Effect of Purine and Pyrimidine Base Analogues on Lymphocyte Proliferation

by

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ABSTRACT

The purine base analogues 8-azaguanine (8-AG) and 6-thioguanine (6-TG) were used to alter RNA synthesis at different times during the activation of mouse splenic lymphocytes with concanavalin A (Con A) and the proliferative response of these cultures was assayed at 48 hours. In 42 hours-old cultures, a 6 hour treatment with 66 μM 8-AG or 6-TG caused an irreversible and total inhibition of ^{3}H-thymidine incorporation while uridine and leucine incorporations were not or little affected. There was also little inhibition of the incorporations of uridine and leucine in resting lymphocytes similarly treated. The inhibition of thymidine incorporation by the analogues could be prevented by the simultaneous addition of guanine but, once expressed, the addition of guanine did not reverse it. Inhibiting protein synthesis also did not affect the inhibitory action of the analogues in proliferating lymphocytes.

Pretreatments of the non-proliferating cultures with the purine analogues, for 6 hours, at any time during activation, whether for the first 6 hours of culture or during G1 caused the same severe inhibition of ^{3}H-thymidine incorporation at 48 hours as for the treatment in proliferating cultures, with uridine and leucine incorporation again little affected. Similar experiments with 66 μM 5-fluorouracil (5-FU) added for 6 hours at different times of activation showed the same irreversible and total inhibition of ^{3}H-thymidine incorporation at 48 hours.

Assay for thymidine transport and autoradiography studies on control, 8-AG or 5-FU treated cultures showed that fewer cells were labelled in the analogue-treated cultures but the amount of ^{3}H-thymidine incorporated in every labelled cell was the same as in the cells from the control culture. Neither the purine analogues nor 5-FU inhibited the essential events characteristic of activation: the 1.75 fold increase in K+ influx, blast formation and disaggregation of the condensed chromatin could be shown in all the cultures where proliferation was inhibited by a previous exposure to the analogues. Thus, the analogues prevented the cells from crossing the G_1/S boundary.

In addition, pretreatment of resting lymphocytes with 8-AG did not affect their ability to respond to Con A and proliferate.

A model is proposed to explain the mitogen-dependant, specific inhibition of lymphocyte proliferation by purine analogues and 5-FU; in cells presenting all the signs of activation.

RESUME

Les analogues de bases puriniques 8-azaguanine (8-AG) et 6-thioguanine (6-TG) ont été utilisés pour modifier la synthèse d'ARN à différents stades de l'activation des lymphocytes de la rate de souris par la concanavaline A (Con A) et la réponse proliférative de ces cultures a été mesurée à 48 heures. Dans des cultures de 42 heures, un traitement de 6 heures avec 66uM 8-AG ou 6-TG provoquait une inhibition totale et irréversible de l'incorporation de ^{3}H-thymidine alors que les incorporations d'uridine et de leucine étaient à peine afrectées. Il y avait aussi très peu d'inhibition des incorporations d'uridine et de leucine dans les lymphocytes au repos, traités de la même façon. L'inhibition de l'incorporation de thymidine pouvait être prévenue par l'addition simultanée de guanine mais une fois exprimée, l'addition de guanine n'a pu la renverser. Si l'on inhibait la synthèse des protéines en présence des analogues, leur action inhibitrice restait tout aussi efficace.

Des prétraitements des cultures non-prolifératives aves les analogues puriniques, pour 6 heures, a n'importe quel moment de l'activation, que ce soit pendant les premières 6 heures de culture ou pendant la phase G1 causaient la même inhibition sévère de l'incorporation de thymidine à 48 heures avec les incorporations d'uridine et de leucine toujours peu affectées. Des expériences similaires avec 66 µM 5-fluorouracil (5-FU), présent pendant 6 heures à différents moments de l'activité, ont démontré la même inhibition totale et irréversible de l'incorporation de thymidine à 48 heures.

Des tests du transport de thymidine et des études d'autoradiographie dans des cultures témoins et celles traitées avec 8-AG ou 5-FU, ont démontré que moins de cellules étaient marquées dans les cultures traitées aux analogues mais les quantités de ^{3}H-thymidine incorporées dans chaque cellule marquée étaient les mêmes que dans les cellules des cultures témoins. Ni les analogues puriniques ni 5-FU n'inhibaient les évènements précoces caractéristiques de l'activation : l'augmentation de 1.75 fois de l'influx de K^{+}, la formation de cellules blastiques et la désaggrégation de la chromatine dense pouvaient être démontrées dans toutes les cultures dont la prolifération avait été inhibée par une préalable exposition aux analogues. L'inhibition s'est manifestée donc avant la transition des cellules du stade G1 au stade S.

D'autre part, un prétraitement des lymphocytes au repos avec 8-AG n'affectait pas leur capacité de répondre au Con A et de proliférer.

Un modèle est proposé pour expliquer la "mitogène-dépendante", inhibition de la prolifération lymphocytaire par les analogues puriniques et 5-FU dans des cellules présentant tous les signes d'activation.

ABBREVIATIONS

Con A	Concanavalin A
PHA	Phytohemagglutinin
PWM	Pokeweed mitogen
8-AG	8-Azaguanine
6-TG	6-Thioguanine
5-FU	5-Fluorouracil
NTP	Nucleoside triphosphate
AMP	Adenosine monophosphate
c-AMP	Cyclic adenosine monophosphate
c-GMP	Cyclic guanosine monophosphate
GMP	Guanosine monophosphate
XMP	Xanthosine monophosphate
UMP	Uridine monophosphate
OMP	Orotidine monophosphate
PRPP	5-Phosphoribosyl pyrophosphate
poly A	Polyadenylic acid

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CHAPTER I

INTRODUCTION

In eukaryotic cells, the transition from quiescence to proliferation is associated with important alterations in RNA metabolism involving changes at both the transcriptional and post-transcriptional levels of gene expression. In studying the mitogenic activation of lymphocytes, a system with great physiological significance because of its relation to the immune response, Kaplan and coworkers have shown that the principal early mitogenic effect on RNA metabolism was to increase the processing and transport of pre-existing transcripts of all classes (Kaplan et al, 1978); however, no change in the overall rate of transcription was observed until 10 to 12 hours after mitogen addition (Mitchell et al, 1978). Further more, evidence indicates that activation of protein synthesis and all of the other early events do not require any new RNA synthesis (Ahern et al, 1974; Quastel et al, 1970). This situation has often been considered analogous to that in sea-urchin embryogenesis where RNAs made in the nonproliferative phase are polyadenylated after fertilization and the fertilized eggs can undergo several divisions in the absence of any RNA synthesis (Slater et al, 1973). However, there has not yet been any conclusive data on whether or not de novo RNA synthesis was an early essential event of

lymphocyte activation.

In this thesis, I attempted to resolve that question and determine at which stage(s) of activation important RNA synthetic events are occurring, by using analogues of purine bases known to interfere specifically with transcription. Unfortunately transcripts containing the modified bases are not necessarily degraded immediately upon removal of the base analogues from the medium and, in such a complex system, the possibility that the presence of altered RNAs can affect the general lymphocyte metabolism had to be considered.

1.1 In Vitro Activation of lymphocytes

The lymphocytes which respond to mitogenic stimulation are non-dividing small cells having a characteristic dense nucleus, rich in heterochromatin, and a thin rim of cytoplasm. The lymphocyte population extracted from peripheral blood or spleen consists of two main cell types with different embryonic origin, functional activities and cell surface properties. The bone marrow derived cells, B lymphocytes, function primarily in humoral immunity and are the precursors of immunoglobulin-producing cells. The thymus derived T lymphocytes are involved in cellular immunity; they constitute a functionally heterogeneous class of cells that include cytotoxic cells, "helper" cells (of B cell activity) as well as suppressor cells.

In cultures, these cells remain quiescent and such cultures

can be maintained for several days; upon addition of mitogens, a proportion of these resting cells will enter the growth cycle in a characteristic sequence of biochemical and morphological changes culminating in DNA synthesis and mitosis 36 to 72 hours later. This phenomenon is termed transformation or blastogenesis and the stages of transformation prior to DNA replication represent the activation process.

Mitogenic substances mostly used in in vitro transformation are the plant lectins, PHA, Con A and PWM, also called polyclonal or nonspecific mitogens from their ability to activate a large proportion of cells.

The mode of action of plant mitogens is not well understood. They agglutinate cells of different types including non-lymphoid cells but their mitogenic action only depends on their interaction with specific glycoproteins at the cell surface. Since different lymphocyte classes have different cell surface properties, they vary in their response to each mitogen: for example, PHA and Con A are primarily T cell mitogens while PWM can stimulate both B and T cells (Ling and Kay, 1975).

Lymphocyte transformation can also be induced by specific antigens to which the donor of lymphocytes has been previously sensitized. Lymphocytes will also enter the growth cycle in vitro when incubated with cells from a genetically different subject (Bain et al, 1964). These two types of response lead to similar biochemical and cytological changes but these occur in fewer

cells and are somewhat delayed as compared to stimulation with polyclonal mitogens.

In general, the biochemical events observed during blastogenesis seem to be a characteristic of all mammalian growth induction systems; however the study, in molecular terms, of lymphocyte transformation can be useful not only for understanding the control of cell proliferation but also for the elucidation of the primary mechanisms of the immune response.

1.2 The Molecular Events of Lymphocyte Transformation.

Lymphocyte transformation is initiated by changes in the function and metabolism of the plasma membrane which plays a vital role in the control of cell growth. Among the early changes that immediately follow the binding of the mitogen are those involving the transport of cations (K^+; Na^+ and Ca^{++}) across the membrane.

A ouabain-sensitive increase in K^+ uptake which does not depend on the synthesis of new K^+ transport sites, has been observed (Quastel and Kaplan, 1970; Averdunk, 1972). This occurs throughout the process and its inhibition, at any time, will prevent subsequent proliferation (Kaplan, 1978). During the early times of activation the increased K^+ uptake is accompanied by an equally increased efflux of K^+ (Segel et al, 1975; Hamilton and Kaplan, 1976) so that the intracellular concentration of the ion is not changed. However, later during the process,

an increased K^+ influx/efflux ratio might occur (Kaplan, 1979).

Concomitant with the enhanced K^+ influx is an increased Na^+ efflux due to the enhanced activity of the common transport site, the ouabain-sensitive Na^+-K^+ ATPase (Averdunk and Lauf, 1975). The role of Na^+ efflux has been less investigated but a number of cellular processes are known which require external Na^+. For example, there is a Na^+- dependant transport of some amino acids that seem to be a characteristic of growth induction in most mammalian cells, including lymphocytes (Van den Berg and Betel, 1973). The early membrane depolarization which is specific for and essential for activated lymphocytes (Kiefer et al, 1980) could be inhibited by a decrease in external Na^+. Also, Na-H exchanges important in the initiation of fertilization of sea-urchin eggs (Johnson et al, 1976) might exist in lymphocytes and contribute to the elevation of PH necessary for early lymphocyte response.

An increase in Ca^{++} uptake can be detected within minutes of mitogen addition (Allwood et al, 1971; Freedman et al, 1975) but its relevance to the subsequent events of transformation is uncertain. Durham (1978) suggested that a small elevation in intracellular Ca^{++}, presumably due to Ca^{++} uptake, could account for all the events of transformation. Parker (1975) reported Ca^{++} influx to be correlated to the degree of stimulation. However, non-mitogenic concentrations of Con A (Hesketh, 1979) as well as the non-mitogenic ionophore X537A (Betel, 1976) induce

Ca^{++} uptake suggesting that Ca^{++} uptake alone would not be sufficient to trigger proliferation although Ca^{++} ions have been implicated in the maintenance of the activated state (Bard. et al, 1979).

Another immediate consequence of mitogen binding to lymphocytes is the change in membrane lipid metabolism, mainly the phospholipids whose composition largely determines membrane function. Increases in diglyceride kinase and phospholipase C activities (Fisher and Mueller, 1971) have been observed that might be responsible for the increased phosphatidyl inositol turnover, an event specific to activated cells as well as necessary for the initiation of RNA and DNA synthesis (Mohrhauer and Holman, 1963). The turnover of lecithin and phosphatidyl ethanolamine, the two main lipids of lymphocyte plasma membranes, is also enhanced as a result of the increased activity of phospholipases A and acyl Co A lysolecithin transferases (Resch, 1979); these enzymes respectively deacylate and selectively incorporate unsaturated fatty acids into the plasma membrane. The net result of these turnovers is then an increased membrane fluidity which might contribute to the increased permeability for ions (Schellenberg and Gillepsie, 1977) and perhaps to the increased transport of amino acids, sugars and nucleotides observed later. Synthesis of membrane lipids, de novo, starts about 6 hours after stimulation (Fisher and Mueller, 1969).

In the cytoplasm, there is a transient rise in cyclic AMP (C-AMP) concentrations in the first 30 minutes which is followed by return to control levels by 2 hours (Parker et al, 1974). Increases in C-AMP concentrations are not specific for activation (Coffey et al, 1977) but they may be relevant to transformation since their absence can prevent the onset of DNA synthesis (Foker et al, 1979). Another increase in C-AMP level occurs in late G1 and persists until the mid-S phase (Wang et al, 1978). These fluctuations in C-AMP levels and the observations that any sustained rise in C-AMP would result in inhibition of the mitogenic response (Quastel and Kaplan, 1970; Diamanstein and Ulmer, 1975) strongly suggest that C-AMP might be both a negative and a positive regulator of activation, depending on the point in the cell cycle. C-AMP and C-GMP have often been suggested as regulators of cellular activities, however, changes in C-GMP in lymphocytes have not been described yet. In any case, if cyclic nucleotides regulate lymphocyte transformation, this is certainly in conjunction with other events, perhaps with Ca^{++} uptake; both C-AMP and Ca^{++} have often been proposed as the second messengers transmitting the activation signal received on the plasma membrane to the nucleus as well as controlling elements for the synthesis of the deoxyribonucleotide precursors involved in DNA synthesis (Whitfield et al, 1976).

Protein synthesis is activated within 3 to 4 hours and continues to increase until 48 to 72 hours (Kay, 1968). The

early increase in protein synthesis is totally independent of de novo RNA synthesis, it results from the translation of pre-existing mRNAs by pre-existing ribosomes (Jagus-Smith and Kay, 1976; Cooper and Braverman, 1977) and is therefore under post-transcriptional control. The changes in RNA metabolism involved in the activation of protein synthesis will be discussed in subsequent sections. At the level of the translational apparatus, a shortage in the activity of initiation factors is the main cause of the deficiency of resting lymphocytes to initiate translation (kay et al, 1970; Ahern et al, 1974). Initiation of translation in eukaryotes involves the dissociation of free ribosomes upon the binding of a factor, eIF_3 and further formation (in presence of other factors) of 80S initiation complex containing mRNA (Freienstein and Blobel, 1975; Schreier et al, 1977; Thomas et al, 1979). An activity identical to eIF_3 dissocation factor exists in resting lymphocytes but free from ribosomes (Resch et al, 1980); upon mitogenic activation, there is a diminution in the free (unbound) acitvity of this dissociation factor, that correlates with an increased dissociation of ribosomes and increased protein synthesis. Furthermore, resting lymphocytes have been shown to contain inhibitors of translation, able to depress protein synthesis in reticulocyte and wheat germ lysates; these inhibitors, thought to act by inactivating some initiation factors (Kay et al, 1978) are themselves inactivated immediately after mitogen binding. Inhibitors of the elongation step of translation have

also been claimed to be present in resting cells (Burrone and Algranati, 1979) but their existence is controversial. In any case, the early protein synthesis is essential for the morphological changes and the final proliferative response.

About 10 to 12 hours contact with mitogen are needed to detect a significant increase in the overall rate of transcription. All classes of RNA are then transcribed and this is paralleled by an increase in the amount of total RNA polymerase. Activation of pre-existing RNA polymerases might also be involved, especially for mRNA where net synthesis can be measured after 6 hours, before the increase in cellular RNA polymerase I. The entry into G1 phase is preceded by small nuclear changes that might be relevant to genome derepression. These include mainly phosphorylation of nuclear proteins (Johnson et al, 1974; Bluthmann, 1978) and acetylation of histones (Pogo et al, 1966).

Thereafter, the cell volume starts to increase and this continues until blast cells, 2 to 3 times the size of resting lymphocytes, are formed. Blast formation is characterized by an increase in cytoplasmic volume, rich in organelles, together with nuclear enlargement and disaggregation of the condensed chromatin (Dardick et al, 1981). During chromatin disaggregation, there is no change in the total hererochromatin volume but rather a reorganization of the condensed chromatin from a few large clumps to many smaller clumps, giving a more dispersed appearance (Setterfield et al, 1981).

At around 30 hours, in the first cell to enter S phase, blast formation is always complete; blast formation is uncoupled from DNA synthesis but the former is a prerequisite for the latter; inhibition of DNA synthesis has no effect on the morphological changes (Setterfield et al, 1980).

The increase in DNA synthesis correlates with the increase of many DNA replicating enzymes such as DNA polymerase (Loeb et al, 1969) thymidine kinase (Wilms and Wilmanns, 1970) and thymidylate kinase (Loeb et al, 1970).

1.3 Changes in RNA metabolism Associated with Lymphocyte Transformation

In animal cells, the primary gene transcripts for all classes of RNA have to be modified or processed to produce mature functional RNA molecules. These post-transcriptional changes do not always immediately follow transcription and pre-RNA molecules may be kept unprocessed or partially processed in the nucleus until they are degraded or transported to the cytoplasm. Thus the increase in cytoplasmic levels of RNA observed during lymphocyte stimulation may be achieved not only through increased precursor synthesis. Indeed, evidence exists indicating increase in cytoplasmic RNA content independent of increased transcription during the early stages of activation. Mitchell and coworkers (1978) have measured an increased accumulation of uridine - labelled RNA in the cytoplasm of cells 6 hours after exposure to Con A, before they could detect increase in label for total

cell RNA. The differences in cytoplasmic labelling they observed were too important to be explained by the mitogen-induced enlargement of NTP pools that result from the increase in uridine transport (Peter and Hausen, 1971) and uridine kinase activity in stimulated lymphocytes (Kay and Handmaker, 1976).

Furthermore, early work by Kay (1968) showed a greater stimulation of uridine-labelling in cytoplasm as compared to labelling of whole cell or nuclear RNA fractions. This also indicated that the mitogen effect on RNA metabolism was to increase the utilization of pre-existing molecules that will contribute, together with the enhanced synthesis, to increase the pool of functional cytoplasmic RNA molecules.

Different classes of RNA are processed differently and their synthesis is stimulated to different extents (Ling and Kay, 1975); therefore their final cytoplasmic levels may be ascribed to different types of control.

rRNA is transcribed as a long 45S precursor that contains the sequences for 18S, 5.8S and 28S mature RNA. This 45S RNA is rapidly methylated and subsequently cleaved, slowly in resting cells, to form a 32S RNA and the final 18S RNA. The 18S RNA is then rapidly transported to the cytoplasm to become the smaller subunit of the ribosome. The 32S intermediate remains in the nucleus for a time before its transformation into mature 28S RNA that will form the larger subunit of the cytoplasmic ribosome.

The maturation of 45S RNA is not only very slow in resting lymphocytes but also inefficient. 28S and 18S RNA are synthesized in equimolar amounts but after exposure to ^{3}H-uridine under the appropriate conditions, the labelling ratio, expected to be 1, shows a marked deficiency of label in the 18S RNA. This has been shown by Cooper (1972) to indicate that a large proportion (at least 50%) of the newly synthesized 18S RNA molecules is completely degraded before even reaching the cytoplasm. The low rate of rRNA synthesis combined with slow maturation and extensive degradation therefore maintain low and almost constant levels of rRNA in the cytoplasm of resting cells.

After mitogen addition, the rate of rRNA processing is accelerated rapidly and the degree of 18S wastage can be shown to decrease at around 2 hours (Cooper, 1969; Rubin, 1970). By 6 hours, maximum rates of processing are obtained together with complete reversal of the wastage. Efficient rRNA maturation has been shown to require synthesis of proteins that are thought to protect 18S RNA from degradation. The observed increase in the rate of maturation in fact coincides with the activation of protein synthesis and the increased movement of proteins from the cytoplasm to the nucleus reported by Johnson and coworkers (1974) in Con A stimulated cells might be related to these post-transcriptional events. Activ ation of rRNA transcription is independent of increased maturation. Efficient rRNA processing can be shown when RNA synthesis has been completely

inhibited and rRNA synthesis can be stimulated when maturation has been totally prevented by inhibition of protein synthesis (Cooper and Gibson, 1971). Between 24 and 48 hours, the degradation of 18S RNA progressively returns to control levels but the rates of synthesis and maturation are still high enough to produce some accumulation of ribosomes (Cooper, 1969b). This situation is comparable to that in resting cells where an excess of rRNA precursors is produced only a limited amount of which is utilized.

Increase in rRNA syntheis is one of the most marked and universal events accompanying growth stimulation in animal cells; however its significance in lymphocyte transformation is not clear. Protein synthesis is not limited by the availability of ribosomes and when rRNA synthesis is selectively inhibited, the rise in the rate of protein synthesis is essentially normal during the first 24 hours after the addition of mitogen (Kay et al, 1969). However inhibition of rRNA synthesis does prevent the initiation of DNA synthesis.

