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LYMPHOCYTE ACTIVATION AND POST-SYNTHETIC
MODIFICATION OF MACROMOLECULES

by

ROCHELLE KAREN SEIDE-KEHOE

A dissertation submitted to the Graduate Faculty
in Biomedical Sciences in partial fulfillment
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1977

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ABSTRACT

LYMPHOCYTE ACTIVATION AND POST-SYNTHETIC
MODIFICATION OF MACROMOLECULES

by

Rochelle Karen Seide-Kehoe

Advisor: Dr. Kurt Hirschhorn

This study was concerned with the effect of ethionine on the biochemical events associated with mitogen induced activation of lymphocytes. This compound allows the progression of stimulated lymphocytes from the G_0 to the G_1 phases of the cell cycle but totally blocks the entry of such cells into the S phase. The inhibition of the initiation of DNA synthesis due to ethionine is totally reversible since DNA replication commences within several hours of the removal of ethionine.

The experiments thus indicated that the commitment of lymphocytes to DNA synthesis was not affected by the presence of ethionine in the culture medium. The early reactions associated with the initiation of DNA synthesis do take place in the presence of ethionine. The rates of both RNA and protein synthesis in cells stimulated in the presence of ethionine were higher than those seen in unstimulated cells, but lower than the rates observed in cells

stimulated with mitogen alone.

The studies further showed that the various types of RNA were synthesized and methylated in the presence of ethionine, although at slower rates than those observed in cells stimulated under normal conditions.

When actinomycin D was present in the culture, but ethionine absent, the stimulated cells showed extensive late methylation of 4S RNA even though virtually no new RNA synthesis could be detected. However, when such actinomycin D exposed cells had been pre-treated with ethionine, and the ethionine then removed, an even greater degree of methylation was observed suggesting that the presence of ethionine had inhibited the usual degree of methylation of the tRNA synthesized previously. Upon the removal of ethionine, such RNA is rapidly methylated.

No evidence could be obtained for either the incorporation of ethionine itself into newly synthesized proteins or the ethylation of macromolecules under any of the conditions studied. Also, no depletion of the cellular ATP levels was seen in the lymphocytes in the presence of ethionine.

Morphologically, as determined by light and electron microscopy, there was no difference between cells activated by PHA in the presence or absence of ethionine.

In sum, the results of these experiments suggest an important role for the participation of RNA methylation, or methylation of other macromolecules, in the initiation of DNA synthesis due to mitogen activation.

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LIST OF ABBREVIATIONS

deoxyribonucleic acid	DNA
ribonucleic acid	RNA
transfer RNA	tRNA
ribosomal RNA	rRNA
heterogeneous nuclear RNA	hnRNA
messenger RNA	mRNA
phytohemagglutinin	PHA
concanavalin A	Con A
pokeweed mitogen	PWM
cyclic 3', 5' adenosine monophosphate	cAMP
cyclic 3', 5' guanosine monophosphate	cGMP
immunoglobulin M	IgM
adenosine diphosphate	ADP
adenosine triphosphate	ATP
absorbance	A
Minimal Essential Medium	MEM
trichloroacetic acid	TCA
sodium dodecyl sulfate	SDS
counts per minute	CPM

INTRODUCTION

Lymphocyte activation by mitogens can be used as a model system for studying the biochemical events involved in the initiation of DNA synthesis in eukaryotic cells. A great variety of metabolic reactions occur as a consequence of mitogen binding to the surface of lymphocytes, including increased rates of synthesis of RNA and proteins, rapid changes in the modification of macromolecules, and increased activity of many enzymes involved in these biosynthetic pathways. These biochemical changes are accompanied by morphologic alterations indicative of lymphocyte blast formation. This well characterized sequence of metabolic changes leads to the onset of DNA synthesis and cell division.

On the basis of the observation that ethionine, the ethyl analog of methionine, reversibly inhibits the initiation of DNA synthesis in activated lymphocytes, several of the proposed mechanisms by which ethionine could act on the array of metabolic reactions in activated lymphocytes leading to the onset of DNA synthesis were studied. These studies were undertaken in order to gain a deeper insight into the process of lymphocyte activation as well as to gain a deeper insight into the mechanisms through which ethionine can provoke a wide variety of biological consequences.

BACKGROUND: LYMPHOCYTE ACTIVATION

Numerous studies of the morphological and biochemical events involved in cell division have been performed since Nowell discovered that phytohemagglutinin (PHA), an extract from kidney beans, could stimulate peripheral blood leukocytes to divide (Nowell, 1960). Subsequent studies showed that the dividing cells were lymphocytes (Hastings et al., 1961; Carstairs, 1961). The early terminology for this phenomenon was lymphocyte transformation, but it is perhaps more accurately termed lymphocyte activation or stimulation.

It is customary to classify the events of lymphocyte activation into several categories: mitogen binding and associated cell surface alterations; biochemical reactions subsequent to surface changes but preceding the initiation of DNA synthesis; and DNA synthesis and cell division.

I. Early Biochemical Events in Lymphocyte Activation

A. Chemical Alterations in the Cell Surface due to Mitogen Binding

The events leading to DNA synthesis in stimulated lymphocytes are initiated by the binding of the mitogen to a receptor on the cell surface (Wigzell, 1970; Möller, 1970). Many lymphocyte mitogens are lectins, proteins that bind sugars, isolated from plants, e.g. phytohemagglutinin (PHA), pokeweed mitogen (PWM), and concanavalin A (Con A). It is generally believed that the receptors to which these mitogens bind are glycoproteins located at the cell surface

(Kornfeld and Kornfeld, 1969). The initiation of lymphocyte activation by lectins is most likely due to their ability to cross-link and aggregate surface receptors with ensuing alterations in membrane fluidity (Cunningham et al., 1974).

This membrane perturbation in some way signals the series of events culminating in cell division to begin. There are mitogens, other than lectins, that stimulate lymphocytes by aggregating surface receptors. This category includes antibodies to cell surface components and antigens that are bound by cell surface immunoglobulins.

There are other membrane active agents that induce lymphocyte activation by means different from receptor cross-linkage. These include enzymes and chemicals that oxidize sugars in cell surface glycoproteins and form free aldehyde groups (O'Brien and Parker, 1976).

Still another group of inducing agents, including calcium ionophore A23187 and cGMP (Maino et al., 1974; Weinstein et al., 1974), do not cause membrane alterations and most likely enter the series of events in lymphocyte activation at a stage later than the surface acting agents. It is assumed, however, that the sequence of biochemical events leading to cell division is similar regardless of the stimulating agent.

The most dramatic chemical changes in the membrane include an increase in the rates of (1) phospholipid and (2) glycoprotein synthesis.

1. Phospholipids. As early as 30 minutes after PHA

stimulation, a significant increase in the turnover rate of cell membrane phospholipid components can be detected. After several hours net increases in the phospholipid content of cell membranes can be measured. These findings are in agreement with the fact that the small lymphocyte greatly enlarges to become a blast cell. Since synthesis of RNA and protein is not necessary for the increase in phospholipid synthesis, it is assumed that the necessary enzymes are present in the resting lymphocyte (Fisher and Mueller, 1968; Kay, 1969).

2. Glycoproteins. Membrane glycoprotein synthesis measured by an increase in radioactive glucosamine incorporation into newly synthesized proteins increases within a few hours after mitogen stimulation (Hayden et al., 1970). In contrast to phospholipid synthesis, new protein synthesis is necessary for glucosamine incorporation. It is not clear, however, whether the new protein synthesis is of glycosyl transferases, the glycoproteins themselves, or both.

B. Functional Alterations in the Membrane Subsequent to Mitogen Binding

Following mitogen stimulation, there is an increased influx of macromolecules and small molecules into the lymphocytes. This influx can be partially explained by increased pinocytotic activity (Hirschhorn et al., 1968) and alterations in transport mechanisms (Quastel and Kaplan 1970, 1971).

Increased rates of potassium, calcium, sugar, amino acid, and nucleoside uptake have been observed. Analysis of the kinetics of potassium transport led to the conclusion that enhanced movement of the ions across the membrane was due to an increase in the available transport sites rather than to an alteration in the activity of individual sites. Furthermore, this effect is also independent of new RNA and protein synthesis and it has been proposed that binding of mitogen to receptors on the cell surface causes conformational changes which expose previously hidden transport sites (Quastel et al., 1970).

The importance of this increased transport has been demonstrated by experiments showing that ouabain inhibition of potassium transport or depletion of calcium from the culture medium (Parker, 1976) resulted in diminished DNA synthesis by stimulated lymphocytes.

C. Cyclic Nucleotides

Following mitogen binding, an increase in cAMP levels occurs (Smith et al., 1971). Both stimulatory and antagonistic roles for cAMP in lymphocyte activation have been proposed. Agents such as epinephrin, prostaglandin E, and cholera toxin which increase cAMP levels by stimulating adenyl cyclase, and others, such as theophylline, which act to increase cAMP levels by preventing its degradation by phosphodiesterase inhibit lectin induced DNA synthesis in lymphocytes. This implies that high, sustained increases

in cAMP levels can, in some way, antagonize the mitogenic response.

It appears, though, that cAMP can also have a stimulatory effect. The earliest metabolic change detectable in lectin stimulated lymphocytes is an approximately two fold increase in cAMP levels. This increase reaches a maximum at 5-15 minutes and persists for up to two hours (Smith et al., 1971). The interesting finding that increases in cAMP produced by lectins and other cAMP stimulators are additive implies that they act on different adenyl cyclases and may explain the apparent contradictory effects of cAMP (Parker et al., 1974).

Further substantiation of a role for cAMP comes from studies of cAMP dependent protein phosphorylation via protein kinases. Stimulation of lymphocytes with various types of mitogens leads to phosphorylation of proteins with a high molecular weight range, whereas non-mitogenic cAMP stimulatory agents induce phosphorylation of proteins with a lower molecular weight range (Parker, 1976).

It has recently been shown that two isozymes of cAMP dependent protein kinase, termed R_{IC} and R_{IIC} , exist and may have separate cellular functions (Rangel-Aldao and Rosen, 1976; Schubart and Rosen, 1976). Activation of lymphocytes by mitogen leads to an increase in type I kinase activity whereas agents that increase cAMP, but inhibit mitogenesis, activate type II kinase activity. (Byus et al., 1977).

It has also been reported by Hadden and coworkers (Hadden et al., 1974) that cGMP levels increase to a larger extent than cAMP levels in the same population of stimulated lymphocytes. The function of increased cGMP levels is unclear, however, and much of the data involving it are controversial (Watson, 1974).

II. Morphological Changes

Resting lymphocytes are small cells approximately 8-10 μ in diameter, with a highly condensed nucleus and meager cytoplasm. Electron microscopic studies have revealed cells with peripherally condensed heterochromatin in the nucleus and a small nucleolus. In addition the cells contain few ribosomes, existing primarily as monosomes, mitochondria, and a poorly developed Golgi apparatus.

Following the addition of mitogens to lymphocytes, numerous morphologic changes have been observed (Douglas, 1972). Under light microscopy, progressive increases in cell size, cytoplasmic basophilia, and number of lysosomal and lipid vacuoles have been seen. Mitotic figures can first be seen approximately 40 hours after PHA addition and reaching a peak at 72 hours (Ling, 1968).

Progressive cellular enlargement occurs following PHA stimulation. As early as four hours after stimulation, morphologic changes occur in the nucleolus consisting mainly of an increase in the number of nucleolar granules and an increase in size (Tokuyasa et al., 1968). These changes re-

late to the increased rate of RNA synthesis that occurs following mitogen stimulation. In addition, there is a progressive reduction in the amount of nuclear heterochromatin during a 48-72 hour period until virtually no heterochromatin remains (Cooper 1973).

Killander and Rigler (1969) have reported an increase in the binding of acridine orange to the DNA of fixed lymphocytes that occurs following lymphocyte stimulation. The amount of acridine orange binding increased three fold within the first 10 minutes after mitogen binding and rose gradually until 60 minutes to a plateau value that remained constant for 12-18 hours. The change was attributable to increased accessibility of the DNA phosphate groups to the dye. However, increased acridine orange binding was also demonstrated in polymorphonuclear leukocytes after PHA treatment even though this lectin caused no increase in RNA synthesis or cell division.

In addition to the nuclear changes seen following stimulation, a striking increase in cytoplasmic volume occurs and the cell undergoes a three to four fold enlargement during a three day period. There is an increase in the number of ribosomes, mainly occurring as polysomes and an increase in the size and complexity of the Golgi apparatus. Numerous mitochondria and an increased number of lysosomes in PHA stimulated lymphocytes have been seen (Douglas, 1972).

In addition, in cultures stimulated with PWM, a second cell type has been observed. This cell is slightly larger

than a resting lymphocyte, has a heterochromatic nucleus, and a well-developed rough endoplasmic reticulum and Golgi apparatus. This cell type is morphologically identical to a plasma cell (Douglas and Fudenberg, 1969).

III. Metabolic Changes Prior to the Initiation of DNA Synthesis

A. Modification of Histones and Non-Histone Proteins

Within 15 minutes after PHA stimulation, an increase in the acetylation of histones was found (Pogo et al., 1966). The acetate groups were localized as N-acetyl lysine residues in the arginine-rich histones. Acetate incorporation into proteins was not dependent on protein synthesis. The authors suggested that modification of nuclear proteins may reduce their binding to DNA to allow the genome to function as templates for RNA synthesis.

An increase in the rates of both histone phosphorylation and dephosphorylation occurs very soon after PHA stimulation (Cross and Ord 1970, 1971).

Increased activity of both histone kinases and phosphorylase were demonstrated that paralleled the turnover of histone phosphate groups. However, in later stages of lymphocyte activation, there is an absolute increase in histone phosphorylation that is associated with the initiation of DNA synthesis.

Following exposure to mitogens, there is a significant incorporation of phosphate groups into the non-histone pro-

teins in addition to that seen in histones (Kleinsmith et al., 1966). Recent experiments have shown that following Con A binding to equine lymphocytes there is an increase in the phosphorylation of non-histone proteins and also a migration of these proteins from the cytoplasm to the nucleus where they bind to the chromatin (Johnson et al., 1974).

B. RNA Synthesis

The rate of incorporation of uridine into total RNA increases within 60 minutes of PHA binding (Cooper and Rubin, 1975; Pogo et al., 1966) and the total cellular RNA content increases 50-100% 24-48 hours after PHA stimulation (Kay, 1966; Cooper, 1973). Increased rates of synthesis of tRNA, rRNA, and hnRNA and increased activities of the enzymes involved in their synthesis have been observed following mitogen stimulation.

1. tRNA

The rate of synthesis of tRNA and a putative tRNA precursor was shown to increase within 90 minutes of PHA stimulation (Kay and Cooper, 1969).

The activity of tRNA methylases is also increased by PHA stimulation (Riddick and Gallo 1971). Cytoplasmic extracts from PHA stimulated cells gave a four to seven fold greater rate and extent of methylation of heterologous substrates than was obtainable from unstimulated lymphocytes. There was a delay of several hours before an increase in tRNA methylase activity could be detected, reaching a maximum at 40-45 hours after stimulation. On the other hand,

Sharma and Loeb (1972) found that the rate of methylation of homologous tRNA in vivo increased within a few hours of PHA stimulation. Furthermore, they found qualitative differences in the pattern of methylation; for example, the ratio of N-2 methylguanosine to N-1 methyladenosine was greatly increased within the first two hours after stimulation. It was not clear how the activity of tRNA methylases was regulated following activation. Puromycin did not affect this early tRNA methylation indicating that protein synthesis was not required for the increased methylase activity. On the other hand, actinomycin D prevented the early increase in methylation, indicating that the synthesis of new tRNA was necessary in the reaction.

2. rRNA

Since rRNA accounts for at least 80% of cellular RNA, the increase in total RNA content following stimulation mainly represents an accumulation of new rRNA molecules (Cooper, 1973). Inhibition of this new rRNA synthesis and the subsequent production of new ribosomes prevents DNA synthesis from occurring in stimulated lymphocytes (Kay et al., 1969), indicating that the accumulation of new ribosomes is essential for cell division as has been suggested in other systems.

The rate of rRNA accumulation seen 20 hours after PHA stimulation was demonstrated to be increased 10-20 fold over the levels seen in resting lymphocytes (Cooper, 1969a). The accumulation of rRNA observed is due to (a) an increase

in the rate of synthesis of the 45S rRNA precursor, (b) an increase in the rate of processing of the 45S precursor to the mature 18 and 28S rRNA species, and (c) a decrease in the wastage of 18S rRNA molecules seen in unstimulated lymphocytes (Kay and Cooke 1971).

In unstimulated lymphocytes, only about one-half of the rRNA molecules synthesized in the nucleolus ever reach the cytoplasm as 18 and 28S RNA (Cooper 1969b & 1969c). The 18S molecules appear to be preferentially degraded and the ratio of 28 to 18S molecules found in resting cells is greatly shifted toward the 28S species. The wastage of rRNA is almost immediately reversed by mitogen stimulation leading to an increase in rRNA accumulation in the cytoplasm even without an increase in RNA synthesis (Cooper, 1970).

3. hnRNA

hnRNA is the most rapidly synthesized species of RNA in both resting and stimulated lymphocytes (Cooper, 1969a; Neiman and Henry, 1971). At any moment, hnRNA synthesis represents 90% of the total RNA synthesis in resting cells and 80% in stimulated cells. Correcting for increases in the specific activity of precursor pools, about a five-fold increase in hnRNA synthesis was shown after 20 hours of PHA stimulation. (Cooper, 1973).

4. Enzymes Involved in RNA Synthesis

There are three main classes of RNA polymerase in eukaryotic cells: polymerase I, which is alpha-amanitin resistant, is found in the nucleolus and transcribes the genes

for rRNA; polymerase II is chromatin associated, alpha-amanitin sensitive and transcribes hnRNA and mRNA genes; polymerase III is alpha-amanitin resistant and transcribes tRNA and 5S RNA genes (Roeder and Rutter, 1969; Weinman and Roeder, 1974). Following PHA stimulation, there is an increase in activity of all three polymerases in accordance with the increased rate of RNA synthesis in the stimulated cells (Cooke and Brown, 1973; Cooke and Kay, 1973). Polymerase I activity was shown to increase rapidly after PHA stimulation to levels 17 fold higher than in resting cells, while polymerase II activity was shown to rise to eight fold higher levels (Handmaker and Graef, 1970; Jaehning *et al.*, 1975). Polymerase III levels also rose 17 fold. These observations are in good agreement with previous data showing that, although the rate of total RNA synthesis increases in stimulated cells, the rates of rRNA and tRNA synthesis increase to a greater extent than does hnRNA.

