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Colchicine, Microtubules, Thymidine Transport and Lymphocyte Function

by

Christopher E. Rudd

A thesis submitted to the School of Graduate Studies of the University of Ottawa in partial fulfilment of the requirements for the degree of Masters of Science in Biology

Ottawa, Ontario, 1980

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To my parents

and

Beth.

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ABSTRACT

The work of this thesis has studied the effect of colchicine on the time course of Con A - induced lymphocyte blastogenesis, microtubule networks, and on thymidine transport in an attempt to elucidate the role of the microtubule in lymphocyte blastogenesis and the molecular mechanism of colchicine action. In human and mouse cultures, colchicine inhibited RNA synthesis as early as 12 hours of culture. With human lymphocytes, the addition of colchicine to stimulated cells at varying times of culture caused marked inhibition of DNA synthesis, provided that sufficient time had been allowed to elapse between the addition of the drug and the assay for DNA synthesis.

Commitment is defined as the period of time after which the removal of Con A from the lymphocyte cell surface no longer prevents the cell from entering DNA synthesis. This

is operationally defined by the addition of the sugar aMM to culture which acts to remove Con A from the lymphocyte cell surface. In my cultures, the addition of aMM after 20 to 30 hours of culture no longer inhibits DNA synthesis. Edelman 's hypothesis that colchicine acts to block commitment was based on what he took to be the similarity between the commitment curve and that produced by the addition of colchicine. (Wang et al, 1975). On the basis of this observation, microtubules were implicated as a candidate for signal-transmission (Edelman, 1976). I have shown that the apparent coincidence of the curves is a factitious consequence of an approximate 20-30 hour lag period before maximal inhibition was observed. The later the time of assay for ^{3}H-thymidine incorporation, the more disparate the aMM and colchicine curves became. In the case of mouse splenocytes, there was no resemblance between the time-course of inhibition by aMM and colchicine. The data on the time course of inhibition by a methyl-D-mannoside and colchicine cannot exclude the possibililty that colchicine may interfere with commitment, but show that colchicine can inhibit well after commitment is complete.

In an attempt to determine the target of inhibition by colchicine, the effect of the alkaloid on microtubule integrity and thymidine transport was examined. Microtubule networks, as detected by tubulin-antibody

immunofluorescence, disappeared in some 70 to 75 percent of the Con A-stimulated leukocytes after a 12 hour incubation with colchicine. In contrast, lumicolchicine had no apparent effect on the presence of microtubules at a concentration as high as 10^{-4}M. At the same time, colchicine was some three-orders of magnitude more inhibitory than lumicolchicine. In other words, colchicine inhibited ^{3}H-thymidine incorporation by 50 percent at 5.0 X 10^{-8}M, while lumicolchicine caused equivalent inhibition at 5.0 X 10^{-5}M. These data suggest that the primary target of colchicine is the cytoplasmic microtubule.

Con A was found to induce the appearance of measureable time-dependent and saturable thymidine transport in human peripheral leukocytes. Transport was not detected in resting cultures. Both colchicine and lumicolchicine inhibited thymidine transport by 50 percent at 5.0 X 10^{-5}M. This suggests that inhibition of thymidine transport by colchicine cannot account for the effect of the agent on ^{3}H-thymidine incorporation. On the other hand, the effect of lumicolchicine on transport may be responsible for its effect on the assay of DNA synthesis.

RESUME

On a étudié l'effect de la colchicine sur la période nécessaire à la blastogénèse des lymphocytes provoquée par la concanavaline A, sur la présence des réseau de microtubules et sur le déplacement de la thymidine, afin de découvrir le role du microtubule dans la blastogénèse des lymphocytes et le mécanisme moléculare de la colchicine. Dans les cultures de cellules d'homme et de souris, la colchicine a entravé la synthèse de l'ARN dès la douzième heure. Avec des lymphocytes humains, l'addition de colchicine à des cellules stimulée à différentes étapes de la culture a provoqué une inhibition marquée de la synthèse de l'ADN, pourvu qu'il s'écoule suffisamment de temps entre l'addition de la droque et le mésure pour la synthèse de l'ADN.

L'hypothèse de Edelman selon laquelle la colchicine agit pour bloquer la détermination était fondée sur ce qu'il croyait être une similitude entre la courbe de determination

et celle produite par l'addition de colchicine (Wang et al, 1975). Par suite de cette observation, on a considéré les microtubules comme l'agent principal d'activation des leucocytes (Edelman, 1976). J'ai montré que la similitude apparente des courbes est une conséquence factice d'une phase de latence d'environ 20 à 30 heures avant que l'inhibition maximale n'ait été observée. Plus le moment du dosage pour l'incorporation de la ^{3}H-thymidine était rétardé, plus les courbes d'aNN et de colchicine devenaient disparates. Dans le cas des splénocytes de souris, il n'y avait aucune ressemblance entre la période nécessaire à l'inhibition par l'aNN et la colchicine. Les données sur le temps nécessaire à l'inhibition par un aNN et la colchicine ne peuvent exclure la possibilité que la colchicine entrave peut-être la détermination, mais elles démontrent que la colchicine peut provoquer l'inhibition bien après la fin de la détermination.

Afin de déterminer la cible d'inhibition de la colchicine, on a examiné les effets de l'alcaloide sur l'intégralité du microtubules et le déplacement de la thymidine. Les réseau de microtubules, tel que décelés par l'immunofluorescence des antocorps pour localiser la tubuline, sont disparus dans quelque 70 pour cent des leucocytes stimulés par la Con A après 12 heures d'incubation avec la colchicine. Par contre, la

lumicolchicine n'avait aucune effet sur la présence des microtubules à une concentration atteignant 10^{-4}M. La colchicine est plus inhibitrice que la lumicolchicine par trois ordres de grandeur. En autres termes, la colchicine entrave de 50 pour cent l'incorporation de la ^{3}H-thymidine, à 5,0 X 10^{-8}M, alors que la lumicolchicine provoque une inhibition équivalente à 5,0 X 10^{-5}M. Ces données semblent indiquer que la cible principale de la colchicine est le microtubule cytoplasmique.

On a découvert que la concanavaline A simule dans les leucocytes périphériques humains un déplacement measureable et saturable de thymidine qui crotit en function du temps. On n'a pu déceler le déplacement dans les cultures au repos. La colchicine et la lumicolchicine entravent de 50 pour cent le déplacement de la thymidine, a 5,0 X 10^{-5}M. Ce qui laisse croire que l'inhibition du déplacement de la thymidine par la colchicine ne peut expliquer l'effet de l'agent sur l'incorporation de la ^{3}H-thymidine. Par contre, l'effet de la lumicolchicine sur le déplacement pourrait être responsable de son effet sur le dosage pour la synthèse de l'ADN.

ABBREVIATIONS

aMM	α methyl-D-mannoside
aMG	α methyl-D-glucoside
BSA	Bovine serum albumin
Con A	Concanavalin A
EGTA	Ethylene glycol bis (B-amino ethyl)-N,N'-tetraacetic acid
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
IgG	Immunoglobulin G
LMGB	Leukocyte medium of glucose, bovine serum albumin
MLR	Mixed lymphocyte reaction
MTOC	Microtubule organising center
NAGO	Neuraminidase-galactose oxidase
PBS	Phosphate buffered saline
PHA	Phytohaemagglutinin
SI	Stimulation index
SB	Stabilisation buffer
TCA	Trichloroacetic acid
TCGF	T cell growth factor

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Chapter I

THE LYMPHOCYTE

One of the key problems in immunobiology is to determine how antigen binding to lymphocyte surface receptors is linked to cell proliferation and the expression of specific immune functions. The thymus dependent T cells can become killer cells or secrete various factors responsible for cell-mediated immune responses. Bone marrow derived B cells mature into antibody secreting plasma cells. Cell response is indicated by the onset of DNA synthesis or certain effector functions such as antibody synthesis, lymphokine secretion or cytotoxicity during culture. Thus, lymphocyte blastogenesis has become important both in the study of the immune response and as a model for cell proliferation.

Lymphocytes respond *in vivo* to antigens with a high level of genetic specificity. As a result, only a small number of clones are generated by the presence of a single antigen. The *in vitro* study of lymphocyte proliferation has been

facilitated by the use of polyclonal mitogens. These substances are relatively nonspecific, activating a significant proportion of the B and/ or T clones to divide (Ling and Kay,1975). This ensures that there are adequate numbers of responding cells available for biochemical study.

The work of this thesis involves the use of the mitogen, concanavalin A. This plant lectin and protein consists of four subunits, each with a saccharide binding site, one transitional metal usually Mn++ and one Ca++ ion (Edelman,1972). The sugars, aMM and aMG competitively remove Con A from the lymphocyte cell surface as monitored by labelled Con A (Novogrodski and Katchalski,1972). This suggests that the Con A receptor has present mannose/glucose residues. Lymphocyte response requires the continual presence of lectin for 18-20 hours (Lindahl-Kiessling,1972). Alternatively, this mitogen need only be present between 0-3 and 15-18 hours of culture to give the same response (Toyoshima et al,1976).

1.1 LYMPHOCYTE RESPONSE TO MITOGENS

Perhaps the greatest disadvantage in using lymphocyte activation as a model for cell proliferation is the asynchronous response of lymphocytes to polyclonal mitogens. Activated cells pass from G0 to G1, and enter DNA synthesis at different times following mitogen addition. As a result,

measurement of biochemical events represents the average of cells at different stages of the cell cycle instead of an average of cells at a common stage of their cycle.

This lymphocyte asynchrony in response to Con A has been partly overcome by the use of the sugars aMM and aMG. By competitively removing Con A from the cell surface, these sugars have been used to determine commitment in the lymphocyte population at various times following lectin addition. Commitment is defined as the time after which the removal of Con A no longer prevents the cell from entering S phase. The period preceeding commitment is important because it corresponds to the time during which initiation of activation is occurring. If aMM is added at various times following lectin addition, the population becomes increasingly refractory to inhibition until 18-20 hours after which the sugar has no effect on subsequent DNA synthesis (Novogrodski and Katchalski,1971 ;Wang et al,1975). The diminished ability of the sugar to inhibit DNA synthesis corresponds to the appearance of increasing numbers of labelled blast cells at 48 hours of culture (Wang et al,1975).

The reason for the asynchronous response of lymphocytes to mitogens is poorly understood. It is probably related both to the existence of various T cell subpopulations

directly activated by the lectin and to subsequent indirect stimulation of T and B cells. In a mixed population stimulated with Con A or PHA, between 10-20 percent of the DNA synthesizing blast cells are B lymphocytes (Phillips and Roitt, 1973). These have been detected using fluorescent anti-Ig coupled with autoradioautography. Two mechanisms have been postulated to explain this B cell activation. One involves the release of a soluble mediator (s) by T cells which in the presence of Con A causes the activation of B cells. Supernatants from resting and activated T cell cultures have been shown to activate purified B cells in the presence of Con A while neither Con A nor the supernatant alone had any effect on the cells (Andersson et al, 1972a). The other proposed mechanism called the "local concentration hypothesis" proposes that T cells concentrate Con A on their cell surface and then present it to B cells in a mitogenic form (Moller, 1970; Mitchison, 1971). Supporting evidence is circumstantial, that is, Con A covalently coupled to solid surfaces directly activated B cells (Andersson et al, 1972b).

In addition to the response of B and T cells, macrophages have been shown to participate in the immune response. Their presence has been found necessary for antigen- (Cline and Swett, 1968) mixed leukocyte- (Alter and Bach, 1970) and mitogen- (Rosenstreich et al, 1976) induced lymphocyte

stimulation. Both B and T lymphocytes cultured in the presence of mitogen but depleted of macrophages failed to enter DNA synthesis. However, if these adherent cells were returned to culture with the sugar, aMM, within 25 hours of mitogen addition, then DNA synthesis began as if the macrophages had been present during the entire culture period (Resch,1979). This suggests that macrophages provide a second signal to activate lymphocytes, which acts independently of the lectin. Macrophages can also be activated directly by Con A but only at concentrations toxic for lymphocytes (Wang and Basch ,1979).

The work reported in this thesis was carried out using mixed populations of T and B cells from human peripheral blood and mouse spleen. Activation must be considered to have occurred in both the T and B cell populations, despite the fact that Con A is conventionally termed a T cell mitogen. Blastogenesis and transformation are defined as the cell cycle of a lymphocyte. In contrast, activation is the stage of blastogenesis prior to DNA replication.

1.2 THESIS RATIONALE

Microtubules have been implicated in the transformation of Con A-stimulated lymphocytes. This contention has been

derived by the use of colchicine, however, there is much disagreement as to when the transforming lymphocyte is sensitive to the anti-microtubule agent. Several investigators maintain that colchicine inhibition is restricted to events known to preceed S phase (Medrano et al,1974; Wang et al,1975; Gunther et al,1976), while others have reported colchicine to inhibit only after the onset of DNA synthesis (Resch et al,1977;Resch,1979). Because these observations have been the basis of the hypothesis implicating microtubules in signal-transmission, it was of interest to study the time-course of lymphocyte sensitivity to colchicine. It was also hoped that such data would shed light on the role of the microtubule cytoskeleton in the transformation of Con A-stimulated mouse and human lymphocytes. Implicated in the molecular basis of lymphocyte transformation and colchicine effects was an investigation of thymidine transport and the effect of colchicine and lumicolchicine on this event.

1.3 EARLY MOLECULAR EVENTS OF LYMPHOCYTE ACTIVATION

The mechanism by which an antigen or mitogen induces blastogenesis appears to be a complex process. Con A, in soluble form, binds to both B and T lymphocytes with approximately the same density but activates directly only certain clones within the T lymphocyte population (Greaves and Janossy,1972). Therefore, although mitogen binding to

the cell surface is a prerequisite to transformation, this event by itself is not sufficient to induce blastogenesis. Puthermore, the fact that lymphocytes require a prolonged period of exposure to the lectin to become committed to transform suggests that the mechanism of activation may lie in the series of early biochemical changes which follow lectin binding. The critical event(s) responsible for activation may be singular, interrelated, in series with or parallel to other pathways (Kaplan,1977).

Increased Ca++ uptake has been reported within minutes of mitogen addition (Alford,1970 ; Allwood et al,1971) due to a decrease in Km (Whitney and Sutherland,1973). A transient influx has also been observed which is completed within 1 minute of Con A addition (Freedman et al,1975).Nevertheless, the role of Ca++ in lymphocyte activation is unclear. Parker (1975) reported Ca++ influx to be correlated to the degree of stimulation, but Hesketh and coworkers (1977) have found no detectable Ca++ influx at optimal mitogen concentrations. However,external Ca++ is reported necessary for the lymphocyte to transform. The presence of the Ca++ chelators, EGTA has been shown consistently to prevent the onset of DNA synthesis (Alford,1970; Whitney and Sutherland,1972) and the inhibition is reversed by Ca++ in the medium. Bard et al (1979) have found that cultures required continuous access to Ca++ the first 20 hours of

culture in order for any cells to move into S phase at about 36 hours of culture.

A oaubain sensitive increase in K+ uptake has also been reported within one hour of PHA addition (Quastel and Kaplan, 1970; Averdunk, 1972); the increase being characterised by an increased Vmax. Quastel and Kaplan (1970) showed that PHA caused an increase in K+ transport even in the presence of inhibitors of protein synthesis. This suggested that the mitogen caused the exposure of former cryptic K+ transport sites. Concomitant with K+ influx is an equally increased efflux of K+ from the cells (Segel et al, 1975; Hamilton and Kaplan, 1976). This suggests that the intracellular concentration of the ion may not significantly change during activation (Hamilton and Kaplan, 1976). The relationship between K+ influx/efflux activity and lymphocyte activation is unclear. The early inhibition of K+ influx by oaubain prevented the onset of S phase (Quastel and Kaplan, 1968).

Other transport events also activated by the presence of mitogen are Na+ efflux; choline, nucleoside and amino acid uptake (Averdunk and Lauf, 1975; Peters and Hausen, 1971; Van den Berg and Betel, 1973).

In addition to transport changes, alterations in cyclic nucleotide content have been reported following addition of mitogen. CAMP transiently increases 1-2 fold within 30 minutes followed by a return to control levels by 1-2 hours (Smith et al,1971; Parker et al,1974; Foker et al,1979). This is followed some 10 hours later by a continous increase until 24-36 hours of culture (Wang et al,1978). The relevance of these changes to lymphocyte activation is questionable. The onset of DNA synthesis can be prevented by an inhibition of the early cAMP rise (Foker et al, 1979). However, wheat germ agglutinin, which is nonmitogenic, has been shown to cause a similar increase in cAMP levels (Coffey et al,1977). In some cases, the addition of exogenous cAMP induced mitosis (Smith et al,1971; Wedner et al,1975). However, others have have found the cyclic nucleotide to be without an effect (Hirschorn et al,1970). Lastly, the addition of cAMP has been reported to inhibit the mitogenic response of lymphocytes to lectin (Quastel and Kaplan,1970; Diamanstein and Ulmer,1975).

CGMP has been reported to undergo a 50 fold increase within 20 minutes of mitogen addition (Hadden et al,1972; Coffey et al,1975,1977), but other investigators have failed to confirm this finding (Parker,1974; Watson,1976). LPS caused an increase in cGMP levels but other B cell mitogens failed to induce any change (Watson,1976). CGMP

has been reported both to activate lymphocytes (Diamanstein and Ulmer,1975) and to be non-stimulatory (Wedner et al,1975), however, when a mitogen is used to induce cells to enter DNA synthesis, the presence of cGMP potentiates the response (Hadden et a l, 1973). CGMP has also been reported to reverse the inhibitory effect of cAMP on the PHA and LPS response (Diamanstein and Ulmer,1975). These data suggest that cAMP and CGMP are respectively negative and postive regulators of mitogen-induced blastogenesis.

If cyclic nucleotides regulate activation it may be accomplished not as a singular event but rather in conjunction with Ca++. Dibutyryl cAMP inhibits mitogen induced Ca++ uptake (Freedman et al,1975;Whitney and Sutherland,1972) while dibutyryl cGMP enhances Ca++ uptake (Freedman et al,1975). Conversely, the ion stimulates guanyl cyclase activity (Shultz et al,1973). With TL cells,Ca++ and cAMP have been reported interdependent. Both the size of the cAMP surge and the susequent DNA synthetic response depend on Ca++ (Boynton and Whitfield,1979).

Accompanying changes in transport and cyclic nucleotides are alterations in the activity of certain membrane associated enzymes. Na+-K+ ATPase activity is enhanced within minutes of PHA and Con A binding (Resch et al,1978). Ca++ATPase activity is increased 100 percent by Con A (

Dormand et al,1978) while Mg++ATPase activity is either activated (Novogrodski,1972) or depressed (Resch et al,1978) during lymphocyte activation. Adenylate cyclase is increased either marginally (Synder and Parker,1977), or not at all (Resch et al,1978). Guanylate cyclase has also been reported activated (Hadden et al,1974).

Enzymes related to membrane composition are also activated by the presence of a mitogen. De novo cholesterol synthesis is increased; however, its inhibition had no effect on transformation (Cuthbert and Lipsky ,1979). Phosphotidyl inositol activity increased within 5-10 minutes of PHA/A23187 addition .. Phospholipase A and acyl coA lysolecithin acyltransferase were also activated due to a change in Vmax (Resch,1979). This latter enzyme selectively incorporated unsaturated fatty acids into the plasma membrane. Subfractionation of the plasma membrane into high and low affinity Con A binding fractions resulted in a co-enrichment of lysolecithin acyltransferase and Na+-K+ATPase in high affinity fractions (Rode et al,1979). This is presumably related to the reported increase in plasma membrane fluidity following lectin binding (Ferber et al,1975).

A final event dependent on membrane fluidity which occurs within 5 minutes of lectin binding is patching and capping

of receptor-ligand complexes.Unbound receptors exist randomly throughout the plane of the lymphocyte membrane. However, the receptor-ligand complexes passively aggregate into a 'patch' followed by their active migration into a 'cap' at one pole of the cell. Cap formation has been shown to occur with several Ig classes,H-2,HLA,O,TL antigens and most lectin receptors. The mechanism is dependent on membrane fluidity and the microtubule/microfilament cytoskeleton. Nonetheless, capping is an insufficent signal for transformation and in fact, may not be involved at all. B and T cells cap Con A and LPS equally well however, only T or B cells are activated, respectively (Loor,1974; Bona et al,1976). On the other hand, both the tetramer and dimer forms of Con A are mitogenic while only the tetramer undergoes cap formation (Edelman,1976).

1.4 LATER MOLECULAR EVENTS OF LYMPHOCYTE BLASTOGENESIS

A detectable increase in RNA synthesis has been reported within 1-6 hours of mitogen addition (Cooper and Kay,1968). This increase then continues for 48 to 72 hours and is accompanied by an increase in uridine kinase activity. DNA replication commences within 24 to 36 hours of culture and also continues for some 48 to 72 hours (Ling and Kay,1975). Thymidine transport and kinase activity appear to start with the onset of S phase (Strauss et al,1977). Unlike most eukaryotic cells, stimulated lymphocytes exhibit large

variations in the length of S phase, from 6 to 30 hours (Younkin, 1975). The length of S phase is inturn thought to be related to the ribosomal content per cell (Darzynkiewicz et al,1979). Protein synthesis has been shown to increase within 1 to 3 hours of Con A/PHA addition and continues to increase until 48 to 72 hours of culture (Kay,1968). Numerous proteins are produced and released into the medium. These include TCGF (Morgan et al,1976) which allows the longterm maintenance of T cells in culture.

1.5 NUCLEOSIDE UPTAKE AND TRANSPORT IN LYMPHOCYTES

The uptake of various nucleosides has been demonstrated in several mammalian cells. This is of special interest because ^{3}H-nucleoside uptake is a prerequisite for the usual assay of DNA and RNA synthesis. However, little is known regarding the mechanism or the appearance of transport systems in either resting or stimulated lymphocytes. Early studies generally measured net uptake of nucleosides, however, it has become increasingly evident that this process must be distinquished from transport(Plagemann and Urbe,1972;Barlow and Ord,1975). Net uptake includes the accumulation and/or backflux of substrate in and out of the cell. In contrast, transport is the inidirectional translocation of substrate into the cell. To measure this, measurements must be made over short intervals where the initial rates are determined and the quantities taken up

extrapolate back to zero (Berlin,1975). With the advent of rapid sampling techniques, it has been possible to take measurements over periods as short as 4 to 20 seconds (Hawkins and Berlin,1969; Strauss et al,1976). This method applied to rat hepatoma cells and murine splenocytes have shown that rates are linear for 10 to 60 seconds (Strauss et al,1977;Hohleuter et al,1978).

Resting murine splenocytes (Strauss et al,1977) and human peripheral lymphocytes exhibit no apparent thymidine transport (Figure 22). In contrast, adenosine transport is quantitatively and qualitatively the same in resting and stimulated mouse splenocytes (Strauss et al,1977). Macrophages also show adenosine transport without the presence of a mitogen (Berlin,1973). Adherence to a glass surface acts to augment nucleoside transport (Pofit and Strauss,1977). In the case of lymphocytes, the presence of Con A *in vivo* induces the appearance of a thymidine transport system as early as 24 hours (Strauss et al,1977). This corresponds to the onset of DNA replication and theoretically, could be related to the commitment of the population to transform.

Chapter II

THE MICROTUBULE

The microtubule is an filamentous aggregate of tubulin subunits found in all eukaryotic cells at some stage in their life cycle (Porter,1966; Margulis,1973). They are usually seen to emanate from a MTOC and to run throughout the cytoplasm and nucleus of various cell types (Biberfeld,1971; Osborn and Weber,1975). In molecular terms, the cytoplasmic microtubule is composed of 13 aligned protofilaments, which in cross-section appear as a tube with an outer diameter of 25 nm , a wall of 5 nm and a core of some 15 nm. Each protofilament consists of an alternating array of two species of 55,000 M.W. tubulin monomers, a and b. These differ in their amino acid compositions (Zee et al,1973) and their degree of projection from the inside surface of the protofilament (Amos and Baker,1979). From X-ray diffraction data, the subunits are displaced from the side horizontal axis by 10. giving rise to a three-start helix with 13 subunits per turn (Erickson,1975).

Microtubules are assembled from free tubulin heterodimers; each consists of an a and b monomer. Assembled tubulin is in a constant state of dynamic equilibrium with free tubulin (Inoué and Sato,1964). Using optical birefringence mitotic fibers that disappeared after cold/colchicine treatment recovered after restoring normal conditions,even in the presence of actinomycin D or puromycin. Inoué and Sato (1967) postulated the existence of a pool of free subunits in equilibrium with the assembled microtubule. Steady state is thought to occur by the addition and loss of tubulin at opposite ends of the microtubule (Margolis and Wilson,1978;Farrell et al,1979).

Besides binding other subunits, the tubulin dimer binds GTP at a ratio of 1:2 and Mg++ at a ratio of 1:1. GTP binding is complicated by the fact that one site contains tightly bound non-exchangeable GDP which is transphosphorylated by GTP bound at the exchangeable site. (Olmsted and Borisy,1973). The exchangeable site becomes non-exchangeable when tubulin is incorporated into the microtubule (Weisenberg et al,1976). Certain exogenous chemicals such as colchicine,vinblastine sulfate and podophyllotoxin also bind the tubulin dimer, at a ratio of 1:1 (Borisy and Taylor,1967).