The precursor for tRNA is about 20 nucleotides longer than the final tRNA and maturation involves extensive methylation as well. Some pre-tRNA methylation occurs in the nucleus but in general tRNA maturation is a cytoplasmic event and pre-tRNA molecules can be found in the cytoplasm after short labelling pulses. Both the rate of synthesis and the rate of pre-tRNA maturation are accelerated after mitogen stimulation but changes

in the pattern and perhaps degree of methylation can be observed within the first 3 hours (Sharma and Loeb, 1973). This new pattern of methylation, which is independent of new protein synthesis is then maintained until 38 to 42 hours when new tRNA methylases are being synthesized and extent of methylation of most tRNAs is further increased. Methylation of tRNA might be an essential preliminary step for tRNA function; it is involved in amino acid acceptance (Shugert et al, 1968), in binding of amino-acyl tRNA to ribosomes (Gefter and Russel, 1969) and in codon response (Capra and Peterkofsky, 1968) and changes in rRNA methylation have often been proposed to participate in the control of the rate and types of proteins synthesized (Stent, 1964; Sueoka and Kano-Sueoka, 1970).

Resting lymphocytes also contain a number of types of low molecular weight RNA other than tRNA; most are methylated and largely confined to the nucleus but their functions are not yet known (Weinberg and Penman, 1968). Low molecular weight nuclear RNAs are also found in stimulated lymphocytes (Howard and Stubblefield, 1972: Hellung-Larsen et al, 1973). Howard and Stubblefield (1972) found one component to be synthesized 48 hours after the addition of PHA, but not by unstimulated lymphocytes or immediately after mitogen addition. However, Hellung-Larsen and coworkers (1973) were unable to confirm this. It was then believed that these RNA species were not influenced by the rate of cell division; however it is still possible

that small changes in the types of low molecular weight RNA synthesized after stimulation occur that have not yet been detected.

mRNA is transcribed from a large precursor molecule that belongs to the class of unstable heterogeneous nuclear RNA (hnRNA). In mammals, the coding sequences for a single mRNA, named exons, are interrupted by non-coding sequences, the introns, present in the hnRNA (Gilbert, 1978). Part of the processing then involves excision of the introns and rejoining of the coding sequences, a process called splicing (Crick, 1979).

The other post-transcriptional transformations of hnRNA involve modifications of the 5'P end by attachment of methyl guanosine to form a "cap", methylations of 2 or 3 nucleotides from the capped end, methylation of some internal adenosines and addition of a stretch of polyadenylic acid (poly A) to the 3'OH end.

The significance of these modifications is not fully understood. There is some evidence that the 5' cap may function in ribosomal binding by the mRNA (Perry and Kelley, 1976) and perhaps facilitate translation (Kozak, 1978). Polyadenylation has been suggested as the triggering change for processing in the viral genome (Darnell, 1979); Marbaix and coworkers (1977) suggested that polyadenylation had a stabilizing effect on mRNAs and facilitated transport to the cytoplasm. However, not all the mRNAs are polyadenylated and some polyadenylation takes place after transport to the cytoplasm.

The first data on poly (A^+) RNA metabolism in lymphocytes was obtained by Rosenfeld's group (1972). They found a 40% increase in poly (A^+) RNA synthesis within 2 hours of PHA addition to human peripheral blood lymphocytes (HPBL) and a 100 to 300% increase by 12 hours; since they found similar increases whether they used uridine or adenosine as label, they concluded that mitogen had no effect on the percent poly (A) content. Increased production of poly (A^+) RNA was also reported by Cooper and coworkers (1974) in 20 hour PHA stimulated HPBL. However they measured a greater increase in ^{3}H-adenosine labelled cytoplasmic poly (A^+) RNA; but in this case, it was possible that part of the increase they reported resulted from polyadenylation of pre-existing transcripts rather than from de novo hnRNA synthesis. Indeed, more recent work by Schaëfer and coworkers (1977) has shown a significant increase in nuclear poly (A^+) content (due to increased rate of polyadenylation) as early as 90 minutes after Con A addition to lymphocytes from bovine lymph nodes, before increase in total RNA content was measured. They also reported that the rate at which poly (A^+) sequences leave nuclei from 3 hour stimulated cells was about 1.2 times higher than that measured in nuclei from resting cells, and at the same time, the cytoplasm from stimulated cells contained about 1.3 times more poly (A^+) sequences. That the early mitogen effect on poly (A^+) RNA metabolism was to accelerate processing, mainly polyadenylation, and transport of pre-existing transcripts

rather than stimulate transcription was further confirmed by Mitchell, Kaplan and coworkers (1978). They found significant differences in accumulation of cytoplasmic ^{3}H-adenosine label in the first 5 hours of mitogen addition, in the complete absence of increase de novo poly (A^+) RNA synthesis as shown by uridine labelling.

Furthermore, the observation by Wettenhal and coworkers (1976) that the template activity of stored mRNA was rapidly improved following mitogen addition strongly suggests that activation of part of the large stable pool of mRNA found in the cytoplasm of resting cells might contribute to the increase in the cytoplasmic levels of functional mRNAs that may be important for the early activation of protein synthesis. Another control point for the levels of poly (A^+) RNA might involve changes in stability as implied by Land and Schaëfer (1977) when they showed a rapid degradation of poly (A^+) RNA in nuclei from resting cells under conditions where this fraction was stable in nuclei from stimulated cells. Also the large decrease in ribonuclease activity (Green, 1977) combined with increase in levels of RNase inhibitors might be implicated.

Post-transcriptional regulation of poly (A^+) RNA levels therefore seem to be a universal feature of anabolic cell activation. This has been observed in sea-urchin egg development (Wilt, 1973), in 3T6 fibroblasts (Johnson, et al, 1976) as well as in AKR cells responding to serum addition (Getz, 1976). The role of post-transcriptional gene expression in lymphocytes has not been clearly

defined yet. New mRNA types might be expected in stimulated lymphocytes to account for the synthesis of new proteins and the development of differentiated lymphocyte functions. This could be achieved either through selective post-transcriptional changes that will favor the translation of certain types of the mRNA transcribed by resting cells or by transcription of previously inactive regions of the genome. Early attempts to resolve that question used RNA: DNA hybridization techniques and failed to detect changes in the types of mRNA synthesized in stimulated cells (Torelli et al, 1968; Clark et al, 1971). This, however was not conclusive because of the limited sensitivity of the method. Since then, the only data on qualitative changes in mRNA synthesis by stimulated lymphocytes has been reported by Milner (1978). He suggested that in mouse splenic lymphocytes, Con A induced the transcription of a new RNA species with a short half-life (3 to 4 hours) and that translation of this message commited the cells to DNA synthesis in absence of further mitogen addition. Jazwinski and coworkers (1976) have also shown the existence, in the cytoplasm of mitogen stimulated lymphocytes, of a new protein of molecular weight larger than 50,000 that can initiate DNA synthesis in nuclei isolated from resting cells. However, these data are controversial and further work is needed.

1.4 Thesis Approach

Earlier data suggesting a dependence of lymphocyte proliferation on RNA synthesis were obtained using inhibitors of transcription (Kay et al, 1969). In the present studies, these inhibitory effects would have to be restricted to specific periods of activation and a reversible inhibitor would be required.

Actinomycin D which has been extensively used in studies of RNA metabolism appears not to be suitable here. This drug blocks RNA polymerase action by binding to DNA (preferentially to nucleolar DNA) and this affinity for chromatin is greatly enhanced by mitogen stimulation (Darzynkiewicz and Ring, 1969); comparison with resting cells will therefore not be possible. Also, there have been reports suggesting that actinomycin D was actively transported and accumulated by stimulated lymphocytes and more recently Mitchell (M.Sc thesis, 1978) has shown the inconsistency of actinomycin D effects in Con A stimulated HPBL. In addition, I found the drug effects in mouse spleen lymphocytes to be irreversible, even after several washes and long incubation in fresh medium.

α-amanitin is another specific inhibitor of transcription, it binds to RNA polymerases, with more affinity for RNA polymerase II. When tested here, even at low doses for relatively short time exposure, the effects were totally irreversible. Milner (1978) claimed that in Con A stimulated Balb/C spleen lymphocytes

the effects observed after a 2 hour exposure to 10^{-4} g/ml α-amanitin would be perfectly reversible. However, he did not mention the degree of inhibition of RNA synthesis he obtained and using his conditions I was unable to show any effect; the effective dose I found was higher and this was irreversible.

In view of the difficulties of finding a specific and reversible inhibitor of RNA synthesis in lymphocytes, I decided to use analogues of nucleic acid bases that should affect specifically RNA synthesis. These drugs in general do not inhibit RNA synthesis to a great extent but by being incorporated into the newly made RNAs (in place of natural bases) will change their functional properties; then if any RNAs transcribed in presence of base analogues were to participate in transformation, its importance will be reflected by an inhibition of the proliferative response and subsequent entry into mitosis.

The use of base analogues has yielded important information concerning RNA metabolism and function. For example, the first indication of a relationship between nucleic acids and protein synthesis was obtained in studies with base analogues (Creaser and Chantrenne, 1956). Analogues have also been used in the past to study the requirements for and localize essential RNA synthetic events involved in many cellular processes such as enzyme synthesis in regenerating rat liver (Szepezi and Friedland, 1970) or hormone-induced liver (Levitan and Webb, 1969) as well as in the development of sea-urchin eggs (Bamberger et al,

(1963). The success of these experiments was based on the ability to discontinue the incorporation of the analogues into RNA and then obtain different effects with exposure at different stages. This was achieved by removal of the analogues from the external medium and/or more effectively by addition of excess of the natural bases.

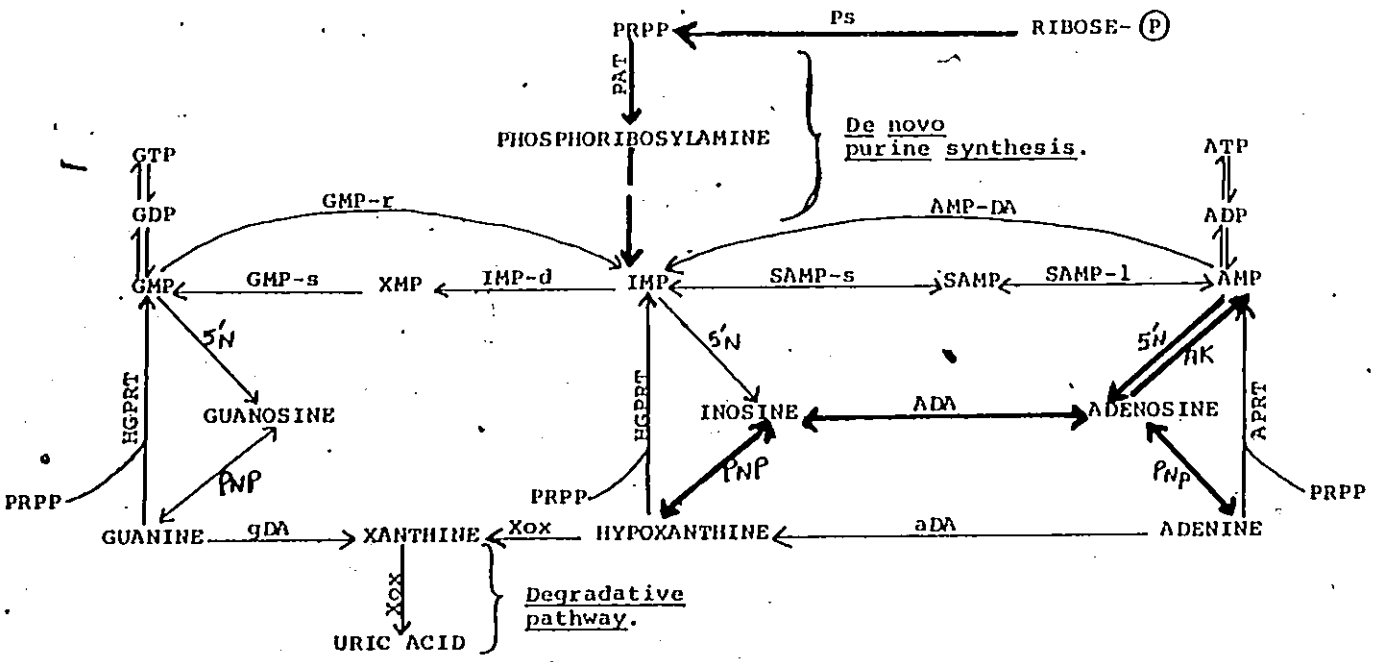
Most of the work in this thesis was carried out using 8-aza-guanine (8-AG), an analogue of guanine which contains a nitrogen atom in place of carbon in position 8 of the guanine ring. The effects of 8-AG seem to be specific with respect to its site of action: reticulocytes, which do not synthesize RNA, are not affected by 8-AG (Zimmerman and Greenberg, 1965) and inhibition of RNA synthesis has been shown to protect against 8-AG (Nelson and Carpenter, 1975). In systems active in RNA synthesis, resistance to 8-AG is in general due to a lack of hypoxanthine guanine phosporibosyl transferase (HGPRT) activity which is required for phosporibosylating purine bases (Demars, 1974); however, high guanine deaminase (Van Diggelen et al, 1979) or 5' nucleo-tidase activities (Fujimoto and Seegmiller, 1970; Williams et al, 1978) that are responsible for the degradation of guanine compounds, have been shown to confer resistance. An examination of the mechanisms by which lymphocytes, quiescent and proliferating, can metabolize purine compounds is thus necessary to ensure biological effectiveness of 8-AG.

1.4.1 Purine Metabolism in Lymphocytes

Lymphocyte purine metabolism has been extensively studied

Figure 1: Pathways of Purine Metabolism in Lymphocytes

The pathways essential for lymphocyte function are shown in heavy lines.
Abbreviations: PRPP: 5-phosphoribosyl Pyrophosphate; S-AMP; Adenylosuccinate.
Ps; PRPP synthetase (EC 2.7.6.); PAT: PRPP amidotranferase (EC 2.4.2.14); IMP-d: IMP dehydrogenase (EC 1.2.1.14) GMP-s: GMP synthetase (EC6.3.4.1); SAMP-l: SAMP lyase (EC 4.3.2.2); SAMP-s: S-AMP synthetase (EC 6.3.4.4); HGPRT: Hypoxanthine phosphoribosyl transferase (EC 2.4.2.8); APRT: Adenine phosporibosyl transferase (EC 2.4.2.7); AK: Adenosine Kinase (EC 2.7.1.20); 5'N: 5'-nucleotiddase (EC 3.1.3.5); PNP: purine nuceloside phosphorylase (EC 2.4.2.1); ADA: Adenosine deaminase (EC 3.5.4.4) AMP-DA: AMP deaminase (EC 3.5.4.6); GMP-7: GMP reductase (EC 1.6.6.8); g DA: guanine deaminase (EC 3.5.4.3); aDA: Adenine deaminase (EC 3.5.4.2)
XOX: xanthine oxidase (EC 1.2.3.2).


PRPP
Ps
RIBOSE-P
PAT
PHOSPHORIBOSYLAMINE
De novo purine synthesis.
GTP
GDP
GMP-r
AMP-DA
ATP
ADP
GMP
GMP-s
XMP
IMP-d
IMP
SAMP-s
SAMP
SAMP-l
AMP
5'N
5'N
5'N
HK
HGPRT
HGPRT
APRT
GUANOSINE
INOSINE
ADA
ADENOSINE
PNP
PNP
PNP
PRPP
PRPP
PRPP
GUANINE
gDA
XANTHINE
Xox
HYPOXANTHINE
aDA
ADENINE
Xox
URIC ACID
Degradative pathway.

(the main pathways are shown in figure 1) but because of the association between defects in the cababolism of adenine nucleotides and impairements of immune function (Gibblet et al, 1975), the metabolism of adenine nucleotides has received more attention than those of the other purine compounds.

There are two potential pathways for the degradation of adenylic acid (AMP) one of which involves formation of adenosine by the action of 5' nucleotidase and the second pathway involves deamination of AMP to inosinic acid (IMP) followed by 5' nucleotidase degradation of AMP to produce inosine (fig. 1). Both adenosine and inosine are generally so rapidly metabolized that they are found in very low amounts in normal cells. Adenosine is either phosphorylated back to AMP by adenosine kinase, deaminated into inosine by adenosine deaminase (ADA) or to some extent degraded by purine nucleoside phosphorylase (PNP) to the base (Snyder and Henderson, 1973; Henderson, 1979). Any inosine formed, either from adenosine or IMP, is used by PNP to generate hypoxanthine. Phosphorylation is generally the favored pathway of adenosine metabolism (Snyder et al, 1976) but when the levels of adenosine are beyond the capacity of the kinase, as in resting cells, in stimulated cells exposed to high levels of adenosine or in cells with abnormally elevated 5' nucleotidase activity (SNyder et al, 1976b), the excess metabolite is removed by the action of ADA. Since inosine at high concentration inhibits ADA activity (Agarwal et al, 1975), its rapid degradation by PNP is necessary to avoid indirect accumulation of adenosine. Adenosine,

at high concentration is particularly toxic to lymphoid cells and among other effects, it has been shown to inhibit the proliferation of mitogen stimulated lymphocytes (Seegmiller et al, 1977; Hirshborn et al, 1970). Therefore, the impairment of immune response observed in ADA and/or PNP deficiency results from the intracellular accumulation of adenosine or of adenine also known to inhibit lymphocyte activation (Quastel and Kaplan, 1970a).

The metabolism of guanine in resting and mitogen-stimulated lymphocytes has been studied mainly by Raivio and Hovi (1978). They reported that after short incubation with radioactive guanine, most of the label associated with the cells was used for nucleotide synthesis and was not immediately externalized. This indicates that the degradation of guanine is verylow and confirms previous reports of the low guanine deaminase acitivity in mammalian cells, (Andrew, 1971).

Nucleotide synthesis from guanine or any other purine base occurs through the purine salvage pathways in which the intact bases are directly converted to nucleotides by phosphoribolsyltransferases in the presence of 5-phosphoribosyl pyrophosphate (PRPP) (figure 1). The transferases, HGPRT and APRT specific for the salvage of guanine/hypoxantine and adenine respectively, have been shown in both resting and proliferating lymphocytes (Hovi et al, 1976) although the efficiency of nucleotide formation from exogenous bases is several fold higher in the proliferating cells than in the resting cells (Raivio and Hovi, 1976). However, because of the correspondingly lower rate of nucleic acid synthesis

in resting cells, the fraction of newly synthesized nucleotides incorporated into nucleic acids might be the same in both the resting and stimulated cells as suggested by studies in cultured HPBL and lymphoblast cell lines (Field and Brox, 1973). Purine salvage pathways are not essential for lymphocyte function: HGPRT-deficient lymphocytes from patients with Lesch-Nyhan syndrome have been shown to have normal responses to PHA, Con. A and PWM (Allison et al, 1978).

The data of Raivio and Hovi (1978) on the fate of exogenously given guanine also show some guanosine formation in lymphocytes. This probably results from the degradation of guanylic acid (GMP) rather than from the ribosylation of guanine, a thermodynamically unfavored reaction since the requisite enzyme, PNP, which also catalyzes the phosphorolysis of nucleosides, has much higher affinity for the nucleoside than for the base (Hartman, 1970). Phosphorolysis, the only route of guanosine metabolism in lymphocytes which usually lack guanosine kinase activity (Friedman et al, 1969; Scholar and Calabresi, 1973) is also enhanced in proliferating cells.

Conversion of GMP into AMP must occur in lymphocytes to explain the presence of label in adenine nucleotides after an exposure to radioactive guanine. This involves reduction of GMP to IMP which is further dehydrated by SAMP synthetase and cleaved by SAMP lyase to yield AMP (figure 1). These interconversions might constitute one of the mechanisms for providing the cell with balanced levels of the various purine nucleotides although

regulation of the rate of de novo synthesis seems to be more efficient. The control of de novo purine synthesis is exerted at the level of the first, presumably rate-limiting, reaction by inhibition of the requisite enzyme PRPP amidotransferase (Henderson, 1972; Kelley et al, 1975) and/or more efficiently through regulation of the levels of PRPP, the necessary substrate in this reaction. A selective control of the synthesis of various types of purine nucleotides has been shown in lymphocytes (Hershfield and Seegmiller, 1976); this is exerted at the level of the IMP branch (figure 1) from where all the purines are synthesized de novo. For example, an excess of guanine nucleotides (in case of high extracellular guanine) has been shown to depress the overall rate of de novo synthesis as well as decrease its own synthesis (inhibition of IMP dehydrogenase), the resultant increased availability of IMP, combined with the stimulatory effect of GMP on SAMP synthetase (the first enzyme committing AMP synthesis from IMP) might then cause an increased synthesis of AMP. Thus, an exogenous supply of guanine or any other purine base might have consequences for the overall purine metabolism.

The rate of de novo purine synthesis is greatly enhanced by mitogen stimulation and this activation correlates with the reported general increase in purine metabolism. Wood and coworkers (1973) have measured up to 100fold activation of de novo purine synthesis as early as 4 hours after PHA addition to HPBL; they proposed that the increase in purine synthesis was due to an increased availability of PRPP rather than to

increased amounts of enzymes. Indeed; increases in PRPP concentrations have been observed both shortly (Hovi et al, 1975) and at later times (Hovi et al, 1977; Wood et al, 1973) during lymphocyte transformation. Increases in the amounts of purine metabolizing enzymes occur but this is part of the increase in protein synthesis per cell and it is too high to explain the activation of purine metabolism; furthermore, no change has been reported on the specific activity (activity per mg protein) of any purine metabolizing enzyme and the report by Holmes and coworkers (1974) on PRPP amidotransferase activation during lymphocyte transformation indicated a modulation of the purine metabolizing enzyme activities by allosteric effects.

