 3uc (3.66mc/mM), (2-^{14}C)-dThd, and harvested at 72ndh of culture.

The results are expressed as c.p.m. (2-^{14}C)-dThd incorporated into DNA per culture.

FIG III-28(a)

c. p. m. ($2-^{14}C$)-dThd incorporated into DNA					
Time of treatment	Control	Only Cyd 4×10^{-4}M	Only 5-azaCyd 4×10^{-5}M	5-azaCyd + Cyd 4×10^{-4}M	5-azaCyd + Cyd 4×10^{-3}M
0-2h	280 270	275 300	22 61	130 132	235 255
2-4h	260 273	280 270	25 55	160 130	240 200
14-16h	270 260	245 250	44 54	160 180	240 235
16-18h	225 200	207 204	33 59	159 161	233 190
24-26h	241 170	250 170	24 41	171 163	211 220
48-50h	260 200	168 250	27 29	133 123	177 199

FIG III - 28(b)

Reversal of growth inhibition by Cyd treated before with or after 5-azaCyd

Standard 2ml cultures containing 1 X 10^6 lymphocytes purified by Rabinowitz' method were incubated under standard conditions (cf. Methods section 2. 2. 1) and the following operations were done:

a) A set of cultures were treated with 3 X 10^{-3}M and 3 X 10^{-4}M Cyd from 0-2h. At 2h the growth medium was changed and the cells were washed (cf. Methods section 2. 5). After washing, the cells were resuspended into 2ml of the growth medium, and 5-azaCyd (4 X 10^{-5}M) was added, incubation was continued for another 2h and at 4h of culture the medium was changed once more and the cells were washed as described above.

b) In this case, the cells were treated with 5-azaCyd (4 X 10^{-5}M) and Cyd (4 X 10^{-3}M) simultaneously from 0-2h of culture, the medium was changed, incubated for another 2h, the medium was changed once more at 4h of culture and the rest of the operations were similar to those described in (a).

c) The procedure in this case was the same as described in (a) except that instead of Cyd, 5-azaCyd (4 X 10^{-5}M) was added at 0h and instead of 5-azaCyd, Cyd (4 X 10^{-4}M) was added at 2h.

In addition, a set of control cultures without Cyd or 5-azaCyd a set with Cyd only and a set with 5-azaCyd only was used. The duration of treatment with Cyd or 5-azaCyd was 0-2h and in all the cases the medium was changed twice at 2nd and 4th h.

Finally, the cells in each culture were resuspended in 2ml of the fresh medium containing PHA-M (0. 005 unit) and reincubated

under standard conditions. The growth was assayed in 72h old cultures as described (cf. Methods section 2.6).

The results are expressed as the c.p.m. (2-^{14}C) - dThd (3uc, 3.66mc/mM) incorporated into DNA for 6h (66-72h) per culture.

FIG III - 28 (b)

c. p. m. ($2\text{-}^{14}C$) - dThd incorporated into DNA							
5-azaCyd concentration	Cyd concentration	Cyd added before	Cyd with 5-azaCyd	Cyd after	Cyd only	5-azaCyd only	Control without Cyd or 5-azaCyd
3×10^{-5} M	3×10^{-3} M	130 106	129 130	37 38	116 97	38 30	126 106
3×10^{-5} M	3×10^{-4} M	67 98	69 91	28 22	91 67	20 22	

4.7.1 Reversal of growth inhibition by Cyd in 5-azaCyd inhibited cells:-

Fig III-28(a) reveals that treatment of lymphocytes with Cyd at 10 to 100 fold higher concentration than 5-azaCyd and simultaneously with the latter for 2h periods at varying time period during the culture reverses the inhibitory effect produced by 5-azaCyd. A similar result has been published by Cihak and Sorm in 1965 who observed that in E. coli complete inhibition of the growth can be reversed by simultaneous addition of Urd, Cyd or dThd but not by uracil or cytosine. Fig III-28(b) reveals that treatment of lymphocytes with 5-azaCyd inhibits the growth of lymphocytes, but if the cells are treated with (X10) or (X100) higher concentration of Cyd compared to 5-azaCyd 2h before or simultaneously with 5-azaCyd treatment the inhibitory effect can be removed. The extent of reversal depends on the concentration of 5-azaCyd and Cyd used.

One the other hand, if Cyd is added 2h after 5-azaCyd treatment, the growth inhibition prevails and Cyd is of little help. This suggests that although equilibriation of 5-azaCytidine with its phosphates occurs within 40 minutes, a second step i. e. incorporation of these phosphates into RNA is complete within 2 hours such that the ensuing inhibition is no longer reversible by the addition of excess of Cytidine.

FIG III - 29

Effect of Cyd or Urd on the inhibition of DNA synthesis is 5-azaCyd treated cells

Standard 2ml cultures containing 2 X 10^6 lymphocytes purified by Boyum's method were incubated after the addition of PHA-M (0.005 unit/culture) under standard conditions (Methods, section 2.2.1) for 66h.

At 66h each culture received 50uc ($^{32}P_i$)-orthophosphate (92mc/mg P_i) and the following additions were made to duplicate sets of cultures, at the same time.

a) Controls

5-azaCyd (4 X 10^{-5}M) to a set of tubes. Cyd (4 X 10^{-3}M) to another set and to the 3rd set 4 X 10^{-3}M Urd. The cultures were reincubated under standard conditions.

b) Tests

Either Cyd (4 X 10^{-3}M) or Urd (4 X 10^{-3}M) was added at 66h: the cells incubated under standard conditions for 2h: 5-azaCyd added at 68h and the cells reincubated as shown above.

5-azaCyd (4 X 10^{-5}M) was added at 66h and either Cyd (4 X 10^{-3}M) or Urd (4 X 10^{-3}M) was added at 68h of culture.

The cells were reincubated under standard conditions.

Tubes were harvested at 70th h (after 4h label) or at 74th (after 8h of label). The amount of ($^{32}P_i$)-orthophosphate incorporated into DNA was measured as described in Methods (section 2.4.2).

The results are expressed as c.p.m. ($^{32}P_i$)-orthophosphate incorporated into DNA per culture during a 4h or an 8h pulse.

FIG III - 29

DNA synthesis

Type of treatment	Remarks	c. p. m. X10^{-3}($^{32}P_i$) incorporated into DNA (66-70h)		(66-74h)		% of control
-PHA	Unstimulated cells	0.6	0.6	0.7	1.0	5
+PHA	stimulated cells	6.9	6.5	16	21	100
+5-azaCyd only	stimulated + 5-azaCyd 4X10^{-5}M 68h	6.5	5.0	4.4	5.9	25
+ Cyd only	+PHA + Cyd 4X10^{-3}M 68h	5.5	5.5	18.5	13.9	90
+ Urd only	+PHA + Urd 4X10^{-3}M 68h	9.6	9.8	22.7	13.4	95
Cyd before 5-azaCyd	+PHA+Cyd(4X10^{-3}M66h) +5-azaCyd(4X10^{-5}M68h)	6.9	7.9	25.4	15.2	100
Cyd after 5-azaCyd	+PHA+Cyd(4X10^{-3}M68h) +5-azaCyd(4X10^{-5}M66h)	4.7	4.3	6.6	6.8	30
Urd before 5-azaCyd	+PHA+Urd(4X10^{-3}M66h) +5-azaUrd(4X10^{-5}M68h)	6.9	5.1	26.8	14.5	100
Urd after 5-azaCyd	+PHA+Urd(4X10^{-3}M68h) +5-azaCyd(4X10^{-5}M66h)	3.9	3.8	8.6	8.1	40

4.7.2 Minimum time period taken by Cyd to reverse the growth inhibition in 5-azaCyd treated cells:-

Fig III-31 reveals that treatment with Cyd as little as 10 minutes later than 5-azaCyd treatment cannot rectify the damage already produced by 5-azaCyd in inhibiting lymphocyte growth. As the time of 5-azaCyd treatment becomes longer, the inhibition of growth is more pronounced and Cyd more helpless in reversing the inhibitory action.

4.7.3 Effect of Cyd or Urd on the inhibition of ($^{32}P_i$)-orthophosphate incorporation into DNA in 5-azaCyd treated cells:-

Because added Cyd and Urd interfere with labelling cells with (^{3}H)-dThd the reversal experiments were repeated following DNA synthesis by labelling with ($^{32}P_i$)-orthophosphate Fig III-30 shows some of the effect of Cyd and uridine on the DNA synthesis in PHA stimulated equine lymphocytes treated with or without 5-azaCyd from 66-74h of culture.

Cyd alone at 4×10^{-3}M has an inhibitory action on cellular growth, but when it is present along with 5-azaCyd it does not produce such an effect during the first 4h of treatment.

Uridine on the other hand seems to be stimulatory since incorporation of ($^{32}P_i$) into DNA is greater in uridine treated than in control cells. Lucas (1967) has reported increased activity of uridine kinase on PHA stimulation.

FIG III - 30

Effect of Cyd or Urd on the inhibition of RNA synthesis is 5-azaCyd treated cells

The experiment is the same as described in Fig III-29

The acid soluble alkali digestable material described in Fig III-29 was counted to measure the extent of rate of RNA synthesis during a 4 and 8h pulse with ($^{32}P_i$) - orthophosphate (Methods section 2. 4. 2. (a).

The results are expressed as the c. p. m. ($^{32}P_i$) - orthophosphate incorporated into RNA per culture during a 4h and 8h pulse.

FIG III - 30

		c. p. m. x 10^{-3} ($^{32}P_i$) incorporated into RNA				% of control
		66-70h		66-74h		
-PHA	Unstimulated cells	3.4	4.2	4.3	5.5	10
+PHA	PHA stimulated control	15.7	18.7	48.7	58.3	100
+5-azaCyd	+PHA 5-azaCyd 0h (4 x 10^{-5}M) 66h	15.0	15.7	15.0	18.4	30
+Cyd	+PHA Cyd (4x10^{-3}M) 0h 66h	16.2	14.9	40.6	49.9	95
+Urd	+PHA Urd (4x10^{-3}M) 0h 66h	58.8	57.6	40.6	56.8	95
Cyd before 5-azaCyd	+PHA Cyd 66h 0h 5-azaCyd 68h	26.8	22.5	65.6	79.5	140
Cyd after 5-azaCyd	+PHA Cyd 68h 0h 5-azaCyd 66h	15.4	17.6	27.3	23.7	50
Urd before 5-azaCyd	+PHA Urd 66h 0h 5-azaCyd 68h	45.0	24.0	68.8	39.4	110
Urd after 5-azaCyd	+PHA Urd 68h 0h 5-azaCyd 66h	13.7	15.7	20.1	21.2	40

He suggested that the induction of uridine kinase is not a direct and immediate consequence of PHA action as the formation of uridine kinase and the incorporation of uridine are under regulatory processes of repression and end product inhibition. It has been shown that uridine kinase synthesis in stimulated cultures grown in 10^{-4}M Cyd is inhibited. Uridine kinase activity in assays invitro is completely inhibited by 10^{-4}M Cyd(Lucas 1967).

Forsdyke (1968) also reported that at certain critical pool concentrations uridine itself can stimulate the rate of its own incorporation. Fig III-30 also reveals that 2h treatment with 5-azaCyd does not inhibit the DNA synthesis measured as an extent of ($^{32}P_i$) - orthophosphate incorporation. On the other hand, treatment for 4h with 5-azaCyd shows a significant inhibition of DNA synthesis and treatment for 8h produces further strong inhibition.

If the cells are treated with Cyd. 2h before 5-azaCyd treatment there is a complete reversal of DNA synthesis, on the other hand treatment two hour after 5-azaCyd does not affect in the reversal either 4 or 8h later.

If the cells are treated with uridine 2h before 5-azaCyd treatment the DNA synthesis returns to normal but the stimulatory effect of uridine observed when uridine alone is in the culture does not prevail in the presence of 5-azaCyd.

FIG III - 31

Time period taken for the growth inhibition in 5-azaCyd treated cells to become resistant to reversal by Cyd

Standard 2ml lymphocyte cultures containing 2×10^6 cells purified by Boyum's method, were stimulated with PHA for 70h under standard conditions (Methods, section 2. 2. 1).

At 70h of culture, 5-azaCyd was added at 4×10^{-5}M concentration and reincubated. A set of cultures received Cyd (4×10^{-3}M) simultaneously with 5-azaCyd or at 10, 20, 40, 80 and 130min following 5-azaCyd. All the cultures were pulse labelled for 2h (72-74h) with 50uc ($^{32}P_i$)-orthophosphate (92mc/mgP_i) per culture and harvested.

The amount of radioactivity incorporated into the DNA was measured as mentioned before (Methods, section 2. 4. (b)).

The results are expressed both as c. p. m. (% of control) and c. p. m. ($^{32}P_i$)-orthophosphate incorporated into DNA per culture during a 2h pulse.

FIG III - 31

Time of treatment	Concn. of substance	Time of treatment	c. p. m. ($^{32}P_i$)X 10^{-3}M incor. into DNA		% of control
Control without Cyd	-		5.0	7.0	100
Control with Cyd	4×10^{-3}M		5.5	7.5	108
+ 5-azaCyd	4×10^{-5}M		2.1	2.8	30
+5-azaCyd + Cyd	4×10^{-5} + 4×10^{-3}M	Simultaneously with 5-azaCyd	7.0	5.0	100
+5-azaCyd + Cyd	$4X10^{-5}+4X10^{-3}$M	10 min after	6.5	5.5	92
+5-azaCyd + Cyd	$4X10^{-5}+4X10^{-3}$M	20 min after	4.9	3.8	67
+5-azaCyd + Cyd	$4X10^{-5}+4X10^{-3}$M	40 min after	4.1	3.2	57
+5-azaCyd + Cyd	$4X10^{-5}+4X10^{-3}$M	80 min after	2.1	2.8	30
+5-azaCyd + Cyd	$4X10^{-5}+4X10^{-3}$M	130 min after	2.3	2.9	30

If uridine is added 2h later than 5-azaCyd, the inhibition of DNA synthesis cannot be reversed, but it is noteworthy that the inhibition is more severe in this case and the values are below the values obtained by Cyd treatment. The above mentioned effects were studied within 2-4h of uridine or Cyd addition.

After 6 to 8h of treatment with Cyd, Urd or 5-azaCyd and during the 8h label with ($^{32}P_i$) - orthophosphate the following results are obtained.

Longer treatment with 5-azaCyd results in a pronounced inhibition of DNA synthesis.

Uridine is not as stimulatory as it was during the first 4 hours.

Cyd and Urd both reverse the inhibition of DNA synthesis produced by 5-azaCyd if these substances are added prior to 5-azaCyd treatments, on the other hand addition of Cyd or Urd 2h later than 5-azaCyd is of no use in reversing the inhibition.

4.7.4 Effect of Cyd or Urd on the incorporation of ($^{32}P_i$) orthophosphate into RNA in 5-azaCyd treated horse lymphocytes:-

In addition to DNA ($^{32}P_i$)-orthophosphate labels RNA, thus from the same cultures a pattern of inhibition of RNA synthesis could be obtained.

Fig III-30 reveals that treatment of cells with cytidine alone does not have a significant inhibitory effect as was the case in DNA synthesis and 5-azaCyd also does not seem to significantly inhibit the RNA

synthesis during the first 2 to 4h of treatment.

Uridine when present alone in the culture has a marked stimulatory effect on RNA synthesis. On the other hand, in the presence of 5-azaCyd, this stimulatory effect of Urd is absent. After 6 to 8h treatment with 5-azaCyd however, the rate of RNA synthesis falls sharply, and is not only reversed but slightly stimulated if Cyd or Urd is added 2h before 5-azaCyd treatment, whereas addition of the normal precursors 2h later does not overcome the inhibitory action of 5-azaCyd.

The striking stimulatory effect of uridine observed during the early period does not prevail 6-8h later.

4.8 The effect of 5-azaCyd on RNA metabolism in PHA stimulated equine lymphocytes:-

Lymphocytes, on stimulation with PHA, show an increased rate of RNA synthesis (Cooper - 1962, Barkhan - 1963). Several workers (Cooper's group and Torelli's group) have studied RNA metabolism in PHA stimulated lymphocytes of different species. Pogo et al, (1966) working on histone acetylation and RNA synthesis have reported on the use of equine lymphocytes.

All the reports in literature agree with the fact that the rate of RNA synthesis increases within hours of PHA addition in lymphocyte cultures invitro. Kay and Cooper (1968) observed a significant increase in the rate of RNA synthesis within half an hour of PHA addition in cultures of human lymphocytes and conclude

Effect of 5-azaCyd on stimulation of RNA synthesis measured as an extent of (^{3}H)-Uridine incorporation

Three sets of standard 2ml cultures containing 2 X 10^6 cells purified by Boyum's method were employed and treated as follows:

To two sets PHA-M at a concentration of 0.005 units per culture was added while the 3rd received 0.2ml of sterile distilled water.

One of the PHA treated cultures received 5-azaCyd (1 X 10^{-5}M) while the other received 0.1ml of water. 5uc (^{3}H)-uridine (28Ci/mM) was added to all the cultures at the beginning of culture and the cells were incubated under standard conditions (cf Methods section 2.2.1). Triplicate cultures from each set were harvested every subsequent hour and the amount of uridine incorporated into RNA and into the acid soluble fraction was measured as described. (Methods, section 2.4.1 (a & b)).

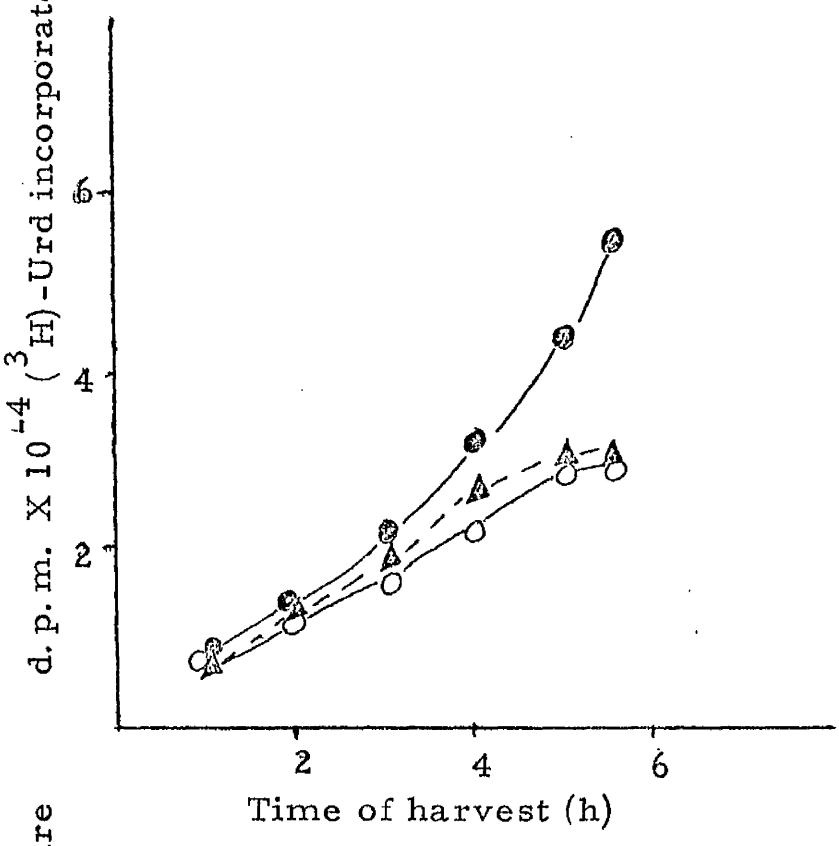
The results are expressed as d.p.m. (^{3}H)-uridine incorporated (a) into RNA or (b) the acid soluble extract per culture.