2.1 THE FUNCTION OF MICROTUBULES IN THE CELL

The microtubule has been implicated in numerous cellular functions. As a constituent of the cytoskeleton, this structure has been shown to both develop and maintain cell shape. In the nerve cell, microfilaments cause the cell to elongate while the microtubule maintains cell shape (Yamada et al,1970). On the other hand, in Ochromonas both the development and maintenance of shape are determined by cytoplasmic microtubules. Following colchicine treatment, the alga becomes rounded with little change in the distribution of organelles (Brown and Bouck,1973). In contrast, leukocytes become more polymorphic with an irregular distribution of organelles (Padawer et al,1975). However, a 50 percent reduction of microtubules around lymphocyte centrioles has no detectable effect on cell shape (Oliver et al, 1980).

As mentioned above (Section 1.3), the capping of ligand/receptor complexes has been thought to be dependent on the microtubule/microfilament cytoskeleton. Anti-tubulin immunofluoresent "subcaps" have been localised under Ig (Gabbiani et al,1977; Yahara and Kakimato-Sameshima,1978) and Con A caps (Albertini and Clark,1975). However, although microtubules have been found associated with caps, it is not clear whether these structures actually determine the location of the cap. Ig cap formation has generally been

found to be insensitive to the presence of colchicine (de Petris, 1975; Unanue et al, 1973; Poste et al, 1975) although, Cohem (1975) has reported marked inhibition. Con A cap formation is promoted by the presence of colchicine (Edelman and McClain, 1976; Oliver et al ,1980). In addition, B cells have been reported to cap more readily by colchicine than T cells (Oliver et al ,1980). At high concentrations of Con A, receptors become immobilised and unable to cap Con A or Ig without the presence of colchicine (Unanue et al, 1972 ; Yahara and Edelman, 1973). Even at low Con A concentraions, PMN cells have been reported to require colchicine in order to cap (Ryan et al, 1974 ; Oliver et al, 1976).

From this observation, models have been developed in which microtubules were shown to exert a transmembrane control over cell surface receptors (Yahara and Edelman,1973;Berlin et al,1974). However, this has been contested by Balla et al (1979) who found that the removal of unbound Con A,Mg++ and Ca++ from the medium induced cap formation without any apparent microtubule disassembly. In addition, Yahara and Kakimoto-Sameshima (1979) claim that there exists a two-way modulatiojn between microtubules and the Con A-receptor complex during cap formation. Microtubules and microfilaments most probably act together in the control of receptor distribution. Colchicine and cytochalasin B inhibit synergistically Ig cap formation (

Poste et al,1975; Taylor et al,1971) and Con A binding (Karsenti et al ,1977).

The ligand-receptor cap is eventually internalised by phagocytosis. In PMN cells, these receptors are selectively removed from the cell surface during phagocytosis. Colchicine and vinblastine don't inhibit phagocytosis per se, but they do prevent the selective removal of lectin receptors from the cell surface (Oliver et al,1974). In a more general sense, colchicine has been shown to interfere with the phagocytosis of certain substances such as starch but not others such as Staphylococcus (Malawista,1975).

Perhaps the most imaginative possibility is the putative role of microtubules in intracellular signal-transmission. This idea has mostly arisen from association, in that microtubules are found in various chemoreceptors (Gaffal and Bassemir,1974) and in organs like the brain (Borisy et al,1974). Models have been proposed in which signals are amplified between adjacent tubulin subunits along the microtubule (Atema,1975). However, there is no direct evidence which supports this hypothesis. Recently, as will be mentioned, this notion has been extended to Con A-induced lymphocyte blastogenesis (Edelman,1976).

2.2 THE EFFECT OF COLCHICINE ON MICROTUBULES

Colchicine, an alkaloid derived from Colchicum autumnale is known to arrest microtubule assembly and eventually to cause microtubule disassembly. However, the mechanism by which this is achieved is poorly understood. Inoué and Sato (1967) postulated that colchicine shifted the monomer-polymer equilibrium toward the monomer state. However, colchicine cannot bind to assembled tubulin directly (Wison and Meza,1973). Instead, colchicine must bind free tubulin dimers at a ratio of 1:1 to form a colchicine-tubulin complex (Borisy and Taylor, 1967) to inhibit microtubule assembly (Margulis and Wilson,1977).

The nature of the colchicine-tubulin complex has been studied principally by Wilson and coworkers (1966, 1967, 1970, 1973, 1974, 1975). Bond formation is slow forming, non-covalent and insensitive to pH and ionic strength (Bryan and Wilson,1971). This latter point suggests that the bound is non-electrostatic. It is also characterised by positive enthalpy,entropy and free energy changes (Wilson,1972). This is consistent with colchicine binding to a hydrophobic, non-polar pocket. Once formed the complex is not readily reversed under normal conditions,with a reported half life of 6-8 hours (Wilson,1974). The rate of decay unlike the rate of formation is very sensitive to pH and ionic strength (Wilson,1974). It is noteworthy that chick

embryo brain tubulin after binding colchicine becomes inactivated,unable to bind the alkaloid again (Wilson,1970). This suggests that the protein undergoes a conformational change causing the colchicine binding site to be no longer accessible. Other agents such as vinblastine sulfate stabilise the tubulin dimer, thereby extending the life of the colchicine-tubulin bound (Wilson 1974). Lumicolchicine, a photoinactivated derivative of colchicine has no effect on colchicine binding or microtubule assembly (Wilson and Freidkin,1966).

The mechanism by which colchicine arrests microtubule assembly and causes disassembly is not fully understood. The colchicine/podophylotoxin-tubulin complex inhibits assembly in a substoichiometric manner (Olmsted and Borisy,1973). Several models have attempted to explain this. Margolis and Wilson (1978) maintain that the complex binds to the growing end of the microtubule. This serves to block assembly, disassembly then occurs at the opposite end resulting the disappearance of the assembled structure. However, in contradiction with this model, Lambier and Engelboroughs (1978) have found the colchicine-tubulin complex to bind reversibly to the microtubule. Sternlicht and Ringel (1979) found that microtubule assembly inhibited by the presence of colchicine/tubulin complexes retained the ability to add tubulin to it's formally growing end.

Chapter III

MICROTUBULE FUNCTION IN LYMPHOCYTE ACTIVATION

In recent years, microtubles have been implicated in the activation of lymphocytes. Using tubulin-antibody immunofluorescence, microtubules have been observed to eminate from a centriolar region and run circumferentially throughout the cytoplasm of resting (Yahara and Katatimoto-Sameshima,1978; Rodgers and Brown,1979) and stimulated lymphocytes (Rogers and Brown,1979; Rudd et al,1979). During lymphocyte transformation, a significant increase of microtubules in the centriolar region has been reported (Biberfield,1971;Thyberg et al,1977; Rudd et al,1979). In contrast, Yahara and Kakatimoto-Sameshima (1978) have failed to stain detectable microtubule networks in stimulated leukocyte cultures.

Microtubules are thought to control the distribution of receptors in the plane of the lymphocyte plasma cell membrane (Taube and Berlin,1972). The presence of high Con

A concentrations has been shown to arrest the redistribution of Ig and Con A receptor/ligand complexes (Yahara and Edelman,1973). It was found that colchicine, brief cold treatments and other anti-microtubule agents induced receptor/ ligand complex redistribution. From this, models were postulated in which the underlying microtubule cytoskeleton exerted a transmembrane control over the distribution of membrane receptors. In an assembled state, the cytoskeleton anchored the receptors while disassembled tubules had no control over receptor mobility (Berlin et al,1974; Yahara and Edelman,1973).

Edelman and coworkers (1975,1976) further postulated a role for microtubules in Con A-induced lymphocyte activation. Their evidence was based on the diminished ability of colchicine to inhibit DNA synthesis when added at the start of culture (Wang et al,1973). Between 0-20 hours, colchicine became increasing less able to suppress the incorporation of thymidine when assayed at 48 hours (Wang et al,1975; Edelman,1976). Futhermore, the curve produced by the addition of colchicine was similar to the curve produced by the addition of aMM (Budd et al,1979). Observing that the inhibitory action of colchicine was restricted to a period of time prior to cell commitment, they concluded that colchicine interfered with the intiation of lymphocyte activation. Colchicine was assumed to act via microtubules

and so it was hypothesised that microtubules were involved in the initiation of lymphocyte activation. The exact nature of this role was never clearly defined (Edelman,1975); however, it was interpreted by other investigators to correspond to the critical second messenger in lymphocyte activation (Betel and Martijnse,1976). It is important to note that at no time was it considered that the similarity between the colchcicine and aMM curves could have been a factitious consequence of the dose and time dependence of colchicine inhibition. Also in these experiments, microtubule disassembly was not actually demonstrated to have resulted from colchicine treatment.

3.1 THE EFFECT OF COLCHICINE ON THE EARLY MOLECULAR EVENTS OF LYMPHOCYTE ACTIVATION

If microtubules are involved in the initiation of activation, it is logical to assume that certain early events causally linked to DNA synthesis would be inhibited by the presence of colchicine. However, unfortunately these events salient to DNA replication have yet to be clearly established Ca++ influx (Greene et al,1976), AIB transport (Greene et al,1976), phosphatidyl lysolecithin turnover (Resch et al,1977) and lymphokine production (Sherline and Mundy,1977; Gunther et al,1976; Resch et al,1977) have been reported unaffected by colchicine. This failure to cause inhibition occured despite the fact that the alkaloid

inhibited thymidine incorporation at later times. Resch and coworkers (1977,1979) have asserted that these data indicate that microtubules are not involved in the triggering of lymphocyte activation. This conclusion, however, is probably premature. The exposure time to colchicine was usually very short;at no time was colchicine actually shown to cause microtubule disassembly. In addition,even if microtubule disassembly was shown it is conceivable that colchicine could interfere with the initiation of activation and not inhibit all events causally related to DNA synthesis. These event(s) would be in series to each other (Kaplan,1977).

In contrast to the above findings, certain events have been found to be altered by the presence of colchicine. The early cAMP surge following binding of lectin has been reported to to be enhanced by the presence of colchicine (Greene et al,1976). Also, phosphatidyl inositol turnover has been found reduced in lymphocyte cultures (Schellenburg and Gillespie,197). These studies have suggested the existence of a role for microtubules in lymphocyte activation. However, the fact that colchicine has been found to bind to isolated membrane fractions points to an alternative target for the action of colchicine (Riordan and Alon,1977; Sherline et al,1977; Gillespie,1971). Therefore, it is possible that colchicine is causing inhibition or

promotion of these early events by some as yet undefined action.

3.2 THE EFFECT OF COLCHICINE ON RNA SYNTHESIS

Literature on the effect of colchicine on RNA synthesis is conflicting. In these cases, the exposure time to the alkaloid is longer and therefore it is probably safer to assume that microtubule disassembly occurred (Rogers and Brown,1979).Betel and Martijnse (1976) using Con A activated rat lymphocytes found that vinblastine and podophyllotoxin when present from the start of culture had no effect on uridine incorporation at 24h. In agreement,Resch et al (1977) using Con A-stimulated rabbit lymphocytes found colchicine to have no effect on RNA synthesis at 4 and 24 hours of culture.Both groups concluded that microtubules are not involved in the triggering of lymphocyte activation.

In contrast to these results,Hausser et al (1976) noted a dramatic inhibition of uridine incorporation into Con A-stimulated bovine lymphocytes until 20h of culture. After this time, the addition of colchicine had no apparent effect. In order to determine if the alkaloid inhibited RNA synthesis directly, colchicine was added to an in vitro transcription system. The alkaloid had no effect on either isolated nuclei or RNA polymerase activity. From this,Hausser et al (1976) concluded that microtubules are

involved in the triggering mechanism of activation, a conclusion similar to that of Edelman (1976). However, their conclusion would appear to be somewhat suspect. Uridine incorporation was assayed only twice after 20h of culture, both within 10 hours of colchicine addition. If colchicine required a long lag time to become inhibitory then inhibition would have gone undetected (Rudd et al, 1979).

The reason for the lack of consensus regarding colchicine's effect on early RNA synthesis is unclear. Forsdyke (1979) claims that it may be related to the concentration of Con A used. He found that colchicine inhibition of uridine incorporation was evident in rat lymphocytes stimulated with 50ug/ml Con A and not with 5ug/ml. This could account for the lack of inhibition reported by Betel and Martijnse (1976). Another possibility could be related to the source of lymphocytes in the different experiments. I have found mouse splenocytes significantly more sensitive to colchicine than human cells (Rudd et al, 1979). The reason for this difference in sensitivity is not known. However, it may be related to the ability of colchicine to enter the cell. Ling and Thompson (1973) have isolated Chinese hamster ovary cells which are colchicine resistant by virtue of reduced permeability. In these cells, microtubule dependent functions such as cappng are unaffected by colchicine. The different sensitivity of

lymphocytes from different sources to colchicine also points to the need to carry out a proper dose response curve before proceeding with an experiment.

3.3 THE EFFECT OF COLCHICINE ON DNA SYNTHESIS

There is also a lack of consensus regarding the effect of microtubule-disrupting agents on DNA synthesis in activated lymphocyte cultures. Most studies have shown that colchicine when present from the start of culture prevented the onset of S phase as assayed by thymidine incorpration (Wang et al, 1975; Milner, 1977). Colchicine, when added at various times prior to DNA synthesis either caused a constant degree of inhibition (Medrano et al, 1974) or became progressively less inhibitory until 20 hours afterwhich there was no effect (Wang et al, 1975)

In contrast to these findings, Betel and Martijnse (1976) reported that vinblastine and podophyllotoxin had no effect in preventing Con A-activated spleen cells from entering S phase. However, this study must be viewed with caution because the number of mitotic figures at 72 hours was unaffected by the presence of these agents, despite colchicine's well documented ability to cause metaphase arrest. Colchicine inhibition of thymidine incorporation was attributed to an effect on transport and not on DNA synthesis. However, the study quoted in support of that

suggestion (Mizel and Wilson,1972) did not examine thymidine transport in lymphocytes and found different Ki values for various cell types. In addition, net thymidine uptake and not transport was measured. Futhermore, these authors found that colchicine inhibited 3H-uridine effect uridine uptake to the samee degree as ^{3}H-thymidine (Wilson,1972). Betel and Martijnse (1976) reported that colchicine had no effect on uridine incorporation at 24 hour.

This does not exclude the possibility that colchicine could be acting on transport.Steen and Limdo (1979) found the measurement of ^{3}H-thymidine as an assay for DNA synthesis to overestimate the degree of colchicine inhibition when using cytofluorometric methods to quantitate DNA.

Reports concerning the effect of colchicine on ongoing DNA synthesis are also conflicting. Inhibition caused by the alkaloid when added after 20-30 hours of culture has been noted in partially synchronised mouse splenocytes (Milner,1976) and in human lymphocytes (Resch,1979) using both thymidine incorporation and cytofluorometry (Steen and Limdo,1978) as an assay for DNA synthesis.

In contrast to the above, Edelman and coworkers (1975,1976) have claimed that human lymphocytes are insensitive to colchicine during S phase. Partially synchronised cells released from the G1/S boundary underwent an early rise in DNA synthesis followed by a return to control levels some 20 hours later. Both colchicine treated and control cells followed the same pattern. They concluded that both ongoing DNA synthesis and thymidine transport in lymphocytes are unaffected by colchicine. However, the interpretation of these results were confounded by the recycling of the stimulated cells and disregarded the possibility that colchicine may require a period of time for inhibition to become manifest (Rudd et al, 1979).

3.4 THE EFFECT OF COLCHICINE ON THYMIDINE TRANSPORT

The effect of colchicine on the assay for DNA replication has been complicated by a possible effect of the alkaloid on thymidine transport (Betel and Martinjse, 1976; Steen and Limdo, 1979). However, it is unclear as to whether colchicine acts on thymidine transport, DNA synthesis or both. Theoretically, the agent could act to limit the amount of ^{3}H-thymidine taken up into the cell to be used in DNA synthesis. Wilson (1972) using several mammalian cell lines found colchicine to inhibit thymidine uptake. In the case of Hela cells, colchicine caused a 50 percent inhibition at a concentration of $6.1 \times 10^{-5}M$. However, it is not clear in

the case of these cells how this effect is related to the effect of colchicine on DNA synthesis. Futhermore, because the Ki values differed significantly between cells from different sources, it is unclear how these values are related to stimulated lymphocytes. Lastly, in this study, thymidine uptake as opposed to transport was measured. Therefore, the colchicine could have affected any number of events linked to uptake such as DNA synthesis itself. In addition to this study, Berlin (1973) has studied the effect of colchicine treatment on adenosine transport in aveolar macrophages. The alkaloid was found to change the temperature transition of transport to that of a low energy state. However, it is not known whether the agent acted via an effect on microtubules. In contrast, Pofit and Strauss (1978) found that micromolar concentrations of colchicine did not inhibit the transport of thymidine in adherent macrophages. The same concentrations inhibited strongly 3H-thymidine incorporation when used as an assay for DNA replication.

Chapter IV

MATERIALS AND METHODS

4.1 PREPARATION OF CELL CULTURES

Human peripheral lymphocytes were purified from heparinised venous blood (100 ug/mL) by Ficoll-Hypaque (Pharmacia) density gradient centrifugation. The heparinised blood was first centrifuged at 400g for 30 minutes, the autologous serum containing platlets was removed and the remaining cell suspension was diluted 1:3 with RPMI 1640 (Flow Labs). The cell suspension was then layered on the Ficoll-Hypaque, centrifuged for 35 minutes at 400g at 18-20°C and the lymphocytes were removed from the cell layer at the Ficoll-RPMI interface. Cells were then washed 3X with RPMI 1640,suspended in culture medium at a concentration of 1.0-1.5 X 10^6 cells/ mL and incubated in either closed 75 cm^2 culture flasks (Corning) to a final volume of 50 mL or in 12 X 75 mm tubes at a final volume of 1 mL. The culture medium consisted of RPMI 1640 supplemented with 10 percent autologous serum,penicillin-streptomycin (100U/mL-100U/mL)

(Difco) and was buffered to pH 7.6 with Hepes buffer (Sigma). Autologous serum was prepared by heating for 30 minutes at 56C and centrifuging for 30 minutes at 2,000g in order to inactivate the complement and heparin and to remove fibrogen and platelets. The purification protocol yielded cultures containing 95 percent lymphocytes with viabilities of usually 92 percent as assessed by trypan-blue exclusion.

Murine lymphocytes were prepared from spleens of Balb/C mice. The mice were killed by cervical dislocation, the spleens excised, and the cells gently teased into RPMI 1640 medium. The cell suspension was layered on fetal calf serum (Gibco Ltd.) to sediment debris.Red blood cells were lysed in NH_4Cl for 7 minutes at 4°C, centrifuged and the lymphocyte pellet washed twice in culture medium. Cells were suspended at a concentration of $1.0-1.5 \times 10^6$ cells/mL in RPMI 1640 supplemented with 6 percent fetal calf serum with penicillin-streptomycin (100 U/mL-100 ug/mL) buffered to pH.6.9 with Hepes buffer. Cultures were incubated in 12 X 75 mm tubes.

4.2 MITOGENS AND INHIBITORS

Concanavalin A (Calbiochem) was added at a final concentration of 20ug/mL and 2 ug/mL to human peripheral and mouse spleen lymphocyte cultures, respectively. This was added prior to distribution to either the flasks or

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

The glycoside, aMM was added at various times of culture to a final concentration of 0.1M. A 50ul aliquot was transfered to 0.5mL volumes of human and mouse lymphocyte cultures from a stock solution.

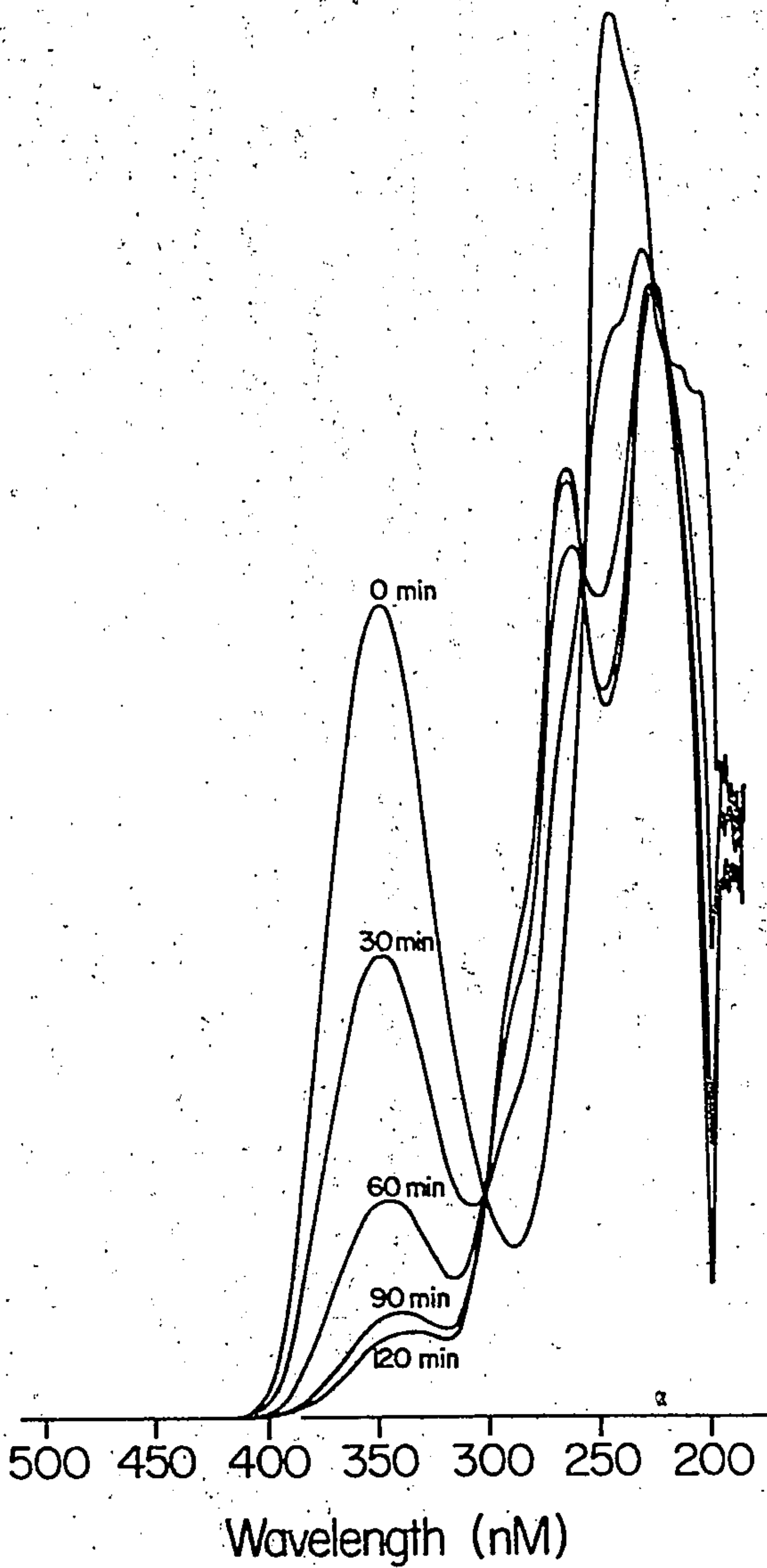
Concentrations of colchicine (Fisher),vinblastine sulfate (Sigma) and lumicolchicine were also added to 0.5mL of cell culture in 50uL aliquots from stock solutions. The concentration of inhibitor used was based on standard dose-response curves (Figures 4-6).

Lumicolchicine was prepared as described by Wilson and Friedkin (1966). Colchicine (Sigma) was dissolved in 100 percent ethanol at the desired concentration placed in a 2.0mL quartz cuvette and placed 2-6" from a long wave ultraviolet light. The setup was in a dark box to avoid extraneous light. The conversion of colchicine to lumicolchicine was followed by U.V.spectrophotometry. Figure 1 shows that between 0 to 120 minutes, the peak absorption at 350nm for colchicine decreased and was replaced by the appearance of another absorption peak at 267nm. The lumicolchicine in ethanol was then evaporated with nitrogen gas to yield a powder. The preparation was occasionally

checked for degradation by resuspending the powder in ethanol and observing the U.V. Absorption Spectra.

Figure 1: Conversion of colchicine to lumicolchicine

Colchicine was dissolved at a concentration of 10^{-4}M and exposed to U.V. light for various periods of time as outlined in Section 5.1.2 of the *Materials and Methods*.


0 min
30 min
60 min
90 min
120 min
500
450
400
350
300
250
200
Wavelength (nM)

4.3 ASSAY FOR RNA AND DNA SYNTHESIS

In experiments measuring RNA synthesis, colchicine was added at the onset of culture. [3H]uridine, labeled at positions 5 and 6 on the molecule (40Ci/mM (1Ci=37 GBq),Amersham) was used to measure RNA synthesis at various times of culture. The isotope was added to cultures 1 hour prior to harvest in 50uL RPMI 1640 to a final radioactivity of 2uCi/mL.

In the assay of DNA synthesis, the inhibitors were added at different times of culture as indicated. The incorporation of [3H]thymidine (40Ci/mM,Amersham) was assayed at 48,60 and 72 h. The isotope was added 2h prior to harvest in 50uL RPMI 1640 to a final concentration of 2uCi/mL.

