NOTICE

The quality of this microfiche is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us a poor photocopy.

Previously copyrighted materials (journal articles, published tests, etc.) are not filmed.

Reproduction in full or in part of this film is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30. Please read the authorization forms which accompany this thesis.

THIS DISSERTATION
HAS BEEN MICROFILMED
EXACTLY AS RECEIVED

AVIS

La qualité de cette microfiche dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de mauvaise qualité.

Les documents qui font déjà l'objet d'un droit d'auteur (articles de revue, examens publiés, etc.) ne sont pas microfilmés.

La reproduction, même partielle, de ce microfilm est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30. Veuillez prendre connaissance des formules d'autorisation qui accompagnent cette thèse.

LA THÈSE A ÉTÉ
MICROFILMÉE TELLE QUE
NOUS L'AVONS REÇUE

EFFECTS OF ULTRAVIOLET LIGHT ON THE ACTIVATION OF HUMAN LYMPHOCYTES

by

Gustavo Castellanos

A thesis submitted to the School of Graduate Studies of the University of Ottawa, as partial fulfillment of the requirements for the degree of M.Sc. in Biology.

University of Ottawa
Ottawa, Ontario, 1980

I hereby declare that I am the sole author of this publication.

I authorize the University of Ottawa to lend this publication to other institutions or individuals for the purpose of scholarly research.

Gustavo Castellanos

I further authorize the University of Ottawa to reproduce this publication by photocopyng or by other means, in total or in part, at the request of other Institutions or individuals for the purpose of Scholarly research.

Gustavo Castellanos

The University of Ottawa requires the signatures of all persons using or photocopyng this publication. Please sign below, and give address and date.

To

My children, wife and mother for their wonderful encouragment and understanding.

ACKNOWLEDGEMENTS

I cannot find words to express my gratitude to my adviser Dr. Gordin J. Kaplan for his wonderful help and encouragment.

I would like to express my deep appreciation to the following people for their valuable assistance: Jack McLean and Trevor Owens for many fruitful discussions and technical help; Christopher Rudd for his help with the thymidine transport technique; Trevor Blaton from the University of Carleton for his help with the EM techniques; Yolanda Barrientos for helping me by taking the electron-micrographs; Christine Boumah for her help in translating the abstract to french; Kevin Doyle and Jennifer Wilson for their valuable help in the English structure of this thesis; Jaques Helie and Paul Brunon for helping me to produce the figures for this thesis and Judy Little for her technical help.

Finally I would like to give special thanks to the Venezuelan Scholarship Programme "Gran Mariscal de Ayacucho" for having economically supported me during my studies for these years.

ABSTRACT

This thesis describes the effects of ultraviolet light on human lymphocytes applied at different times after Con A stimulation.

Among the parameters of stimulation examined were DNA, RNA, and protein synthesis. Reductions in the incorporation of ^{3}H-thymidine, ^{3}H-leucine and ^{3}H-uridine were observed in Con A stimulated human lymphocytes which had been irradiated with 84 ergs/mm^2 /sec. This reduction could not be accounted for by the decrease in cell viability.

DNA synthesis was about equally sensitive to UV whether cells were irradiated at 24 hr or 44 hr after addition of Con A; in these experiments, DNA synthesis was assayed at either 48 or 72 hr. DNA synthesis was most sensitive to irradiation when this was administered at the onset of culture with Con A (DNA synthesis assayed at 72 hr). Since at 0 hr, cells are some 30 hr from S phase, UV irradiation affects most strongly the activation process, rather than DNA synthesis itself. Attempts to show repair by allowing an interval of 24 h between irradiation and addition of mitogen were unsuccessful.

As far as protein synthesis is concerned, uptake of H-leu was inhibited by 96% in those cells irradiated prior to addition of Con A. This process was also sensitive in the resting cells.

Thus, certain of the most striking effects of UV on the stimulation of lymphocytes are independent of its well documented production of pyrimidine dimers in DNA.

UV produced a significant reduction in the Con A activated potassium influx in human peripheral lymphocytes irradiated with 84 ergs/mm^2/sec immediately prior to the addition of mitogen. Potassium influx in resting cells was not significantly ($p=0.1$) inhibited. Potassium influx measured 2.5 hours after Con A addition, using ^{86}Rb as a tracer dropped after UV from 6.8 to 1.7 fmoles/cell/hr.

UV also produced a significant reduction ($p = 0.001$) in the binding of ^{3}H-Con A to the lymphocyte cell surface. The number of binding sites per cell at 40 min was $6.6 \pm 0.4 \times 10^6$ in the control cells and $4.2 \pm 0.4 \times 10^6$ in UV treated.

UV therefore inhibits early events, essential for activation which are produced at the lymphocyte cell surface. However, UV irradiation had no effect on thymidine transport, measured at 48 hr after addition of Con A. One could reasonably conclude that approximately the same amount of ^{3}H-thymidine is being made available for DNA incorporation in control and UV irradiated cells.

Therefore the main effects of UV is on the appearance of the system that synthesizes DNA during S phase of the cell cycle and not on the function of this system or of that which translocates thymidine across the lymphocyte membrane.

It was observed that UV prevented nuclear decondensation, measured at 24 hr in cells which had been irradiated at 0 hr (prior to Con A addition), whereas in those cells irradiated 16 hrs after Con A addition, nuclear decondensation continued to occur.

Since a low UV dose produces irreversible and apparently irreparable damage, it may be a useful tool for the study of cell function.

RESUME

Cette thèse décrit les effects des rayons ultraviolets, sur des lymphocytes humains, à différentes périodes après la stimulation au Con A.

Certains des paramètres de stimulation examinés étaient les synthèses d'ADN, d'ARN et de protéines. Des réductions dans les incorporations de ^{3}H-Thymidine, ^{3}H-leucine et ^{3}H-Uridine ont été observées dans les lymphocytes humains stimulés au Con A et irradiés avec 84 ergs/mm^{2}/sec. Ces réductions ne pourraient s'expliquer par une baisse de viabilité des cellules.

La synthèse d'ADN était presqu'aussi sensible aux rayons U.V. que les cellules aient été irradiées à 24 heures ou à 44 heures après l'addition du Con A; dans ces expériences, la synthèse d'ADN a été mesurée à 48 heures ou à 72 heures. La synthèse d'ADN était plus sensible à l'irradiation quand celle-ci était donnée au moment de l'addition du Con A dans la culture (la synthèse d'ADN étant mesurée à 72 hrs).

Vu qu'à 0 heure, les cellules sont à peu près à 30 heures de la phase S, l'irradiation aux U.V. affecte beaucoup plus le processus d'activation que la synthèse d'ADN elle-même. Les tentatives de prower une réparation dans un intervalle de 24 heures entre l'irradiation et l'addition du mitogène étaient infructueuses.

En ce qui concerne la synthèse des protéines, l'entrée de ^{3}H-leucine était inhibée à 96% dans ces cellules irradiées avant l'addition de Con A. Ce processur était aussi plus sensible dans les cellules au repos.

Donc, certains des effets les plus marqués des rayons U.V. sur la stimulation des lymphocytes sont indépendants de leur si bien connu effet à savoir la production de diamètre pyrimidiques dans l'ADN.

Les rayons U.V. produisent une réduction importante dans l'activation

de l'influx de potassium dû au Con A, dans les lymphocytes humains périphéraux irradiés avec 84 ergs/mm^2/sec juste avant l'addition du mitogène. L'influx de potassium des cellules au repos n'était pas considérablement inhibé (P=0.1).

L'influx de potassium mesuré 2.5 heures après l'addition de Con A, avec ^{86}Rb comme marqueur administré après les rayons U.V. de 6.8 à 1.7 fmoles/cellule/heure.

Les rayons U.V. produisent aussi une réduction importante ($p = 0.001$) dans la liaison de ^{3}H-Con A à la surface cellulaire du lymphocyte.

Le nombre de sites d'attachement par cellule à 40 min. était 6.6 ± 0.4 ($\times 10^6$ dans les cellules témoins et $4.2 \pm 0.4 \times 10^6$ dans celles traitées aux U.V.

Donc les rayons U.V. inhibent les évènements primordiaux, essentiels à l'activation qui sont produits à la surface cellulaire du lymphocyte. Cependant, l'irradiation aux U.V. n'a aucun effet sur le transport de thymidine, mesuré à 48 hrs après l'addition de Con A. Il serait donc raisonnable de conclure qu'approximativement la même quantité de ^{3}H-thymidine est disponible pour incorporation dans l'ADN des cellules témoins et celui des cellules irradiées aux U.V.

Donc, les effect principaux des rayons U.V. sont sur l'apparition du système qui synthétise l'ADN pendant la phase S du cycle cellulaire et non sur la fonction de ce système ou de celui qui transporte la thymidine à travers la membrane du lymphocyte.

On a observé que les rayons U.V. empêchaient à 24 heures dans les cellules qui ont été irradiées à 0 heure (avant l'addition de Con A), tandis que dans celles irradiées 16 heures après l'addition de Con A, la decondensation nucléaire continuait normalement.

Vu qu'une faible dose de rayons U.V. produit des dommages irréversibles et apparemment irréparables, cela pourrait constituer un système utile pour l'étude de la fonction cellulaire.

TABLE OF CONTENTS

LIST OF FIGURES

page

LIST OF TABLES

CHAPTER I
INTRODUCTION

The lymphocyte (one of the most investigated groups of cells) is found in virtually all higher animals which have developed an immune system. This cell exists in a resting state with little metabolic activity, and under certain conditions it can be induced by agents to undergo a variety of morphological and metabolic changes (called blastogenesis) until it is a fully activated immune cell.

Although the lymphocytes are small they are very motile and are recognized to be an integral part of the human defense system against foreign cells and materials, being responsible for virtually all specific immune reactions (Roitt. 1974).

The major emphasis and purpose of this work has been to attempt to elucidate the cause (s) by which UV inhibits blastogenesis of lymphocytes.

1.A. Effects of Ultraviolet Radiation on animals cells

It has been known for many years that the maximal bactericidal effectiveness of ultraviolet radiation is in the UV-C region(200-290 nm). The absorption spectra of many bacteria have a peak near 280 nm, where aromatic amino acid residues of their proteins have maximum absorption.

The bactericidal action spectrum in most experimental conditions corresponds to the absorption spectrum of the nucleic acids DNA and RNA, both peaking at about 260 nm. It has been concluded that the primary event in UV inactivation or killing of bacteria results from photochemical alteration of the cellular DNA. Furthermore in many experimental circumstances, DNA replication is inhibited by lower doses of ultraviolet radiation than is RNA synthesis or protein synthesis. For this reason, research has been concentrated primarily on the effects of UV absorption by nucleic acids and proteins occurring at wavelengths shorter than 300 nm.(Parrish,et al. 1978).

In vitro and in vivo, in both prokaryotic and eukaryotic cells, one major molecular consequence of exposing DNA to ultraviolet radiation is the formation of pyrimidine dimers. Thymidine dimers have received the attention, perhaps because DNA is easily labeled with (^{3}H) thymidine, and the dimers are an acid stable DNA photoproduct that may be easily isolated.

In some systems, however, other dimers, such as cytosine-cytosine and cytosine-thymidine, make up the largest percentage of dimers. (Setlow and Carrier 1966).

Other DNA photoproducts are known to occur, including other dimers, hydrated pyrimidines, and cross-links between two strands of DNA, as well as between DNA and protein. (Smith, K.C. 1974).

DNA strand breakage, local disruption of hydrogen bonds, and changes in RNA also occur when cells are irradiated. Changes in RNA similar to those in DNA certainly occur and may be more numerous events. However, changes in RNA are not as evident or as easy to measure as those in DNA, because multiple copies of each species of RNA may exist within a given cell, whereas one, or, at most, a few copies of a DNA sequence may be present in bacteria.

Cell protein structure may also be altered by UV irradiation. Such protein changes would usually have to be extensive and numerous to affect the cell as severely as do changes in DNA. However, many enzyme activities can be shown to be decreased following UV irradiation. Proteins may be primarily affected and enzymes may be either activated or inactivated. The effects on proteins may result from photochemical alterations of the individual amino-acids of which the proteins are composed. Decarboxylations, deaminations, and ring breakage occur. Among the more sensitive targets are the aromatic amino acids, especially tryptophan. The disulfide bonds of cystine can be broken by ultraviolet radiation. (Parrish, et al. 1978).

Ultraviolet-induced changes in macromolecular synthesis may have significant effects on cellular metabolism.

At radiation doses that have little or no effect on oxigen consumption, there may be derangements in the synthetic metabolism of the cell. These derangements affect DNA synthesis, RNA synthesis, and therefore protein and enzyme synthesis. The eventual changes in the cell may therefore be profound. Because of DNA's essential role as the template for synthesis of RNA, which in turn codes for protein synthesis, many of these other processes induced by ultraviolet radiation are less important (Parris, et al., 1978).

Funtional enzymes, however, are necessary for the repair of damaged DNA. The effects of ultraviolet on animal cell are complicated, and hundreds of UV-induced changes have been noted. The effects usually mediated by wavelengths shorter than 320 nm have been summarized by Giese (1965) and Painter (1970).

Organisms have evolved a series of enzymic repair mechanisms for the repair of DNA base damage. Three major pathways have evolved in prokaryotes and most eukaryotes for that purpose:

1. Photoreactivation of cyclobutane-type pyrimidine photodimers.
2. Excision repair.
3. Postreplication or recombination repair.

All these three enzymic repair mechanisms are fully described elsewhere, Hart, R.et al (1978).

1. Photoreactivation:

The photoreactivation repair system is specific for monomerization of UV-induced cyclobutane type pyrimidine dimers. The photoreactivation enzyme binds to irradiated DNA at a cyclobutane-type pyrimidine dimer. In the presence of photoreactivating light (300-600 nm), the enzyme is activated, converting the dimer into two monomers. Of all the many photoproducts made by UV irradiation of cellular DNA, only the cyclobutane-type pyrimidine dimers are affected by enzymatic photoreactivation. Thus, photoreactivation is a diagnostic tool that may be used to discern the role of selected alterations in the secondary structure of DNA with regard to their biological function.

2. Excision Repair:

In its simplest form, excision repair involves the removal of damaged parental DNA by a complex of enzymes. The damaged segment is removed and replaced by DNA synthesized using the opposite strand as a template. Excision repair may be measured by assaying: (1) the removal of known lesions such as thymidine dimers. DNA-DNA crosslinks, or DNA carcinogen complexes; (2) the production of single-strand breaks after treatment of cellular DNA with semi-site specific of crude repair endonucleases; or (3) the measurement of repair synthesis following excision.

In human, but not in many rodent cell systems, at the single-cell level, repair synthesis following exposure of cells to UV radiation is equated with unscheduled DNA synthesis(Hart and Setlow, 1974). This in turn is measured by the incorporation of radioactive precursors into DNA during periods in the cell cycle when DNA is normally not being synthesized or into cells whose normal semi-conservative DNA synthesis has been retarded by hydroxyurea (Hart and Setlow, 1976).

2.a Recognition and Incision:

The process of excision repair involves four general steps: incision, excision, polymerization and ligation. The first step in the excision repair process involves recognition of the damaged site in the DNA by an endonuclease or N-glycosidase. These enzymes can be used in the quantitation of DNA damage. Further by examining the disapperance of specific forms of DNA damage as a function of cell cycle, a determination as to the mutagenic potential of specific forms of DNA lesions with regards to DNA replication can be made.

The N-glycosidases catalyze the hydrolysis of the N-glycosidic bonds of the damaged nucleotide residues but, unlike endonucleases, do not cleave phosphodiester bonds.

The reaction products after the action of one type of N-glycosidase are a free uracil and partly depyrimidinated DNA of unaltered chain length (Lindahl, 1976). It has been suggested that in bacterial systems, endonuclease II and N-glycosidase may act together with endonuclease I nicking the DNA backbone at the apyrimidinic site (Kirtikar and Goldthwait, 1974).

The endonucleases involved in excision repair may act on many types of damage, although there seems to be specificity for the types of damage they act upon (Hart and Trosko, 1976).

2.b Excision:

The second step in the excision process involves the activity of an exonuclease that degrades denatured but not native DNA (Kaplan et al, 1971). Following site recognition and nicking by an endonucleases, there is subsequent degradation by an exonuclease, which results in release of the damaged region as well as a limited number of the other nucleotides.

2.c. Repair replication:

The third step in the excision repair process involves the activity of a DNA repair polymerase.

The DNA repair polymerases isolated from either M. luteus or E. coli are associated with exonuclease activity, and have the ability to excise pyrimidine dimers from incised UV-irradiated DNA. In order for the DNA polymerase to act in excision repair, a 3'-Oh site must be produced followed by the reinsertion of nucleotides prior to termination by a polynucleotide ligase. To date no single enzyme has been isolated from mammalian cells with the combined excision and polymerizing activities equivalent to E. coli DNA polymerase I (Hanawalt, et al., 1979).

2.d Ligation:

The final step of the excision repair process is the sealing of the newly synthesized DNA to the parental DNA by the action of a polynucleotide ligase. This step in excision repair might lead to mutation by abortive repair when nicked DNA is resealed by ligase action prior to removal of the damaged region, thereby enhancing the probability of either misincorporation or subsequent misrepair.