● ——● PHA stimulated cells

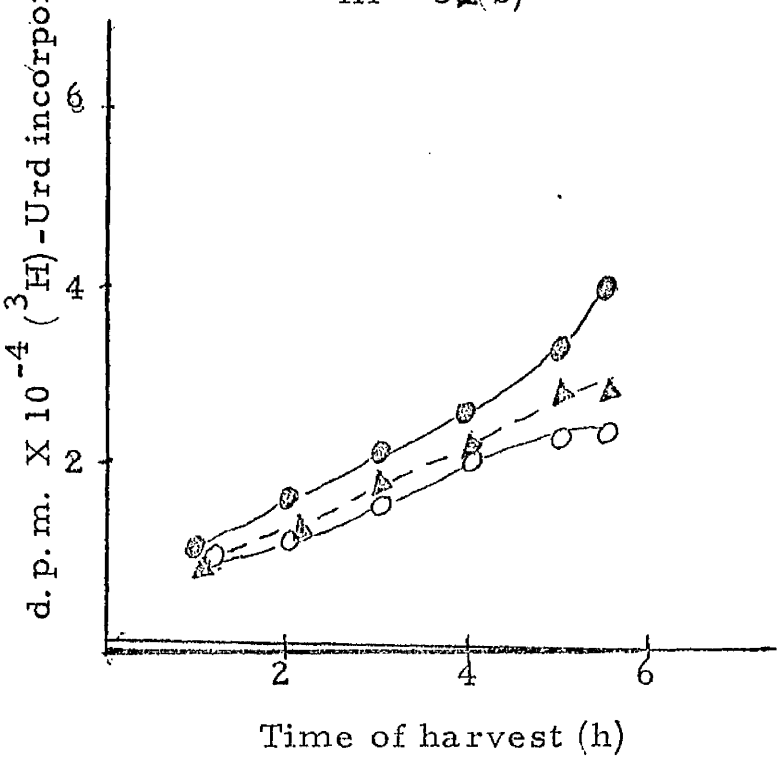
○ ——○ Unstimulated cells

△ ——△ PHA and 5-azaCyd treated cells

III - 32 (a)



III - 32 (b)



that acceleration of rRNA synthesis is an early event in the transition from a resting to a growing state in lymphocytes.

The work described in this section was done in order to study the RNA metabolism in equine lymphocytes and the effect of 5-FUra and 5-azaCyd on it.

4.8.1 Stimulation of RNA synthesis in equine lymphocytes and effect of 5-azaCyd on it:-

Fig III-32 shows the stimulation of horse lymphocytes after adding PHA-M into invitro cultures.

In a communication Kay (1967) reports the stimulation of RNA synthesis studied as a measure of (^{3}H)-Urd incorporation is apparent within half an hour of PHA addition. Fig 32 (a) reveals that addition of PHA-M to horse lymphocyte cultures (invitro) does not reveal any increased Urd incorporation into the RNA for at least 3h. Addition of 5-azaCyd to these cells. Simultaneously with PHA results in an inhibition of Urd incorporation into the acid insoluble fraction. The same effect is observed in the acid soluble fraction (Fig III-32(b)). Doskocil, Paeces and Sorm (1967) reported that general inhibition of RNA synthesis in E. coli is not observed by 5-azaCyd, but Raska, Jurovcik, Sarmova and Sorm (1966) noted the inhibition of RNA synthesis in the isolated nuclei of calf thymus. At the same time it has been shown that 5-azaCyd is

FIG III - 33

Effect of 5-FUra on RNA synthesis

Standard 2ml cultures containing 1 X 10^6 lymphocytes purified by Rabinowitz method were used. The cultures were treated with 4 X 10^{-3}M 5-FUra for 2h at 1-3h; 18-20h or 28-30h of culture.

After treatment with the drug for 2h the medium was changed and the cells washed. (cf. Methods section 2.5). The controls without drug were also treated similarly during washing and media change. Finally, the cells were resuspended in 2ml of fresh 10% HPEHM containing 0.005 unit of PHA-M and reincubated under standard conditions. The cultures were harvested at the time periods shown in Fig III-32 after labelling with (5-^{3}H)-uridine 2uc (23Ci/mM) for 2h, immediately before harvest. The effect of 5-FUra on RNA synthesis was assayed by measuring the incorporated of the isotope in RNA of lymphocytes (cf. Methods section 2.4.1).

The results are expressed as c.p.m. (^{3}H)-uridine incorporated into RNA per culture.

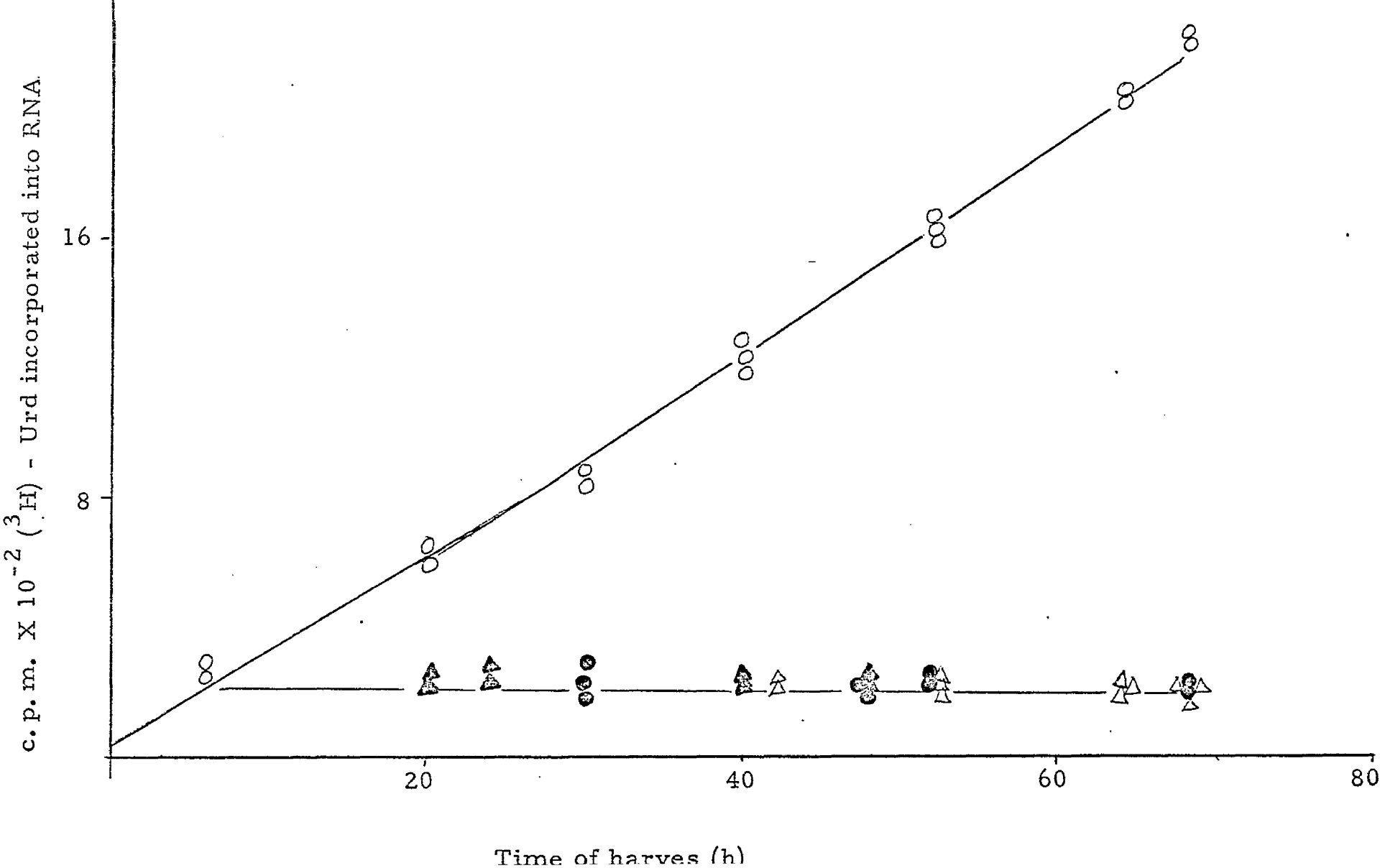
○ ——— ○ Control cells

△ ——— △ Cells treated with 5-FUra at -3h of culture

◎ ——— ◎ Cells treated with 5-FUra at 18-20h of culture

△ ——— △ Cells treated with 5-FUra at 28-30h of culture

Fig III - 33


24
16
8
c. p. m. X 10^{-2} (^{3}H) - Urd incorporated into RNA
20
40
60
80
Time of harves (h)

incorporated into DNA and RNA of plants, E coli and various mammalian tissues. Raska et al, - 1965 and 1966 (a), (b), Cihak et al - 1966, Kalousek et al - 1966.

4.8.2 Effect of 5-Fluorouracil on RNA synthesis in PHA stimulated equine lymphocytes:-

In order to see if 5-FUra has any inhibitory effect on RNA synthesis in PHA stimulated equine lymphocytes, we studied the effect of 5-FUra after short treatments, on RNA synthesis in lymphocytes at different time periods during the culture. Fig III-33 reveals that treatment with 5-FUra completely inhibits the increased incorporation of (^{3}H)-Urd into RNA, no matter at what time period during the first 30h of culture the cells are treated with the drug, the extent of inhibition is the same at all times during 68h of culture. Imrie and Robinson using human lymphocyte treated the cells with 5-FUra for 3h, but unlike our results they could detect a slight inhibition of uptake of (^{3}H)-Cyd into lymphocyte RNA. They showed that 5-FUra is incorporated into lymphocyte RNA.

4.8.3 Effect of 5-azaCyd on ($^{32}P_i$) - orthophosphate incorporation into RNA:-

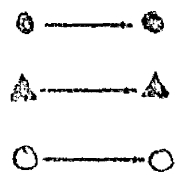
While conducting the binding studies of 5-azaCyd and reversal of 5-azaCyd inhibited growth with Uridine we came to learn that Urd can itself stimulate the incorporation of (^{3}H)-Urd (Forsdyke 1967). In addition Urd incorporation studies could

FIG III - 34

Effect of 5-azaCyd on Stimulation of RNA synthesis measured by ($^{32}P_i$) - orthophosphate incorporation

The experiment was exactly similar to that described in Fig III-32 except that 50uc ($^{32}P_i$) - orthophosphate (92Ci/mg P_i) was used for labelling the cells instead of (^{3}H)-Uridine. The effect of 5-azaCyd on the stimulation of RNA synthesis was followed for six hours. The cells were harvested every subsequent hour following incubation of 0h and the amount of ($^{32}P_i$) - orthophosphate incorporated into RNA was measured (c. f. Methods section 2. 4. 2. (a).

The results are expressed as c. p. m. ($^{32}P_i$) - orthophosphate incorporated into RNA per culture.



PHA stimulated cells

5-azaCyd treated cells

Unstimulated cells

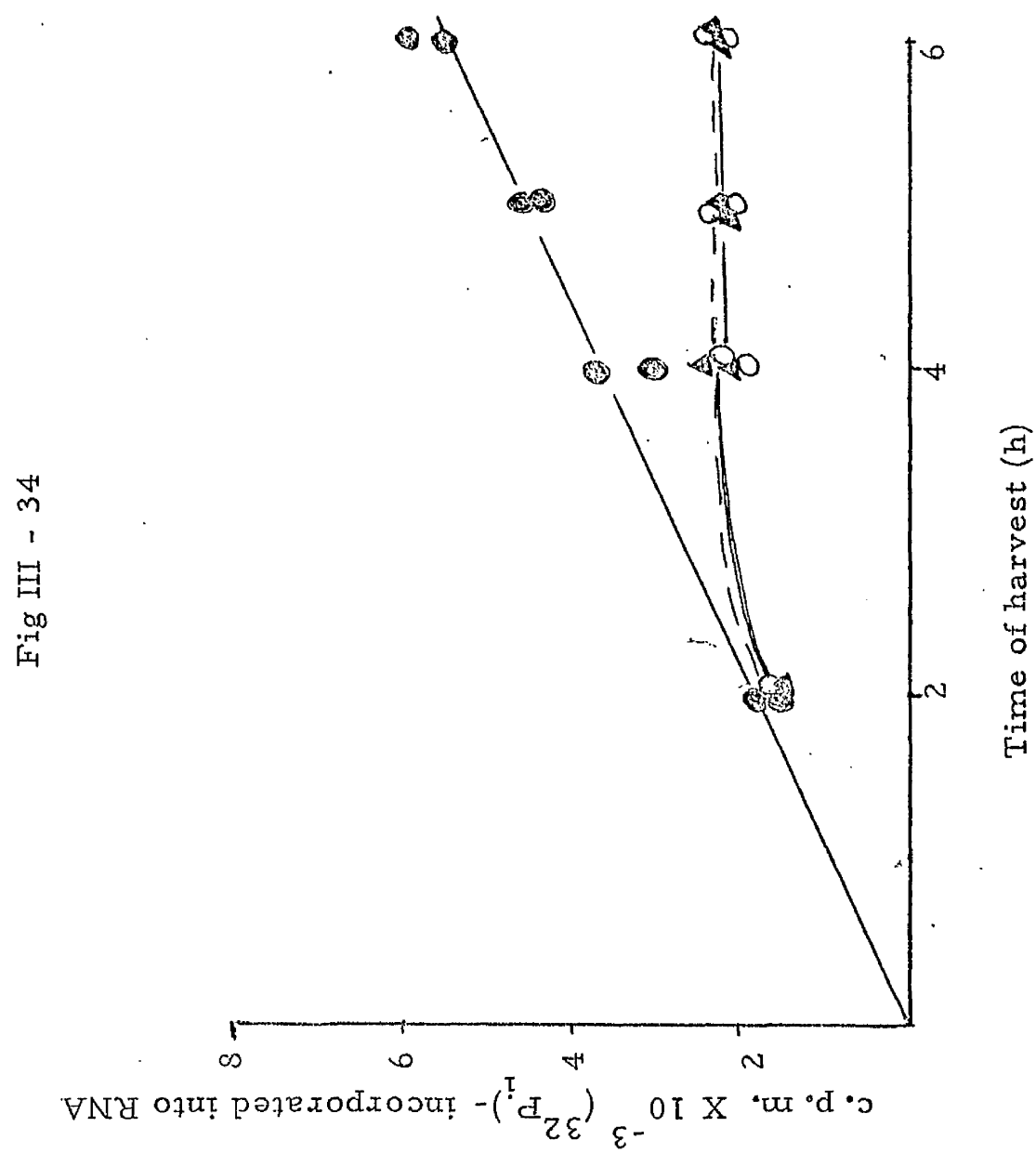

c. p. m. X 10^{-3} ($^{32}P_i$)- incorporated into RNA
2
4
6
8
2
4
6
Time of harvest (h)

Fig III - 34

not give us a definite answer with regard to inhibition of the rate of RNA synthesis by 5-azaCyd. Since 5-azaCyd can effect the activity of Uridine kinase and the uptake of exogenous Urd can be slowed down by competition of the drug for the kinases (Sorm et al, - 1966). Hence to clear the above mentioned doubts we labelled the cells with ($^{32}P_i$)-orthophosphate. Fig III-34 reveals that 5-azaCyd inhibits the rate of lymphocyte RNA synthesis within 3h of its application to the cultures.

4.8.4 Induction of uptake and phosphorylation of Uridine in PHA stimulated equine lymphocytes and the effect of 5-azaCyd on it:-

This study was made to provide evidence that the inhibition of RNA synthesis measured as an incorporation of (^{3}H)-Uridine into RNA is not due to the impaired uptake of (^{3}H)-Uridine.

In order to assess the Uridine kinase activity, the radioactive nucleotides formed following incubation of lymphocytes with (^{3}H) Uridine were separated by chromatography.

Fig III-35 reveals that PHA induces an increased rate of uptake and phosphorylation in equine lymphocytes. Unlike the stimulated cells, the unstimulated lymphocytes do not have a big UMP pool. Short treatment (3h) with 5-azaCyd does not inhibit this increased uptake and phosphorylation induced by PHA, but treatment for a longer period (8h) with 5-azaCyd results in the inhibition of both uptake and

FIG III - 35

Effect of 5-azaCyd on phosphorylation and uptake of Uridine in lymphocytes stimulated with PHA

Four sets of 10ml cultures containing 10×10^6 lymphocytes purified by Boyum's method were used. (Methods section 2.2 (ii)) At 0h of culture 3 sets received 0.025 unit of PHA-M, while the 4th received 0.5ml of sterile distilled water. The cultures were incubated under standard conditions (Methods, section 2.2.1).

At 64th h of culture to one of the sets containing PHA 5-azaCyd (4×10^{-5}M) was added while the set without PHA and one with PHA received 0.5ml of sterile distilled water. The last set containing PHA received 5-azaCyd (4×10^{-5}M) at 69th h of culture.

At 70th h 100uc of ($6\ ^3$H)-uridine (31Ci/mM) was added to all the cultures and the cells were incubated for two more hours.

The cultures were harvested at 72h of culture and washed three times with cold Earle's BSS extracted once with 1ml of 5% TCA (^{w}/v) followed by three ether extractions. Uptake and phosphorylation of Uridine was measured by chromatography of 100ul of acid soluble fractions as described in Methods section 2.10 (b).

The results are expressed as the d.p.m. (^{3}H)-Uridine present in the intracellular uridine, UMP and CMP.

The amount of label present in intracellular uridine gives an extent of uptake of labelled Uridine and the amount of label present in UMP and CMP gives an indication of phosphorylation and Uridine kinase activity.