In all cases, the incorporation of isotope was measured by depositing cells on Whatman GF1A filters, washing with PBS, ice-cold 5 percent trichloroacetic acid and methanol. The dried filters were counted in 7 mL of scintillation fluid in a Beckman liquid scintillation counter. At least four replicates were counted for each sample; mean counts are presented with their standard deviations.

4.3.1 Immunofluorescence

Immunofluorescent staining was based on a modification of the method of Osborn, Webster and Weber (1975) as outlined in Rudd et al (1979). Briefly, cells were pipetted onto coverslips coated with poly-Llysine, washed twice with stabilzation buffer consisting of 0.1M piperazine-N,N'-bis (2-ethane sulfonic acid), 1mM ethylene glycol-bis (2-aminoethyl ether) -N-N'-tetraacetic acid (EGTA) and 4 percent polyethylene glycol. Cells were then extracted for 5 minutes with stabilization buffer containing 1 percent Triton X-100. The cytoskeletons were washed twice for 30 seconds with SB, fixed for 10 minutes with 1 percent glutaraldehyde in SB and reduced twice with 2mg/mL sodium borohydride in PBS. The cell preparations were washed thrice in PBS, incubated for 45 minutes with antiserum to tubulin, washed thrice with PBS and finally, incubated for 45 minutes with fluorescein-conjugated goat anti-IgG to rabbit IgG (Hyland Laboratories). The tubulin-antibody serum was generously provided by Dr. V.I. Kalnins and Dr. J.A. Connolly, University of Toronto ; the other supplies were provided by our colleague, Dr. D.L. Brown.

4.4 MEASUREMENT OF THYMIDINE TRANSPORT

Thymidine transport in human peripheral lymphocytes was measured by modification of the rapid sampling technique of

Strauss (Strauss et al,1976). Cells were removed from Corning incubation flasks, washed twice and suspended in a incubation medium (LMGB) at a concentration of 10.0 X 106 cells/mL. LMGB consisted of 10mM sodium phosphate buffer, pH.7.5,0.9 percent Nacl,0.1 percent bovine serum albumin,5X crystallized (Sigma) and 5mM glucose (Fisher). Transport was measured by mixing equal volumes (0.25 X 0.25 mL) of cell suspension with thymidine at the desired concentration at 15 or 30uCi/mL in LMGB. After the appropriate incubation time (15-60 seconds),200 uL of reaction mixture was layered into a 400uL microfuge tube (Canlab) containing 50uL of 7 percent perchloric acid as the bottom layer and 150uL of silicone 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 scinitillation vial for counting. Cell digestion was accomplished overnight with 1.0mL of Protosol (New England Nuclear) at 37C. This was then neutralised with 100.0uL glacial acetic acid and counted in 7 mL of scintillation fluid (Scintiverse, Fisher) with a Beckman Scintillation counter. Counting efficencies,which ranged between 24-35 percent, were determined by internal quench correction.

Chapter V

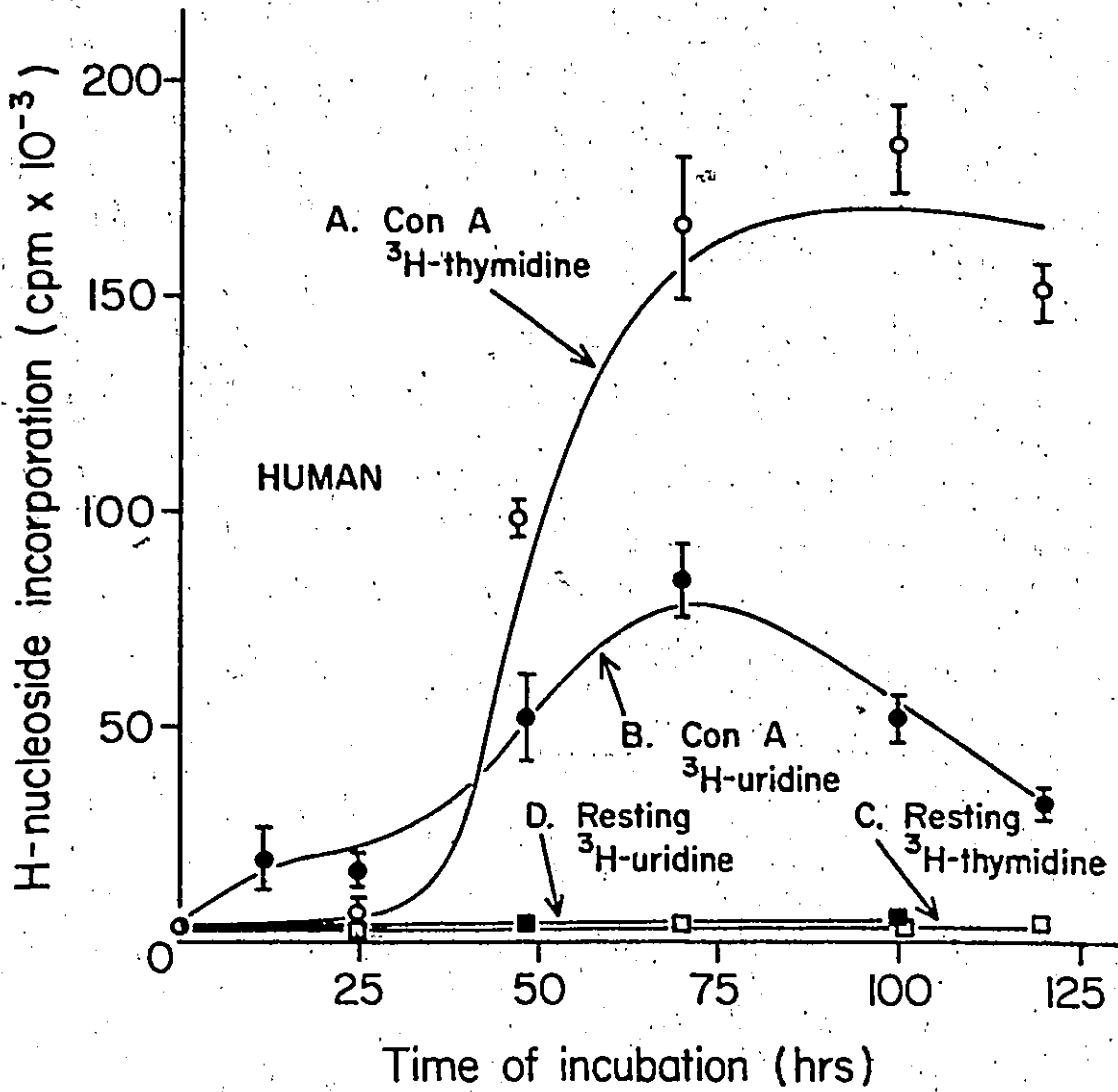
RESULTS

5.1 MITOGENIC RESPONSE OF HUMAN AND MOUSE LYMPHOCYTES TO CON A

Figures 2 and 3 show the mitogenic response to Con A of human peripheral blood and mouse spleen leukocyte cultures, respectively. In both cases, ^{3}H-uridine incorporation increased as a early as 12 hours and reached a maximal level of incorporation by between 48 to 72 hours of culture. The incorporation of ^{3}H-thymidine incorporation increased between 24 to 36 hours and peaked by between 48 to 100 hours. Human leukocyte cultures were usually seen to commence DNA synthesis later than mouse splenocyte cultures;that is at 30 to 36 hours as opposed to 24 to 30 hours. Human cell cultures contained at least 92 percent viable cells for the full duration of incubation (120 hour), as assayed by trypan blue exclusion. In the case of mouse splenocytes,viabilities dropped to about 45 to 60 percent during the first 24 hours of culture in both resting and Con A -stimulated cultures. This was followed by a

Figure 2: The Incorporation of ^{3}H-Uridine and ^{3}H-Thymidine into Con A-Stimulated Human Leukocytes

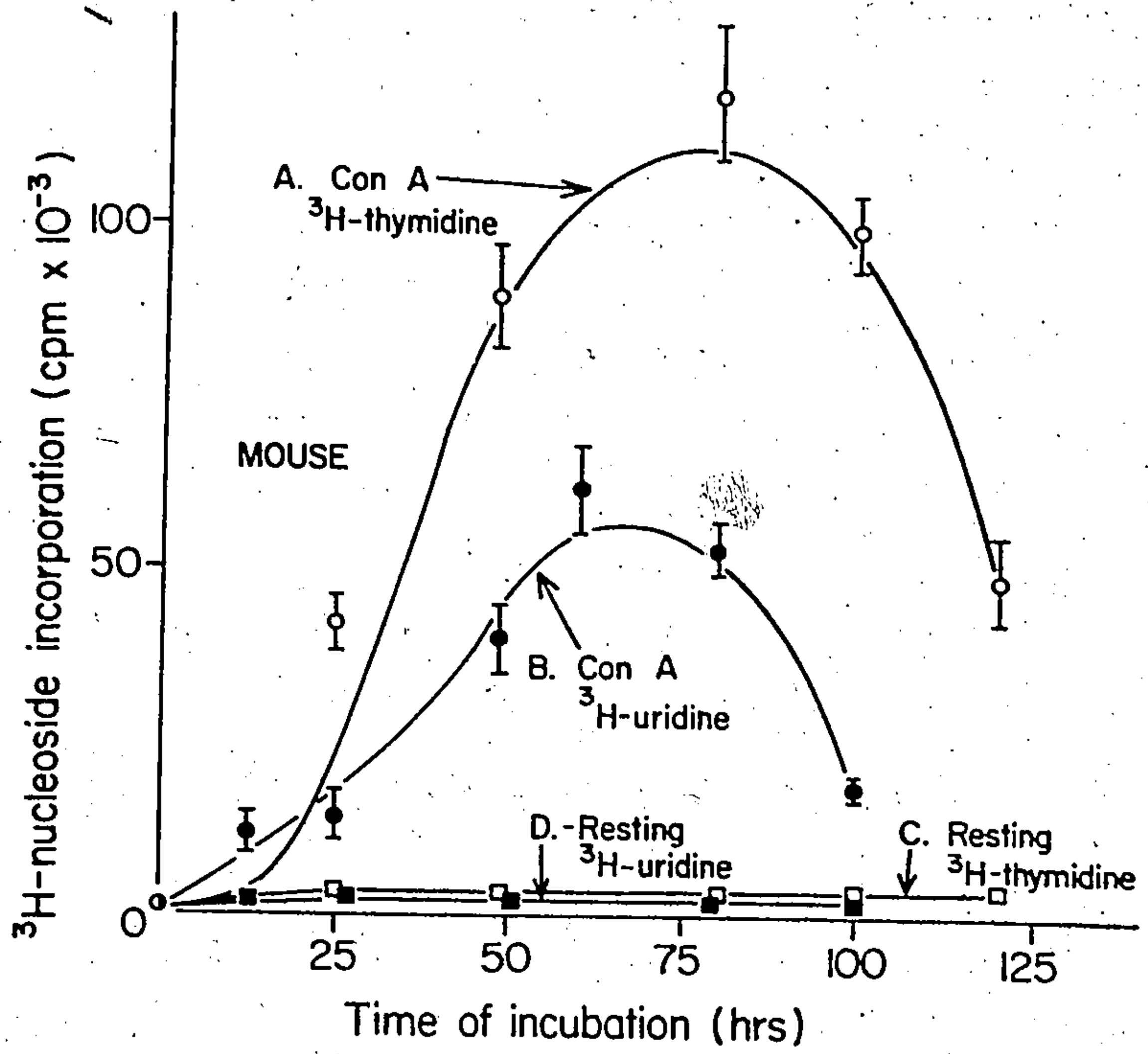
Human leukocyte cultures were prepared as outlined in <u>Materials and Methods</u>. The incorporation of ^{3}H-uridine and ^{3}H-thymidine (2uCi/mL/2h) was assayed at different times following Con A addition.resting control:2.0-3.0 X 10^{3} cpm.



HUMAN
A. Con A
^{3}H-thymidine
B. Con A
^{3}H-uridine
D. Resting
^{3}H-uridine
C. Resting
^{3}H-thymidine
H-nucleoside incorporation (cpm x 10^{-3})
Time of incubation (hrs)
0
25
50
75
100
125
150
200

Figure 3: The Incorporation of ^{3}H-Uridine and ^{3}H-Thymidine into Con A-Stimulated Mouse Splenocytes

Mouse splenocytes were cultured as previously outlined in <u>Materials and Methods</u>. The incorporation of ^{3}H-uridine and ^{3}H-thymidine (2uCi/mL/2h) was assayed at various times following Con A addition.resting control:2.0-3.0 X 10^{3} cpm.



A. Con A
^{3}H-thymidine
MOUSE
B. Con A
^{3}H-uridine
D.-Resting
^{3}H-uridine
C. Resting
^{3}H-thymidine
^{3}H-nucleoside incorporation (cpm x 10^{-3})
0
50
100
25
50
75
100
125
Time of incubation (hrs)

gradual increase in cell number by 48 to 72 hours in stimulated cultures (data not shown), presumably due to the presence of second generation cells(Ling and Kay, 1975).

5.2 DOSE-DEPENDENT COLCHICINE INHIBITION OF LYMPHOCYTE TRANSFORMATION

Colchicine when present from the start of culture caused a dose-dependent inhibition of ^{3}H-thymidine incorporation when measured at 60h of culture. Figure 4 shows that at 10^{-5}M to 10^{-7}M colchicine reduced the incorporation of ^{3}H-thymidine into Con A-stimulated human lymphocytes by 70-90 percent. At concentrations between 10^{-7}M-10^{-8}M colchicine, the lymphocytes exhibited a sharp dose-dependent inhibition of ^{3}H-thymidine incorporation;at 10^{-7}M colchicine the nucleoside incorporation ranged from 70-90 percent of stimulated control while at 10^{-8}M there was generally no inhibition.Further experiments were carried out at 10^{-6}M colchicine in order to avoid variablility which could result from slight changes in alkaloid concentration. Colchicine at 10^{-6}M had no detectable effect on ^{3}H-thymidine incorporation into resting human lymphocytes(data not shown).

Figure 5 shows a similar dose-dependent inhibition of ^{3}H-thymidine incorporation into Con A-stimulated human lymphocytes caused by the presence of vinblastine sulfate.

In this case, thymidine incorporation was also reduced about 70-90 percent between concentrations 10^{-5}M and 10^{-7}M. In addition, between concentrations 10^{-7}M and 10-9M there was a sharp dose-dependence. Human cells appeared slightly more sensitive to the effects of this alkaloid since concentrations less than 10^{-8}M caused significant inhibition.

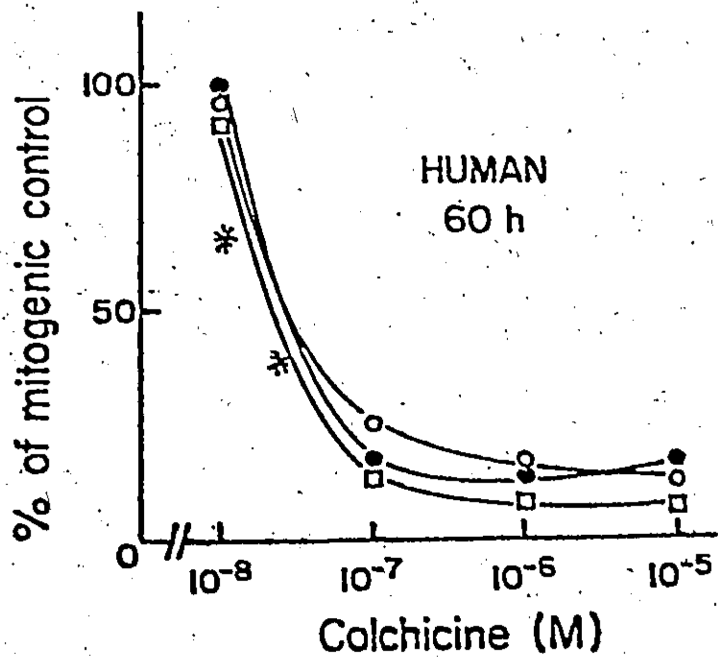
Figure 6 shows the dose-related inhibition of ^{3}H-thymidine incorporation caused by colchicine in Con A activated mouse spleen lymphocytes. The mouse cells exhibited a sensitivity to the alkaloid in a manner similar to that of human cells and hence, 10^{-6}M colchicine was selected for further experiments.

Figure 4: Effect of Different Concentrations of Colchicine on Stimulated Human Leukocytes

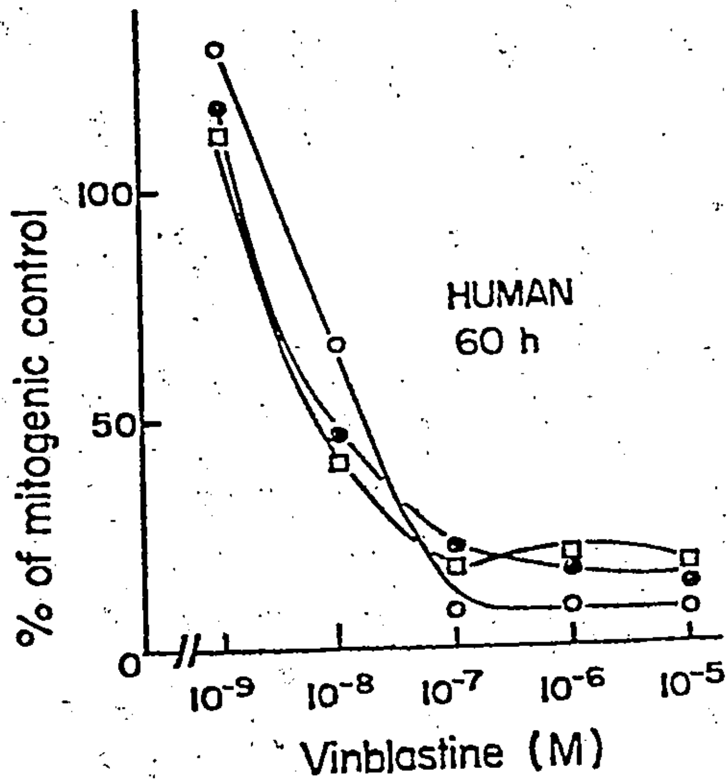
Colchicine at concentrations between 10^{-8} to 10^{-5}M was added at the start of culture to Con A-stimulated human leukocytes. The incorporation of ^{3}H-thymidine (2uCi/mL/2h) was assayed at 60 hour of culture. Con A control: 1.75-2.25 X 10^{5} cpm; resting control: 2.0-3.0 X 10^{3} cpm.

Figure 5: Effect of Different Concentrations of Vinblastine Sulfate on Stimulated Human Leukocytes

Vinblastine sulfate at concentrations between 10^{-9} to 10^{-5}M was added at the start of culture to Con A-stimulated human leukocyte cultures. The incorporation of ^{3}H-thymidine (2uCi/mL/2h) was assayed at 60 hour of culture. Con A control: 1.75-2.00 X 10^{5} cpm; resting control: 2.0-3.0 X 10^{3} cpm.



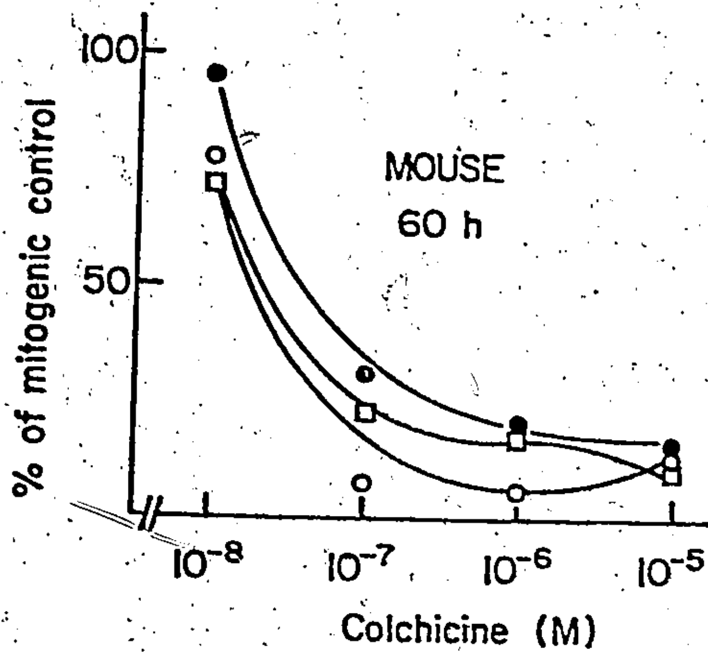
100
50
0
% of mitogenic control
HUMAN
60 h
10^{-8}
10^{-7}
10^{-6}
10^{-5}
Colchicine (M)



100
50
0
% of mitogenic control
HUMAN
60 h
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
Vinblastine (M)

Figure 6: Effect of Different Concentrations of Colchicine on Stimulated Mouse Splenocytes

Colchicine at concentrations between 10^{-8} to 10^{-5}M was added at the start of culture to Con A-stimulated mouse splenocyte cultures. The incorporation of ^{3}H-thymidine was assayed at 60 of culture. Con A control:1.50-1.75 X 10^{5}cpm.


100
50
% of mitogenic control
MOUSE
60 h
10^{-8}
10^{-7}
10^{-6}
10^{-5}
Colchicine (M)

5.3 COLCHICINE EFFECTS PRIOR TO AND AFTER THE ONSET OF DNA SYNTHESIS

Next it was of interest to define the stage of Con A-induced lymphocyte transformation sensitive to colchicine. As mentioned, several reports have claimed that lymphocytes are sensitive to colchicine only prior to S phase (Medrano et al, 1974; Wang et al, 1975). Other reports have maintained that colchicine is inhibitory only after the onset of DNA synthesis (Resch et al, 1977). Figures 7 and 8 show that colchicine when present from the start of culture prevented the onset of S phase in both Con A-stimulated human and mouse lymphocyte cultures, respectively. This is in agreement with Wang et al (1975) and has been interpreted to indicate that colchicine may inhibit early event(s) causally linked to DNA replication.

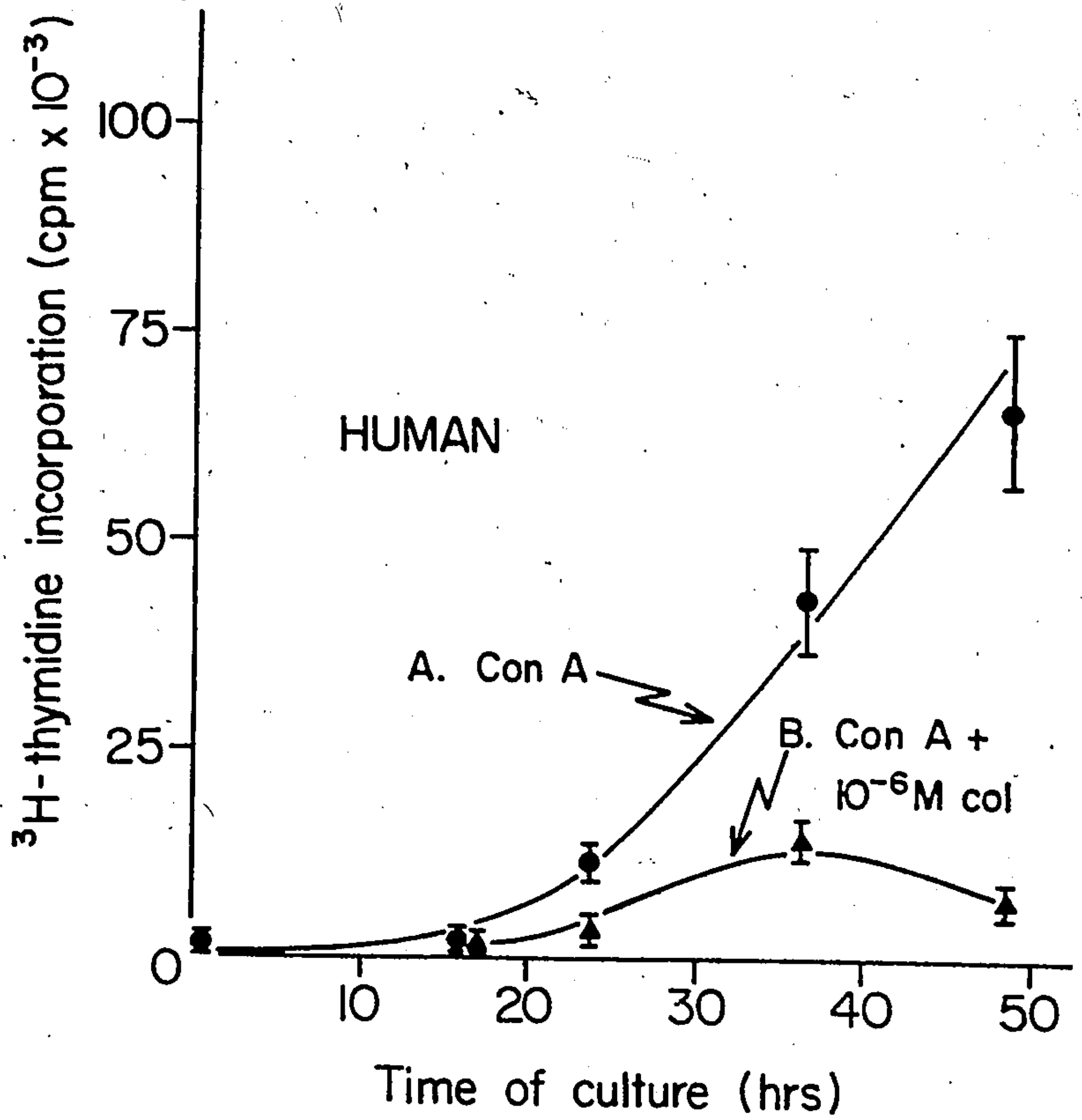
Since colchicine prevented the onset of DNA synthesis in most of the population, it was of interest to measure directly the effect of the alkaloid on an event which occurs before 24 hours. Table 1 shows that a 12 hour exposure to colchicine caused an inhibition in RNA synthesis after 12 hours of culture. ^{3}H-uridine incorporation was inhibited about 50-60 percent at 12 and 20/24 hours in both stimulated human and mouse cells. There was little or no inhibition of RNA synthesis in resting human cultures. In

the case of resting mouse splenocytes, colchicine inhibited RNA synthesis by approximately 60 percent.