The activities of other enzymes involved in RNA precursor utilization, such as uridine kinase (Lucas, 1967), are also increased in stimulated lymphocytes as are the activities of two of the enzymes involved in de novo pyrimidine synthesis, carbamyl phosphate synthetase and aspartate transcarbamylase (Ito and Uchino, 1971, 1973).

C. Protein Synthesis

As early as 15 minutes and certainly by four hours after mitogen stimulation, the rate of protein synthesis, as

measured by amino acid incorporation, increases in lymphocytes. This increase in rate probably involves new mRNA synthesis as it is sensitive to inhibitors of both protein and RNA synthesis. (Hirschhorn et al., 1963; Pogo et al., 1966; Hirschhorn and Hirschhorn, 1974)

There is an increased synthesis of many cellular proteins by lymphocytes after mitogen stimulation (Hirschhorn and Hirschhorn, 1974). Many of these proteins are enzymes involved in RNA, DNA, and precursor synthesis, such as RNA polymerases, DNA polymerase, uridine kinase, thymidine kinase, etc. Most lysosomal and many non-lysosomal enzymes show an increase in activity after stimulation and some show changes in isozyme patterns.

Depending upon the composition of the lymphocyte population, and the type of mitogen used, stimulated lymphocytes in culture produce and secrete immunoglobulins (mainly 19S-IgM) (Parkhouse et al., 1972). In addition, stimulated lymphocytes synthesize de novo various mediators of cellular immunity, such as macrophage inhibition factor, interferon and blastogenic factor. It appears that the production of many of these proteins has no bearing on events leading to cell division and most likely reflects the differentiated state of the lymphocyte.

In agreement with these data about increased protein and rRNA synthesis is an increase in the number active ribosomes which become involved in protein synthesis. Utilizing both ultrastructural and biochemical means, it has been

shown that the preponderance (70%) of cytoplasmic ribosomes exist as free, easily dissociable, 80S monomers in resting lymphocytes (Cooper, 1973, 1976; Douglas, 1971). After 10-15 hours of PHA stimulation, free, inactive, ribosomes account for about 40% of the total, decreasing to 30% at 24 hours. (Cooper, 1973)

IV. DNA Synthesis

The initiation of DNA synthesis is the primary characteristic of the mitogenic response of lymphocytes. DNA synthesis, measured by thymidine incorporation, begins 24 hours after PHA and ConA stimulation and reaches a maximum by 72 hours.

A. Enzymes Involved in DNA Synthesis

Enzymes involved in DNA synthesis greatly increase in activity after lymphocyte stimulation. It is essential to differentiate DNA replication prior to mitosis from DNA synthesis due to repair processes. Resting lymphocytes normally possess enzymes capable of repairing chemically or physically induced damage to DNA. Since the polymerases involved in DNA replication (and not those involved in repair) are sensitive to hydroxyurea and cyclohexamide, these agents have been used to suppress the small amount of DNA replication in resting cells so that repair activity can be measured (Lieberman et al., 1971 a, 1971 b).

Using calf thymus DNA as template and studying the incorporation of labelled nucleosides into DNA, it was found that increased DNA directed DNA polymerase activity in cell

extracts could first be detected at 20 hours, increasing to a maximum around 60 hours (Loeb et al., 1968; Tyrsted et al., 1973). The increase in DNA polymerase activity paralleled the increase in thymidine incorporation into DNA in intact cells. Cyclohexamide abolished the increased polymerase levels indicating that de novo synthesis of the enzyme occurred during stimulation.

An RNA-dependent DNA polymerase activity has also been reported present in PHA stimulated lymphocytes (Penner et al., 1971; Bobrow et al., 1972). This enzyme activity preceded that of the DNA directed polymerase and did not transcribe the heteropolymeric regions of 70S RNA from RNA tumor viruses, and was thereby distinguishable from the "reverse transcriptase" of leukemic cells and tumor viruses. It was postulated (Penner et al., 1971) that the enzyme may function in gene amplification since it appeared subsequent to increased RNA synthesis but prior to the largest increase in DNA synthesis.

Other enzymes that are involved with DNA synthesis, such as thymidine kinase and deoxycytidine kinase, also increase in activity (Pegoraro and Bernengo, 1971) beginning around 24 hours after PHA stimulation and reaching a plateau around 60 hours.

B. Initiation of DNA Synthesis

Blastogenesis is not synonymous with eventual cell division and blast-like cells that do not divide revert back to

the appearance of resting lymphocytes after several days (Polgar and Kibrick, 1970).

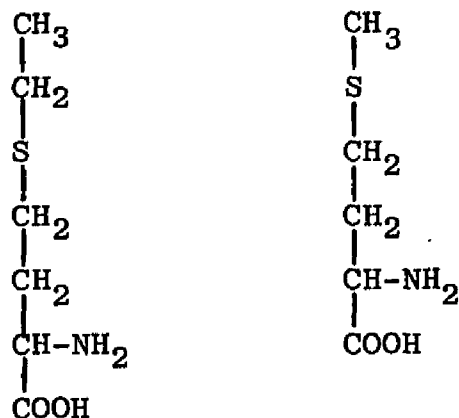
The onset of DNA synthesis and subsequent mitosis in stimulated lymphocytes occur asynchronously. That is, not all of the population of cells that are committed to division start DNA synthesis at the same time and only a fraction of the population will be synthesizing DNA at any given time point. Therefore, the measurement of cell cycle time in stimulated lymphocytes has been very difficult (Cooper, 1973).

Several agents have been used to try to synchronize stimulated lymphocytes so that the length of various stages of the cell cycle could be determined (Cave, 1969; Steffen and Stolzmann, 1969). Among these agents are those that exert their inhibitory effect by interfering with the synthesis of nucleic acid precursors, such as amethopterin, S-fluorodeoxyuridine, and excess thymidine. These agents prevent cells in G₁ from entering S and their effect can be eliminated by their removal or addition of a required metabolite. Ethionine can also, reversibly inhibit DNA synthesis in stimulated lymphocytes by undefined mechanisms that are at present under study.

ETHIONINE

Despite the vast body of information known regarding the reactions involved in lymphocyte activation, those reactions leading to the initiation of DNA synthesis in these cells are not completely known. It was found that ethionine could cause reversible inhibition of the initiation of DNA synthesis in mitogen stimulated lymphocytes.

Ethionine, the ethyl analog of methionine,



Ethionine

Methionine

was chosen because the mechanisms by which it can affect cellular metabolism are mostly known and there are profound biological consequences that result from ethionine administration.

I. Mechanisms by which Ethionine can Affect Cellular Metabolism

It is known that ethionine has the potential to affect cellular metabolism by at least four mechanisms: (1) inhibition of post-synthetic modification of macromolecules, (2) ethylation of macromolecules, (3) incorporation into proteins in place of methionine and (4) depletion of

cellular ATP pools (Farber, 1971)

A. Inhibition of Post-Synthetic Modification of
Macromolecules

There are a number of experiments showing the occurrence of post-synthetic modification of macromolecules which may have important biological consequences. For example, the post-synthetic modification of histones by acetylation, phosphorylation, and methylation is believed to affect, in some way, histone binding to DNA and may be involved in regulating gene expression (Allfrey, 1970, 1971; Borun, 1975; Dixon et al., 1975). In addition ADP-ribosylation of histone H1 has been described (Smith & Stocken 1973) and may be concentrated in transcriptionally active sites of G₀ liver chromatin (Ord & Stockton 1975).

Phosphorylation of non-histone proteins also occurs and several lines of evidence suggest that phosphorylation and dephosphorylation of non-histone proteins play a major role in the regulation of gene expression by these proteins (Kleinsmith, 1975). Another post-synthetic modification that occurs mainly on membrane and exported proteins is glycosylation. In mitogen stimulated lymphocytes, the production and secretion of 19S molecules of IgM is accompanied by alterations in glycosylation (Melchers and Andersson, 1973) and this glycosylation may be a necessary prerequisite for secretion.

In addition to protein modification, post-synthetic modification of nucleic acids occurs. The pioneering work

of Borek and coworkers (Fleissner and Borek, 1962) demonstrated that modification (methylation) of tRNA occurred post-transcriptionally and, to date, this appears to be the rule for all RNA and DNA modification. Methylated nucleotides have been demonstrated in all classes of RNA except 5S rRNA and numerous regulatory functions have been proposed for these modifications (Kerr and Borek, 1972; Nau, 1976; Maden and Salin, 1973; Shatkin, 1976). Post-synthetic methylation of bacterial DNA has been shown to protect specific base sequences from cleavage by restriction endonucleases (Arber 1974; Messelson et al., 1972). However there is no evidence for restriction enzymes in mammalian cells. A number of investigators have speculated that methylation of cytosine residues in DNA might serve to regulate gene expression. (Holliday & Pugh, 1975, Sager and Kitchin, 1975; Scarano, 1967).

The methylation of macromolecules is accomplished by the transfer of methyl groups from S-adenosylmethionine to various substrates by the action of specific methyl transferases (Fleissner and Borek, 1962; Borek, 1963; Borek and Srinivasan, 1966). It has been demonstrated that S-adenosylethionine, one of the metabolites of ethionine, inhibits a number of methyl transferases including tRNA (Moore & Smith, 1969; Pegg, 1971; Moore, 1970) and histone (Byvoet and Baxter, 1975) methyl transferases. In addition exposure of cells to ethionine has been shown to result in the synthesis of undermethylated tRNA (Rajalakshmi, 1973; Wainfan et al.,

1975; Wainfan and Maschio, 1975) and undermethylated DNA (Lark, 1968; Sneider et al., 1975; Cox and Irving, 1977) and in the degradation of a presumably undermethylated rRNA precursor (Swann et al., 1975).

B. Ethylation of Macromolecules

Ethylated nucleotides have been found in tRNAs and DNA (Ortwerta and Novelli, 1969; Swann et al., 1971) isolated from organs of ethionine treated animals. In addition, post-synthetic ethylation of histones (Baxter & Byvoet 1974) and other nuclear proteins (Friedman et al., 1969) has been demonstrated.

C. Incorporation of Ethionine into Proteins

Ethionine can be incorporated into proteins in place of methionine under conditions of severe methionine deprivation, thereby, possibly causing alterations in protein stability and function (Levine and Tarver, 1951).

D. Depletion of ATP Pools

The formation of S-adenosylethionine has been implicated in the rapid decrease in hepatic ATP levels seen following ethionine administration to rats (Stekol, 1963; Shull, 1962). The ATP depletion arises as a result of S-adenosylethionine formation at a rate faster than hepatic cells could regenerate adenosine. In other words, S-adenosylethionine had an "adenosine-trapping" effect. Marked inhibition of hepatic protein synthesis (Villa-Trevino, et al., 1963),

RNA synthesis (Villa-Trevino et al., 1966), and disorganization of hepatic cell nucleoli (Shinozuka et al., 1968) have been among the consequences observed as a result of ethionine induced decreases in rat liver ATP levels. These effects could be reversed by administration of adenine or methionine.

II. Biological Consequences Resulting from Ethionine Administration

Ethionine was first synthesized by Dyer (1938), who reported that young rats fed a diet of casein, supplemented with ethionine, showed a significant weight loss and subsequently died.

The carcinogenic effect of ethionine was first demonstrated in 1953 by Popper and coworkers (Popper et al., 1953) who fed rats an ethionine containing diet. They reported that chronic feeding of ethionine to rats resulted in tumor-like nodules in their livers. Carcinogenesis, resulting from chronic feeding of ethionine was confirmed by Farber (1956). Ethionine has been reported to induce a wide variety of biochemical and morphological effects in bacteria and higher animals (Farber, 1963). It is well established as a potent carcinogen, but little is known about its mode of action (Farber, 1973).

Beside the induction of hepatocarcinomas in rats, various other consequences of ethionine administration to animals and cells in culture have been reported. Induction of increased α -fetoprotein levels was demonstrated in rats

fed for only three days with an ethionine containing diet (Hancock et al., 1976). The authors speculated on various mechanisms by which ethionine might activate embryonic genes and subsequently might induce malignancy. Borek and co-workers (Sharma et al., 1976) reported that ethionine administered to immature chicks induced ovalbumin synthesis in their oviducts. They subsequently reported (Sharma and Borek, 1977) that ethionine induced elevated progesterone levels in the immature chicks that, in turn, stimulated oviduct maturation and ovalbumin synthesis. Ethionine administration to Friend erythroleukemia cells (FLC) in culture induces them to synthesize globin (Christman et al., 1977) and accumulate globin mRNA. The exocrine elements of the pancreas were shown to be destroyed by ethionine, whereas the endocrine portions were unaffected (Payne et al., 1977). As is described here, ethionine reversibly inhibits the initiation of DNA synthesis in mitogen stimulated lymphocytes (Zabos, et al., 1977).

RATIONALE

Although many of the events associated with lymphocyte activation are known, the precise biochemical reactions leading to the initiation of DNA synthesis in these cells are, as yet, undefined.

Since it was found that ethionine could reversibly inhibit the initiation of DNA synthesis in mitogen stimulated lymphocytes, an exploration into the effects of ethionine in this system was made. The effect of ethionine on commitment to DNA synthesis, RNA synthesis and modification, and ATP pool levels was studied.

These studies were initiated to understand more fully the modes of action of ethionine as well as to learn more about the process of lymphocyte activation.

The events involved in lymphocyte stimulation which culminate in the initiation of DNA synthesis and subsequent mitosis are extremely complex. It is very difficult to discern which of the many metabolic processes that occur subsequent to mitogen binding are directly involved in the initiation of DNA synthesis. By using ethionine, which allows the progression of stimulated lymphocytes into an active G₁ stage, but prevents their transition into the S phase, the specific reactions occurring in G₁ that are necessary for the initiation of DNA synthesis may be elucidated.

MATERIALS AND METHODS

I. Cells and Methods of Isolation

Both mouse spleen and human circulating lymphocytes were used for these experiments and gave qualitatively similar results. The source of cells used in various experiments is given below, in the Results, and in the legends to Figures and Tables.

A. Human Circulating Lymphocytes

Lymphocyte rich plasma was obtained from the Blood Bank at the Mt. Sinai Hospital (courtesy of Dr. E. Ambender and Ms. Gail Button). Each sample, obtained from a single healthy donor, was diluted with an equal volume of sterile isotonic saline and further purified by buoyant density gradient centrifugation according to the method of Bøyum (1968). Sterile Lymphocyte Separation Medium (LSM), with a density of 1.077, was obtained from Bionetics and was used according to manufacturer's directions. Briefly, three volumes of LSM were layered under four volumes of diluted cells in a 50 ml sterile plastic tube (Falcon Plastics). The gradients were centrifuged for 30 minutes at 1000 x g at room temperature in an International model UV centrifuge. The top layer of plasma was discarded and the mononuclear cells and platelets at the interface were collected. The cells were washed twice by suspension in saline followed by centrifugation at 1000 x g for 10 minutes.

After washing, the cells were resuspended in 25-40 ml

of Minimal Essential Medium (MEM, Grand Island Biological Co.) and a small aliquot (0.05-0.1 ml) was removed, diluted 100-200 fold and counted in a hemocytometer. Only nucleated cells were scored. Cell viability, as determined by Trypan Blue exclusion, was always 95-100%. Cell quantities obtained from a single donor ranged from $1-3 \times 10^9$ cells.

B. Mouse Spleen Lymphocytes

Spleens from individual six to nine week old (18-25 gm) Swiss-Webster mice were used. Spleens from more than one mouse were never mixed to eliminate the possible occurrence of mixed lymphocyte reactions.

The spleens were aseptically removed and placed in six ml of MEM in a sterile 60 mm plastic Petri dish (Falcon Plastics). A single cell suspension was prepared by mashing the spleen with the plunger of a sterile syringe, then forcing the suspension through a 25G needle. Lymphocytes were isolated by the Boyum method described above.

Approximately 10^8 lymphocytes could be obtained from single spleens. Cell viability was greater than 95%.

II. Culture Conditions

For culture, mouse spleen or human circulating lymphocytes were suspended at a concentration of $1-2 \times 10^6$ cells per ml in MEM made with Earle's salts (Grand Island Biological Co.), supplemented with 10% heat-inactivated fetal bovine serum (Grand Island Biological Co.), $10^{-5}M$ 2-mercaptoethanol (Eastman Organic Chemicals), 2 mM glutamine (Grand

Island Biological Co.), 100 u/ml penicillin and streptomycin (Grand Island Biological Co.), and 1% MEM non-essential amino acids (Grand Island Biological Co.). The concentration of L-methionine in the medium was 0.1 mM for all experiments except those involving RNA isolation, where it was 0.01 mM.

The cultures were incubated in sterile 12 x 75 mm plastic tissue culture tubes (Falcon Plastics) or in sterile 250 ml plastic centrifuge tubes (Corning) at 37°C. in a humid 5% CO₂ atmosphere. Lymphocytes were stimulated by 1-2 µg/10⁶ cells phytohemagglutinin (PHA) (Burroughs-Wellcome Co., HA-16) or with 8-10 µg/ml concanavalin A (Con A) (Pharmacia). A sterile stock solution of 0.1 M DL-ethionine (Sigma Chemical Co.) in saline was used in these experiments. Media and ethionine were used within two weeks of preparation or discarded. PHA and Con A were diluted in sterile saline and stored at -20°C. between use. Sterilization of media and all other reagents was performed by filtration through 0.2 µ Millipore filters. Each separate experiment was performed using cells from a single individual.

III. Conditions for Isotope Incorporation

To measure the rate of DNA synthesis, cells were pulse labeled with 2.5 µCi/ml ³H-thymidine (New England Nuclear, sp. act.: 6 Ci/mmol) or with 50 µCi/ml ³²Pi (New England Nuclear). RNA synthesis was measured by ³H-uridine (New England Nuclear) incorporation (2.0 µCi/ml; sp. act.: 60

Ci/mmmole). When RNA was to be isolated for simultaneous measurement of synthesis and methylation, the cells were labeled with $0.1\mu\text{Ci/ml}$ ^{14}C -uridine (New England Nuclear, sp. act.: 60 mCi/mmmole) and $5\mu\text{Ci/ml}$ methyl- ^3H -methionine (New England Nuclear, sp. act. 15 Ci/mmmole) in MEM containing 0.01 mM L-methionine. Adenosine ($20\mu\text{M}$), guanosine ($20\mu\text{M}$), and sodium formate (20 mM) were included in the medium to prevent label from the methyl- ^3H -methionine to be incorporated into bases via the C-1 pathway (Perry and Kelley, 1974). Protein synthesis was determined by ^3H -leucine (New England Nuclear, sp. act.: 50 Ci/mmmole) incorporation ($2\mu\text{Ci/ml}$). Ethylation of macromolecules or ethionine incorporation into proteins was determined by incorporation of $5\mu\text{Ci/ml}$ ethyl- ^3H -ethionine (New England Nuclear, sp. act.: 75mCi/mmmole) into macromolecules.