1.4.2 Pyrimidine Metabolism

Since purine analogues can interfere with purine synthesis thereby inhibit lymphocyte prolieration independently from entering RNA, experiments were carried out with pyrimidine base analogues, which also enter ribonucleic acids but do not affect purine metabolism as severely as purine analogues might. Furthermore, no disorder in pyrimidine metabolism has been found associated with impairment of the immune function.

The analogue used, 5-fluorouracil (5-FU) is like uracil, taken up in mammalian cells by facilitated diffusion and is further utilized for nucleotide synthesis by the same pathways as uracil. The only difference in the metabolism of 5-FU is that the analogue is not converted into cytosine or thymine nucleotides, F-UTP and F-dUMP being not good substrates for CTPSase and TMPSase, the

enzymes involved in amination of UTP and methylation of dUMP respectively (Hiedelberg, 1964). Synthesis of uracil nucleotides from uracil occurs, like in the salvage of purine bases, mainly through the phosphoribosylation reaction. Uracil is converted directly to UMP by the action of orotidylate phosphoribosyltransferase (OMPppase) in the presence of PRPP. OMPppase is also involved in the de novo synthesis of pyrimidine nucleotides (figure 2) and this enzyme activity has been suggested as the rate-limiting component in the synthesis of uridine nucleotides in several mammalian cells (Ullman and Kirsch, 1979). The alternative pathway of pyrimidine salvage which involves formation of the nucleoside (and further phosphorylation) is used for nucleotide synthesis from cytosine and thymine but for uracil, the uracil phosphorylase has more affinity for uridine than for the base and is rather utilized in degradation.

Both, the de novo and salvage pathway of synthesis of uracil nucleotides utilize PRPP (figure 2) and PRPP is also a substrate for purine phosphoribosyltransferases. Therefore, one way by which pyrimidines or their analogues might affect purine metabolism is by reducing the level of PRPP available for purine synthesis and purine and pyrimidine biosynthesis might be coordinately regulated by PRPP levels. Indeed, orotic acid which increases the intracellular concentration of pyrimidine nucleotides has been shown to slightly reduce the synthesis of purines (Fox and Kelley, 1971) and sublethal doses (50 μM) of adenine, adenosine

and inosine, in turn have been shown to decrease the concentration of pyrimidine nucleotides in lymphoblasts (Astrin et al, 1977). However, studies with the HGPRT-deficient lymphoblasts derived from Lesch-Nyhan patients failed to show any change in the concentration of pyrimidine nucleotides as compared to normal lymphoblasts and there is no clear evidence for a coordinate regulation of purine and pyrimidine synthesis by PRPP. The intracellular concentration of pyrimidine nucleotides, like that of purine nucleotides, is more effectively controlled by modulation of the enzymes involved in the de novo pathway of synthesis. For example, high pyrimidine nucleotides feedback inhibit CPSase (the first enzyme in the pathway) and this enzyme can in turn be stimulated by purine nucleotides.

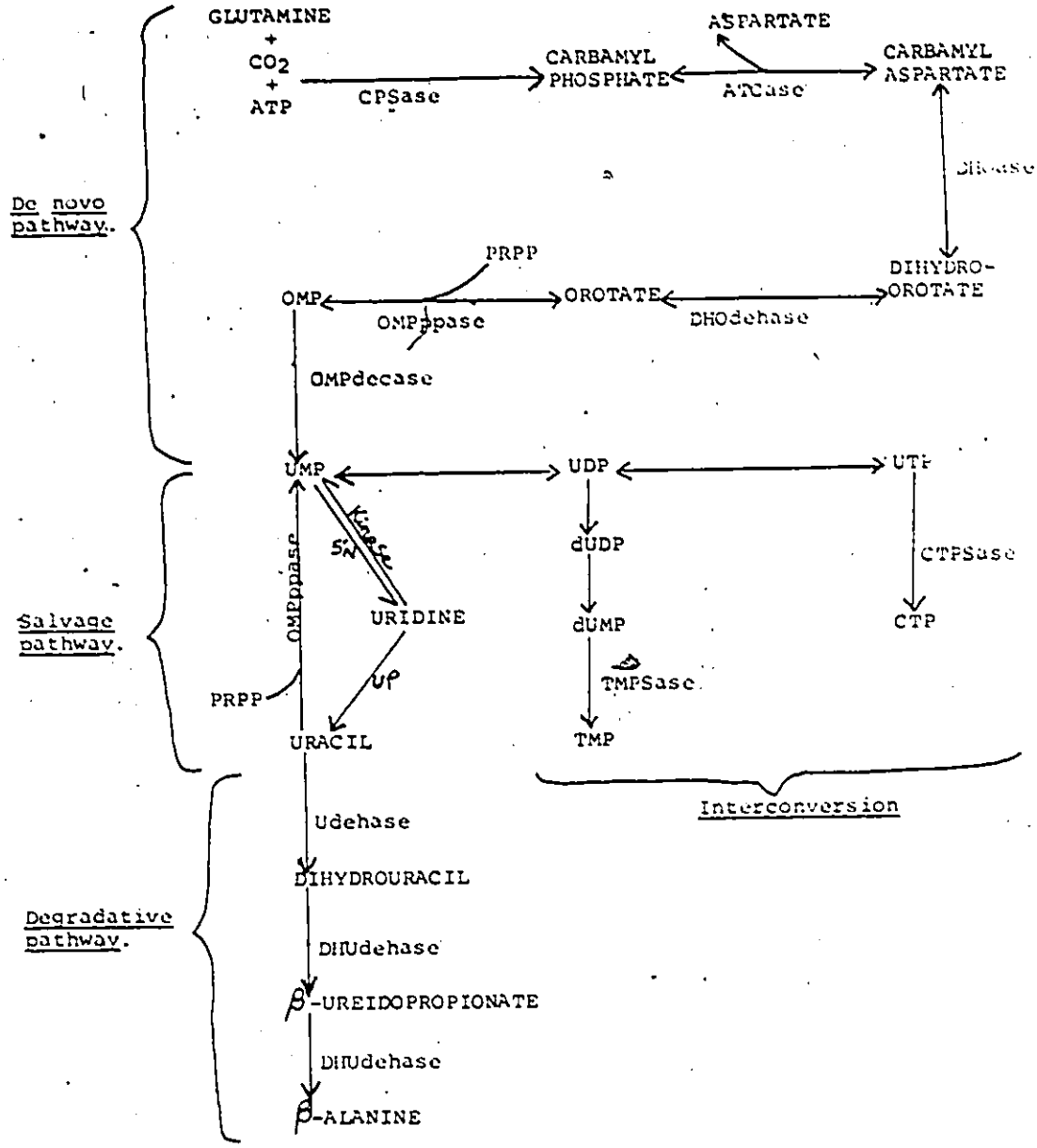
The degradation of pyrimidine nucleotides, like that of purine nucleotides, involves the sequential action of 5'-nucleotidase and uracil phosphorylase to yield the base. The base is then cleaved and degraded by the action of dihydrouracil dehydrogenase to β-alanine (or its β-fluoro derivative in the case of 5-FU), the main degradative intermediate released by animal cells.

The changes in the metabolism of uracil after mitogenic stimulation of lymphocytes are not well documented but increases in uridine kinase and the reported enhanced activity of ATCase and OMPppase in regenerating rat liver and tumor cells (Cohen and Marshall, 1962), together with the enormous increase in nucleic acid synthesis associated with lymphoctye transformation, strongly suggest an enhancement of pyrimidine metabolsim during mitogenic stimulation.

Figure 2: Pathways of Pyrimidine Metabolism in animal cells.

Abbreviations:

CPSase: carbamyl phosphate synthetase.
ATCase: Aspartate transcarbamylase.
DHOase: Dihydro orotase.
DHOdehase: Dihydro orotate dehydrogenase.
OMPppase: Orotate phosphoribosyl transferase.
OMPdecase: OMP decarboxylase.
CTPSase: CTP synthetase
UP: uracil phosphorylase.
Udehase: uracil dehydrogenase.
DHUdehase: Dihydrouracil dehydrogenase.
TMPSase: TMP synthetase.


GLUTAMINE
+
CO_2
+
ATP
CPSase
CARBAMYL PHOSPHATE
ASPARTATE
ATCase
CARBAMYL ASPARTATE
DHOase
DIHYDRO-OROTATE
DHOdehase
OROTATE
PRPP
OMP
OMPppase
OMPdecase
De novo pathway.
UMP
UDP
UTP
Kinase
ATP
OMPppase
URIDINE
dUDP
dUMP
CTPSase
CTP
TMPSase
TMP
PRPP
UP
URACIL
Salvage pathway.
Interconversion
Udehase
DIHYDROURACIL
DHUdehase
β-UREIDOPROPIONATE
DHUdehase
β-ALANINE
Degradative pathway.

CHAPTER II

MATERIALS AND METHODS

2.1 Culture of Mouse Splenic Lymphocytes

2.1.1 Preparation of Cells

Male Balb/C mice (Bio-Breeding labs), 8-10 weeks old, were killed by cervical dislocation. Their spleens were removed and disrupted on a wire screen, into a drop of calf serum (Flow Labs), then suspended in medium RPMI 1640 (Flow).

The cell suspension was layered on calf serum to sediment debris. Red blood cells were removed by lysis with NH_4Cl (0.83%) for 7 minutes at 4°C. Resultant suspensions (95% lymphocytes) were washed once with RPMI 1640 before use.

2.1.2 Culture Conditions

Cells were cultured in RPMI 1640 supplemented with 6% fetal calf serum (Flow), penicillin-streptomycin (100 units/ml - 100 µg/ml; Difco) and 2 mM L-glutamine, buffered with pH 7.0 with 20 mM Hepes buffer.

All cultures were at a density of 2.5 - 3 x 10^6 cells/ml. Cells were incubated in plastic tubes (Falcon), at 0.5 - 1.0 ml/tube (depending on the experiment); or in plastic flasks (Corning) at 10 - 20 ml/flask.

Containers were kept sealed and cells were cultured at 37°C.

2.1.3 Mitogen

Concanavalin A (Calbiochem, Sigma) was added to cultures at a final concentration of 3 μg/ml.

This was added prior to distribution in flasks or tubes, or after inhibitor treatment (depending on the experiment).

Dose-response curves were occasionally done to check the Con A stock for mitogenic activity.

2.2 Bases, Base Analogues and Other Inhibitors

In all cases, inhibitors were given for 6 hours, at times indicated for various experiments. When necessary, the treated and control cells were washed twice in RPMI 1640 containing 2 mM L-glutamine and resuspended in fresh culture medium.

All the bases and base analogues were obtained from Sigma.

In some experiments (figure 5) adenine (132 μM) and uracil (132 μM) were added along with purine base analogues, from 42 to 48 hours of culture with mitogen.

Guanine (132 μM) dissolved in 0.5% KOH was added either together with 8-azaguanine and 6-thioguanine or after treatment with these analogues.

8-azaguanine was dissolved in 0.5% Na_2CO_3 and added to cultures at indicated times while control cultures had an equal volume of 0.5% NA_2CO_3 added (100 μl; pH adjusted to 7.0).

6-Thioguanine (2-amino-6-mercaptopurine) (in 0.5% Na_2CO_3) and 5-Fluorouracil were added under same conditions as 8-azaguanine.

The concentration of analogues used was based on standard dose-response curves (fig. 3) carried out with cells of high metabolic activity (42 - 48 hours ConA).

Stable solutions of anisomycin (Pfizer, diagnostic division) were prepared as described by Schwarz et al (1971). The base was dissolved in water, diluted with an equivalent amount HCl to the desired concentration (0.5 mg/ml) and finally adjusted to pH 6.5 for maximum stability at 4°C.

A concentration of 200 ng/ml anisomycin, added from 42 to 48 hours to Balb/c splenocyte cultures, was sufficient to bring about 70 - 85% inhibition of protein synthesis and the inhibition was found to be reversible upon washing.

2.3 Measurement of Thymidine Transport

Lymphocytes were treated with 66 μM 8-Azaguanine after 42 hours in ConA and the thymidine transport assay was carried out at 48 hours. Thymidine transport in Balb/c splenocytes was measured by the rapid sampling technique of Strauss (Strauss et al., 1976) as modified by Rudd (MSC thesis, 1980). Cells were removed from Corning incubation flasks, washed twice and suspended in LMGB (Leukocyte medium, pH 7.4 - 7.6, containing glucose and bovine serum albumin) at a concentration of 10 x 10^6 cells/ml. LMGB consisted of 10 mM sodium phosphate buffer (pH 7.5), 0.9% naCl, 0.1% bovine serum albumin 5 x crystallized (Sigma) and 5 mM glucose (Fisher).

Transport was measured by mixing equal volume (0.5 x 0.5 ml) of cell suspension with thymidine at the desired concentration at 20 μCi/ml in LMGB. After the appropriate incubation time (30 seconds), 200 μl of reaction mixture was layered into a 400 μl microfuge tube (Canlab) containing 50 μl of 7% perchloric acid as the bottom layer and 150 μl of silicon oil (12 volumes of Dow Corning 550 fluid and 13 volumes of Dow Corning 510 fluid). The tube was spun at 10,000 rpm for 20 seconds using a Beckman microfuge. The microfuge tubes were frozen in ethanol/dry ice and the tips sliced into a scintillation vial for counting. Cell digestion was accomplished overnight with 10 ml of Protosol (New England Nuclear) at 37°C. This was then neutralized with 100μl glacial acetic acid and counted in 7 ml scintillation fluid (Scintiverse; Fisher) with a Beckman scintillation counter model LS 233.

Non-specific associated label was determined by the method of Rudd (C.E. Rudd, M.Sc. Thesis) which accounts for non-specific adsorption to the cell surface as well as for label trapped in the intracellular space.

Values in this range were subtracted from cell associated radioactivity to determine the actual transport values.

2.4 Potassium Influx

^{86}RB was used as a tracer for potassium. 8-Azaguanine (66 μM) was added at 6 hrs and potassium fluxes measured (in presence of the analogue) by 12 hrs since the increased fluxes

of mouse cells are best detected 12 to 14 hrs after mitogen addition (T. Owens, Ph.D. thesis).

One hour before the assay, 40 μCi (in 25 μl) of ^{86}RbCl (New England Nuclear) was added to each ml of culture, in plastic tubes.

Cells were sampled by centrifugation of 200 μl aliquots through 100 μl of silicone oil using a microfuge. The tips of the tubes were cut off and the radioactivity in the pellets counted in a gamma counter.

Conversion of the ^{86}Rb data to K+ uptake was performed according to equation 1 and 2:

1. $$\text{SRM (counts/fmole } K^+) = \frac{\text{Counts/ml}}{Z \text{ (fmoles/ml)}}$$

where SRM is the specific radioactivity of the medium and Z is the concentration of K^+ in the supernatant, 5.4×10^9 fmoles/ml.

2. $$K^+ \text{ uptake (fmoles/cell)} = \frac{\text{(Counts/pellet) - (counts in trapped medium)}}{\text{SRM X viable cells/pellet}}$$

Correction for trapped space was done using a zero time uptake. Cells and isotope were pre-cooled at 4°C for 15 min. mixed rapidly, immediately sampled and spun with 15-30 seconds of mixing.

2.5 Assay for DNA Synthesis and Chromatin Decondensation:

2.5.1 Measurement of ^{3}H-Thymidine Incorporation

To measure DNA synthesis, the incorporation of (^{3}H)-thymidine (51 Ci/mM, Amersham) was assayed at 24 and

48 hrs. The isotope was added 2 hrs prior to harvest, in 50 µl PBS (Phbsphate Buffer Saline) to a final concentration of 2 µCi/ml.

The incorporation of isotope was measured on a manifold (Millipore) equipped with glass microfibre filters (Whatman type GF/C). Cells were deposited on the filters, washed with isotonic saline (0.85% NaCl), ice-cold 5% trichloroacetic acid and methanol. The dried filters were counted in 7 ml of Scintilene in a scintillation counter for 2 min. At least 3 replicates were counted for each sample, mean counts were presented with their standard deviations.

2.5.2 Autoradiography and light Microscopy

To ascertain the effects of base analogues on DNA synthesis, autoradiographic grain counts were also performed.

At specified times after treatment with the analogues, cells were labelled with ^{3}H-thymidine (10 µCi/ml) 1 hr prior to fixation.

Fixation was in 2% glutaraldehyde-2% formaldehyde in 0.05M sodium cacodylate buffer (NaCac, pH 7.4) for 1 hr at room temperature. After 3 washes in 0.05 M NaCac buffer, cells were post-fixed in 1% osmium tetroxide in 0.05M NaCac buffer for 1 hr at 4°C, then washed 3 times in buffer and dehydrated in 70% ethanol at 0°C for 10 min. Cells were resuspended twice in pure dioxane, then in dioxane-epon araldite mixtures (1:1, 1:3) and finally embedded in

pure epon araldite and incubated at 80°C for 2 days. The hardened blocks were then processed for light autoradiography.

The same preparation was also used in studies of nuclear decondensation patterns observed herein by light microscopy.

For both autoradiography and light microscopy, 0.5 μm thick sections were cut with glass knives on a Reichert OmU_2 ultramicrotome, expanded with xylene vapours and placed on gelatin coated slides (0.1% gelatin).

Light autoradiographs were prepared by coating slides, with Ilford L_4 emulsion diluted 1:1 with distilled water. After exposure times of 1, 6 and 10 days, the slides were developed in Dektol: water 1:1 for 4 min (Eastman Kodak, Ltd) and stained with toluidine blue pH 9.0.

Slides were mounted in immersion oil under coverslips for scoring under bright field optics using a Zeiss photomicroscope.

300 nuclei/slide were scored for nuclear morphotype distribution and silver grains counted in 500 cell samples.

2.6 Assay for RNA and Protein Synthesis

In experiments measuring RNA and protein synthesis, the incorporations of (5, 6-^{3}H) uridine (42 Ci/mM; Amersham) and L-(4, 5-^{3}H) leucine (65 Ci/mM; Amersham) were assayed at 12, 18, 24 or 48 hrs depending on the experiment.

The isotopes were added 2 hrs before harvesting, in 50 µl PBS to final concentrations of 5 µCi/ml for ^{3}H-uridine and 10 µCi/ml for ^{3}H-leucine, respectively.

Trichloroacetate precipitate material was collected using a manifold, then counted in a scintillation counter.

2.7 Blast Transformation

After the treatment with base analogues, cells were washed, incubated in fresh culture medium and the distribution of cell sizes determined between 44 and 50 hrs, either using a Coulter counter or by phase contrast microscopy on viable cells.

Percentages of blast cells in culture were calculated on the basis of the number of cells with a diameter greater than 8 µm.

2.8 Determination of Mitotic Indices

Cells were cultured in flasks. After 48 hours, colcemid (0.1 g/ml. Difco) was added for 6 hours. Cells were collected by centrifugation, washed in PBS and gently resuspended in 0.075M KCl for 10 minutes. They were then fixed in methanol: acetic acid (3:1), and spread on glass slides.

Spreading was accomplished by gently blowing on the slide as it dried under an incandescent light bulb. Slides were subsequently stained with 2% Giemsa solution for 15 minutes then dried; metaphase spreads were scored over 1000 nuclei/slide.

CHAPTER III

RESULTS

The activation of mouse splenocytes by Con A is accurately assayed by measuring the incorporation of radioactive thymidine, uridine and leucine, all of which increase to reach peak values approximately 48-60 hours after initiation of culture.

The increase in ^{3}H-leucine and ^{3}H-uridine incorporations are detected as early as 12 hours while ^{3}H-thymidine incorporation cannot be measured earlier than 24-30 hours.

Since it was necessary to determine simultaneously the sensitivities of protein, RNA and DNA synthesis to purine analogues, fully activated cells (40-48 hours) were used to establish the conditions required for maximum impairment of transcription in cultured lymphocytes.

3.1 Purine Analogues in Metabolically Active Cells

3.1.1 Dose Response Curve

From the proposed mechanism of action of 8-AG in animal cells (Roy-Burman, 1970), incorporation of that analogue into RNA was expected to be revealed by a decrease in protein synthesis.

The length of exposure to 8-AG was arbitrarily fixed at 6 hours and the conditions for maximum incorporation into RNA established for that length of time. Figure 3

shows the effects of increasing concentrations of 8-AG on the incorporations of ^{3}H-thymidine, ^{3}H-uridine and ^{3}H-leucine measured at 48 hours.

After the 6 hours exposure, there was little or no inhibition of ^{3}H-leucine or uridine incorporations while the incorporation of ^{3}H-thymidine was severely affected. A concentration of 6.6×10^{-6} M 8-AG was sufficient to decrease thymidine incorporation by 40% and maximum inhibitions (80%) were achieved with doses of 6.6×10^{-5} M or higher which decreased ^{3}H-leucine and uridine incorporation by no more than 20%.

For purpose of comparison, similar experiments were carried out with 6 -TG, another analogue of guanine, having a more complex mode of action than 8-AG. Unlike 8-AG, 6-TG can enter both RNA and DNA and also inhibits some of the pathways involved in DNA synthesis (Lepage and Jones, 1961).

The results obtained with 6.6×10^{-5} M 6-TG are shown in Table I. Here again, ^{3}H-thymidine incorporation was more sensitive (90% inhibition) than were leucine and uridine incorporations which were inhibited by 70 and 40% respectively. There was very little inhibition of biosynthesis of RNA and protein in resting cells.

Degrees of inhibition observed with 6-TG were in general higher than with 8-AG. In a few experiments somewhat

Figure 3: Effect of Different Concentrations of 8-AG on the Incorporation of ^{3}H-Thymidine ^{3}H-uridine and ^{3}H-Leucine in Resting and Con A Stimulated Mouse Splenocytes.