In addition, table 2 shows that with Con A-stimulated human lymphocytes studied over 12 hour intervals at varying durations of culture, colchicine was equally inhibitory both prior to and after the onset of S phase. Colchicine inhibition of RNA synthesis was not restricted to a period prior to DNA synthesis as has been previously reported (Hauser et al,1976).

Figure 7: Effect of Colchicine on the Onset of DNA Synthesis in Stimulated Human Leukocytes

Colchicine at a concentration of 10^{-6}M was present from the start of culture. The incorporation of ^{3}H-thymidine was assayed at between 16 to 48 hours following the addition of Con A. resting control: 2.0-3.0 X 10^{3} cpm.



^{3}H-thymidine incorporation (cpm x 10^{-3})
100
75
50
25
0
HUMAN
A. Con A
B. Con A +
10^{-6}M col
10
20
30
40
50
Time of culture (hrs)

Figure 8: Effect of Colchicine on the Onset of DNA Synthesis in Stimulated Mouse Splenocytes

Colchicine at a concentration of 10^{-4}M was present from the start of culture. The incorporation of ^{3}H-thymidine was assayed at between 24 and 48 hours following the addition of Con A. resting control:2.0-3.0 X 10^{3} cpm.

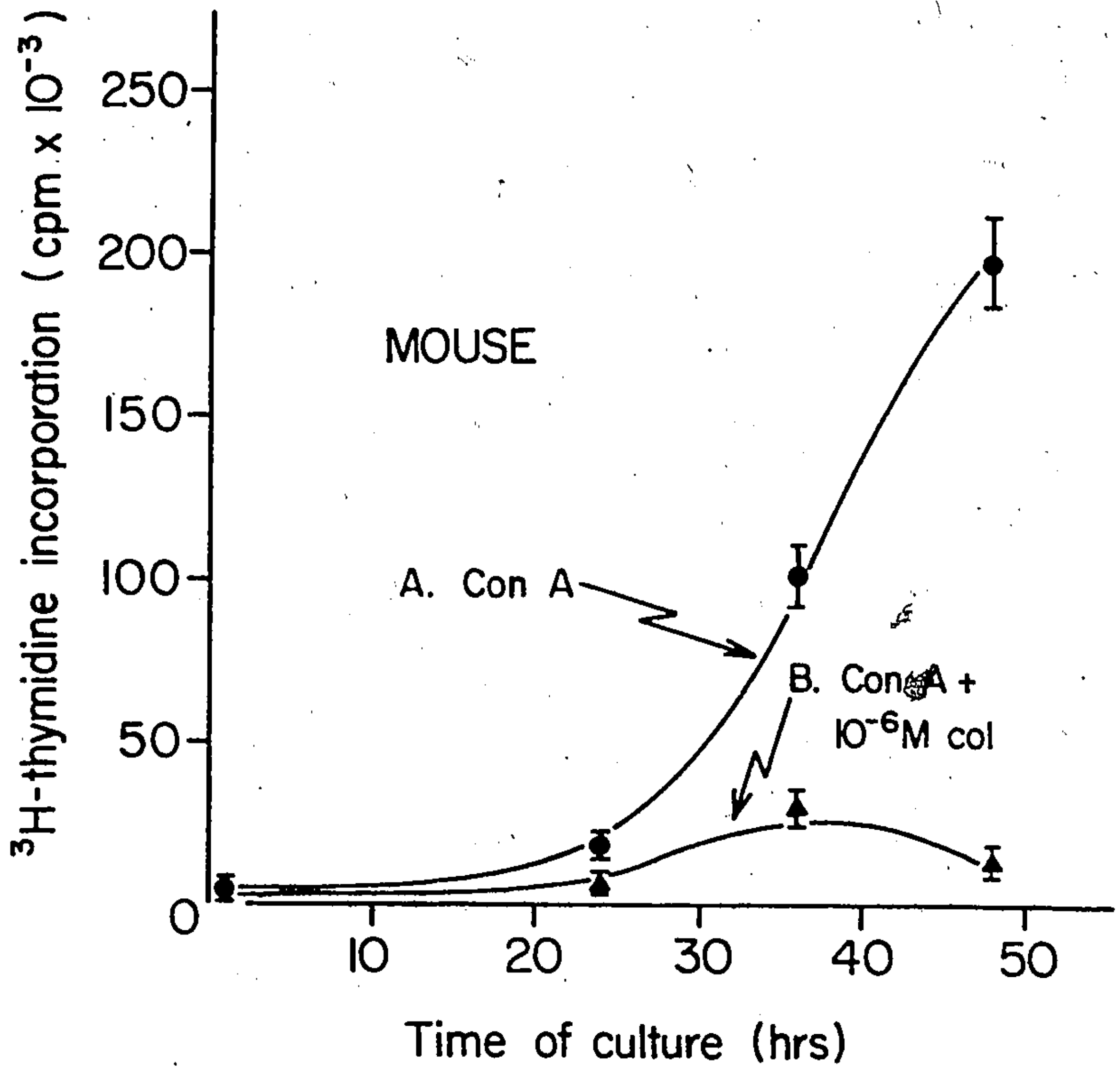

MOUSE
A. Con A
B. Con A +
10^{-6}M col
^{3}H-thymidine incorporation (cpm x 10^{-3})
250
200
150
100
50
0
10
20
30
40
50
Time of culture (hrs)

TABLE 1: EFFECT OF COLCHICINE ON EARLY RNA SYNTHESIS IN HUMAN PERIPHERAL AND MOUSE SPLEEN LYMPHOCYTES

CELL CATEGORY	HUMAN		MOUSE	
	12 H	24 H	12 H	20 H
RESTING CONTROL	1400 ± 50	2500 ± 120	6450 ± 700	4500 ± 150
RESTING COLCHICINE	1400 ± 200	1650 ± 120	4050 ± 400	2100 ± 200
CON A CONTROL	14200 ± 1900	22800 ± 1450	46800 ± 1500	28700 ± 1200
CON A COLCHICINE	6100 ± 1400	6550 ± 550	22700 ± 400	14000 ± 500

Table 2: Effect of a 12 Hour Exposure of 10^{-6}M Colchicine at Various times on ^{3}H-Uridine Incorporation in Con A - Stimulated Human Lymphocytes.

Exposure Period(h)		Resting Cpm	Resting % of Control	Con A-Stimulated Cpm	Con A-Stimulated % of Control
0-12	-Col	3562 ± 797	89 ± 13	5731 ±137	62 ± 8
	+Col	3165 ± 158		3567 ±761	
2-14	-Col	3190 ± 2104	92 ± 42	9506 ±461	47 ± 3
	+Col	2924 ± 595		4424 ±194	
4-16	-Col	4516 ± 632	75 ± 8	17690 ±1107	51 ± 3
	+Col	3403 ± 128		9068 ±49	
6-18	-Col	4697 ± 183	82 ± 9	21068 ±682	48 ± 2
	+Col	3866 ± 616		10203 ±153	
8-20	-Col	4204 ± 256	82 ± 10	21524 ±2104	49 ±10
	+Col	3462 ± 583		10502 ±2044	
10-22	-Col	4442 ± 822	103 ± 22	22183 ±1690	57 ± 7
	+Col	4563 ± 1132		12568 ±1212	
22-34	-Col	3967 ± 797	77 ± 13	53328 ±4322	51 ± 5
	+Col	3865 ± 223		27040 ±1159	
34-46	-Col	2218 ± 388	101 ± 15	127600±4742	61 ± 3
	+Col	2244 ± 256		77425 ±2153	

5.4 RELATION OF COLCHICINE INHIBITION TO CELL COMMITMENT

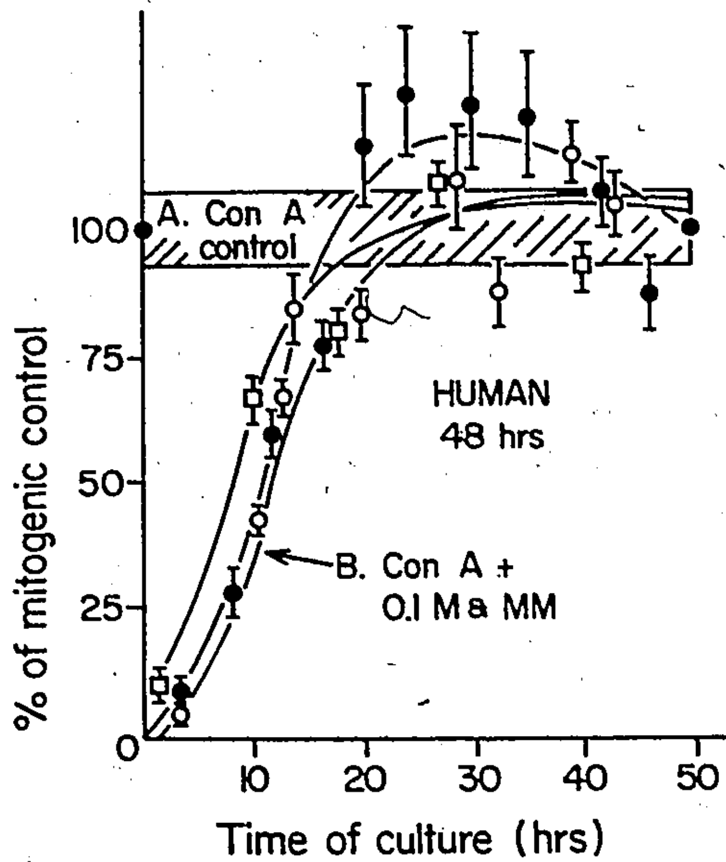
Having shown that colchicine could inhibit lymphocyte activation prior to S phase, it became of interest to investigate whether this inhibition was related to commitment of lymphocytes during blastogenesis. This is important because commitment approximates the period of time during which initiation of activation is occuring. Removal of Con A prior to the completion of the commitment period prevents the cell from transforming. Figures 9 and 10 show the commitment kinetics of the human and mouse lymphocytes, respectively. The sugar, aMM was added at different times following Con A addition.From the beginning of culture to 20-30 hours later, the lymphocyte population became increasingly refractory to the presence of the sugar. If the sugar was added 12 hours after Con A then the incorporation of ^{3}H-thymidine at 48-60 hours was 50 percent of Con A activated control. After 20-30 hours of culture, the addition of aMM had no effect on subsequent ^{3}H-thymidine incorporation indicating that the entire population had been commited to transform. This effect has been well documented (Novogrodski and Katchalski, 1972) and has been shown related to the progressive appearance of blast cells (Gunther et al, 1976).

Figure 9: Effect of Adding aMM at Various Times on Stimulated Human Leukocytes

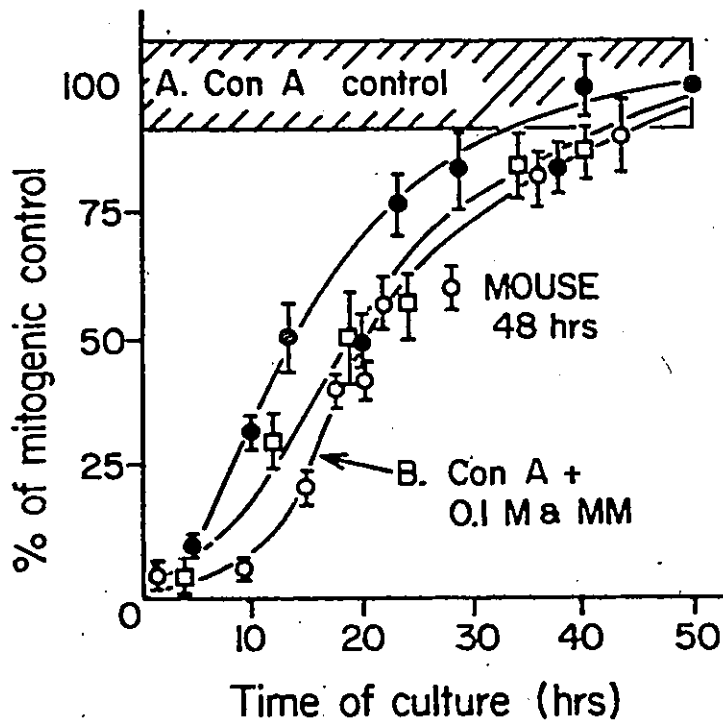
The sugar, aMM at a final concentration of 0.1M was added at various times after the addition of Con A. The incorporation of ^{3}H-thymidine was assayed after 48 hours of culture. Three separate experiments are shown. Con A control: 1.50-2.00 X 10^{5} cpm; resting control: 2.0-3.0 X 10^{3} cpm.

Figure 10: Effect of Adding aMM at Various Times on Stimulated Mouse Splenocytes

The sugar, aMM at a final concentration of 0.1M was added at various times following the addition of Con A. The incorporation of ^{3}H-thymidine was assayed after 48 hours of culture. Three separate experiments are shown. Con A control: 1.50-1.75 X 10^{5} cpm; resting control: 1.5-2.0 X 10^{3} cpm.



A. Con A
control
100
75
50
25
0
HUMAN
48 hrs
B. Con A +
0.1 M a MM
% of mitogenic control
10
20
30
40
50
Time of culture (hrs)



A. Con A control
100
75
50
25
0
MOUSE
48 hrs
B. Con A +
0.1 M a MM
% of mitogenic control
10
20
30
40
50
Time of culture (hrs)

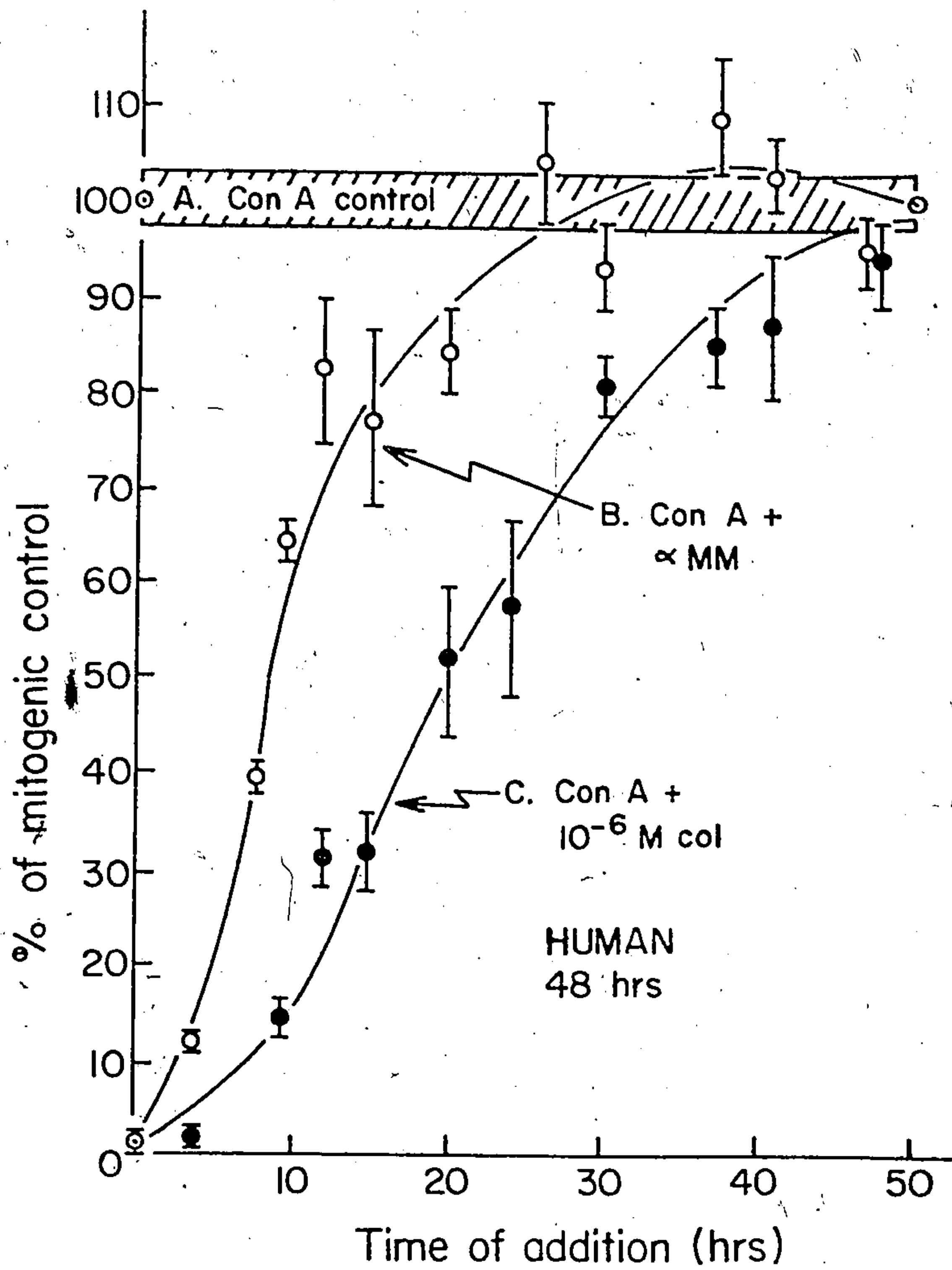
The relation of colchicine inhibition of lymphocyte transformation to the progressive commitment of Con A-stimulated human lymphocytes was investigated by adding colchicine at various times of culture in a manner similar to aMM. Figure 11 shows that when ^{3}H-thymidine incorporation was assayed at 48 hours of culture, the addition of aMM and colchicine produced curves which although similar are not identical. The similarity between these curves had been previously interpreted by Edelman and coworkers(1975) as indicating that colchicine blocked lymphocyte transformation at the point of cell commitment. However, Figures 12 and 13 illustrate that if ^{3}H-thymidine incorporation was assayed at later times, 60 and 72 hours respectively, the curve produced by colchicine became progressively disparate relative to the aMM curve which remained more or less the same. The colchicine curve was shifted a time-frame which approximated the time difference between ^{3}H-thymidine assays. Futhermore, the shape of the curve generated by the addition of colchicine remained more or less the same regardless of the time of assay. It is evident that there exists a lag time , in the range of 20-30 hours, between the addition of the alkaloid and manifestation of inhibition of ^{3}H-thymidine uptake. It would appear that the similarity between the aMM and colchicine curves when the assay took place at 48 hours was a factitious consequence of the lag time required for colchicine to become inhibitory. Finally,

it is also clear that colchicine could inhibit lymphocyte transformation well after the time of commitment, provided that sufficient time be allowed to elapse for inhibition to become manifest.

Figure 14 shows the same experiment done on mouse spleen lymphocytes after 48 hours of culture. In this case, there was an even greater difference between the curves generated by the addition of the sugar and colchicine. Also, as seen in Figure 15, the discrepancy between the aMM curve and the colchicine curve increased when ^{3}H-thymidine incorporation was assessed at 72 hour, as in the case of human lymphocytes. At both times of assay the lag time required for the alkaloid to exert fully its inhibition was about 15 hours.

Figure 11: Effect of Adding aMM and Colchicine At Various Times on Stimulated Human Leukocytes At 48h

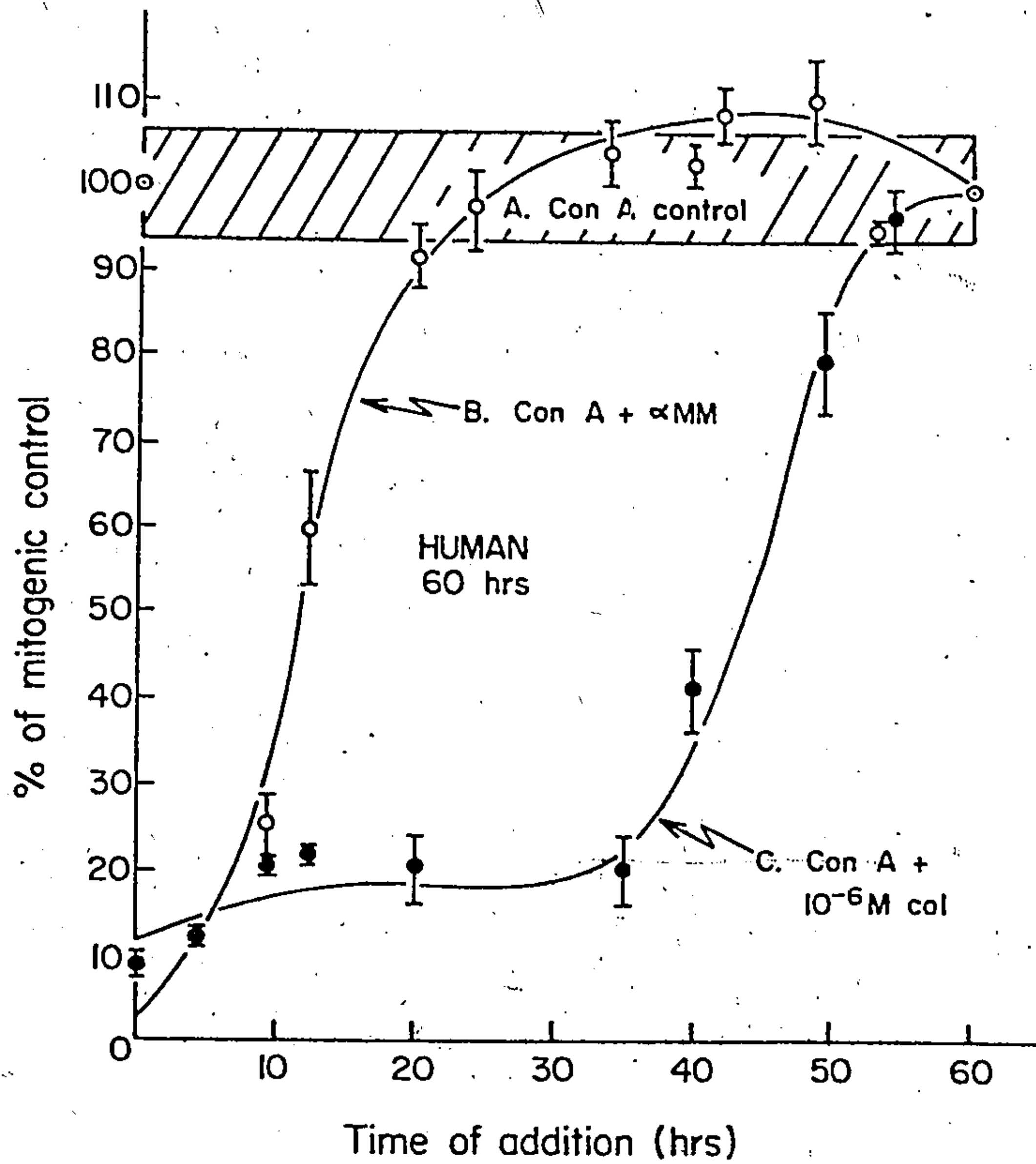
The sugar, AMM and colchicine at final concentrations of 0.1M and 10^{-6}M respectively were added at various times following the addition of Con A. The incorporation of ^{3}H-thymidine was assayed at 48 hour of culture. Con A control: $1.75-2.25 \times 10^{5}$ cpm; resting control: $2.0-3.0 \times 10^{3}$ cpm.



110
100
90
80
70
60
50
40
30
20
10
0
10
20
30
40
50
A. Con A control
B. Con A + ∝ MM
C. Con A + 10⁻⁶ M col
HUMAN
48 hrs
% of mitogenic control
Time of addition (hrs)

Figure 12: Effect of Adding aMM and Colchicine at Various Times on Stimulated Human Leukocytes at 60h

The sugar, aMM at 0.1M and colchicine at 10^{-6}M were added at various times following the addition of Con A. The incorporation of ^{3}H-thymidine was assayed at 60 hours of culture. Con A control: 1.5-1.75 X 10^3 cpm; resting control: 2.0-3.0 X 10^3 cpm.



% of mitogenic control
A. Con A control
B. Con A + ∝MM
HUMAN
60 hrs
C. Con A +
10⁻⁶M col
Time of addition (hrs)

Figure 13: Effect of Adding aMM and Colchicine at Various Times on Stimulated Human Leukocytes at 72h

The sugar, aMM and colchicine at final concentrations of 0.1M and 10^{-6}M respectively were added at various times following the addition of Con A. The incorporation of ^{3}H-thymidine was assayed at 72 hours of culture. Con A control:1.50-1.75 X 10^{5} cpm; resting control:1.50-3.00 X 10^{3} cpm.