3. Postreplication Repair:

Postreplication repair, which presumably may be either error-fre or error-prone, is a process in which DNA, newly synthesized from a defective or damaged template, is repaired.

Postreplication repair is usually studied by observing that the newly-synthesized DNA from damaged templates contains gaps that disappear with time. In human cells, the the number of gaps approximately equals the number of lesions. This system is important in actively dividing cells and cells that have been induced to divide. XP "variant" cells, which are normal for excision repair, have been shown to be defective in postreplication repair. However, in cell cultures that have been treated with either physical or chemical mutagens and DNA replication blocked following such treatment, the mutation frequency per dose decreases as a function of the duration of mitotic blockage. These data suggest a role in mammalian cell mutagenesis for an error-prone postreplication repair system as well as misinsertion during DNA replication.

4. Measurements of Excision-Repair (Cleaver, J.E. 1980)

4.a DNA repair pathways

Exposure to DNA damaging agents is often followed by excision repair during which cells attempt to remove damaged oligonucleotide regions and resynthesize small replacement patches. It was convenient to think of two main branches: nucleotide and base excision repair.

Nucleotide excision repair occurs following exposure to UV light and many agents that form large adducts in DNA (Cleaver 1973; Regan & Setlow 1974). This kind of repair is defective in 7 complementation groups of XP, and was thought to be initiated by endonucleolytic cleavage 5' to to the dimer and result in a relatively large patch up to 100 bases, whose size may be regulated by chromatin structure (Cleaver 1977,1980). Base excision repair occurs after exposure to alkylating agents or thermal deamination. No base excision defective mutants are known in human or other mammalian cells. The repair system is initiated by a glycolytic cleavage that releases the damaged base and results in a relatively small patch of a few bases. Recent observations indicate that the initial step of excision repair of UV induced pyrimidine dimers by enzymes from T4 infected E. coli or M. luteus occurs by glycolytic cleavage of the 5' thymidine of the dimer (Haseltine et al., 1980). They propose that strand scission at a dimer site by the M. luteus enzymes requires two activities, a pyrimidine dimer DNA glycosylase and a apyrimidinic/apurinic endonuclease. If this mechanism occurs in eukaryotic cells the distinction between nucleotide and base excision repair is blurred and some of the ob - served experimental differences between these pathways will need revised interpretation. Work by a number of investigators suggests that normal human cells repair radiation damage by two rather distinction mechanisms. The distinction is clearly seen with a repair assay which yields information on the size of the repaired region. (Regan, et al., 1971).

In the repair of DNA single strand breaks such as those induced by ionizing radiation, after the initial break there is very limited excision and replacement of bases; highest estimates suggest perhaps three nucleotides are involved. The break is quickly closed by ligase. This "short patch" repair is completed within sixty minutes or less after a dose of 10 Krad. In the repair of ultraviolet (UV) damaged human DNA, the DNA is not broken by the irradiation but the symmetry of the helix is disorted in the region of the pyrimidine dimers induced by the UV irradiation. Normal human cells apparently possess an endonuclease capable of recognizing this distortion and of making a single strand break near the dimer. There follows an extensive excision and replacement of bases ($\sim$100). This "long pach" repair may take 20 or more hours to complete after doses of UV irradiation of from 50-150 ergs/mm^2/sec (Regan and Setlow. 1975).

Cell cultures developed from most patients with xeroderma pigmentosum (Cleaver, J.E. 1968), show greatly reduced "long patch" repair after UV irradiation but are capable of normal "short patch" repair after ionizing radiation. Results with a number of chemicals capable of damaging DNA suggest that such chemicals induce radiomimetic repair processes. (Regan and Setlow. 1974). That is, they induce either long or short repair in human cell DNA. Those agents which induce long repair in normal cells produce greatly reduced repair in xeroderma pigmentosum (XP) cells.

If human cell cultures are γ-irradiated under nitrogen, certain UV-like damages appear to be induced as evidenced by reduced repair of these damages in XP cells.

1.B Effects of UV Radiations on Immunologic Functions.

There are two reports in the literature suggesting that UV radiation has local, as well as systemic, immunological effects. In 1963, Haniszko and Suskind reported that UV irradiation of guinea pig skin rendered it unreactive to topical challenge doses of DNCB.

Challenging an unirradiated site on the same animal produced an apparently normal delayed hypersensitivity reaction. This implied that only the local effector portion of the reaction was inhibited and that systemic immunity was unaltered. Unfortunately, this interesting observation has not been pursued, and the mechanism responsible for it is unknown.

A more recent report by Horowitz et al. (1974) described what may be a similar phenomenon in a human subject. This individual exhibited a prior delayed hypersensitivity reaction to a bacterial antigen (Streptokinase-streptodornase).

UV irradiation of the skin-test site with a germicidal lamp (mainly 254 nm radiation) six hours after injection of the eliciting dose of antigen markedly reduced the local reaction. In contrast to Haniszko and Suskind's finding, however, topical UV treatment of the test site at other times (i, e., prior to injection of the challenge dose) did not dimminish the reaction intensity (Horowitz et al., 1974).

1.B.1 UV irradiation of lymphocytes in vitro

This observation by Horowitz et al.,(1974) is interpreted by them as illustrating the lethal effect of UV radiation for lymphocytes in vivo.

In this same study (Horowitz et al., 1974), these authors show that T lymphocytes from both mouse and man are more sensitive to killing by UV irradiation in vitro than are B lymphocytes (antibody-forming cells and their progenitors). Thus, topical irradiation of a skin-test site within a few hours after antigen challenge might inactivate the T lymphocytes that accumulate at the site of injection, thereby preventing the subsequent chain of events constituting a positive skin test reaction. Although this interpretation is consistent with the in vitro lymphocyte inactivation studies, there are many possible interpretations that are equally likely.

In addition to the lethal effects of UV light on lymphocytes, there is a more pertinent and subtle effect, which was described first by Lindahl Kiessling and Safwenberg (1971) and later by others (Lafferty et al.,1974: Röllinghoff and Wagner, 1975; Wagner and Röllinghoff, 1976). Irradiation of lymphocytes in culture with sublethal dose of UV light from a germicidal lamp rendered them incapable of serving as stimulator cells in an allogeneic mixed lymphocyte reaction. Measurements of surface antigens of the major histocompatibility complex indicate that the failure of these cells to stimulate allogenic lymphocytes to proliferate in vitro was not due to loss or alteration of these antigens (Lindahl-Kiessling and Safwenberg, 1971; Lafferty et al:, 1974).

Instead, there seemed to be loss or blocking or inactivation of the lymphocyte-activating determinants(Ia antigens) that are required for the initiation of the primary proliferative response of T lymphocytes, and for the primary induction of cytotoxic T-effector lymphocytes (Röllinghoff and Wagner, 1975). Thus, <u>in vitro</u> UV irradiation of lymphoid cells has the peculiar feature of selectively inactivating the cell-surface determinants that are essential for certain cell interactions in the immune response.

The implications of this phenomenon for the in vitro deficiencies exhibited by various UV-irradiated subjects are still unclear. However, if this phenomenon also occurs following in vivo irradiation, it is quite probable that it would perturb some of the cellular interactions that occur during the course of an immune response. Ia antigens, which seem to be the target of UV irradiation of lymphocytes in vitro, also occur on macrophages and are thought to constitute part of antigen- "recognition structure" complex that triggers T lymphocytes to initiate an immune response (Benacerraf & Germain, 1978). Recently, Ia antigens also have been found on Langerhans cells, which reside in the epidermis (Klareskog et al., 1977; Rowden, et al., 1977; Stingl, et al., 1978). It is tempting to speculate that the defect in antigen processing that they described in UV-irradiated mice (Jessup, et al., 1978) might be related to the destruction of Ia antigens on macrophages or Langerhans cells residing in or circulating through UV-irradiated skin (Kripke, M. 1980).

The altered responsiveness of UV-irradiated mice, as measured by delayed type hypersensitivity (DTH) and primary in vitro plaque-forming cell (PFC) response to T-dependent antigens, has recently been correlated with a functional defect in the splenic adherent cell (SAC) population of these animals (Greene, et al., 1979; Letvin, et al., 1980).

In the report published by Letvin, et al., splenocytes from mice receiving whole-body UV irradiation do not make a normal primary in vitro plaque forming cell response to the soluble T-dependent antigen trinitrophenylated poly (L-glutamic acid^{80}L-alanine^{60}L-tyrosine^{10}).

This impared immune response results from a selective loss of antigen-presenting cell (APC) function in the splenic adherent cell population of the UV-treated mice.

1.C. Thesis Approach.

This thesis used lymphocytes as a model of cell proliferation in an attempt to elucidate the mechanism by which UV causes the inhibition of DNA synthesis.

We were hoping to be able to demonstrate nuclear damage and repair on lymphocytes, but this was unsuccessful.

We then attempted to demonstrated reproducible UV effects on the cell surface. Another purpose of this thesis was to try to show whether the activation process was affected by UV irradiation or whether the effect was mainly directly on the nuclear DNA or on its replication and transcription. It was also a purpose of this thesis to determine if cells close to S phase were more sensitive to UV than cells in other stage of the activation process.

CHAPTER II
MATERIALS AND METHODS

2.A. Culture of human peripheral blood lymphocytes.

2.A.1 Preparation of cells:

Peripheral venous (120 ml.) from healthy human donors was withdrawn into 60 ml. syringes containing 2-3 ml. of sodium heparin (100 U/ml). The content of each syringe was mixed with 40 ml of RPMI - 1640 culture medium (Flow Labs). This diluted blood mixture was gently layered onto 15 ml of Ficoll paque (Pharmacia) in 50 ml plastic (Falcon) tubes.

These tubes were then spun at 400 g for 35 min at 18-20°C. Following centrifugation each tube contained a yellowish upper layer of mostly plasma and platelets, a distinct band of lymphocytes, a clear area of Ficoll,and finally a pellet containing predominantly agglutinated red cells and granulocytes. The plasma layer was quickly removed to within 3-4 ml above the lymphocyte band.

Lymphocytes were removed by using a sterile Pasteur pipette and transferred to clean, sterile 50 ml tubes. Between 25-30 mls of RPMI 1640 culture medium was added to each 20 ml of lymphocytes-rich solution, mixed well and centrifuged at 400 g for 15 min at 18-20°C, the supernatant was removed and the lymphocyte pellet resuspended in fresh culture medium in (50) ml sterile, plastic (Falcon) tubes.

A second wash was carried out spinning at room temperature for 5 min after which the cells were resuspended in RPMI-1640 culture medium containing 15% fetal calf serum and 1% penicillin (100 IU/ml) streptomycin (100mg/ml) (Difco Labs). Before adding the cells to medium, the pH was adjusted to 7.2 by using 7.5% $NaHCO_3$.

Cells were cultured in plastic 75 cm^2 culture flasks (Corning) at a cell concentration of $1x10^6$ lymphocytes/ml in incubator at 37°C. Sterile conditions were maintained throughout the separation and culturing procedure.

2.A.2 Cell concentration and viability determination.

Cell concentrations were determined using a standard hemocytometer. Before counting, cells were diluted (1:20) with 0.1% gentian violet in 0.2% acetic acid which stains the nucleus and lyses any remaining red cells. At least 200 cells were counted to determine cell concentration for each sample. The purification protocol yielded cultures containing 95% lymphocytes with viabilities of usually 92-95% as assessed by trypan-blue exclusion.

2.A.3 Mitogen

Concanavalin A(Con A) (Calbiochem) was added at a final concentration of 25 µg/ml to human peripheral lymphocytes.

This was added either prior to distribution to the flasks or disposable tubes, or before or after UV irradiation of the cells.

Dose response curves were occasionally done to check the Con A stock for mitogenic activity. Cells were cultured with Con A for different intervals of time (22,36,44 hrs),depending on the time of UV irradiation.

2.B. UV irradiation:

Prior to the exposure of lymphocytes to UV they had been in Con A for different time as described above. To remove the cells from flasks a rubber policeman was used, so that few or no cells were left in the flask, since Con A stimulated lymphocytes stick to flask walls.

After the cells were centrifuged, (400 g for 5 min) and counted, they were resuspended in sterile phosphate buffered saline (PBS) at 2 x 10^6 cells/ml. 5mls of cell suspension were poured into small sterile petri dishes (60 mm).

Lymphocytes were exposed to UV light for varying times at a rate of 6 ergs/mm^2/sec. The UV light source was located 66.5 cms above the cell suspension.

The UV light source was a General Electric germicidal lamp (G15T8), 15 watts U.S.A., which was turned on for 20-30 min. before cell exposure. Calibration of the lamp was carried out using a Black-Ray ultraviolet Meter, Model J-225 (Ultraviolet Product, Inc. San Gabriel California, U.S.A) which was borrowed from Dr. Peter Fitt.

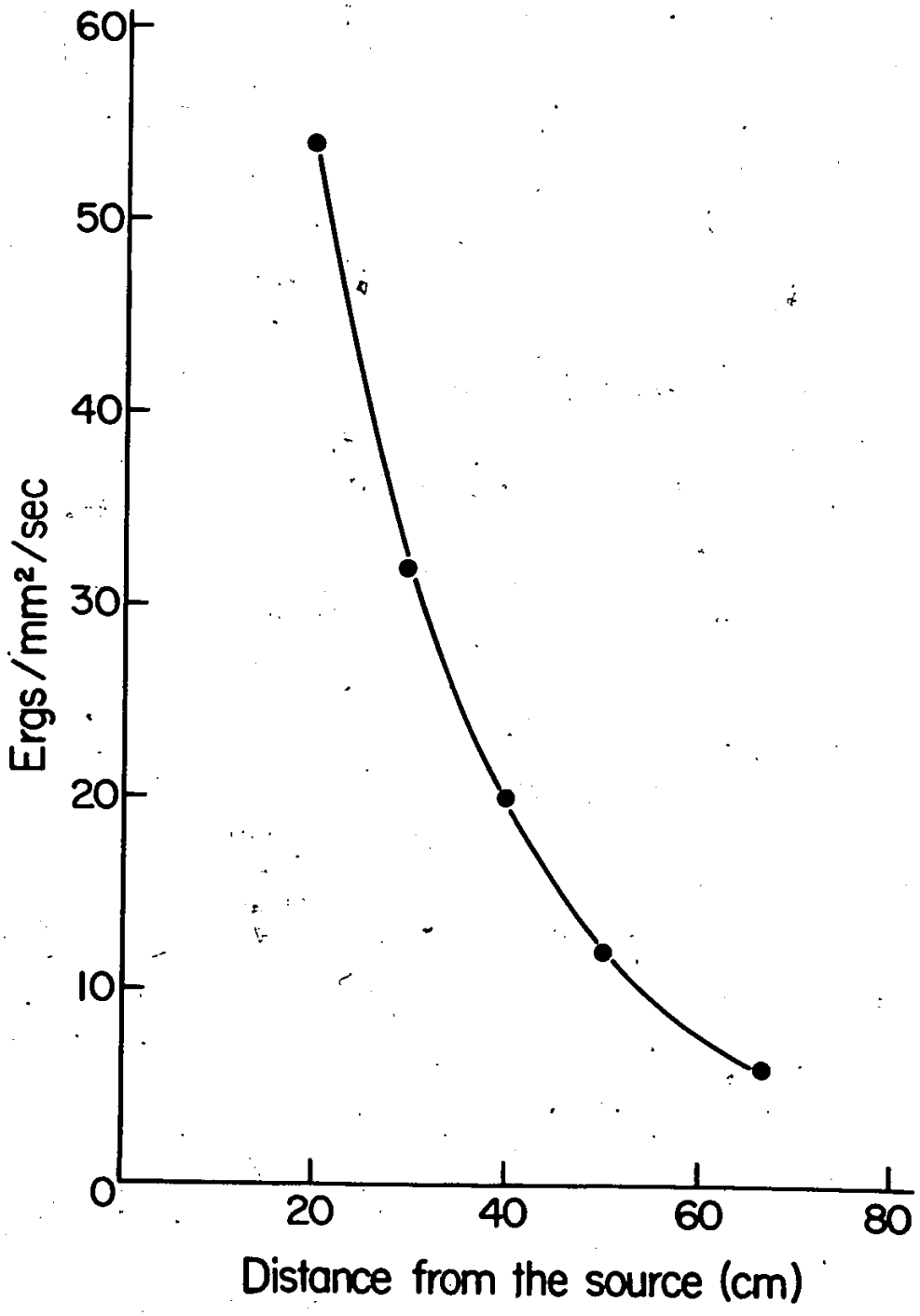
Fig. 2.1 shows the calibration of the lamp.

2.C Assay for DNA synthesis

To measure DNA synthesis, the incorporation of (^{3}H)-thymidine (40Ci/mM, Amersham) was assayed at 24, 48,96 and 120 hrs. The isotope was added 2 hrs prior to harvest in 50µl PBS to a final concentration of 2µCi/ml. Cells were kept in an incubator at 37°C.

To harvest the cells, either manifold or harvester was used; when I used the harvester, the stimulation index was usually greater than that obtained using the manifold. By using the harvester, lymphocytes were lysed by pumping water through the filters, which were partially dried by vacuum, removed from the harvester and left to air dry. Dried filters were placed in small plastic vials, filled with 4 ml of scintillation solution (Scintilene, Fisher) and counted on the Beckman model LS233 liquid scintillation counter for 2 min.