8-AG was added at 42 hours and the incorporation of radioactive precursors measured at 48 hours, in presence of 8-AG. Results from 3 experiments are combined, the bars represent standard deviations.

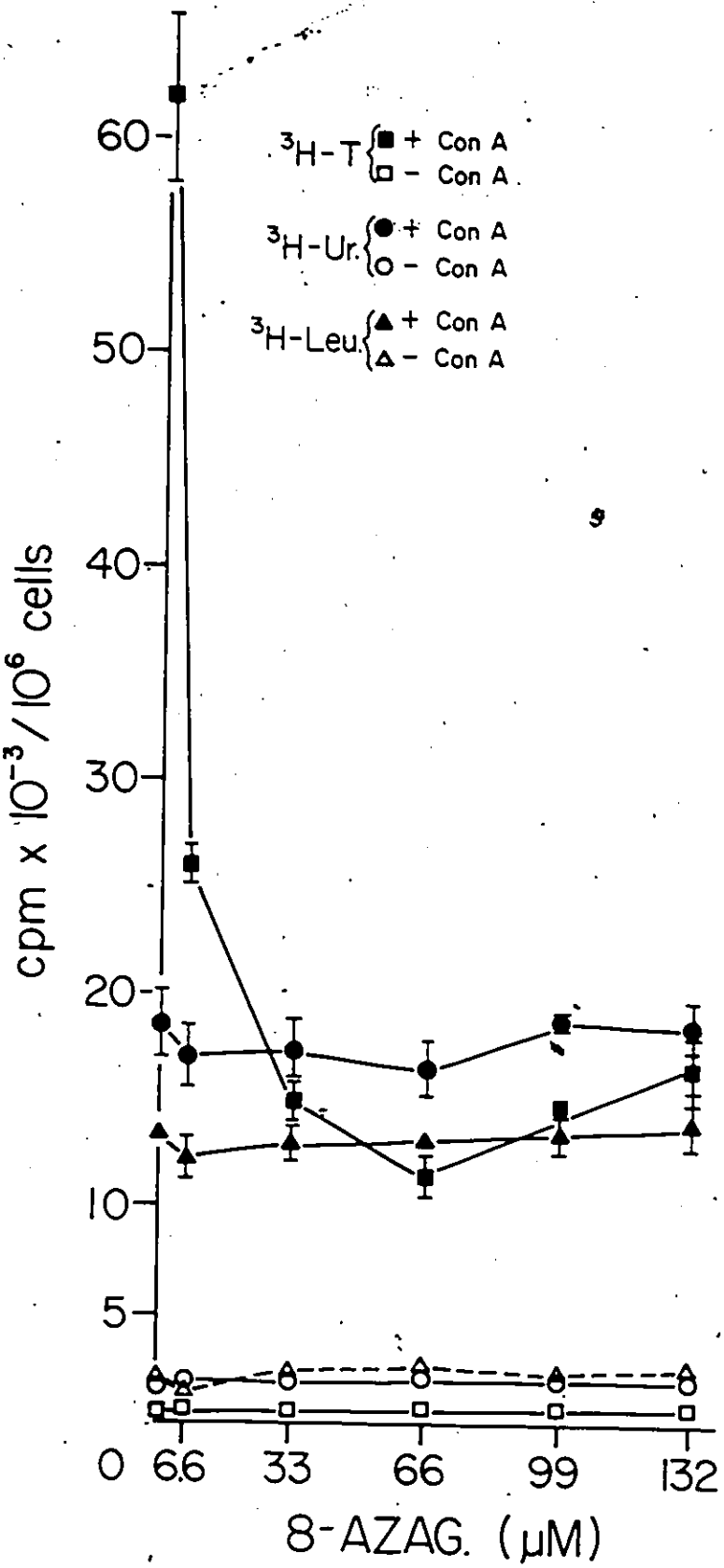

60
50
40
30
20
10
5
0
6.6
33
66
99
132
cpm x 10⁻³ / 10⁶ cells
8-AZAG. (μM)
³H-T
+ Con A
- Con A
³H-Ur.
+ Con A
- Con A
³H-Leu.
+ Con A
- Con A

TABLE I

Effect of 6-TG on the Incorporation of ^{3}H-leucine, ^{3}H-Uridine and ^{3}H-Thymidine in proliferating Mouse Splenocytes.

Treatment	^{3}H-leucine	^{3}H-Uridine	^{3}H-Thymidine
Resting control	2819 ± 27	2470 ± 99	1051 ± 47
Resting 6-TG	2213 ± 275 (22%)	1919 ± 31 (23%)	676 ± 13 (30%)
Con A control	18489 ± 452	19930 ± 816	85589 ± 2214
Con A 6-TG	5782 ± 474 (69%)	12311 ± 41 (38%)	4888 ± 162 (84%)

6-TG (6.6×10^{-5}M) was given 42 hours after the initiation of culture. Incorporation of radioactive precursors, expressed as cpm /10^{6} cells, was measured at 48 hours in presence of 6-TG.

Values are means of triplicates ± SD

Numbers in bracket represent percent inhibition over control.

lower levels of inhibition (approximately 60%) were observed with both analogues but the stronger effect was always on the incorporation of ^{3}H-thymidine.

A concentration of 6.6×10^{-5} M was then selected for all the experiments with guanine and other base analogues.

3.1.2 Thymidine Transport

The action of 8-AG on thymidine incorporation might result from an inhibition of the Con A - dependent activation of thymidine transport in stimulated cells (Rudd, 1980).

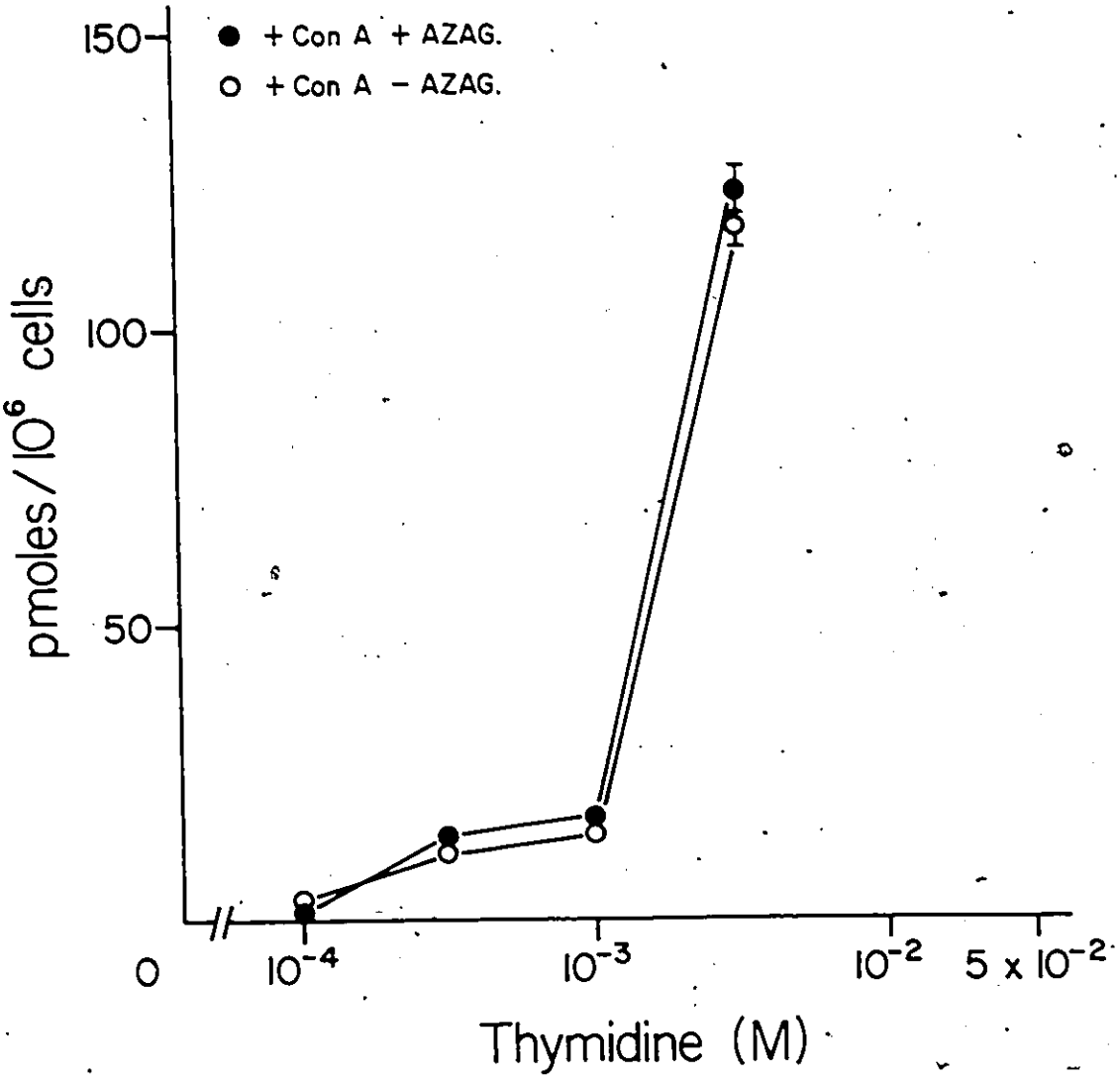
Thymidine transport was assayed at 48 hours in cells in which the ^{3}H-thymidine incorporation was maximally inhibited (80%) with 6.6×10^{-5} M 8-AG. Figure 4 shows that, at any extracellular concentration of thymidine and for comparable specific radioactivity, the same amount of radioactive thymidine was transported whether or not 8-AG was present and ^{3}H-thymidine incorporation inhibited. This indicates that approximately the same amount of ^{3}H-thymidine is made available for DNA incorporation in control and 8-AG treated cells.

3.1.3 Effect of Exogenous bases on the activity of purine analogues

The presence of natural bases during exposure to base analogues can affect the expression of their effects. In this and the following experiments, the expression of 8-AG or 6-TG effects was assessed by the degrees of inhibition

Figure 4: Thymidine Transport in Con A Stimulated Mouse Splenocytes Exposed to 8-AG.

Con A stimulated cells were exposed to 8-AG from 42 to 48 hours then suspended in prewarmed LMGB at a cell concentration of 10.0×10^6 cells/ml. A volume of 500 µl of cell suspension was then mixed with an equal volume of LMGB containing concentrations of thymidine between 10^{-4} to 10^{-2} M. The final specific activity of radioactive thymidine remained constant at 20 µci/ml. A 200 µl aliquot of reaction mixture was then centrifuged using the microcentrifugation technique outlined in the Materials and Methods


● + Con A + AZAG.
○ + Con A − AZAG.
150
100
50
0
pmoles/10^6 cells
10^-4
10^-3
10^-2
5 x 10^-2
Thymidine (M)

in thymidine incorporation. Figure 5 shows the changes in 8-AG dose-response curve when 13.2×10^{-5} M guanine, adenine or uracil were added together with 8-AG. It is clear that both guanine and adenine almost completely prevented the inhibitory effects of 8-AG. At any concentration of 8-AG, with guanine (curve B) or adenine (curve D) present, the incorporation of thymidine measured represented more than 85-90% control values while in the absence of the bases (curve A), the counts did not exceed 15-20% control.

Similarly (Table 2), the 90% inhibition of thymidine incorporation caused by 6.6×10^{-5} M 6-TG was reduced to less than 10% by the simultaneous addition of 13.2×10^{-5} M guanine.

However, in presence of uracil (curve C), the dose-response curve for 8-AG effects was not changed. The degrees of inhibition were the same, with the same inhibitory maximum (80%) reached with 6.6×10^{-5} M analogue, whether or not uracil was present.

Guanine and adenine alone did not affect the incorporation of thymidine, nor did uracil in the conditions used here. The prevention of the inhibitory effects of guanine analogues by adenine and not only by guanine can be explained by the intracellular interconversions between purine nucleotides or by the use of common metabolic pathways by all purines (Hershfield et al, 1976).

Figure 5: Effect of Exogenous Bases on the Inhibition of ^{3}H-Thymidine Incorporation by 8-AG.

Guanine, adenine or uracil (13.2×10^{-5} M) were added along with the different concentrations of 8-AG, at 42 hours. Incorporation of thymidine was measured at 48 hours. Points represent the means of 3 experiments. The bars are standard error of the mean. Resting counts ranged between $1.5 - 3.0 \times 10^{3}$ cpm.

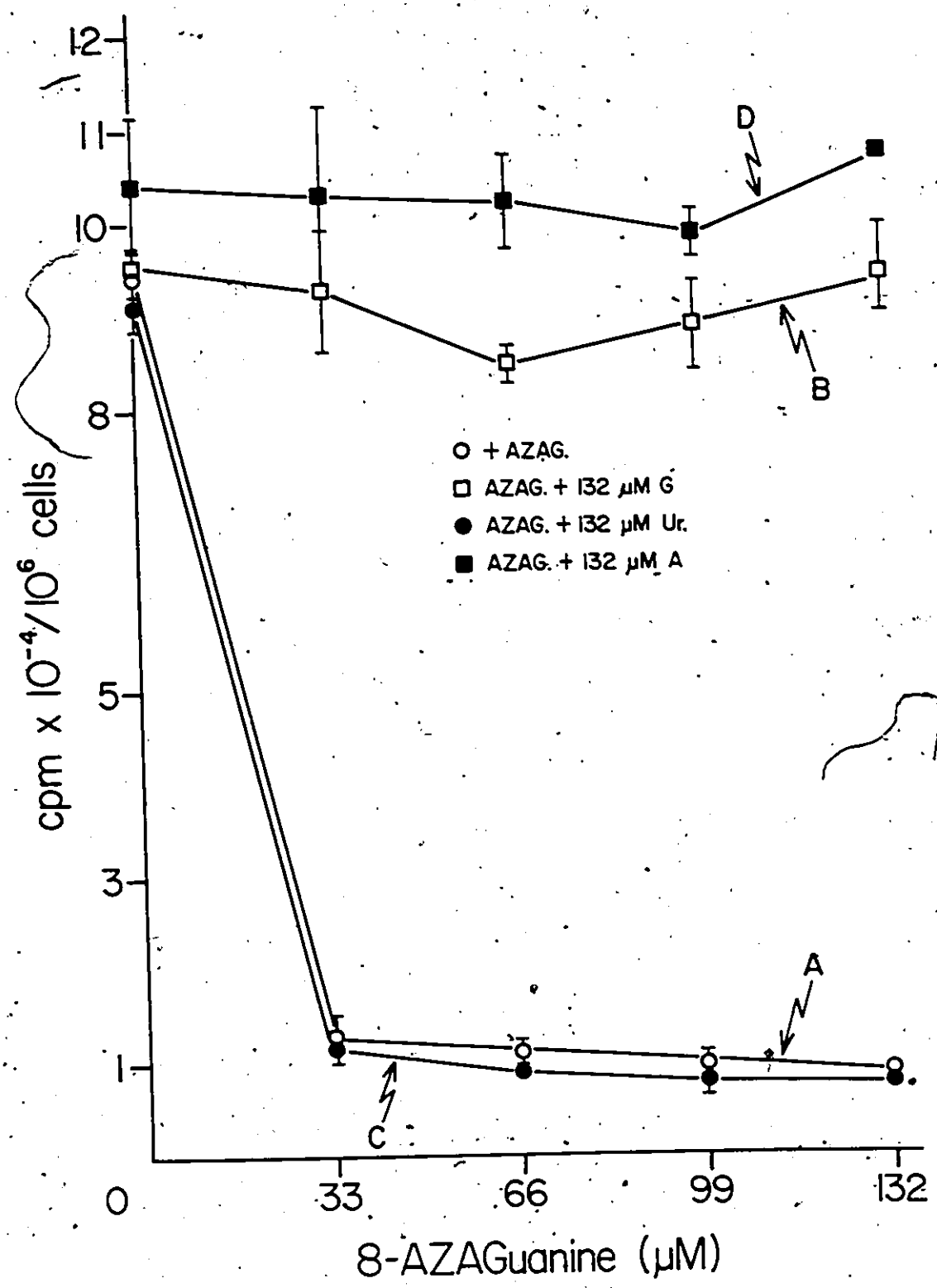

12
11
10
8
5
3
1
0
33
66
99
132
D
B
A
C
O + AZAG.
□ AZAG. + 132 μM G
● AZAG. + 132 μM Ur.
■ AZAG. + 132 μM A
cpm x 10⁻⁴/10⁶ cells
8-AZAGuanine (μM)

TABLE 2

Inhibition by Guanine of the Effects of 6-TG in Con A - treated Mouse Splenocytes.

cultures	^{3}H-Thymidine Incorporation	
	- Guanine	+ Guanine
Con A	59132 ± 189	61914 ± 930
Con A 6-TG	5800 ± 31 (90%)	51727 ± 308 (8%)

Guanine (13.2×10^{-5} M) was added along with 6-TG (6.6×10^{-5} M) in 42 hours Con A treated cells.

^{3}H-thymidine incorporation (cpm /10 cells) was measured at 48 hours.

Resting counts ranged between $0.5 - 1.0 \times 10^3$ cpm.

Thus, all the effects of guanine analogues in proliferating lymphocytes must result from their ability to mimic guanine and, as shown in figure 6, there is a competition between the base and it analogues for some function related to DNA synthesis. The degree of inhibition of thymidine incorporation by 6.6×10^{-5} M 8-AG was inversely proportional to the concentration of guanine until the ratio added guanine: added 8-AG was equal to 1, the minimum ratio at which the effects of the analogue were no longer expressed.

However, once the effects of guanine analogues were expressed (6 hour-exposure in the absence of excess purine bases) they were no longer sensitive to the presence of exogenous purines. In fact, the inhibition of thymidine incorporation by 8-AG or 6-TG was irreversible. Figure 7 illustrates the persistence of 8-AG effects after washing and incubation of the cells in fresh (analogue-free) medium containing 13.2×10^{-5}M guanine. A general increase in thymidine incorporation with time of incubation was observed. In the cultures previously treated with 8-AG and then washed, the reincubation with guanine (curve B) for 4 hours seemed to increase slightly the incorporation of thymidine as compared to the cultures incubated in the absence of guanine (curve A). Nonetheless these counts never reached those of control non-azaguanine cultures (curves A and B) which increased proportionally in such a way that the degree of inhibition remained

Figure 6: Effect of Different Concentrations of Guanine on the Inhibition of Thymidine Incorporation by 8-AG.

Guanine was added along with 8-AG (6.6×10^{-5}M) from 42 to 48 hours in Con A stimulated mouse splenocytes. Thymidine incorporation was measured at 48 hours in presence of 8-AG and guanine. Points represent the means of 3 experiments. Resting counts ranged from 1.0 to 1.5×10^3 cpm.