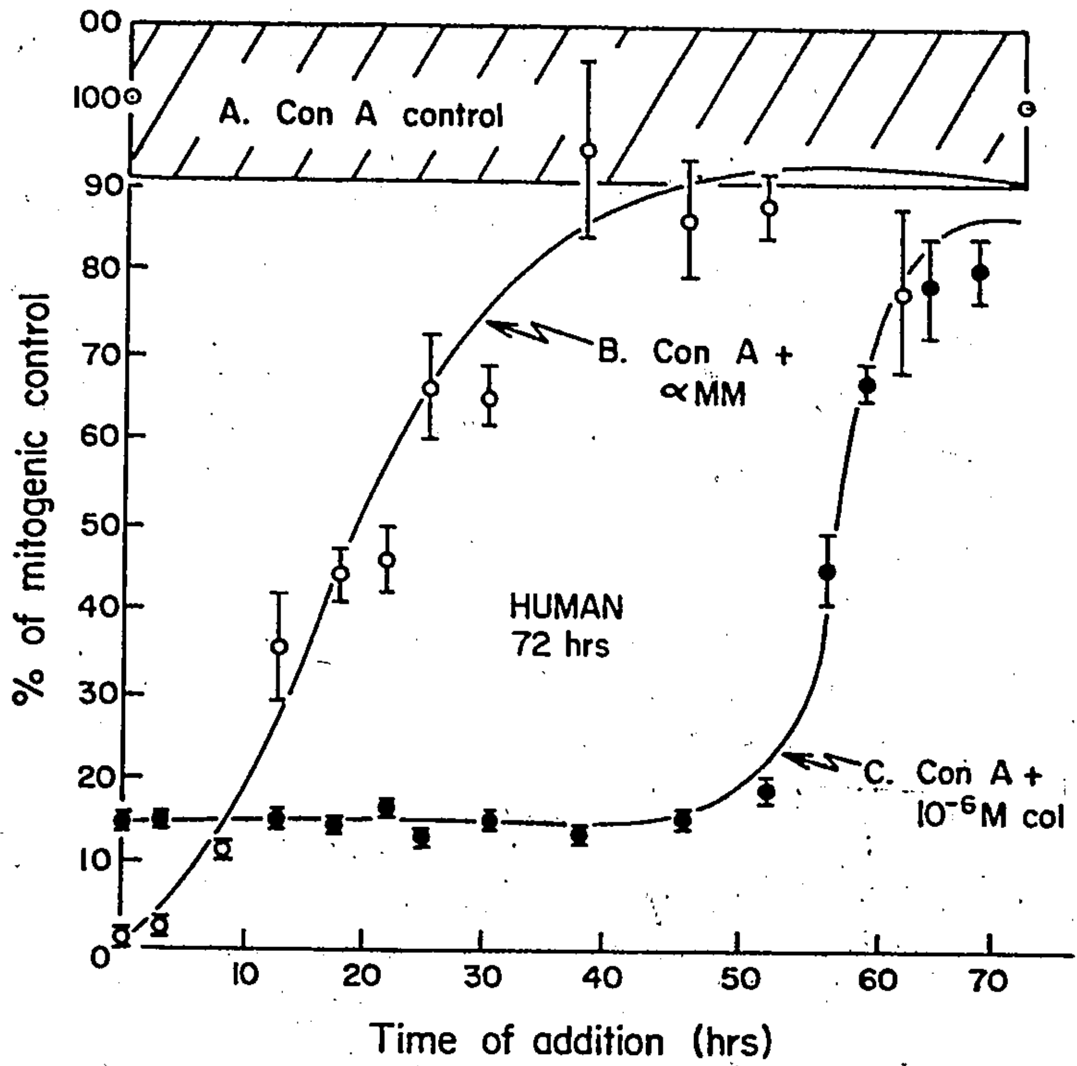
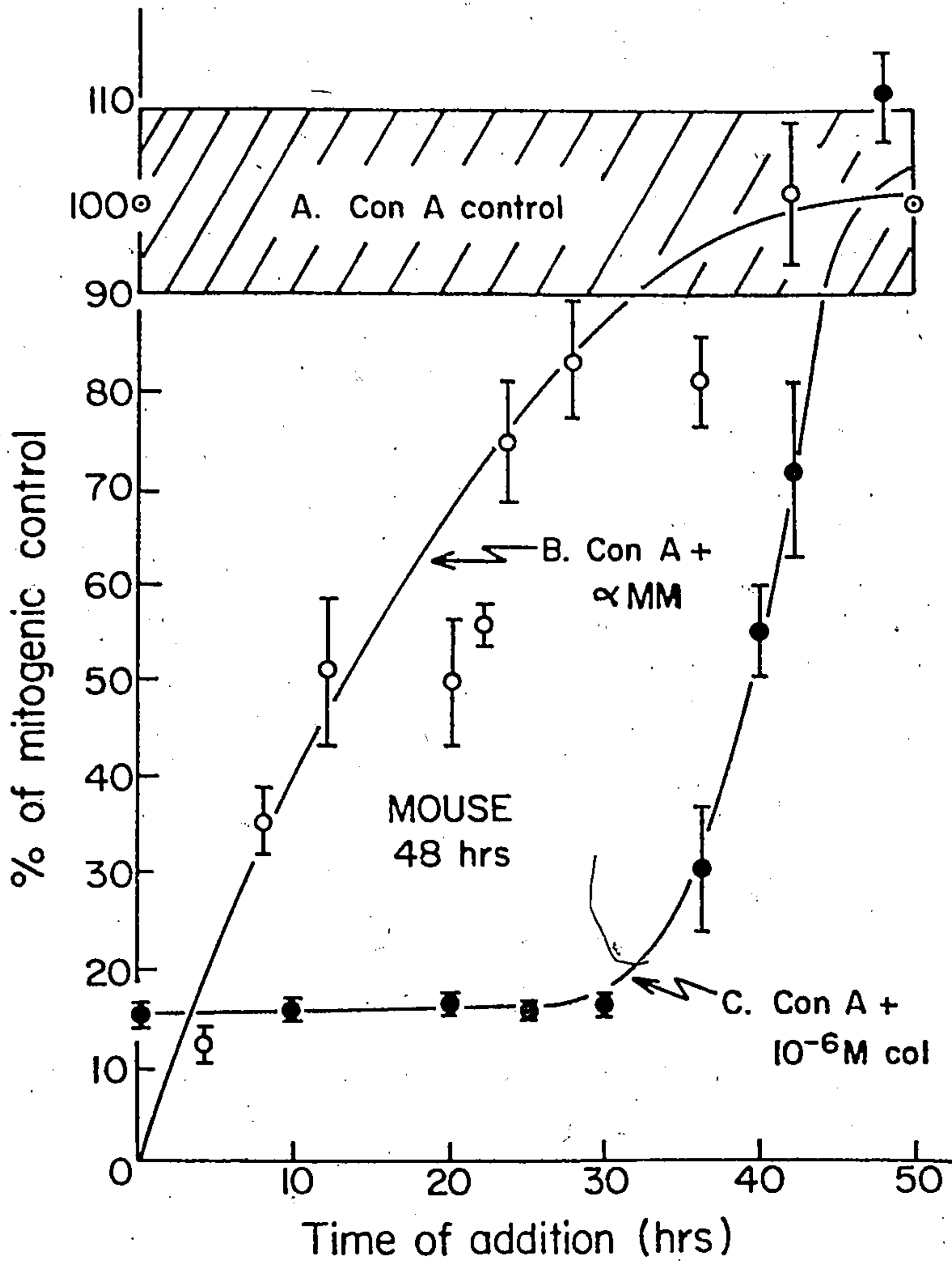

A. Con A control
B. Con A + ∝MM
C. Con A + 10⁻⁶M col
HUMAN
72 hrs
% of mitogenic control
Time of addition (hrs)
00
100
90
80
70
60
50
40
30
20
10
0
10
20
30
40
50
60
70

Figure 14: Effect of Adding αMM and Colchicine at Various Times on Stimulated Splenocytes at 48h

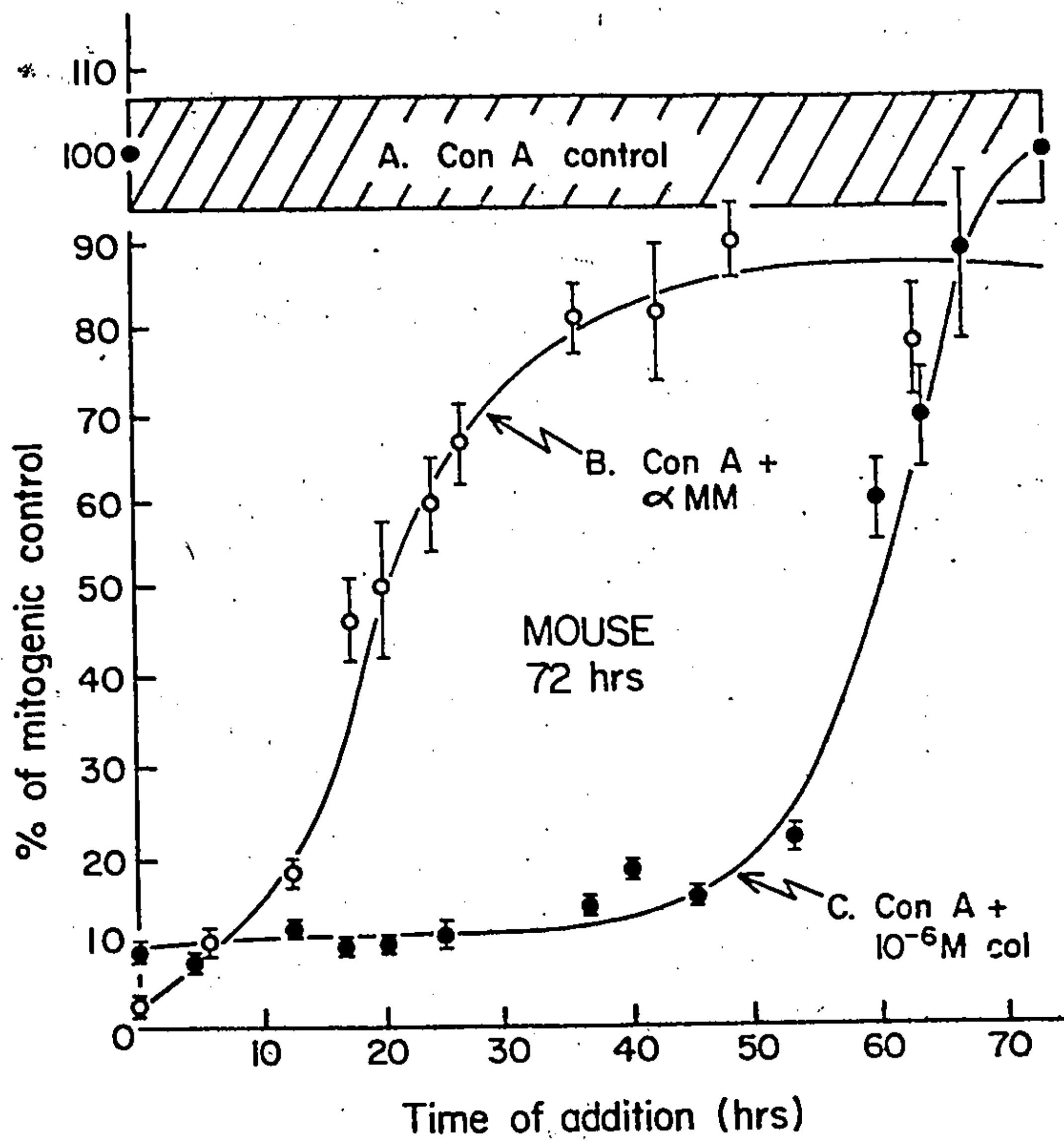
The sugar, αMM and colchicine at final concentrations of 0.1M and 10^{-4}M respectively were added at various times after the addition of Con A. The incorporation of ^{3}H-thymidine was assayed at 48 hour of culture. Con A control:1.25-1.75 X 10^5 cpm;resting control:2.0-3.0 X 10^3 cpm.



110
100
90
80
70
60
50
40
30
20
10
0
10
20
30
40
50
A. Con A control
B. Con A +
∝MM
MOUSE
48 hrs
C. Con A +
10⁻⁶M col
% of mitogenic control
Time of addition (hrs)

Figure 15: Effect of Adding aMM and Colchicine at Various Times on Stimulated Mouse Splenocytes at 72h

The sugar, aMM and colchicine at final concentrations of 0.1M and 10^{-6}M respectively were added at various times after Con A addition. The incorporation of ^{3}H-thymidine was assayed at 72 hour of culture. Con A control: 1.50-2.00 X 10^{5} cpm; resting control: 1.50-2.00 X 10^{3} cpm.


110
100
A. Con A control
90
80
70
60
50
40
30
20
10
0
% of mitogenic control
B. Con A + α MM
MOUSE
72 hrs
C. Con A + 10^{-6}M col
10
20
30
40
50
60
70
Time of addition (hrs)

5.5 TIME- AND CONCENTRATION-DEPENDENCE OF COLCHICINE INHIBITION

The discrepancy between the curves generated by the addition of aMM and colchicine could be a consequence of the concentrations of the agents used. In order to investigate this possibility, different concentrations of each agent were added at various times of culture and ^3H-thymidine incorporation assayed at 60 hour. Figure 16 shows that at $10^{-6}M$, colchicine required some 6-8 hours to cause any detectable inhibition of ^3H-thymidine uptake. An additional 20 to 30 hours was necessary for the alkaloid to cause maximal inhibition. At $10^{-7}M$ colchicine, some 10 to 15 hours were required for first detectable inhibition and 30 hours for maximal inhibition. An extension of this graph is seen in Figure 17. At 5.0 X $10^{-8}M$ colchicine, maximal inhibition was apparent only after 30-40 hour of incubation while at 2.5 X $10^{-8}M$ colchicine a 50-60 hour incubation was necessary. Due to the sharp concentration dependence in the range of 10^{-8} to $10^{-7}M$, there was some variation in the time course of inhibition from one experiment to the next. Nonetheless, the curve produced by the addition of 2.5 X $10^{-8}M$ was seen frequently to lie to the left of the commitment curve on the same time axis. Finally, the final level of maximal inhibition was the same at all colchicine concentrations used, 70-90 percent of stimulated control.

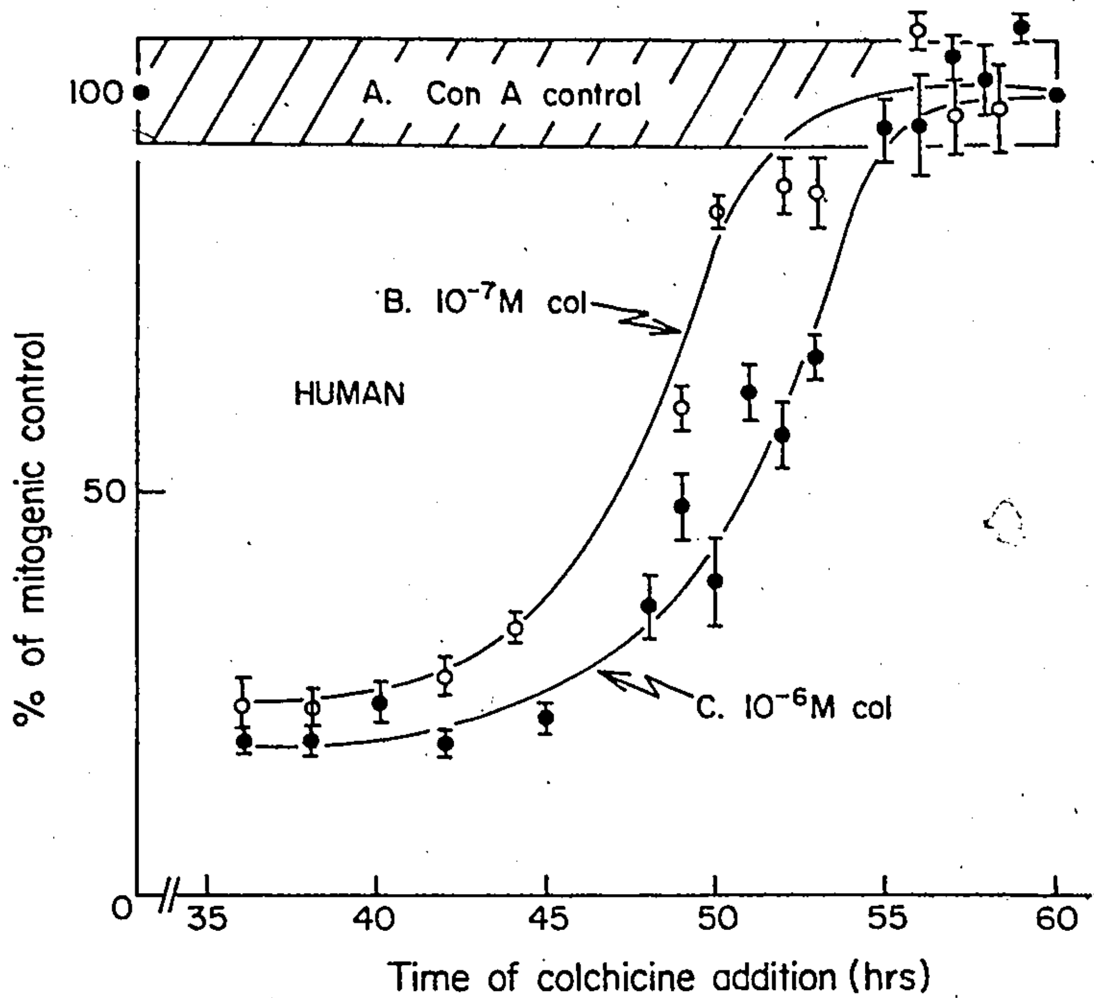
Figure 18 shows that the addition of aMM at concentrations of 0.05 to 0.1M produced the same curve. However, below these concentrations at 0.02M the sugar appeared to have little if any effect on the subsequent uptake of ^{3}H-thymidine. On the other hand, at 0.2M aMM the sugar appeared to have a general suppressive effect on ^{3}H-thymidine uptake, by about 50 percent.

5.6 REVERSIBILITY OF STIMULATED LYMPHOCYTES TO COLCHICINE

Figure 19 shows that Con A-stimulated human peripheral lymphocytes recovered fully from an 8 or 24 hour exposure to colchicine at 10^{-6}M within 100 hours of resuspension in colchicine-free medium.Exposure times of 8 and 24 hours were selected because they corresponded to the period necessary for the alkaloid to cause any detectable inhibition and maximal inhibition,respectively. This findings show that human cells were neither permanently damaged nor killed by the alkaloid. In the case of mouse splenocytes,recovery was usually only partial(data not shown). It was for this reason that human cells were used in subsequent studies.

Figure 16: Time-Dependent Inhibition by 10^{-6} to $10^{-7}M$ Colchicine on Stimulated Human Leukocytes at 60h

Colchicine at concentrations 10^{-6} to $10^{-7}M$ was added at various times after Con A addition to human peripheral leukocyte cultures. The incorporation of ^{3}H-thymidine (2uCi/mL/2h) was assayed after 60 hours of culture. Con A control: 1.5-2.0 X 10^{5} cpm;resting control:2.0-3.0 X 10^{3} cpm.



A. Con A control
100
50
0
B. 10^{-7}M col
C. 10^{-6}M col
HUMAN
% of mitogenic control
35
40
45
50
55
60
Time of colchicine addition (hrs)

Figure 17: Time-Dependent Inhibition by 10^{-7} to $2.5x10^{-6}M$ Colchicine on Stimulated Human Leukocytes at 60h

Colchicine at concentrations between $10^{-7}M$ and $2.5 \times 10^{-6}M$ was added at various times to Con A (20ug/mL) stimulated human peripheral lymphocyte cultures. The incorporation of ^{3}H-thymidine (2uCi/mL/2h) was assayed after 60 hours of incub ation. Con A control:1.5-2.0 X 10^{5} cpm;resting control:2.0-3.0 X 10^{3} cpm.

B. Con A + 0.02 M αMM

A. Con A control

C. Con A + 0.05 M αMM

D. Con A + 0.1 M αMM

E. Con A + 0.2 M αMM

F. Resting cells

HUMAN
60 hrs

% of mitogenic control

0
25
50
75
100

10 20 30 40 50 60

Time of addition (hrs)

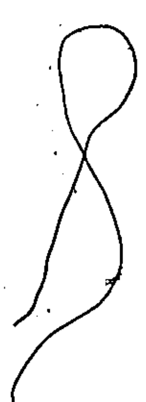


Figure 18: Effect of Adding Different Concentrations of αMM on Stimulated Human Lymphocytes at 60h

The sugar, αMM at concentrations between 0.02 to 0.2M was added at various times to Con A (20ug/mL) stimulated human peripheral lymphocytes. The incorporation of ^{3}H-thymidine (2uCi/mL/2h) was assayed after 60 hours incubation. Con A control: $1.5-2.0 \times 10^{5}$ cpm; resting control: $2.0-3.0 \times 10^{3}$ cpm.

2

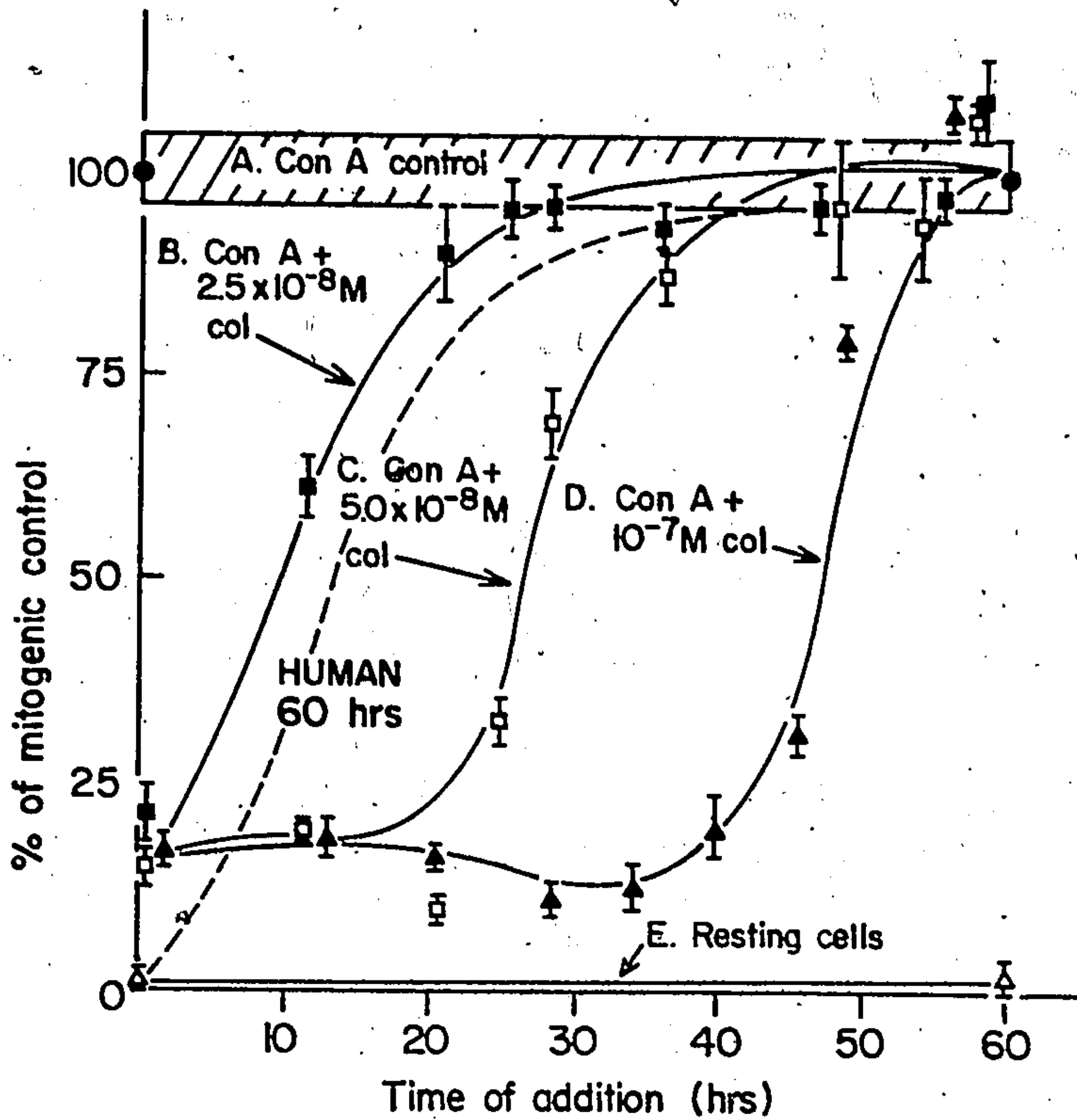
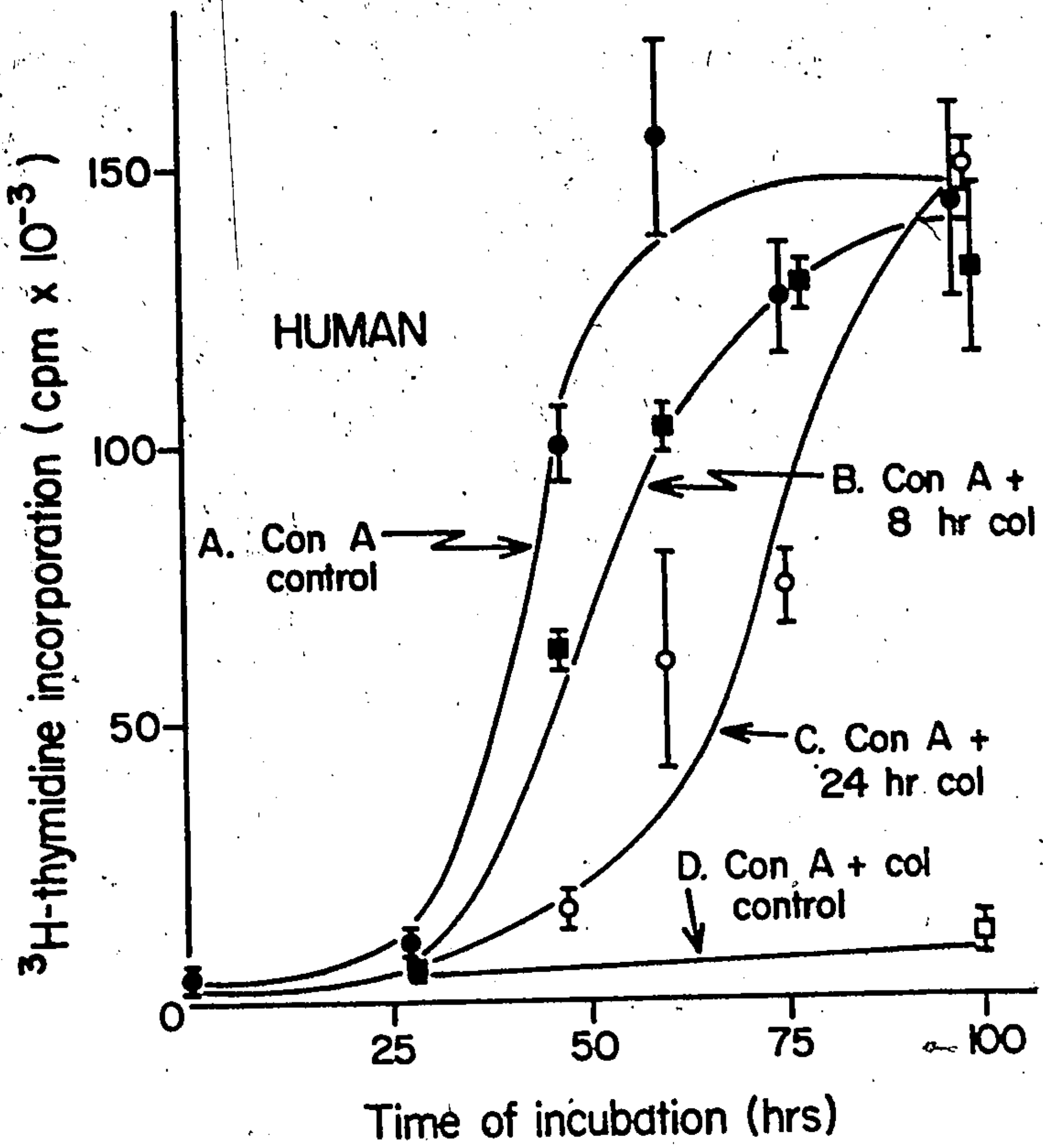


Figure 19: Reversibility of Con A-Stimulated Human Lymphocytes to Colchicine

Colchicine at 10^{-4}M was added to Con A-stimulated human lymphocytes from the start of culture for 8 or 24 hours. Cells were washed 3X in prewarmed medium and resuspended in colchicine-free medium with Con A. The incorporation of ^{3}H-thymidine was assayed at 24 to 120 hours of culture. Resting control: 1.5-3.0 X 10^{3} cpm.