○——○ Unstimulated cells

●——● PHA stimulated control cells

△——△ 3h 5-azaCyd treated cells

▲——▲ 8h 5-azaCyd treated cells

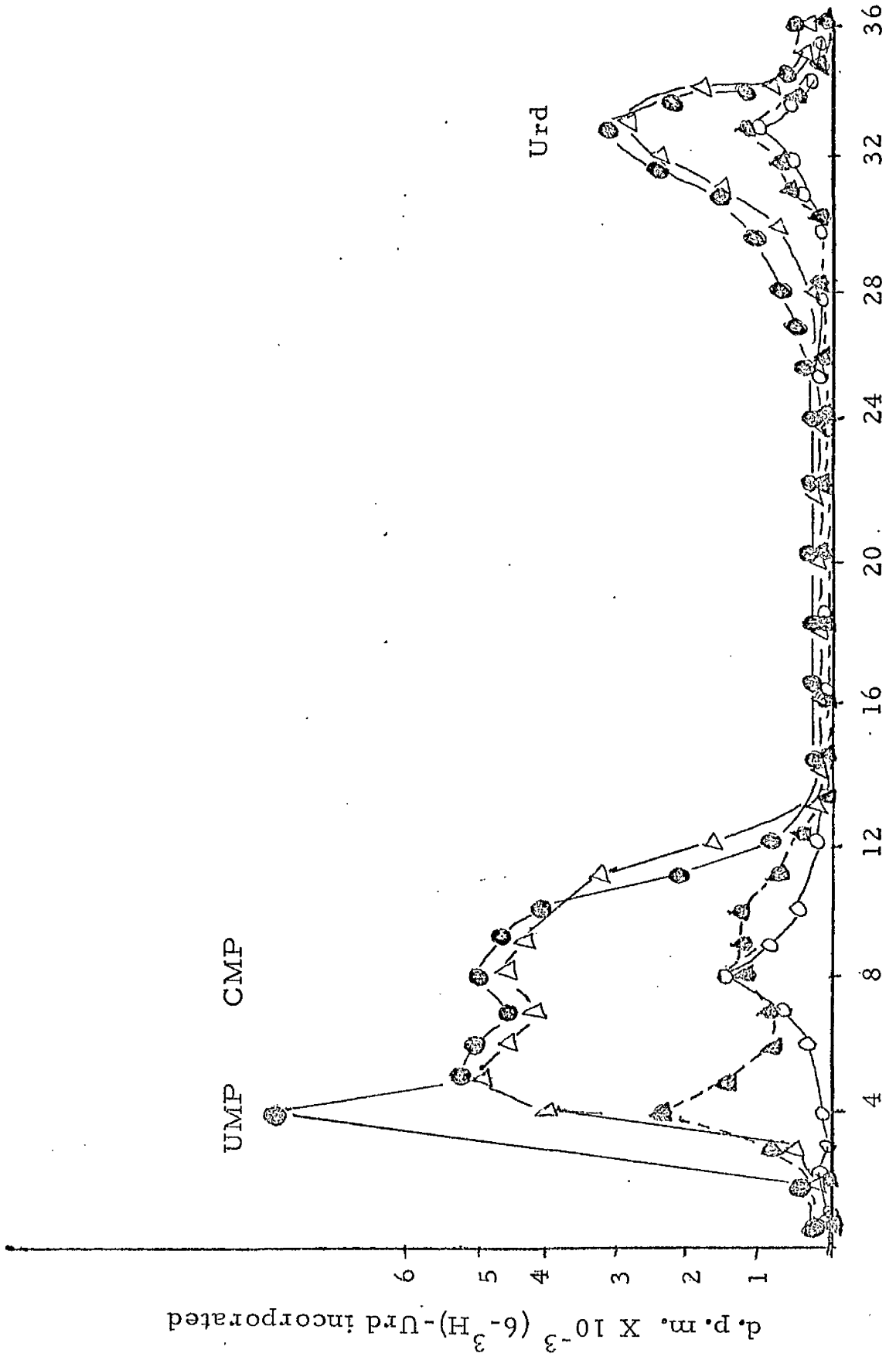

UMP
CMP
Urd
d.p.m. X 10^{-3} ($6\text{-}^{3}H$)-Urd incorporated
1
2
3
4
5
6
4
8
12
16
20
24
28
32
36
Strip No.

Fig III - 35

phosphorylation of (^{3}H)-Urd, in the cells. The level of nucleotides in 5-azaCyd inhibited cells comes down to almost the same level as in unstimulated cells except that 5-azaCyd treated cells contain an extra amount of UMP (and/or its higher phosphates).

Raska et al, 1965 reported that in the cell free extract of liver cells the phosphorylation of Cyd is not substantially inhibited by 5-azaCyd, whereas for the same substrate inhibitor ratio the phosphorylation of 5-azaCyd is inhibited by Cyd.

4.8.5 The effect of 5-azaCyd on RNA polymerase activity in PHA stimulated lymphocytes:-

Once we came to know that the synthesis of RNA is inhibited by 5-azaCyd, we were anxious to know the reason. In order to learn whether RNA polymerase is inhibited by 5-azaCyd resulting in the inhibition of RNA synthesis, we studied the affect of 5-azaCyd on lymphocyte RNA polymerase in cell free extract. As has already been shown, the uptake of (^{3}H)-Uridine into the intracellular nucleotide pool is increased considerably as a result of PHA addition. Incorporation of (^{3}H)-Urd into the lymphocyte RNA increases tremendously and a rapid induction of RNA synthesis takes place.

Induction of RNA polymerase activity is thus expected after treatment with PHA, Hausen, Stein and Peter (1969) showed that in human lymphocytes activities of both deoxyribonucleoprotein bound and soluble RNA polymerase activities increase as a

FIG III - 36

Effect of 5-azaCyd on RNA polymerase activity in cell free system in (PHA stimulated horse lymphocytes and L929 strain of mouse fibroblast).

Lymphocytes purified by Boyum's method were grown for 72h in Winchester bottles after stimulating with PHA. (Methods section 2. 2(iii)) The cells were harvested and washed as described (Methods section 2. 9. 2). and pure nuclei were prepared. The nuclei were frozen at -70°C for 3 weeks. The experiment was conducted using 1 X 10^9 nuclei pooled from 3 different experiments.

Approximately equal number of nuclei from logorithmically growing mouse fibroblasts (L929 strain) were also treated similarly.

The RNA polymerase activity in PHA stimulated horse lymphocytes and L929 strain of mouse fibroblast cells was determined as described in Methods (cf. Section 2. 11. 1). In addition, the effect of 5-azaCyd on nuclear RNA polymerase in a cell free system in both types of cells was tested by including in the assay mixture 5-azaCyd at the concentrations shown in Fig III-36.

The amount of (^{3}H) CTP (1uc 4. 39 Ci/mM) incorporated into the acid insoluble fraction, under standard assay conditions over a 15min period was determined.

A unit of enzyme activity is expressed as n mole of (^{3}H)-CTP incorporated per hour per mg of nuclear proteins.

The assay mixture contained
25 µmole of Tris pH 8.0
2 " " " mercaptoethanol.
3 " " " $MgCl_2$
5 " " " KCl
Sorted Amm.SO_4

1 µc CTP (sp. activity 4.39 Ci/mM)
0.02 µmole cold CTP
0.2 " " each of ATP, GTP & UTP
in a final vol. of 0.25 ml.
0.13 ml being enzyme from 6×10^6 cells
& 0.12 ml cocktail.

FIG III - 36

Units of RNA polymerase activity in		
Concentration of 5-azaCyd	PHA Stimulated lymphocytes	L929 Cells
Blank	0.299	0.352
Control	1.831	1.876
4×10^{-5} M	2.05	1.866
8×10^{-5} M	2.23	1.833
4×10^{-4} M	1.88	1.673

result of PHA stimulation.

We expected a similar result in equine lymphocytes, and did not perform the preliminary experiments to show that RNA polymerase activity increases as a consequence of PHA stimulation. To perform such enzymic assays a large quantity of lymphocytes is required. We could not show the effect of 5-azaCyd on the induction of RNA polymerase. The important thing we were interested in was the effect of the drug on RNA polymerase in the cell free system.

Fig III-36 reveals that 5-azaCyd does not inhibit the RNA-polymerase activity from PHA stimulated equine lymphocytes or L929 strain of mouse fibroblast cells, in a cell free system, even at 10 fold higher concentration to that used in the culture.

4.9 Effect of 5-azaCyd on the induction of rRNA synthesis:

Cooper (1969) has suggested that regulation of rRNA metabolism is the controlling factor in lymphocyte growth.

Most of the RNA which is synthesized in the resting lymphocytes is nonribosomal (Cooper and Rubin - 1964). When PHA is added to lymphocyte cultures, within 6h of its addition rRNA production starts and by 24h the absolute rate of RNA production has been shown to be 50 fold higher than in resting cells.

In order to understand the mechanism of 5-azaCyd action, we were interested to know whether 5-azaCyd

FIG III - 37

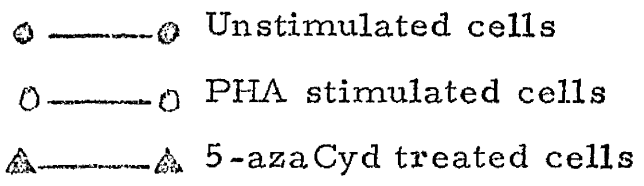
The effect of 5-azaCyd on the induction of rRNA synthesis

Standard 10ml lymphocyte cultures, containing 20 X 10^6 purified by Rabinowitz' method were used. To one set of tubes 5-azaCyd (4 X 10^{-5}) was added at 0h of culture along with PHA. After treating the cells with the drug for 2h, were washed and the medium changed as described (Methods section 2.5). The cells were reincubated under standard conditions 0.025 unit of PHA-M. Other cultures which served as unstimulated or PHA stimulated controls, also underwent similar operations of change in media.

The cells were pulse labelled with 50uc of (6^3H)-Uridine (1160mc/mM) for 2h immediately before harvesting at 20h of culture.

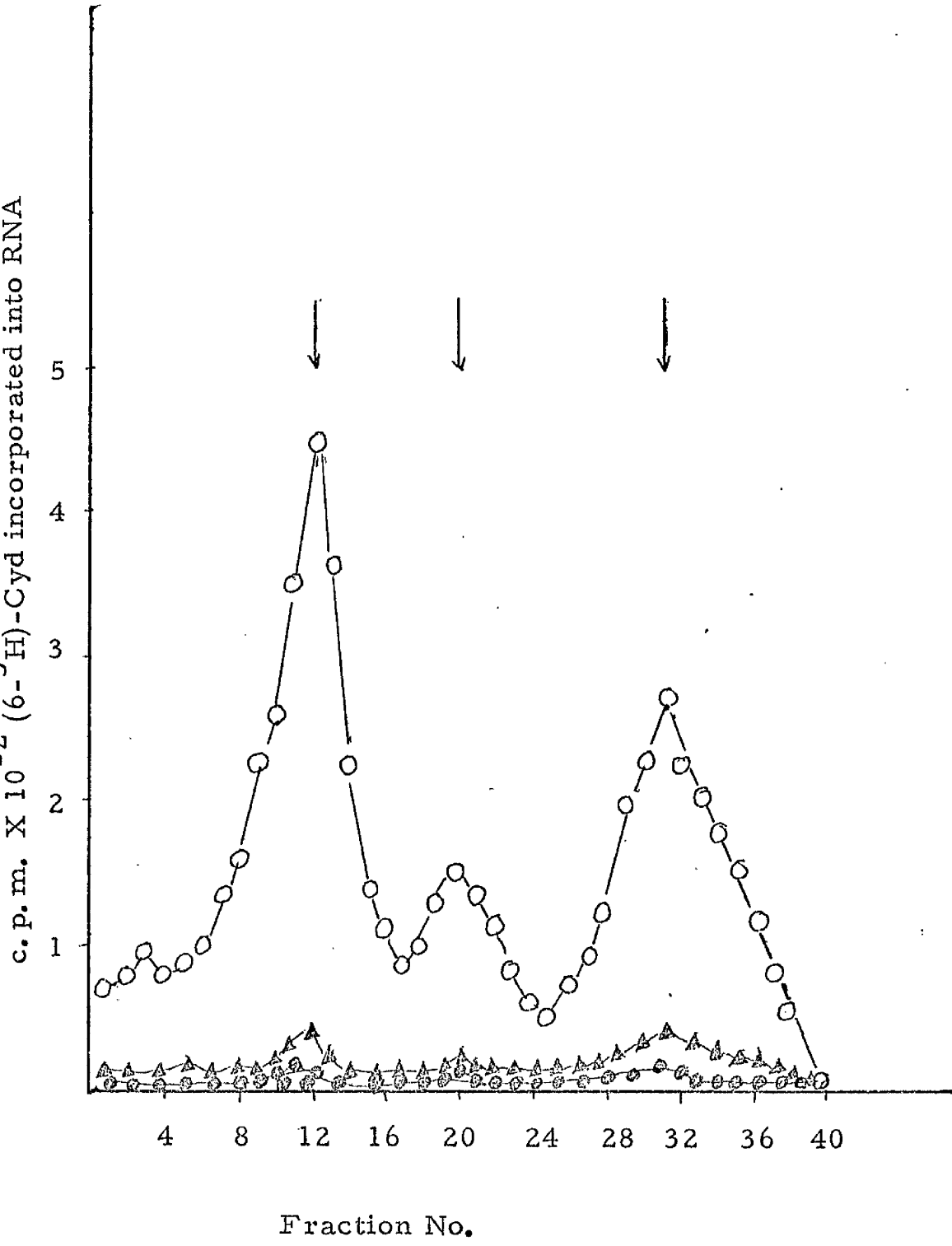
The RNA was extracted as described in Methods (Section 2.12a) and dissolved in 0.5ml of the buffer (2.12 (c)) and 0.25ml layered on the top of a 5-20% (^{w}/v) sucrose gradients. (The sucrose solutions were prepared in the same buffer).

The gradients were centrifuged at 49K r.p.m. for 2h in a Beckman model L_2 ultracentrifuge at 0-4°C. After the run 3 drop fractions were collected, by puncturing the bottom of the tube, and the amount of acid insoluble radioactivity in each fraction was measured in 10ml of toluene scintillation fluid.



The direction of sedimentation in the graph is from right to left. The results are expressed as c.p.m. X 10^{-2} (^{3}H)-Cyd incorporated into the acid insoluble fraction. The arrows indicate the absorbance markers provided by the ribosomal RNA from rat liver post mitochondrial supernatant.

Fig III - 37


c. p. m. X 10^{-2} ($6-^3$H)-Cyd incorporated into RNA
1
2
3
4
5
4
8
12
16
20
24
28
32
36
40
Fraction No.

inhibits, the induction of rRNA synthesis. Since the latter has been shown to be essential for the growth of these cells.

Fig III-37 reveals that 2h treatment with 5-azaCyd prevents the induction of rRNA synthesis, normally observed after PHA addition, and that all the species of RNA in 5-azaCyd treated cells remain at almost the same level as in resting lymphocytes.

4.10 The effect of 5-azaCyd on rRNA formed as a consequence of PHA stimulation:-

As we have seen the induction of rRNA is inhibited if 5-azaCyd is added along the inducer PHA. In order to see in what way 5-azaCyd effects the rRNA already formed the effect on the nuclear and cytoplasmic RNA of lymphocytes after 5-azaCyd treatment for 1, 2 and 4h was studied.

Fig III-38 (a) reveals that labelling rRNA does not appear in the cytoplasm during a 1 hour label but cytoplasmic RNA sedimenting in the 4S region is heavily labelled. 5-azaCyd inhibits the production of low mol. wt. RNA sedimenting in the 4S region.

Fig III-38 (b) reveals that after an hour almost all the label appears in the heterogeneously sedimenting nuclear RNA, but no clear 28S or 18S rRNA peaks are apparent. The label is greater in the low mol. wt. 4S region in 5-azaCyd treated cells, while the control cells show a reduced amount in the last 3 tubes. Otherwise treatment for 1h with 5-azaCyd has little effect on the pattern of nuclear RNA synthesis.

FIG III - 38, 39 and 40 (a & b)

The effect of 5-azaCyd on rRNA formed as a consequence of PHA stimulation

Standard 10ml lymphocyte cultures containing 20 X 10^6 cells purified by Boyum's method were utilised. The cultures were incubated after the addition of PHA-M (0.025 unit/culture) under standard conditions for 60h.

At 60h 3 sets of cultures were treated with 5-azaCyd ($4X10^{-5}$M) while three more served as controls. At the same time, 250uc ($^{32}P_i$) orthophosphate (92mc/mg P_i) was added to each culture and the cells were reincubated under standard conditions.

One set each of control and test cultures were harvested every hour for the subsequent 4 hours (cf Methods 2.3.3). The extraction of RNA was carried out as described in Methods (cf Methods section 2.12 a & b). The cytoplasmic RNA collected by cold phenol extraction and the nuclear RNA by the hot phenol method were precipitated at -20°C, washed once with cold ethanol containing 2% Potassium acetate and dissolved in 0.5ml of buffer (cf Methods, section 2.12 (b)).

0.25ml of the RNA solution was layered carefully over a 10-30% ($^{w}/_{v}$) sucrose gradient and fractionated as described in Methods (cf Methods section 2.12 (iii)).

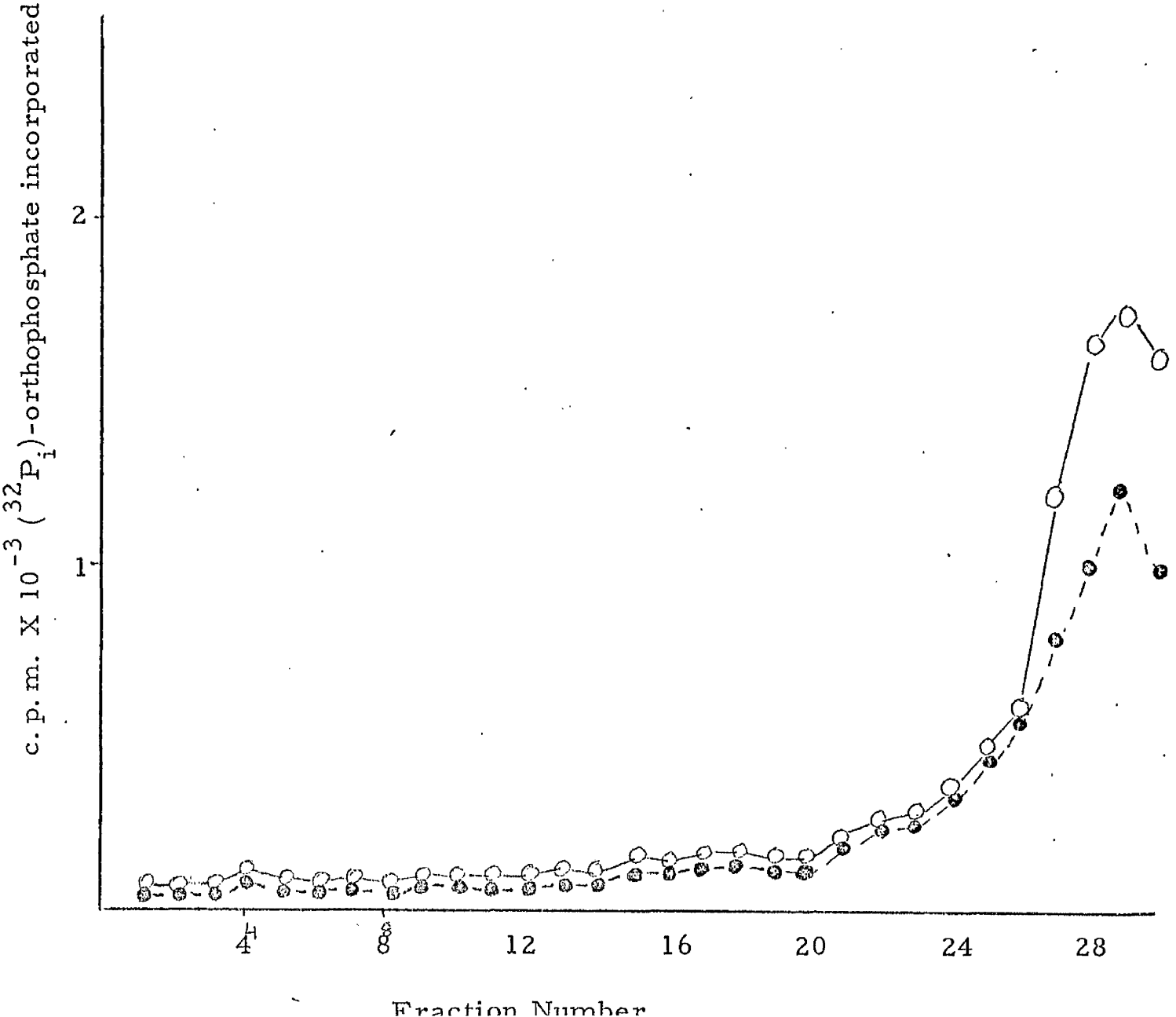
○——○ Control cells

●——● 5-azaCyd treated cells

The results are expressed as c.p.m. ($^{32}P_i$)-orthophosphate incorporated into RNA per fraction.