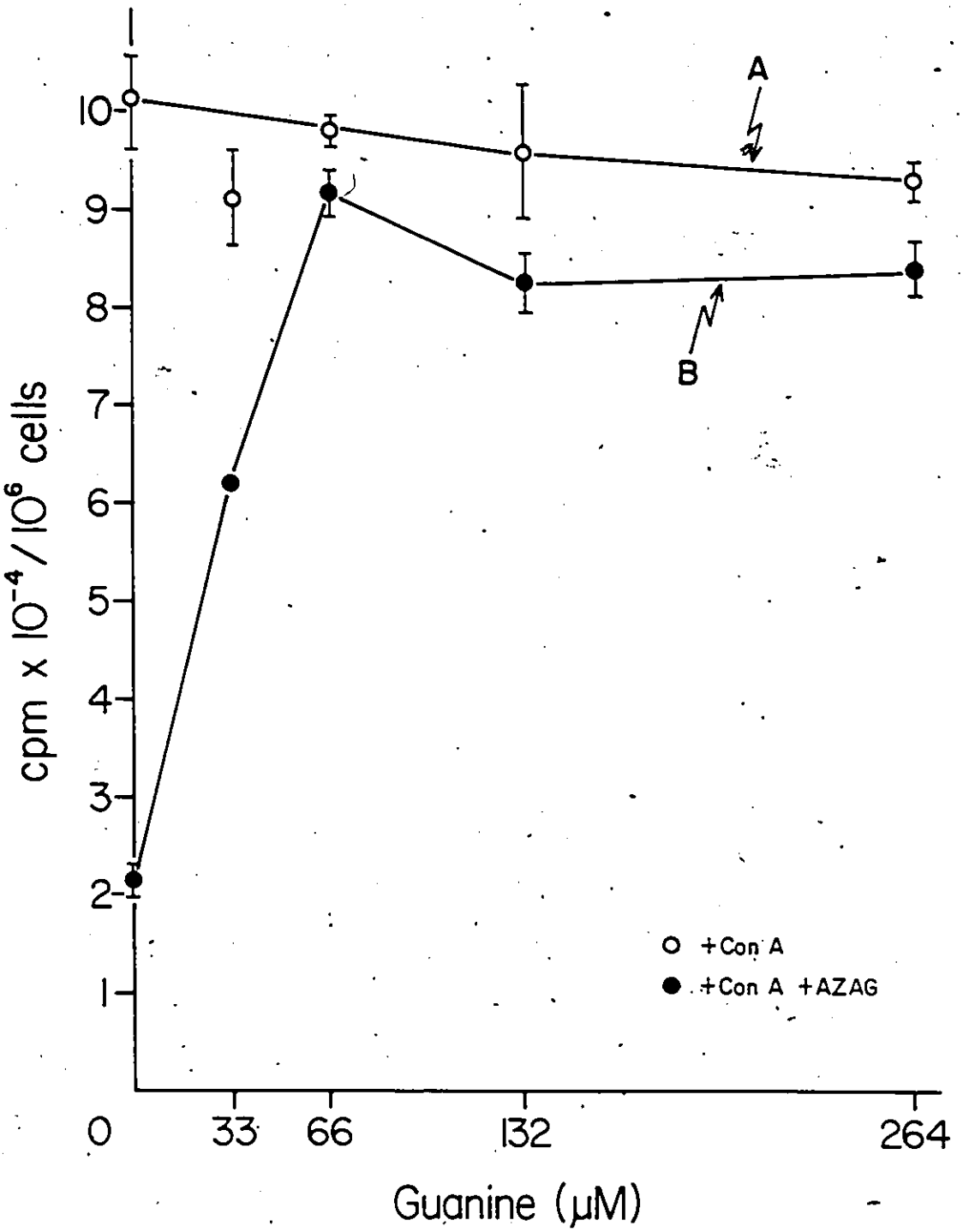
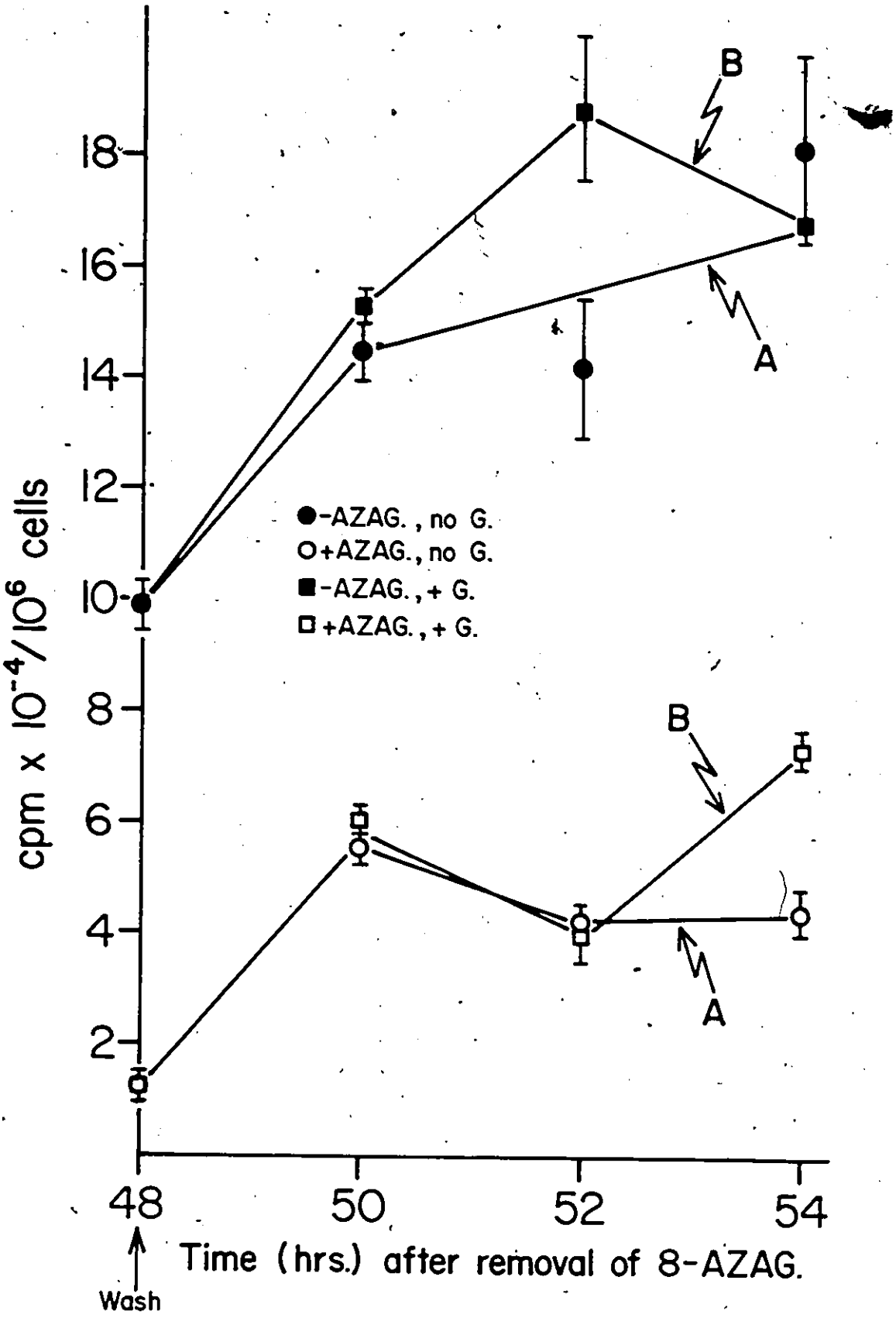

A
B
10
9
8
7
6
5
4
3
2
1
cpm x 10^{-4} / 10^6 cells
0
33
66
132
264
Guanine (μM)
+Con A
+Con A +AZAG

Figure 7: Persistence of 8-AG Effect on Thymidine Incorporation after Wash and/or Addition of Guanine.

8-AG (6.6×10^{-5}M) was added between 42 and 48 hours after Con A addition, then cells were washed and reincubated with or without guanine (13.2×10^{-5}M). Thymidine incorporation was measured at 48 hours and after wash.


B
A
18
16
14
12
10
8
6
4
2
cpm x 10^{-4}/10^6 cells
●-AZAG., no G.
○+AZAG., no G.
■-AZAG., + G.
□+AZAG., + G.
B
A
48
50
52
54
Wash
Time (hrs.) after removal of 8-AZAG.

almost unchanged 6 hours after removal of the analogues from the medium.

3.1.4 Onset of the Inhibition of Thymidine Incorporation

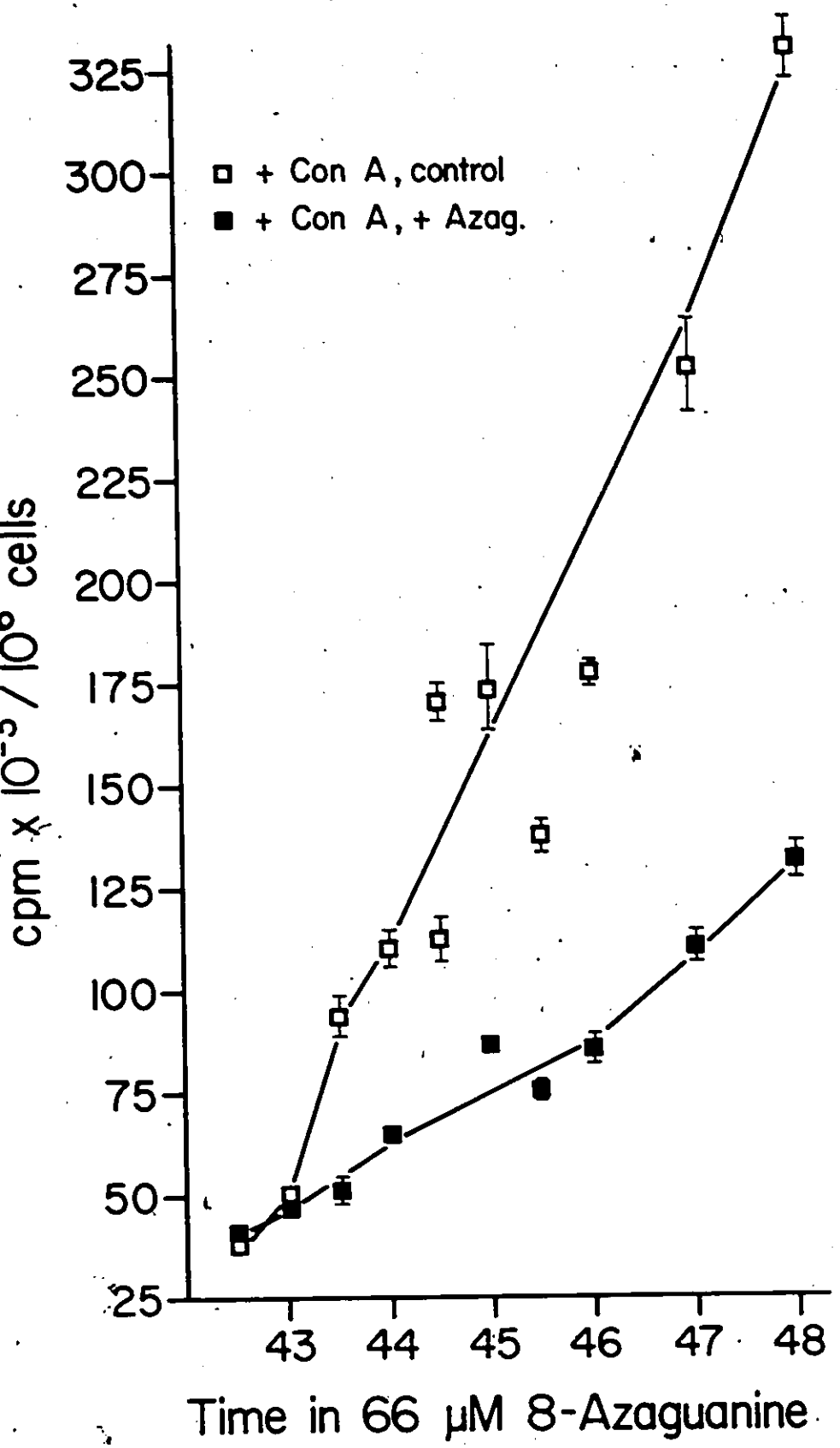
Inhibition of DNA synthesis following an exposure to 8-AG, is usually an indirect effect resulting from the inhibition of protein synthesis.

To determine whether in proliferating lymphocytes, the effects of 8-AG on DNA synthesis were also indirect, 5μ ci/ml ^{3}H-thymidine was added along with 8-AG at 42 hours and the changes in thymidine incorporation were followed every 30 minutes for the duration of the exposure.

Figure 8 shows that significant difference ($p=0.001$) from the control non-azaguanine treated cultures was observed as early as 1.5 hours after the addition of 8-AG, at which time DNA synthesis had decreased by about 45%. Increases in ^{3}H-thymidine incorporation with time were still observed in the cultures treated with the analogue but these were very low compared to the control cultures. This indicated that the progression of the cultures in or beyond the S phase was greatly reduced but there was still some replication in presence of 8-AG, that could have resulted from the activity of few, unaffected cells or from the combined equal and partially inhibited activities of all the cells in the cultures.

Figure 8: Time-course of the Effects of 8-AG on Thymidine Incorporation.

Radioactive thymidine (5 μci/ml) was added together with 8-AG (6.6×10^{-5} M) 42 hours after Con A addition. Incorporation of thymidine was measured every 30 minutes up to 48 hours. Points represent means of 3 experiments ± SI


□ + Con A, control
■ + Con A, + Azag.
cpm x 10^{-3} / 10^6 cells
325
300
275
250
225
200
175
150
125
100
75
50
25
43
44
45
46
47
48
Time in 66 μM 8-Azaguanine

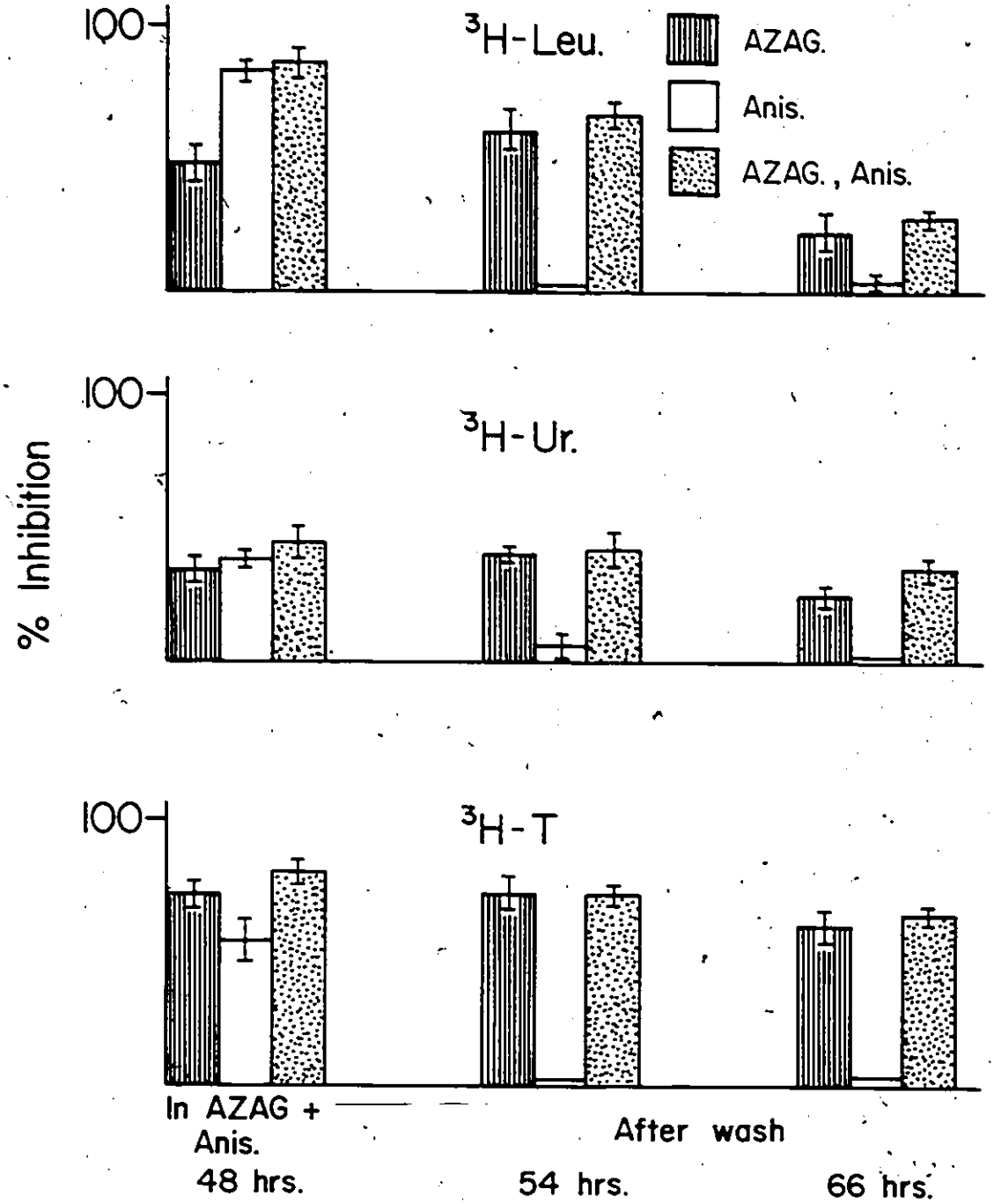
3.1.5 Non-Requirement for Protein Synthesis

The overall protein synthesis being only partially inhibited by 8-AG, the synthesis of abnormal or toxic proteins in presence of the analogues could explain the observed increase with time of the inhibition of DNA synthesis (figure 8). It was thus important to investigate the role of protein synthesis in the action of 8-AG on DNA synthesis.

Anisomycin, a reversible inhibitor of protein synthesis, was added along with 8-AG for 6 hours and the effects of the analogue assessed in presence of the inhibitor and after recovery of protein synthesis. Figure 9 shows that all the inhibitory effects of 8-AG were independent of protein synthesis. Anisomycin alone decreased the incorporation of leucine by 82%, that of uridine by 40% and the incorporation of thymidine by 48%. The effects of anisomycin were apparent within 30 minute incubation (data now shown). 8-AG alone showed the usual pattern of inhibition i.e. 45, 30 and 75% for leucine, uridine and thymidine incorporation respectively. When added together, the effects of the 2 drugs were not additive although the degree of inhibition of ^{3}H-thymidine was slightly higher than that observed for each drug separately.. After washing (to restore protein synthesis) and addition of excess guanine, the levels of inhibition were identical in all the cultures exposed to 8-AG whether or not protein synthesis had been inhibited.

Figure 9: Effect of Anisomycin on 8-AG Action in Con A Stimulated Mouse Splenocytes.

Anisomycin (200 ng/ml) was added between 42 and 48 hours in presence or absence of 8-AG. Cells were then washed and reincubated in presence of guanine. Incorporation of radioactive precursors was measured in presence of the drugs at 48 hours and after wash. Results are expressed as percent inhibition over control non 8-AG, non-anis. and/or non-8-AG-anis. cultures.


100
^{3}H-Leu.
AZAG.
Anis.
AZAG., Anis.
100
^{3}H-Ur.
% Inhibition
100
^{3}H-T
In AZAG + Anis.
48 hrs.
After wash
54 hrs.
66 hrs.

This suggests that the inhibition of DNA synthesis by 8-AG is not due to the synthesis or accumulation of toxic proteins.

3.2 Effects of guanine analogues on the entry into S phase.

Exposure of fully activated lymphocytes to guanine analogues resulted in severe inhibition of DNA synthesis accompanied by a decresse in the synthesis of proteins and RNA. The inhibition of protein and RNA synthesis can be explained by the incorporation of the analogues into ribonucleic acids. However, the effect on DNA synthesis apparently does not require protein synthesis and is so rapid and pronounced that it might not be a consequence of impaired transcription.

One possibility by which such an immediate inhibition of replication could occur is through incorporation of the analogues into DNA, althogh the entry of 8-AG into DNA has never been demonstrated in animal cells. Therefore, exposure to these analogues prior to the onset of DNA synthesis and also before the increase in transcription may give some insight into the actual mechanism of the inhibition of replication and perhaps may reveal the importance of transcripts made at various times during trahsformation, for the subsequent proliferation.

The prevention of the action of guanine analogues by guanine was exploited to limit the interference of the analogues at selected periods.

3.2.1 Similarities in the inhibition patterns for different times of exposure

8-AG was given for the standard 6 hours at different times after Con A addition and the incorporation of thymidine, uridine and leucine measured after removal of the analogue from the medium and incubation with 13.2×10^{-5} M guanine for up to 48 hours.

The results obtained after an exposure to 6.6×10^{-5} M 8-AG for the first 6 hours following Con A addition are shown in Table 3. Protein and RNA synthesis were measured at 12, 24 and 48 hours; DNA synthesis was measured at 24 and 48 hours. It appears that all the 3 parameters, protein, RNA and DNA synthesis were permanently decreased after the treatment with 8-AG. At 12 hours, the incorporation of leucine and uridine were similarly affected; they both represented about 50% of the incorporations in control cultures. The inhibition of uridine incorporation decreased later while that of leucine remained constant and by 48 hours the final degrees of inhibition were 50 and 30% for leucine and uridine incorporation respectively. In the case of thymidine incorporation, there was a profound inhibition (80%) at 48 hrs. The degree of inhibition in resting cells similarly treated varied from 30% for leucine incorporation to 40% for the basal thymidine incorporation.

Tables 4 and 5 show the results of exposure to 8-AG for 6 to 12 hours of culture and from 12 to 18 hours, after

TABLE 3

Incorporation of ^{3}H-leucine, ^{3}H-Uridine and ^{3}H-Thymidine at Different Times after an Exposure to 8-AG from 0 to 6 hours.

Tissue of Assay (hours)	^{3}H-leucine			^{3}H-Uridine			^{3}H-Thymidine		
	Control	+8-AG	% Inhibition	Control	+8-AG	% Inhibition	Control	+8-AG	% Inhibition
12	5764 ±113	2772± 159	52	19783± 455	9118 ± 696	54	–	–	–
24	7717± 159	4018± 342	48	25452± 1357	19423± 591	24	7019± 409	3697± 145	47
48	14604± 685	7436± 281	49	32680± 2539	23310± 514	29	66321± 602	12551± 1170	81

Cells were incubated with Con A and 8-AG for 6 hours, then washed 3 times with RPMI 1640, and resuspended in medium containing Con A and guanine.

TABLE 4

Effect of an Exposure to 8-AG from 6 to 12 hours on Con A Response at 48 hours.

Treatment	^{3}H-leucine	^{3}H-Uridine	^{3}H-Thymidine
Resting	2789 ± 89	3860 ± 42	1710 ± 63
Resting - 8-AG	2527 ± 139 (9%)	3096 ± 254 (20%)	154[illegible] ± 97 (10%)
Con A	14736 ± 1032	31356 ± 1852	48593 ± 754
Con A 8-AG	12712 ± 400 (14%)	30544 ± 1675 (3%)	19048 ± 516 (61%)

Cells were treated as in the experiment Table 3.

The numbers in brackets represent percent inhibition over control, non-azaguanine treated cultures.

TABLE 5

Effect of an Exposure to 8-AG from 12 to 18 hours on Con A Response at 48 hours.

Treatment	^{3}H-leucine	^{3}H-Uridine	^{3}H-Thymidine
Resting	2969 ± 106	4354 ± 327	2079 ± 89
Resting 8-AG	1882 ± 89 (36%)	4012 ± 258 (8%)	1850 ± 128 (11%)
Con A	12838 ± 391	54497 ± 4767	55056 ± 1267
Con A 8-AG	6826 ± 92 (57%)	33519 ± 1717 (36%)	9598 ± 79 (83%)

Cells were treated as in the experiment Table 3.

The numbers in brackets represent percent inhibition over control, non-azaguanine treated cultures.

the increase in transcription has began. Here again, when measured at 48 hours, there was a much stronger inhibition of thymidine incorporation (60 to 85%) than of leucine (10 to 45%) or uridine incorporation (3 to 35%).