Figure 2.1 Calibration of UV lamp. Its calibration was carried out using a Black-Ray ultraviolet Meter, Model J-225.


60
50
40
30
20
10
0
Ergs/mm²/sec
20
40
60
80
Distance from the source (cm)

2. D. Assay for RNA Synthesis

In experiments measuring RNA synthesis, the incorporation of (5,6-^{3}H) uridine (40 Ci/mM. Amersham) was assayed at 3, 10, 24, 48, 72 and 96 hrs.The isotope was added in 100 µl PBS to a final concentration of 5 µCi/ml to the culture 2 hrs before harvesting.

2.E. Assay for Protein Synthesis

In the assay of protein synthesis,leucine-depleted medium (no leu) from Gibco was used (only for those irradiated prior to addition of Con A) to avoid competition with cold leucine from RPMI 1640 medium, as suggested by Dr. Patrick Lai.

The incorporation of L-(4,5-^{3}H) leucine (Amersham) was assayed at 3, 10,24, 48, 72 and 96 hrs. The isotope was added to culture 2 hours prior to harvest in 100 µl PBS to a final concentration of 10µCi/ml.

In the last two cases, manifolds (Millipore) containing twelve wells each were used to layer cells onto glass microfibre filters (Whatman type GFC), having a diameter of 2.4 cm. Before sampling each filter was moistened with phosphate buffered saline (PBS). A 0.5-1 ml sample of cells was washed onto the filters using 20-30 ml of cold PBS.

Finally all cells were lysed with 5% cold trichloracetic acid and methanol while layered on the filters so that the intracellular isotope which was not incorporated into the RNA or protein was washed through the filter.

Filters were partially dried by vacuum, removed form the manifold and left to air dry. Dried filters were placed into glass vials, filled with 8-10 ml of scintillation solution.

The bars in figures indicate the range of values observed. In all cases survival curves were followed at the times when those parameters were measured and irradiated (84,120 ergs/mm^2/sec.

2.F Assay for (^{3}H)-Con A binding

After lymphocytes were irradiated, centrifuged, resuspended and counted to have a final concentration of 5×10^6 cells/ml in calcium-and-magnesium-free phosphate buffered saline (CMF-PBS), they were labelled with 100 µl (^{3}H)-Con A(New England Nuclear) isotonic saline(.25% NaCl) for different intervals of time(5, 10, 20, 40 and 60 min) to a final concentration of 1 µCi/ml. Finally both unirradiated and irradiated cells were incubated at 37°C.

Manifolds (Millipore) containing twelve wells each, were used to layer samples of lymphocytes onto glass microfibre filters (Whatman GFC). Prior to sampling, the filters were moistened with 0.5 ml of 0.5% bovine serum albumen (BSA) and vacuumed through the filter. This BSA treatment of the filters was repeated 3 times to reduce non-specific sticking of the isotope to the filters.

When 1 ml samples of cells were placed in the wells on the filters, the empty tubes that contained the samples were filled 3 times with ice cold CMF-PBS and emptied into the wells. The supernatant and washes were then vacuumed and washed through the filters with 20-30 ml ice cold CMF-PBS, leaving the labelled cells to be counted (Jones, G. 1973).

The filters were dried, placed into vials, and counted as described. To determine the percentage counts from sticking or binding of the (^{3}H) concanavalin A(^{3}H-Con A), filters treated first with BSA, and then with ^{3}H-Con A in CMF-PBS were routinely used as blanks for all the binding experiments (two for each sampling time). When just culture medium and the ^{3}H-Con A (no cells), were used the same background was recorded. This meant the background counts were due mainly to sticking of the ^{3}H-Con A to the filters (Mike H. Repacholi Ph D thesis).

2.F.1 Determination of the number of Con A Binding Sites on Human Lymphocytes

To determine the number of binding sites for Con A on human lymphocytes, it was first necessary to calculate the counting efficiency of the liquid scintillation counter.

This was achieved by adding 1 µCi of Con A to a filter paper within the counting vial, drying, and then adding the same volume of scintilene as with experimental samples.

Counting efficiency (C.E.) was then determined using

$$C.E. = \frac{\text{cpm (1 µCi counted in system)}}{\text{dpm (from 1 µCi)}}$$

The counting efficiency was found to be $(12.24 \pm 0.65) \times 10^{-2}$.

The number of Con A binding sites on the lymphocytes was determined knowing the activity of ^{3}H-Con A bound to cells, as measured by the liquid scintillation counter. This was converted to total desintegrations/min bound to the total number of cells/ml by dividing by the counting efficiency.

Converting the desintegrations/min to Ci and dividing by the specific activity of the ^{3}H-Con A (Ci/µlMol) one obtains the number of m Mol of ^{3}H-Con A in cells, or the number of m Mol/cell by dividing this by the total number of cells/ml.

If the number of atoms of Con A bound per cell is equivalent to the number of binding sites/cell, then multiplying Avogadro's number (atoms/mMol) by the number of mMol of Con A/cell gives the number of binding sites for Con A/cell.

2.G. Potassium influx

After lymphocytes were irradiated with 84 ergs/mm^2, they were concentrated to a 1.5 x 10^6 cells/ml and Con A was added for 2.5 hr.

At specified times after mitogen addition approximately 40µCi (in 25 µl) of ^{86}RbCl were added to each ml sampled by centrifugation of 200 µl aliquots of culture through a mixture of corn oil (Mazola) and n, n-dibutylphthalate (10:3) on a Beckman microfuge (Oliver and Paterson, 1971)

The tips of the tubes were cut off and radioactivity in the pellet and the supernatant counted in a gamma counter.

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

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

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

2. K^+ uptake (fmoles/cell)

$$= \frac{(\text{counts/pellet}) - (\text{counts in trapped medium})}{\text{SRM x viable cells/pellet}}$$

In this experiment, correction for trapped space was done using a zero time uptake; cells and isotope were pre-cooled at 4°C for 15 min mixed rapidly and immediately sampled and spun within 15-30 sec of mixing.

Means of Rb influx presented herein are accompanied by their standard errors (SEM).

2.H. Preparation for Electron Microscopy.

Resting and stimulated human lymphocytes were irradiated (84 ergs/mm^2) and prepared for electron microscopy using standard fixation, dehydration and embedding procedures.

In brief the following procedures were conducted:

A Fixation:

i. Irradiated and non-irradiated were placed in an equal volume of 2% formaldehyde, 2% glutaraldehyde in 0.05M sodium cacodylate buffer (pH. 7.2) at room temperature for 1 hour.

ii. Cells were washed three times (5 min each) in 0.05M sodium cacodylate buffer (pH. 7.2).

iii. Cells were pelleted and resuspended in 1% OsO_4 in 0.05M sodium cacodylate at 4°C for 1 hour.

iv. Cells were washed three times as described in step 2.

B. Dehydratation:

i. Cells were dehydrated in (25,50,70%) ethanol. Lymphocytes were kept 15 min in each alcohol after they had been centrifuged.

Cells were left in the refrigerator overnight.

ii. Two changes in 95% Ethanol for 15 min each.

iii. Two changes in absolute ethanol for 15 min each.

iv. Two changes in propylene oxide for 15 min each.

v. Warm to room temperature.

C. Embedding:

Lymphocytes were left in :

i. Propylene oxide /resin mixture (3:1) for 1 hour.

ii. Propylene oxide /resin mixture (1:1) for 1 hour.

iii. Propylene oxide /resin mixture (1:3) overnight.

iv. Pure resin mixture for 24 hours.

v. Place tissue in capsules filled with fresh resin mixture.

vi. Place capsules in ordinary oven at 78°C. Allow polimerization to proced for 48 hours.

2.G Measurement of thymidine transport:

Lymphocytes were irradiated (84ergs/mm^2/sec) after 44 hrs in Con A and the thymidine transport assay was carried out at 48 hours.

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 LMGB (leukocyte medium pH: 7.4-7.6, containing glucose and bovine serum albumen) at a concentration of 10.0×10^6 cells/ml. LMGB consisted of 10mM sodium phosphate buffer, (pH. 7.5), 0.9% NaCl. 0.1% bovine serum albumen 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 30µCi/ml in LMGB, after the appropriate incubation time (15-60 seconds) 200 ul of reaction mixture was layered into a 400 µl microfuge tube (Canlab) containing 50µl of 7% perchloric acid as the bottom layer and 150 µl of silicone oil (12 volumes of Dow Corning 550 fluid and 13 volumes of Dow Corning 510 fluid).

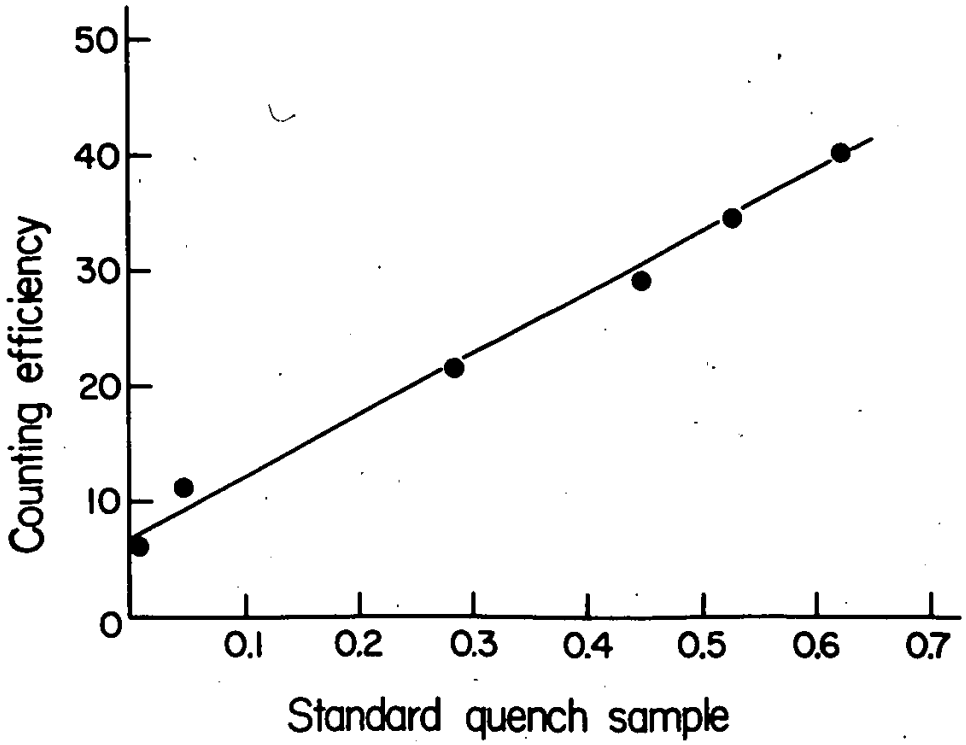
The tube was spun at 10,000 rpm for 20 seconds using a Beckman Microfuge. The microfuge tubes were frozen in ethanol /dry ice and the tips sliced into a scintillation vial for counting. Cell digestion was accomplished overnight with 1.0 ml of Protosol (New England Nuclear) at 37°C. This was then neutralised with 100 µL glacial acetic and counted in 7 ml of scintiverse. Counting efficiencies, which ranged between 24-35% were determined by internal quench correction.

A method of determining non-specific associated label was considered better than subtracting the intercellular volume because it accounts for non-specifific adsorption to the cell surface as well as for label that might be found in the intercellular space. Values in this range were routinely subtracted from cell-associated radioactivity at lower external thymidine concentrations in order to determine transport value (Rudd, C,M.Sc. Thesis).

Figure 2.3 shows the external quench curve, where S represents standard of quench samples, given by the counter machine. To get the counting efficiency for each samples,one has to divide the counts per minute (Cpm) over the desintegration per minute of each sample and multiply by 100.

N.B. Photolyase enzyme responsible for the monomireza-tion of thymidine dimers on DNA damaged by UV irradiation, has a weak activity on leukocytes. Therefore, it cannot be accounted any possible reduction in dimers on DNA that may result from its action. The photolyase enzyme also requires special conditions for its action such as: Special medium, temperature etc.

Figure 2.2: Thymidine transport quench curve:
Quench samples were counted in a Beckman Scintillation counter for 10 min counting efficiencies were determined by dividing CPM over DPM and multiplying by 100 as described in Materials and Methods.


Counting efficiency
50
40
30
20
10
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
Standard quench sample

2.H. Statistics

The modified t test was used to determine the statistical significance between two points. This test takes into account any differences that may occur in the standard deviations (a_1 , a_2). If the standard deviations are not compatible (a conservative assumption), then the degrees of freedom (df) for the t test determined using the formula:

$$df = \frac{\left[a_1^2/(N_1-1) + a_2^2/(N_2-1) \right]}{\left[a_1^2/(N_1-1) \right] /(N_1+1) + \left[a_2^2/(N_2-1) \right] /(N_2+1)}$$

Where N_1 and N_2 were the number of samples used to determine the means $\bar{X}_1$ and $\bar{X}_2$, and standard deviations a_1 and a_2 respectively.

The value of t was determined using:

$$t = \frac{\bar{X}_1 - \bar{X}_2}{\sqrt{a_1^2/(N_1-1) + a_2^2/(N_2-1)}}$$

With the values of t and df known, the level of significance for the two-tail test was then determined by referring to tables listing the distribution of t for given degrees of freedom.

The Kolmogorov-Smirnov two samples test (Siegel 1956) compares the distributions as a whole by finding the maximum difference between the two emperical distributions and determining whether this difference is significant, given that the population distributions were equal.

2.I. DNA repair synthesis- Autoradiography

To determine if UV light produced repair to damage in the DNA, the following procedure was undertaken:

(i) Lymphocytes were isolated and stimulated with Con A for 20 hr, then

(ii) Cells resuspended in PBS and exposed to dose of 84 ergs/mm^2 UV. Immediately after, cells were centrifuged and labelled with ^{3}H-thy(10 uCi/ml) for 2 hr.

(iii) Cells were washed twice in cold PBS, resuspended in 1 ml PBS + 1 ml 2% gluteraldehyde in 0.1 M PO_4 (pH 7.2) and left overnight.

(iv) Cells were pelleted, resuspended in cold PBS and left 30 min (On ice).

(v) Step (iv) was repeated twice again

(vi) Cells were pelleted, and resuspended in 4-5 drops of fetal calf serum.

(vii) Cells were smeared onto clean slides and air dried overnight.

(viii) Following 2 washes in PBS and 2 washes in distilled water, the cells were air dried.

(ix) Slides were dipped in diluted (1:1 water + 2% glycerol) nuclear emulsion (Kodak NTB-3), dried, wrapped light tight and kept in the refrigerator(4°C) for 2-3 weeks.

(x) Following development, fixing and washing, the cells on the slides were stained in a 2% solution of Giemsa for up to 15 min, then dried for silver grain counting.

CHAPTER III
RESULTS

3.A Gross effects of UV irradiation on growth and survival curves: Irradiation at 0 h, 24 h, and 44 h incubation with mitogen.

Figures 3.1, 3.2 and 3.3 show the effect of different doses of UV light on DNA synthesis in Con A stimulated human lymphocytes when exposed at 0 (immediately prior to addition of Con A), 24 or 44 hrs respectively. In 0 hr UV cells, Con A was removed at 22hr.

Survival was determined in all cases at 24, 48 and 72 hours. The figures show ^{3}H-thymidine incorporation for each group of treated cells at the times described above.

In figure 3.1 where lymphocytes were irradiated at 0 hr with 30, 42 and 60 ergs/mm^2/sec the incorporation of ^{3}H-thy was reduced more than 60% compared with control cells. In the case of cells treated with 84, 120, and 150 ergs/mm^2/sec, the inhibition of ^{3}H-thy was even greater (95%).

As can be seen in the same figure, cell survival dropped steadily to 45-50% in cells exposed to the highest doses of UV light (120, 150 ergs/mm^2/sec), while it remained above 60 per cent in cells treated with lower doses.

Fig. 3.2 shows that in the case of cells irradiated at 24 hr UV had almost no effect in lymphocytes exposed to a dose of 30 ergs/mm^2/sec, its effect starts to be seen in cells irradiated with 42, 60 and 84 ergs/mm^2/sec, and is well marked in those cells that were given a dose of 120, 150 ergs/mm^2/sec. Also it can be seen that cell survival dropped rapidly to 45-60% in cells exposed to 84,120 and 150 ergs/mm^2/sec, whereas it remained above 70 per cent at the other doses.

In fig. 3.3, lymphocytes irradiated at 44 hrs shows that at low doses, (30, 42, 60, ergs/mm^2/sec) there was some inhibition from which the cells were able to recover. But the inhibition was well marked at higher doses (84,120, 150 ergs/mm^2/sec) from which the cells did not recover. Also in the same figure cell survival dropped quickly to about 70 per cent in cells irradiated with 120 and 150 ergs/mm^2/sec, while it remained above 75 per cent in cells treated with lower doses.

The difference in percentage survival might be explained by the time at which cells were irradiated. For cells treated at 44 hrs, survival was only followed for another 24 hrs (Fig. 3.3). However, cells given UV light at 0 or 24 survival curves were followed longer, that is, for 72 and 48 hrs after irradiation respectively (Figs. 3.1, 3.2). In all fig, DNA synthesis decreased as the dose of UV light increased reaching its peak at 48 hrs. The effect was independent of either the length of time in Con A or when cells were treated with UV light.