For quantitating ^3H -thymidine and ^3H -uridine incorporation, the incubation was stopped by chilling the cells on ice, washing with ice cold Dulbecco's phosphate buffered saline (PBS, Grand Island Biological Co.), and precipitating macromolecules with 5% trichloroacetic acid (TCA). After precipitation, the cells were washed onto Millipore filters and washed three more times with 5% TCA. For determining ^3H -leucine and ethyl- ^3H -ethionine incorporation into proteins, and additional step prior to TCA precipitation was necessary. The cells were treated with dilute NH_4OH , pH 10, for 15 minutes at 37°C . to remove any amino acids attached to tRNA.

More elaborate methods were required to quantitate in-

corporation of ^{32}Pi into DNA. Nuclei, isolated as described below, were suspended in nuclear isolation buffer (NIB: 10 mM Tris-HCl, pH 8.0; 1 mM MgCl_2 ; 0.25 M sucrose) at a concentration of 1.4×10^7 nuclei/ml and incubated with 40 $\mu\text{g}/\text{ml}$ proteinase K (Beckman) at 37°C for 30 minutes. The mixture was then brought to 0.1 N with NaOH and incubated for an additional 16 hours at 37°C . to hydrolyze RNA. DNA was precipitated by adding an equal volume of 50% TCA and collected on a Millipore filter.

In all labeling experiments, filters containing washed precipitates were dried under a heat lamp. Radioactivity was determined in a Nuclear Chicago liquid scintillation counter using OCS (Amersham/Searle) as fluor.

IV. Isolation of Nuclei

Lymphocytes were washed once with PBS and once with NIB containing 2×10^{-3} M 2-mercaptoethanol and 5×10^{-4} M spermine (supplemented NIB). The cells were resuspended in this solution and quickly frozen in an alcohol-dry ice bath. The suspension was thawed and adjusted to a final concentration of 0.5% Triton X-100 (Sigma Chemical Co.) with gentle mixing. After five minutes at 4°C ., nuclei were pelleted by centrifugation at $1000 \times g$ for five minutes. The nuclei were resuspended in supplemented NIB for 24 hours at 4°C ., centrifuged, and suspended for use at a concentration of 4×10^7 nuclei/ml in supplemented NIB.

V. Isolation of RNA

For all experiments, RNA was prepared from 1.5×10^8 PHA stimulated lymphocytes that had been cultured at a concentration of 2×10^6 /ml in MEM containing 0.01 mM L-methionine in the presence and absence of 5 mM DL-ethionine. Other additions have been listed previously.

RNA was prepared by the hot phenol-sodium dodecyl sulfate (SDS) method of Cooper and Kay (1969). After labeling, the cells were harvested by centrifugation at $1000 \times g$ for 10 minutes at 4°C . Polyvinylsulfate ($20 \mu\text{g}/\text{ml}$, Eastman Organic Chemicals) was added to inhibit the action of nucleases. The cells were washed once with ice cold Tris-saline-ethylenediamine tetraacetic acid (EDTA), pH 7.4 (0.01 M Tris-HCl, pH 7.4; 0.15 M NaCl; 0.001 M EDTA) containing $20 \mu\text{g}/\text{ml}$ polyvinylsulfate. After centrifugation, the cells were resuspended in ice cold 0.01 M acetate buffer, pH 5 and diethylpyrocarbonate (Sigma Chemical Co.) was added to a 1% final concentration. Immediately, enough 10% SDS solution was added to the cell suspension to make a concentration of 0.5%. The suspension was mixed on ice for 30 seconds to lyse the cells, after which time the lysate was transferred to a 60°C -water bath where an equal volume of acetate buffer-saturated, freshly distilled phenol was added. The lysate-phenol mixture was kept at 60°C . with intermittent mixing for five minutes. The emulsion was chilled in an ice bath and the aqueous and phenol phases were separated by centrifugation at $13,000 \times g$ (Sorvall RC 2B superspeed centrifuge, HB 4 rotor) for 10 minutes at 4°C . The aqueous layer was

carefully removed, without disturbing the precipitate at the interface, and transferred to another tube and kept in an ice bath. The phenol layer was reextracted at 60°C. for one minute with a half volume of acetate buffer. After centrifugation, the two aqueous layers were combined.

The aqueous layer was brought to 0.1 M NH_4OH , pH 10, and incubated for 15 minutes at 37°C. to remove any methyl- ^3H -methionine esterified to tRNA in an aminoacyl linkage. Potassium acetate was added to a final 5% concentration prior to the addition of 2 volumes of -20°C. absolute ethanol. This mixture was then placed at -20°C. overnight to allow the RNA to precipitate.

The RNA precipitate was harvested by centrifugation at 13,000 x g for 10 minutes at 4°C. and washed twice with ethanol to rid the precipitate of phenol. The precipitate was drained of excess alcohol and dried under vacuum for 10-15 minutes.

After drying, the precipitate was dissolved in 0.5 ml sucrose gradient buffer (0.01 M Tris-HCl, pH 7.4; 0.1 M NaCl; 0.001 M EDTA; 0.5% SDS). A small aliquot (25 μl) was diluted to one ml with water to determine the absorbance at 260 and 280 nm and TCA precipitable radioactivity in the sample. An acceptable $A_{260\text{ nm}}/A_{280\text{ nm}}$ ratio of 2 was usually obtained.

Determination of the incorporation of ^{35}S -methionine (Schwartz-Mann, sp. act.: 1Ci/mmole, 1 $\mu\text{Ci/ml}$) into RNA isolated from PHA stimulated cells exposed to the label for 24 hours indicated that, after this isolation procedure, fewer

than one molecule of methionine per 2000 tRNA molecules remained bound in an aminoacyl linkage.

To rid the sample of any acid precipitable radioactivity due to methyl-³H-methionine incorporation into contaminating protein, the RNA sample was treated with 400 µg/ml proteinase K for two hours at 37°C.

VI. Sucrose Gradient Analysis

Following isolation, the total RNA sample (0.5 ml) was layered onto a gradient of 5-20% sucrose made in sucrose gradient buffer. Samples were centrifuged in a preparative ultracentrifuge (Beckman Spinco, model L3-50) for 19 hours at 20,000 rpm at 17°C. in an SW 27.1 rotor (Beckman Spinco).

Gradients were fractionated by pumping them through an ISCO gradient fractionator and ultraviolet analyzer (ISCO model UA 2 or UA 5). Approximately 30-35 0.5 ml fractions were collected from each gradient and precipitated in the cold with 5% TCA. The fractions were washed onto Millipore filters with ice cold 5% TCA. Filters were handled and counted as described previously.

VII. Preparation of tRNA

Bulk tRNA was separated from other RNA species by dissolving the dried ethanol precipitated in 1M NaCl and incubating overnight at 4°C. The sample was then centrifuged at 13,000 x g for 10 minutes at 4°C. to remove any precipitated material (higher molecular weight RNA species). The supernatant was applied to a Sephadex G-100 column equilibrated in

1 M NaCl. Fractions were monitored by $A_{260\text{nm}}$ or by determining the radioactivity in a small aliquot. Transfer RNA and 5S ribosomal RNA chromatograph within the included volume of the column and are separable from one another (Christman and Goldstein, 1969)

VIII. Morphologic Examination of Cells

A. Preparation of Cells for Electron and Light Microscopy

For all morphological examinations, 1×10^7 lymphocytes ($2 \times 10^6/\text{ml}$) were used. PHA was added at $4\mu\text{g}/\text{ml}$ and DL-ethionine, where used, was added at a concentration of 5 mM. Following incubation, the cells were centrifuged, washed once with saline, and fixed overnight at 4°C . in 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4. Fixed cells were prepared for microscopy by the method of Marovitz (Schulte et al., 1977).

The following steps were carried out at 4°C . unless otherwise noted. The fixed cells were washed with 0.1 M cacodylate buffer and were then stained for 90 minutes with 1% osmium tetroxide made in the same buffer. After staining, the cells were washed in cacodylate buffer and stained again overnight with 0.5% uranyl acetate. The fixed, stained cells were then washed in buffer and dehydrated in ethanol solutions of increasing concentrations: 25% ethanol (10 minutes), 50% ethanol (15 minutes), and 70% ethanol (30 minutes or longer). Further dehydration steps were performed

at room temperature: 95% ethanol (15 minutes) and 100% ethanol (one hour, three changes). Cells were cleared at room temperature in two changes of propylene oxide (20 minutes) and then placed for two hours in a 1:1 mixture of propylene oxide: epon 812 mixture (preparation of epon 812 mixture below). The dehydrated cells were placed in fresh 100% epon 812 mixture overnight. After changing the epon 812 mixture, the blocks were polymerized in a 60°C. oven for 72 hours.

For light microscopy, sections of 2-3 μ were cut with a glass knife on a microtome (Ultratome III[®], LKB Instruments), and heat mounted onto slides. Following staining with 1% Toluidine Blue in 1% sodium borate, the cells were observed using a Zeiss Photomicroscope III[®]. For electron microscopy, 500 Å sections were cut with a diamond knife (DuPont Instruments) on an Ultratome III[®]. Grids were examined and photographed with a Zeiss 9S-2 electron microscope.

B. Preparation of Epon 812 Mixture

Fifty ml of epon 812 mixture were prepared as follows: 19.75 ml epon 812 (Shell Chemicals) plus 17.5 ml DDSA (dodecyl succinic anhydride) were mixed in a beaker for 15 minutes. Ten ml NMA (nadic methyl anhydride) and 2 ml DBP (dibutyl phthalate) were added and the mixture was stirred for another 30 minutes before the addition of 0.75 ml DMP-30 (tri(dimethylaminomethyl) phenol). After adding the DMP-30, the complete mixture was stirred for at least 30 minutes more.

Prior to use, the epon 812 mixture was degassed overnight under vacuum to remove bubbles introduced during mixing.

IX. Determination of ATP Levels

ATP levels in mouse lymphocytes were measured by the firefly luciferase assay (Stanley and Williams, 1969). Desiccated firefly lantern extract (FLE) was obtained from Sigma Chemical Co. and stored according to manufacturer's directions. Prior to use, a 50 mg vial of the extract was diluted with 5 ml water and stored at 4°C. overnight to reduce any background light emission due to endogenous ATP.

The lymphocytes were sedimented by centrifugation for 10 minutes at 1000 x g at 4°C. The supernatant medium was discarded and the cells were resuspended to a concentration of 2×10^6 /ml with cold 0.04 M Tris-borate buffer, pH 9.2. The cells were lysed by flash freezing in a dry ice-acetone bath followed by immersion in a boiling water bath for 5 minutes. They were then cooled and centrifuged at 2500 rpm for 10 minutes at 4°C. The cell extract (supernatant) was removed and kept on ice.

ATP levels in the extracts were determined in duplicate 50µl samples the same day. Fifty µl samples were added to pre-cooled plastic scintillation vials containing three ml of phosphate (0.01 M potassium phosphate, pH 7.4, containing 4 mM MgSO_4) and arsenate (0.1 M sodium arsenate, pH 7.4) buffer. Fifty µl of the diluted FLE were added to one vial at a time. The vials were capped and shaken and light

emission was determined by counting each vial two times for 0.1 minute in a Packard Tri-Carb liquid scintillation counter. The second set of counts for each sample was used for experimental values since background luminescence due to the vials was lower. A standard ATP concentration curve was prepared by diluting a frozen stock solution of ATP (Sigma Chemical Co.) (10^{-3}M) with 0.04 M Tris-borate buffer analyzing as above.

A linear decrease in the amount of measurable light was seen over the range of 10^{-9} to 10^{-12} moles of ATP in the samples. Results are given as moles of ATP/ 10^6 cells.

RESULTS

I. Effect of Ethionine on DNA Synthesis

A. Inhibition of DNA Synthesis by Ethionine

Lymphocytes, at a concentration of 1×10^6 cells/ml were cultured in MEM supplemented with 10% heat- inactivated fetal bovine serum. PHA was added at a concentration of 2 $\mu\text{g}/10^6$ cells. DL-ethionine, in increasing concentrations up to 16 mM, was added to the PHA stimulated lymphocytes at the initiation of culture. DNA synthesis was measured by ^3H -thymidine incorporation in a pulse labeling 40-42 hours after the initiation of culture. As can be seen from Table 1, the rate of DNA synthesis in PHA stimulated lymphocytes was inhibited 80% by the addition of 2 mM DL-ethionine, while in the presence of 4 mM DL-ethionine, the rate of DNA synthesis was the same as that seen in unstimulated lymphocytes. With higher concentrations of ethionine, the rate of DNA synthesis was even lower than that seen in unstimulated lymphocytes.

Evidence that incorporation of ^3H -thymidine is a true reflection of the rate of DNA synthesis in the presence of ethionine comes from studies of ^{32}Pi incorporation into DNA by Con A stimulated lymphocytes (Table 2). After labeling, the cells were treated with proteinase K and hydrolyzed in NaOH at pH 10 to remove acid precipitable label due to protein and RNA. It was found that increasing concentrations of DL-ethionine inhibited the incorporation of ^{32}Pi into the

Legend to Table 1.

Human circulating lymphocytes were incubated for 42 hours at a concentration of 1×10^6 cells/ml. Labeling with ^3H -thymidine ($2.5\mu\text{Ci/ml}$ was from 40-42 hours. The incubation period was terminated by the addition of cold PBS and the cells were processed as described in the Materials and Methods Section. All experiments were done in duplicate.

Table 1. Effect of Increasing Concentrations of DL-Ethionine on DNA Synthesis in Lymphocytes

Addition	CPM ^3H -Thymidine Incorporated per 10^6 cells
None	1600
PHA (2 $\mu\text{g}/\text{ml}$)	29,762
PHA plus:	
2 mM DL-Ethionine	5996
4 mM DL-Ethionine	1625
8 mM DL-Ethionine	578
16 mM DL-Ethionine	335

Legend to Table 2.

Mouse spleen lymphocytes were incubated for 44 hours at a concentration of 1.5×10^6 /ml. Labeling with ^3H -thymidine incorporation was determined as in Table 1 and Methods. ^{32}P i incorporation into the TCA insoluble residue of nuclei treated with proteinase K and NaOH was determined as described in the Material and Methods Section. All experiments were performed in duplicate.

Table 2. Effect of DL-Ethionine on DNA Synthesis: Comparison of ^3H -Thymidine and ^{32}P i Incorporation into DNA

Addition	CPM ^3H -Thymidine per 10^6 Cells	CPM ^{32}P i in DNA of 10^6 Cells
Con A (8 $\mu\text{g}/\text{ml}$)	50,334	4650
Con A plus 8 mM DL-Ethionine	2214	214

DNA in mitogen stimulated lymphocytes to the same extent as ³H-thymidine incorporation. Thus, the simpler method of thymidine incorporation was used for all subsequent determinations of the rate of DNA synthesis.

B. Reversibility of Inhibition

Although DL-ethionine inhibited DNA synthesis in mitogen stimulated lymphocytes, the cells were still viable and, as can be seen from the data in Table 3, the inhibitory effect of ethionine was completely reversible. In PHA stimulated lymphocytes, the rate of DNA synthesis measured at 22 and 46 hours after a two hour thymidine incorporation was significantly higher than the rate seen in unstimulated lymphocytes. This increase was abolished when 4mM DL-ethionine was included in the culture along with the PHA. However, when PHA stimulated lymphocytes, that had been cultured for 24 hours in the presence of 4mM DL-ethionine, were washed free of the ethionine and cultured for another 24 hours in fresh medium, the rate of DNA synthesis seen was of the same magnitude as that observed at 48 hours for lymphocytes cultured with PHA alone. As a further control, PHA was added to lymphocytes that had been cultured without any additions to the MEM for 24 hours. After another 24 hours, the rate of DNA synthesis observed was similar to that seen at 24 hours in culture when PHA was present from the beginning (Table 3.) This rate of synthesis was much less than that seen 24 hours after the removal of ethionine.

Legend to Table 3.

Human circulating lymphocytes ($1 \times 10^6/\text{ml}$) were incubated for the first 24 hour period with the indicated additions. At 22 hours ^3H -thymidine ($2.5 \mu\text{Ci}/\text{ml}$) was added to half of the cultures. After two hours the incubation was terminated as described previously. The other half of the cultures were centrifuged for five minutes at $400 \times g$, washed with saline, and incubated in fresh MEM for another 24 hours with the additions described in the table. ^3H -thymidine incorporation was assayed at 46 hours for a two hour period, and the cells processed as described previously. All experiments were performed in duplicate.

Table 3. Reversibility of Ethionine Inhibition of DNA Synthesis

Additions		CPM ^3H -Thymidine Incorporated per 10^6 Cells	
<u>First Incubation</u>	<u>Second Incubation</u>		
0-24 hr	24-48 hr	22-24 hr	46-48 hr
None	None	1100	4700
PHA (2 $\mu\text{g}/\text{ml}$)	None	8980	21,950
PHA plus 4mM DL-Ethionine	PHA plus 4mM DL-Ethionine	1500	4816
PHA plus 4mM DL-Ethionine	None	1320	28,010
None	PHA	----	6325

These data are compatible with the premise that, although the continuing presence of ethionine in the culture with the PHA stimulated lymphocytes prevented DNA synthesis, some of the early reactions leading to the initiation of DNA synthesis could take place. As depicted in Figure 1, when ethionine was removed from the PHA stimulated culture at 17, 40, or even 60 hours (not shown) of incubation, an increase in the rate of DNA synthesis could be measured within several hours of ethionine removal. On the other hand, if PHA alone was added at either the initiation of culture (0 time), or 40 hours after the beginning of the culture, an increase in the rate of DNA synthesis was observed only after a 22-24 hour lag period. Continued presence of DL-ethionine in the culture medium prevented any significant increase in the rate DNA synthesis for as long as 4 days in culture. The rapidity with which an increase in DNA synthesis was initiated after ethionine removal, again suggested that at least some of the early reactions leading to initiation of DNA synthesis in these cells could take place.