The direction of sedimentation in the graphs is from right to left and the arrows indicate the optical density markers obtained from the rRNA of rat liver post mitochondrical supernatant fraction.

Fig III - 38 (a)


c.p.m. X 10^{-3} ($^{32}P_i$)-orthophosphate incorporated into RNA
2
1
4
8
12
16
20
24
28
Fraction Number

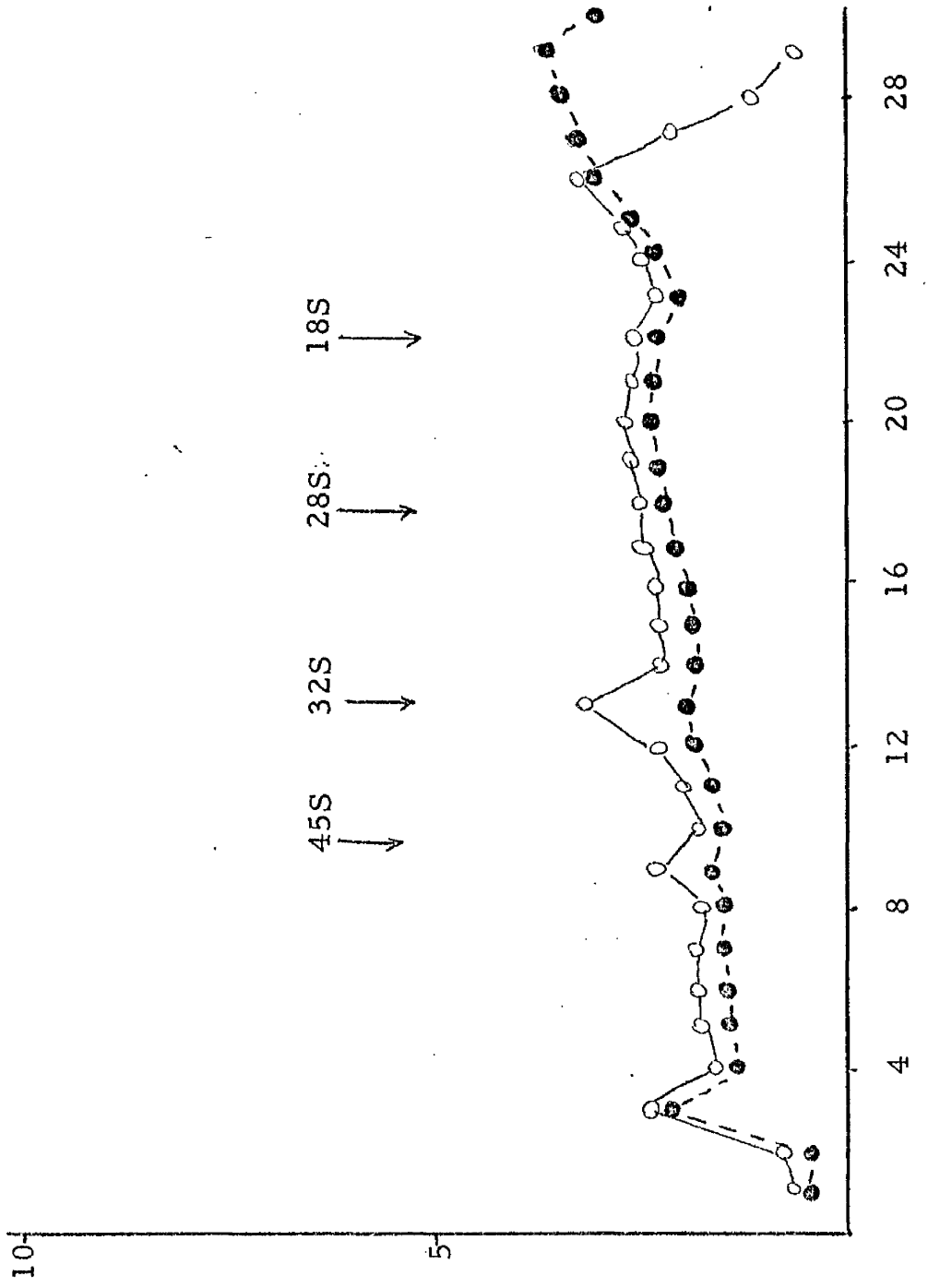

c.p.m. X 10^{-2} ($^{32}P_i$) incorporated into RNA
10
5
45S
32S
28S
18S
4
8
12
16
20
24
28
Fraction No.

Fig III - 38 (b)

Fig III-39 (a) reveals that after 2h label with ($^{32}P_i$)-orthophosphate small peaks of 28S and 18S rRNA appear in the cytoplasm. The 4S RNA peak is comparatively huge. Except for the 4S peak where there is about 44% reduction in the peak height in 5-azaCyd treated cells, there is no effect of 5-azaCyd on cytoplasmic rRNA at 2h.

Fig III-39 (b) reveals that there is not much inhibition in the heterogeneously labelled nuclear RNA in the 5-azaCyd treated cells. The label incorporated is almost twice that found during the one hour pulse, and small peaks sedimenting at 45S, 32S, 28S and 18S RNA are apparent in the control cells. 5-azaCyd treated cells do not show any peak in the 45S and 32S region and the counts incorporated in this region are a bit lower than in the controls. The height of 28S RNA peak is reduced, but there is no effect on 18S RNA peak. Whereas the control cells do not show any peak in the 4S region, 5-azaCyd treated cells do show a small peak which is probably due to degradation.

Fig III-40 (a) reveals that after 4h of labelling definite peaks of rRNA appear in the cytoplasm. A huge 28S rRNA peak and a small 18S peak are apparent with a high 4S RNA peak, in the control cells. 4h 5-azaCyd treated cells on the other hand show a reduction of about 60% in the 28S rRNA peak height and about 50% reduction in the 18S rRNA peak height. There is almost negligible difference in the control and 5-azaCyd treated cells in the peak heights in the 4S RNA region.

Fig III - 39 (a)

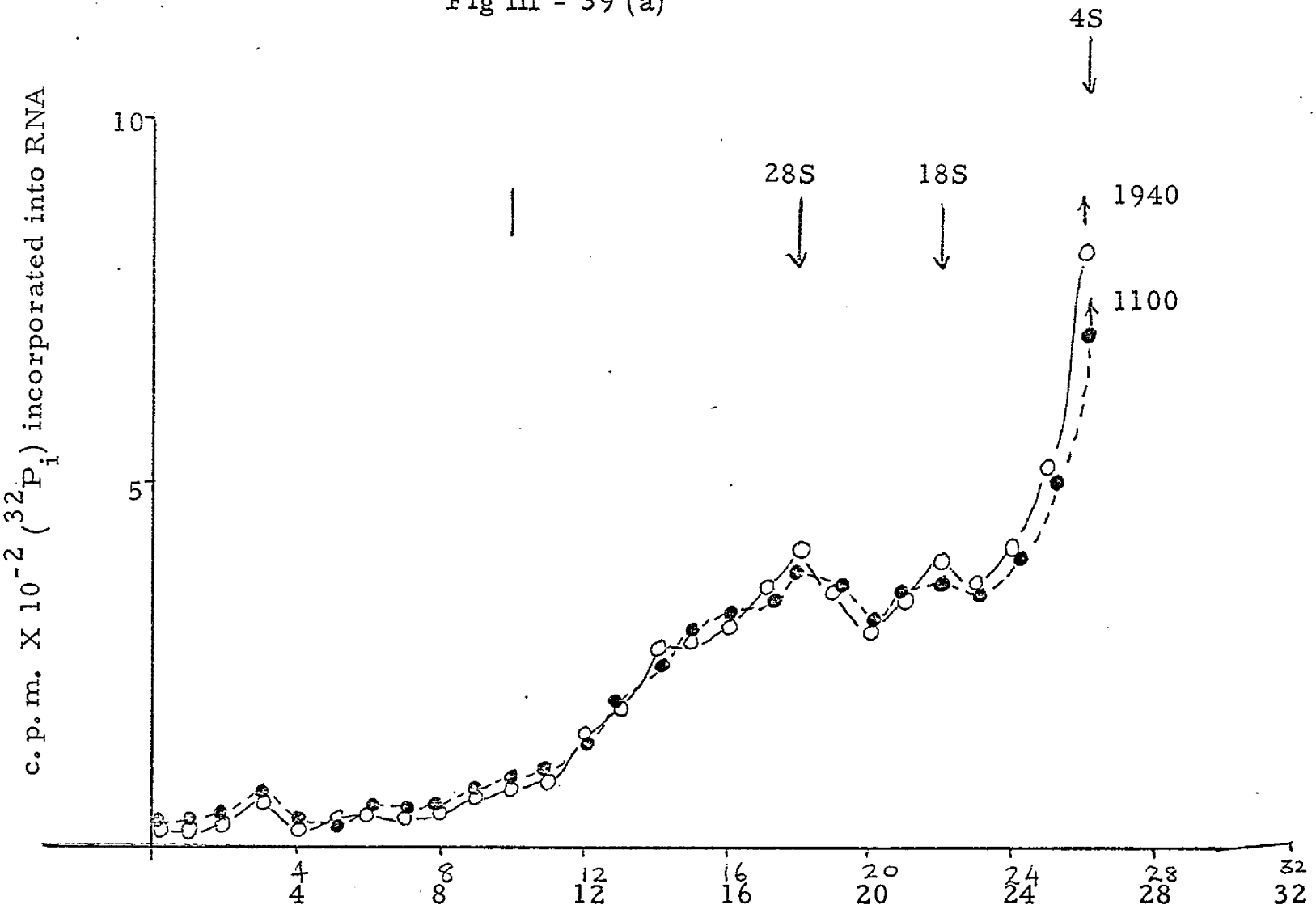

4S
28S
18S
1940
1100
10
5
c.p.m. X 10^{-2} ($^{32}P_i$) incorporated into RNA
4
8
12
16
20
24
28
32
Fraction Number

Fig III - 39 (b)

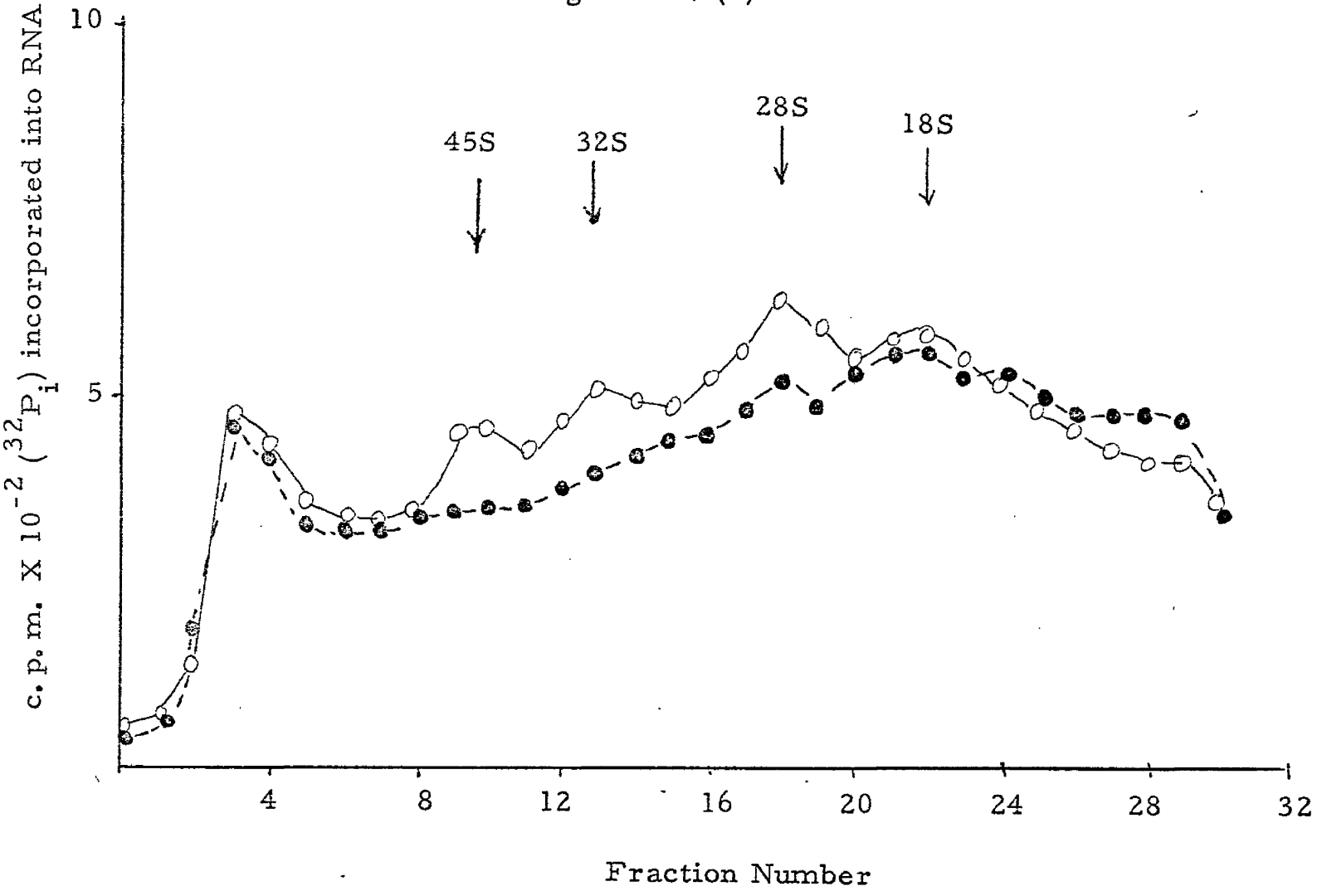

10
5
45S
32S
28S
18S
c. p. m. X 10^{-2} ($^{32}P_i$) incorporated into RNA
4
8
12
16
20
24
28
32
Fraction Number

Fig III - 40 (a)

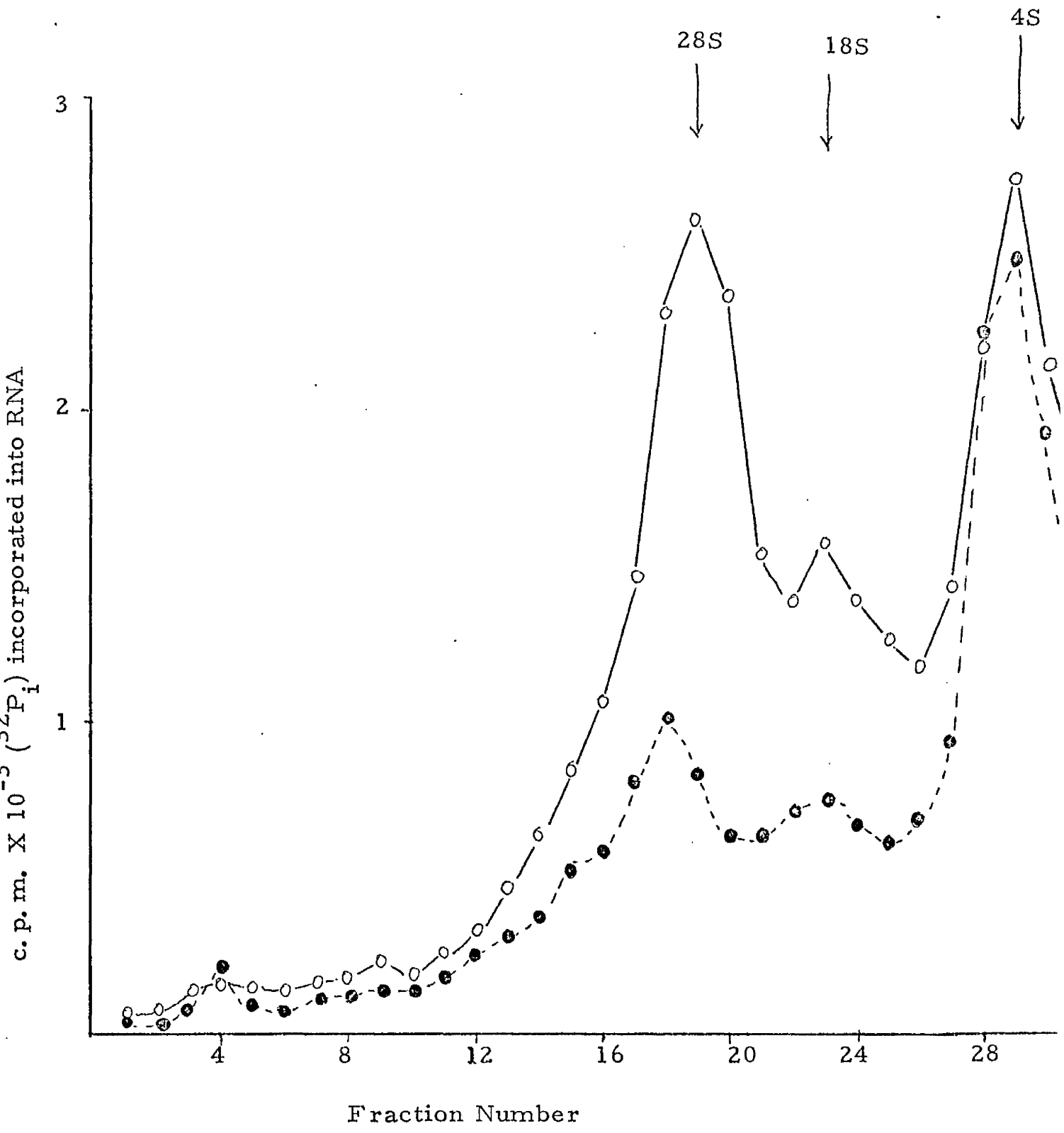

28S
18S
4S
3
2
1
c. p. m. X 10^{-3} ($^{32}P_i$) incorporated into RNA
4
8
12
16
20
24
28
Fraction Number