Figure 3.1: Incorporation of ^{3}H-thymidine into Con A stimulated human peripheral lymphocytes irradiated at 0 hrs, and their survival curves.

Human lymlhocyte cultures were prepared as described Materials and Methods. Incorporation of ^{3}H-thymidine (2µCi/ml/2 hrs) was assayed at different time points following UV irradiation and Con A addition. UV doses varied. Resting counts were 1.2-3.0 x 10^{3} c.p.m.

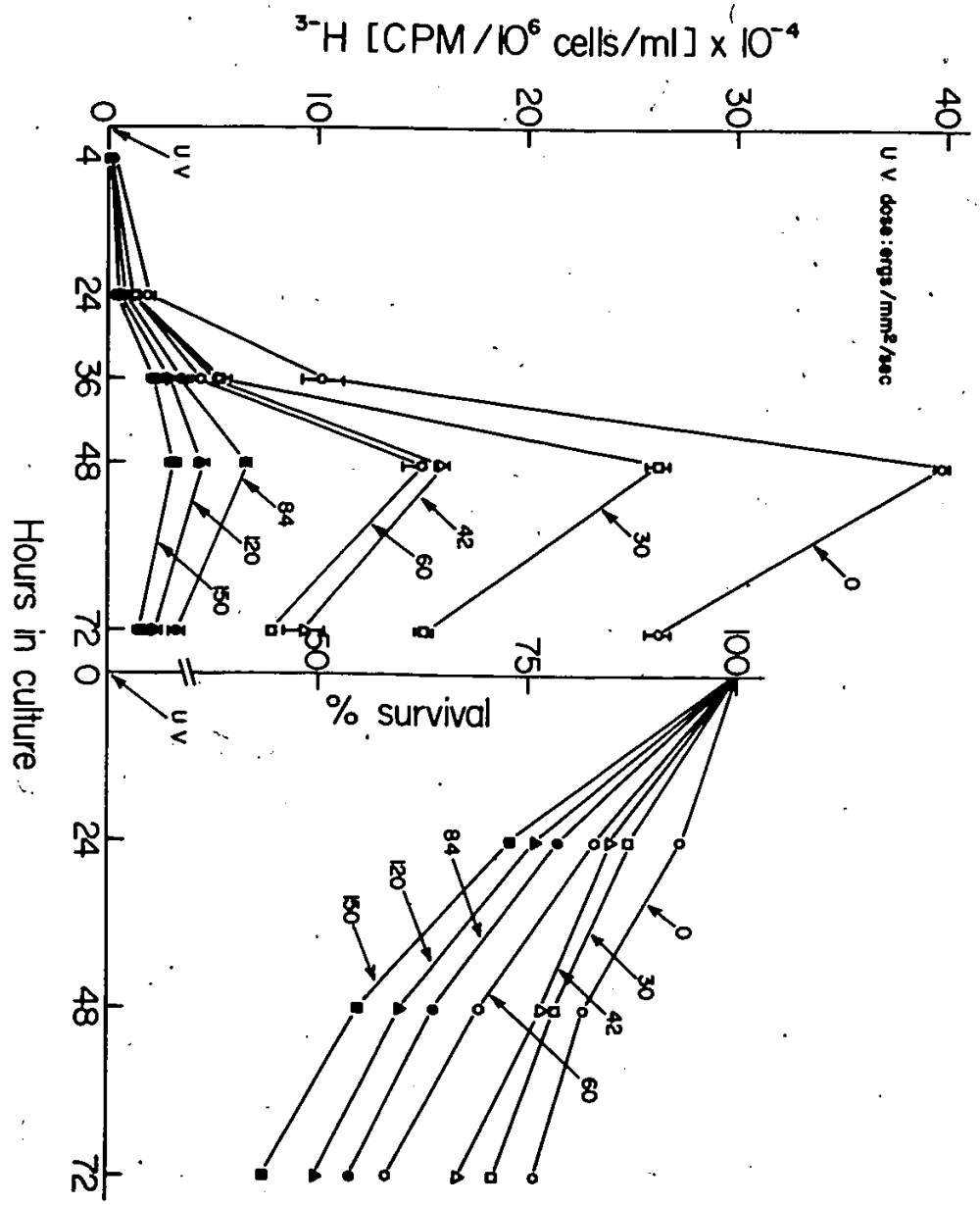

^{3}H [CPM / 10^{6} cells/ml] x 10^{-4}
0
10
20
30
40
uv
4
24
36
48
72
uv dose: ergs/mm^{2}/sec
84
120
150
60
42
30
0
Hours in culture
0
50
75
100
% survival
uv
24
48
72
84
120
150
0
30
42
60

Figure 3.2: Incorporation of ^{3}H-thymidine into Con A stimulated human peripheral lymphocytes irradiated at 24 hrs, and their survival curve.

Human lymphocyte cultures were prepared as outlined in Materials and Methods. The incorporation of ^{3}H-thymidine (2μCi/ml/2 hrs) was assayed at different time points following UV irradiation and Con A addition. UV doses varied. Resting counts 1.5-2.5 x 10^{3} c.p.m.

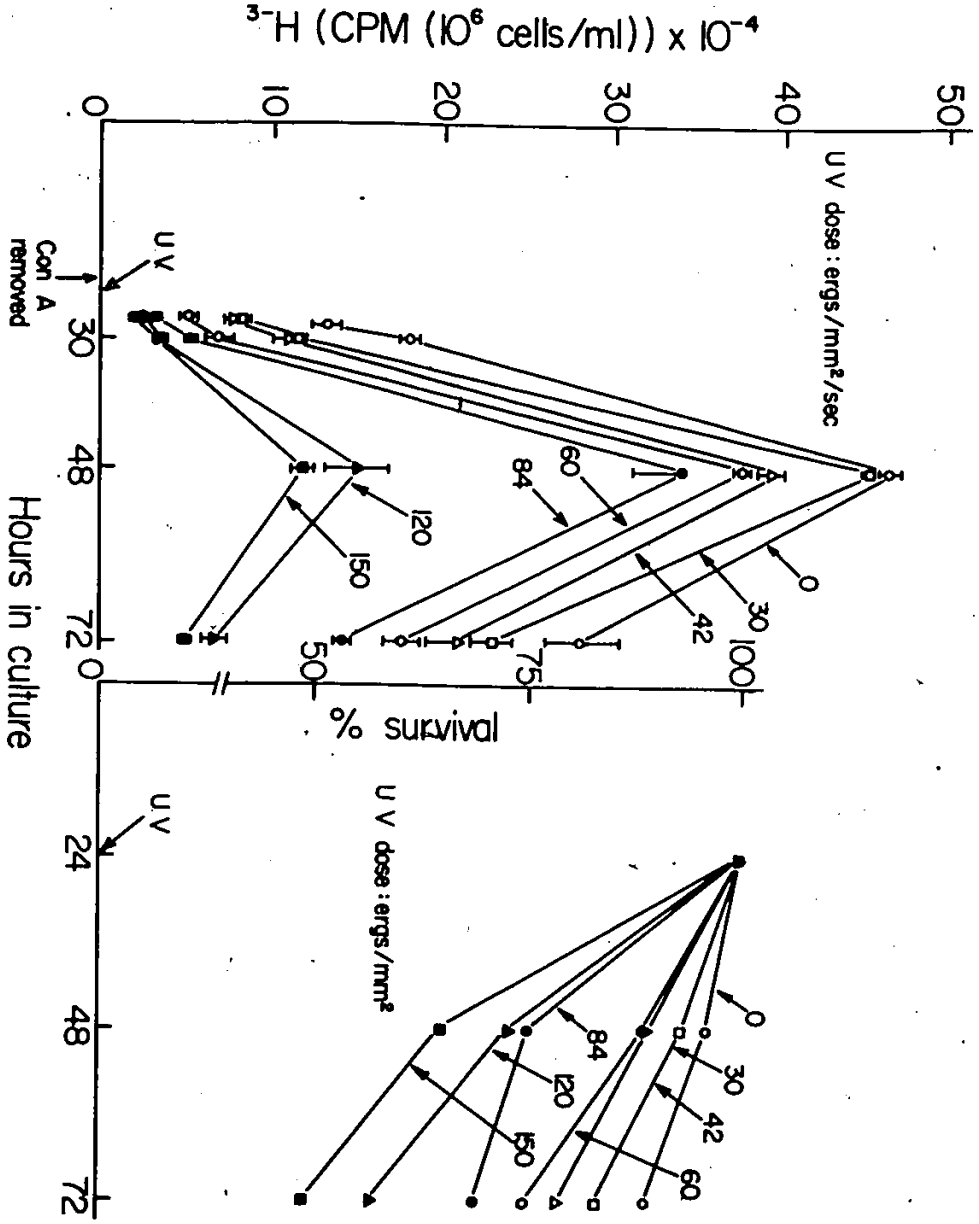
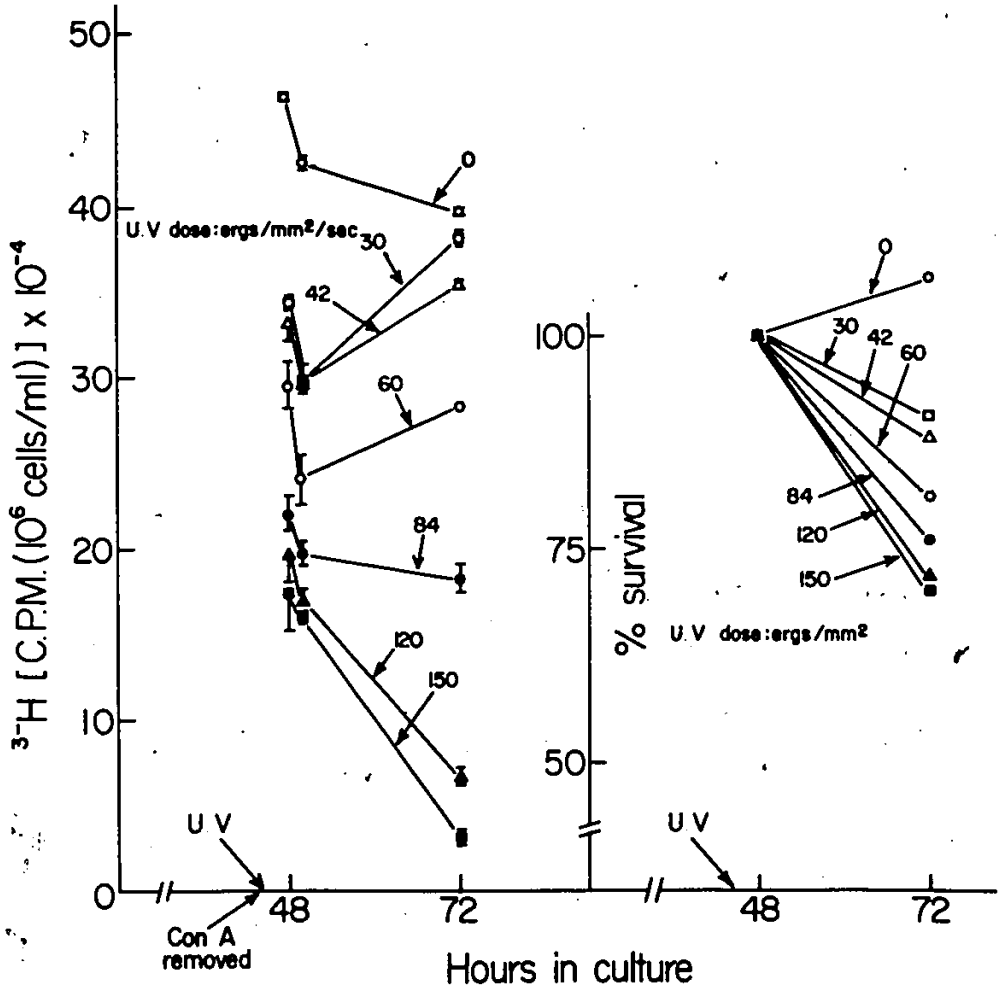

^{3}-H (CPM (10^{6} cells/ml)) x 10^{-4}
0
10
20
30
40
50
UV dose : ergs/mm²/sec
UV
Con A removed
30
48
72
Hours in culture
0
50
75
100
% survival
0
120
150
60
84
42
30
UV
24
48
72
UV dose : ergs/mm²
0
84
120
150
30
42
60

Figure 3.3: Incorporation of ^{3}H-thy into Con A stimulated human lymphocytes irradiated at 44 hrs, and their survival curves.

Human lymphocytes cultures were prepared as described in Materials and Methods. The incorporation of ^{3}H-thy (2µCi/ml/2 hrs), was assayed at different time points following UV irradiation and Con A addition.
UV doses varied. Resting counts ranged between 4.0 - 4.4 x 10^{3} c.p.m.


50
40
30
20
10
0
^{3}H [C.P.M.(10^6 cells/ml)] x 10^{-4}
U.V dose:ergs/mm^2/sec
0
30
42
60
84
120
150
U V
Con A removed
48
72
Hours in culture
100
75
50
% survival
U.V dose:ergs/mm^2
0
30
42
60
84
120
150
U V
48
72

3.B. Dose response curve:

The data of figures 3.1, 3.2 and 3.3 are plotted in figures 3.4 and 3.5 as a percentage of control incorporation of ^{3}H-thy assayed at 48 and 72 hr versus dose of UV.

In figures 3.4 and 3.5, cells irradiated prior to addition of Con A showed a sharp decrease of label uptake as a function of UV dose lower than 84 ergs/mm^2/sec, above which the slope of the inactivation curve decreased.

In the same figures, statistical analysis of experimental results of lymphocytes harvested at 48 and 72 hrs showed that cells irradiated either prior to addition of Con A or 24 or 44 hr after being in the presence of Con A were significantly different ($p = 0.001$) each other.

As in the case of the stimulated cells assayed at 48 hr, DNA synthesis at 72 hr was most sensitive to UV when this was administered at the onset of culture with Con A there was little difference between the sensitivity of the cells irradiated at 24 h or at 44 hr culture.

Since the 0 h cells are 30 h away from S phase, the UV affects most strongly the activation process rather than the DNA synthesis itself.

Figure 3.4: Incorporation of ^{3}H-thy as a percentage of control at 48 hours.

Human peripheral lymphocytes irradiated at 0, 24, or 44 hrs pulsed with ^{3}H-thy (2μCi/ml) for 2 hrs were harvested at 48 hrs as described in Materials and Methods.