C. Commitment to DNA Synthesis

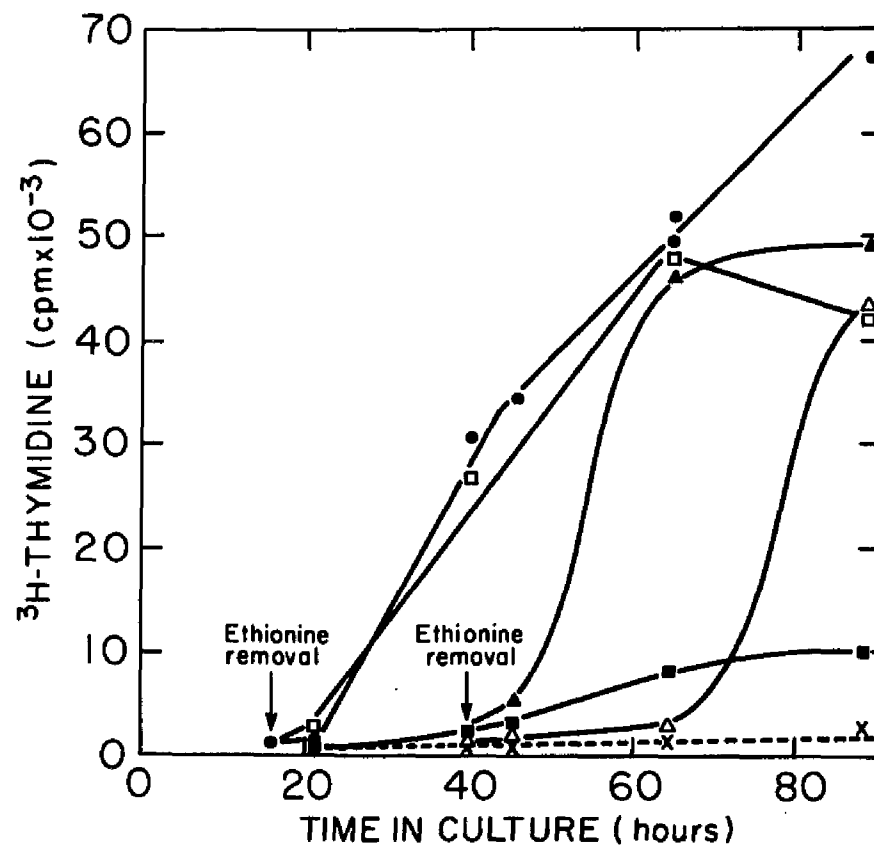
As was discussed in the background section, it has been shown that commitment to DNA synthesis is initiated by the mitogen (lectin) binding to its receptor (most likely a glycoprotein) on the cell surface (Kornfeld and Kornfeld, 1969). Mitogen must be present for a certain minimal time period in order for commitment to DNA synthesis to occur.

Figure 1. Rate of DNA Synthesis in PHA-Stimulated Lymphocytes, after Removal of Ethionine.

Human circulating lymphocytes (1×10^6 cells/ml) were incubated with the following additions during the time periods. PHA concentration was $1\mu\text{g/ml}$; DL-ethionine concentration, 4 mM;

PHA, 0 hr to assay ($\square-\square$), 40 hrs to assay ($\Delta-\Delta$); PHA and ethionine, 0 hr to assay ($\blacksquare-\blacksquare$); 0-17 hrs ($\bullet-\bullet$); 0-40 hrs ($\blacktriangle-\blacktriangle$); no additions (X-X).

To remove unbound PHA and ethionine, cells were washed as described in Table 3. Points indicate start of 2 hr labeling with ^3H -thymidine. Incubation was terminated and cells processed as described in the Materials and Methods Section.



Utilizing radioactive Con A, Edelman and coworkers (Gunther et al., 1974) demonstrated that Con A bound to lymphocytes could be removed by the addition of α -methylmannose. If lymphocytes were exposed to Con A for less than 12 hours, no DNA synthesis occurred after its removal. With longer exposure, however, its removal did not affect the ensuing DNA synthesis and mitosis, indicating that the commitment had occurred during this time.

The data in Table 4 indicate that commitment to DNA synthesis could occur in the presence of DL-ethionine. As can be seen, removal of bound Con A from lymphocytes at 24 hours did not significantly alter the rate of DNA synthesis as compared with the cultures where Con A was not removed. On the other hand, continuing presence of ethionine in the culture, irrespective of whether Con A was present or not for the full incubation period, resulted in total inhibition of DNA synthesis. However, when ethionine and the bound Con A were removed from the cells at 24 hours, the rate of DNA synthesis seen at 64 hours was similar to that seen when the cells were incubated in the presence of Con A alone, indicating that commitment to DNA synthesis had occurred in the presence of ethionine.

II. Blast Formation in the Presence of Ethionine

A. Biochemistry

The early reactions in the blastogenic response include an increased rate of RNA and protein synthesis. As can be

Legend to Table 4.

Mouse spleen lymphocytes (1×10^6 cells/ml) were exposed to Con A (10 μ g/ml) or Con A plus ethionine (8 mM) for 22 hours. The cells were then centrifuged at 400 x g, and resuspended in MEM. α -methylmannose (0.1 M) was added to alternate cultures. After 90 minutes incubation at 37° the cells were centrifuged, washed with saline, resuspended in MEM and incubated with or without ethionine as indicated. Forty hours later, 2.5 μ C/ml ^{3} H-thymidine was added for a two hour period. The reaction was terminated, and the cells processed as described in the Materials and Methods Section.

Table 4. The Commitment of Lymphocytes to DNA Synthesis in the Presence of
Ethionine

Additions		CPM, ^3H -Thymidine/ 10^6 cells	
<u>First Incubation</u>	<u>Wash with</u>	<u>Second</u>	
0-22 hr	α -methyl mannose	<u>Incubation</u> 24-66 hr	64-66 hr
—	—	—	2514
Con A	—	—	42,471
Con A	+	—	30,138
Con A plus ethionine	—	ethionine	1874
Con A plus ethionine	+	ethionine	2287
Con A plus ethionine	—	—	56,770
Con A plus	+	—	41,500

seen in Table 5, at any of the time points shown, the rates of RNA and protein synthesis, as measured by ^3H -uridine and ^3H -leucine incorporation respectively, were higher in the ethionine treated lymphocytes than in unstimulated cells, but lower than the rate seen in lymphocytes cultured with PHA alone. This is in marked contrast to the total inhibition of DNA synthesis seen in ethionine treated cells. Removal of ethionine from the cultures resulted in an increased rate of protein and RNA synthesis, that occurred, however, after the onset of DNA synthesis. In most instances, substantial DNA synthesis could be detected four to eight hours after the removal of ethionine. At that time, the rates of RNA and protein synthesis were only slightly increased over the levels seen in the presence of ethionine and did not reach a maximum until about 20 hours after the removal of the ethionine. A reasonable explanation for the increased rate of RNA and protein synthesis seen in the presence of ethionine (as compared with the rates in unstimulated cells) is that some of the early biochemical reactions necessary for the initiation of DNA synthesis already occurred. This is further indicated by the rapidity with which the onset of DNA synthesis begins after ethionine removal.

B. Morphology

1. Light Microscopy

Lymphocytes were cultured for 24, 48, and 72 hours with PHA, PHA plus ethionine, and no additions. In addition,

Legend to Table 5.

Human Circulating Lymphocytes (1×10^6 cells/ml) were incubated in MEM with PHA ($2 \mu\text{g/ml}$) and DL-ethionine (8 mM) (added as indicated). At 14 hours the cells were centrifuged at $400 \times g$, washed with saline, resuspended in fresh MEM and further incubated with the additions shown. ^3H -uridine ($2 \mu\text{Ci/ml}$) or ^3H -leucine ($2 \mu\text{Ci/ml}$) was added for two hours at the indicated times. The labeling process was terminated and the cells processed as described in the Material and Methods Section.

N.D., not determined.

Table 5. Effect of DL-Ethionine on the Rates of Protein and RNA
Synthesis in PHA Stimulated Lymphocytes

Additions		CPM/10 ⁶ Cells					
First Incubation	Second Incubation	³ H-Uridine			³ H-Leucine		
		<u>Labeling Period (hours)</u>					
		<u>16-18</u>	<u>22-24</u>	<u>40-42</u>	<u>16-18</u>	<u>22-24</u>	<u>40-42</u>
----	----	4607	4656	3760	2146	2238	3260
PHA	----	31,193	41,460	58,035	6500	8916	16,389
PHA + ethionine	PHA + ethionine	13,973	23,273	36,945	3760	4859	8214
PHA + ethionine	----	N.D.	26,946	54,222	N.D.	5470	12,435
----	PHA	N.D.	N.D.	35,112	N.D.	N.D.	7246

cells stimulated with PHA in the presence of ethionine for 24 hours were washed, resuspended in fresh medium, and cultured for further 24 and 48 hour periods. At the indicated times, cells were washed, pelleted, fixed in glutaraldehyde, and processed for electron microscopy.

Cell pellets were first observed by light microscopy after thick sections had been cut from the blocks and stained with toluidine blue. Figures 2 and 3 show unstimulated lymphocytes at 24 and 72 hours. At both times, small, round cells with a densely stained heterochromatic nucleus surrounded by a small rim of cytoplasm were seen. After the addition of PHA, the cells became progressively larger with increased volume of both the nucleus and cytoplasm (Figures 4, 5, and 6). The cytoplasm became quite vacuolated while the nucleus became progressively more euchromatic and prominent nucleoli could be seen. At 72 hours a small percentage of cells in mitosis was seen. Cells stimulated in the presence of ethionine appeared similar to cells incubated with PHA alone. (Figures 7, 8 and 9) They were increased in size and possessed numerous vacuoles. These cells did not, however, become as large by 72 hours as those stimulated with PHA alone. In addition, no mitotic figures were apparent in these cells. However, the presence of euchromatic nuclei and prominent nucleoli persisted in these cells (Figure 9).

Figures 10 and 11 show stimulated lymphocytes 24 and 48 hours after ethionine had been removed from the culture. The cells appeared very similar to those from cultures

Figure 2. Light Micrograph: Non-Stimulated
Lymphocytes after 24 Hours in Culture.
(X2000)

Human circulating lymphocytes ($2 \times 10^6/\text{ml}$) were cultured without mitogen for 24 hours. At the end of the incubation, the cells were washed once with saline and fixed in 2% glutaraldehyde. The fixed cells were processed for electron microscopy as described in the Materials and Methods section. Sections of 2-3 μ were cut and stained with 1% Toluidine Blue. Photographs were taken with a Zeiss Photomicroscope III.®

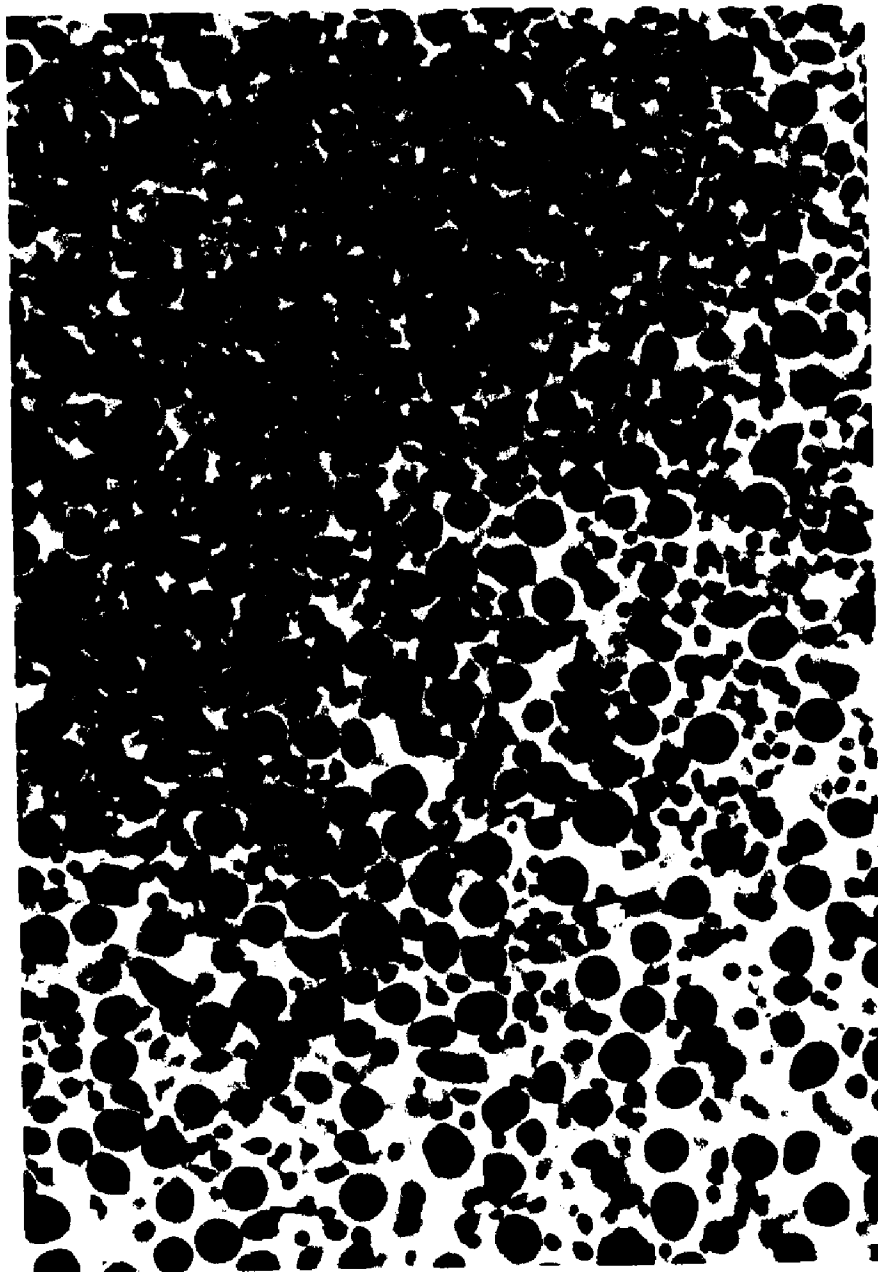


Figure 3. Light Micrograph: Non-Stimulated
Lymphocytes after 72 Hours in Culture.
(X2000)

Conditions other than time were the same as
described for Figure 2.

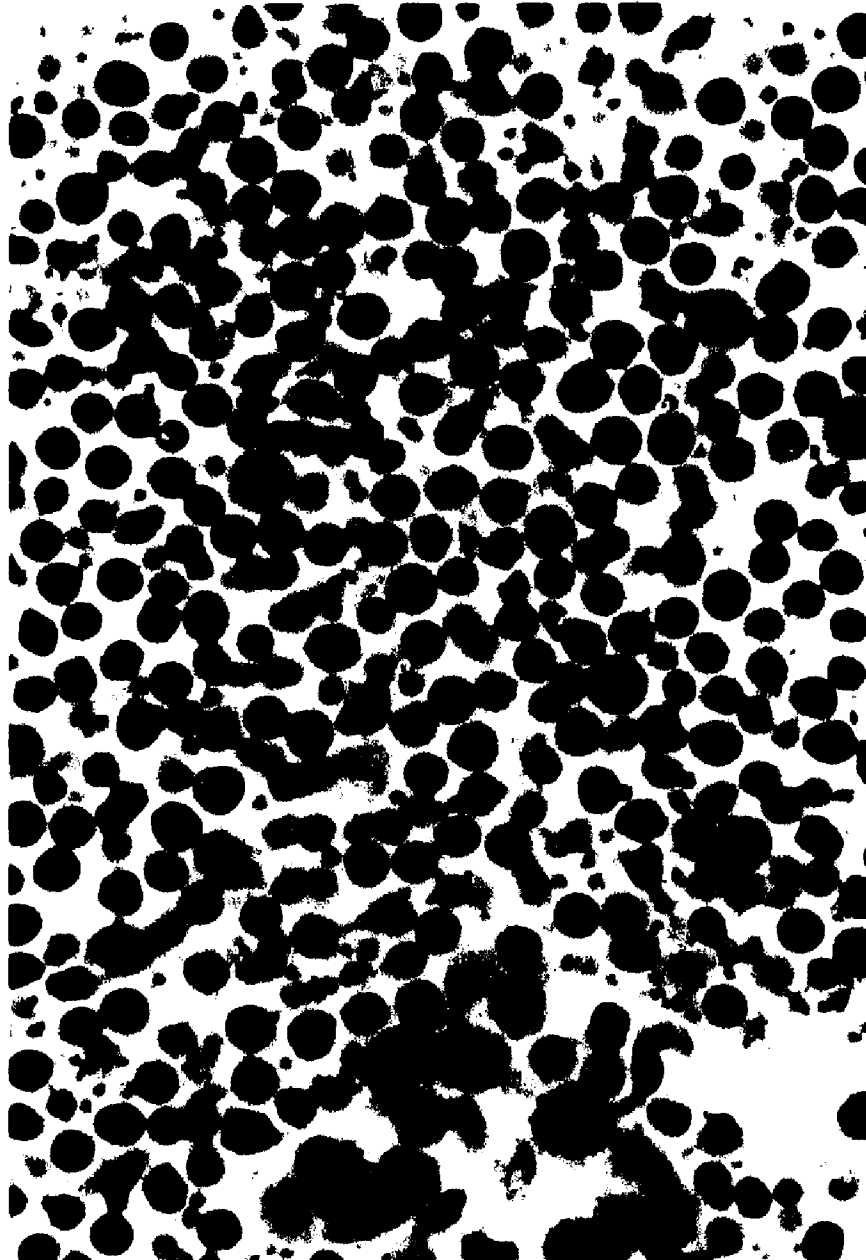


Figure 4. Light Micrograph: PHA Stimulated
Lymphocytes after 24 Hours in Culture.
(X2000)

Conditions the same as described in Figure 2
except that PHA (4 μ g/ml) was added at the initiation
of culture.



Figure 5. Light Micrograph: PHA Stimulated
Lymphocytes after 48 Hours in Culture.
(X2000)

Conditions the same as described in Figure 4
except that PHA was present for 48 hours.



Figure 6. Light Micrograph: PHA Stimulated
Lymphocytes after 72 Hours in Culture.
(X2000)

Conditions the same as described in Figure 4
except PHA was present for 72 hours. Note the pres-
ence of mitotic figures (M).



Figure 7. Light Micrograph: PHA Stimulated
Lymphocytes after 24 Hours in Culture
in the Presence of Ethionine. (X2000)

Conditions the same as Figure 4 except that
DL-ethionine (5mM) was present in the culture medium.



Figure 8. Light Micrograph: PHA Stimulated
Lymphocytes after 48 Hours in Culture
in the Pressence of Ethionine. (X2000)

Conditions the same as Figure 7 except that
the incubation period was 48 hours.