In comparing the effects of the different times of exposure to 8-AG (figure 10) or 6-TG (figure 11) on the proliferative response at 48 hours, it is evident that exposures either before increased transcriptional activity or any time after (even after the onset of DNA synthesis) resulted in similar patterns of inhibition at 48 hours, with the same severe effect on DNA synthesis. There was some variability in degree of inhibition in different experiments performed with one analogue under the same conditions. This is illustrated in figures 10 and 11 by the two sets of data (labelled 1 and 2) obtained after exposures from 42 to 48 hours of culture: in some experiments, the incorporation of leucine and uridine were not, or only slightly affected and the inhibition of thymidine incorporation was less marked (60-65%).

Whether or not the data in figures 10 and 11 exclude the incorporation of the analogues into DNA as a mechanism of inhibition of DNA synthesis is not evident. The presence of analogues might have indirectly affected some earlier events essential for proliferation and the inhibition of DNA synthesis could have been a consequence of the abnormal activation.

Figure 10: Effect of 8-AG Treatments at Different Times of Activation on Con A Response at 48 hours.

Cells were exposed to 8-AG at times indicated, for 6 hours, then washed and reincubabed in presence of excess guanine, with or without Con A (depending on the time of treatment) until 48 hours. 2 separate sets of results are presented for the treatments between 42 and 48 hours to show the experimental variability; each set of results represent means of 3-4 different experiments.

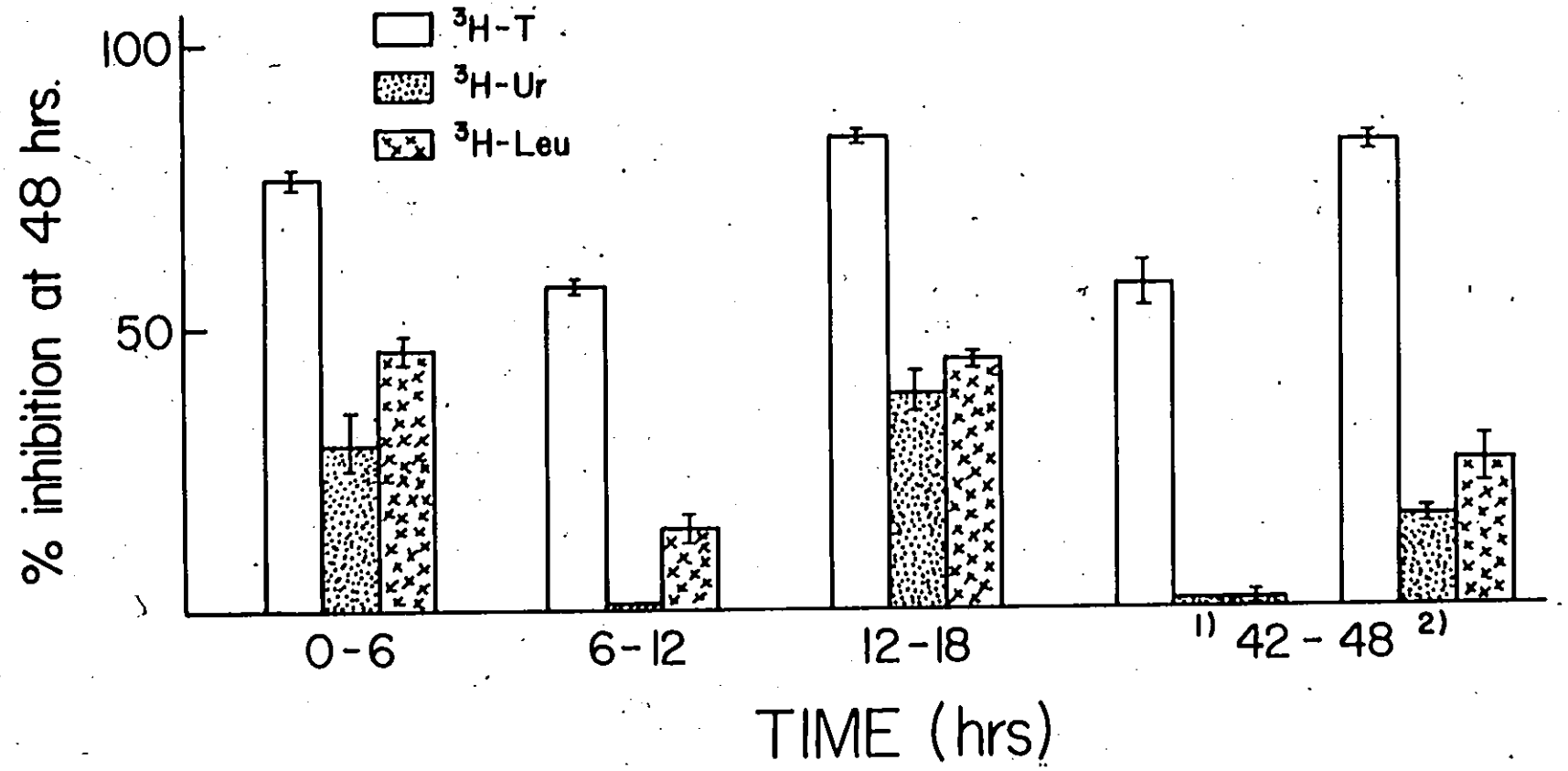
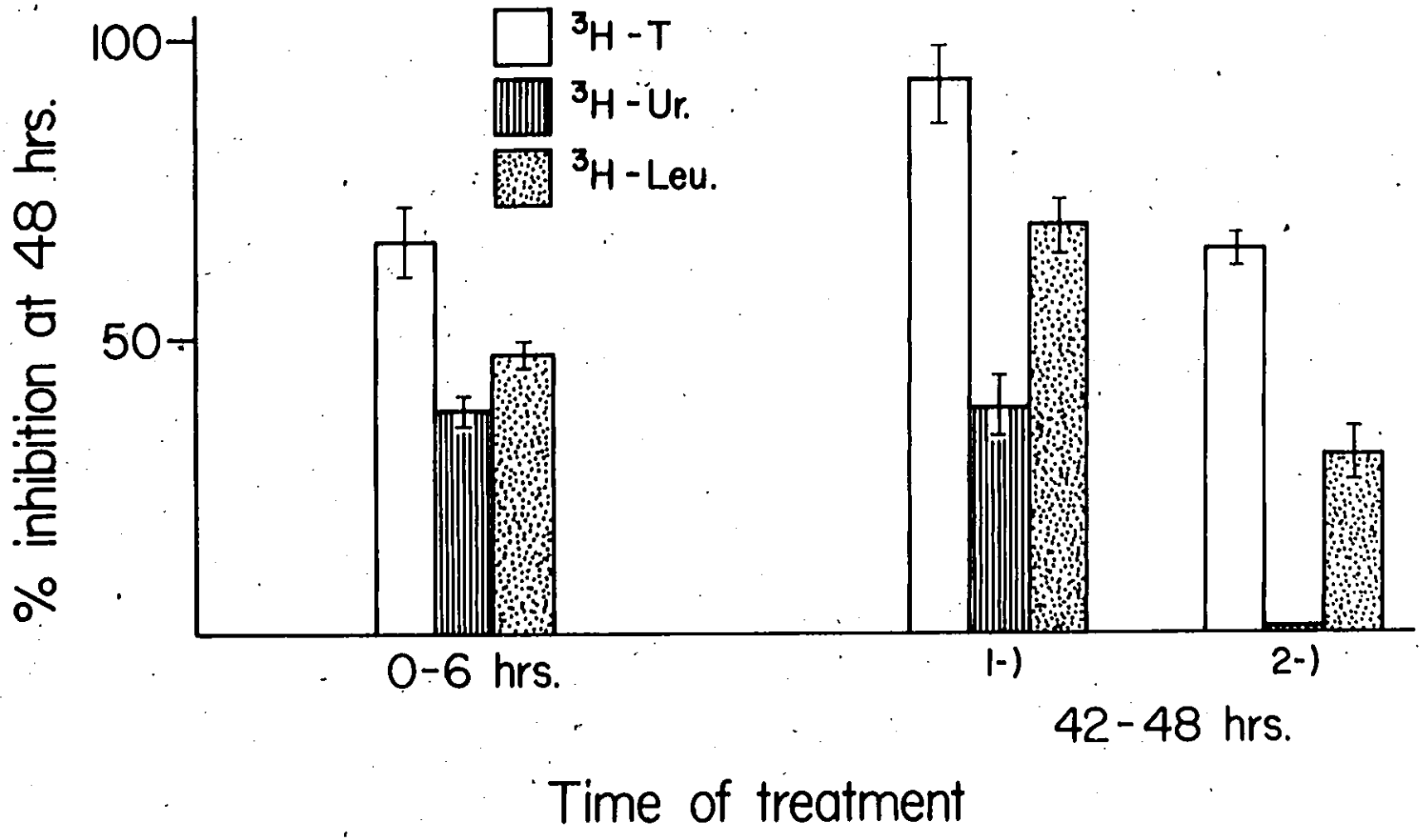

100
50
% inhibition at 48 hrs.
^{3}H-T
^{3}H-Ur
^{3}H-Leu
0-6
6-12
12-18
1) 42-48 2)
TIME (hrs)

Figure 11: Effect of 6-TG Treatments at Different Times of Activation on Con A Response at 48 hours.

Cells were exposed to 6-TG from 0 to 6 hours or from 42 to 48 hours after Con A addition. Incorporation of ^{3}H-thymidine, ^{3}H-uridine and ^{3}H-leucine was measured at 48 hours after wash and incubation of cells with guanine alone for the S phase treatments and guanine plus Con A for the early treatments. The 2 sets of results presented for the 42-48 hour treatment illustrate the experimental variability.


100
50
% inhibition at 48 hrs.
^{3}H - T
^{3}H - Ur.
^{3}H - Leu.
0-6 hrs.
1-)
2-)
42-48 hrs.
Time of treatment

3.2.2 Potassium Uptake

The activation of K+ influx was assessed at 12 hours in Con A-treated cells exposed to 8-AG at 6 hours and whose thymidine incorporation at 48 hours was inhibited by 75-80%.

Table 6 shows that the influxes of K+ in 8-AG and control cultures were not significantly different ($p=.3$) from each other. In both Con A control and Con A - 8-AG cells, the uptake of K+ was about 1.8-fold greater than that of resting cells. This corresponds to uptakes of 7.2-7.4 fmoles/cell/hr in the stimulated cultures as compared to 4.0-4.2 fmoles/cell/hr in resting cultures. The influx measured at 48 hours (data not shown) after early treatment with 8-AG were also the same as control. It is thus clear that 8-AG treatment does not inhibit early stages of activation.

3.2.3 Blastogenesis

The morphological changes induced by mitogens after 20 hour culture are also essential for the subsequent entry into S phase. It was then necessary to investigate the effects on early exposure to 8-AG might have on the Con A induced increase in cell size and decrease in chromatin aggregation.

Figure 12 compares the Coulter 'channelizer' size distributions at 48 hours of control cultures with those of cultures exposed to 8-AG from 0 to 6 hours. The stimulated cultures, both control (curve A) and 8-AG treated (curve B) showed

TABLE 6

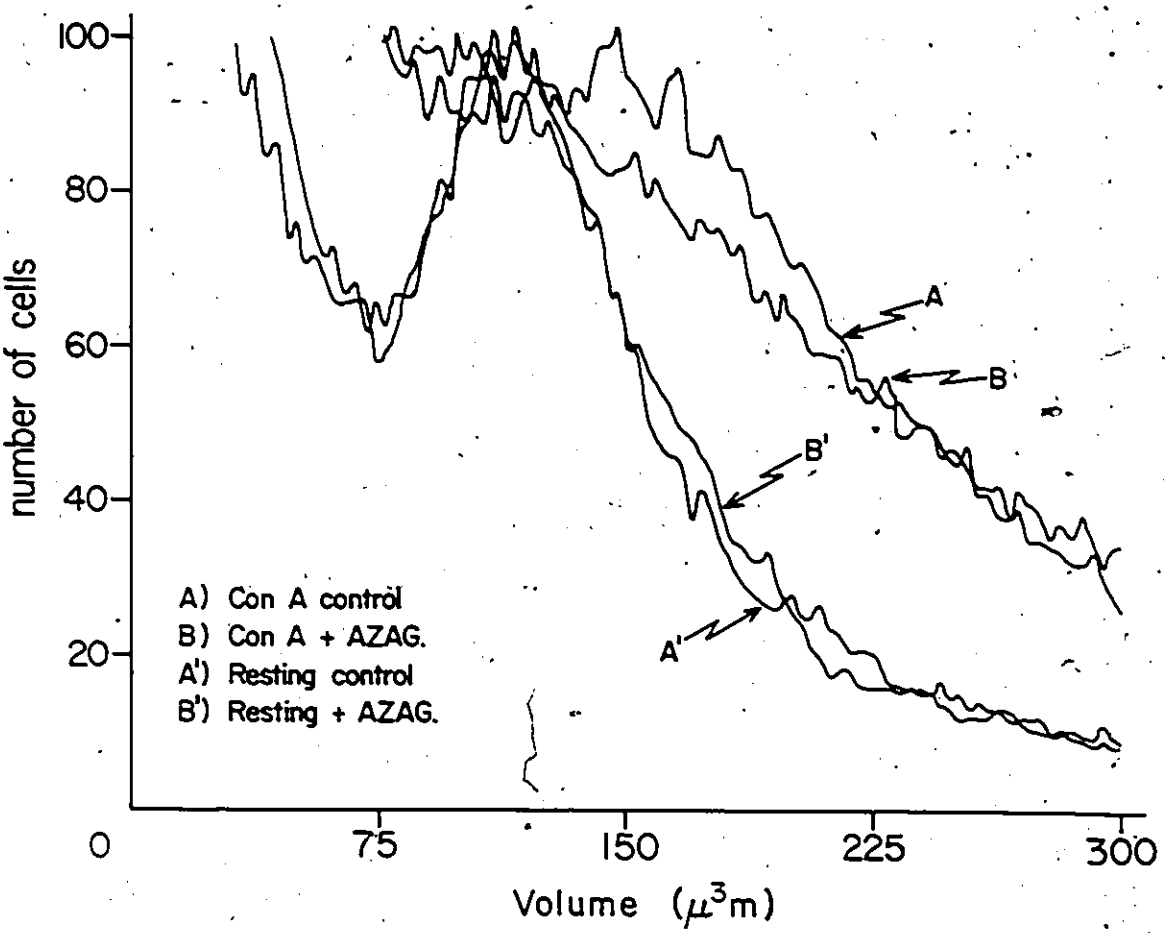
Con A - induced Activation of Potassium Influx in presence of 8-AG.

Treatment	Potassium Influx (f moles/cell/hr)	
Resting	4.2 + 3.8;	4.11 + .02
Con A	7.4 + 15;	7.23 + .10
Resting 8-AG	4.06 + .18;	4.03 + .05
Con A 8-AG	7.27 + .12	7.22 + .03

Potassium Influx was measured at 12 hours (as outlined in Materials and Methods) in cells exposed to 8-AG for the last 6 hours.

Results from 2 experiments are shown; each was performed in quadruplicate.

Figure 12: Coulter Channelizer Cell Size Distributions of 48 hours Cultures of Resting and Con A Stimulated cells Exposed or not to 8-AG from 0 to 6 hours.


100
80
60
40
20
0
75
150
225
300
number of cells
Volume (μ^3m)
A) Con A control
B) Con A + AZAG.
A') Resting control
B') Resting + AZAG.
A
B
B'
A'

a wider distribution of cell sizes (ranging from 75 μm^3 to 300 μm^3 volume) than the resting cultures (curves A^1 and B^1) where most cells had a volume around 114-120 μm^3. Although there were slightly more cells with a volume in the 150 to 225 μm^3 range in the Con A control, the mean cell volume in these 48 hours stimulated cultures was around 230 μm^3 whether or not they had been exposed to 8-AG. The mean cell volume in resting cultures was 160 μm^3 and 170 μm^3 for control and 8-AG treated respectively.

It was then concluded that exposures to 8-AG during the early stages of activation had no effect on blast transformation measured at 48 hours, even if protein synthesis was decreased by 50% and RNA synthesis by 35%. In these experiments, DNA synthesis was decreased by 85% at 48 hours.

Similar results were obtained when blast transformation was assessed by counting the proportion of cells with a diameter greater than 8 µM (Table 7).

3.2.4 Con A Response after Pretreatment of Resting Cells with 8-AG.

Guanine analogues had very little effect on the activities of resting cells but their presence during the first hours of activation prevented the entry of cells into S phase.

It was thus interesting to determine whether resting cells previously treated with these base analogues could respond to mitogens.

TABLE 7

Proportion of Blast Cells in Cultures in which the proliferative Response to Con A has been Inhibited by an Early Exposure to 8-AG.

Cultures	Percent Blasts	^{3}H-Thymidine Incorporation
Resting	17 ; 15	1033 + 106 ; 1485 + 151
Resting 8-AG	14 ; 18	941 + 87 ; 1209 + 34
Con A	48 ; 44	42178 + 1267 ; 54183 + 915
Con A 8-AG	40 ; 47	8236 _ 102 ; 8587 + 79

Cells were treated with 8-AG for the first 6 hours following Con A addition, then washed 3 times in RPMI 1640 and resuspended in medium containing Con A and guanine.

Blast cells were defined as cells greater than 8μm in diameter.

Results from 2 experiments are shown; values for thymidine incorporation represent means of triplicates + SD.

Table 8 compares the incorporation of thymidine, uridine and leucine measured at 48 hours in cells exposed to 8-AG before Con A addition with those measured when 8-AG was added together with Con A. The difference in the responses in the two assays is evident: the counts measured in the first case represented more than 90% of the control values while in the second case, low counts, typical of 8-AG effects in activated lymphocytes (80, 13 and 42% inhibition of DNA, RNA and protein synthesis respectively) were obtained with a stimulation index (ratio thymidine incorporated in stimulated cells: thymidine incorporated in resting cells) of 8.3. The stimulation index in cultures treated with 8-AG before the addition of Con A were the same as in control, untreated, cultures (19 fold).

Blast transformation (figure 13) could also be demonstrated in these cultures indicating that resting cells previously exposed to 8-AG can respond normally to mitogens.

3.3 Pyrimidine Analogues in Con A Response

An intact purine metabolism is essential in the immune response and purine analogues have been shown to inhibit de novo purine synthesis (McCollister et al, 1964). To exclude the possibility that the inhibition of lymphocyte proliferation observed with 8-AG and 6-TG resulted from their ability to interfere with purine metabolism, the effects of pyrimidine analogues, which also interfere with transcription but have a less pronounced effect on purine

TABLE 8

Comparison of the Response of Cultures Treated with 8-AG Before Con A Addition with that of Cultures Exposed to 8-AG in presence of Con A.

Pretreatment	Addition along with Con A	^{3}H-leucine	^{3}H-Uridine	^{3}H-Thymidine	S.I.
none	none	12108 ± 52	30112 ± 2214	37437 ± 1104	18.7
8-AG	none	10840 ± 450	31400 ± 672	32240 ± 1354	19.2
none	8-AG	7131 ± 114	25982 ± 823	7480 ± 468	8.3

Cells were treated with 8-AG for 6 hours, then washed 3 times in RPMI 1640 and resuspended in medium containing Con A and guanine.

S.I (stimulation index) = ^{3}H-thymidine cpm in Con A-treated cells / ^{3}H-thymidine cpm in resting cells

Values are means of triplicates + S.D.

Figure 13: Comparison of Cell Size Distribution at 48 hours between Cultures Exposed to 8-AG before Con A Addition and Stimulated Control Cultures, non-azaguanine Treated.