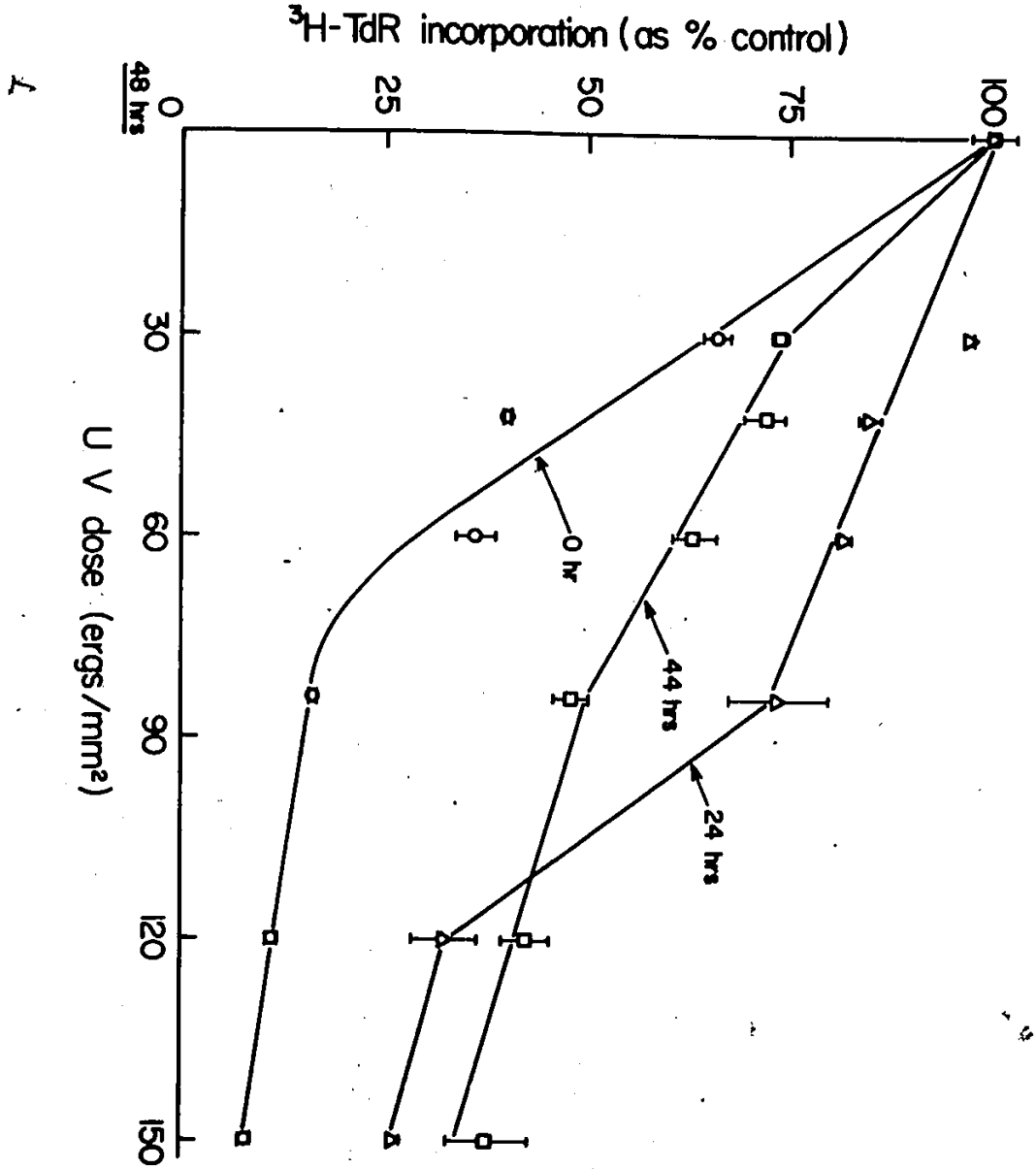
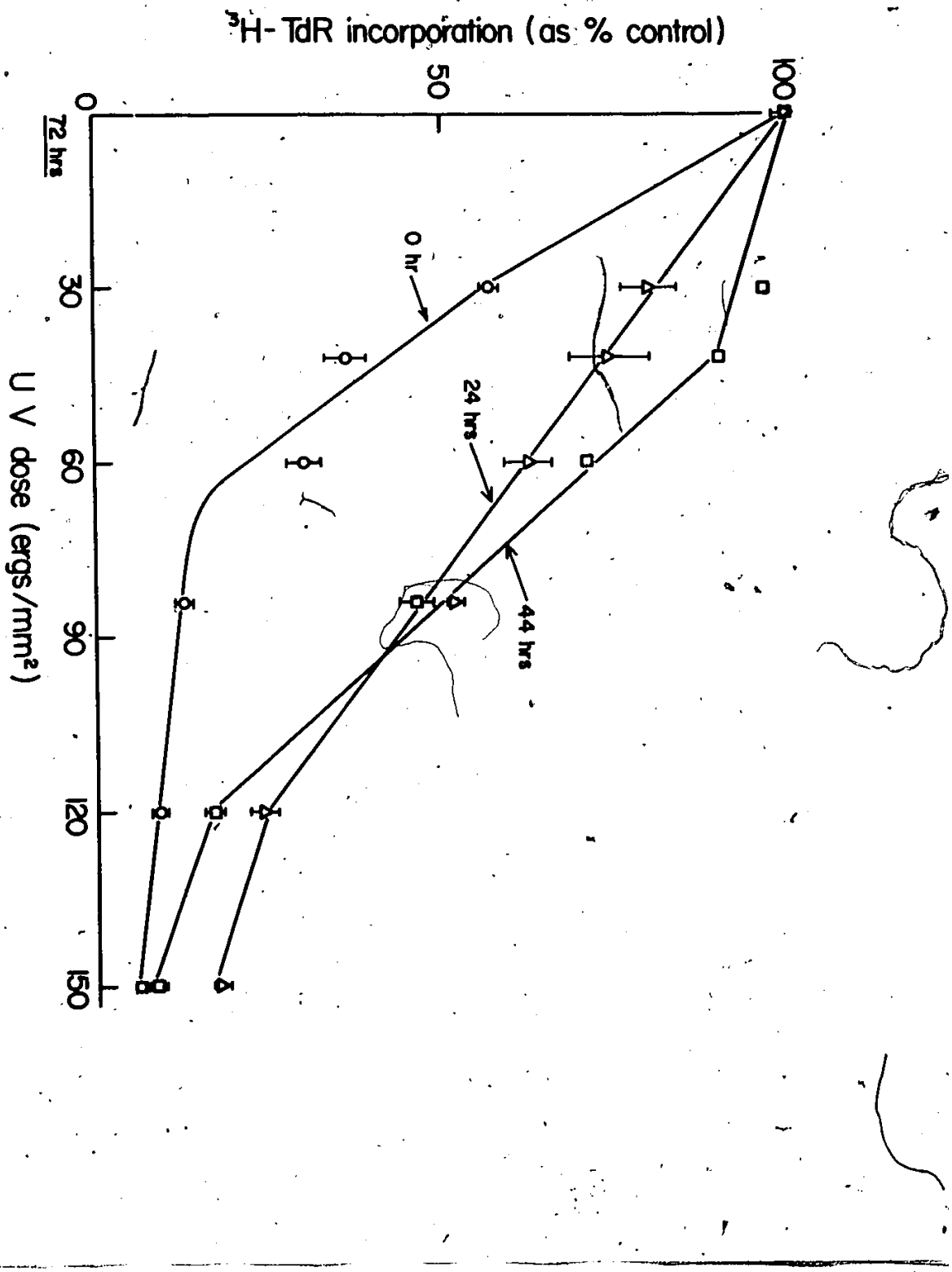

^{3}H-TdR incorporation (as % control)
0
25
50
75
100
48 hrs
30
60
90
120
150
U V dose (ergs/mm²)
0 hr
44 hrs
24 hrs

Figure 3.5: Incorporation of ^{3}H-thy as a percentage of control at 72 hours.

Human peripheral lymphocytes irradiated at 0, 24 or 44 hrs were labelled with ^{3}H-thy (2µCi/ml) for and harvested at 72 hrs as described in Materials Methods.


^{3}H-TdR incorporation (as % control)
0
50
100
72 hrs
0 hr
24 hrs
44 hrs
30
60
90
120
150
U V dose (ergs/mm^2)

3.C Comparative effect of UV irradiation on DNA, RNA, and Protein Synthesis.

The previous section demonstrated a marked effect of UV on DNA replication. We wished to to extend this observation to determine the effect of UV light on RNA and protein synthesis by measuring the incorporation of appropriate radioactive precursors (^{3}H-uri for RNA, ^{3}H-leu for proteins) into Con A stimulated human peripheral lymphocytes *in vitro* in an attempt to determine a threshold level of exposure and to obtain a reproducible end-point for the evaluation of the possible mechanisms of action of UV light.

It was also of interest to me to determine wheter UV light could affect these events at early stages of activation (i.e. when irradiation is given at 0 hr).

Figures 3.6 to 3.8 show the results of the incorporation of the appropriate radioactive precursors in lym - phocytes exposed to Con A for varying lengths of time prior to exposure to 84 ergs/mm^2/sec UV light.

Figures 3.6 shows the incorporation of ^{3}H-leu and ^{3}H-uri into proteins and RNA of lymphocytes exposed to Con A for 21 or 35 hr and to a 84 ergs/mm^2/sec dose of UV light.

In control cells for the 0 hr irradiation (D) for protein, (E) for RNA and for the 22 hr (A) and 36 hr (B) irradiation periods, Con A was removed at 21 hr and 35 hr respectively. The cells were washed in PBS and re - placed in fresh RPMI-1640 medium.

The same procedure was given to the cells irradiated at 0 hr (D')(E'), 22 hr (A') and 36 hr (B'). In relation to protein and RNA synthesis, there was virtually total inhibition of incorporation of ^{3}H-leu(D') and ^{3}H-uri (E') in the cells irradiated at 0 hr, showing only 5% compared to control. The effect of UV on RNA and protein synthesis was detectable 4 hr after irradiation.

In cells irradiated at 22 or 36 hr, the effect of 84 ergs/mm^2/sec UV dose on protein synthesis seemed negligible since label incorporation levels were practically the same as those of the unirradiated controls at 96 hr.

Nevertheless, cells irradiated at 22 and 36 hr showed a great decrease in DNA synthesis (Figures 3.4 and 3.5). This indicates that not only does UV affect an early event but also that it upsets one or more events at a later stage in the cell activation that affect DNA but not RNA or protein synthesis.

The data of figure 3.6 are plotted in figure 3.7 as incorporation of ^{3}H-leu as a percentage of control incorporation, versus time of incubation(hrs).

In figure 3.7 cells irradiated (84 ergs/mm^2/sec) immediately prior to addition of Con A are the most affected showing a severe inhibition of incorporation of the radio label from which cells are not able to recover during the time of incubation (96 hr). On the other hand cells irradiated at 24 and 36 hr show very little effect of UV irradiation.

As far as protein synthesis is concerned, UV light seems to affect cells irradiated at 0 hr but not cells exposed to irradiation at 24 or 36 hr(Figures 3.6 and 3.7).

Thus UV affects the activation process leading to increased protein synthesis but not this synthesis itself.

Figure 3.6: Incorporation of ^{3}H-leu and ^{3}H-uri in Con A stimulated human lymphocytes irradiated at 0,24 or 36 hr.

Human peripheral lymphocytes were irradiated with 84 ergs/mm^{2}/sec and labelled with ^{3}H-leu (10 µCi/ml) and ^{3}H-uri(5 µCi/ml) as described in Materials and Methods. In non-irradiated cells Con A was removed at 21 hr (A),(D),(E)and at 35 hr(B).
In D',E',A'and B'Con A was removed at the time of irradiation: 0, 22 and 36 hr respectively.

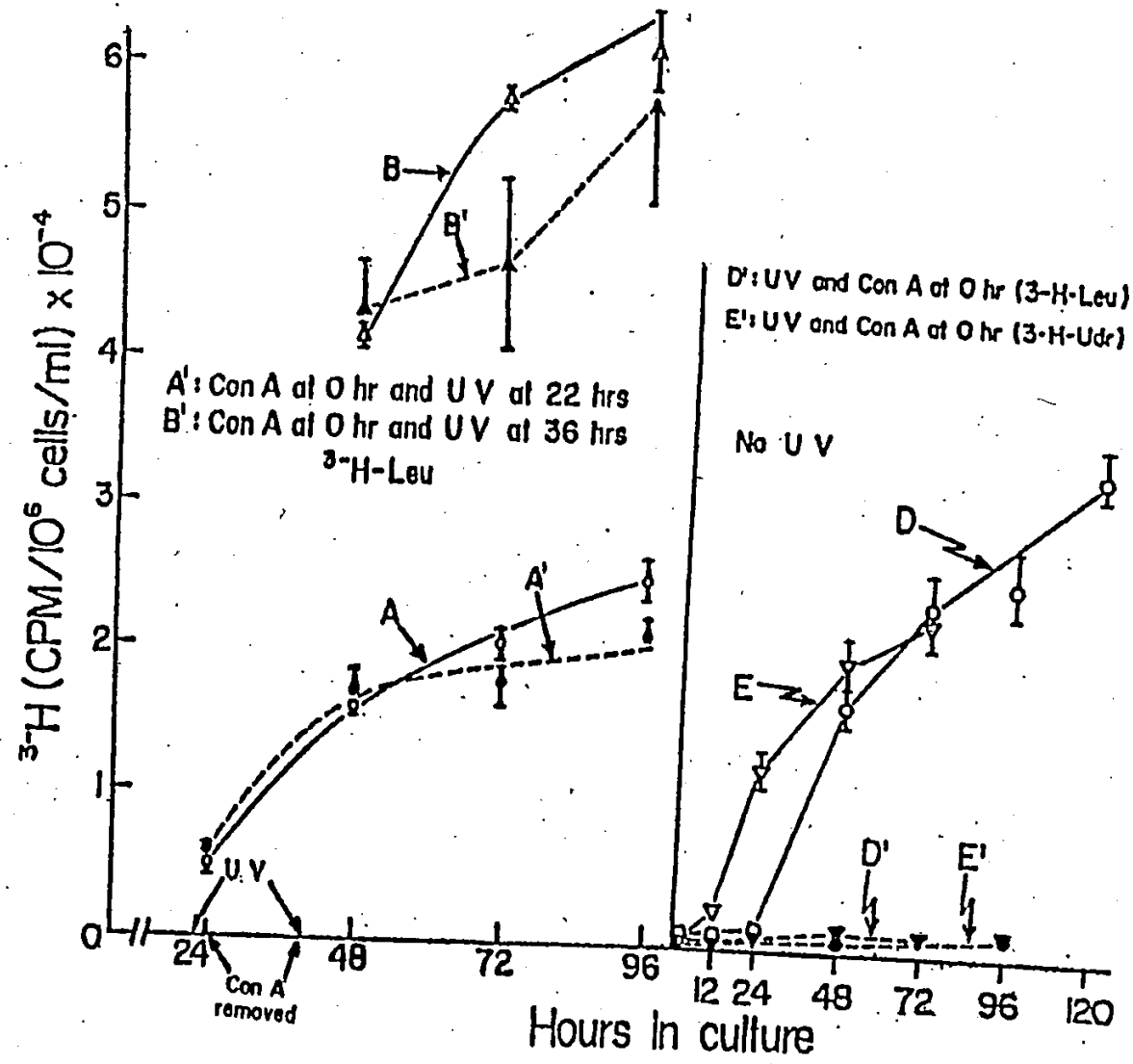
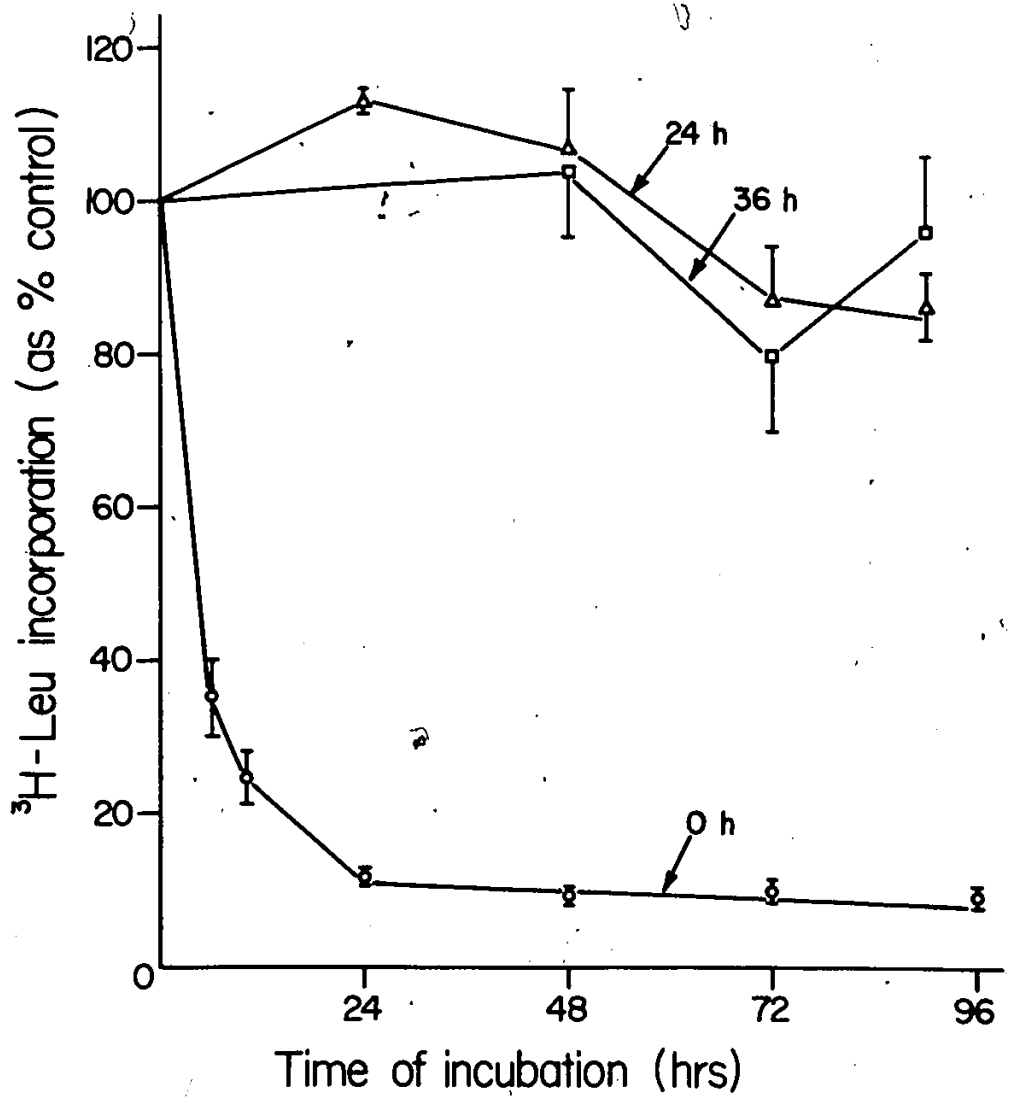

^{3}H (CPM/10^6 cells/ml) x 10^{-4}
6
5
4
3
2
1
0
B
B'
A'
A
A': Con A at 0 hr and U V at 22 hrs
B': Con A at 0 hr and U V at 36 hrs
^{3}-H-Leu
D': UV and Con A at 0 hr (3-H-Leu)
E': UV and Con A at 0 hr (3-H-Udr)
No U V
D
E
D'
E'
U V
24
Con A removed
48
72
96
12 24
48
72
96
120
Hours in culture

Figure 3.7 Incorporation of ^{3}H-leu as a percentage of control.

Human peripheral lymphocytes were irradiated with 84 ergs/mm^2/sec prior to addition of Con A, 24 and 36 hrs and labelled with ^{3}H-leu (10 µCi/ml) for 2 hours as described in Materials and Methods.