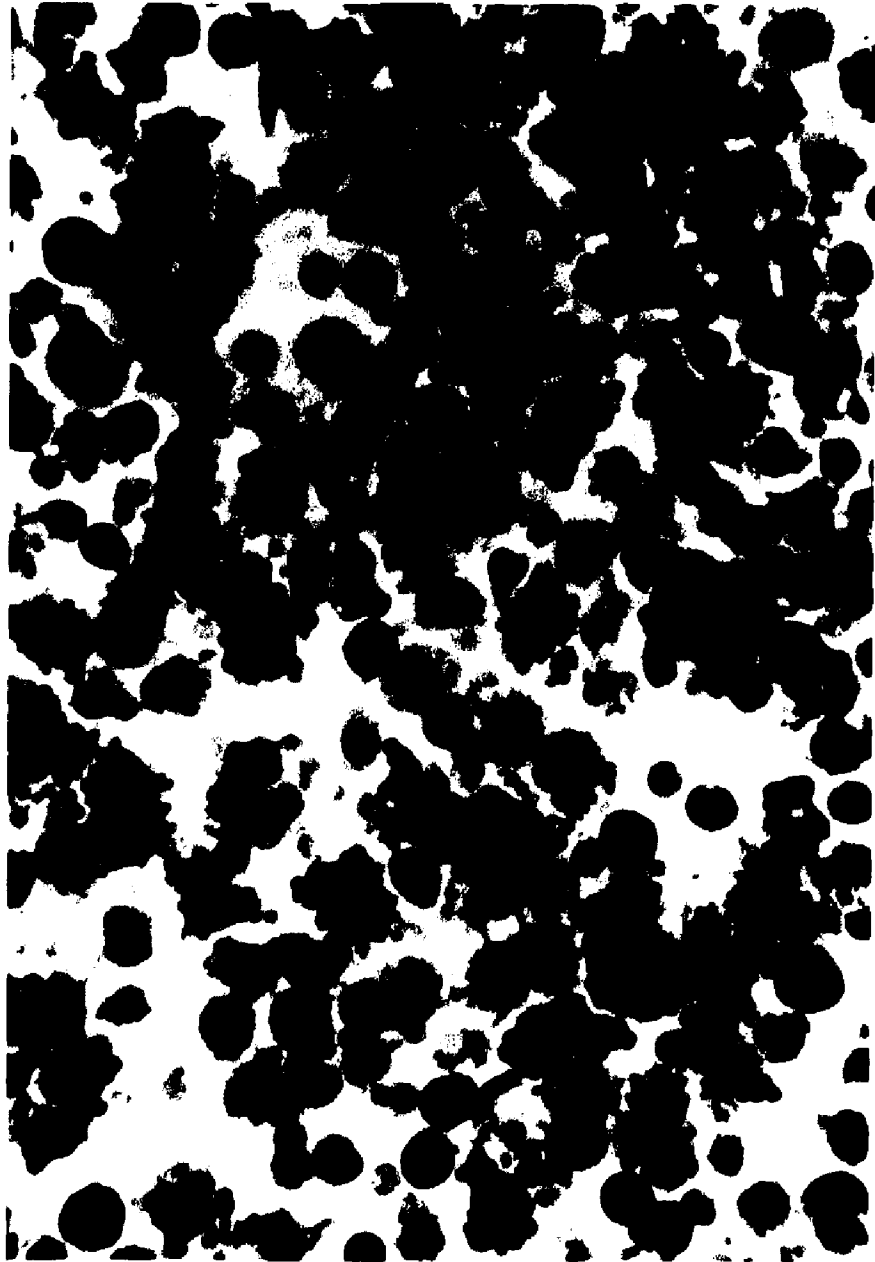


Figure 9. Light Micrograph: PHA Stimulated
Lymphocytes after 72 Hours in Culture
in the Presence of Ethionine. (X2000)

Conditions the same as Figure 7 except that
the incubation period was 72 hours.



Figure 10. Light Micrograph: PHA Stimulated
Lymphocytes 24 Hours after Removal
of Ethionine.

Lymphocytes were cultured with PHA and ethionine for 24 hours, washed, then cultured in fresh ethionine-free medium for an additional 24 hours prior to being processed for microscopy.

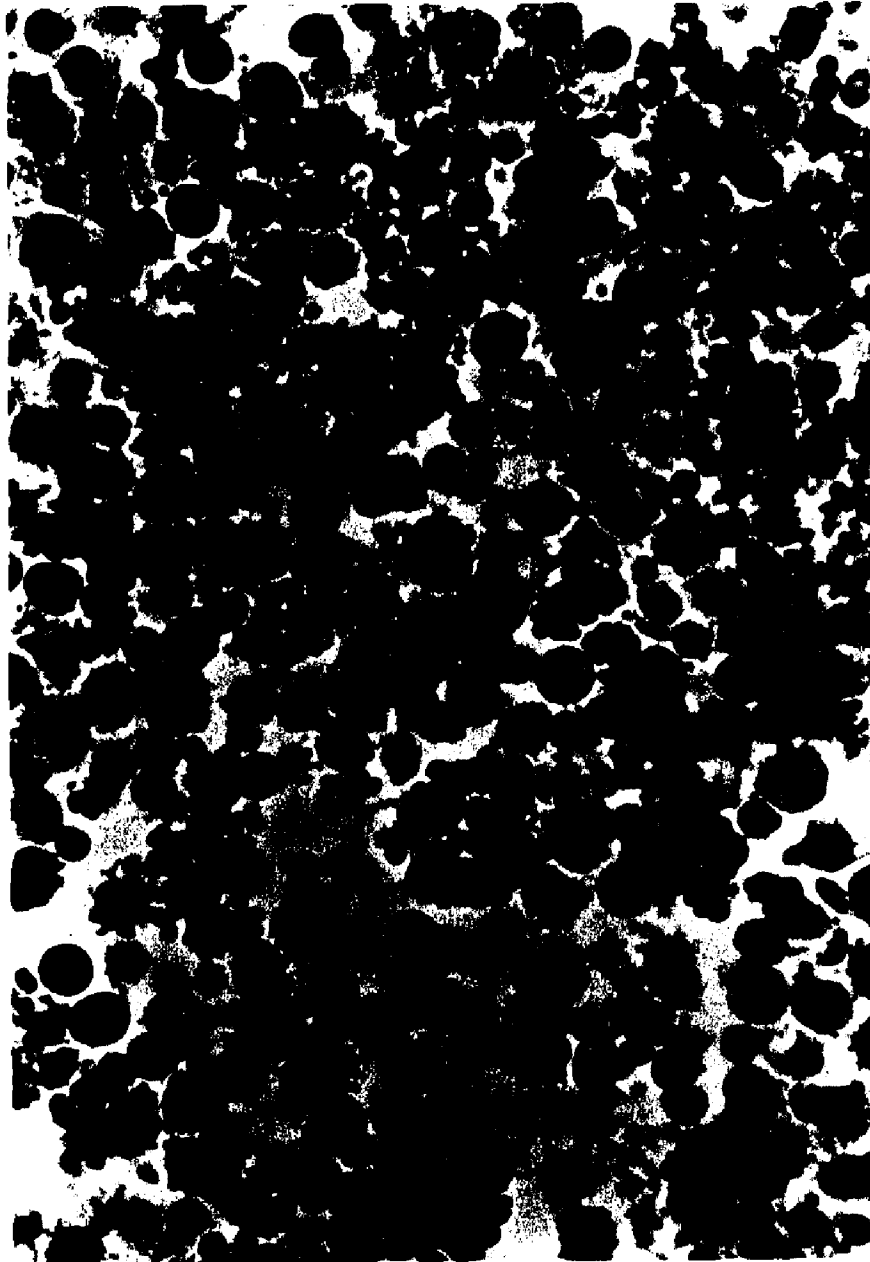


Figure 11. Light Micrograph: PHA Stimulated
Lymphocytes 48 Hours after Removal
of Ethionine. (X2000)

Conditions same as figure 10 except that the
additional culture period lasted 48 hours. Note
the presence of mitotic figures (M).



stimulated with PHA alone and observed at 48 and 72 hours. They increased greatly in size, became vacuolated, their nuclei showed large amounts of euchromatin, and their nucleoli became increasingly prominent. Mitotic cells were seen 48 hours after ethionine removal.

Approximately 40-50% of the cells in any of the PHA stimulated cultures underwent blastogenesis whether ethionine was present or not. The light microscopic appearance of ethionine treated cells thus appears to corroborate the biochemical data. The cells appear blast-like, undergoing enlargement indicative of increased RNA and protein synthesis. They do not, however, appear to synthesize DNA and undergo subsequent cell division as indicated by the lack of mitotic figures. Removal of ethionine reverses this inhibition; the cells are then able to initiate DNA synthesis and divide.

2. Electron Microscopy

Thin sections were observed in the electron microscope. Figure 12 is an unstimulated lymphocyte 24 hours after the initiation of culture. As can be seen, the nucleus contains highly condensed heterochromatin and a discernable nucleolus. Most of the ribosomes exist in the cytoplasm as monosomes. Figures 13 and 14 depict cells stimulated for 24 hours with PHA and with PHA and ethionine, respectively. No significant differences can be seen between the cells in the two figures. Prominent nucleoli can be seen in the

Figure 12. Electron Micrograph: Non-Stimulated
Lymphocytes after 24 Hours in Culture.
(X19,500)

Conditions the same as for Figure 2 except that
500 Å sections were prepared for microscopy.



Figure 13. Electron Micrograph: PHA Stimulated
Lymphocyte after 24 Hours in Culture.
(X19,500)

Culture conditions the same as for Figure 4.



Figure 14. Electron Micrograph: PHA Stimulated
Lymphocyte after 24 Hours in Culture
in the Presence of Ethionine. (X19,500)

Culture conditions the same as for Figure 7.



nuclei of both cells and a greater number of ribosomes are present as polysomes in these cells than was seen in the unstimulated cells.

III. Effect of Ethionine on RNA Synthesis and Methylation

The data in Table 5 show that even in the presence of ethionine, the rate of RNA synthesis, measured by ^3H -uridine incorporation, was increased relative to unstimulated cells. Since it has been reported that there may be a link between new RNA synthesis and the ability to initiate DNA replication (Cooper 1973), it was of interest to determine the kinds of RNA synthesized. Since ethionine is known to affect post-synthetic methylation of RNAs, the extent of methylation of RNAs synthesized in the presence of ethionine was also studied.

Human peripheral blood lymphocytes, at a concentration of $2 \times 10^6/\text{ml.}$, were stimulated by PHA in MEM containing 0.01 mM methionine with and without the addition of 5mM DL-ethionine. A comparison of the RNA species synthesized in the presence or absence of ethionine by mitogen stimulated lymphocytes was undertaken. At various times during the culture period, the cells were incubated with ^{14}C -uridine and methyl- ^3H -methionine to allow the simultaneous determination of the kinds of RNA being synthesized and their extent of methylation. To prevent the incorporation of label from methionine into the ring structure of the bases via the C-1 pathway, sodium formate (20mM), adenosine (20mM), and

guanosine (20mM) were added to the cultures (Perry and Kelley, 1974).

Figures 15 and 16 are sucrose gradient profiles of radiolabeled RNAs isolated from lymphocytes 20 hours after activation by PHA with and without ethionine. In this experiment methyl- ^3H -methionine and ^{14}C -uridine were present for the entire culture period. As can be seen, 4, 18, and 28S RNAs were all synthesized and methylated in the presence of ethionine, whereas, the rate of synthesis of these RNAs appeared to be greatly reduced. The data in Table 6 shows that the synthesis of 4S RNA, as measured by ^{14}C -uridine incorporation, in lymphocytes exposed to ethionine appeared to be decreased by 75% as compared with the 4S RNA from untreated lymphocytes. Similarly, the synthesis of 18 and 28S RNAs appeared to be reduced by 80 and 85%. The methylation of the RNAs as measured by incorporation of methyl- ^3H from methionine, is also greatly reduced in the presence of ethionine. There was, however, essentially no difference in the ratio of methyl to uridine incorporation in the 18 and 28S RNAs. By the same criteria, methylation of the 4S RNA appeared to be slightly inhibited in the presence of ethionine (Table 6).

It can also be seen that the amount of 4S RNA, as determined by absorbance at 260 nm, was greater relative to the amounts of 18 and 28S RNA in mitogen-stimulated lymphocytes exposed to ethionine for 20 hours than it was in unexposed cells (Figure 17). These absorbance profiles were

Figure 15. Sucrose Gradient Profile of Radio-labeled RNA Isolated from PHA Stimulated Lymphocytes after 20 Hours in Culture.

Human circulating lymphocytes ($2 \times 10^6/\text{ml}$) were incubated in 75 ml of MEM containing 0.01mM L-methionine, 20mM sodium formate, 20mM adenosine, and 20mM guanosine. PHA ($4\mu\text{g}/\text{ml}$) was added at the initiation of culture. The cells were labeled with $0.1\mu\text{Ci}/\text{ml}$ ^{14}C -uridine (sp. act. 60mCi/mM) and $5\mu\text{Ci}/\text{ml}$ methyl- ^3H -methionine (sp. act 15Ci/mM) for 20 hours. After incubation, RNA was extracted and analyzed for TCA precipitable radioactivity on 5-20% sucrose gradients as described in the Materials and Methods Section. Sedimentation is from left to right.

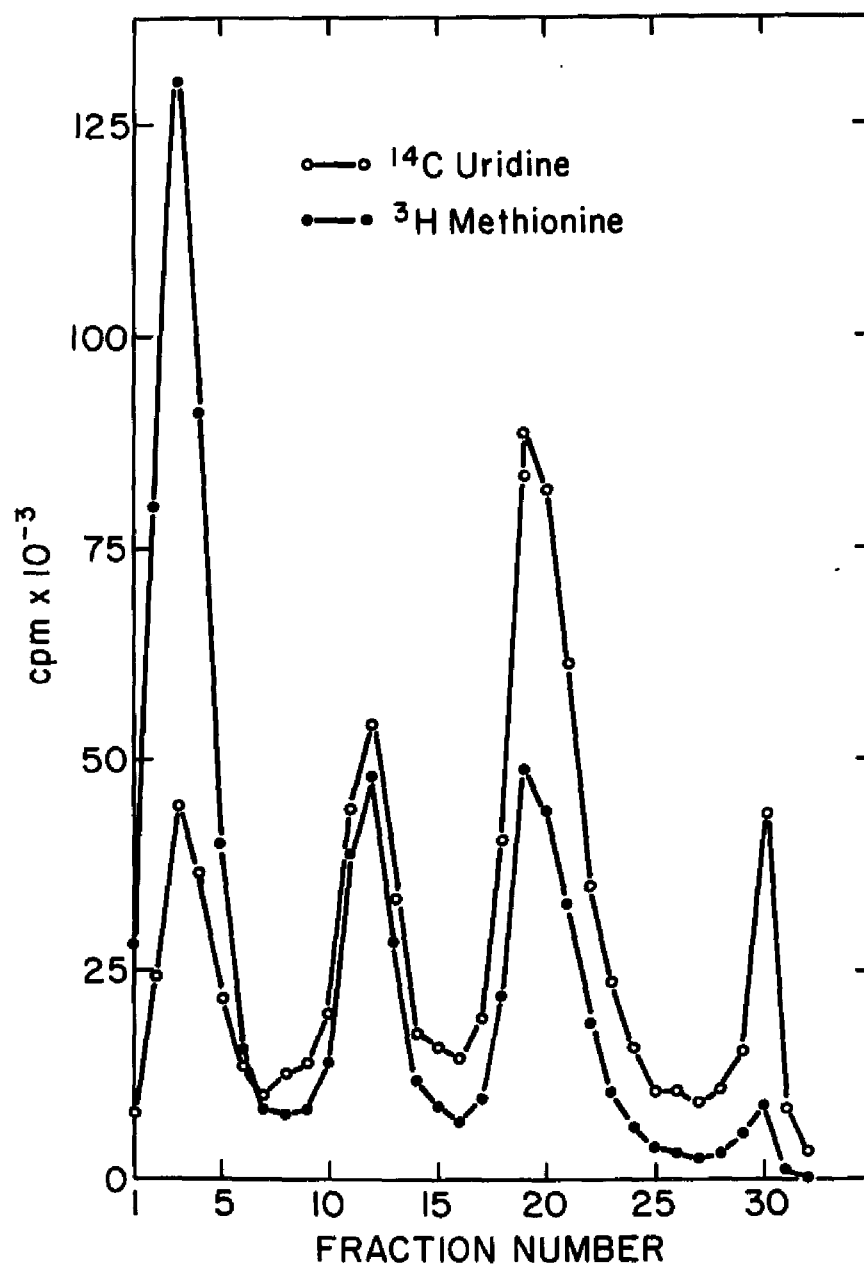
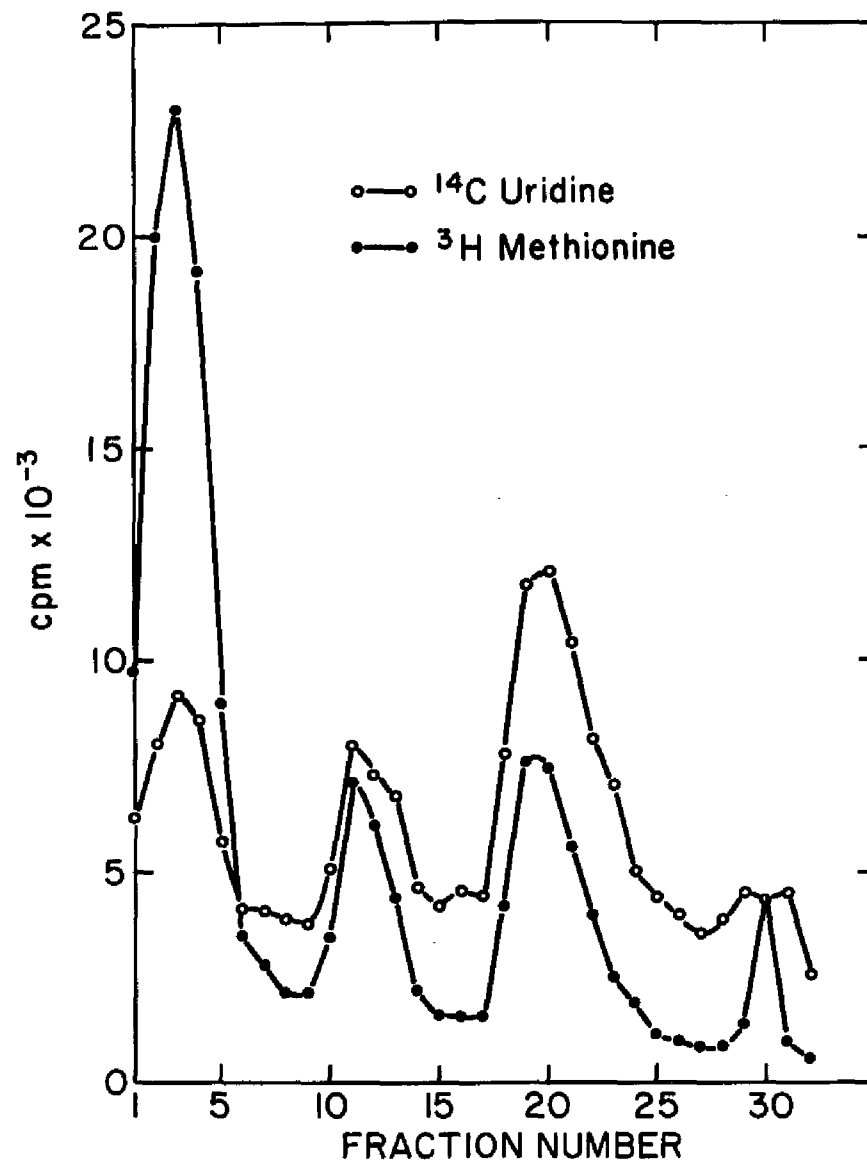


Figure 16. Sucrose Gradient Profile of Radiolabeled
RNA Isolated from PHA Stimulated
Lymphocytes after 20 Hours in Culture in
the Presence of Ethionine.