Fig III - 40 (b)

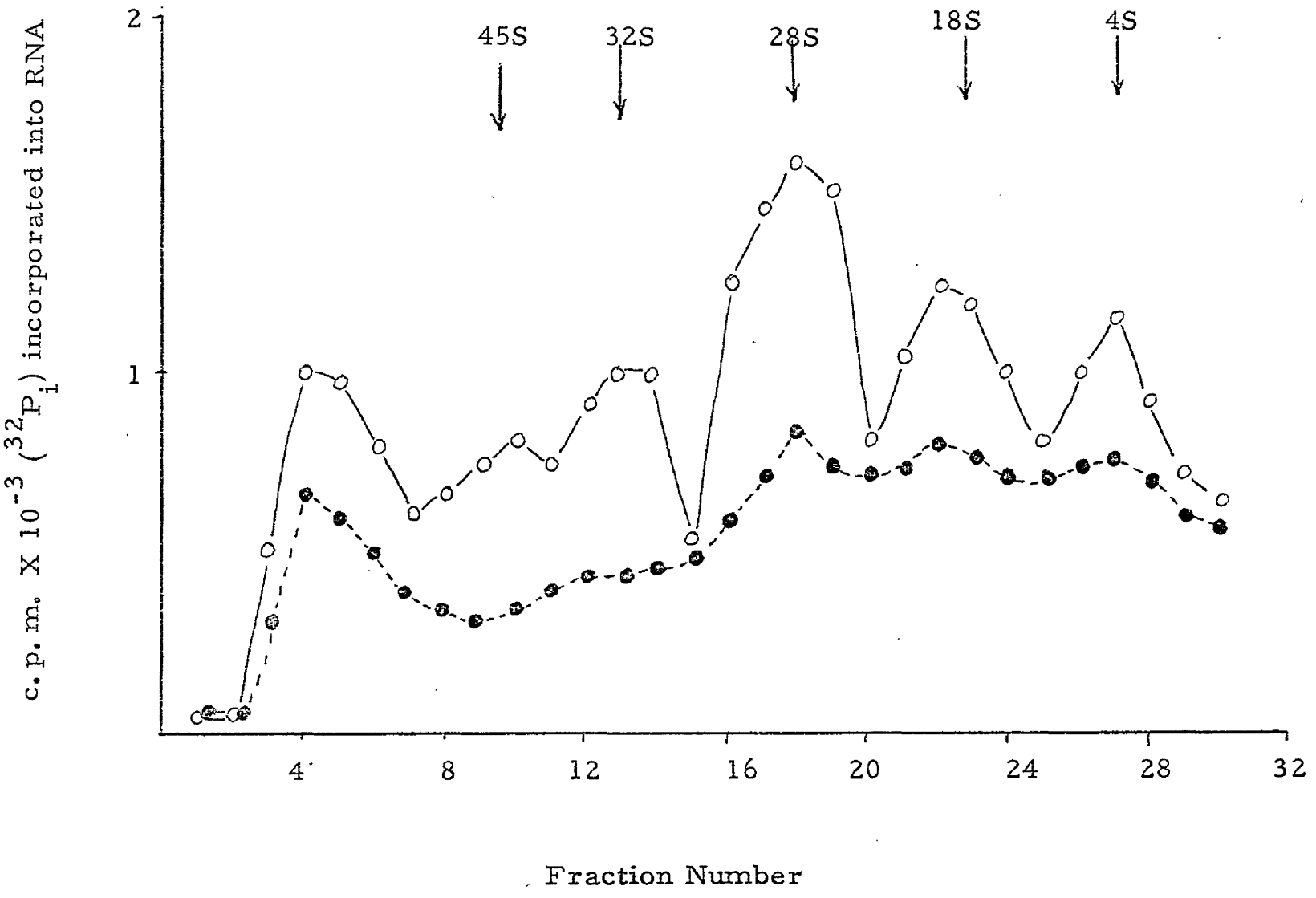
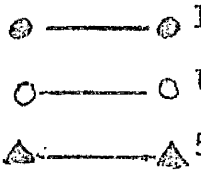

45S
32S
28S
18S
4S
2
1
c.p.m. X 10^{-3} ($^{32}P_i$) incorporated into RNA
4
8
12
16
20
24
28
32
Fraction Number

FIG III - 41

Effect of 5-azaCyd on Protein Synthesis in PHA stimulated lymphocytes

Standard 2ml cultures containing 2 X 10^6 cells purified by Boyum's method were used. They were stimulated with 0.005 unit of PHA-M and incubated under standard conditions (cf. Methods section 2.2.1). At 48h of culture to the test cultures 5-azaCyd (4 X 10^{-5} M) and 0.1ml of water was added to the control cultures. 10uc (methyl-^{3}H)-L-Leucine (22.2 Ci/mM) was added at 48h of culture and the cells were reincubated under standard conditions. The cultures were harvested every subsequent hour. The extraction procedure was similar to that described in Methods (cf. section 2.4.1), except that 10% (^{w}/v) TCA containing cold leucine was used, and ethanol and ether extractions were avoided. After 5 extractions with 10% TCA the acid insoluble precipitate was dried overnight at 37^{o}C, dissolved in hyamine hydroxide and washed into the scintillation vials with 10ml of toluene scintillator and counted in Phillips liquid scintillation spectrometer.

The results are expressed as d.p.m. (^{3}H)-L-Leucine incorporated into proteins per cultures.



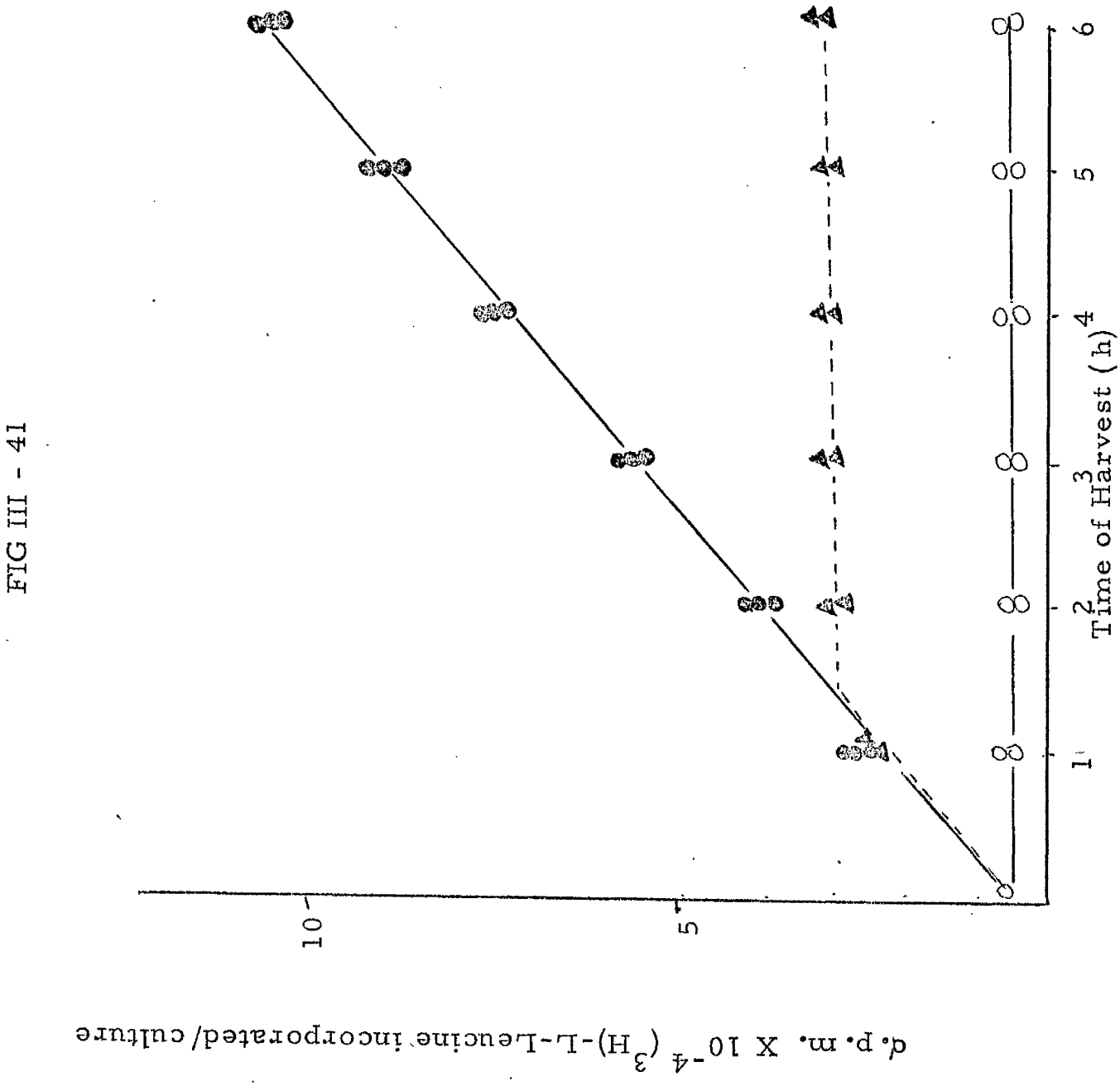

d.p.m. X 10^{-4} (^{3}H)-L-Leucine incorporated/culture
10
5
1
2
3
4
5
6
Time of Harvest (h)

FIG III - 41

Fig III-40 (b) shows clearly differentiated peaks of 45S, 32S, 28S, 18S and 4S RNA. In addition a peak of very high mol. wt. RNA appears at the junction of 2M sucrose and 30% sucrose solution in the gradient. In 4h 5-azaCyd treated cells, almost all the species of nuclear RNA are inhibited nearly to the same extent. There is no 45S and 32S peak and in the test the peak heights are small compared to the controls.

Even the 4S RNA peak is inhibited after 4h treatment with 5-aza-Cytidine. These results demonstrate that within 4h of treatment with 5-azaCyd the synthesis of all the species of rRNA and their precursors is inhibited by 5-azaCyd.

4.11 Effect of 5-azaCyd on protein metabolism:-

4.11.1 The effect of 5-azaCyd on the stimulation of protein synthesis in PHA stimulated horse lymphocytes:-

It is a well established fact that as a consequence of treatment with PHA lymphocytes start to synthesize proteins at a higher rate (Bach and Hirschhorn - 1963, Kay - 1966, Forbes and Henderson - 1966, Scheurlen 1968, Turner and Forbes - 1966).

Fig III-41 reveals that proteins are continuously synthesized at a higher rate in cells stimulated by PHA, the incorporation of (^{3}H)-Leucine is very high in PHA stimulated lymphocytes compared to the unstimulated controls.

FIG III - 42

Time of inhibition of protein synthesis following 5-azaCyd treatment in PHA stimulated horse lymphocytes

Standard 2ml cultures containing 2 X 10^6 lymphocytes purified by Boyum's method were set up. After adding 0.005 unit of PHA-M the cultures were incubated under standard conditions for 40h (cf. Methods section 2.2.1).

At 40th h 5-azaCyd (4 X 10^{-5}M) was added to the test cultures, while the controls received 0.1ml of sterile distilled water. All cultures received 0.1ml of a solution containing 2uc each of 4, 5 (^{3}H)-L-Leucine (22Ci/mM) and (2-^{14}C)-Urd, (62Ci/mM) and were incubated under standard conditions (cf. Methods section 2.2.1). The cells were harvested at the time periods shown in Fig III-42.

The cells were extracted with 10% TCA X 3, 1:1 ethanol ether X2 and the amount of radioactivity incorporated per culture was measured as described in Methods section 2.4.1.

The Results are expressed as d.p.m. X 10^{-4}.

a) (4, 5-^{3}H)-L-Leucine incorporated into Proteins

b) (2-^{14}C)-Urd incorporated into RNA per culture.

● = PHA stimulated control cells

△ ▲ = 5-azaCyd treated cells

Fig III - 42 (a)

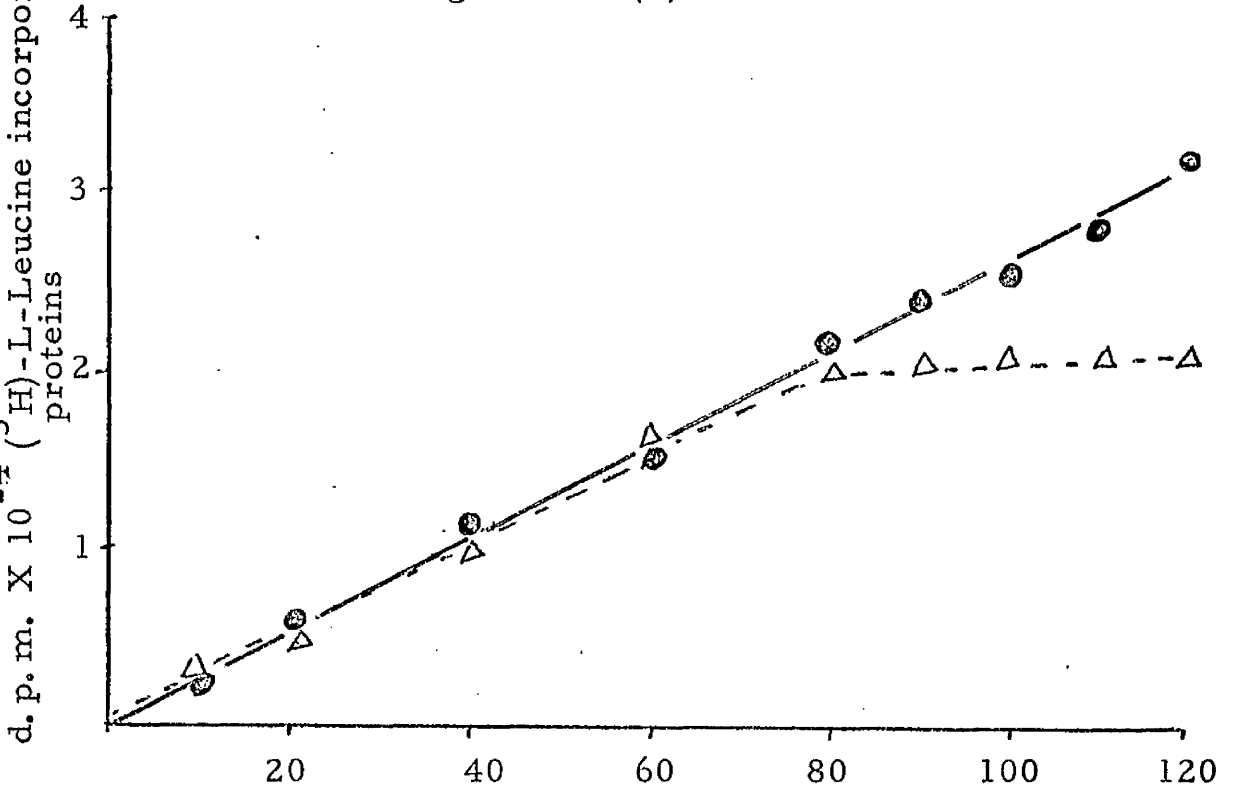
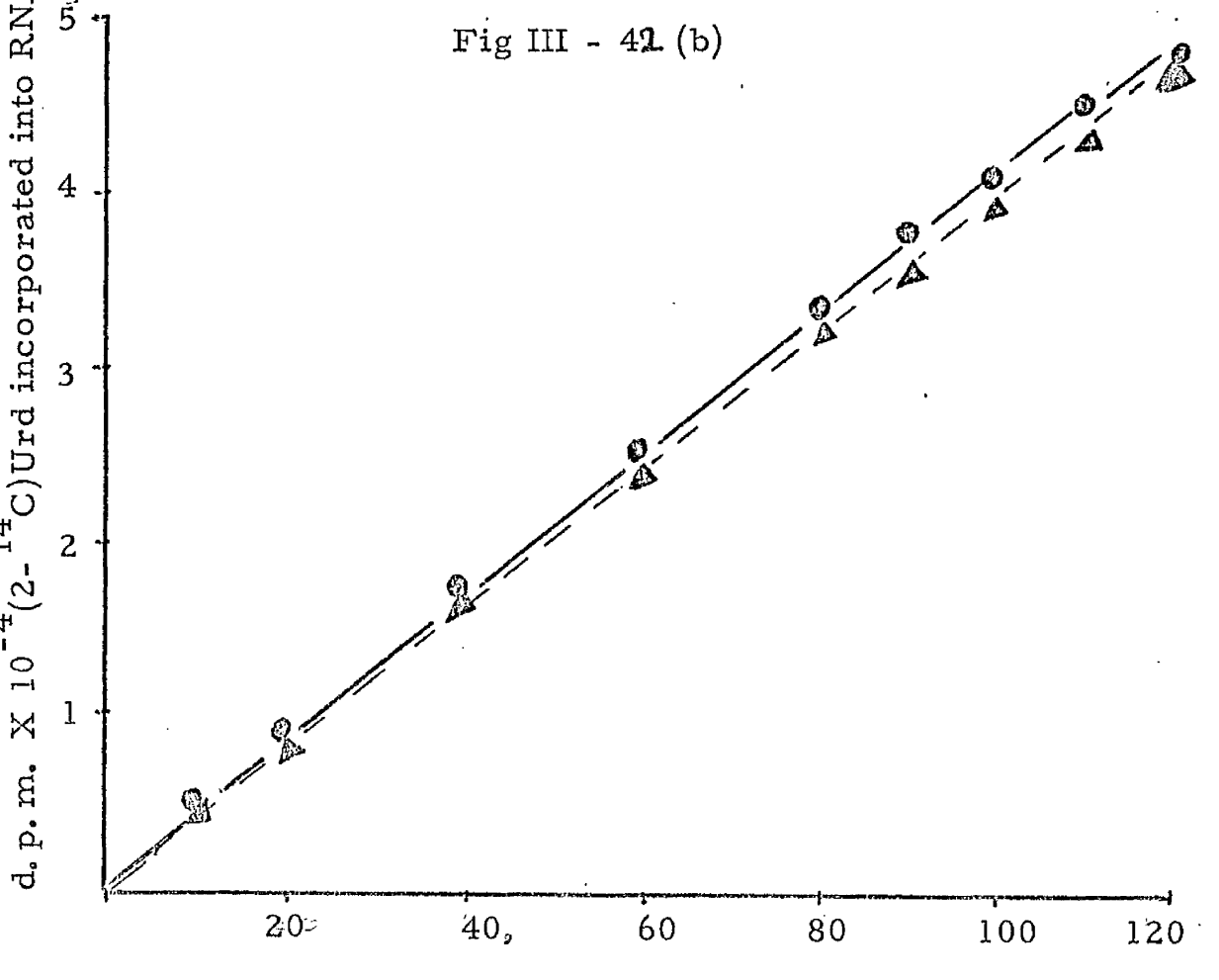


Fig III - 42 (b)



On the other hand if the cells are treated with 5-azaCyd the cells synthesize proteins at the normal rate for an hour but shortly after this there is an inhibition of protein synthesis. Study for the following 4h reveals that the rate of protein synthesis in the control cells increases steadily while the 5-azaCyd treated cells stop synthesizing.