120
100
80
60
40
20
0
24 h
36 h
0 h
^{3}H-Leu incorporation (as % control)
24
48
72
96
Time of incubation (hrs)

3.D. Membrane Effects

3.D.1 Effects of UV irradiation on potassium influx

To determine whether irradiation altered the K^+ influx across the cell membrane, experiments were undertaken to measure the amount of ^{86}Rb transported into cells following a UV dose of 84 ergs/mm^2/sec. Isotope was added 2.5 hr after incubation with Con A.

Figure 3.8 shows the UV effects on potassium influx in both resting and Con A-activated human peripheral lymphocytes irradiated with 84 ergs/mm^2/sec immediately prior to addition of Con A. Inhibitory effects were noted even after 30 min of incubation with ^{86}Rb (3 hr after Con A addition). Potassium influx in resting cells was not significantly ($p = 0.1$) inhibited.

Potassium influx, measured 2.5 hours after Con A addition, using ^{86}Rb as a tracer, was inhibited from 6.8 to 1.7 fmoles/cell/hr by UV irradiation prior to Con A addition.

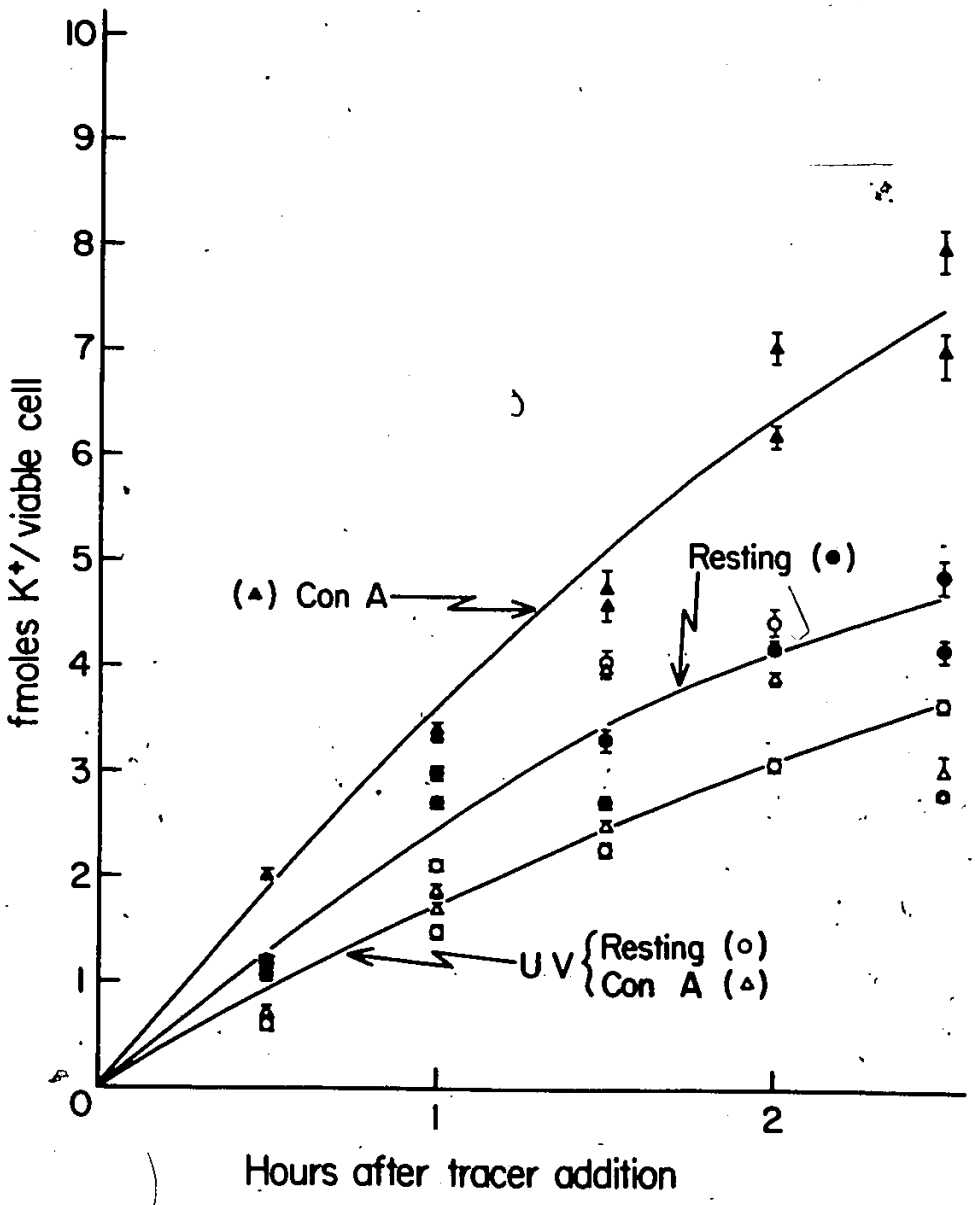
It can be seen that the effect of UV on stimulated cells is so drastic that influx of K^+ into the cells was reduced to levels lower than those of non-activated lymphocytes.

Thus, UV inhibits at least certain of the essential early events of activation at the lymphocyte cell surface.

Figure 3.6: Activation of potassium uptake in human peripheral blood lymphocytes, measured within 3 hours of addition of Con A.

Cells were irradiated with 84 ergs/mm^2/sec at 0 hr. ^{86}Rb (40 μCi/ml) was added 2.5 hr after beginning the incubation with Con A.

Two different experiments are shown. Quadruplicate determinations were performed at each time points of ^{86}Rb uptake.


10
9
8
7
6
5
4
3
2
1
0
fmoles K+/viable cell
(▲) Con A
Resting (●)
UV {Resting (○)
Con A (△)
1
2
Hours after tracer addition

3.D.2 Time Course of Tritiated Con A binding

In an attempt to investigate whether or not UV light affected Con A binding, resting lymphocytes were incubated with 1 μCi/ml of H-Con A for varying periods of time. The results are shown in figure 3.9 and table 3.1. The binding of Con A to the lymphocyte surface membrane was a time dependent process.

Reduction in rate of binding was evident even after 5 min. of incubation with labelled mitogen, where the difference was significant at p= 0.01. At 20 min there was a 25% reduction in binding, the difference being significant at p = 0.01. At 60 min there was a reduction of 28% in binding, the difference being significant at p = 0.02.

The number of binding sites per cell at 20 min was $6.6 \pm 0.5 \times 10^6$ in the unirradiated and dropped, to $4.2 \pm 0.4 \times 10^6$ in the irradiated, a reduction of 35%.

There are a number of possibilities that should be considered to explain this effect. For example, could the reduced binding be due to an increase in the rate of internalization, breakdown and release of the tritiated structure of the Con A molecule?. Do the lymphocytes quickly shed instead of internalizing the ^{3}H-Con A?. Both these possibilities could explain the observed result.

TABLE 3.1 TIME COURSE OF ^{3}H-CON A BINDING IN HUMAN PERIPHERAL LYMPHOCYTES AFTER UV LIGHT EXPOSURE OF 84 ERGS/MM^2

SAMPLING TIME(MIN.)	C P M* UNIRRADIATED	IRRADIATED	% DIFFERENCE
5	4192 ± 553	2092 ± 118	50 ± 3[a]
10	5928 ± 366	4977 ± 347	16 ± 2[b]
20	6957 ± 244	5186 ± 180	25 ± 2[c]
40	6050 ± 350	3655 ± 275	40 ± 4[d]
60	4604 ± 531	3325 ± 133	28 ± 3[e]

* UV irradiation immediately prior to addition of ^{3}H-Con A.

The difference between UV irradiated and non-irradiated controls were significant at the following levels:

a: $p = 0.01$

b: not significant at $p = 0.2$

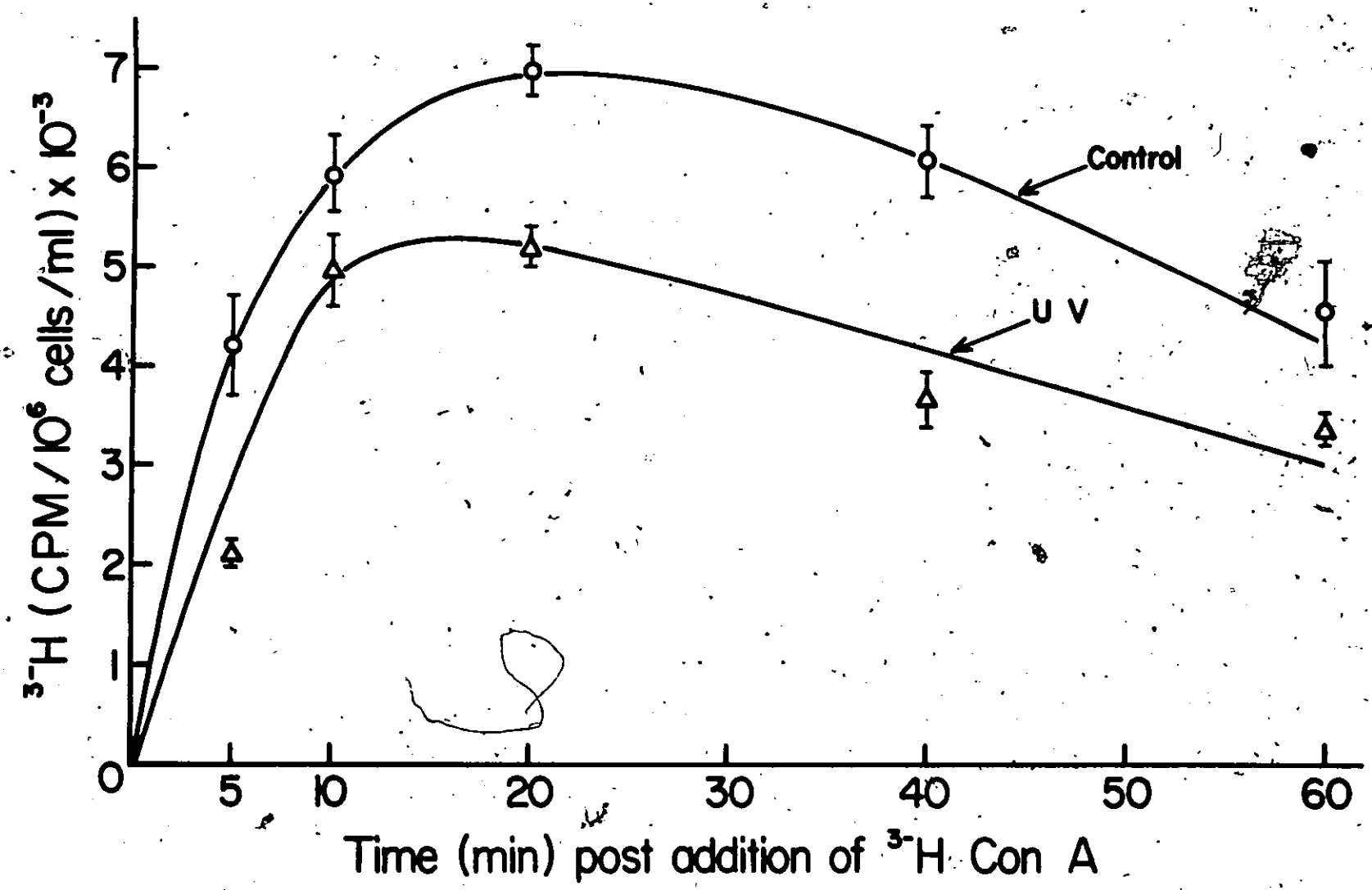
c: $p = 0.01$

d: $p = 0.01$

e: $p = 0.02$

Figure 3.9 : Time course of ^{3}H-Con A binding to human peripheral lymphocytes following exposure to 84 ergs/mm^2/sec at 0 hr.

Cells were resuspended in CMF-PBS at 5×10^6 cells/ml after UV dose and were incubated for different intervals of times at a temperature of 37 °C, as described in Materials and Methods.


^{3}H (CPM / 10^6 cells / ml) x 10^{-3}
0
1
2
3
4
5
6
7
5
10
20
30
40
50
60
Time (min) post addition of ^{3}H Con A
Control
U V

3.D.3 Thymidine Transport

In view of the inhibition of K^+ transport and Con A binding by UV, it seemed possible that the effect of UV on blastogenesis was due to an indirect effect on thymidine transport rather than on DNA synthesis. With that in mind, a series of experiments were conducted to measure the amount of thymidine translocated into cells that had been incubated with Con A for 44 hrs and then irradiated with 84 ergs/mm^2/sec.

Thymidine transport experiments were carried out at 48 hrs. As shown in table 3.2 when UV was given at that time, the incorporation of ^{3}H-thy into the DNA was inhibited 58 ± 2%, the difference being significant at $p = 0.001$.

To determine whether there was a difference in rate of transport at different thymidine concentrations, cells were incubated with label (20μCi/ml) at a number of concentrations of unlabeled thymidine at constant radioactivity. Lymphocytes were exposed to the isotope for 20 seconds to permit measurement of initial rates of transport uncomplicated by backflux of label (Strauss, et al. 1977).

Figures 3.10 and 3.11 show that there was no significant difference in transport of varying concentrations of ^{3}H-thy between the control and the UV treated cells.

Figure 3.10 shows that both control and UV-treated cells exhibit practically the same pattern of thymidine uptake. Thymidine uptake was saturable with increasing concentrations of cold nucleoside. At thymidine concentrations at or above 1.0 mM, there was no further decrease in cell associated radioactivity. Values in this concentration range were considered undiluted radioactivity. These counts were probably due to trapped medium and/or to adsorption of the nucleoside to cell membrane.

Figure 3.11 shows the thymidine transport expressed in pmoles/10^6 cells in cells both unirradiated and irradiated with 84 ergs/mm^2/sec. As can be seen, UV irradiation does not affect the transport of thymidine into cells. The transport of cold thymidine as a function of external concentration increased in a linear fashion until 10^{-4}M, starting to reach a plateau between concentrations of 2.5×10^{-4}M and 7.5×10^{-4}M.

One may reasonably conclude from these data that approximately the same amount of ^{3}H-thy is being made available for DNA incorporation in control and UV -irradiated cells.

Figure 3.10: Saturation of thymidine uptake in Con A stimulated human leukocytes irradiated with 84 ergs/mm^2/sec at 46 hr.

Con A stimulated human peripheral leukocytes cultured for 48 hours were suspended in pre-warmed LMGB at 10×10^6 cell/ml. The cell suspension was then mixed with an equal volume of LMGB containing thymidine concentrations between 5×10^{-5} to 5×10^{-2} M. The final radioactivity remained constant at 20uCi/ml. A 200ul aliquot o reaction mixture was then centrifuged using the microcentrifugation technique as outlined in Materials and Methods.