Conditions as described for Figure 15 except that
DL-ethionine (5mM) was present in the culture medium.



Legend to Table 6.

Human Lymphocytes (2×10^6 /ml) were incubated with $2\mu\text{g}$ PHA/ 10^6 cells in MEM containing 0.01mM methionine, 20mM adenosine, 20mM guanosine, and 20mM formate, with 5mM DL-ethionine added as indicated.

The cells were labeled with methyl- ^3H -methionine and ^{14}C -Uridine for 20 hours. RNAs purified from 1.5×10^8 cells were separated by sucrose gradient centrifugation as described in the Materials and Methods Section. Radioactivity incorporation data was corrected for overlap.

Table 6. Effect of DL-Ethionine on the Rates of RNA Synthesis and Methylation During a 20 Hour Labeling Period

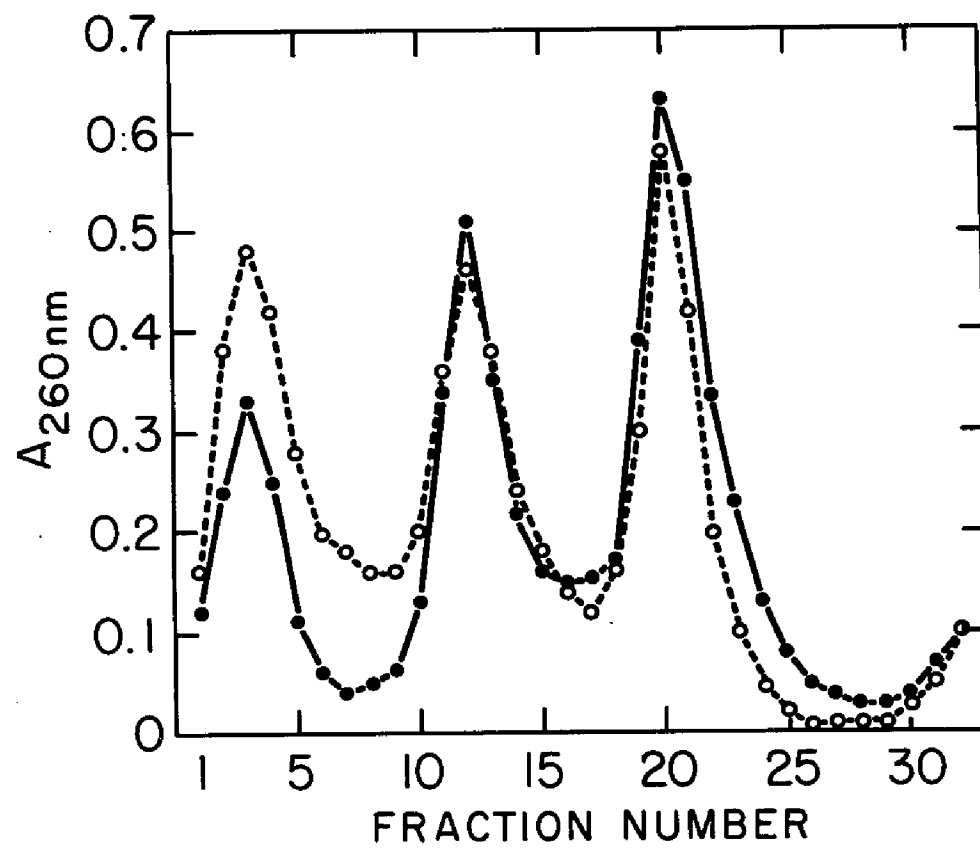
RNA Size Class	Additions: * None			DL-Ethionine		
	<u>CPM Incorporated</u>		M/U ⁺	<u>CPM Incorporated</u>		M/U ⁺
	³ H-Methionine	¹⁴ C-Uridine		³ H-Methionine	¹⁴ C-Uridine	
4S	386,770	148,430	2.61	83,484	42,088	2.03
18S	150,485	182,376	0.83	26,130	35,776	0.73
28S	186,480	352,023	0.53	33,720	61,964	0.54

* Additions other than PHA

+ Ratio of Methylation (M) to Synthesis (U)

Figure 17. Absorbance Profiles of RNA Isolated
from Lymphocytes Stimulated in the
Presence and Absence of Ethionine.

Culture conditions and sucrose gradient
analysis the same as described for Figures 15 and 16.
Absorbance was monitored at 260nm (●—●, PHA; O----O,
PHA + Ethionine).



typical of the ones obtained from PHA stimulated lymphocytes cultured in the presence and absence of ethionine.

When lymphocytes were exposed to methyl-³H-methionine and ¹⁴C-uridine three to six hours after the addition of mitogen, the picture was quite different (Figures 18 and 19). There was no difference in incorporation of uridine into 4S RNA by cells incubated in the presence and absence of ethionine. However, the uridine labeling of 18 and 28S RNAs was reduced by approximately 50% in the presence of ethionine (Table 7). The methylation of all three species of RNA was inhibited by 75% in the presence of ethionine (Table 7). The comparison of the ratios of methyl-³H-methionine and ¹⁴C-uridine incorporation in the presence and absence of ethionine indicated that all three species of RNA were undermethylated with the 4S RNA being most severely affected.

The synthesis and methylation of RNAs in the presence and absence of ethionine were also examined 18-21 hours after mitogen addition (Figures 20 and 21), during a more active period of RNA synthesis (Cooper, 1969a). In contrast to the earlier time period, no significant differences in the extent of methylation of the RNAs synthesized in the presence or absence of ethionine were seen (Figures 20 and 21, Table 8), although the absolute incorporation of methyl from methionine into the various RNAs was severely decreased.

This finding would seem to indicate that the RNAs synthesized in the presence of ethionine were, in fact, not undermethylated. However, when mitogen treated lymphocytes

Figure 18. Sucrose Gradient Profile of Radiolabeled
RNA Isolation from Lymphocytes Labeled from
Three to Six Hours after PHA Stimulation

Conditions the same as described for Figure 15 except that the labeling was carried out for a three hour period three to six hours after the addition of mitogen. RNA was isolated immediately following the termination of the pulse period.

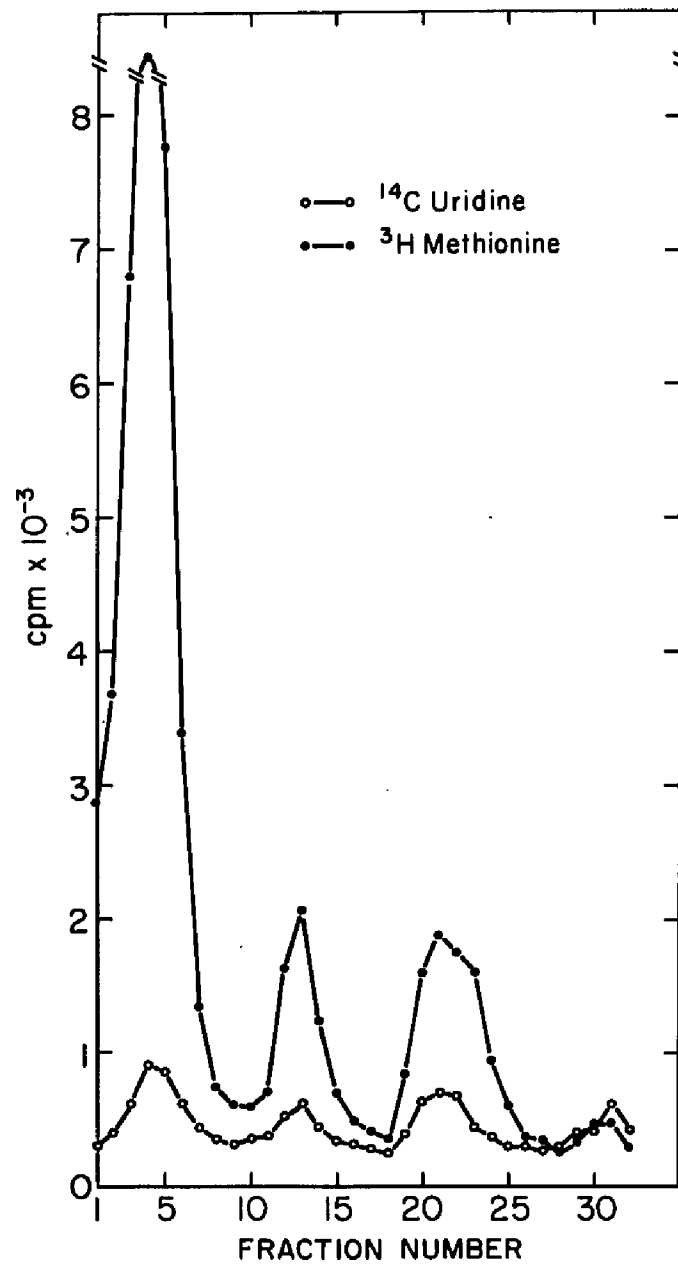
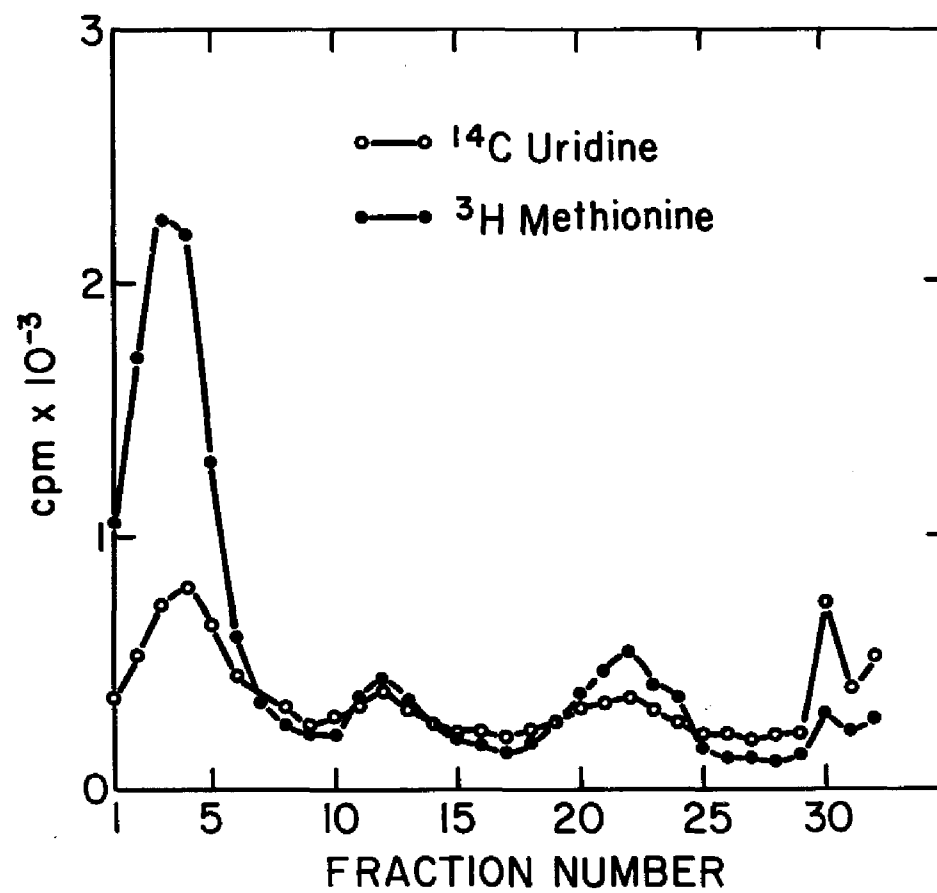


Figure 19. Sucrose Gradient Profile of Radiolabeled RNA Isolated from Lymphocytes Labeled from Three to Six Hours after PHA Stimulation in the Presence of Ethionine.

Conditions the same as described for Figure 18 except that DL-ethionine (5mM) was present during the culture period.



Legend to Table 7.

Same as Table 6 except that the labeling period was from three to six hours after addition of PHA.

Table 7. Effect of DL-Ethionine on the Rates of RNA Synthesis and Methylation
During a Labeling Period Three to Six Hours after Mitogen Stimulation

Additions: *		None		DL-Ethionine		
RNA Size Class	<u>CPM Incorporated</u>		M/U ⁺	<u>CPM Incorporated</u>		M/U ⁺
	³ H-Methionine	¹⁴ C-Uridine		³ H-Methionine	¹⁴ C-Uridine	
4S	34,160	2782	9.03	9083	3537	2.57
18S	6945	2688	2.58	1738	1725	1.01
28S	9245	2831	2.41	2203	1802	1.22

* Additions other than PHA

+ Ratio of Methylation (M) to Synthesis (U)

Figure 20. Sucrose Gradient Profile of Radiolabeled
RNA Isolated from Lymphocytes Labeled 18-
21 Hours after PHA Stimulation.

Culture conditions the same as described for Figure 15 except that the cells were washed and resuspended in serum-free medium prior to labeling. Labeling was carried out for three hours 18-21 hours after the addition of mitogen. RNA was isolated immediately following the termination of the pulse period.

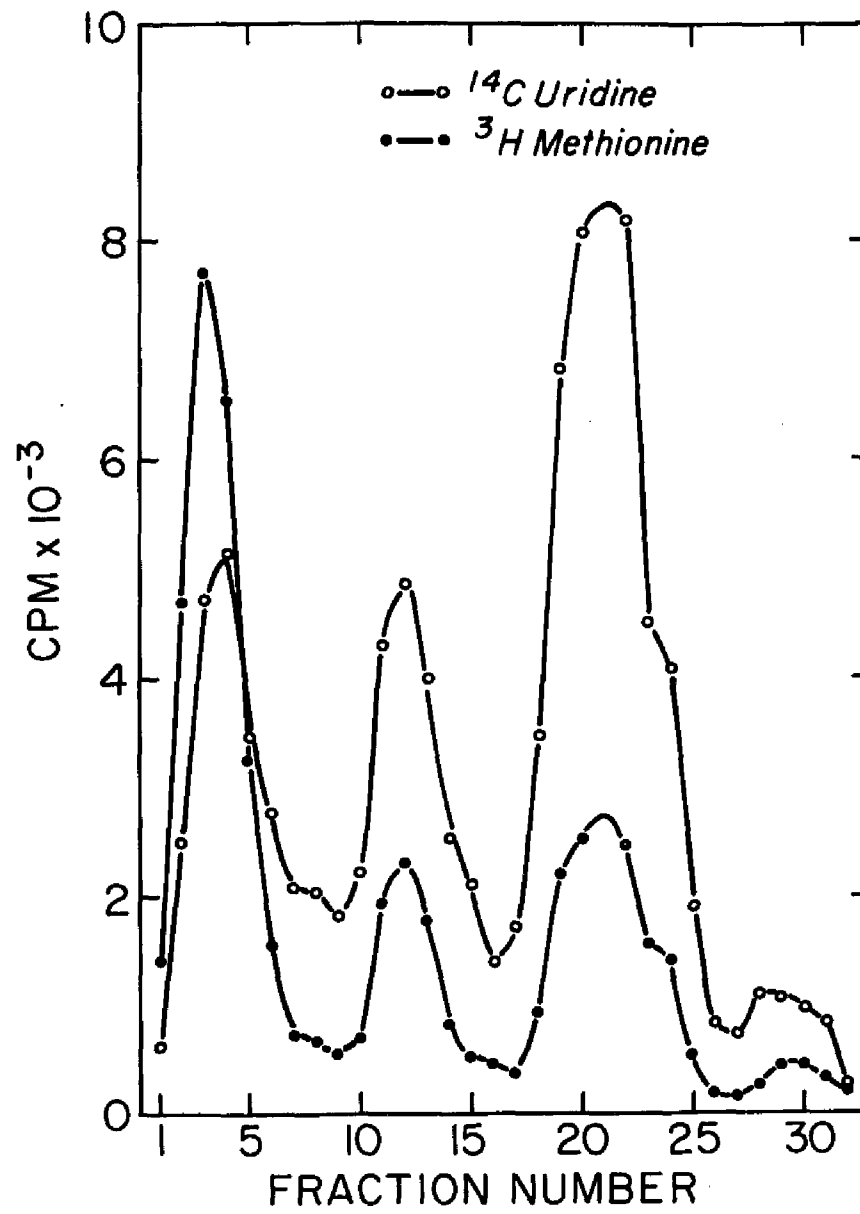
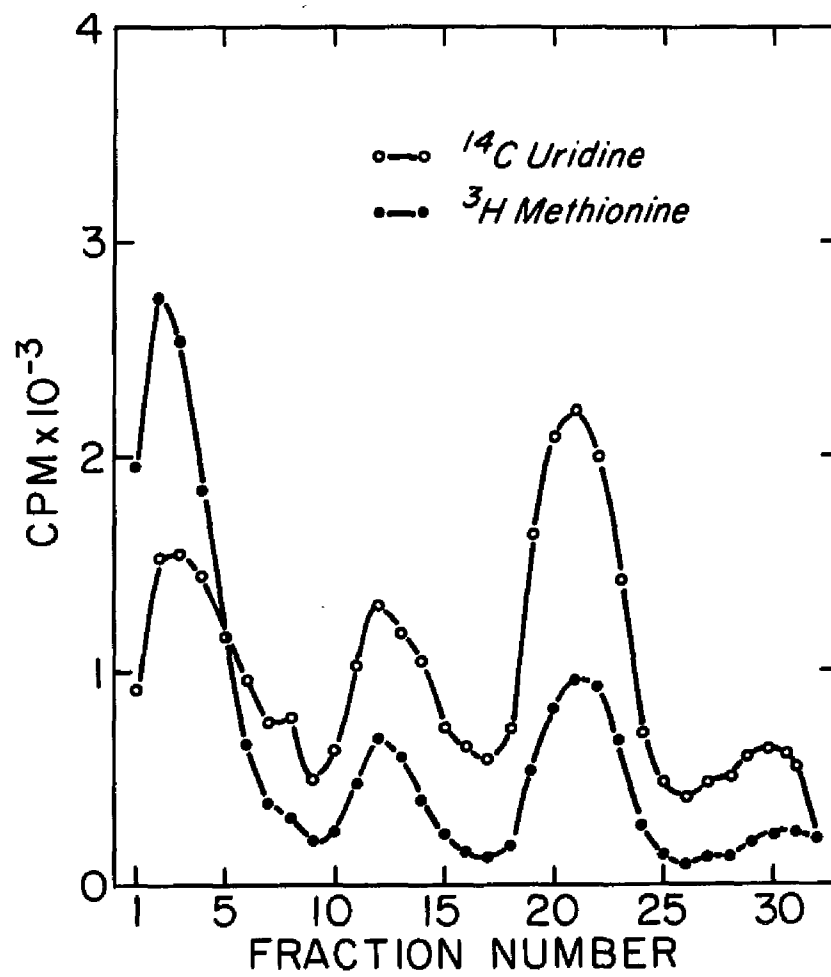


Figure 21. Sucrose Gradient Profile of Radiolabeled
RNA Isolated from Lymphocytes Labeled 18-
21 Hours after PHA Stimulation in the Pre-
sence of Ethionine.