4.11.2 Time of inhibition of Protein synthesis:-

Fig III-42 reveals that protein synthesis stops sometime between 70 to 90 minutes of the addition of the drug.

Studies on the effect of 5-azaCyd on protein synthesis both in bacteria and in mammalian cells have been carried out by Sorm and co-workers.

Doskocil, Paeces and Sorm (1967) reported that when 5-azaCyd is added to an E. coli culture induced to synthesize B-Galactosidase, 5-azaCyd completely inhibits the synthesis of the enzyme within 12 min while total protein synthesis was inhibited to 6% of the control.

Levitan and Webb (1969) reported that 5-azaCyd inhibits the hormonally induced rises in the activity of tryptophan pyrrolase but tyrosine transaminase is not affected by the drug.

Raska et al, (1966) working with the isolated nuclei of calf thymus reported that protein synthesis in this system is inhibited by 5-azaCyd only after a lag of 90 minutes.

FIG III - 43

Effect of 5-azaCyd on protein synthesis in Unstimulated lymphocytes

Standard 2ml cultures containing 4×10^6 freshly isolated lymphocytes purified by Boyum's method were set up.

To one set 5-azaCyd (4×10^{-5}M) was added, while the other set served as a control and received 0.1ml of sterile distilled water. To all the cultures 10uc of (methyl-^{3}H)-L-Leucine (22.2 Ci/mM) was added, and the cells were incubated under standard conditions (c.f. Methods section 2.2.1).

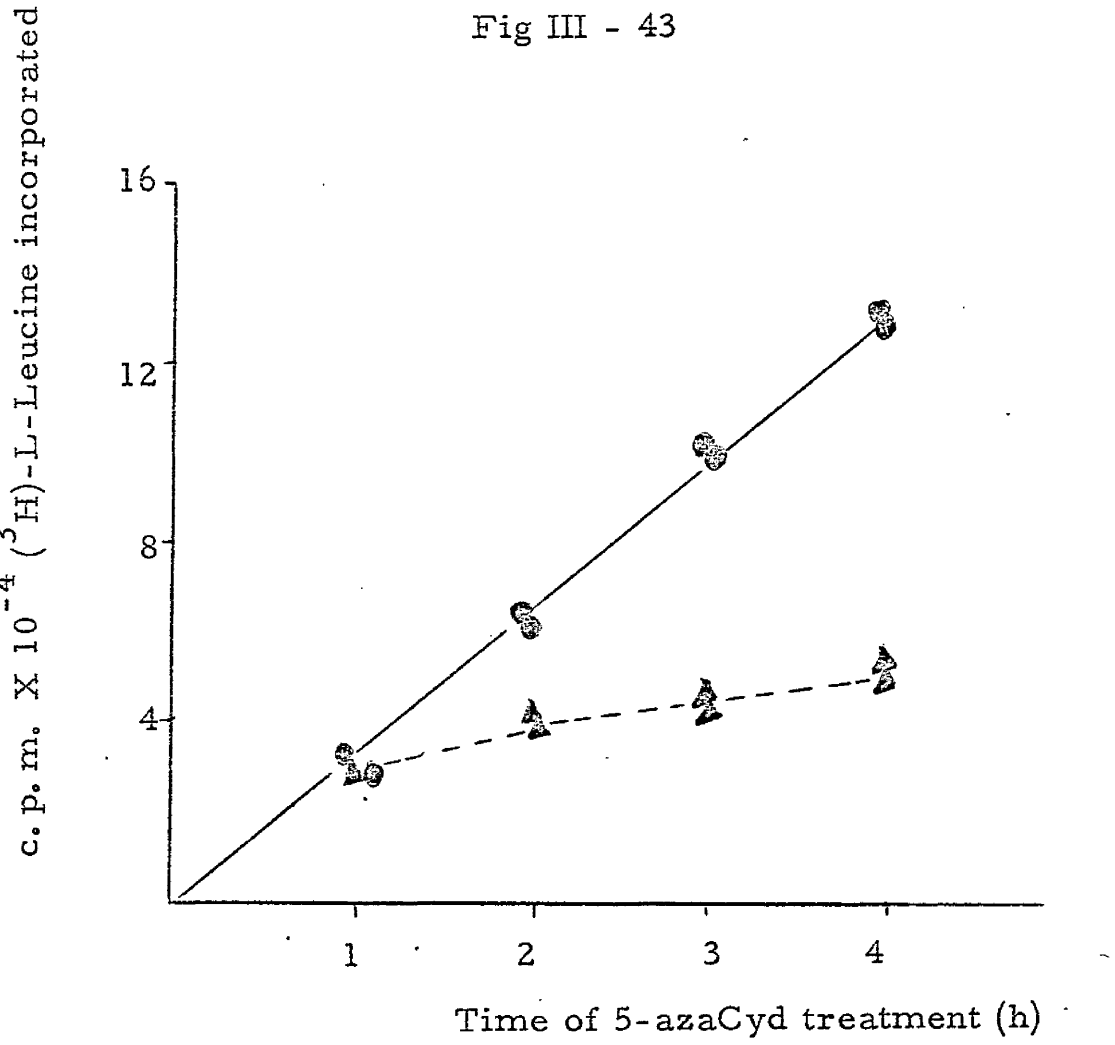
The extraction and measurement of radioactivity was carried out as mentioned in the last experiment.

The results are expressed as d.p.m. (CH_3-^{3}H)-L-Leucine 10uc (22.2 Ci/mM) incorporated into proteins per culture.

Unstimulated control cells

Unstimulated cells treated with 5-azaCyd

Fig III - 43


c. p. m. X $10^{-\frac{3}{4}}$ (^{3}H)-L-Leucine incorporated
16
12
8
4
1
2
3
4
Time of 5-azaCyd treatment (h)

The most widely accepted mechanism of 5-azaCyd action today is that the drug is incorporated into mRNA producing faulty messengers, which results in the production of enzymes either with inferior quality or low in quantity. The inhibition of enzymes is selective i. e. tryptophan pyrrolase induction is inhibited while tyrosine transaminase is not affected. Thus the fraudulent messengers are responsible for the disturbance in cellular metabolism leading to growth inhibition.

4. 11. 3 Effect of 5-azaCyd on protein synthesis in unstimulated cells: -

In PHA stimulated lymphocytes synthesis of a number of new messengers and proteins is expected and accordingly 5-azaCyd should inhibit protein synthesis. Fig III-41 and Fig III-42 show such a result .

As far as unstimulated lymphocytes are concerned the cells are less active in this respect and these cells therefore may be less susceptible to the action of 5-azaCyd. Fig III-43 reveals that this is not the case and 5-azaCyd equally inhibits protein synthesis in the unstimulated lymphocytes.

DISCUSSION

CHAPTER IV - DISCUSSION

1. SUITABILITY OF EQUINE LYMPHOCYTES IN TISSUE CULTURE:-

1.1 Separation, purification and preservation of equine lymphocytes:-

Horse lymphocytes purified by Boyum's method (Boyum - 1968), on stimulation with PHA, provide the most suitable and reproducible preparations for the study of blastogenesis.

The biochemical studies involved in my work required a blood sample of at least 200ml containing $3\text{-}4 \times 10^8$ lymphocytes. Samples had to be available at regular intervals and had to be as similar as possible to reduce incontrollable variations.

Many workers have used human blood taken from normal or leukemic patients. PHA stimulated lymphocytes in many ways resemble leukemic lymphocytes and, to help understand the basis of leukemias, I wished to understand the essential metabolic changes and mechanism of blastoid transformation. I have therefore used PHA stimulated lymphocytes as a substitute for leukemic lymphocytes. When it is impossible to arrange a supply of the desired quantities of fresh normal human blood, it is out of question to think of obtaining such quantities of leukemic blood. The alternatives allowed were adequate quantities of normal human blood which was being discarded from the blood banks (i. e. 2 to 3 weeks old and no longer responsive to PHA) or small volumes (20ml) taken from volunteers around the laboratory.

Hence it was decided to use horse lymphocytes after considering the disadvantages of other animals. A horse, because of its size, can more readily supply 300ml of blood at weekly intervals. Moreover it is a relatively simple matter to obtain samples from a docile horse. Other workers (Forsdyke - 1968) have used pig blood, but this has only been obtained following slaughter of the animals. The response of Sheep lymphocytes was tested, but the difficulty confronted in the purification of the lymphocytes was the small size of sheep erythrocytes, which made it very difficult to separate them from lymphocytes.

In the early studies Rabinowitz method of isolation and purification of lymphocytes was used, as granulocytes are removed by this method. It had been shown by Milton, Cooper and Halle-Panneko (1965) that granulocytes degrade the thymidine in the cultures to dehydrothymidine thereby making it unavailable for DNA synthesis. Thus, radioactive thymidine cannot be used in such cultures for assaying the lymphocyte growth.

The presence of the erythrocytes in the lymphocyte preparation was a problem because:

i) In studies connected with protein synthesis attempts were made to isolate and characterize by polyacrylamide gel electrophoresis the various proteins synthesized by lymphocytes, but I found the lymphocyte proteins to be completely masked by the high concentration of erythrocyte proteins.

ii) Haemoglobin from erythrocytes caused quenching of tritium counts in the liquid scintillation counting.

On the otherhand erythrocytes do not synthesize DNA, hence they do not interfere in such assays. Many authors consider the presence of erythrocytes in the lymphocyte cultures beneficial, as:

i) The increased bulk of cells present facilitates processing of the cultures.

ii) Pentycross (1968) has shown that erythrocytes act as a buffer in stabilizing the pH of the nutrient media.

Attempts to lyse the erythrocytes selectively by giving hypotonic shocks (Fallon - 1962; Thomas, Bull and Robinson - 1966) were unsuccessful because it was not possible to lyse all the erythrocytes without some of the lymphocyte also getting lost. The erythrocyte concentration was reduced by using leukocyte rich plasma as the starting material and using Boyum's Ficoll-triosil method of lymphocyte purification.

Boyum's method proved more satisfactory in many respects. The cells obtained are of high purity, only 3-5% of the cells were erythrocytes compared to levels ranging from 5-30 red blood cells per white blood cell (Ling - 1968) or 4 red blood cells per white blood cell (Hugh and Caspray - 1970). The contamination with granulocytes is almost negligible (0-1%). Moreover, the yield is very high compared to Rabinowitz method. In addition Boyum's method proved simpler, as the reagents are easily available and all that is required is to mix and layer the sample, compared with the preparation of a sterile glass bead column, which involves washing the beads, drying and sterilizing them. Because of the size

of the column, it is inconvenient to wash the tube containing the concentrated cell suspension, moreover washing involves consuming more autologous plasma. If the tube is not washed, a large quantity of cells is wasted, thus reducing the yield.

Boyum's method relies for the separation of lymphocytes on the basis of the difference in the densities of red and different species of white blood cells. Centrifugation of whole blood against high density fluids has been reported in the literature. Some author's (Faucet and Valley - 1952; Szenberg and Shortman - 1966) have used bovine serum albumin but the procedure is laborious and the use of albumin is not only expensive but liable to produce complications. Since lymphocytes are stimulated by antigens and allergens, and they play an active role in immunological reactions, while bovine serum albumin is often used as an antigen.

The reagents employed in Boyum's method are stable, inexpensive and have been reported (Boyum - 1968) to be nontoxic to the cells. However, we have shown (Fig III-9) that the continuous presence of triosil or separation fluid in the growth medium is toxic to the cells. This effect can be removed by washing the cells despite the fact that Rabinowitz (1964) and Elves (1965) have shown that centrifugation causes damage to the cells. Caron (1967) demonstrated reduced transformation of the cells after washing. Our studies (Figs III-8, III-10) do not demonstrate any such effects.

The greatest advantage of using Boyum's method was that such cultures were essential for studies connected

with protein metabolism. Erythrocytes do not synthesize DNA, but they do synthesize proteins, and hence, there is the possibility of the radioactive aminoacids being incorporated into erythrocyte proteins. Comparative studies, using growth inhibitors, can give misleading results.

1.2 Conditions for culturing and growth of equine lymphocytes:-

Basically the culture conditions chosen were those described by Tormey and Mueller (1965), but modified when necessary.

Cultures were labelled with thymidine for relatively short periods (6h for ^{14}C-dThd and 2h for (^{3}H)-dThd) because Milton, Cooper and Halle-Panneko (1965) have shown that dThd is broken down by granulocytes in tissue cultures. However, our cultures had very low contamination with granulocytes, and no breakdown of tritiated thymidine was observed even when we labelled the cells continuously for four days for autoradiographic experiments. The incorporation was very high on the third day compared to the first two days, and all the radioactivity was incorporated into the nucleus.

1.2.1 Growth Media:-

Although NCTC-109 proved to be the best and most efficient medium, we used EHM (Eagle's MEM c.f. Materials section 1.2 b.) because it is simple and easily available in our laboratory. Addition of extra dThd to EHM had no effect on the growth of lymphocytes, and the low incorporation of radioactive dThd into DNA

can be explained by the lowering of the specific activity of radioactive dThd.

1.2.2 Sera:-

The extent of blastogenesis as measured by the uptake of radioactive thymidine is dependant on the type and concentration of the added sera. Shellekens and Eijsvoogel (1968) state that the volume of the plasma employed should be 20-33% while Ling used 10-25%. On the otherhand, the results shown in Fig III-2 reveal that 10% of the plasma or serum is quite adequate. Though higher concentrations of homologous horse serum were effective as shown by increased incorporation of radioactive dThd into DNA, autologous horse plasma at higher than 10% concentration was inhibitory. Autologous horse serum at higher than 10% concentration neither stimulated nor inhibited the growth. Probably the presence of heparin in the plasma, at higher concentrations is inhibitory towards the cellular growth. At the same time we cannot rule out the possibility of the presence of certain inhibitory factors which are removed when the blood is allowed to clot.

10% autologous horse serum gives the best response, under our experimental conditions, but we used 10% autlogous horse plasma. This is because, it is economical and handy to use the plasma separated from the same blood sample which is used to collect the lymphocytes.

Moreover, use of autologous plasma or serum is advantageous since it does not expose the cells to foreign antigens, cytotoxic or growth-promoting bodies etc. Cooper et al (1964) have shown the presence of inhibitory factors in normal sera. Foetal calf serum has been shown to be stimulatory towards human lymphocytes by many workers. i. e. Shellekens and Eijsvoogel (1968), Woodliff and Onseti (1968), Wilson (1965) Johnson and Russel. Sabesin (1965) observed the blast transformation in rabbit and guineapig lymphocytes invitro in the presence of either foetal calf serum or autologous serum .

In contrast horse lymphocytes incorporated less (^{14}C)-dThd when foetal calf serum was used than when autologous horse plasma or serum of the same concentration was used.

The higher activity of autologous horse plasma or serum compared to other types of sera can be a result of spontaneous transformation of the cells in addition to the action of PHA, as we did not study this effect in the absence of PHA. At the low concentrations of sera used by us probably foetal calf serum is ineffective in producing the spontaneous transformations.

1. 2. 3 Anticoagulant:-

Ling (1968) recommended avoidance of the use of citrate as an anticoagulant. Boyum (1968) suggested the use of EDTA as an anticoagulant

as this led to better purification. Since autologous plasma was utilised in this work, the presence of EDTA (a chelating agent) was considered undesirable as the presence of various metallic ions is necessary for cellular growth. In order to avoid both citrate and EDTA, heparin was used as an anticoagulant.

1.2.4 PHA-M:-

Studies connected with the dose response of PHA-M reveal that our dose response curve is similar to that of Tormey and Mueller (1965). It is difficult to compare the quantities used by different authors, since there is no fixed unit of blastogenic activity described, and it is misleading to assign a standard unit to a definite quantity of the PHA since nobody knows what is the blastogenic factor and it is unlikely that a fixed quantity of different preparations will contain a definite and fixed amount of the blastogenic principle. Tormey and Mueller (1965) described the assay for mitogenic activity and used lower levels of PHA-M compared to those used by other workers. They suggested the possibility of selective cell damage in the presence of high levels of PHA. Thus we used lower concentrations of PHA compared to other workers, but levels which stimulate the DNA synthesis optimally. The same levels of PHA-M have been reported by Mueller and Le Mahieu (1965) to be optimal for the induction of RNA synthesis.

Recently Robbins and Levis (1970) studied the effect of concentrations of PHA on the time course of DNA synthesis. They report that if a high concentration of PHA is used the peak of DNA synthesis can occur before 72h. However the peaks can occur after 72h if either the PHA-M concentration or cell concentration is reduced, leading to delayed peaks of DNA synthesis.

1.2.5 pH:-

Personal experience has shown that compared to Hela cells or L929 cells in tissue culture, lymphocytes are more sensitive to changes in pH. Paul (1960) has reported that the hydrogen ion concentration of the nutrient effects the metabolic rate. Whether screw capped tubes were used or silicone stoppered tubes, it was difficult to control the leakage of the gases, and this lead to unequalities in pH between various tubes. Gassing the cultures daily increased the rate of contamination, but use of Co_2 incubator was more satisfactory as it is a very simple method of stabilizing the pH.

Unlike Tormey & Mueller (1965), I processed the radioactive cells (i. e. extractions with TCA, ethanol and ether and solubilisation of acid insoluble precipitate) in the same culture tubes in which cells were grown, thereby cutting down the risk of loss of cells during transfer from one tube to the other.

1.3 Comparison between PHA-M and Pokeweed mitogen:-

One disadvantage with PHA stimulated cultures is the leukoagglutination, which makes it difficult to accurately count the cell number at varying times during the culture. On the otherhand pokeweed mitogen does not elicit such an effect. Though it is impossible at the moment, to compare the two on a weight for weight basis our results reveal that if a greater volume of PWM is added it produces an equivalent degree of blastogenesis in lymphocytes. However, PWM was not used in these studies because it is not known whether both the substances act by a similar mechanism. It has been reported that some of the blast cells obtained by stimulation with PWM differ from the blast cells obtained by PHA treatment. (Chessin, Borjesson, Welsh and Cooper - 1967).

1.4 Effect of Cyclic AMP on lymphocyte growth:-

Nowell (1961) suggested the probability that the action of PHA might be just changing the permeability of the cell membrane, so that some substances enter the cells and induce blastogenesis.

Whitfield, Rixon, Perris & Youdale have shown that in rat thymocytes Ca^{++} increase the mitotic activity of these cells.

If the action of PHA is just changing the permeability of the cell membrane then any other substance, which can alter the membrane permeability should bring about blastogenesis. Cyclic AMP has been shown to have effects on cell membrane permeability (Rasmussen et al 1968) and the dibutyryl cyclic AMP has been shown to be

active in many tissues where Cyclic AMP is not (Posternak, Sutherland & Henion - 1962). It may be that PHA exerts its effect by raising the level of Cyclic AMP in the cell membrane and indeed a stimulation of adenylcyclase has been reported within minutes of PHA addition.

Even though a wide concentration range was tested, we did not get any stimulation of growth in our system. In contrast to our results Cross & Ord (1970) working with the pig lymphocytes reported that Cyclic AMP at 10^{-6} - 10^{-8}M concentration brings about similar metabolic changes as that of PHA.