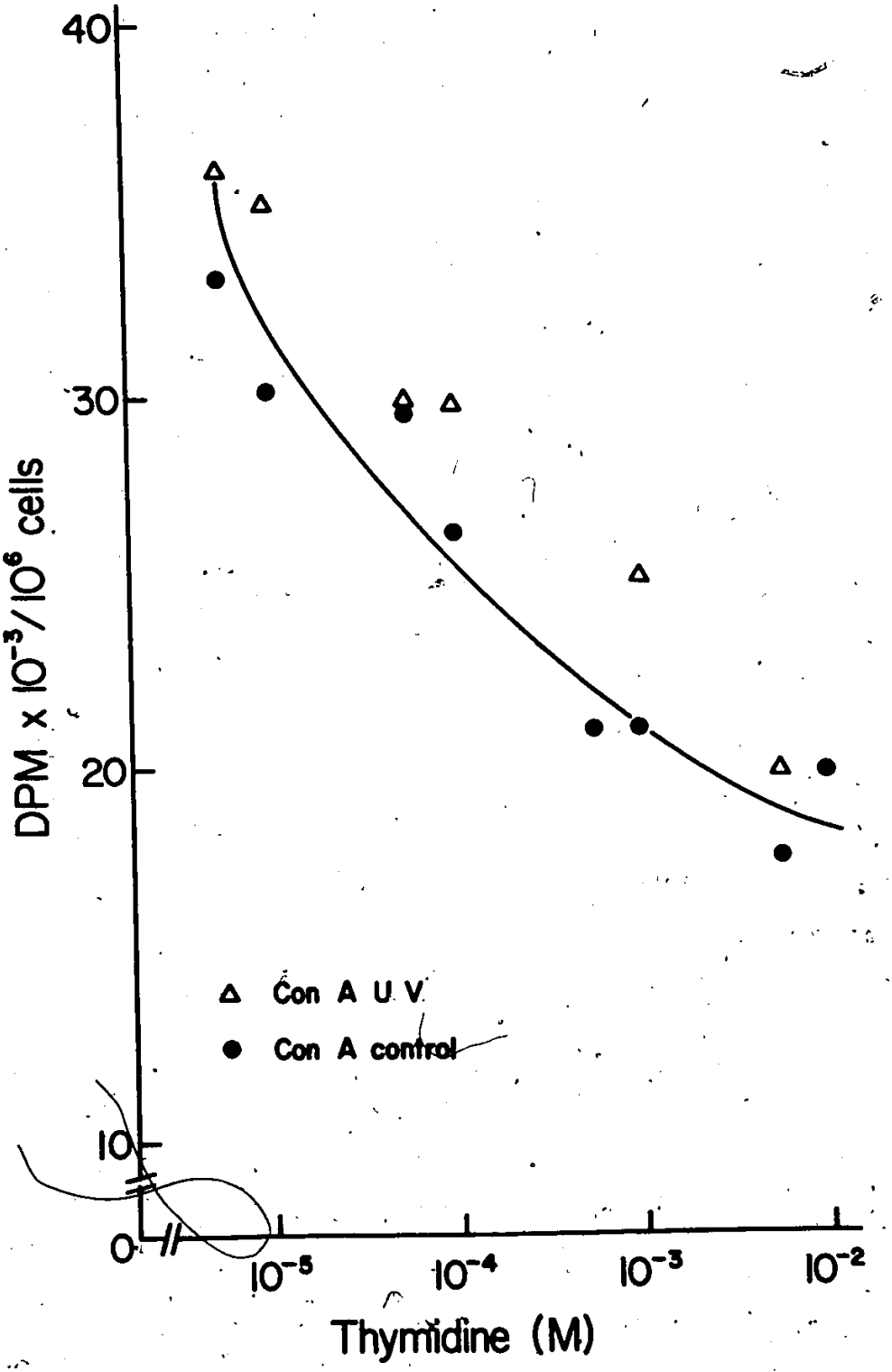

40
30
20
10
0
DPM x 10^{-3}/10^6 cells
Con A U.V.
Con A control
10^{-5}
10^{-4}
10^{-3}
10^{-2}
Thymidine (M)

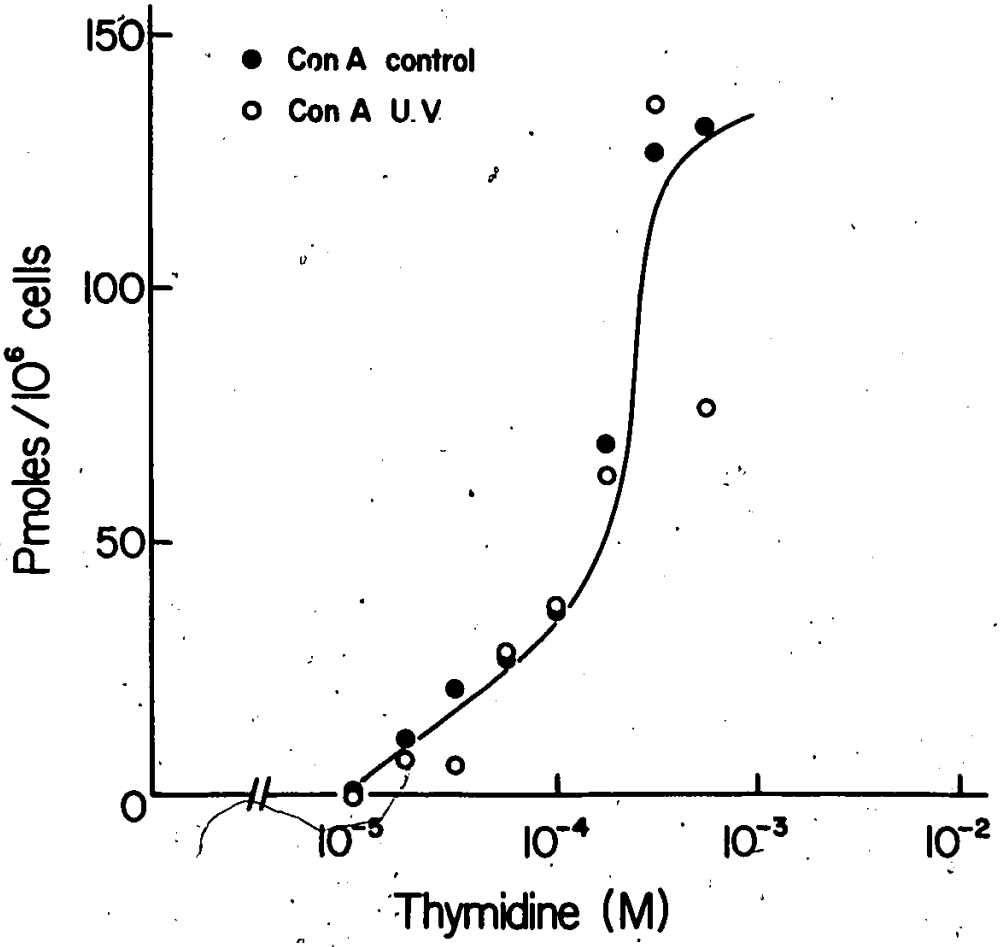
TABLE 3.2: INCORPORATION OF ^{3}H-THYMIDINE IN CON A STIMULATED HUMAN LYMPHOCYTES IRRADIATED WITH 84 ERGS/MM^2/SEC.

EXPT. N°.	C P M STIMULATED CONTROL	C P M* STIMULATED UV	% DIFFERENCE
1	465583 ± 32302	270568 ± 10639	58 ± 2
2	462841 ± 1649	220733 ± 9552	48 ± 2
3	291203 ± 16744	157249 ± 12522	54 ± 4

* UV irradiation was given at 44 hrs. The difference between UV irradiated and non-irradiated control were significant at the level, $p = 0.001$ in all three experiments.

Figure 3.11: Thymidine transport in Con A stimulated leukocytes as a function of external thymidine concentration, irradiated with 84 ergs/mm^2/sec at 46 hr.

Con A stimulated human peripheral leukocytes after 48 hours of culture were suspended in pre-warmed LMGB at a concentration of 10 x 10^6 cells/ml. The cell suspension was then mixed with an equal volume of LMGB containing thymidine concentrations between 10^{-5} to 5.0 x 10^{-3} M. The final radioactivity remained constant at 20uCi/ml. A 200ul aliquot of reaction mixture was then centrifuged using the microcentrifugation technique as outlined in the Materials and Methods.


150
100
50
0
Pmoles/10⁶ cells
Con A control
Con A U.V
10⁻⁵
10⁻⁴
10⁻³
10⁻²
Thymidine (M)

3.D.4. Time-Dependence of Thymidine Uptake

It was demonstrated by Rudd (M. Sc. Thesis) that a thymidine transport component exists in human lymphocytes cultured *in vitro* with Con A for 48 hours.

Figure 3.12 shows the presence of time-dependent thymidine net transport in human peripheral leukocytes cultures after a 48 hours incubation with Con A.

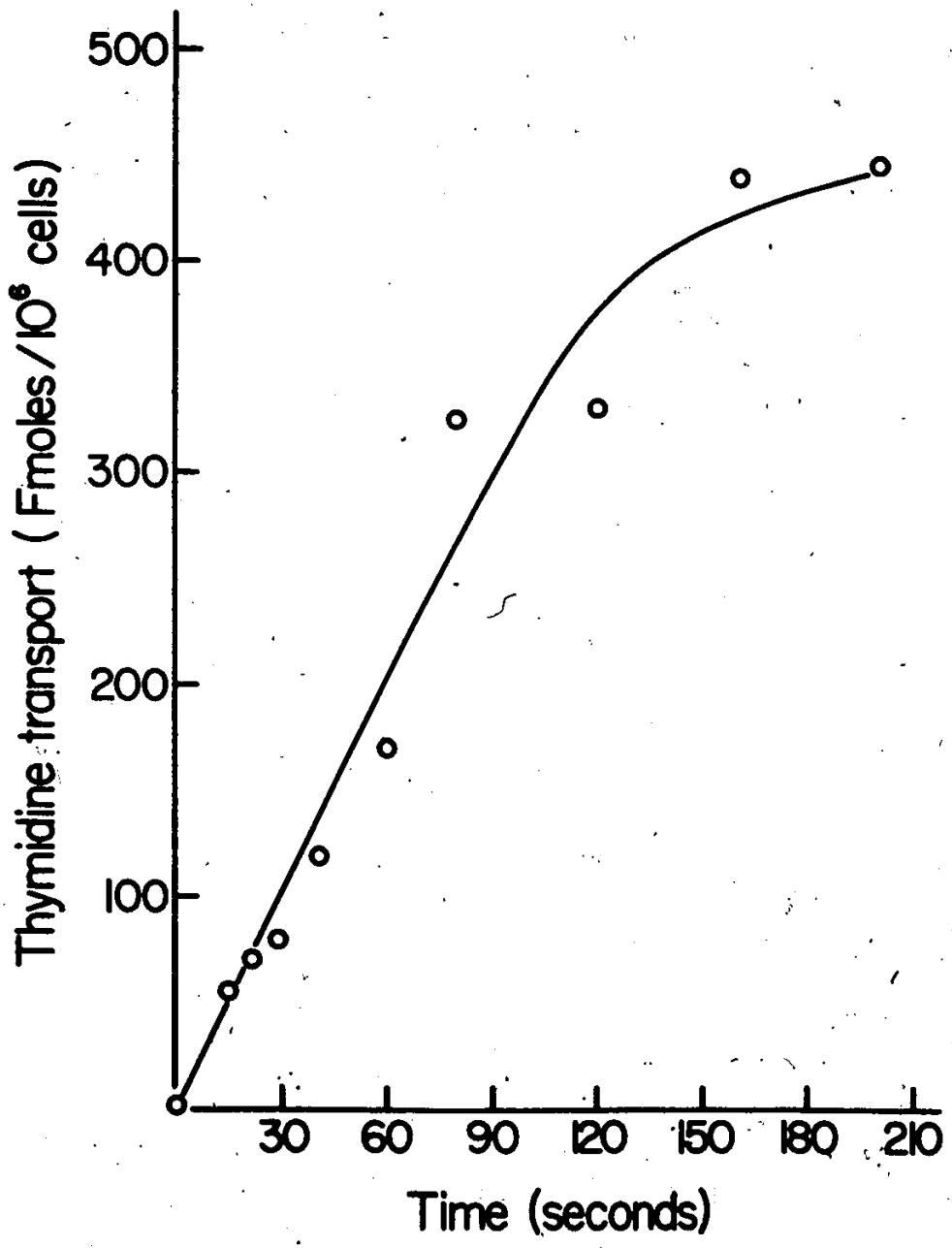
Thymidine transport was observed to continue in a linear fashion during the 60 sec period tested and lasted for approximately 100 sec, after which thymidine transport started to reach a plateau. The plateau corresponds to the point at which equilibrium of thymidine across the membrane has been attained; thus use of 15 sec experimental period is justified.

3.D.5 Electron-Microscopy Studies

After having shown that UV inhibits not only DNA but also RNA, and protein synthesis, lymphocytes were irradiated either prior to addition of Con A or at 16 hrs. with 84 ergs/mm^2/sec to see if UV prevents nuclear decondensation as well. The 16 h point was done since it is at this time that the first signs of decondensation of chromatin are visible (Setterfield & Kaplan, personal communication).

Figure 3.12: 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 10×10^6 cells/ml. The cell suspension at a volume of 250µl was then mixed for different periods of time with an equal volume of LMGB containing 2.0×10^{-7}M thymidine (final radioactivity: 20µCi/ml). A 200µl aliquot was then centrifuged using the microcentrifugation technique as described in the Materials and Methods.


500
400
300
200
100
Thymidine transport (Fmoles/10⁶ cells)
30
60
90
120
150
180
210
Time (seconds)

They were fixed for EM studies at 24 hr. Table 3.3 shows that after counting 200 cells for each treatment, lymphocytes that were exposed to this dose of UV at 0 hr did not show nuclear decondensation, whereas stimulated cells irradiated at 16 hrs did show nuclear decondensation as compared to stimulated controls. All lymphocytes irradiated (0, 16 hr) showed "sick" nuclei as a typical characteristic. Electromicrographs of both irradiated and non-irradiated cells are shown in fugres 3.13 and 3.14.

From these data and the electromicrographs one may conclude that UV light prevents nuclear decondensation if it is given to the cells early during stimulation, but not after the onset of decondensation.

3.D.6 Unsheduled DNA synthesis

After cells were in the presence of Con A for 20 hr, they were irradiated (84 ergs/mm^2), labelled with ^{3}H-thy(10 µCi/ml) for 2 hr. When autoradiografyc studies were carried out and the slides developed: no silver grains over nuclei were observed; in these preliminary experiments no positive controls were employed.

TABLE 3.3: EFFECTS OF UV LIGHT ON NUCLEAR DECONDENSATION FROM HUMAN PERIPHERAL LYMPHOCYTES FIXED AT 24 HRS.

SAMPLE	N°. CELLS	NUCLEAR TYPE ***		
		1	2	3
Resting Control	200	0	67	133
Stimulated Control	200	54	106	40
Resting UV*	200	2	44	154
Stimulated UV*	200	3	53	144
Resting UV**	200	4	120	76
Stimulated UV**	200	60	101	39

* Irradiated at 0 hr.

** Irradiated at 16 hr.

1 Blast
2 Intermediate
3 Condensed

*** Nomenclature from the Laboratory of Dr. Setterfield.

Figure 3.13: Electronmicroscopy of untreated resting and stimulated human lymphocytes.

A: Resting human lymphocytes fixed at 24 hrs. The nucleus is completely condensed.

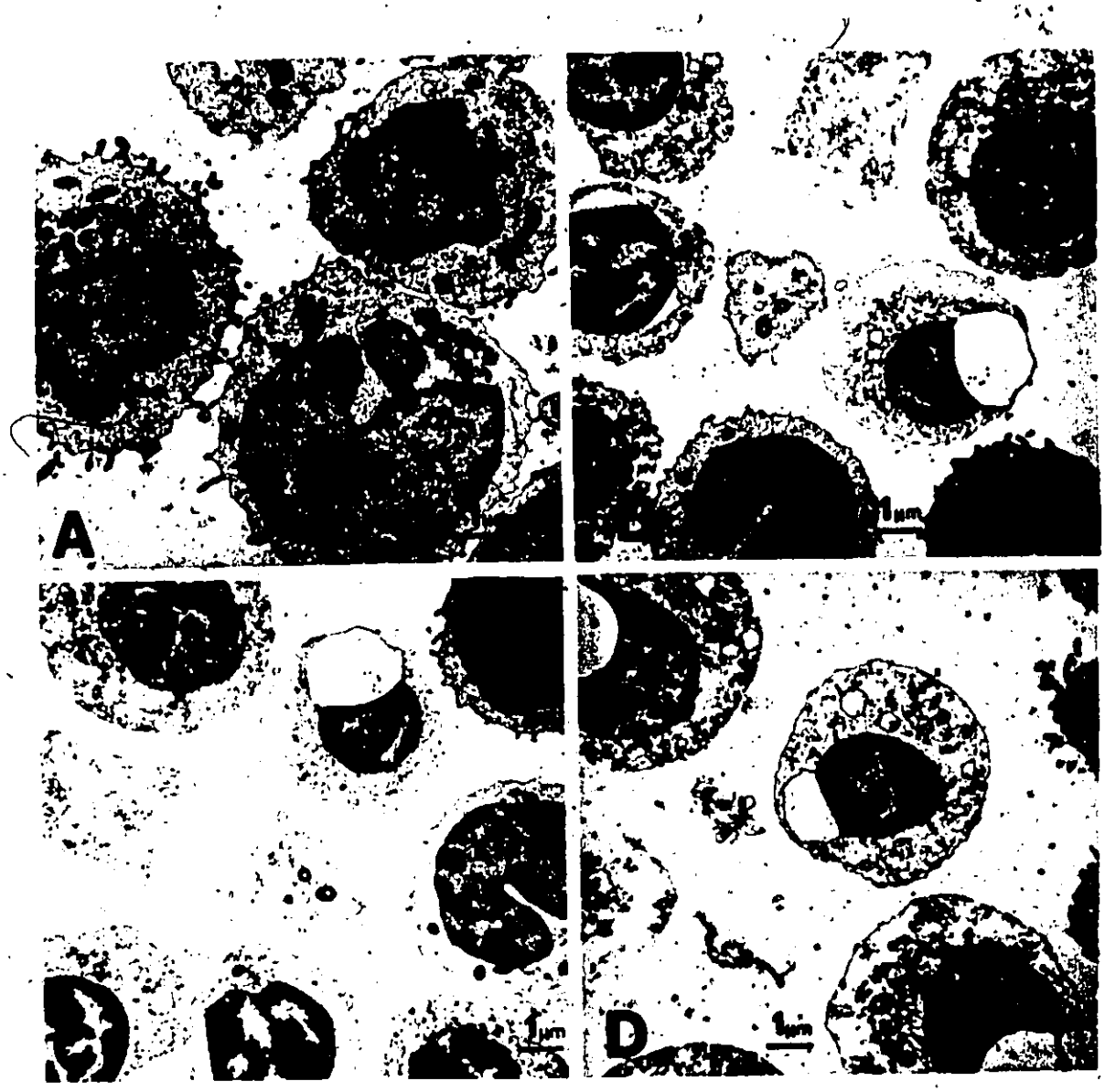
B,C: Stimulated human lymphocytes fixed at 24 hrs. Cells increased their volume and size. The nucleus is partly decondensed and a nucleolus is visible.

A
1μm
B
1μm
C
1μm

Figure 3.14: Electronmicroscopy of both resting and stimulated human lymphocytes irradiated (84 ergs/mm^2/sec): at O hr.