HUMAN
³H-thymidine incorporation (cpm x 10⁻³)
150
100
50
0
25
50
75
100
Time of incubation (hrs)
A. Con A control
B. Con A + 8 hr col
C. Con A + 24 hr col
D. Con A + col control

5.7 COLCHICINE AND LUMICOLCHICINE EFFECTS ON MICROTUBULES IN HUMAN LEUKOCYTES

It was essential to determine whether the effect of colchicine was exerted via disassembly of microtubules. For this reason, the effect of colchicine and the photo-inactivated derivative, lumicolchicine on immunofluorescence-stained microtubule networks in Con A-stimulated human leukocytes was examined (Osborn and Weber, 1975,1976; Rodgers and Brown,1979). Table 3 illustrates the effect of colchicine and lumicolchicine when present from the start of culture on human leukocytes after a 12 hour incubation. Later time points were avoided because of the difficulty in visualising microtubule networks in stimulated cells later in culture(Rodgers and Brown,1979;Rudd et al,1979). In the control samples, it is evident that 70 to 80 percent of the cells in the population had stained tubulin filaments. After a 12 hour exposure to 10^{-4}M lumicolchicine, it is apparent that the percent of cells with stained networks remained the same. In contrast,only 20 percent of the cells which had been treated with 10^{-4} to 10^{-6}M colchicine had positively stained filaments after 12 hours. This suggests that the lumicolchicine preparation had little if any anti-microtubule activity. However, the possibility exists that residual colchicine at very low concentrations may be present in the lumicolchicine preparations.

Figure 20 shows the relative effects of different concentrations of colchicine and lumicolchicine on ^3H-thymidine incorporation into stimulated human lymphocytes after 48 hours of incubation. While colchicine caused 50 percent inhibition of incorporation at $5.0 \times 10^{-8}M$, lumicolchicine required a concentration of $5.0 \times 10^{-4}M$ to cause equivalent inhibition. In other words, there were some 4 orders of magnitude difference in the ability of the alkaloids to cause inhibition of thymidine incorporation. Again it is possible that the inhibitory effect of lumicolchicine was due to the presence of residual quantities of colchicine;it is also possible that very high concentrations of lumicolchicine have an inhibitory effect. In any case, these data are consistent with the hypothesis that colchicine inhibits DNA synthesis of Con A-stimulated lymphocytes by virtue directly or indirectly of its effect on microtubule assembly.

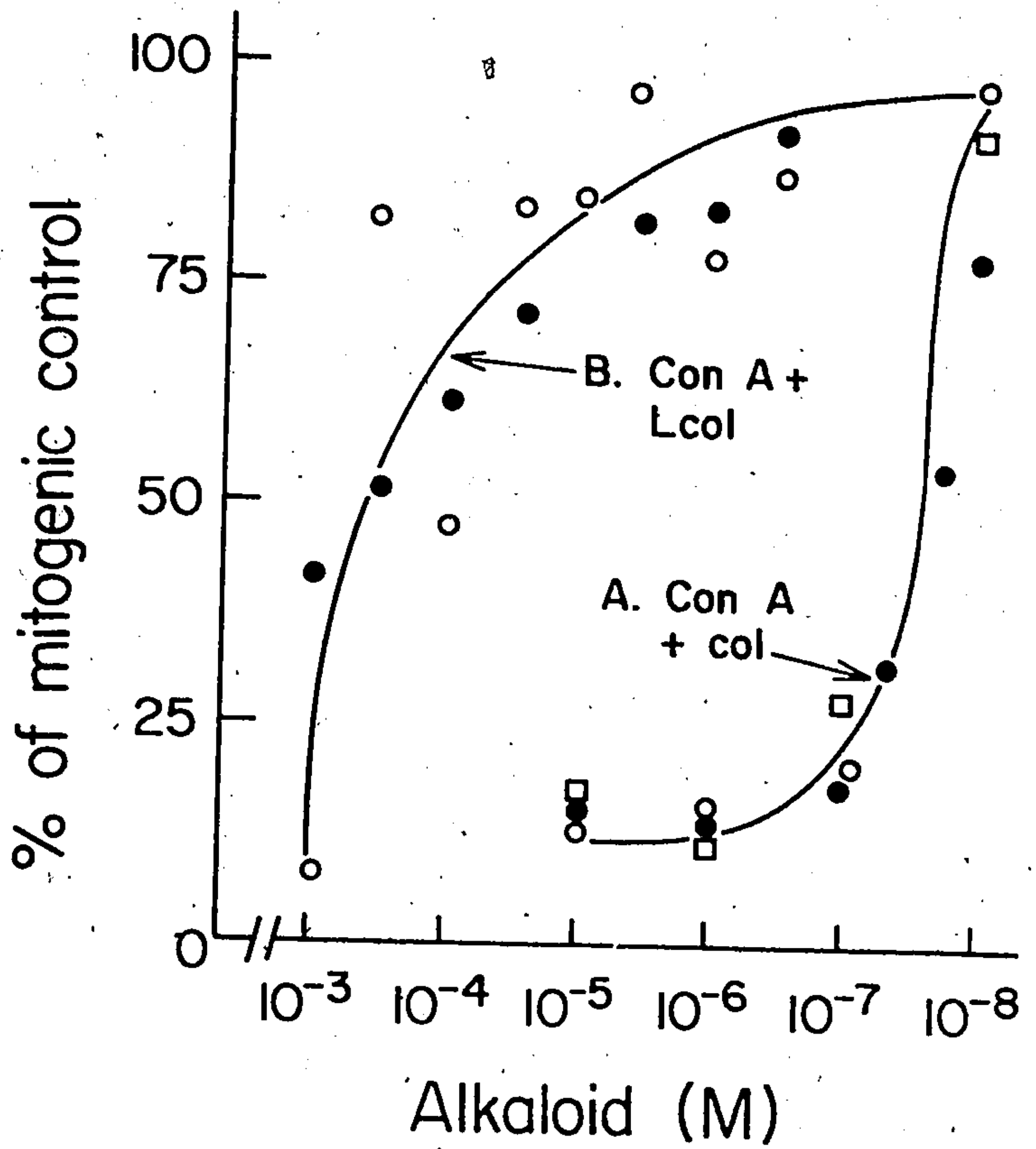
TABLE 3: Effect of Colchicine and Lumicolchicine on microtubule Networks in Con A-Stimulated Human Peripheral Lymphocytes

	% Positive	% Negative*
Con A Control	70 ± 3	30
Colchicine (10^{-4}M)	11 ± 1	89
Colchicine (10^{-6}M)	12 ± 2	88
Lumicolchicine (10^{-4}M)	62 ± 6	38

* Includes ≤ 2 stained filaments

Figure 20: Effect of Concentrations of Lumicolchicine and Colchicine on Stimulated Human Leukocytes at 48h

Lumicolchicine and colchicine at concentrations between 10^{-3} to 10^{-8}M were added at the start of culture to Con A-stimulated human peripheral leukocyte cultures. The incorporation of ^{3}H-thymidine (2uCi/mL/2h) was assayed at 48 hour of culture. Con A control: 1.50-2.00 X 10^{5} cpm;resting control:2.00-3.00 X 10^{3} cpm.


100
75
50
25
0
% of mitogenic control
B. Con A +
Lcol
A. Con A
+ col
10^{-3}
10^{-4}
10^{-5}
10^{-6}
10^{-7}
10^{-8}
Alkaloid (M)

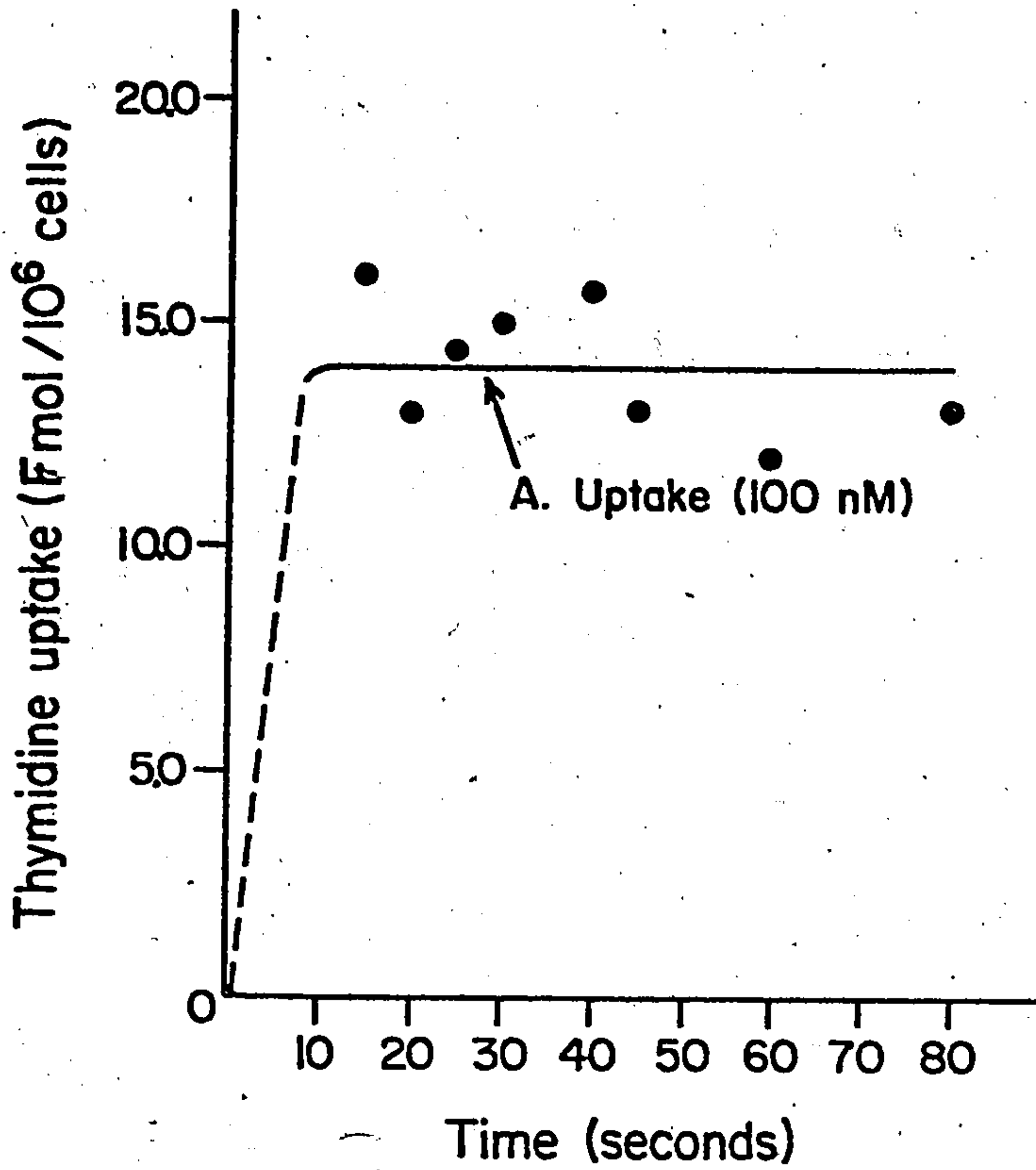
5.8 THYMIDINE UPTAKE AND TRANSPORT IN LEUKOCYTE CULTURES

5.9 TIME-DEPENDENCE OF THYMIDINE UPTAKE

In view of the reports suggesting an alternative target for the action of colchicine, it was necessary to investigate the effect of colchicine and lumicolchicine on thymidine transport. To due this, it was important first to demonstrate the existence of a transport component in human lymphocytes cultured *in vitro* with or without Con A. As seen in Figure 21, time-dependent thymidine uptake could not be demonstrated in resting human leukocyte cultures for a period as long as 80 seconds. In contrast, human leukocytes after a 48 hour, incubation with Con A showed a marked increase in thymidine uptake as a function of time. As shown in Curves A and B of Figure 22, the uptake and transport of thymidine at a final external concentration of 100nM increased in a linear fashion for about 45 seconds. In this and other experiments, the increased uptake continued to be linear for approximately 80 to 100 seconds after which radiolabel associated with the cell pellet began to level off. The plateau corresponds to the time at which equlibrium of thymidine across the plasma membrane has been attained. The linear phase, during which the data points extrapolate to the origin (Curve B) represent the time during which the translocation of substrate is unidirectional and

Figure 21: Thymidine Uptake in Resting Human Leukocytes

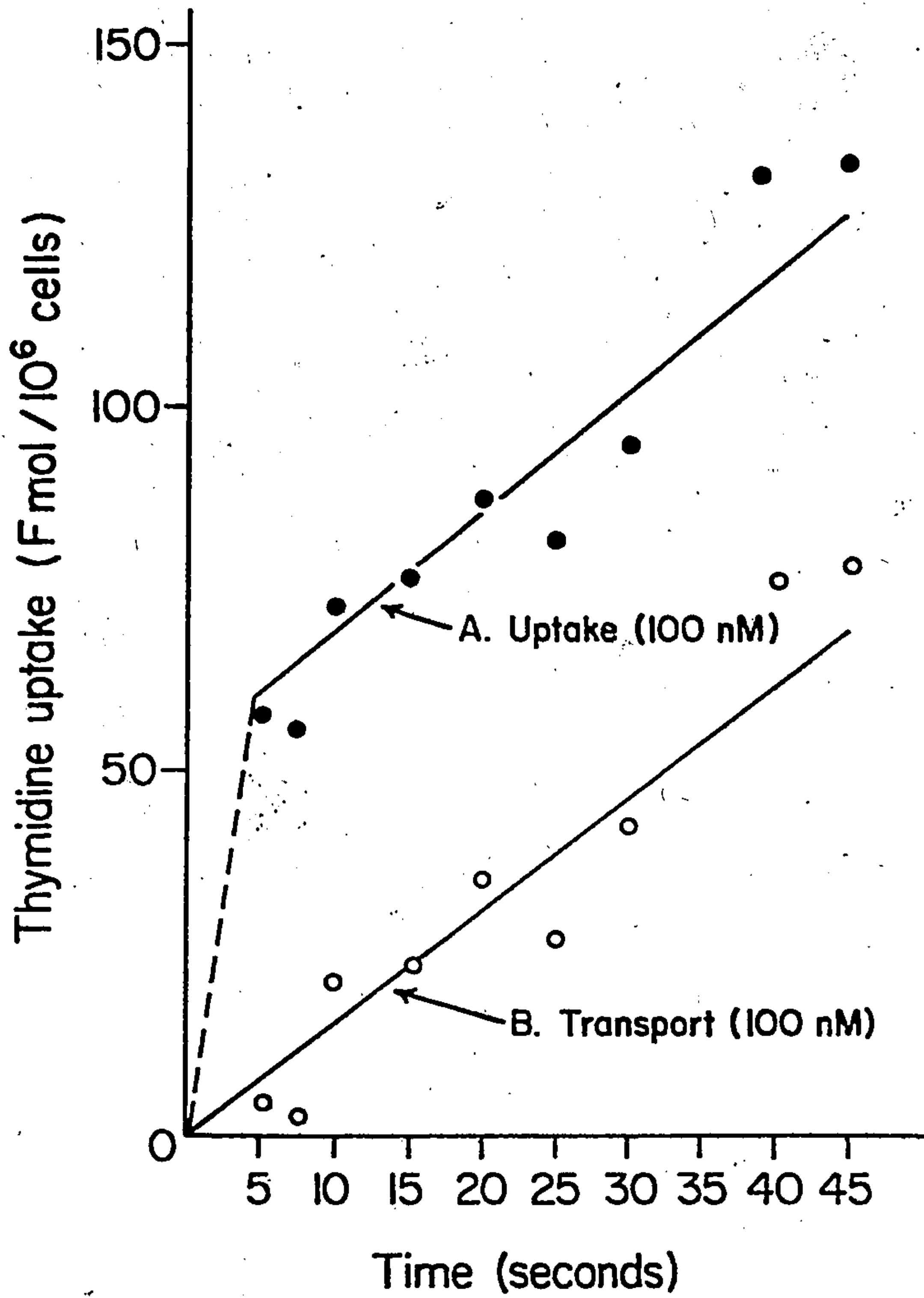
Resting human leukocytes at a cell concentration of 10.0 X 10^6 cells/mL in 250uL LMGB was mixed for different time periods with an equal volume of LMGB with 2.0 X 10^{-7}M thymidine (Final Radioactivity: 15 uCi/mL). A 200uL aliquot was then centrifuged using the microcentrifugation technique as outlined in Materials and Methods



20.0
15.0
10.0
5.0
0
Thymidine uptake (Fmol / 10⁶ cells)
A. Uptake (100 nM)
10
20
30
40
50
60
70
80
Time (seconds)

Figure 22: Time-Dependent Thymidine Uptake and Transport in Con A-Stimulated Human Leukocytes

Con A-stimulated human peripheral leukocytes after 48 hours of culture were suspended in pre-warmed LMGB at a cell concentration of 10.0 X 10^{6} cells/mL. The cell suspension at a volume of 250uL was then mixed for different periods of time with an equal volume of LMGB containing 2.0 X 10^{-7}M thymidine(Final Radioactivity:20uCi/mL).A 200ul aliquot was then centrifuged using the microcentrifugation technique as outlined in the Materials and Methods.


150
100
50
0
Thymidine uptake (F mol / 10^6 cells)
A. Uptake (100 nM)
B. Transport (100 nM)
5
10
15
20
25
30
35
40
45
Time (seconds)

uncomplicated by backflux (Berlin and Oliver, 1975). Similar time-dependency was also demonstrated at external concentrations as high as 10^{-3}M. All subsequent experiments utilised a 15 to 20 second time point in order to be sure that no backflux of the substrate was occuring.

5.10 SATURATION OF THYMIDINE UPTAKE

A kinetic component of thymidine uptake in Con A-stimulated human leukocytes was found to be saturable and this component was defined as transport. Figure 23 shows that in medium containing a constant amount of isotope, the presence of increasing concentrations of unlabeled thymidine reduced the amount of radioactive thymidine associated with the pellet until a concentration of 1.0mM was reached. Above this concentration, no further decrease in radiolabel with the pellet was observed. This non-diluted residual radioactivity was taken to represent contaminating label either due to diffusion, adsorption to the cell surface and/or in the extracellular space. It was subtracted from the total pellet counts at lower external thymidine concentrations to give values due to transport alone (Curve B, Figure 22).

5.11 KINETICS OF THYMIDINE TRANSPORT

After the subtraction of the non-specific cell associated label, a double reciprocal plot of the data yielded a normal Michaelis Menton curve (Figure 24). Data was fitted to an equation derived from the method of least squares. Contrary to the results of Strauss and coworkers (1976,1979), the correlation coefficient was maximized when a single line was determined for all points. Thus a single Km value was estimated from the asymptotic curve and from the Linweaver-Burk plots which ranged from 200 to 500uM, varying with the experiment and presumably, the donor. Vmax values ranged from 5 to 8 pmoles/ cell/ second.

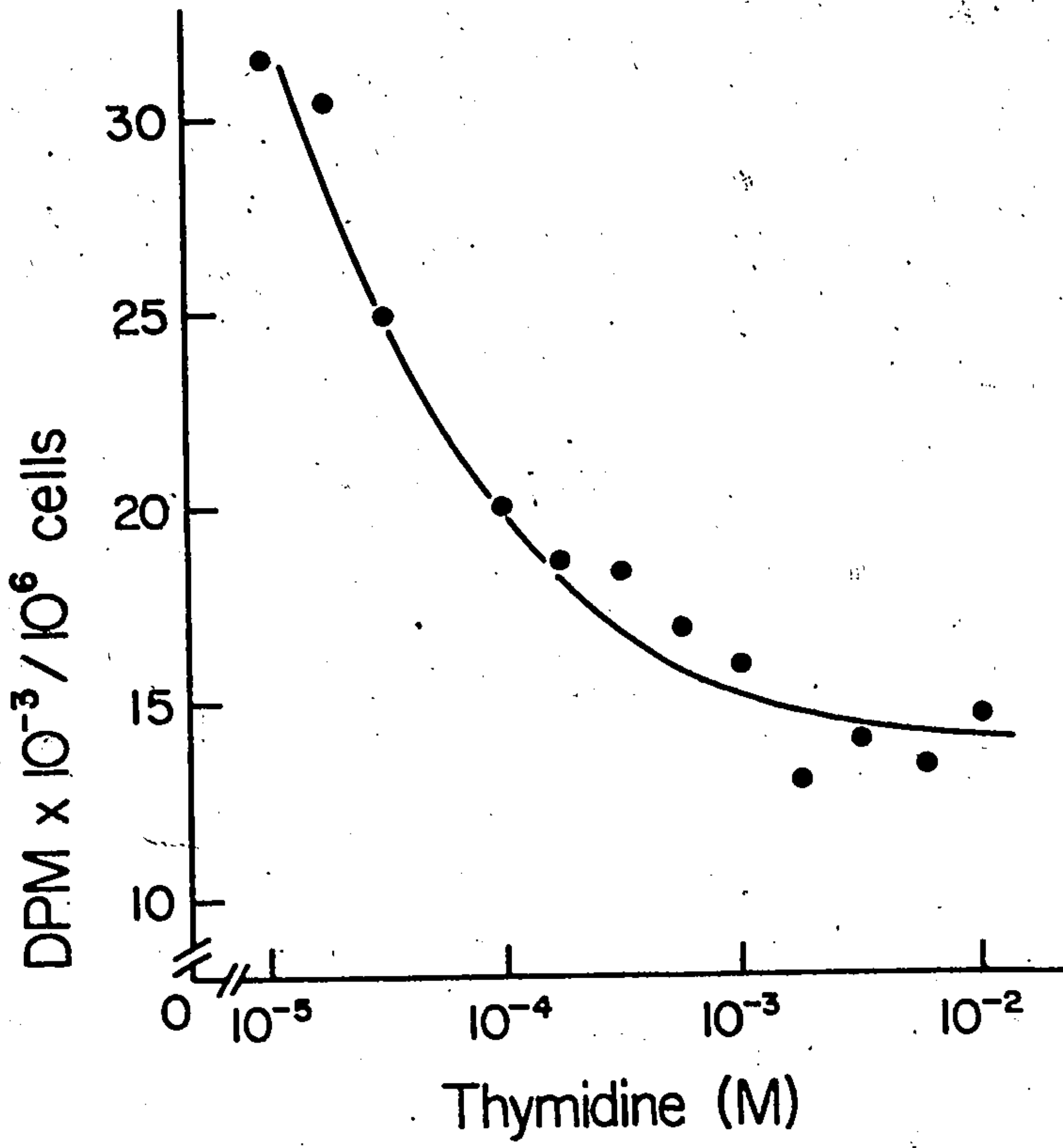
5.12 LUMICOLCHICINE AND COLCHICINE EFFECTS ON THYMIDINE TRANSPORT

Figure 25 shows that colchicine when present over the 15 second transport interval caused significant inhibition of thymidine transport in stimulated human leukocyte cultures. The alkaloid reduced transport by 50 percent at a concentration of 5.0×10^{-5}M. Futhermore, Figure 26 shows that lumicolchicine caused the same degree of inhibition as observed from the parent compound. In contrast, as previously shown in Figure 20, colchicine caused a 50 percent inhibition of ^{3}H-thymidine incorporation at 5.0×10^{-8}M whereas lumicolchicine did so at about 5.0×10^{-3}M.This is consistent with the hypothesis that

lumicolchicine inhibited ^{3}H-thymidine uptake as a result of its effect on transport.