Smith, Steiner, Newberry and Parker (1969) studied the action of 10^{-4}M dibutyryl Cyclic AMP on PHA treated human lymphocytes. They report that 10^{-4}M dibutyryl Cyclic AMP inhibits the uptake of (^{3}H)-dThd, and the inhibition is more marked during the first hour of a 72h incubation. They did not note any inhibitory effect of DC AMP whenadded after 24h of stimulation.

Fig III-6c reveals that DC AMP in equine lymphocytes has no inhibitory effect on the growth measured at 72h of culture if it is added along with PHA. But, if it is added at a time period later than 24h of culture inhibition takes place and is more marked the later it is added during a 72h period. Probably like in other systems Cyclic AMP does take part in the regulation of cellular metabolism in PHA stimulated lymphocytes, and the level of this nucleotide is optimum in PHA treated cells. If exogeneous DC AMP is added to the cultures this raises the concentration above optimum in the cells causing inhibition of cellular growth. Thus the inhibitory effect is

more pronounced, in PHA stimulated cells when measured shortly after its addition, whereas at longer time periods the concentration of the nucleotide in the cells is maintained optimum either by the degradation of excessive quantities of the nucleotide or may be by some feedback mechanism regulating the rate of its synthesis. Thus PHA seems to act like a hormone, utilising cyclic AMP as a secondary messenger, and one of the action of PHA is to regulate the synthesis of intracellular cyclic AMP. Probably PHA increases the activity of adenylcyclase, thus increasing the intracellular concentration of the nucleotide. It seems that in unstimulated cells the activity of this enzyme is low because of the concentration of the nucleotide is low, in a tissue it may be either due to low concentration of adenylcyclase, the enzyme which converts the AMP to cyclic AMP, or due to the high concentration of the enzyme phosphodiesterase which hydrolyses cyclic AMP (Sutherland & Rall - 1958).

Adams (Personal communications) has shown that Theophylline which inhibits the enzyme phosphodiesterase, does not stimulate growth in unstimulated lymphocytes. This means that unstimulated equine lymphocytes, obtained from the peripheral blood do not have high adenyl cyclase activity and PHA induces this activity and regulates the intracellular cyclic AMP concentration. .

To summarise, we have standarised a suitable and convenient method for the isolation, purification, preservation and growth of equine lymphocytes, suitable for metabolic studies.

This system is not only parallel to the various tissue culture systems used for the studies of growth control mechanisms like cultures of L929 cells, Hela cells, rabbit kidney Cortex cells etc. but is more specific for studies connected with leukemias and other lymphocyte disorders. In addition, it provides a means for studies involved in understanding the metabolic patterns of lymphocyte growth, their immunological behaviour and significance of their presence and their role in an organism.

2. ACTION OF PHA ON EQUINE LYMPHOCYTES INVITRO, COMPARISON WITH LYMPHOCYTES FROM OTHER SPECIES:-

As far as response towards PHA is concerned, equine lymphocytes behave in many ways like human lymphocytes.

In both cases various metabolic and morphological changes take place when PHA is added e. g.

2. 1 Leukoagglutination ensues (Fig III-12)

2. 2 Acridine orange binding:-

Killander and Rigler (1959) have reported that on stimulation with PHA human lymphocytes show an increased capacity to bind the dye acridine orange. Fig III-12 reveals the changes in acridine binding capacity in equine lymphocytes.

2. 3 Autoradiographic studies:-

It has been reported that between 48-72h of culture blastoid cells appear. Fig III-12(f) show the marked increase in the size of cells on the 3rd day of PHA stimulation, autoradiographic studies reveal that more than 90% of the cells synthesize DNA on the 3rd day of culture. (Fig III-13 & Fig III-14)

2.4 DNA synthesis:-

Studies involving the incorporation of radioactive dThd reveal that DNA synthesis begins 24-30h after the addition of PHA (Fig III-7). Similar observation was made by Michalowski (1963) and MacKinney, Stohlman and Brecher (1962).

2.5 Protein Synthesis:-

Kay (1966) studying the effects of PHA on human lymphocyte cultures reported that an increase in the rate of protein synthesis as determined by (^{14}C)-leucine incorporation, takes place following PHA treatment. The same result has been obtained in equine lymphocytes (Fig III-42).

RNA synthesis:-

RNA synthesis measured by incorporation of (^{3}H)-uridine or ($^{32}P_i$)-orthophosphate (Fig III-32) again confirm that equine lymphocytes are not different from human (Kay - 1966). pig (Forsdyke - 1968) or bovine (Hausen et al, - 1969) lymphocytes in this respect.

2.6 DNA and Protein content:-

Hausen et al, (1969) determined the changes in DNA, RNA and protein content of bovine lymphocytes treated with or without PHA during the first thirty hour of culture. The initial DNA and protein content of equine lymphocytes is very similar to that in bovine lymphocytes. The difference in the results however is that, in our experiments the DNA content falls down during the first 30h of culture. Tullis (1953) reported the fall in cell

number during the 1st 2 days of preservation. On the other hand Hausen et al, do not report any such change. Bovine lymphocytes used in the experiments were derived from lymphnodes, while the equine lymphocytes were derived from the blood. It is a known fact that blood is comprised of a heterogeneous population of lymphocytes originating from different parts. Maybe some cells are of short half life which die thus decreasing the DNA content over the first 24 hour. This is further depicted in the decreased protein content during the first 24h of culture. The initial protein content of equine lymphocytes and bovine lymphocytes is similar.

2.7 Enzymes:-

As expected from the induction of DNA synthesis, various enzyme activities participating in the DNA metabolism are also increased. We have shown that Thymidine kinase, dTMP kinase, dTDP kinase and DNA polymerase activities are increased manifold in PHA treated cells compared to the untreated controls.

2.7.1 DNA polymerase:-

This finding is in accord with that of Loeb et al (1970) who have shown similar changes in human lymphocytes treated with PHA. The difference however is that Loeb et al, report a fall in DNA polymerase activity on the 4th day of culture, whereas in our system this enzyme activity rises steadily for 4 days. This can be explained by the fact that incorporation of dThd or ($^{32}P_i$)-orthophosphate into the DNA also rises continuously for four days in our system.

Depending on the initial leukocyte counts in the blood we get a 20-150 fold increase in the DNA polymerase activity. Like Loeb et al, (1968) and unlike Rabinowitz, McCluskey, Wong and Wilhite (1969) we could not demonstrate a significant increase in the DNA polymerase activity on the first day of culture. This again may be due to the difference in culture techniques and a reflection of different quantities of PHA used at different cell concentration. (Robins and Levis - 1970).

The additional features of our studies connected with the enzyme DNA polymerase are the following.

It has been reported in literature that the physical state of the DNA primer influences the DNA polymerase activity in a number of tissues. Mukundan, Devi and Sarkar (1963) have shown that high speed supernatant (HSS) fractions of regenerating rat liver contain an enzyme which is highly active with native DNA. In contrast we have shown that in PHA stimulated equine lymphocytes the DNA polymerase of high speed supernatant fraction shows 3-4 fold higher activity when denatured DNA is used as a primer (Fig III-25a). This result is in agreement with the finding of Yoneda and Bollum (1965) who were working with calf thymus gland. A similar result has been reported by Lindsay and Adams (1968) for the enzyme from mouse fibroblast cells. We have shown that the enzyme activity obtained from HSS fraction is unstable compared to the nuclear enzyme activity and is lost on preservation of extracts at

-20°C for a fortnight. Lindsay (1969) has shown that DNA stabilises the enzyme activity, thus the nuclear enzyme is more stable than the enzyme from HSS fraction.

Whereas Lindsay and Adams (1968) found a 3-4 fold preference for native DNA primer by the nuclear enzyme from L929 cells, nuclei from PHA stimulated lymphocytes do not show any preference for native DNA primer. The nuclear enzyme activity was more stable compared to the supernatant enzyme activity. There was no loss of activity, inspite of preserving it for 3 weeks at -20°C.

We compared Loeb's (1968) method of DNA polymerase assay to that of Keir and Shepherd (1965). The two methods differ from each other by virtue of the difference in the amount of various components of assay mixture and the pH at which the assay is performed. (cf.Methods section 2.11.i.). We have shown that if Keir's method with denatured DNA primer is used it is possible to detect the increase in the denatured DNA primed enzyme activity of the HSS fraction. We have also shown that even if crude cell homogenate is used as the source of enzyme, in the presence of denatured DNA primer the enzyme activity rises if Keir's method is used, whereas Loeb's method does not give such a result. Probably the assay mixture used by Keir has optimum conditions required to get maximum supernatant enzyme activity, thereby making it useful as a means of detecting both the nuclear and supernatant enzyme activity.

2.7.2 Thymidine kinase:-

Loeb, Agarwal and Ewald (1970) have shown that the activities of thymidine kinase and thymidine monophosphate kinase multiplies by 2-10 fold on stimulation of human lymphocytes with PHA. We did not assay the induction of the thymidine kinase activity invitro, but on the basis of intracellular nucleotide pools separated by paper chromatography (Fig III-22), our results show increased uptake and phosphorylation of thymidine on the 3rd day of culture in PHA treated cells compared to the unstimulated control cells. The phosphorylation of thymidine to dTMP invivo is increased 24 fold in stimulated cells compared to the unstimulated cells which gives a reflection of thymidine kinase activity.

2.8 RNA synthesis: -

Studies connected with various aspects of RNA metabolism again are in agreement with the findings reported in literature. Equine lymphocytes on treatment with PHA show an increased rate of RNA synthesis. Kay (1966) detected an increase in the rate of RNA synthesis in human lymphocytes within an hour after PHA treatment. But, we could not detect an increase in RNA synthesis in equine lymphocytes at least for 2h after PHA addition. Monjardino and MacGillivray on the otherhand report an increase in the RNA synthesis, which starts after 4h of incubation of human lymphocytes with PHA. The differences in these results are probably due to the use of different concentrations of PHA derived from different

sources. Monjardino and MacGillivray (1970) have shown the dependance of the content of total RNA upon PHA concentration.

Studies connected with the uptake and phosphorylation of uridine have given some information about the metabolism of uridine in these cells. Unstimulated lymphocytes do synthesize RNA and exogeneous supply of radioactive Urd to these cells results in the uptake, and phosphorylation of the nucleoside leading to its incorporation into RNA. On stimulation with PHA, the rate of uptake, phosphorylation and incorporation of Urd into RNA increases considerably. (Fig III-35).

The intracellular pool of pyrimidine phosphates in unstimulated and PHA stimulated lymphocytes differs considerably.

Our results (Fig III-35) show a major difference in the intracellular pyrimidine nucleotide pools. In unstimulated cells there is no accumulation of the phosphorylation products of uridine, whereas PHA stimulated cells are characterized by a big intracellular pool of uridine nucleotides. This phenomenon probably signifies some connection with the regulation of RNA synthesis in these cells.

In unstimulated lymphocytes control is probably exerted on the uptake of uridine, though thymidine kinase is present and is not the rate limiting step. The regulated uptake of uridine probably results in building of very small pool of uridine phosphates. This low concentration of uridine phosphate is probably stimulatory

towards cytidine synthetase, the enzyme which converts UTP to CTP. Thus as soon as the UTP is formed it is either utilized in RNA synthesis or removed by the cytidine synthetase and not allowed to accumulate in the cell. Lucas (1967) has shown that 10^{-4}M CTP inhibits the uridine kinase activity in an invitro assay. Thus the CTP pool in the cells probably regulates the uridine kinase activity.

On stimulation with PHA the uptake of uridine increases. The presence of high concentration of intracellular uridine may stimulate the uridine kinase and cytidine synthetase is then unable to cope with the removal of this high concentration of the uridine phosphates. This will lead to a build up of a pool of uridine nucleotides. Thus uridine itself stimulates the uridine kinase activity.

2.8.1 Sucrose gradient analyses of RNA:-

Sucrose gradient analyses of the RNA from unstimulated and PHA stimulated lymphocyte reveals that, the RNA synthesised by unstimulated cells is mainly of nonribosomal type, whereas stimulation with PHA results in the stimulation of the synthesis of all classes of RNA (Fig III-37). A major product is the low mol. wt. RNA sedimenting in the 4s region and the rRNA and its precursors. (Fig III-38, 39, 40). In addition (Fig III-40(b) in PHA stimulated equine lymphocytes some high mol. wt. RNA sedimenting in higher than 45s region is also found. These findings are similar to those reported by Monjardino and MacGillivray (1970) in human lymphocytes.

3. INHIBITION BY 5-AZACYD OF PHA INDUCED STIMULATION AND GROWTH IN EQUINE LYMPHOCYTES:-

5-azaCytidine inhibits the stimulation and growth of lymphocytes by PHA. If the cells are treated with the drug at the beginning of the culture, further events in the stimulation and growth do not take place.

This situation seems similar to that described by Cihak et al, (1967) where administration of the drug to rats hormonally induced to synthesize tryptophan pyrrolase, results in the inhibition of the induction of enzyme activity.

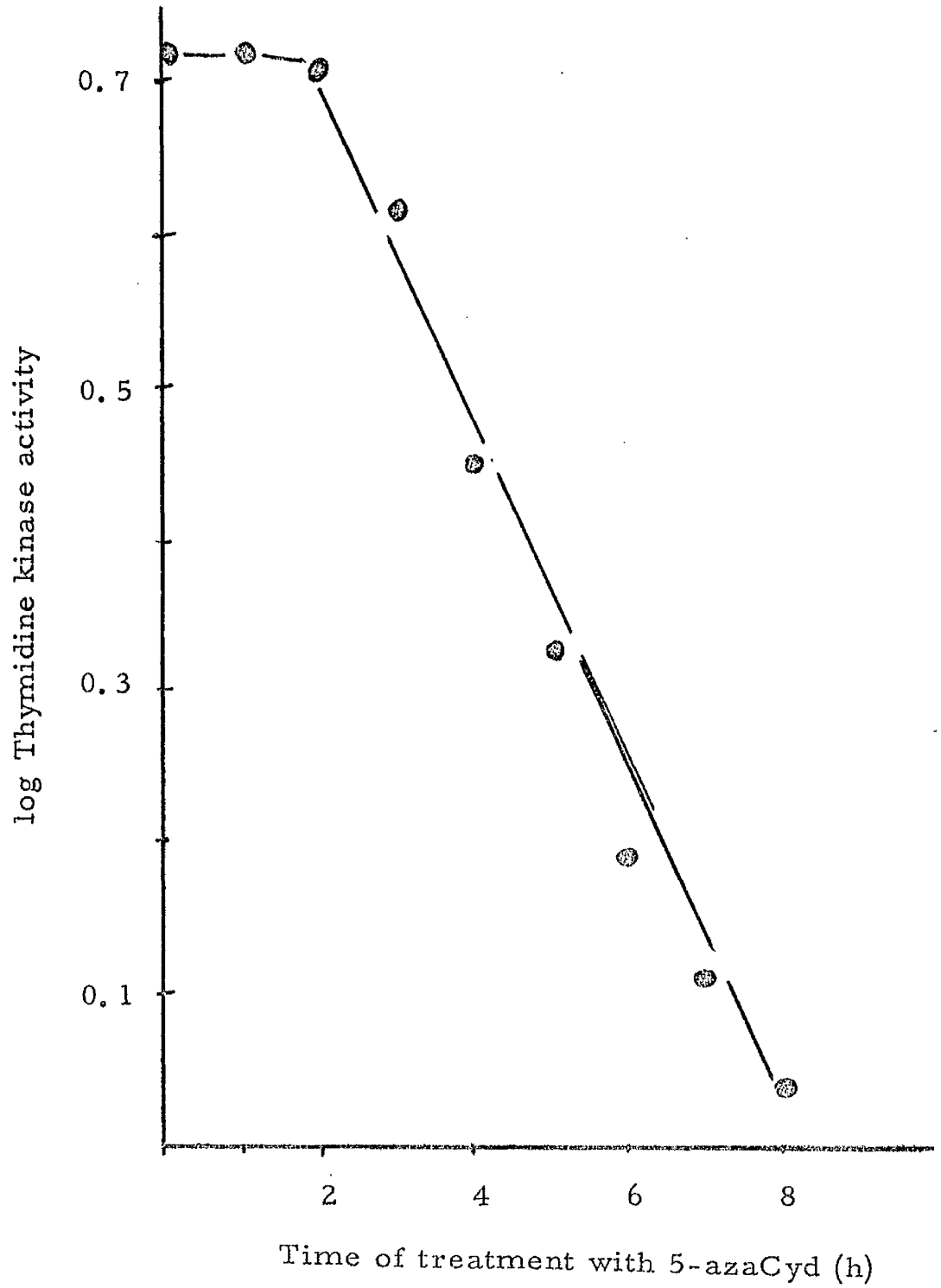
On the otherhand, if the lymphocytes are treated with the drug when they are actively engaged in DNA synthesis, the rate of DNA synthesis falls slowly after an hour's lag and inhibition is complete in about 7h. Thus the site of 5-azaCyd action for the growth inhibition in equine lymphocytes is not the inhibition of DNA synthesis. On testing the action of the drug on thymidine kinase and DNA polymerase in an invitro assay, the activity of these enzymes does not seem to be inhibited by the drug even at a hundred fold higher concentration to that used in the cultures. Thus inhibition of DNA synthesis is not a result of inhibition of the enzyme activity, but results from the inhibition of the synthesis of these enzymes. The rate limiting step in the incorporation of radioactive d'Thd into DNA is the enzyme thymidine kinase. If we calculate the total thymidine phosphorylated, we can take this as a reflection of thymidine kinase activity. However, this may not be a true picture of the enzyme activity since control is exerted on the enzyme activities invivo, and especially on the levels of nucleotide synthesis. (Davidson- 1969).

FIV IV - 1

The experiment is the same as described in Fig III-21.

The sum of d. p. m. incorporated into acid soluble and acid insoluble fractions is taken as a measure of thymidine kinase activity.

Fig IV - 1


0.7
0.5
0.3
0.1
log Thymidine kinase activity
2
4
6
8
Time of treatment with 5-azaCyd (h)

Thus the enzyme activity may be lower than the enzyme activity assayed in an invitro assay. However, on plotting the results from Fig III -21 in a different way, Fig IV-I shows that after a lag of one hour, the thymidine kinase activity falls exponentially and the half life of the enzyme is about $2\frac{1}{2}$h.

Hartwell, Vogt & Dulbecco (1965) describe a similar situation in Mouse kidney cells infected with Polyoma virus. On treatment with puromycin, an inhibitor of protein synthesis (4h pulse, 16-20h after infection), the thymidine kinase activity decays rapidly, but DNA polymerase activity does not, suggesting the thymidine kinase to be an unstable enzyme. This agrees with our results. The site of 5-azacyd action is not the inhibition of DNA synthesis per se but inhibition of thymidine kinase (Protein) synthesis.

We have shown that protein synthesis is inhibited by 5-azaCyd within ninety minutes of its addition to the culture. A similar observation was made by Raska et al (1966) in the isolated nuclei of calf thymus, where the protein synthesis was measured as incorporation of ^{14}C leucine into proteins.