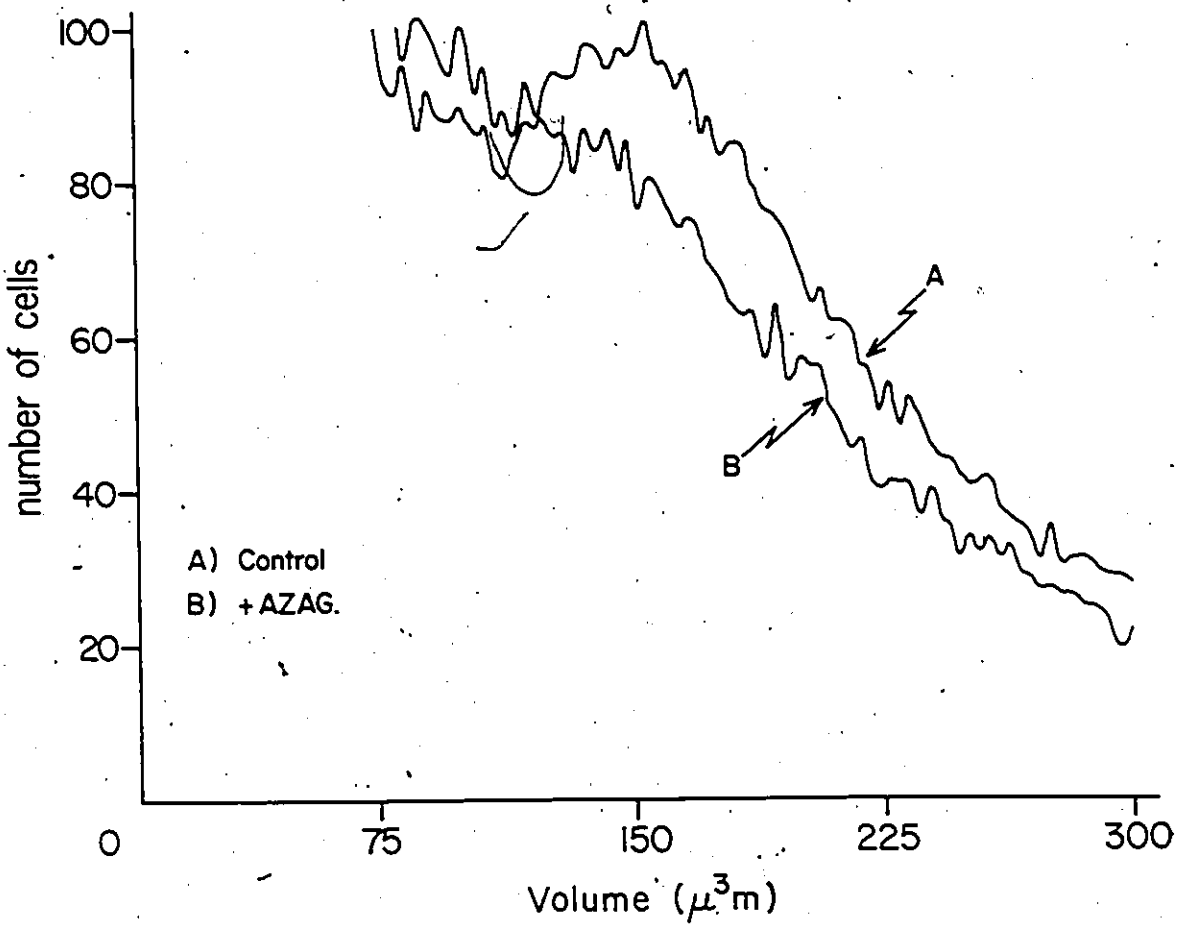
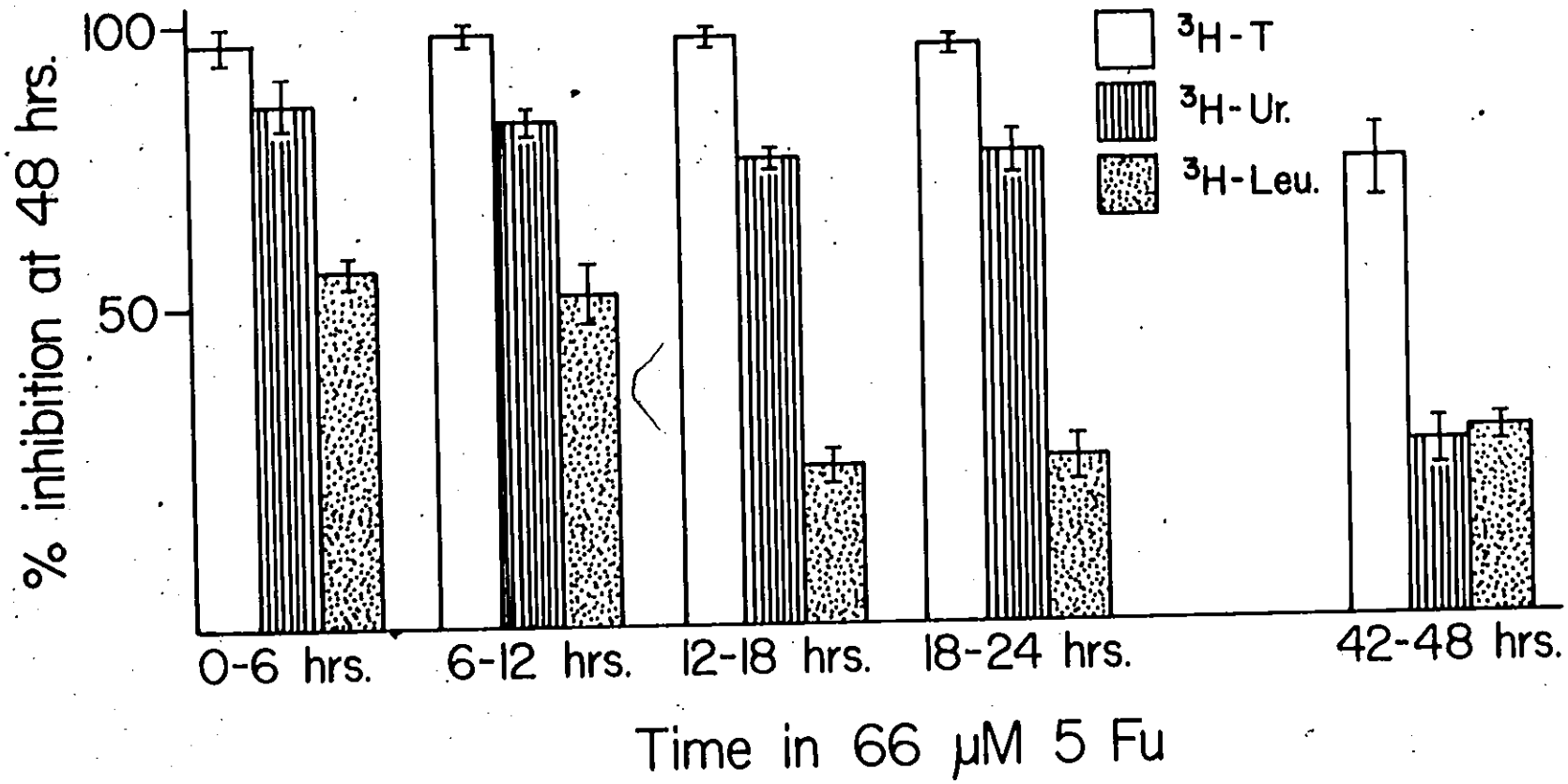

100
80
60
40
20
0
number of cells
75
150
225
300
Volume (μ^3m)
A
B
A) Control
B) +AZAG.

Figure 14: Effect of 5-FU Treatments at Different Times of Activation on Con A Response at 48 hours.

Cells were exposed to 5-FU for 6 hours, at times indicated, then washed and reincubated in presence of uracil, with or without Con A, until 48 hours. Results are means of 3 experiments.


100
50
% inhibition at 48 hrs.
^{3}H-T
^{3}H-Ur.
^{3}H-Leu.
0-6 hrs.
6-12 hrs.
12-18 hrs.
18-24 hrs.
42-48 hrs.
Time in 66 μM 5 Fu

synthesis were investigated.

3.3.1 Effects of 5-Fluorouracil.

5-Fluorouracil (5-Fu) was used to study the effects of pyrimidine analogues on the lymphocyte response to Con A. As in the case of the experiments with purine analogues, the cells were exposed for 6 hours to 6.6×10^{-5} M 5-Fu at various times after Con A addition and the proliferative response assessed at 48 hours. Figure 14 shows the inhibition of thymidine, uridine and leucine incorporations at 48 hours for the different times of exposure. Under all conditions, the incorporation of ^{3}H-thymidine was the most sensitive parameter. Leucine incorporation seemed more affected by exposure before 12 hours (60% inhibition as opposed to 30% for treatments at later times) and there was a smaller effect on uridine incorporation in the 42 to 48 hours treatment. Despite these differences and a stronger inhibition of uridine incorporation (75-80%), the general patterns of inhibition by 5-Fu in Con A activated lymphocytes resembled those observed with the analogues of guanine, and DNA synthesis was still the parameter the most affected in any 6 hour exposure.

3.3.2 Blast Transformation.

Figure 15 and table 9 show that blast transformation occurred in cultures exposed to 5-Fu during the first hours after activation. The distribution of cell size was typical

Figure 15: Coulter Channelizer Cell Size Distribution of 48 hours Cultures of Resting and Con A Stimulated Cells Exposed to 5-FU from 0 to 6 hours.

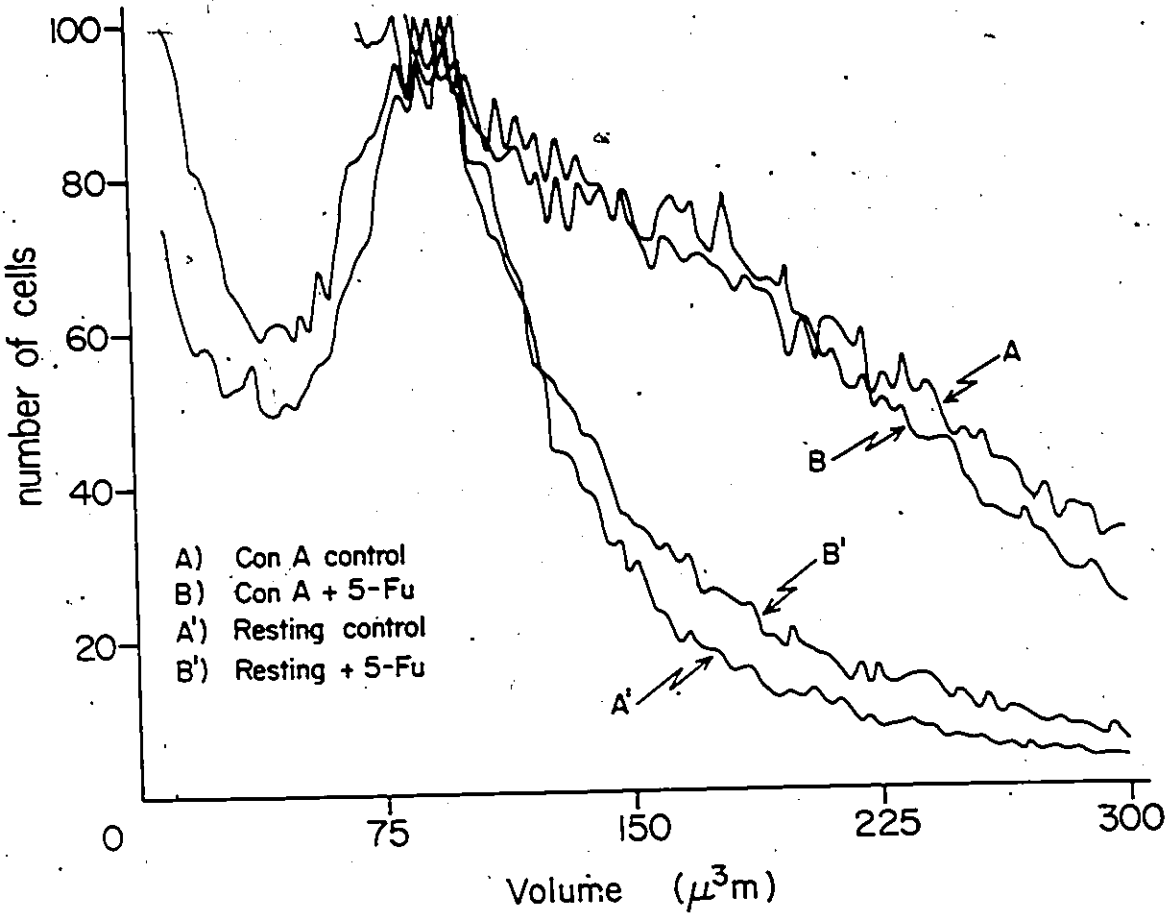

100
80
60
40
20
0
number of cells
75
150
225
300
Volume (μ^3m)
A) Con A control
B) Con A + 5-Fu
A') Resting control
B') Resting + 5-Fu
A
B
B'
A'

TABLE 9

Proportion of Blast Cells in Cultures in which the Proliferative Response to Con A has been Inhibited by an Early Exposure to 5-FU

Cultures	Percent Blasts	H-Thymidine Incorporation
Resting	9 ; 11	1346 ± 137 ; 1519 ± 88
Resting 5-FU	10 ; 8	905 ± 78 ; 876 ± 39
Con A	47 ; 42	53760 ± 3371 ; 77612 ± 3562
Con A 5-FU	39 ; 36	1233 ± 134 ; 3912 ± 27

Cells were treated with 5-FU for the first 6 hours following Con A addition, then washed and resuspended in medium containing uracil and Con A. Blast cells were defined as cells greater than 8 μm in diameter.

Results from 2 experiments are shown.

of Con A stimulated cells with a mean cell volume around 230 μm^3 and the proportion of cells with a diameter above 8 μm was the same in control and 5-FU treated cultures (about 40%).

3.4 Nuclear Changes

Both purine and pyrimidine analogues inhibit the proliferation of lymphocytes with no apparent effect on the increase in cell size.

The following experiment was intended to analyse the changes in nuclear size and in degree of chromatin aggregation and to relate them to the increase in cell volume. Table 10 shows the increase, with time of incubation, of the proportion of cells having large, decondensed nuclei, in control cultures as well as in cultures exposed to 8-AG or 5-FU during the early stages of activation. Protein, RNA and DNA synthesis were inhibited by 40; 25 and 80% in 8-AG treated cultures and by 60, 80 and 94% in the cultures treated with 5-FU. The nuclei were arbitrarily classified into 3 groups of morphotypes based on the appearance of chromatin in the light microscope (Dardick et al, 1981). Representative nuclei are shown in figure 16. Morphotype I represents the small nuclei with very condensed chromatin; characteristic of unstimulated cells, morphotype 2 the intermediate degrees of chromatin disaggregation and nuclear size and morphotype 3 represent the large nuclei, typical of blast cells, with little condensed chromatin.

24 hours after addition of Con A, about 50% of the nuclei in all the cultures were still type I, with only 1 to 3% type 3

TABLE 10

Percentage of Nuclei of Specific Morphotypes (Classified as to degree of Chromatin dissaggregation) in Cultures of Resting, Con A and Con A plus Base Analogues - treated Mouse Splenoctyes.

Incubation hours	Nuclear Morphotypes (%)											
	Resting			Con A			Con A 8-AG			Con A 5-FU		
	1	2	3	1	2	3	1	2	3	1	2	3
24	90	8	2	52	44	4	49	48	2	51	48	1
36	87	11	2	40	40	20	27	59	14	27	68	5
48	82	17	1	19	31	50	13	47	40	19	60	21

Cells were exposed to 8-AG or 5-FU for the first 6 hours following Con A addition.

Morphotype 1: condensed chromatin (mature lymphocytes).

Morphotype 2: intermediate degree of chromatin disaggregation.

Morphotype 3: very disaggregated chromatin, large nucleus (lymphocytes).

300 nuclei were scored on each slide; the numbers represent mean counts from 3 slides.

nuclei. At 36 hours, the proportion of nuclei of morphotypes 2 and 3 increased at the expense of the type I nuclei which represented only 27% of the nuclei in the cultures treated with base analogues. By 48 hours, the percentage of large nuclei was even greater, representing 50% of the nuclei in control cultures and 40% in 8-AG cultures. The progression toward complete nuclear disaggregation seemed arrested at the intermediate stages in 5-Fu cultures where only 20% type 3 nuclei were counted; this predominance of nuclear morphotypes 2 in 5-Fu cultures (also evident in figure 16-C) could be explained by the lower synthesis of proteins and RNA.

In any case, the proportion of inactive, condensed, nuclei at 48 hours was the same (14-20%) for the 3 culture conditions suggesting that the increase in size of the cells treated with base analogues must have been associated with some chromatin disaggregation and enlargement of nuclear volume as would have been expected for a typical blast cell. Hence, all the evidence suggests that the purine and pyrimidine analogues do not inhibit the early stages of blastogenesis but act mainly on the later stages.

85 to 90% of the nuclei in resting cultures remained small and condensed after 48 hours incubation.

3.5 Utilization of Exogenous Thymidine

Measuring thymidine incorporation to assay for DNA synthesis can be misleading when drugs with complex sites of action are being used (Drach et al, 1981).

Figure 16: Light Micrographs of 48 hour Con A stimulated mouse lymphocytes showing the different nuclear morphotypes in control cultures (panel A) and in cultures treated with 8-AG (panel B) or 5-FU (panel C) for the first 6 hours of activation.

Note the presence of large, type 3 nuclei (lymphoblasts) in 8-AG cultures and the predominance of type 2 nuclei (activated but not full-size lymphoblasts) in 5-FU cultures.

Bars are 5μm in panels A and B and 10 μm in panel C.


A
B
C

The base analogues tested here did not affect the transport of thymidine (figure 4), however, they still might act by inhibiting the incorporation of thymidine into DNA simply by interfering with some steps in the utilization of thymidine after its entry in the cell.

To exclude this possibility, DNA synthesis was assayed by autoradiography (grain density) in both 8-AG and 5-Fu treated cultures, under optimum inhibitory conditions i.e. drugs added for 6 hours at the onset of the cultures, along with Con A.

Figure 17 shows the labelled cells found at 48 hours in each culture after 1 hour pulse wiht 5 μci/ml ^{3}H-thymidine. All the labelled cells contained approximately the same number of grains over their nucleus indicating that all the cells able to replicate DNA incorporated the same amounts of ^{3}H-thymidine. However, the proportion of labelled cells was found different for every culture condition. Table II compares the counts measured for ^{3}H-thymidine incorporation with the percentage of labelled cells at 48 hours in resting cultures and stimulated cultures treated or not with 8-AG or 5-FU. Although there was no simple arithmetic proportion between the differences in the percentage of grain-containing cells and the differences in the counts measured for ^{3}H-thymidine incorporation, it was still clear that there was a correlation between incorporation of ^{3}H-thymidine on the one hand and number of labelled nuclei on the other hand.

TABLE 11

Effect of Base Analogues on the Proportion of ^{3}H-Thymidine Labelled Cells and on the Incorporation of ^{3}H-Thymidine in 48 hours Con A Stimulated Mouse Splenocytes

Treatment	Percent cells with grains	^{3}H-Thymidine Incorporation cpm/10^{6} cells	Percent control
Resting	0.4	1848 ± 77	3
Con A	40.8	73379 ± 505	100
Con A 8-AG	18.7	22103 ± 968	31
Con A 5-FU	0.2	1692 ± 173	2

The proportion of grain-containing cells was obtained from 500 cell samples in each culture.

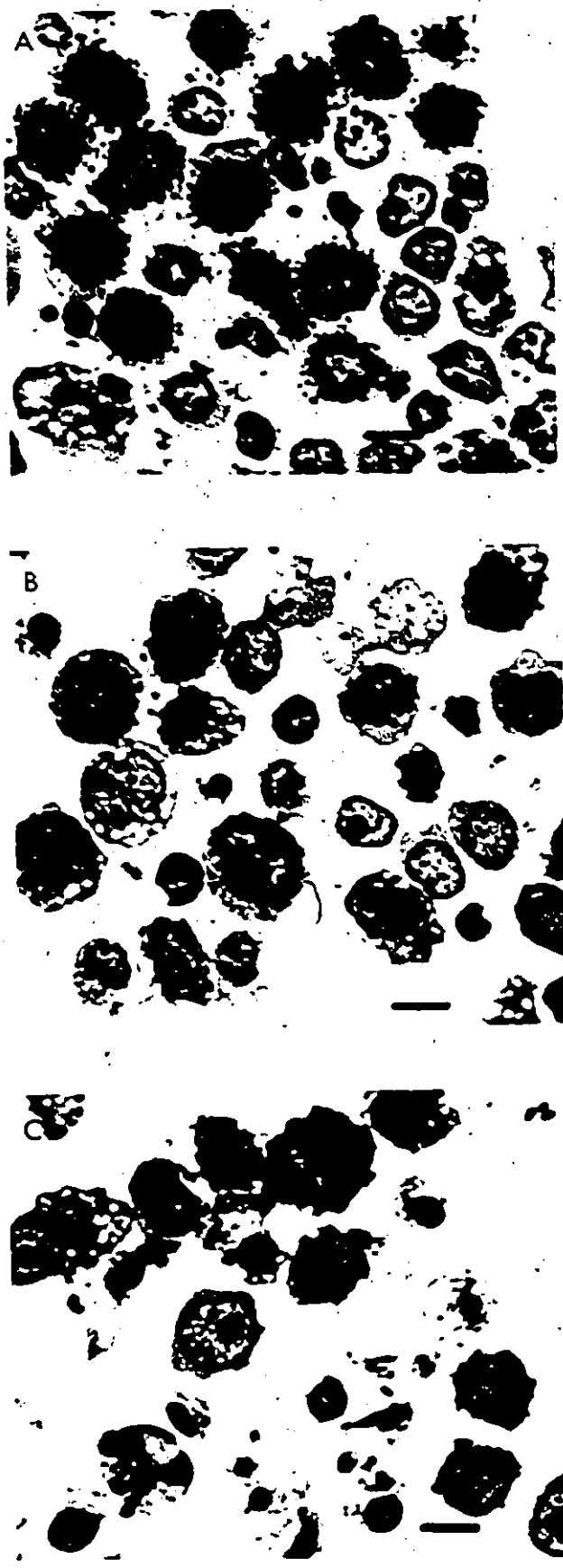
Figure. 17: Light Autoradiographs of 48 hours Con A Stimulated mouse splenocytes.

Panel A: control

Panel B: 8-AG treated

Panel C: 5-FU treated,

Note the "all or none" presence of grains and the similar heavy labelling of cells in the 3 cultures. The proportion of grain-containing cells is different in the 3 cultures (Table II). Bars are 10 µm.


A
B
C

3.6 Inhibition of Mitosis

Table 12 shows the mitotic indices at 54 hours for resting, Con A and Con A plus analogue-treated cultures.

As expected from the previous observations, cells exposed to base analogues and which did not replicate much DNA could not enter mitosis. Cells were probably arrested to late G1 by the early exposures and in S phase if the base analogues were present after the onset of DNA synthesis.

TABLE 12

Effect of Base Analogues on the Con A Induced Increase in the Number of Metaphase Cells.

Cultures	Mitotic Index
Resting	2
Con A	34
Con A 8-AG	4
Con A 5-FU	1

Mitotic Index = number of metaphase spreads per thousand nuclei.