Figure 23: Saturation of Thymidine Uptake in Con A-Stimulated Human Leukocytes

Con A-stimulated human peripheral leukocytes after 48 hours of culture were suspended in prewarmed LMGB at a concentration of 10.0×10^6 cells/mL. The cell supension was then mixed with an equal volume of LMGB containing thymidine concentrations between 10^{-7} to 5.0×10^{-2}M. The final specific activity of radioactive thymidine remained constant at 20.0uCi/mL. A 200ul aliquot of reaction mixture was then centrifuged using the microcentrifugation technique as outlined in the _Materials and Methods_.



30
25
20
15
10
0
10^{-5}
10^{-4}
10^{-3}
10^{-2}
DPM x 10^{-3} / 10^{6} cells
Thymidine (M)

Figure 24: Kinetics of Thymidine Transport in Con A-Stimulated Human Leukocytes

Con A-stimulated human peripheral leukocytes after 48 hours of culture were suspended in prewarmed LMGB at a cell concentration of 10.0 X 10^6 cells/mL. A volume of 250uL of suspension was then mixed with an equal volume of LMGB containing concentrations of thymidine between 10^{-2} to 10^{-5}uM. The final specific activity of radioactive thymidine remained constant at 20uCi/mL. A 200uL aliquot of re action mixture was then centrifuged using the microcentrifugation technique outlined in the <u>Materials</u> <u>and</u> <u>Methods</u>. The dotted lines represent separate experiments for which the plotted points are not shown.

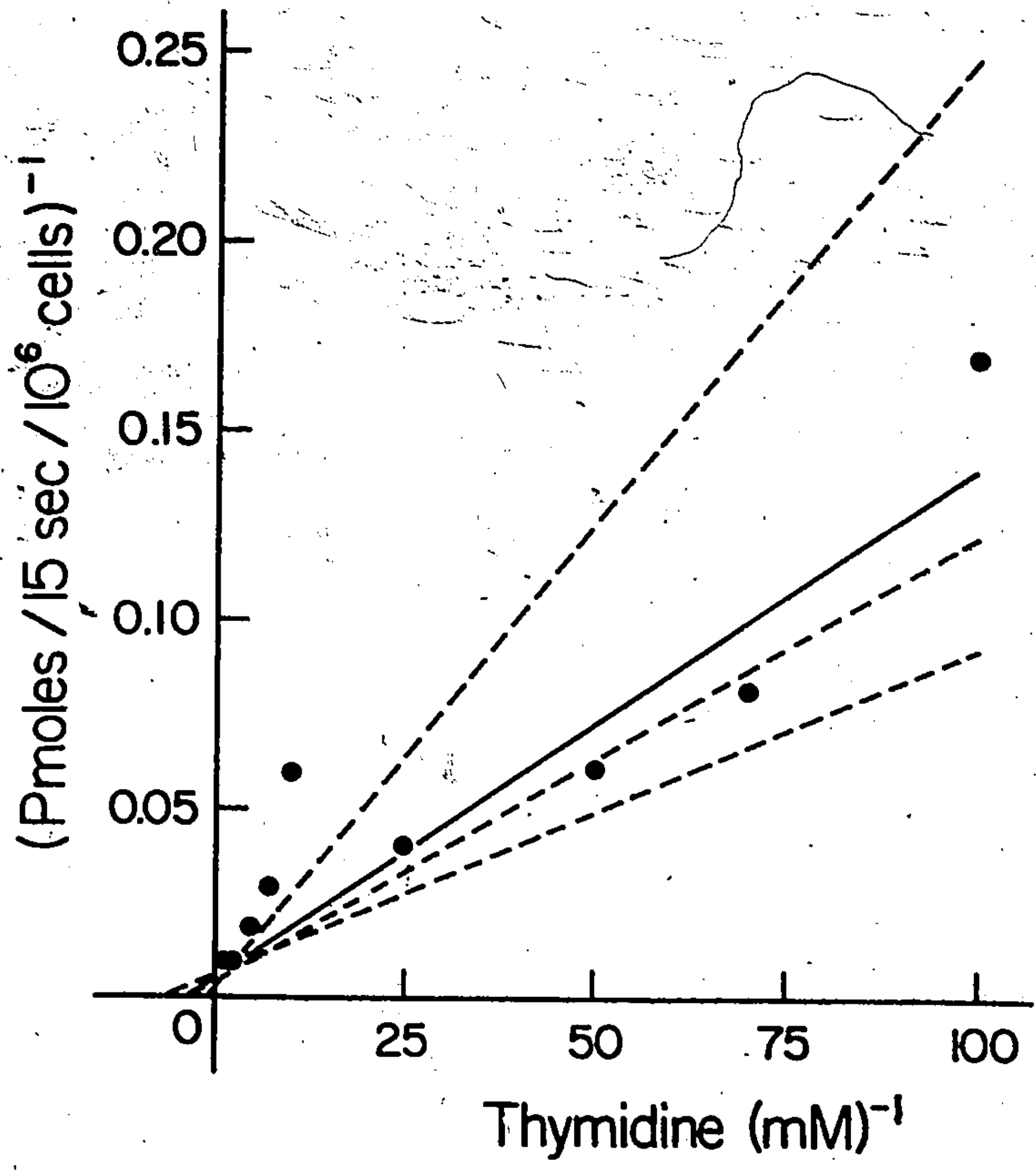

(Pmoles / 15 sec / 10^6 cells)^-1
0.25
0.20
0.15
0.10
0.05
0
25
50
75
100
Thymidine (mM)^-1

Figure 25: Effect of Colchicine on Thymidine Transport in Con A-Stimulated Leukocytes

Con A-stimulated human peripheral leukocytes after 48 hours of culture were suspended in prewarmed LMGB at a cell concentration of 10.0 X 10^{6} cells/mL. A volume of 250uL of cell suspension was then mixed with an equal volume of LMGB containing 2.0 X 10^{-7}M thymidine (Final Radioactivity: 20.0uCi/mL). Colchicine at concentrations between 10^{-3} to 5.0 X 10^{-6}M was present over the 15 second incubation period. A 200uL aliquot of reaction mixture was then centrifuged using the microcentrifugation technique outlined in the <u>Materials</u> <u>and</u> <u>Methods</u>.

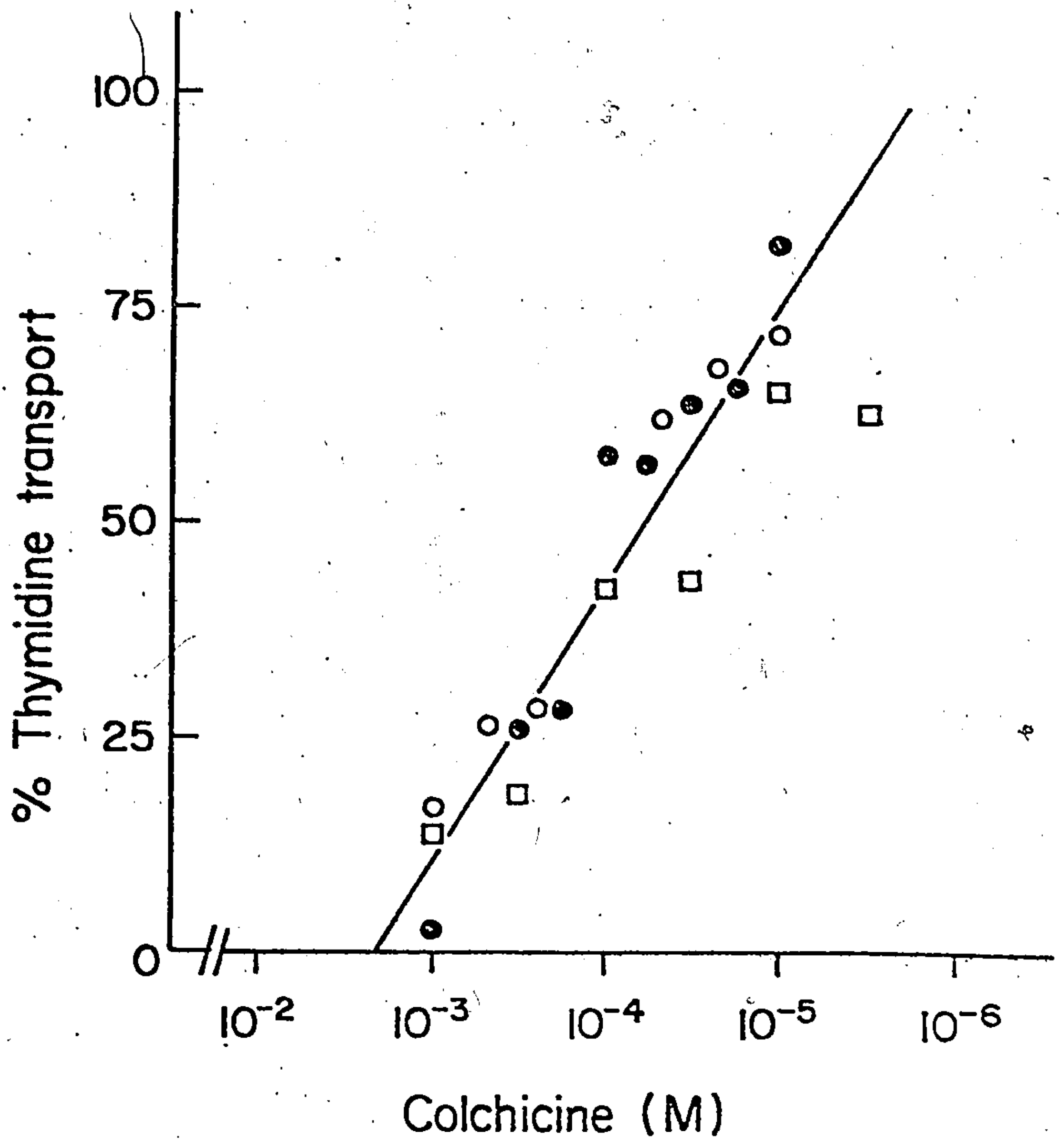
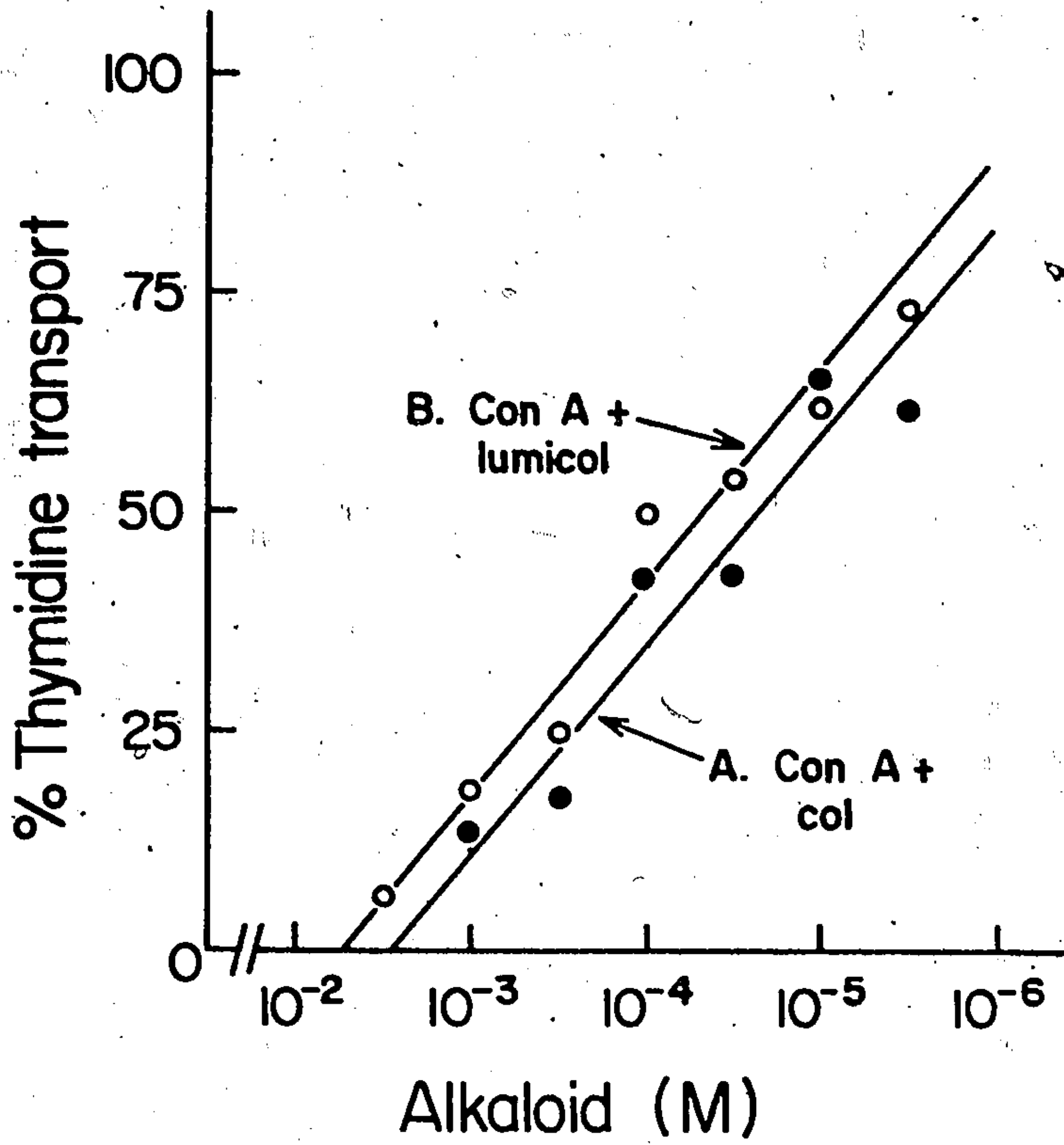

100
75
50
25
0
% Thymidine transport
10^{-2}
10^{-3}
10^{-4}
10^{-5}
10^{-6}
Colchicine (M)

Figure 26: Effect of Lumicolchicine and Colchicine on Thymidine Transport in Con A-Stimulated Human Leukocytes

Con A-stimulated human peripheral lymphocytes at 48 hours of culture were suspended in prewarmed LMGB at a cell concentration of 10.0 X 10^6 cells/mL. A volume of 250uL of suspension was then mixed with an equal volume of LMGB containing 2.0 X 10^{-7}M thymidine (Final Radioactivity: 20uCi/mL). Lumicolchicine or colchicine at concentrations between 10^3 to 10^5M was present over the 15 second incubation period. A 200ul aliquot of reaction mixture was then centrifuged using the microcentrifugation technique outlined in the _Materials and Methods_.


100
75
50
25
0
% Thymidine transport
B. Con A +
lumicol
A. Con A +
col
10^{-2}
10^{-3}
10^{-4}
10^{-5}
10^{-6}
Alkaloid (M)

Chapter VI

DISCUSSION

6.1 THE EFFECT OF COLCHICINE ON THE DIFFERENT STAGES OF LYMPHOCYTE TRANSFORMATION

The contention that inhibition of lymphocyte blastogenesis by colchicine was restricted to event(s) preceeding S phase was based primarily on the gradual loss in the ability of the alkaloid to inhibit ^{3}H-thymidine incorporation at 48 hours of culture (Medrano et al,1974; Wang et al,1975). In addition, colchicine, when added after the onset of DNA synthesis, was found to have little if any effect on the incorporation of the nucleoside (Medrano et al,1974; Wang et al,1975). My data have shown that colchicine can inhibit Con A-stimulated lymphocyte transformation before DNA replication. This has been observed in both human and mouse lymphocyte cultures where colchicine prevented the onset of DNA synthesis (Figures 7 and 8) and inhibited RNA synthesis by about 60 percent as early as 12 hours of culture (Table 1). However, it is

important to note that my findings also show that the alkaloid can inhibit lymphocyte transformation after S phase has started (Figure 11-15). In addition, the degree of inhibition of RNA synthesis over various 12 hour intervals prior to and during S phase was more or less the same (Table 2). This is consistent with the concept that inhibition by colchicine occurs by the same mechanism, irrespective of the stage of lymphocyte transformation.

My findings also indicate that the action of colchicine is both time-and concentration-dependent. In fact, it is this observation which may explain the discrepancy between my findings and those which were taken to indicate that inhibition occurs only prior to S phase (Medrano et al,1974; Wang et al,1975). In the experiments of Medrano et al (1974), the longest incubation of cells in S phase with colchicine was 3 hours, a period clearly too brief to cause a detectable reduction of ^{3}H-thymidine incorporation. Wang et al (1975) found that ^{3}H-thymidine incorporation into Con A-stimulated lymphocytes fell within 16 hours of a hydroxyurea-induced G1/S block in both colchicine-treated and -untreated cultures. The authors interpreted this to indicate that colchicine had no effect on DNA synthesis. However, my data indicate that inhibition would have been detectable if sufficient time had been allowed to elapse before the assay of DNA synthesis.

From the data of Wang et al (1975), one could extrapolate that 10^{-6}M colchicine required some 10-15 hours before inhibition was detectable. My findings have shown inhibition by colchicine to be evident within 6-8 hours of incubation; however, it is possible that this latent period could vary with differerent culture conditions. For example, colchicine has been reported to bind to components of serum (Wilson,1972;Trnavska et al,1979). Both Medrano et al (1974) and Wang et al (1975) used 20 percent foetal calf serum, in contrast to 10 percent autologous serum used in my cultures. By binding colchicine, serum could act to modulate the amount of colchicine available to enter the cell.

Colchicine has also been shown to augment the response of lymphocytes to periodate (Stenzel et al,1978) and to allogeneic cells (Suthanthiron et al, 1980). A one-half hour pretreatment of cells with 10^{-6}M colchicine increased the DNA synthetic response by 250 percent. However, when the same cells were stimulated with Con A or PHA, the colchicine pretreatment actually inhibited ^{3}H-thymidine incorporation. Unfortunately, in the former study , the kinetics of DNA synthesis were not followed, which makes it difficult to determine whether the effect of colchicine was to actually inhibit S phase or to alter the kinetics of the response, that is, to synchronise the population so that

more cells happened to be in S phase relative to control cultures at the single time point examined. In the case of the enhanced MLR response, Suthanthiron et al (1980) found that pretreating the reponders and/ or the stimulators potentiated the MLR response and the subsequent generation of specific cytotoxic cells. In contrast, Ranney and Pincus (1976) found that a 2 hour pretreatment of stimulators with colchicine or vincristine inhibited the MLR by inhibiting the stimulator cells. Clearly, further study will be necessary to verify these observations and to determine the target of colchicine. From their study, Novogrodski and coworkers attributed the action of colchicine to an effect on microtubules. However, Rogers and Brown (1979) and Oliver and coworkers (1980) have found more than half of the lymphocyte population with microtubules after a half hour exposure to 10uM colchicine.

Human lymphocytes have also been observed to differ in their sensitivity to colchicine over 72 hours depending on the mitogen used to stimulate the population. Rasmussen and Davis (1977) found human lymphocytes, when stimulated with Con A, to be some two orders of magnitude more sensitive to colchicine than the same cells stimulated with NAGO. Interestingly, colchicine inhibited ^{3}H-thymidine incorporation into NAGO-stimulated human lymphocytes by 50 percent at a concentration of 5.0 X 10^{-5}M. This is close to

the concentration of colchicine which I have found to inhibit thymidine transport by 50 percent (Figure). Therefore, it may be that the ability of colchicine to inhibit ^{3}H-thymidine incorporation in NAGO-stimulated human cultures is due to an effect on thymidine transport, and not on the microtubule.

My data show that colchicine can inhibit Con A-induced lymphocyte blastogenesis both prior to and after the onset of DNA replication, provided that sufficient time be allowed to elapse for inhibition to become detectable. However, it is possible that human cells stimulated with NAGO may be less sensitive to colchicine than Con A-stimulated cells (Rasmussen and Davis ,1977) and that a brief colchicine pretreatment may actually augment the response in the MLR and to periodate (Suthanthiron et al,1978).

6.2 IS THE EFFECT OF COLCHICINE RELATED TO COMMITMENT?

Edelman's hypothesis that colchicine acts to block lymphocyte transformation near or at the point of cell commitment was based on what he took to be the similarity in the time course of inhibition of DNA synthesis by aMM and colchicine (Wang et al,1975; Gunther et al,1976). On the basis of this observation, microtubules were implicated as a candidate for signal-transmission (Edelman,1976). I have shown that the apparent coincidence of the curves is a

factitious consequence of an approximate 20-30 hour lag period before maximal inhibition of thymidine inhibition was observed (Figure 11,16). The later the time off assay for thymidine, (e.g. 60 and 72 hours (Figures 12 and 13), the more disparate the aMM and colchicine curves became.

In the case of mouse splenocytes, the lag period between the addition of colchicine and the manifestation of inhibition is considerably shorter than with human lymphocytes. For this reason, the aMM and colchicine curves were different even when thymidine was assayed as early as 48 hours (Figure 14). As in the case of human lymphocytes, the later the time of assay the more disparate the aMM and colchicine curves became (Figure 15). Hence, in both human and mouse lymphocyte cultures, inhibition by colchicine was evident well after the populations were fully commited (Rudd et al,1979). This is in agreement with the results of Milner (1977) who used partially synchronised mouse splenocytes and by Resch (1979) who used human cells. The principal difference between my findings and those of Resch and coworkers is that the latter claimed that early events of lymphocyte activation were unaffected by colchicine (Resch et al,1976). However, recently they have found that colchicine inhibited early RNA synthesis in human lymphocytes (Resch, personnal communication).

The effect of colchicine on lymphocyte transformation cannot be due exclusively to an effect on chromosome movement in mitosis. Colchicine has been widely used for many years to inhibit the cell cycle by blocking cells at metaphase, prior to chromsome movement at anaphase (Inoué and Sato, 1967). This effect cannot explain most of the inhibition observed in my experiments, since it was noted at times when cells would be expected to enter S phase for the first time. In human cells in culture, no second round mitosis was noted before X hours of culture in the presence of Con A (Dular, this laboratory, personn. commun.). It is also clear that the inhibitory effect of colchicine cannot be ascribed to an action on cell viability. The drug did not cause significant cytoxicity as routinely assessed by trypan blue dye exclusion. This confirms the previous findings of others (Medrano et al,1974; Wang et al,1975; Greene et al,1976).

Human peripheral lymphocytes were found to recover from the effects of an 8 or 24 hour exposure to 10-[illegible]M colchicine within 50-75 hours after resuspension in colchicine-free medium. These incubation periods were selected because they correspond to the exposure times necessary for colchicine to cause the first detectable inhibition and maximal inhibition of DNA synthesis. The ability of these cells to recover supports further the claim that the alkaloid is not

cytotoxic. The length of time required for complete recovery is consistent with the slow-decay kinetics reported for the dissociation of the colchicine-tubulin complex (Wilson et al,1974). However, the molecular mechanism by which the lymphocyte recovers from colchicine treatment is unknown.

Therefore, my data show that colchicine inhibition cannot be due solely to an effect on commitment. Otherwise, the curves generated by the addition of aMM and colchicine would be related in a manner independent of the time of the thymidine assay. However,while there is no evidence that colchicine inhibition has anything to due with commitment, my data cannot exclude such an effect. They show that colchicine can inhibit at any time of culture, at or after commitment, provided sufficient time be allowed to elapse between the addition of the drug and the assay for DNA synthesis.