5-azaCyd does inhibit the total RNA synthesis in equine lymphocytes, measured as the extent of (^{3}H)-Urd or ($^{32}P_i$)-orthophosphate incorporation. However, RNA synthesis is not inhibited for at least more than 2 hours after drug addition (Fig - 34) and is probably inhibited as a result of the inhibition of protein synthesis. The inhibition of RNA synthesis is not a result of inhibition of the enzymes RNA polymerase or uridine kinase. Studies connected with uptake and phosphorylation of uridine reveal that these processes are not effected drastically for at least $2\frac{1}{2}$h, after treating the

cells with the drug (Fig III - 35

In an invitro assay conducted to test the effect of 5-azaCyd on RNA polymerase activity, it was found that even at a 10 fold higher concentration of 5-azaCyd (to that used in the culture) the drug does not inhibit the RNA polymerase activity. One possibility is that 5-azaCyd-5-po_4 is probably responsible for inhibition and 5-azaCyd does not exert any effect whatsoever. However, in the assay system we used, there are possibilities of 5-azaCyd-5-po_4 formation, since the source of enzyme used is a crude lymphocyte homogenate. Thus RNA polymerase is not inhibited by 5-azaCyd invitro (Fig III-36) and it is the inhibition of the enzyme synthesis rather than the inhibition of already made enzyme, which is the rate limiting step.

What is the causative factor for the inhibition of protein synthesis is not clearly understood. Sucrose gradient analysis of the lymphocyte RNA reveals that rRNA synthesis is not inhibited for 2h, but that synthesis of low. mol. wt. RNA (sedimenting in the 4S region) is inhibited within an hour.

Kalousek et al (1966) showed that the acceptor activity for different aminoacids is inhibited in tRNA isolated from 5-azaCyd treated mice, and one of the most probable modes of action of 5-azaCyd depends on its incorporation into tRNA (see later).

3.1 Mechanism of 5-azaCyd action in equine lymphocytes:

The various mechanisms of 5-azaCyd action reported so far are:

1. Competition of the drug with Cyd or Urd for the kinases:-

   Raska et al (1966) suggested that the drug competes with the normal nucleosides for the kinases involved

in the biosynthesis of nucleic acids.

This mode of action does not seem to be the major factor causing growth inhibition within hours. The pool of phosphorylated bases (Uridine and Cytidine) does not seem to be effected for $2\frac{1}{2}$h, whereas inhibition of protein synthesis ensues within 90 minutes.

2. Inhibition of denovo pyrimidine synthesis:-

It has been reported that 5-azaCyd-5-phosphate inhibits the enzyme orotidylic acid decarboxylase, thus inhibiting the denovo pyrimidine synthesis.

We have shown that the intracellular pool of uridylate and cytidylate is not effected for at least $2\frac{1}{2}$hours. If the inhibition of cellular growth is by virtue of inhibition of this enzyme, then total RNA synthesis should be inhibited before protein synthesis is being inhibited. If pyrimidine nucleotides are the rate limiting factors then addition of Urd or Cyd no matter at what time it is added should reverse this effect, but the inhibition cannot be reversed by Cyd or Urd after 80 minutes of drug treatment. (Fig III-31).

3. Incorporation into DNA:-

Incorporation of 5-azaCyd does not seem to be the responsible factor in the inhibition of lymphocyte growth. Lymphocytes do not synthesize DNA for at least 25-30h after PHA addition. But the RNA and protein synthesis is inhibited if the PHA stimulated cells are treated with 5-azaCyd during the

first 30h of culture. Protein synthesis is inhibited in cultures of unstimulated lymphocytes where the cells do not synthesize DNA.

4. Incorporation into RNA:-

As far as the action of 5-azaCyd on equine lymphocyte is concerned, our data favours this mode of 5-azaCyd action.

Our data suggests that 5-azaCyd inhibits the stimulation and growth of equine lymphocytes, by virtue of getting bound into the cells and probably into RNA.

Results from the experiments conducted to see the reversal of growth inhibition produced by Cyd or Urd suggest that the drug is getting incorporated into RNA and then exhibiting its inhibitory effects.

If the cells are treated with the drug before the addition of Cyd or Urd, the damage produced cannot be reversed by Cyd or Urd even at 100 fold higher concentrations of Cyd or Urd.

On the otherhand simultaneous treatment with Cyd or Urd helps in reversing the growth inhibition studied as late as 72h after treatment. Though we could not obtain radioactive 5-azaCyd to show its incorporation we have no reason to doubt the results of Sorm and coworkers, who have shown the incorporation of radioactive 5-azaCyd into various species of RNA.

Our data gives support to the view that in equine lymphocytes the primary effect of 5-azacytidine action is the inhibition of protein synthesis, which takes place after

a lag of $1\frac{1}{2}$h. Fig III-31 reveals that 80 minutes treatment with 4×10^{-5} M 5-azaCyd is sufficient to bring about an irreversible growth inhibition to 30% of the control measured after 72h treatment with the drug. Treatment for shorter times results in lesser inhibition of growth.

4.1 Inhibition via mRNA:-

The question is how is the drug after incorporation into RNA, able to inhibit growth, and which species of RNA is responsible in bringing about such an effect.

According to the view held so far by various authors everyone believes that incorporation into mRNA is responsible for the inhibitory effects of 5-azaCyd. Paeces et al, (1968) studying the kinetics of 5-azaCyd incorporation into DNA and various species of RNA reported that when 5-azaCyd is present during the synthesis of mRNA the synthesis of B galactosidase is completely inhibited, whereas if messenger formed before its addition is utilized, little inhibition is observed.

The most widely accepted view is that 5-azaCyd is incorporated into mRNA and produces faulty messengers, which results in disturbances in the cellular metabolism.

Levitan and Webb (1969) reported that 5-azaCyd inhibits the hormonal induction of tryptophan pyrrolase, but not that of tyrosine transaminase. This evidence fits well with the concept of incorporation of the drug into mRNA, thus effecting the production of healthy proteins at the translation level. i. e. the coding property of mRNA is altered, depending on the position of 5-azaCyd in the mRNA. In the same animal some proteins are effected while the

others are not.

Cihak and Vesely (1968) reported that 5-azaCytidine causes a massive breakdown of polysomes into monomers and dimers in the regenerating rat liver. Levitan and Webb (1969) observed a similar effect in intact livers of adrenalectomized rats.

8-azaguanine has been reported to behave in a similar manner (Webb - 1967, Kwan and Webb - 1967), and it has been suggested that this drug also acts by virtue of its incorporation into mRNA. The same concept of mRNA involvement is mentioned by Raska et al (1966). They noted the inhibition of protein synthesis in nuclei of calf thymus and suggest the probability of faulty mRNA formation. They refer to Allfrey and Mirsky's (1963) finding that inhibition of mRNA synthesis following 60-90 minutes of treatment with actinomycin-D results in the inhibition of protein synthesis in the isolated nuclei.

The situation seems to be different in the case of lymphocytes. If the drug is getting incorporated in mRNA, no matter at what time period during the culture 5-azaCyd is added, it is active. This implies that the drug is acting on a mRNA which is continuously synthesized throughout the culture period. Yet, were this so, then why would 90 minutes treatment at any time lead to complete inhibition of growth?

If the drug is acting via mRNA then at a time when many new mRNAs are being synthesized a greater inhibition would be expected.

However, this is found not to be so, unstimulated lymphocytes are much less active in producing new messengers and should therefore be less susceptible to the

drug. Fig III-43 shows that the rate of protein synthesis is effected equally both in the stimulated as well as unstimulated cells.

Wilson and Hoagland (1967) have shown that at least a part of mRNA is relatively stable. The half life of mRNA in rat liver has been shown to be 3-3½h for the labile portion, which comprises ⅔ of the total mRNA, while the other ⅓ is stable with a half life of at least 80h. Since inhibition of protein synthesis is taking place within 1h of drug treatment, it seems unlikely that synthesis of faulty mRNA could be the major factor responsible.

4.2 Involvement of rRNA:-

On the basis of breakdown of polysomes in the regenerating rat liver Levitan and Webb (1969) suggested that incorporation of 5-azaCyd into rRNA interferes with the binding and/or synthesis of ribosomal particles. The structure of the drug reveals that it cannot be methylated in the 5th position, as instead of a $-\overset{|}{\underset{|}{C}}-$atom a $-\overset{|}{N}-$atom is present. This might change the affinity of the molecule or the presence of a methyl group on the cytidine moiety is probably essential for the aggregation of ribosomal particles.

However, this lack of methylation of rRNA does not seem to be responsible for inhibition of protein synthesis. The synthesis of rRNA does not seem to be effected for 2 hours, while protein synthesis is inhibited within 90 minutes moreover, little rRNA appears in the cytoplasm during the first 2h. It is difficult to see how a small proportion of defective ribosomes could so effectively disrupt protein synthesis.

Production of ribosomes is not the rate limiting step, since ribosomes have a long life span, the average half life of an adult liver ribosome is 4-6 days (Loeb, Howell and Tomkins - 1965).

How many ribosomes containing 5-azaCyd can be transported to cytoplasm in an hour? and how this small amount of faultyribosomes be fatal for the cells is difficult to consider. Thus production of faulty ribosomes does not seem to be responsible for the inhibition of protein synthesis. However one experiment to exclude this possibility would be to check the efficiency of the ribosomes isolated from 5-azaCyd treated and control lymphocytes in an invitro protein synthesizing system. Using the pH 5.0 enzyme and tRNA from rat liver, and employing the synthetic poly U as the exogeneous mRNA the efficiency of the polysomes can be easily checked.

4.3 Possible mode of 5-azaCyd action via tRNA:-

Another species of RNA involved in protein synthesis and worth mentioning is tRNA. The involvement of tRNA in the regulation of growth has been considered for quite some time. This implies that growth control is exerted at the translation level. If 5-azaCytidine is inhibiting the stimulation of lymphocyte growth by virtue of its incorporation into pre tRNA, whose synthesis and maturation has been reported to be increased in the presence of PHA (Kay and Cooper - 1969), there is a possibility to think that maturation of tRNA is altered in the presence of 5-azaCyd, as the 5-azaCyd molecules cannot be methylated. In Hela and BHK cells deprivation of L-Valine, an essential aminoacid results in reduced RNA

and protein synthesis, whereas methionine starvation seems to have deleterious effects (Burdon - 1971).

Our results FigIII38&39reveal that5-azaCyd does inhibit the 4S cytoplasmic RNA synthesis in an hour and two. The lack of inhibition after 4h treatment with the drug can be explained by considering that probably rRNA containing 5-azaCyd is less stable and degrades to give rise to low mol. wt. species which sediment in the 4S region. tRNA is synthesized both in the resting and PHA stimulated lymphocytes. Monjardino and MacGillivray (1970) and Kay and Cooper (1969) have shown that resting lymphocytes do synthesize RNA which sediments in the 4S region on sucrose density gradient analysis.

tRNA has shown to be relatively stable, and the recent data of Blobel and Potter (1968) indicates the half life of rat liver tRNA to be 90h. Unless the continuous production of tRNA is essential for cellular growth it is difficult to visualise how the inhibition of tRNA synthesis, can result in the inhibition of protein synthesis so quickly.

5-azaCyd may be incorporated either internally into tRNA molecule or into the pCpCpA 3-hydroxyl terminal tri nucleotide of tRNA.

4. 3. 1 Internal incorporation:-

Incorporation of 5-azaCyd internally into tRNA molecule in place of cytidine may have deleterious effect for several reasons.

Because of the different charge distributions it may:

a) Change the nature of the anticodon

Kalousek et al, (1966) showed that the aminoacid acceptor activity of tRNA containing 5-azaCyd (isolated from mice after injecting the radioactive drug) is reduced in an invitro assay, whereas they could get no evidence of anticodon damage after the incorporation of the drug into tRNA.

b) Affect on the secondary structure of tRNA:-

The substitution of aza analogue probably alters the secondary and tertiary structure of the tRNA molecule. Burdon (1971) has recently suggested the possibility of the role of alterations in secondary and tertiary structure of tRNA molecules on the control of protein synthesis.

c) Preclude proper maturation or function of the tRNA by changing its affinity for the enzyme:-

Probably substitution of 5-azaCyd for Cyd results in loss of specificity of the tRNA molecule and the specific aminoacyl tRNA synthetases are unable to select the specific tRNA molecules for aminoacylation, leading to the loss of aminoacid acceptor activity.

Because of the presence of a -$\overset{1}{N}$- in the 5th position of Cytidine ring, methylation cannot occur. Phillips (1969) proposed a generalised structure for tRNAs. He has shown the presence of a methylcytosine molecule in position 48 (ref Fig I - Phillips 1969). near the pentanucleotideloop. If the presence of this

methyl group is essential for maintainance of the structure of tRNA molecule, then lack of ^{a}-CH_3 group probably results in lack of activity of the molecule.

However, the average half life of tRNA molecule is 90h (Blobel & Potter - 1968) and thus in 1h treatment with 5-azaCyd only about 1% of the tRNA molecules will be defective. Unless the continuous production of tRNA molecules is vital for protein synthesis it is difficult to see how such a small portion of defective molecules could so drastically affect protein synthesis. The possibility however does exist that one essential species of tRNA has a very much shorter half life than average, and that a large proportion of this tRNA is rendered inadequate by short treatment with 5-azaCyd.

4. 3. 2 Terminal Incorporation:

Compared to the whole tRNA molecule, the 3 hydroxyl terminal pCpCpA trinucleotide of tRNA turns over very rapidly. Scholtissek[1962] has shown that the terminal adenosine of rat liver tRNA has a rapid turnover invivo. Merits[1965] has shown that cytidylic acids adjacent to the terminal A display a slight but measurable turnover. Thus within an hour, in the presence of saturating levels of 5-azaCyd the terminal sequences of tRNA will contain the irregular base. This may lead to rapid inhibition of protein synthesis for reasons discussed below.

However, if the 3-hydroxyl terminal pCpCpA trinucleotide continues to turnover rapidly even when

some of the Cytidine molecules are replaced by 5-azaCytidines, then we should expect a rapid rapid reversal of inhibition on removal of the drug or on addition of excess cytidine. This we do not find, suggesting that if this is the initial site of action of 5-azaCytidine, that substitution of Cyd by 5-azaCyd results in a stabilisation of the pCpCpA end group of tRNA, which results in irreversible inhibition of protein synthesis. Berg (1968) has the important and involvement of the turnover of pCpCpA end in protein synthesis.

This can be easily tested, simply by labelling the œlls with ($^{32}P_i$)-orthophosphate for a short time ($\frac{1}{2}$h) in the presence or absence of 5-azaCyd. Washing of the cells and studying the amount of label present in tRNA during the following few hours (3h) in the presence of excess of unlabelled phosphate.

If the pCpCpA sequence is not stablised, then the label in the RNA should decrease as the turnover of the group is fast.

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SUMMARY

Biological effects of 5-azaCytidine on PHA stimulated equine lymphocytes

by B. Sayeeda Zain

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PHA stimulated lymphocytes offer a convenient system for the study of growth inhibition, cellular proliferation and cell differentiation. Because of the scarcity of human blood for experimental purposes, horse blood was utilised as a substitute.

A standard method of culture and growth of equine lymphocytes invitro has been developed. In addition, studies connected with the isolation, purification and preservation of equine lymphocytes have been made.

To understand the basic metabolic pattern of lymphocytes biochemical studies were carried out. It has been shown that on stimulation with PHA, horse lymphocytes, in many ways behave like human lymphocytes both morphologically and metabolically.

On incubation with PHA, changes in the nuclear chromatin take place, and increase in the rate of proteins, RNA synthesis ensues. The quiscent cells start synthesizing DNA after 28-30h of incubation with PHA. The activities of the enzymes involved in the synthesis of RNA and DNA is increased.

The uptake and phosphorylation of dThd and Urd is increased, which in turn reflects an increased thymidine kinase and uridine kinase activity. The DNA polymerase activity is increased by 50-150 fold.

The DNA polymerase activity in the high speed supernatant fraction prefers the denatured DNA primer, while the nuclear enzyme has no specific requirements. The supernatant enzyme is less stable compared to the nuclear enzyme.

Uridine has a stimulatory effect on RNA synthesis in PHA stimulated equine lymphocytes. An increased rate of RNA synthesis starts within 4h of PHA addition, and unlike in unstimulated cells rRNA accumulates.

In an attempt to elucidate the mechanism of PHA stimulation cyclic AMP was used. Our results reveal the possibility of PHA acting as a hormone, utilizing cyclic AMP as the secondary messenger.

In order to understand the growth control mechanisms in these cells 5-azaCytidine was used.

It has been shown that 5-azaCyd inhibits the growth of these cells by virtue of getting incorporated into RNA and inhibiting the protein synthesis. If 5-azaCyd is added to the cultures for a short time and then removed, the growth does not resume after drug removal upto at least 50h.

Addition of Cyd or Urd (at a concentration 10-100 fold higher than 5-azaCyd) simultaneously with or before 5-azaCyd treatment reverses the inhibitory affect of the drug; on the other hand addition of Cyd or Urd, 80 minutes after 5-azaCyd addition is of no use in reversing the growth inhibition.

We have shown that the primary site of 5-azaCyd action is not the DNA metabolism, as DNA synthesis is inhibited slowly after a lag of an hour and is complete in 6-8 hours. The uptake and phosphorylation of radioactive dThd is not affected, for 2 hours, measured by looking at the intracellular pool of radioactive dThd and its phosphorylation products

To avoid the possibility of dThd kinase involvement, ($^{32}P_i$)-orthophosphate label was used but the same results were obtained.

5-azaCyd does not inhibit the enzyme DNA polymerase invitro but it does inhibit the induction of the enzyme invivo. Thus basing on this data, we believed that 5-azaCyd does not primarily affect DNA synthesis, but the DNA synthesis is inhibited because of inhibition of protein synthesis.

Studies connected with RNA metabolism revealed that 2h treatment with 5-azaCyd inhibits the total RNA synthesis in equine lymphocytes.

We have shown that the uptake and phosphorylation of Urd is not affected for $2\frac{1}{2}$h. The enzyme RNA polymerase is not inhibited invitro, but total RNA synthesis is inhibited within 3-4h. Treatment of the cells with the drug simultaneously with PHA for 2 hours at the beginning of the culture, washing off the drug and reincubation of the cells with fresh medium containing PHA inhibits the expected induction of rRNA synthesis.

Fractionation of nuclear and cytoplasmic RNA from cells treated with the drug for 1, 2 and 4h on sucrose gradients reveal that neither the rRNA precursors, nor the rRNA itself is inhibited during the first 2h of treatment. By 4h all the species of RNA are inhibited. The cytoplasmic RNA sedimenting in the 4s region shows inhibition during the first two hourse of treatment, but there is no inhibition at 4th h.

We have shown that 5-azaCyd also inhibits the protein synthesis in unstimulated lymphocytes.

The mechanism of 5-azaCyd action on the inhibition of protein synthesis, after getting incorporated into RNA is discussed. The possibility of the involvement of tRNA rather than the mRNA is discussed in detail.