CHAPTER IV

DISCUSSION

My original intention in studying the effects of purine and pyrimidine analogues was to affect reversibly the synthesis of normal RNA transcripts during the early stages of lymphocyte activation and thus to determine whether transcription played a significant role during these early events. This intention was frustrated by the fact that the analogues used here (8-AG, 6-TG and 5-FU) or some intermediates in their metabolism (perhaps the modified transcripts synthesized in their presence) were interfering irreversibly with the cell metabolism and these agents could therefore not be used to answer the question that I originally thought I would answer. However, the effects of the analogues were interesting and unexpected in several respects. The first I have already referred to, namely that the effects of a relatively brief treatment (6 hours) could not be washed out, even by an excess of the natural base after the treatment. The second unexpected finding was that a 6 hour treatment with the analogues caused a severe degree of irreversible inhibition of DNA synthesis at all periods of the culture of lymphocytes stimulated with Con A, even the early stages remote by 24 hours and more from the onset of S phase. The third is that pretreatment with the analogues early in culture caused inhibition of none of the early events such as stimulation of K+ transport, increase in nuclear and

cytoplasmic volume (blast formation), chromatin disaggregation and the development of the thymidine transport system. Furthermore the increase in RNA and protein synthesis, measured by incorporation of labelled uridine and leucine were but little affected. Essentially the only parameter of the stimulated state that was irreversibly and severely prevented was the entry of most of the pretreated cells into S phase of DNA synthesis and the subsequent mitosis - that is the final stages of proliferation. Another unexpected finding was that pretreatment of resting cells with the analogues did not affect their response to Con A. These findings seemed of considerable interest and explain why I decided to consecrate my thesis to their study.

4.1 Effects of Base Analogues in Proliferating Lymphoctyes.

4.1.1 RNA and Protein Synthesis in Presence of Base Analogues.

In all the experiments with proliferating lymphocytes, a pretreatment with 6.6×10^{-5} M 8-AG, 6-TG or 5-FU had little or no effect on RNA synthesis while protein synthesis, in some cases was inhibited by as much as 70% (Table I, figure II). These data are consistent with those expected from an incorporation of the analogues into ribonucleic acids, an action that has been unequivocally shown to cause partial inhibition of protein synthesis (Zanen and Pechere, 1966) when the overall RNA synthesis was either unaffected (Zanen and Pechere, 1966; Szepezi and Freedland, 1970), slightly depressed (Carrico and Sartorelli, 1977a) or even stimulated (Chantrenne and Devreux, 1960) depending on the cell type and experimental conditions. The inhibition of RNA synthesis observed in some experiments (Table I and figures 10 and 11),

I believe was due to the presence of toxic metabolites of the analogues in the soluble pool that could cause some decrease in nucleotide synthesis (Heidelberg, 1974); the variability in degree of inhibition observed with different experiments using a same analogue (figures 10 and 11) could thus be explained by the differences in intracellular levels of toxic metabolites that might result form variability in mitogenic stimulation of purine or pyrimidine metabolism (Raivio and Hovi, 1978). In any case, there was always a good correlation between inhibition of RNA synthesis and that of protein and DNA synthesis: the lowest inhibition of protein synthesis (3% with 8-AG, 35% with 6-TG or 5-FU) and DNA synthesis (60-67%) being observed when RNA synthesis was not affected (figures 10, 11 and 14).

4.1.2 Inhibition of DNA synthesis

In the conditions where RNA and protein synthesis were only partially inhibited, DNA synthesis was found totally and almost immediately depressed by the presence of analogues in proliferating lymphocytes (figure 8).

Whether this resulted also from the incorporation of the analogues into RNA was questionable since for all the systems studied so far, inhibition of DNA synthesis following incorporation of 8-AG or 6-TG into RNA, after short time exposures, was found negligeable and even with very high doses of analogues, never exceeded that of RNA synthesis (Mandel et al, 1965). Using 6×10^{-5} M 8-AG, for 12 hours, Zimmerman and Greenberg (1965) found DNA synthesis in

Hela cells to be inhibited by only 6% when RNA synthesis was depressed by 36% and protein synthesis by 75%. With respect to the effects on protein and RNA synthesis, these data are compatible with mine, but the strong inhibition I found on DNA synthesis, which was not an effect on the transport (figure 4) or utilization (figure 17) of the thymidine used in the assay, must reflect some specific action of the analogues in the lymphocyte system. That this might be on the DNA replicating system itself is suggested by the fact that this action was expressed with a short lag period of 1.5 hours (figure 8) corresponding to the time period reported by Kwan and coworkers (1973) for RNA-containing 6-TG to be synthesized and manifest themselves in the translational apparatus of Sarcoma 180 ascites cells.

In the case of 5-FU, conversion to the deoxyribonucleotide 5-FdUMP was possible and has previously been implicated in the inhibition of DNA synthesis via inhibition of the enzyme thymidylate synthetase (Heidelberg, 1964) that converts uridine to thymidine nucleotides. However, Spiegelman and coworkers (1980) showed this effect of 5-FU not to be significantly expressed in the mammalian cells having high levels of thymidine kinase. Thymidine kinase is one of the enzymes greatly activated by mitogens (Hoywood et al, 1975) and its activity would probably be high enough in 48 hours stimulated lymphocytes for the cells to have a

source of dTTP other than that from thymidylate synthetase, especially in presence of exogenous thymidine. Therefore, the inhibition of DNA synthesis observed after a pretreatment of proliferating lymphocytes with 5-FU might occur by mechaism(s) similar to those of 8-AG or 6-TG action.

4.1.2.1 Availability of Nucleotides.

Both the guanine analogues and 5-FU can alter the levels of nucleotides in the cells, however this cannot account for the severe and almost selective inhibition of DNA synthesis here, since inhibition of purine or pyrimidine biosynthesis and/or utilization would lead to similar decreases in the synthesis of all the nucleic acids and probably of protein synthesis as well. Furthermore, inhibition of the synthesis of nucleic acids through lack of purines or pyrimidines has been shown to be immediately reversible upon the addition of exogenous bases (Hryniuk, 1975; Sriram and Taylor, 1977) and my results showed that the effects of the analogues on lymphocyte proliferation persisted after the addition of the adequate natural bases (figures 7 and 9).

4.1.2.2 Activity of DNA Replicating Enzymes.

Strong inhibition of DNA synthesis by purine analogues is usually a long term effect, associated with the lack of enzymes involved in DNA replication (Bamberger et al, 1963; Carrico and Sartorelli, 1976).

That absence of enzymes is not responsible for the total decrease in DNA synthesis observed after a treatment of proliferating lymphocytes with base analogues, is implied by the fact that a 1.5 hour exposure was sufficient to inhibit the incorporation of thymidine by about 45% (figure 8). Plus for cells already in S phase, the enzymes involved in DNA replication must already be present and even if they continue to be synthesized, their mRNAs could have all been transcribed at earlier times and just translated after the onset of DNA synthesis (Loeb, 1973), in which case they would be synthesized normally since the presence of 8-AG (Zimmerman, 1963), 6-TG (Carrico and Sartorelli, 1977b) or even 5-FU (Carrico and Glazer, 1978) in rRNA or tRNA would not significantly depress translation and the amount of functional ribosomes and tRNA is usually not limiting in lymphocytes. The decrease in overall protein synthesis then observed involves proteins such as some ribosomal proteins (Kay, 1980) whose mRNAs are transcribed and translated during S phase. If some mRNA for the enzymes of DNA replication was transcribed after the onset of DNA synthesis, the observed increase in the degree of inhibition of DNA synthesis with time of incubation in 8-AG (figure 8) would be explained by a progressive decrease in enzyme activity. (although the time-course for inhibition of

protein synthesis by 8-AG was not investigated here), however, the rapid 45% inhibition of DNA synthesis would still have to be considered. The results in figure 9 where 8-AG was added along with the inhibitor of protein synthesis, anisomycin, also indicate, (in addition of showing that synthesis or accumulation of toxic proteins was not the mechanism of inhibition of DNA synthesis by 8-AG) that at least 38% of the decrease in DNA synthesis was not due to a decrease in protein synthesis: in the combination 8-AG plus anisomycin, the inhibition of leucine incorporation was about the same as that caused by anisomycin alone, i.e. about 80%, but the inhibition of thymidine incorporation due to the anisomycin action was about 38% less than that observed with the combined drugs. Furthermore, anisomycin inhibited the synthesis of proteins from all the mRNAs translated at that time as suggested by its site of action (Schwarz et al, 1971) while the effects of 8-AG on protein synthesis, shown by Roy-Burman (1970) and Langen (1975) to result entirely from its presence in mRNA, were restricted to the de novo mRNAs transcribed in its presence.

4.1.2.3 Incorporation of the Analogues into DNA

Another possibility by which immediate inhibition of DNA syntheis could be achieved is through incorporation of the analogues into DNA after their conversion to deoxynucleotides.

Incorporation of 8-AG into DNA has only been reported for B. cereus (Mandel, 1958) but its significance was uncertain since most of the inhibitory effects observed could have resulted from the presence of the analogue into RNA. 5-FU nucleotides, like uracil nucleotides are usually not incorporated into DNA and in case of error, enzymes have been found in lymphoblasts that will immediately excise any misincorporated 5-FU or uracil (Ingraham et al, 1980). However, the fact that the patterns of inhibition by 8-AG and 5-FU were similar to those observed with 6-TG which is known to enter DNA, lead me to consider that possibility. There are a number of reports claiming that the toxic effects of 6-TG in animal cells are solely due to its incorporation into DNA. Lepage (1963) showed a correlation between sensitivity to 6-TG and its incorporation into DNA. Nelson and Carpenter (1975) reported that the selective inhibition of DNA synthesis prevented 6-TG cytotoxicity while inhibition of RNA synthesis failed to. However, the expression of this toxicity was shown to be greatly delayed (Tidd and Paterson, 1974) and the short-term significance of 6-TG in DNA is not clear. Beikirch and coworkers (1972) reported that transcription of synthetic deoxynucleotide polymers containing 6-TG was inhibited compared to the transcription of corresponding polymers containing guanine. Wotring and Roti (1980)

have found an inhibition of DNA replication in L210 mouse leukemic cells shortly after their exposure to 6-TG; this was reversible and shown to result from an inhibition of purine synthesis and the authors suggested that the presence of 6-TG in DNA would not interfere with the completion of DNA synthesis./ Carrico and Sartorelli (1976) came to the same conclusion when they showed that exposure to 6-TG before the onset of DNA synthesis in regenerating rat liver inhibited DNA synthesis through interference with transcription and inhibition of the enzymes necessary for replication (thymidine kinase, deoxycytidylate deaminase, cytidine diphosphate reductase and DNA polymerase) while exposure after DNA synthesis had started had little or no effect.

My data, although indirectly, also prove that incorporation into DNA is not necessary for 6-TG, or any of the analogues used here, to inhibit DNA synthesis. Figures 10, 11 and 14 showed that exposures to base analogues any time before the onset of DNA synthesis, when no significant replication could be detected, resulted in similar inhibition patterns as with the exposures in S phase.

Therefore, incorporation into DNA by the analogues, if it occurred is probably not responsible for the effects observed at 48 hours in proliferating lymphocytes after

a 6 hour exposure to analogues; and while not unequivocably proven by the present studies (difficulty to obtain radioactively labelled analogues), incorporation of the analogues into RNA is the likely explanation for their ability to inhibit DNA synthesis in lymphocytes.

4.2 Inhibition of Lymphocyte Proliferation by Exposure to Analogues at Different Stages of Activation.

The data presented in this thesis showed that any 6 hour exposure to 8-AG (figure 10), 6-TG (figure 11) or 5-FU (figure 14) after Con A addition to mouse splenocytes resulted in a strong inhibition of proliferation (up to 98%) at 48 hours. The effects observed when the analogues were given at time of Con A addition were in general as severe as when the analogues were present in G1 or S phase.

Although base analogues are not necessarily phase specific drugs, studies with other growth induction systems have shown that there was always a period where the presence of the analogues had very little consequences for cell activities, and this made it possible to determine the stages during which essential transcripts were synthesized. In these experiments, an excess (13.2×10^{-5}M) guanine or uracil was added after each treatment and the possibility for continuous reutilization of the toxic nucleotides (generated by RNA degradation) for subsequent transcription was minimized. However, my data do not prove that transcription throughout the whole transformation is necessary for the lymphocyte to proliferate and enter mitosis, but rather reflect the complexity

and peculiarities of the lymphocyte system. Even if a 6 hour exposure was too long to detect the "analogues-insentive" periods and assuming the effects of analogues here are, like in other systems, expressed mainly by absence of enzyme activities, one would still expect some differences in the degree of inhibition of protein synthesis with treatments at different times but this is not the case here, at least with the guanine analogues. This suggests that inhibition of protein synthesis is not responsible for the total inhibition of DNA synthesis observed after pretreatment with analogues in G_0 or G_1 phases: the mRNAs transcribed during activation are different from one stage to another (Ling and Kay, 1975) thus different proteins are affected in the different treatments; even if these proteins were all essential, would their messages be transcribed exclusively in 4 to 5 hours?

In the case of 5-FU, RNA synthesis at 48 hours was far more affected by the presence of the analogue any time before S phase than when it was present after the onset of DNA synthesis. This might be due to the inhibition of rRNA processing and transport (Wilkinson et al, 1975) and also to the inhibition of polyadenylation of mRNA precursors (Glazer and Peale, 1979) which perhaps by retaining the defective transcripts in the nucleus could affect the synthesis of additional RNAs. This explanation would be consistent with the observation that the greatest inhibition of RNA synthesis (87%) was obtained with treatment in the first 6 hours of activation and the minimum inhibition (30%) with the exposure in S phase (figure 14). 8-AG or 6-TG would not have

the same effect on RNA synthesis because they inhibit RNA processing to a lesser extent than 5-FU (Weiss and Pitot, 1974).

4.3 Resting Cells Pretreated with 8-AG can Respond to Con A.

Table 8 shows that exposure to 8-AG prior to the addition of Con A had little or no effect on the subsequent proliferative response while exposure at time of Con A addition strongly inhibited DNA replication. This suggests that the effects of the analogue on lymphocyte transformation are related, directly or indirectly, to the action of the mitogen. Sensitivity to base analogues is largely determined by the rate of analogue uptake (Harris and Whitmore, 1973) and conversion into nucleotides (Berman et al, 1980). Mitogens increase the rate of incorporation of exogenous purine or pyrimidine bases into nucleic acids but this effect cannot account for the present data. First, although there is little information concerning transport of bases in resting and activated lymphocytes, evidence indicates that in mammalian cells, guanine and consequently 8-AG at concentrations below 1mM enter the cell by simple diffusion and at higher concentrations, they are taken up by facilitated diffusion (Zylka and Plagemann, 1975; Rivera et al, 1979); therefore, a difference in 8-AG uptake between resting and activated cells cannot be invoked here. Second, the rate of nucleotide synthesis in general correlates with the rate of nucleic acid synthesis in such a way that the proportion of synthesized nucleotides entering nucleic acids is the same in both resting and activated lymphocytes. Third, there was

always some inhibition of RNA and protein synthesis in the resting cells, which at least with 8-AG was not significantly different from that observed in the stimulated cells.

4.4 Involvement of RNA in DNA Replication.

All together my data seem to indicate that the base analogues, probably after their incorporation into ribonucleic acids, affect some component(s) in the activated state that is specifically required for DNA replication (none of the preliminar events of transformation were inhibited). As previously mentioned, this does not involve the synthesis of faulty mRNAs and inhibition of protein synthesis but possibly that of an RNA species that participates more directly in replication. One such RNA could be the primer RNA required for initiation of DNA replication (for reviews see Kornberg, 1976; Zechel, 1978). It is well established that DNA polymerases cannot initiate chains de novo, they need a free 3'-hydroxyl end that is provided by an RNA primer synthesized by special RNA polymerases, the primases. Primers exist in all DNA replicating systems and have often been isolated attached to the 5' end of nascent DNA fragments. The primers isolated from in vitro replication systems from animal cells, including lymphocytes (Tseng and Goulian, 1975; 1977) are 8-11 nucleotides long. They have apparently no specific sequence but carry adenine or guanine residues at their 5' end. There is no cellular pool of primer RNAs as initially suggested by Goulian (1969), the primers are short-lived molecules, transiently synthesized at time of replication; however, there is

evidence that certain cellular tRNAs in eukaryotic cells serve as primers for the DNA synthesis on the genomes of avian myeloblastosis virus (Verma et al, 1971; Brown and Armentrout, 1977).

Since replication is discontinuous, several primers are formed along the DNA template and each nascent DNA fragment is elongated until the DNA polymerase reach the 5' end of the next primer in line. At this point, the primers are removed by the ribonucleases, hybridases in eukaryotes or by the 5'→3' exonuclease activity of DNA polymerase I in prokaryotes. The gaps left by primer excision are subsequently filled in by DNA polymerases (β and γ in eukaryotes and I and II in prokaryotes) and the discontinuous Okazaki fragments are rejoined by ligases to form the natural DNA. Excision of the primer is an essential step in DNA replication, E. coli temperature sensitive mutants defective in the 5'→3' exonuclease activity cannot replicate DNA at the non-permissive temperature (Konrad and Lehman, 1974). One could suppose that a small RNA or oligonucleotide that normally turns over rapidly resists degradation by nucleases if it contains base analogues; this hypothetical molecule inhibits passage of the G1-S boundary.

4.5 Conclusion

From the available data, I have attempted to construct a model concerning the mechanism of inhibition of lymphocyte proliferation by purine and pyrimidine base analogues. It accounts for the following observations:

1. The presence of base analogues at any time during transformation causes a severe and irreversible inhibition of DNA synthesis that cannot be totally explained by inhibition of protein synthesis or accumulation of toxic proteins. It has been suggested that the entry of cells in the S phase of growth must be preceded by the synthesis of specific proteins in late G1 (Sheinin and Humbert, 1978) but again nothing is known about the period their mRNAs are transcribed and it is unlikely the presence of analogues in G_0 or S phase will totally inhibit synthesis of these proteins.
2. All the events prerequisite for proliferation such as increase in ion fluxes, cell volume enlargement and nuclear decondensation are essentially normal in cells whose proliferation has been totally inhibited by a previous exposure to analogues.
3. The degree of inhibition of basal RNA and protein synthesis by the analogues in resting lymphocytes is in general not significantly different from that obtained in the stimulated lymphocytes.
4. Resting lymphocytes can respond to Con A after exposure to 8-AG while activated cells, still in G_0 cannot: the inhibition of DNA synthesis is related to the action of the mitogen.

4.5.1. The Model

The mitogen induces the synthesis of an RNA species of low molecular weight, this RNA is unstable but continuously transcribed during transformation. Its function is not clear,

it might serve to prepare the cells for an eventual entry in S phase. In presence of base analogues, all the RNA in process of transcription are being affected including that species: in mRNA, it can be seen as inhibition of the overall protein synthesis; in rRNA or tRNA, the effects will not be as apparent in lymphocytes because of the excess ribosomes and functional tRNAs they contain. In this low molecular weight RNA, the main consequence will be a change in the half-life: the substitution of the analogues for natural bases makes it no longer accessible to the nucleases. The persistance of this species in the vicinity of the DNA shuts off further synthesis of other small RNAs. The consequence is that, at time of replication when primers are required, these low - molecular weight RNA species substitute for them and the Okazaki fragments are probably elongated from them; but when they have to be exised, the hybridases cannot degrade them, neither can the discontinuous DNA fragments be rejoined and the cycle of replication is irreversibly arrested.

In this model, I assumed that incorporation of analogues into RNA can change its half-life; this has been often proposed for 5-FU and 6-TG (Spiegelman et al, 1980; Carrico and Sartorelli, 1976b) and it would not be surprising 8-AG has the same effect. Spiegelman and coworkers (1980a, 1980b) have also suggested that the presence of 5-FU in a primer RNA could irreversibly interrupt the cycle of DNA replication.

Regulation of cellular activities by small RNAs or oligoribonucleotides seem to be a common phenomenon in both prokaryotes and eukaryotes. DNA replication of the E. coli plasmid Col E1 is negatively controlled by an unstable small RNA that acts by inhibiting the formation of functional primers. Similarly, bacteria starved for amino acids have been shown to synthesize oligonucleotides, the so-called "Magic spots" to limit the synthesis of additional ribosomes and tRNAs. In animal cells, the production of interferon after viral infection is the main defense mechanism developed by the cell to inhibit viral replication; this is mediated mainly by a small oligonucleotide that is induced by the interferon and that acts to degrade viral mRNA by activation of a nuclease.

4.5.2 Possible Alternatives

There are a number of other alternatives to my model which could explain some of the data:

1. For example, the effects of base analogues in activated lymphocytes resemble those of hydroxyurea, a specific inhibitor of DNA synthesis. Setterfield and coworkers (1981) have shown that blast formation in lymphocytes was not affected by hydroxyurea while their DNA synthesis was severely inhibited. This drug inhibits ribonucleotide reductase and it is quite possible that base analogues, somehow, irreversibly affect that enzyme and the resultant lack of deoxynucleotide triphosphates could account for some of my data. However, preliminary work by Wenda Greer (personal Commun-

ication) failed to show any change in the intracellular levels of nucleoside triphosphates after exposure of Con A stimulated mouse splenocytes to 5-FU.

2. Another possibility, although unlikely because base analogues normally do not cause free radical formation, is that of direct damage of the DNA by the analogues. This could be investigated by one of these new techniques for rapid detection of DNA strand breaks (Birnboim et al, 1981).

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