6.3 COMMITMENT IN LYMPHOCYTE BLASTOGENESIS

In this thesis, commitment, the period of time during which Con A is required on the lymphocyte cell surface for the cell to enter DNA synthesis, is operationally defined by the addition of aMM to culture. This sugar has been shown to remove the lectin from the cell surface (Novogrodski and Katchalski, 1971; Stenzel et al,1978). The suspension of

cells is said to be committed when the addition of aMM causes no inhibition of DNA synthesis when this is subsequently assayed by ^{3}H-thymidine incorporation. If aMM is added to culture at various times after the addition of Con A then the population becomes increasingly refractory to the sugar until 20 hours afterwhich aMM has no effect on the subsequent incorporation of ^{3}H-thymidine. This corresponds to the appearance of an increasing number of blast cells when measured at 48-60 hours (Gunther et al, 1976). In addition, the period necessary for the complete commitment of the population varies with the concentration of Con A used (Milner,1977; Stenzel et al,1978). However, my data have shown that this straight-forward interpretation of the commitment kinetics of the population can become complicated when DNA synthesis is measured late in culture. When ^{3}H-thymidine incorporation is assayed at 48 hour, the addition of aMM after 20-30 hours of culture resulted in a level of incorporation equal to that found in the Con A control cultures. This applies to both the human and mouse cells. However, when DNA synthesis is assessed at 72 hours, the addition of aMM after commitment resulted in a level of ^{3}H-thymidine incorporation some 10 to 20 percent less than that observed in the Con A control cultures.

The reason for this discrepancy may be related to the effect of aMM on the stimulation of second generation cells.

If Con A is necessary for their entry into S phase, the addition of the sugar after commitment to the first round of DNA synthesis would be expected to inhibit the incorporation of ^{3}H-thymidine measured at 72 hours to a degree equal to the contribution of the second generation cells to the overall level of ^{3}H-thymidine incorporation at this time. In my mouse and human cultures, the addition of aMM after commitment reduced ^{3}-thymidine incorporation some 10 to 20 percent relative to that in Con A control cultures. This compares well with the number of second generation cells which have been reported at 72 hours in Con A control cultures of human cells (Dular, personnal commun.). Therefore, besides explaining the difference between Con A control and aMM treated cells when DNA synthesis is measured at 72 hour, this observation provides is consistent with the hypothesis that Con A is required for the stimulation of second generation cells into S phase.

An alternative explanation is that there is a subpopulation of cells which between 40 to 60 hours of culture enter DNA synthesis for the first time. The presence of aMM would prevent this population from entering DNA synthesis. It is noteworthy that if this is the case, one cannot exclude the possibility that colchicine could also prevent their entry into S phase by acting at the point of commitment. However, colchicine when present between 40 and

72 hours inhibited DNA synthesis by 80 percent indicating that at least for the majority of cells responding to Con A, colchicine inhibits after commitment.

6.4 IS INHIBITION BY COLCHICINE DUE TO AN EFFECT ON MICROTUBULES?

Having established that colchicine could inhibit Con A-induced lymphocyte blastogenesis after the onset of DNA replication and commitment, it became essential to ascertain the target of colchicine. This was of particular importance because it had been assumed by most investigators that the effect of colchicine was directed to the microtubule (Medrano et al ,1974; Wang et al,1975; Gunther et al,1976; Greene et al, 1976), however, in only one case was microtubule disruption actually demonstrated (Thyberg et al, 1977) and this was at a colchicine concentration that was toxic in my experiments. Futhermore, several investigators suggested that the effect of colchicine was to inhibit thymidine transport into the cell (Betel and Martijnse, 1976; Steen and Lindmo, 1978).

The first evidence implicating the microtubule as the primary target of colchicine involved a comparison of the effect of vinblastine sulfate and colchicine on lymphocyte blastogenesis. Vinblastine sulfate prevents microtubule assembly by binding to tubulin at a site distinct from the

colchicine binding site (Wilson et al, 1974). As seen in Figures 7 and 8, both agents ,when present from the start of culture, caused a similar concentration-dependent inhibition of DNA synthesis. Futhermore, this inhibition was evident at concentrations as low as 10^{-8}M suggesting a common specific target for the agents. However, caution is needed in the interpretation of these data since millimolar concentrations of vinblastine sulfate have also been shown to precipitate membrane proteins (Wilson et al ,1976). At present, the effect of vinblastine sulfate at concentrations as low as 10^{-8}M on cell surface proteins is unknown.

Colchicine was also found to have a significant effect on the presence of microtubule networks in Con A-stimulated human leukocytes. Microtubule networks, as detected by tubulin-antibody immunofluorescence, disappeared in 70 to 75 percent of the population after a 12 hour incubation with 10^{-6}M colchicine. At the same time, this alkaloid inhibited ^{3}H-thymidine incorporation by 70-90 percent when measured at 60 hours of culture. In contrast, lumicolchicine, an inactive or weakly active congener of colchicine (Wilson et al, 1974), had no effect on the presence of microtubule networks after a 12 hour incubation at concentrations as high as 10^{-4}M. It also was some three orders of magnitude less inhibitory of the incorporation of ^{3}H-thymidine than colchicine. These combined data suggest that at

concentrations between 10^{-6} and 10^{-8}M, the primary target for the action of colchicine is the microtubule. As will be discussed, at higher concentrations, colchicine caused significant inhibition of thymidine transport. However, since colchicine inhibited lymphocyte blastogenesis at concentrations as low as 10^{-8}M, it is unlikely that the effect of colchicine on thymidine transport is responsible for the drugs effect on ^{3}H-thymidine incorporation.

The observation that lumicolchicine inhibits the incorporation of ^{3}H-thymidine into Con A-stimulated lymphocytes has been reported in only one other study (Greene et al, 1976). The mechanism of inhibition is not known; however, it is noteworthy that inhibition occurred under conditions where there was no apparent effect on microtubule integrity. In addition, this derivative caused 50 percent inhibiton at a concentration of 5.0-7.5 X 10^{-5}M which is close to the concentration of the agent required to inhibit thymidine transport. Therefore, while I cannot exclude the possibility that residual quantities of colchicine remained present in my colchicine preparation, and that over 48 hours this could have inhibited the incorporation of ^{3}H-thymidine, my data suggest that lumicolchicine probably inhibited incorporation by a direct effect on thymidine transport. From the spectophtometric curves, it was not possible to estimate the concentration of

colchicine in the lumicolchicine preparation, when the colchicine concentration was below 10^{-7}M.

After having established that the primary target of colchicine was most likely the microtubule, it became of interest to correlate the time-course of microtubule disassembly to the inhibition of DNA synthesis. Rogers and Brown (1979) had previously shown that the effect of colchicine on the microtubule networks in resting human and mouse cells was time-dependent. At concentrations between 10^{-4} and 10^{-6}M, colchicine required only 1 hour to cause a 50 percent reduction in the number of cells with detectable microtubule networks. In comparison, my data show that at 10^{-6}M, colchicine required some 15 hours to cause a 50 percent reduction in the incorporation of ^{3}H-thymidine into Con A-stimulated human lymphocytes. Assuming that the kinetics of disassembly in stimulated cells is similar to that of resting lymphocytes, a lag time of some 15 hours exists between the disappearance of microtubules and the manifestation of inhibition. Viewed from another angle, my data with Con A-stimulated human lymphocytes indicate that after a 12 hour exposure to colchicine, the majority of stained microtubule networks disappeared from the stimulated population. Therefore, a lag period of at least 15 hours exists between the disappearance of microtubules in stimulated human cells and demonstrable inhibition of DNA

synthesis. Thus, while the primary target of inhibition by colchicine seems to be the microtubule, the lag time between cause and effect suggests that its inhibition of Con A-induced lymphocyte transformation is only indirectly related to its effect on microtubule integrity.

6.5 THIMIDINE TRANSPORT IN STIMULATED HUMAN LYMPHOCYTES

The work of this thesis has demonstrated that Con A, when present in culture for 48 hours, can induce thymidine transport in human peripheral leukocytes. The translocation of this substrate also appears to be carrier-mediated since it is both time-dependent and saturable. In addition, in one experiment, deoxycytidine inhibited thymidine transport, suggesting a common carrier for both nucleotides. Thymidine uptake increased with time in a linear fashion until 45-60 seconds, afterwhich it started to reach a plateau. The early linear increase represents transport, that is, the time during which uptake is unidirectional and uncomplicated by backflux (Berlin and Oliver, 1975). This period is comparable to that in mouse splenocytes (Strauss et al, 1976). In contrast, transport in Novikoff hepatoma cells at 18°C lasted for several minutes while in non-phosphorylating Novikoff cells, thymidine achieved equilibrium across the membrane within 10-12 seconds (Plagemann et al, 1976; Wohlheuter et al,1978). Several other studies have attempted to measure transport over periods of 10-20

minutes, which probably corresponds to the net uptake of substrate (Breslow and Goldsby,1969, Plagemann and Erbe, 1972; Barlow and Ord, 1975). In Novikoff cells, the measurement of thymidine uptake over 5 minutes underestimated both the Km and Vmax of thymidine transport (Wohlheuter et al, 1978). This observation shows the need to measure the initial rates of thymidine uptake.

Thymidine transport was also found to be saturable with increasing concentrations of external thymidine. Unlabelled thymidine decreased the rate of isotope incorporation until about 1.0mM, afterwhich no further label was found associated with the cell pellet. A double-reciprocal plot of the initial rates of uptake against the concentration of external thymidine yielded a normal Michaelis-Menton curve. Data was fitted to an equation derived from the method of least squares. Contrary to the results of Strauss and coworkers (1976,1979), the correlation coefficient was maximized when a single line was determined for all points. This resulted in the estimation of a single Km value that ranged from 200-500uM, and which varied with the experiment and presumably, the donor. These values compare well with those derived from other systems; 85uM for rat Novikoff hepatoma cells (Wohlueter et al,1978) and 160uM for mouse splenocytes stimulated <u>in vivo</u> with Con A (Strauss et al,1977). In the case of AKR cells of leukaemic mice, a

transport system with a Km of 34uM was reported. With the addition of Con A, an additional system with a Km of 212uM appeared. Signifigantly, this system which appeared directly as a result of the addition of Con A has the same affinity as that observed for Con A-stimulated human cells (Strauss et al,1979).

Strauss and coworkers (1977) have described an additional low affinity system with a Km of 4mM in mouse splenocytes stimulated *in vivo* with Con A and 1.6mM for unstimulated AKR cells (Strauss et al,1977; Strauss et al,1979). However, the identity of this entity as a transport system is suspect. For example, in the case of AKR cells, where a system with a Km of 1.6mM was reported, time-dependent uptake of thymidine in this concentration range could not be demonstrated. Therefore, instead of corresponding to a transport system, it may represent something like a binding protein such as has been described for glucose transport in chick fibroblasts (Lee and Lipman,1977).

From a comparison with other studies(Strauss et al,1977; Wohlheuter, 1978), it is evident that the Vmax for thymidine transport in human leukocytes stimulated *in vitro* with Con A is significantly greater than that found in other cells. While the Vmax for human leukocytes ranged from 75 to 125 pmoles/ 10^6 cells/ 15 seconds, mouse splenocytes stimulated

in vivo with Con A transported the nucleoside with a Vmax of 20 pmoles/ 6.7 X 10^5 cells/ 20 seconds. In addition, Novikoff cells had a velocity of 0.13 pmoles/ 1.3 X 10^6 cells/ 60 seconds. Unfortunately, it is not possible to correlate the Vmax of thymidine transport to the magnitude of DNA synthesis for different cell cultures because of the different methods used to measure ^{3}H-thymidine incorporation. In fact, Strauss et al (1977) claim that the used of TCA precipitation to measure DNA synthesis can overestimate ^{3}H-thymidine incorporation by as much as 700 percent relative to DNA extraction techniques.

Resting human leukocytes showed no apparent transport of thymidine over the period of time tested, an obervation which agrees with that found for resting mouse splenocytes (Strauss et al,1976). The inability to detect thymidine transport in resting cells may be a related to a lack of thymidine kinase activity in these cells. Thymidine kinase activity has been reported to increase exclusively during S phase of the cell cycle (Littlefield, 1966; Hoywood et al,1975). In the case of lymphocytes stimulated by PHA, there is a correlation between ^{3}H-thymidine incorporation and thymidine kinase activity (Peters and Hausen, 1971; Barlow and Ord, 1975). In addition, recent evidence suggests that thymidine transport involves a tandem arrangement of translocation and subsequent phosphorylation,

with phosphorylation as the rate-limiting step. Wohlheuter et al (1978) found that transport in non-phosphorylating Novikoff hepatoma cells achieved equilibrium much more rapidly than the phosphorylating cells. At 18°9, thymidine attained equilibrum in non-phosphorylating cells within 8-12 seconds, while the same phosphorylating cells required several minutes. A comparable situation could exist with resting leukocytes which have been reported to have a low level of thymidine kinase activity (Barlow and Ord, 1975). Thymidine could attain equilibrium across the lymphocyte plasma membrane so quickly as to be undetectable when assayed over 15 seconds. Any further thymidine uptake as a function of time would be negligible because there would be no synthetic activity to use the available thymidine.

6.6 COLCHICINE AND LUMICOLCHICINE INHIBITION OF THYMIDINE TRANSPORT

My data have shown colchicine to inhibit thymidine transport in Con A-stimulated human lymphocytes in a concentation-dependent manner, during a 15 second interval (Figure 25). Thymidine transport was inhibited by 50 percent at a concentration of 5.0-7.5 X 10^{-5}M. Futhermore, inhibition was microtubule-independent because it occurred over 15 seconds, a period too short for the significant formation of the colchicine-tubulin bond (Wilson,1970) and because lumicolchicine inhibited thymidine transport to the same degree as colchicine (Figure 26).

These findings agree in part with the report of Mizel and Wilson (1972). These investigators found colchicine to inhibit competitively nucleoside uptake in several cell lines during a 10 minute incubation period. Colchicine inhibited thymidine uptake in HeLa cells by 50 percent at a concentration of 4.0×10^{-5}M. This value is quite comparable to the inhibition constant of thymidine transport of Con A-stimulated human leukocytes, that is, $5.0-7.5 \times 10^{-5}$M. These investigators also noted that colchicine did not preferentially inhibit ^{3}H-thymidine incorporation into TCA-insoluble material relative to TCA-soluble material. They concluded that the putative inhibition of RNA and DNA synthesis by colchicine claimed by previous workers (Hell and Cox,1963;Ilan and Quastel,1966; Fitzgerald and Brehaut,1970) was in fact an effect on transport rather than on nucleic acid synthesis.

It is here that the interpretation of my findings differs from those of Mizel and Wilson (1972). My data suggest that colchicine inhibition of thymidine transport in Con A-stimulated human leukocytes is unlikely to be responsible for colchicine inhibition of ^{3}H-thymidine into DNA. While both colchicine and lumicolchicine inhibited transport by 50 percent at 5.0×10^{-5}M, colchicine inhibited ^{3}H-thymidine incorporation into TCA-insoluble material to the same degree at 5.0×10^{-8}M. In other words, there were three orders of

magnitude difference between the alkaloid's effect on transport relative to incorporation into DNA. Therefore, since colchicine inhibited DNA synthesis at concentrations at which it had no effect on thymidine transport, inhibition of DNA synthesis cannot be due to an effect on transport.

Another possibility is that colchicine, when present for 48 hours, could inhibit thymidine transport at concentrations lower than would be required to cause equivalent inhibition over a 15 second period. This could be related to the binding kinetics of the drug to the lymphocyte plasma membrane (Riordan and Alon, 1977). However, I would argue against this possibility for the following reason. Since colchicine and lumicolchicine are structurally similar (Wilson and Freidkin, 1966), and interact with the plasma membrane with similar properties (Riordan and Alon, 1977) and inhibit the transport of thymidine over 15 seconds to the same degree (Figure 26), it is reasonable to assume that both agents interfere with thymidine transport by a common mechanism. Therefore, if the sensitivity of thymidine transport to the presence of colchicine increased with time, then this should occur with lumicolchicine as well. However, as seen in Figures 20 and 26, lumicolchicine inhibited the incorporation of ^{3}H-thymidine for 48 hours to about the same degree as over 15 seconds. In both cases, lumicolchicine inhibited thymidine

transport/incorporation by 50 percent at a concentration of approximately 5.0 X 10^{-5} M. Therefore, the increased sensitivity of Con A-stimulated human leukocytes to the presence of colchicine over a 48 hour period as opposed to 15 seconds is probably not related to the microtubule independent effect of colchicine on thymidine transport. Instead, it is probably related to colchicine's previously demonstrated effect on microtubule integrity.

Colchicine could still interfere with the transport of thymidine into the lymphocyte; however, this would be expected to occur indirectly due its effect on the microtubule. Berlin and coworkers (1972,1975) have shown colchicine to interfere with the selective exclusion of certain proteins during phagocytosis (Berlin and Oliver, 1972; Taube and Berlin,1975). Therefore, contrary to the conclusion of Mizel and Wilson (1972), the ability of colchicine to inhibit thymidine transport, independent of the microtubule is unlikely to account for the diminished incorporation of ^{3}H-thymidine into DNA.

Our understanding of the mechanism by which colchicine and lumicolchicine inhibit thymidine transport in Con A-stimulated human lymphocytes is limited by the lack of knowledge concerning the molecular basis of nucleoside transport in mammalian cells. It is not known whether

thymidine transport is coupled to phosphorylation as with sugar transport in several bacterial systems (Kaback,1970). Plagemann and collaborators (1978) found the transport of thymidine into non-phosphorylating cells to be more rapid, less specific and to have a higher Km than transport into phosphorylating cells. This suggests that phosphorylation is the rate-limiting step in transport. On the other hand, thymidine kinase activity measured *in vitro* has an activity several fold greater than that necessary for thymidine phosphorylation (Plagemann and Erbe, 1972). In addition, the majority of thymidine taken up by the lymphocyte appears to be immediately phosphorylated (Strauss et al, 1977). It is my belief that both steps in the translocation process may be rate-limiting since several agents such as persantin which have no effect on the enzyme activity can inhibit transport (Plagemann et al, 1972). Theoretically, Con A-induced thymidine transport in human leukocytes could result from either increased thymidine kinase activity, *de novo* synthesis of a protein carrier(s) or alternatively, from derepression of previously cryptic transport sites, as in the case of PHA-induced K+ transport (Quastel and Kaplan,1970).

Thus, colchicine and lumicolchicine could potentially interfere with any of several aspects of thymidine transport. However, it is probably not the result of general

membrane damage since many other membrane-related events such as Ca^{++} transport, lectin binding and CoA lysolecithin acyltransferase are uneffected by colchicine (Greene et al, 1976 ; Resch et al, 1977). In addition, it seems unlikely that colchicine would inhibit the _de novo_ synthesis of transport proteins or thymidine kinase activity over a period as short as 15 seconds. This conclusion could not be derived from studies where the effect of colchicine on thymidine uptake was measured over a period as long as 1 hour (Wilson,1972). From my data, colchicine and lumicolchicine are more likely to interact directly with membrane transport components. As outlined in Section 2.1, colchicine-tubulin bond formation is thought to involve hydrophobic interactions (Wilson,1970). It has also been suggested that transport proteins consist of hydrophobic areas (Lieb and Steen,1971). The molecular basis of transport mechanisms in lymphocyte blastogenesis could make for interesting future study. Colchicine could become a useful tool in delineating the rate-limiting steps of thymidine transport.

6.7 MODEL

From the available data, I have attempted to construct a model concerning the role of colchicine, microtubules and thymidine transport in lymphocyte blastogenesis. It is based on the following facts:

(1) Colchicine can inhibit Con A-induced lymphocyte blastogenesis before and after the onset of DNA synthesis, provided sufficient time be allowed to elapse for inhibition to become manifest. In the case of human cells, approximately 20 to 30 hours are necessary for maximal inhibition of DNA synthesis, while with mouse splenocytes 10 to 15 hours is required.

(2) The primary target for the action of colchicine appears to be the microtubule. A secondary target is the plasma membrane (Riordan and Alon, 1977) and the membrane components involved in the transport of thymidine. In the case of the microtubule, the existence of a lag period between the disappearance of the microtubule networks in Con A-stimulated human leukocytes and subsequent inhibition of DNA synthesis suggests that colchicine that while acting by affecting microtubule integrity produces its inhibitory effect via indirect (and possibly remote) consequences of this primary action.

(3) Brief exposures to colchicine may augment the response of human lymphocytes to periodate (Stenzel et al, 1978) and to allogeneic cells (Suthanthiron et al, 1980). In contrast, the same pretreatment has been shown to inhibit the response of cells to Con A and PHA. However, it is not certain that the actual target responsible for potentiation of response is the microtubule, since the exposure time to colchicine was too brief to permit such a conclusion.

(4) NAGO-stimulated cells may be some 2 orders of magnitude less sensitive to prolonged exposures to colchicine than the same cells stimulated with Con A (Rasmussen and Davis, 1977). This suggests indirectly a role for microtubules in the Con A induced activation of lymphocytes. However, there is no direct evidence to support this theory. Edelman's hypothesis that colchicine acts to block lymphocyte blastogenesis at the point of cell commitment has been shown to be a factitious consequence of a 20 to 30 hour lag period required before maximal inhibition is achieved. In the case of mouse splenocytes, there is no apparent similarity between the colchicine and commitment curves. While my data cannot exclude a role for microtubules in signal transmission or commitment, there is no evidence that inhibition by colchicine and thus microtubule integrity has anything to due with either of these events. A 12 hour exposure of cells to colchicine inhibited RNA synthesis to the same degree before and after

the onset of DNA synthesis. In addition, colchicine has not been found to inhibit many of the early events associated with lymphocyte activation. These include Ca++ influx (Greene et al, 1976); lectin binding (Greene et al, 1976); CoA lysolecithin acyltransferase (Resch et al, 1978). The effect of colchicine cannot be due exclusively to interference with Con A capping. If this was the case, then the time-course of inhibition by colchicine would be the same as cytochalsin B, which inhibits only when present within 2 hours of Con A addition (Medrano et al, 1974). These data, while they do not exclude a role for the microtubule in signal-transmission, suggest that microtubule integrity is simply a general requirement for the maintenance of lymphocyte function.

(5) Con A, when present for 48 hours, can induce time-dependent and saturable thymidine transport in human peripheral leukocytes. Transport in resting cells is undetectable.

(6) Colchicine and lumicolchicine can inhibit thymidine transport in human peripheral leukocytes, independent of the microtubule. However, in the case of Con A-stimulated cells, this inhibition of transport cannot account for the effect of this agent on the assay for DNA synthesis. In contrast, colchicine inhibition of thymidine transport could be responsible for inhibition of ^{3}H-thymidine incoporation in NAGO-stimulated cultures.

(7) The effect of lumicolchicine on the transport of thymidine can account for the inhibitory action of this agent on the assay for DNA synthesis.

From the available data, it is clear that the initial event in lymphocyte blastogenesis is the binding of the mitogen to the cell surface. However, although necessary, this step by itself is insufficient to activate the lymphocyte. Binding is followed by capping of the

ligand/receptor complexes, which does not appear to be necessary for activation. Therefore, by some yet undefined process (receptor-receptor cooperativity, a specific receptor etc.), a mitogen induces a cascade of early molecular events, which eventually lead to protein, RNA and DNA synthesis. These events have already been described in terms of their possible role in lymphocyte activation (Section 1.3). At present, it is not known which of these many events are or is causally linked to DNA replication.

The role of the microtubule in lymphocyte activation remains elusive, but there no longer exists any direct evidence to support the Edelman hypothesis implicating the microtubule in signal-transmission. Microtubule integrity is required both before and after the onset of DNA replication. Futhermore, the time lag between colchicine addition, microtubule disassembly and inhibition of lymphocyte blastogenesis suggests that whatever role the microtubule does play in blastogenesis, it is only indirectly related to the DNA synthesis.

Colchicine has been found to interfere with the activity of few early molecular events associated with activation (Section 3.1). Theoretically, microtubules could be involved in activation due an interaction with the cyclic nucleotides. Greene and coworkers (1976) have reported

that colchicine enhances and prolongs the early cAMP surge following lectin binding. Futhermore, cAMP when present *in vitro* has been noted to inhibit calf tubulin assembly (Garland, 1979). Therefore, both these molecular events may regulate each others activity. Colchicine was reported to have no effect on cGMP levels. However, agents known to increase cGMP levels have also been shown to antagonise Con A capping (Oliver et al, 1976). This is presumed to occur by an effect on microtubules.

Colchicine has been found to disrupt numerous events thought to be dependent on microtubules. These include determination of cell shape (Brown and Bouck, 1973), phagocytosis (Oliver et al, 1974), export of proteins (Ukena and Berlin, 1972) and the topographic distribution of cell surface receptors (Oliver et al, 1974). On this basis, the time-lag of colchicine inhibition could be the result of an effect on any number of functions. In the case of leukocytes, microtubule disassembly could have resulted in inhibition of TCGF production (Morgan et al, 1976), or the enhanced release of prostaglandin E (Gemsa, 1979).

While the basis for inhibition is poorly understood, it appears that the primary target of colchicine action is the microtubule. Although this agent has been found to bind membrane, it does not appear to grossly damage the cell

membrane since events such as Con A binding and early Ca++ transport are unaffected by the presence of the agent (Greene et al, 1976). However, colchicine can inhibit thymidine transport in a manner independent of microtubules. This inhibition is probably the result of a direct interaction with transport carrier components. In addition, this effect does not appear to be the cause of the inhibition of the assay for DNA synthesis.

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