Culture conditions the same as described for Figure
20 except that DL-ethionine (5mM) was constantly pres-
ent.



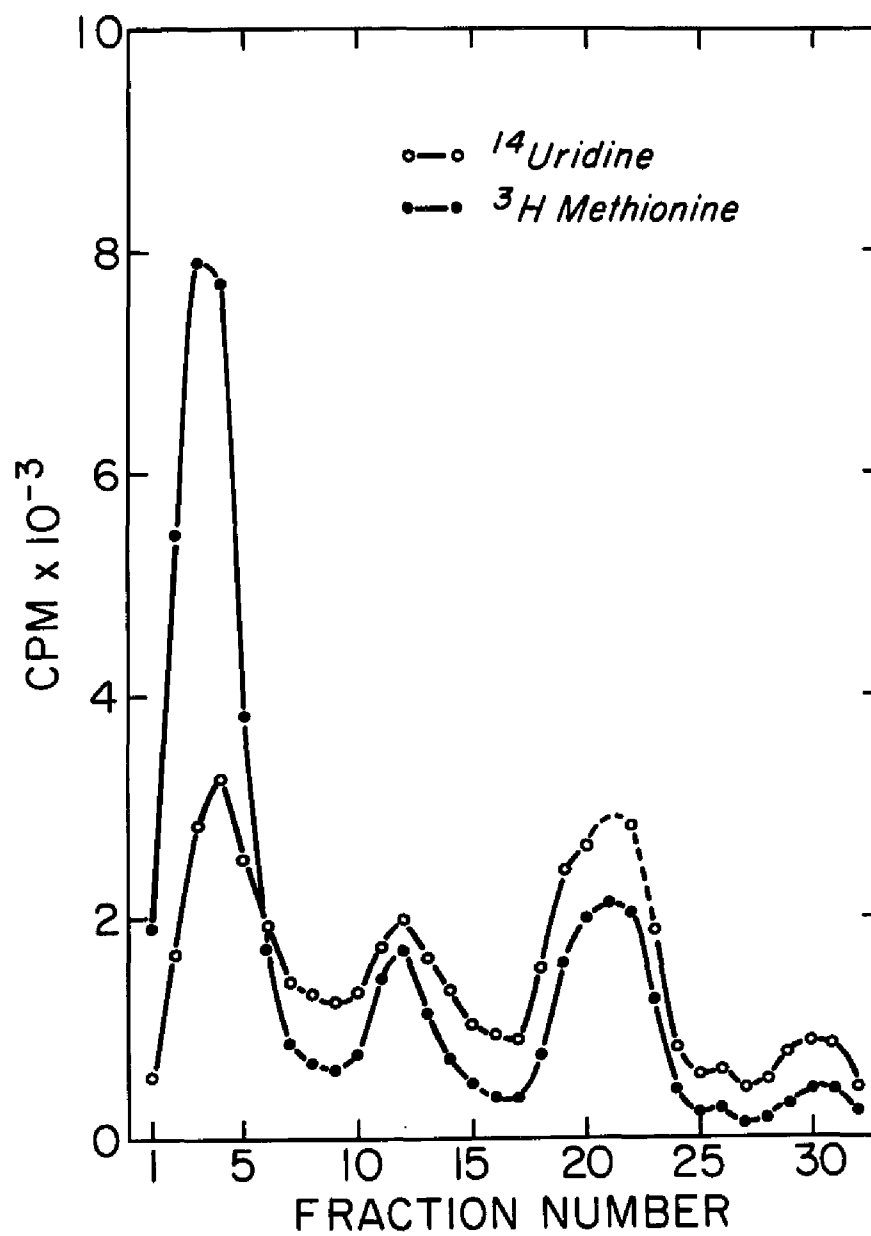
cultured in the presence of ethionine for 18 hours were washed free of ethionine immediately prior to labeling, the methylation of all types of RNA increased to a greater extent than their rate of synthesis (Figure 22, Table 8), a result which might indicate the presence of undermethylated RNAs or RNA precursors.

The rate of uridine transport, the rate of its phosphorylation and the size of the uridine pool have been shown to increase markedly during lymphocyte activation (Cooper, 1972). Thus, the rate of radio-labeled uridine incorporation into RNA, especially during short exposures, may not be an accurate reflection of the rate of RNA synthesis in mitogen stimulated lymphocytes. Since a similar effect of ethionine on uridine utilization might explain the apparently paradoxical results seen, an alternative method was used to determine whether methyl deficient RNA was synthesized in the presence of ethionine.

In parallel cultures PHA stimulated lymphocytes were incubated in the presence and absence of ethionine, as above, for 18 hours. During the final hour of incubation, actinomycin D (1 $\mu\text{g}/\text{ml}$) was added to inhibit further RNA synthesis. The cells were then washed to remove PHA and ethionine from the cultures. Actinomycin D was present during the washing period. The cells were then exposed to ^{14}C -uridine and methyl- ^3H -methionine for three hours in the presence of actinomycin D (1 $\mu\text{g}/\text{ml}$) to prevent synthesis during this time. Figure 23 is a sucrose gradient profile of the labeled RNAs

Figure 22. Sucrose Gradient Profile of Radiolabeled RNA Isolated from PHA Stimulated Lymphocytes Labeled after the Removal of Ethionine

Conditions same as described for Figure 21 except that DL-ethionine was omitted during the labeling period.



Legend to Table 8.

Human lymphocytes were incubated as described in the legend to Table 6. However, prior to labeling, all cells were washed with saline and resuspended in serum-free medium containing the constituents described. Ethionine (5mM) was added as indicated. The cells were labeled with methyl-³H-methionine and ¹⁴C-uridine from 18-21 hours after the addition of PHA. All subsequent steps were as given in Table 6 and the Materials and Methods Section.

Table 8. Effect of DL-Ethionine on the Rates of RNA Synthesis and Methylation
During a Labeling Period 18-21 Hours after Mitogen Stimulation

Additions:		Prior to Labeling;	None	DL-Ethionine		DL-Ethionine	
		During Labeling;	None	DL-Ethionine		None	
RNA Size Class	<u>CPM Incorporated</u>		<u>CPM Incorporated</u>		<u>CPM Incorporated</u>		
	³ H- Methionine	¹⁴ C- Uridine	³ H- Methionine	¹⁴ C- Uridine	³ H- Methionine	¹⁴ C- Uridine	
4S	25,176	19,215	10,899	7578	28,542	12,730	
18S	8165	19,778	2646	5934	6255	9070	
28S	14,794	45,488	4574	11,266	10,703	12,910	

isolated after actinomycin D treatment. Although new RNA synthesis, as measured by ^{14}C -uridine incorporation, was essentially absent under these conditions, 4S RNA was extensively methylated.

It was clear from the results of this experiment that methylation of tRNA could occur in the virtual absence of new RNA synthesis. This was probably a reflection of "late" modifications of tRNA (Munns and Sims, 1975) that continue for several hours after RNA synthesis is completed, a situation analogous to that observed during DNA synthesis (Adams and Hogarth, 1973). In any case, in the presence of actinomycin D, almost twice as many methyl groups were incorporated into the 4S RNA of cells pre-treated with PHA and ethionine as into the 4S RNA treated with PHA alone (Table 9, Figure 23). Thus it appeared that at least some of the tRNA synthesized in the presence of ethionine contained sites whose methylation was prevented by the presence of ethionine and which could be methylated once ethionine was removed.

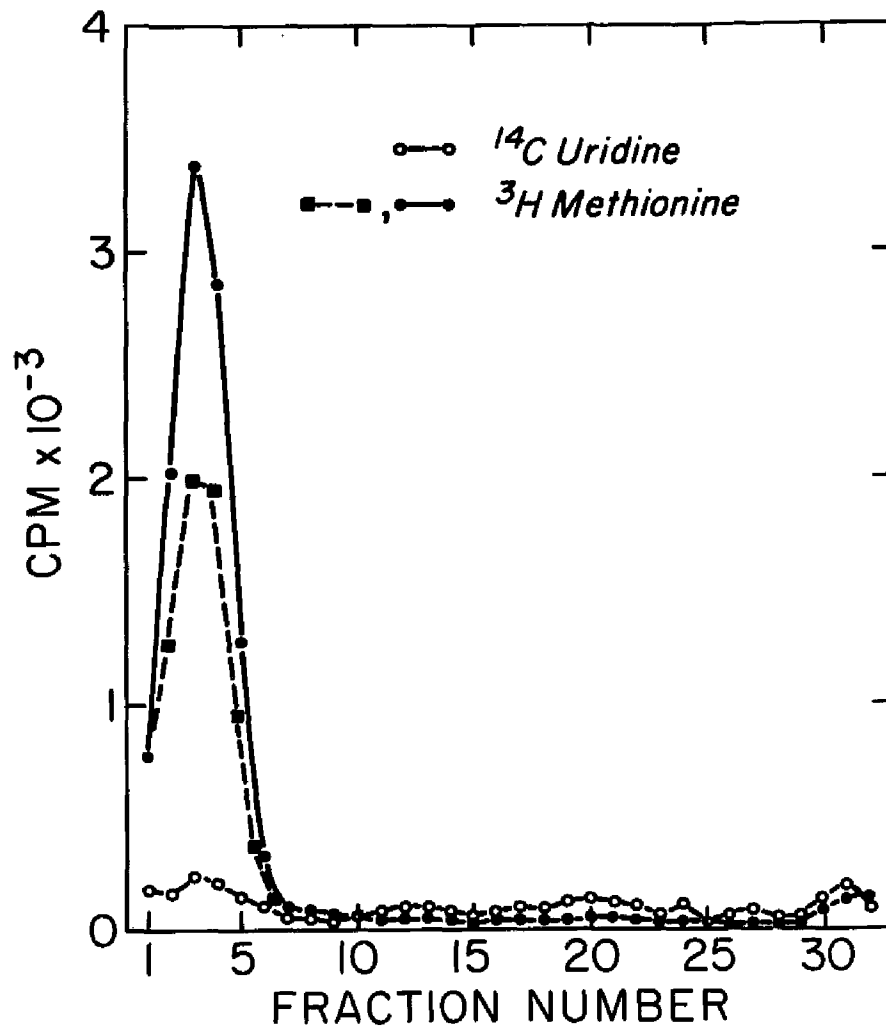
It should be noted that the acid precipitable methyl label found in the 4S region of the gradient was sensitive to ribonuclease digestion. Furthermore, the methyl label found in this region did not represent methionine in an aminoacyl linkage to tRNA since determination of ^{35}S -methionine esterified to tRNA isolated from PHA stimulated lymphocytes indicated that less than one molecule of methionine per 2000 tRNA molecules remained esterified during RNA isolation

Figure 23. Sucrose Gradient Profile of Radiolabeled RNAs Isolated from Lymphocytes Labeled in the Presence of Actinomycin D.

Two different gradient profiles are displayed in this figure. In one case (● ————— ●) 1.5×10^8 lymphocytes had been incubated as described for Figure 20. Actinomycin D ($1 \mu\text{g/ml}$) was present during the last hour of this incubation period and throughout the washing and pulse label period.

In the second case (■ ————— ■) the conditions were the same except that DL-ethionine was present during the incubation period but not during the labeling period.

As discussed in the text, the lack of ^{14}C -uridine incorporation (O ————— O) indicated that virtually no new RNA synthesis occurred.



Legend to Table 9.

The conditions of this experiment were the same as described in the legend to Table 8 except that actinomycin D (1 μ g/ml) was added to two separate cultures as indicated. Actinomycin D was also present during the washing period. Further details are given in the text and in the Materials and Methods Section.

Table 9. Effect of Actinomycin D on 4S RNA Synthesis and Methylation

Additions:	Prior to Labeling; During Labeling;		None Actinomycin D		DL-Ethionine Actinomycin D	
	³ H- Methionine	¹⁴ C- Uridine	³ H- Methionine	¹⁴ C- Uridine	³ H- Methionine	¹⁴ C- Uridine
CPM Incorporated	25,167	19,215	6795	1345	10,607	1090
% Original Label	100	100	27	7	42	5.6

(Materials and Methods).

No further methylation of 18 and 28S RNA was detected in either preparation in the presence of actinomycin D (Figure 23). It is well known that the bulk of rRNA methylation occurs as the 45S precursor is being transcribed and that this methylation is necessary for proper processing (Maden and Salim, 1974). In several systems described, interference with proper methylation of rRNA led to preferential degradation (Vaughn *et al.*, 1967; Swann *et al.*, 1975). Although in the absence of RNA synthesis, there appeared to be no further methylation of the rRNA, the possibility of the presence of undermethylated rRNA or precursor cannot be ruled out. A further indication for the presence of hypomethylated rRNA was seen in the experiment where ethionine was removed and RNA synthesis was allowed to proceed. In that instance the extent of rRNA methylation markedly increased after ethionine removal.

IV. Ethylation of Macromolecules and Ethionine Incorporation into Proteins

It has been proposed that ethionine may mediate its many effects by modifying the functional properties or the stability of nucleic acids and proteins by post-synthetic ethylation of these molecules or by the substitution of ethionine for methionine in proteins. To evaluate the importance of ethylation and ethionine substitution in regard to inhibition of lymphocyte activation, the extent to which

radioactivity from ethyl-³H-ethionine could be incorporated into macromolecules was determined. Under standard experimental conditions, where the concentration of DL-ethionine was 40-80 fold higher than the L-methionine concentration, incorporation of ethyl-³H-ethionine into acid precipitable material by mitogen stimulated lymphocytes was not significant. The radioactivity found in these cells was barely above background levels and was considered to be artifactual.

V. Effect of Ethionine on ATP Levels in Mitogen-Stimulated Lymphocytes

It has been reported that ethionine administration to rats could cause a depletion of hepatic ATP concentration to 20% of its original level within two hours (Villa-Trevino et al., 1966). These authors suggested that this excessive trapping of the adenosine moiety of the nucleoside as S-adenosylethionine accounts for the observed inhibition of macromolecular synthesis in the livers of the treated animals. The extent of depletion of the ATP pool was dependent on the dose of ethionine and could be counteracted by adenine or methionine administration.

In all of the experiments presented above, the media contained methionine. Furthermore, adenosine, which was present in the culture media where RNA methylation was examined had no effect in relieving ethionine mediated inhibition of DNA synthesis. Even though these observations,

taken together with the evidence that increased rates of RNA and protein synthesis by mitogen stimulated cells could occur in the presence of ethionine, seemed to rule out ATP depletion as the mechanism by which ethionine caused the observed inhibition of DNA synthesis, it was believed to be of value to directly examine the effect of ethionine on the ATP level under the experimental conditions used here.

Direct determination of the ATP levels in lymphocytes was accomplished using the luciferase method (Stanley & Williams, 1969). Measurement of ATP levels in mouse spleen lymphocytes, made after 20 hours in culture, gave values of 2.07×10^{-10} moles/ 10^6 cells in the uninduced cultures; 3.50×10^{-10} moles/ 10^6 cells in cultures stimulated by PHA; and 2.62×10^{-10} moles/ 10^6 cells in cultures stimulated by PHA in the presence of 10mM DL-ethionine. It is worth noting that although the ATP level in mitogen stimulated, ethionine treated cells was higher than in resting lymphocytes, they incorporated ^3H -thymidine into DNA at a slower rate (see Table 1).

Although there did not appear to be a direct correlation between the level of ATP in the cells and the rate of DNA synthesis, as measured by ^3H -thymidine incorporation, it was clear that the increase in the level of ATP due to mitogen stimulation of lymphocytes was inhibited by the presence of ethionine. Consequently, the possibility that a critical level of ATP required for the initiation of DNA synthesis or for the induction of specific enzymes was not

achieved in the presence of ethionine cannot be ruled out.

DISCUSSION

In this project the effect of ethionine on lymphocyte activation was studied. It was seen that ethionine inhibited the initiation of DNA synthesis in mitogen stimulated lymphocytes. The inhibition was, however, reversible; removal of ethionine resulted in a rapid initiation of DNA synthesis in these cultures. Biochemical and morphologic evidence suggested that mitogen stimulated lymphocytes could, in the presence of ethionine, enter an active G_1 state of the cell cycle, with increases in the rates of protein and RNA synthesis. They were, however, blocked from entering the S phase. In trying to determine the nature of this block, several of the mechanisms by which ethionine is believed to affect cellular metabolism were studied.

Because animal models used to study ethionine effects are, by necessity, quite complex, it has been difficult to determine which of the many metabolic consequences of ethionine administration lead to alterations in cellular control processes and ultimately lead to malignancy. Since lymphocytes, under proper stimulation, can pass from a quiescent to actively dividing state, they provide a very good system for studying agents that may affect biochemical events involved in cell division. The effect of ethionine on lymphocyte activation was studied to gain further insight into the mechanism involved in lymphocyte activation, as well as to understand more fully the mode of action of ethionine.

It was found that the presence of ethionine in the culture could completely inhibit DNA synthesis by mitogen stimulated lymphocytes. Irrespective of the cell source used (mouse spleen or human circulating lymphocytes), a similar ethionine effect was seen. This effect of ethionine on DNA synthesis was completely and rapidly reversed when ethionine was removed from the culture.

These data appeared compatible with the supposition that many of the reactions necessary for the initiation of DNA synthesis by these cells had occurred, even though DNA synthesis itself was blocked. Several lines of evidence support this proposal:

(1) Rapidity with which the onset of DNA synthesis occurs after ethionine removal. Measurable DNA synthesis occurred within four hours after ethionine removal (Figure 1) at 17, 40, or 60 hours (not shown) in culture. Contrary to this finding an increase in the rate of DNA synthesis occurred only after a 24 hour lag period when PHA alone was added at 0 or 40 hours in culture.

(2) Commitment to DNA synthesis. As the data in Table 4 showed, the commitment to DNA synthesis could take place even in the presence of ethionine. Ethionine did not prevent Con A binding and initiating lymphocyte stimulation. Furthermore, when Con A was removed from the lymphocyte surface by α -methylmannose after sufficient time to initiate lymphocyte activation (Gunther et al., 1974), the rate of DNA synthesis was similar in cultures that had been

stimulated in the presence and absence of ethionine.

(3) Blast transformation. The rates of protein and RNA synthesis in the presence of mitogen and ethionine were substantially higher than those seen in unstimulated cells, but lower than the rates observed in lymphocytes stimulated in the absence of ethionine (Table 5). Ethionine removal also resulted in increased rates of synthesis of these macromolecules, although maximum synthesis occurred considerably after the onset of DNA synthesis.

These findings were in contrast to the striking inhibition of RNA and protein synthesis in rat liver following ethionine administration as shown by Farber and coworkers (Villa-Trevino et al., 1963, 1966) who proposed that their results were due to a rapid depletion of the cellular ATP concentration due to the trapping of the adenosine moiety as S-adenosylethionine. In those experiments, methionine intake was restricted, whereas in the conditions used here, a normal methionine concentration in the medium was maintained. The ATP pool was not depleted and, in fact, increased to some extent in response to mitogen, even though ethionine was present.

Even though the ATP levels in mitogen stimulated cells in the presence of ethionine were higher than in resting cells, these lymphocytes incorporated thymidine into their DNA at rates equal to or lower than non-stimulated cells. Taken together with the increased protein and RNA synthesis, it seems unlikely that a decrease in ATP pool size caused

the inhibition of DNA synthesis by ethionine, although the possibility that a critical ATP concentration required for DNA synthesis was not reached cannot be ruled out.

(4) Morphologic changes. The light micrographs of non-stimulated lymphocytes revealed cells that were very small, had a highly condensed nucleus, and contained meager cytoplasm. Even in the presence of ethionine, morphologic changes characteristic of mitogen stimulation could be seen. However, the cells treated with ethionine never progressed as far as the control cells, and at 72 hours no cells in mitosis were seen. On the other hand, 48 hours after ethionine removal, (Figure 11), the cells were indistinguishable from cells cultured for 72 hours in the presence of PHA alone.

Thus it appears that ethionine permits the transition of stimulated lymphocytes from the quiescent G_0 stage into an active G_1 phase of the cell cycle, but blocks entry of the cells into S. It was hoped that studies of the reactions affected by ethionine could yield information about the processes involved in the initiation of DNA synthesis, not only in stimulated lymphocytes, but in the initiation of the S phase in general.

It is generally believed that the degree of chromatin condensation is an indication of the amount of gene activity (Allfrey, 1971). The degree of chromatin condensation greatly decreased in mitogen stimulated lymphocytes, despite the presence of ethionine. In the electron

microscope prominent nucleoli with morphologic characteristics of increased rRNA synthesis were seen in the ethionine treated cells. In addition, in the mitogen stimulated cells, both in the presence and absence of ethionine, significantly higher numbers of ribosomes were present as polysomes than were seen in unstimulated cells.

The morphologic observations taken together with the biochemical data indicated that, in contrast to various animal models, ethionine did not appear to cause gross toxicity in these cells. Indeed, the rapidity with which the cells recover from the effects of ethionine, merely by its removal by washing, argues against a toxic effect. In contrast, in rats, an acute toxicity, seen within a few hours was caused by ethionine administration (Shinozuka et al., 1968). The authors found gross morphologic alterations, attributable to drastic ATP depletion, in the rat hepatocytes. Nucleoli underwent disorganization and fragmentation and chromatin condensation was evident. These changes could be reversed by replenishment of the cellular ATP levels by adenine administration or by methionine.

These morphologic discrepancies could possibly be explained by the fact, as was mentioned above, that in ethionine treated lymphocytes, no decrease in ATP levels occurred. The levels of ethionine used could be a critical factor; much higher concentrations were used to yield the toxic effect in rats than were used to inhibit DNA synthesis in lymphocytes. In addition, in many of these experiments,

especially when RNA was isolated and in the morphologic studies, adenosine was included in the culture medium.

In view of the finding that label from radioactive ethionine was not significantly incorporated into acid precipitable material in the culture conditions used here, it seems unlikely that ethionine incorporation into protein or ethylation of various macromolecules is the cause of the inhibition of DNA synthesis. Since one of the major effects of S-adenosylethionine formation in cells treated with ethionine is a decreased methylation of macromolecules mediated by S-adenosylmethionine (Shull et al., 1966; Smith and Salmon, 1965) it was of interest to look at the effect of ethionine on macromolecular methylation in stimulated lymphocytes. It is possible that ethionine may act to inhibit DNA synthesis in mitogen stimulated lymphocytes by the inhibition of post-synthetic methylation of some molecules necessary for the initiation of that synthesis.

Since DNA methylation occurs after synthesis (Adams and Hogarth, 1973), it seemed unlikely that the complete inhibition of DNA synthesis seen in the ethionine treated cells was due to decreased DNA methylation. There is no entry of label from methyl-³H methionine into lymphocyte DNA during the first 24 hours after mitogen stimulation (P. Zabos personal communication), so it appears unlikely that a DNA hypomethylation is causing the inhibition of synthesis in this system.

The effect of ethionine on RNA synthesis and methylation

was studied. Ethionine has been shown to interfere with RNA methylation in many systems (Rajalakshmi, 1973; Wainfan et al., 1975; Wainfan and Balis, 1975). Whether studied during an early (three to six hours) or later (18-21 hours) pulse following mitogen stimulation, RNA methylation was inhibited by the presence of ethionine in the culture. Also, the overall methylation (20 hour labeling) of RNA was decreased in the presence of ethionine.

In consideration of the data presented here, 4S and tRNA have been used synonymously. The peak on sucrose gradients corresponding to "4S", in fact, contains a small amount of 5S rRNA (about 10% of the peak) that cannot be separated by this technique. However, this RNA species is not methylated and can in effect be ignored for the purposes of discussion of methylation. (see page 129A)

Differences in the rates of RNA synthesis measured by ^{14}C -uridine incorporation in the presence and absence of ethionine were more difficult to evaluate. It is conceivable that the presence of ethionine may affect the rate of uridine transport, pool size, and utilization which all increase in response to mitogen (Cooper, 1972). Especially early in the response, these factors could affect the apparent rate of RNA synthesis.

However, as seen in Figures 17 and 18, from three to six hours after PHA stimulation there was little difference between the apparent rates of synthesis of tRNA in the ethionine treated and untreated cells. On the other hand,

In addition to the 5S RNA that contaminates the 4S peak on sucrose gradients, there may also be a small amount of low molecular weight mRNA present. These RNA's could contribute to the data on methylation because they may have methylated 5' termini. The presence of these RNA molecules has not been ruled out.

there was extensive methylation of the tRNA in the absence of ethionine that was greatly reduced when ethionine was present. The synthesis and methylation of the rRNAs both appeared reduced at this time. The high rate of methylation of 4S RNA seen at this time is in good agreement with the findings of Sharma and Loeb (1972) who reported an increased rate and altered pattern of tRNA methylation within several hours after addition of PHA that was dependent upon new tRNA synthesis.

The difference in the rate of RNA synthesis and methylation between ethionine treated and untreated cells was more pronounced at a later time (18-21 hours) after stimulation. Only at the earlier time (three to six hours after PHA addition) was methylation more severely inhibited than RNA synthesis, as measured by uridine incorporation. The synthesis of 4S RNA appeared least affected by the presence of ethionine. The amount of 4S RNA, as measured by absorbance at 260 nm, relative to the other species appeared to be greater in the presence of ethionine.

Internal comparisons of the extent of methylation to RNA synthesis in the presence and absence of ethionine (Tables 6-8) indicated, especially for tRNA, that undermethylated species were present. Further evidence for hypomethylated RNA species in the ethionine treated cells was obtained when cells were labeled after the removal of ethionine from the culture (Table 8). A two fold increase in the rate of tRNA and rRNA methylation occurred immediately

following the removal of ethionine at 18 hours after PHA stimulation. This marked increase in RNA methylation was accompanied by only a slight increase in the rate of RNA synthesis. Undermethylated tRNA, produced in the presence of ethionine, has been demonstrated in several systems by an increased in vitro acceptance of methyl groups from S-adenosylmethionine mediated by cytoplasmic methyl transferase enzymes isolated from a homologous source (Wainfan et al., 1975; Rajalakshmi, 1973). To show unequivocally that undermethylated tRNA exists in ethionine treated lymphocytes it will be necessary to isolate unlabelled tRNA from ethionine treated and untreated lymphocytes and measure their methyl acceptor ability in a homologous in vitro methylating assay. There are, however, some technical difficulties associated with this assay for lymphocytes. Lymphocytes have a large amount of nuclease activity which coisolates with the crude cytoplasmic methylase preparation used for most in vitro studies. Purification of tRNA methylases is extremely difficult, and as yet no one has been able to obtain a homogeneous preparation (Nau, 1976). In addition, many of the agents used to inhibit nuclease activity also inhibit methyl transferase activity. However, it would be possible to use enzymes isolated from mouse and human tissues other than lymphoid. For example, enzymes prepared from mouse L cells could be used in an in vitro assay to methylate tRNA isolated from mouse spleen lymphocytes.

The demonstration of the existence of undermethylated rRNAs was more difficult, although their presence was suggested by the greater increase in the extent of methylation than of synthesis of rRNA following ethionine removal. The lack of rRNA methylation in the absence of synthesis was not surprising since it has been well documented that the majority of rRNA methylation occurs as ribose methylations while the 45S precursor rRNA is being transcribed (Maden and Salim, 1974). Although a small number of base methylations occurs after the molecule is cleaved during maturation, the methods used here may not have been sensitive enough to detect any methylation after synthesis was stopped. It is thus a matter of conjecture that undermethylated rRNA was produced. It is likely that, as has been described in other systems (Vaughn et al., 1967; Swann et al., 1975), undermethylated rRNA precursors were not processed but rather degraded. The 18 and 28S RNA that appeared in the cytoplasm may have been normally methylated whereas any undermethylated rRNA made in the presence of ethionine would not be seen because of degradation.

The concentration of actinomycin D used in these experiments did not totally inhibit tRNA synthesis regardless of whether the cells had a prior treatment with ethionine. Thus, the rates of tRNA synthesis were approximately 5% of the rates seen before actinomycin D inhibition (Table 9). It has been reported previously that the synthesis of tRNA is more resistant to inhibition by actinomycin D than that

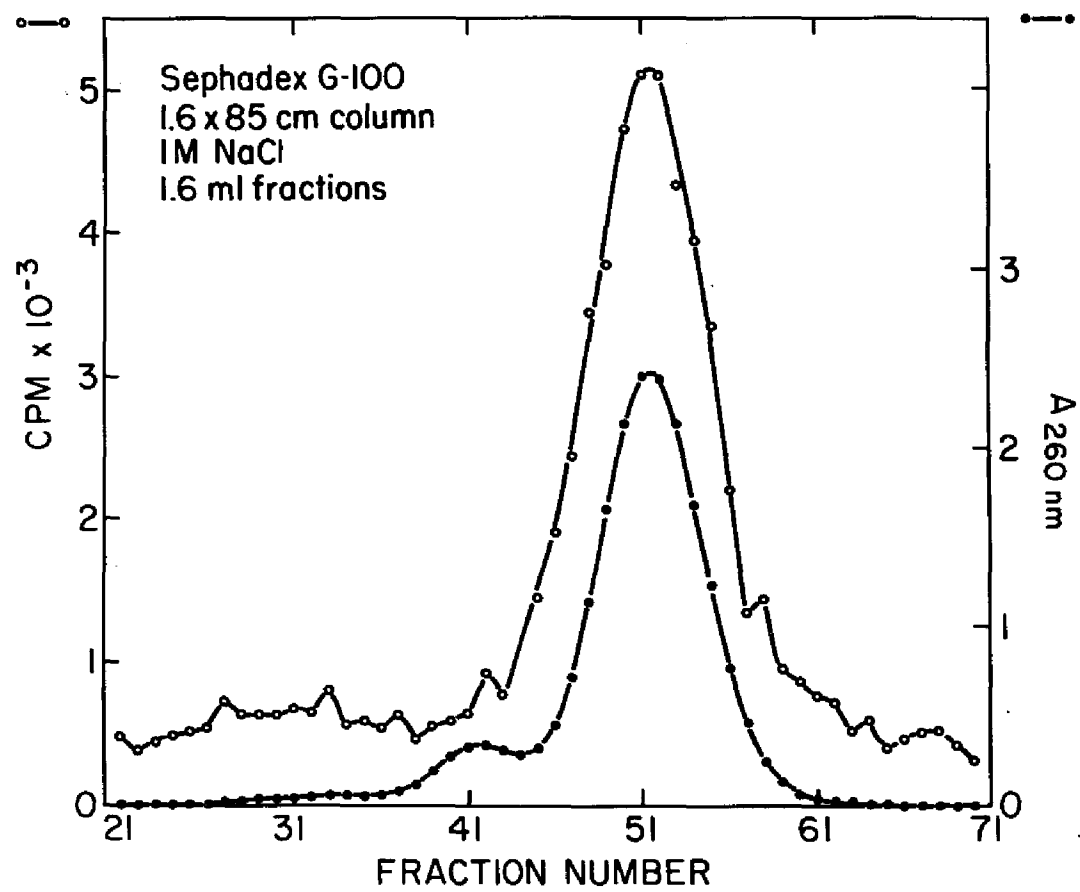
of rRNA (Franklin, 1963). Although the residual rate of tRNA synthesis in the presence of actinomycin D was approximately the same in both cell populations, there was increased incorporation of methyl groups into the tRNA from the ethionine treated cells (Figure 23) indicating the presence of more hypomethylated tRNA.

There are several pieces of evidence indicating that the label seen in the 4S peak on the gradients was due to methylation of tRNA. (1) The label was not due to charged tRNA. Incubating cells with ^{35}S -methionine showed that under the conditions of culture and RNA isolation, the label was not due to methionine attached to tRNA in an aminoacyl bond. (2) The radioactivity seen in the 4S peak was not due to DNA breakdown because incubation of this sample with ribonuclease eliminated the major part of acid precipitable material. (3) The sedimentation profile of the RNA from actinomycin treated cells showed a sharp peak in the 4S region. If the radioactivity was from methyl- ^3H -methionine incorporated into large RNAs that were subsequently degraded, a broad, disperse peak would be seen. Further, methyl- ^3H -methionine labeled tRNA isolated from actinomycin D treated cells cochromatographs with E. coli tRNA on a Sephadex G 100 column (Figure 24).

It was of interest that a significant amount of methylation could occur on the tRNA from cells not exposed to ethionine several hours after synthesis. It has been suggested (Tidwell and Howard, 1972) that post-transcriptional

Figure 24. Gel Filtration of tRNA

4S RNA isolated from lymphocytes treated as described in Figure 23 (O—O) cochromatographs with tRNA from E.coli B (●—●). See the Materials and Methods Section for additional experimental details.



tRNA modifications in regenerating rat liver fall into two classes: (1) modifications that are tightly coupled to tRNA synthesis and (2) those that are not. Cells may normally contain undermethylated, supposedly nonfunctional, tRNA molecules that may be converted to active molecules by methylation under proper stimulus. A potential use of ethionine treatment and removal in stimulated lymphocytes could be a test of this hypothesis.

Furthermore, a temporal sequence of tRNA methylation has been described (Munns and Sims, 1975). Three classes of tRNA methylations have been shown: early (1-methylguanosine, N 2, 2-dimethylguanosine, and 7-methylguanosine), intermediate (5-methylcytidine, 5-methyluridine, and 1-methyladenosine) and late (N 2-methylguanosine and 2' O-methylcytidine). However, the relationship of these reactions to tRNA maturation is still not known. It would be of considerable interest to determine the patterns of methylation in actinomycin D treated lymphocytes to see whether ethionine selectively inhibits any of these methylations. Differences in tRNA methylation patterns may yield alterations in their functions. Sharma and Loeb (1972) found a change in the pattern of tRNA methylation in lymphocytes that may possibly reflect a change in functional characteristics. A late methylation, N-2 methylguanosine, was increased in amount after PHA stimulation with a concurrent decrease in the amount of 1-methyladenosine.

Future experiments will involve studying the effect of

ethionine treatment on these particular modifications.

There have been reports from several laboratories that ethionine selectively reduces the amount of several modified nucleosides (including N2-methylguanosine, N2, 2-dimethylguanosine, 5-methylcytidine and 5-methyluridine) found in rat liver tRNA after short and long term ethionine administration (Lu et al., 1976; Friedman, 1977). It will be of interest to see whether the amount of any of these modified nucleosides are decreased in the tRNA isolated from mitogen stimulated lymphocytes treated with ethionine, especially in view of the alteration in tRNA methylation that occurs following stimulation (Sharma & Loeb, 1972). Base analysis of the tRNAs synthesized in the presence and absence of ethionine and after ethionine removal (with and without the presence of actinomycin D) could help elucidate a possible relationship between tRNA methylation and DNA synthesis.

The degree of modification of specific tRNAs may affect not only their role in protein synthesis, but also their ability to play other regulatory roles (Singer et al., 1972). Therefore, it is not implausible that alterations in tRNA methylation may, in some way, affect the initiation of DNA synthesis in mitogen stimulated lymphocytes. Although a lack of correlation between tRNA methylation and DNA synthesis in mitogen stimulated lymphocytes has been reported (Chang and Coward, 1975) these studies looked at inhibition of tRNA methylation after the initiation of DNA synthesis. It would seem that studies such as those presented here look-

ing at early reactions will yield more information, and discover a possible role of RNA methylation in the initiation of DNA synthesis in mitogen stimulated lymphocytes.

However, it is most likely that the effect of ethionine on lymphocyte activation is not at the level of new protein and RNA synthesis in stimulated cells, but rather at the level of post-synthetic modification of specific macromolecules believed to be involved in regulating gene expression. Examples might be ethionine induced alterations in the modification of histones and non-histone proteins seen in activated lymphocytes.

CONCLUSIONS

It has been demonstrated that ethionine can reversibly inhibit the initiation of DNA synthesis by mitogen stimulated lymphocytes. Ethionine did not affect the morphological changes characteristic of blast transformation. Complete commitment to DNA synthesis could take place in the presence of ethionine. Some of the early reactions in lymphocyte activation could take place in the presence of ethionine as evidenced by substantial increases in the rates of RNA and protein synthesis and the rapidity with which the onset of DNA synthesis occurred after the removal of ethionine.

It was shown that ethionine was not incorporated into macromolecules, either as a substitute for methionine in proteins or as post-synthetic ethylation. Furthermore, ethionine did not deplete lymphocyte ATP levels, although it did inhibit, to some extent, the ATP levels seen in mitogen-stimulated lymphocytes. Finally, it was shown that ethionine interfered with the methylation of tRNA. However, a direct connection between tRNA methylation and regulatory events necessary for the initiation of DNA synthesis has not yet been established.

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