A: Resting human lymphocyte fixed at 24 hrs. Cells show a "sick" nuclei (polylobular).

B, C, D: Stimulated human lymphocytes fixed at 24 hrs. Cells show a nucleus completely condensed.


A
1μm
1μm
D
1μm

CHAPTER IV
DISCUSSION

The original intention of this study was to investigate the effects of UV irradiation on DNA of both resting and Con A stimulated human peripheral lymphocytes. When resting stage cells or stimulated cells were irradiated with 84 ergs/mm^2 UV no evidence of unsheduled DNA synthesis was observed.

These results were unexpected because UV has been shown to cause unsheduled DNA synthesis in a number of cell systems including unstimulated human monocytes(Lake, R. et al. 1980). Furthermore these results must be interpreted with caution since no positive control was used.

However, a possible explanation for these results is that excision repair synthesis due to UV-induced lesions occurs at a very slow rate under the conditions used in our experiments.

In cells irradiated 24 hr before Con A addition it was impossible to show any repair replication when this was assayed 20 hr after contact with the lectin(i.e. 44 hr after UV).

4.A. Dose response curve:

The data of figures 3.1, 3.2 and 3.3 are plotted in figures 3.4 and 3.5 as a percentage of control incorporation of ^{3}H-thy, assayed at 48 and 72 hrs respectively, versus dose of UV.

In these figures, an immediate and reproducible, statistically significant ($p = 0.001$) difference in the inhibition of incorporation of labeled thymidine was observed in cells stimulated either immediately after irradiation(0 hr) or in stimulated cells irradiated at 24 or 44 hr. DNA synthesis whether assayed at 48 or 72 hr, was more sensitive to UV when this was administered at the onset of culture with Con A; there was little difference between the sensitivity of the cells irradiated at 24 hr or 44 hr culture. Since the 0 hr cells are some 30 hr away from S phase, UV preferentially affects the activation process rather than the DNA synthesis itself. However in cells irradiated either at 24 or 44 hr DNA synthesis was inhibited by 95% as assayed by ^{3}H-thy incorporation (figures 3.2 and 3.3).

The lethal effect of UV radiation has generally been found to be greatest in late G_1- early S phase, in HeLa cells (Downes and Johnson. 1979), in human D98/A9 (Erikson and Szybalski. 1963), in Chinese hamster V79 (Sinclair and Morton 1965), CHO-KI cells (Burg, et al. 1977) and in mouse L cells Rauth and Whitmore. 1966). But in our case, cells irradiated prior to Con A addition (G_o cells) were the most sensitive to UV.

4.B. Comparative effect of UV irradiation on DNA, RNA, and Protein synthesis.

4.B.1 DNA synthesis:

Ultraviolet irradiation is known to inhibit DNA synthesis in mammalian cells, although the mechanism of this inhibition is not known.

One of the purposes of this thesis was to investigate the effect of UV light, using the incorporation of ^{3}H-thy into Con A stimulated human peripheral lymphocytes *in vitro* as an assay, in an attempt to determine some of the cause(s) of that inhibition.

When stimulated cells were exposed to varying doses of UV at 0,24 or 44 hrs after addition of Con A, the incorporation of ^{3}H-thy into the DNA is severely reduced and DNA synthesis never reached the control levels. (Figures 3.2 and 3.3).

It was reported by Friedberg, et al.(1978) that thymidine containing pyrimidine dimers produced in mammalian cell DNA by UV doses lower than 10 J/m^2, 90% of them were removed in one hour, and with a dose of 40 j/m^2 less than 50% of dimers were removed after 48 hrs of irradiation.

An early attempt at a kinetic analysis by Cleaver, 1967 concluded that replication halted as discrete blocks were reached, but those blocks were only about 1/10 as frequent as pyrimidine dimers (i,e, dimers were probably not the blocks)

Cleaver's study, however, was presented prior to the demonstration that in mammalian cells many replicons along each DNA fiber are active at any one time (Huberman and Riggs 1968). Klímek and Vlăsínová (1966) noted that DNA synthesis was not completely blocked after UV, and suggested that continued synthesis was partly due to the presence of multiple replication units.

In experiments conducted to measure the time course of DNA repair replication in HeLa cells (Edenberg and Hanawalt, 1973), it was noted that after UV, repair replication made a significant contribution to the total thymidine incorporation. For this reason, Edenberg, H. (1976) re-examined the kinetics of the inhibition of semi-conservative DNA synthesis in UV irradiated HeLa, using density labeling to distinguish semi-conservative synthesis from incorporation due to repair.

An analysis of these results in terms of our present knowledge of the many replicons active in mammalian cells (for review see Edenberg and Huberman, 1976) shows that the inhibition could be explained by replicon forks halting (or pausing) at pyrimidine dimers (Edenberg, 1975). This analysis could not itself rule out the alternate possibilities that the rate of replication fork movement was uniformly slowed, or that the initiation of new replicon was inhibited.

To directly approach these problems, Edenberg, H.J. 1976, studied post-UV DNA synthesis by a different technique-DNA fiber autoradiography. The results demonstrated that the inhibition of DNA replication is due to a halt (or long pause) when replication forks reach discrete lesions in the template DNA, and that these lesions are present in approximately the frequency of pyrimidine dimers.

Another possible explanation of our results could be that UV produces irreversible damage, which does not produce as much cell death. As it was pointed out before, cells irradiated at 0 hr were the most sensitive to UV, which indicates that UV affects the activation process.

On the other hand, the inhibition in the uptake of ^{3}H-thy into DNA, seen in lymphocytes irradiated either at 24 hr (late G_1) or 44 hr (S phase) could be explained by replicon forks halting as they encounter lesions (dimers) in the DNA, so that DNA synthesis cannot continue. At these times UV may be affecting DNA synthesis itself rather than the activation process.

The unsheduled DNA synthesis data showing negligible repair replication and hence perhaps faulty dimer removal, although of a very preliminary values, support the above hypothesis.

4.B.2 RNA and protein

Data from experiments measuring the incorporation of (^{3}H) uridine and (^{3}H) leucine into cells irradiated prior to addition of Con A (0 hr) suggest that UV causes an immediate depression of protein and RNA synthesis (Fig.3.6 and 3.7). Such a depression of both protein and RNA synthesis could have a contributory effect on the inhibi - tion of DNA synthesis. In order for the cell to synthesi- ze DNA, it requires RNA and protein.

Since UV is inhibiting their synthesis, no RNA is available for the translation of protein and none of the proteins which are essential for DNA replication to occur later on in the activation process (S phase) are being ma- de.These proteins include DNA.polymerase and RNA polymerase.

Before a resting lymphocyte stimulated by a mitogen enters the cell cycle and divides, complex molecular changes occur in the cell membrane, the nucleus and the cytoplasm.

One of the earliest events in this transformation pro- cess, occurring just after the changes in membrane permeabi- lity (i, e, 2-3 hr after mitogen addition), is an increase in the rate of protein synthesis (Kay, et al. 1971).

This appears to be due to an increased rate of translation of pre-existing mRNA by pre-existing ribosomes. Studies on lymphocyte cytoplasm indicate that the low rate of protein synthesis in unstimulated cells is due to the low activity of one or more protein synthesis initiation factors; the activity of inhibitors of these factor is decreased after activation by mitogens (Kay, et al.1978, 1979).

Early de novo protein synthesis may be responsible, at least in part, for the observed increases in DNA polymerase and RNA polymerase activities. Along with a doubling of amino acid incorporation, the number of polysomes is increased one hour after stimulation (Hauser, et al,1978).

Resting lymphocytes maintain a basal level of RNA synthesis. When lymphocytes are activated by a mitogen, there is no increase in total RNA synthesis for some 4-6 hr. Thereafter, it increases steadily for the next 40 hr until a plateau is reached, during S-phase(Kaplan, et al. 1979). The earliest response of lymphocytes to mitogen appears not to be the initiation of new RNA chains, (Mitchell, et al. 1978), but rather increased RNA processing, including polyadenylation, splicing, methylation and capping, RNA export to the cytoplasm, and RNA binding to and translocation by polysomes(Shafer & Mitchell, 1979).

It is known that a suppression of RNA synthesis will cause a corresponding decrease in protein synthesis in lymphocytes(Ling and Kay 1975). Low amounts of actinomycin D (0.02 µg/ml), an inhibitor of RNA synthesis, when added within 6 hr of mitogen addition entirely block the increase in polymerase activity which is normally observed at 14 hr. This suggests that this increase in enzyme requires RNA synthesis. Yet, when the same amount of actinomycin D is added 14 to 72 hr after PHA addition, polymerase activity increases without interruption (Agarwarl & Loeb 1972). Moreover, experiments presently in progress in our laboratory indicate that when cells in a high DNA synthetic phase (42 hr) are exposed to 8-azaguanine (8-azag), an inhibitor of RNA synthesis, for 2 hr, an inhibition of DNA synthesis is still observed. This inhibition is probably due to incorporation of the 8-azag into the RNA, leading to the production of false messages and thus non-functional proteins (Christine Boumah, personal communication).

Studies on the effects of protein synthesis inhibitors on stimulated lymphocytes indicate that the observed increase in polymerase activity is dependent on concomitant protein synthesis.

Thus, cycloheximide, when added to cultures of stimulated lymphocytes at concentrations sufficient to prevent 95% of the PHA-mediated enhancement of protein synthesis, prevents the increase in polymerase activity (Quastel, & Kaplan. 1970b).

Our studies have shown that when lymphocytes are irradiated at 0 hr (prior to addition of Con A) RNA and protein synthesis are inhibited by 95% (Fig. 3.6), whereas in cells irradiated at 24 or 36 hr no apparent inhibition is observed (Fig. 3.7), although DNA synthesis was inhibited in those cells irradiated at times described above. It seems that UV does not directly affect protein and RNA synthesis, but rather inhibits the activation process leading to these syntheses. This indicates that there is a critical step in the activation process, which is blocked by UV. It also appears that when lymphocytes are UV-irradiated at later stages of cell transformation, DNA synthesis is the parameter most affected.

Effects of UV irradiation on RNA synthesis in mammalian cells have been studied in a few cases only. No studies on <u>in vitro</u> transcription of UV-irradiated mammalian DNA by mammalian RNA polymerases have been reported yet. Painter(1970) has shown that the rate of RNA synthesis in HeLa cells decreases non-exponentially with UV dose given to the cells and that the decrease is exaggerated in bromodeoxyuridine grown cells(Painter, et al.1970).

It was shown by Willems, et al., (1968) that synthesis of rRNA was preferentially inhibited by UV irradiation of HeLa cells. Previously, ultraviolet microbeam irradiation had been used to selectively inhibit the synthesis of rRNA in the nucleolus (Perry, et al., 1961). Synthesis of RNA in basal cells of mouse epidermis is reduced following UV irradiation (Epstein, et al., 1970).

Membranes have -SH groups, some of which are necessary for cell function. They are apparently necessary for the integrity of the membrane since cells from various sources treated with a thiol oxidising agent become ruptured. The -SH groups of the erythrocyte membrane are implicated in the Na^+/K^+pump, glucose difusion and the prevention of haemolysis. The membrane (Na^+,K^+)-ATP-ase of red cells and kidney cells is inhibited by mercurials which in turn prevent the dependent Na^+/k^+exchange (Sutherland, et al., 1967;Vansteveninck,et al., 1967).

It is known that thiols and disulphides can protect against the biological damage caused by ionizing radiation. In view of that, Sabovljev in 1976 did a series of <u>in vitro</u> experiments to see if their protective action could be ex - tended under the condition of UV irradiation. His results shows that -SH and S-S groups of proteins are among the groups most vulnerable to attack, not only by ionizing but also by UV radiation.

Another report on the effect of UV on proteins is one carried out by Lawler, et al., (1976) on blood platelet proteins to correlate changes in platelet functions and proteins. Their results indicate that platelet associated fibrinogen is sensitive to UV.

4.C Membrane Effects

4.C.1, Effects of UV irradiation on potassium influx

Having shown that UV light not only affects DNA synthesis, but also RNA synthesis, all possibly via an affect on the activation process, experiments were carried out to determine whether irradiation altered the rate of K^+influx across the cell membrane. Previous work by Quastel & Kaplan (1970) showed that one of the earliest biochemical events following activation of human lymphocytes by the mitogen PHA, was a marked increase in the rate of uptake of $^{42}K^+$. It was subsequently shown that the increased uptake of this radioisotope was manifest within 30 sec of adding PHA to lymphocyte culture (Averdunk, R. 1972).

Mitogen-induced increase in K^+influx has been confirmed (Segel,G. and Lichtman, M. 1976) and increased Na^+ efflux (due to the activity of the Na^+-K^+-ATP-ase of the lymphocyte membrane) has been demonstrated (Averdunk and Lauf. 1975).

The inhibitory effects of UV were noted as soon as after 2.5 hr of incubation with Con A (Figure 3.8). Potassium influx, measured using ^{86}Rb as tracer, was decreased by prior UV irradiation from 6.8 to 1.7 f/moles/cell/hr. It can be seen that the effect of UV on resting and on stimulated cells was so drastic that influx of K^+ into the cells was reduced to levels lower than those of non-activated lymphocytes.

This indicates that UV inhibits at least one of the essential early events of activation at the lymphocyte cell surface. Unfortunately there is only one report in the literature about UV effects on Na^+-K^+-ATP-ase enzyme. This investigation was done by Lieberman, E. (1967). UV radiation, besides having electrophysiological effect on nerve fibers, was shown to inhibit the Na^+K^+-activated ATP-ase system, prepared from crab nerves.

His experimental results demonstrated that wavelengths of 285 nm and 255 nm, which block nerve conduction, also block the Na^+-K^+-activated ATP-ase. In the same series of experiments, it is seen that the Mg^{2+}-activated ATP-ase activity was not or only minimally affected by the radiations. Enzymes, characteristically, require irradiation with 10^5 ergs/mm^2 of ultraviolet light for photoinactivation to occur (Setlow and Pollard. 1962).

This implies that UV is not inactiviting the enzyme (Na^+-K^+-activated ATP-ase), but rather UV is affecting an earlier event(s) of the activation process.

4.C.2 Binding of Con A to cell surface:

Ultraviolet was shown to act at the cell surface by significantly ($p= 0.01$) reducing the binding of ^{3}H-Con A (Figure 3.9). The time course of binding of ^{3}H-Con A indicated that the time required for saturation of sites was 20-30 min at 37°C.

A large reduction in the total number of Con A binding sites occurred in the irradiated samples since 25-50% less Con A was bound to the cell surface. Binding sites / cell at 20 min were $6.6 \pm 0.5 \times 10^6$ in the control cells and $4.2 \pm 0.4 \times 10^6$ in the UV treated, a reduction of 54%. There is evidence (Boldt, et al. 1975) that the number of receptor sites for any lection on the surface of human lymphocytes is the same for each type of lymphocyte.For example, Stubo, et al. (1972), and Anderson, et al. (1972)have found that there are between $0.8\text{-}3 \times 10^7$ molecules of Con A bound per cell for saturation of murine thymocytes. However only 2.5×10^6 molecules of mitogen or approximately 20% are needed to initiate blast transformation and DNA synthesis. In another report, Boldt, et al (1975) claim that the number of Con A receptor sites on T, B and Null cells is 1.7×10^6/cell.

This value is in reasonable agreement with that found in other studies (Boldt, et al. 1972, Wands, et al. 1976, and Krug, et al. 1973, Repacholi, M. Ph.D thesis 1980). UV, therefore, affects the number of receptor sites on the surface of human lymphocytes. This indicates that it is the linkage between events occurring at the cell surface and inner mechanisms for cell activation which is affected by UV. It seems probable that the reduction observed in (^{3}H)-Con A binding occurs because UV damages or distorts the molecular configuration of the receptor sites on the cell surface to the extent that many of the binding sites do not recognize the Con A molecule (Repacholi, M. Ph.D thesis 1980). Unfortunately there is no report on UV effects on binding sites by a mitogen on human lymphocytes.

4.C.3 Thymidine Transport

Having shown that UV inhibits K^+ uptake and Con A binding, it was necessary to exclude the possibility that UV prevents thymidine incorporation by interfering with transport of the nucleoside into the cell.

Ultraviolet exposure under the conditions described in chapter II did not appear to alter the initial rates of transport of ^{3}H-thy irradiated cells (Fig. 3.10 and 3.11),

even though the incorporation of the label into DNA was inhibited (58%) when cells were harvested and counted as described in Materials and Methods, the difference being significant at $p = 0.001$ (Table 3.2). The control rates of transport were found to agree with those of Christopher Rudd, (M.Sc. thesis 1980).

One cannot conclude from these experiments that the amount of radioactive precursor available to both control and irradiated cells was the same since the experiments provided no information on the internal cell pools of the precursor. However, if the initial rates of thymidine transport were unaltered by UV, it seems reasonable to suggest that both irradiated and control cells had the same amount of ^{3}H-thymidine available for incorporation.

These results are very similar to those found for sonicated lymphocytes by Michael Repacholi (Ph.D thesis 1980). There are no reports in the literature of experiments conducted to determine if UV altered transmembrane transport of ^{3}H-thy.

4.C.4 Effects of UV on nuclear decondensation

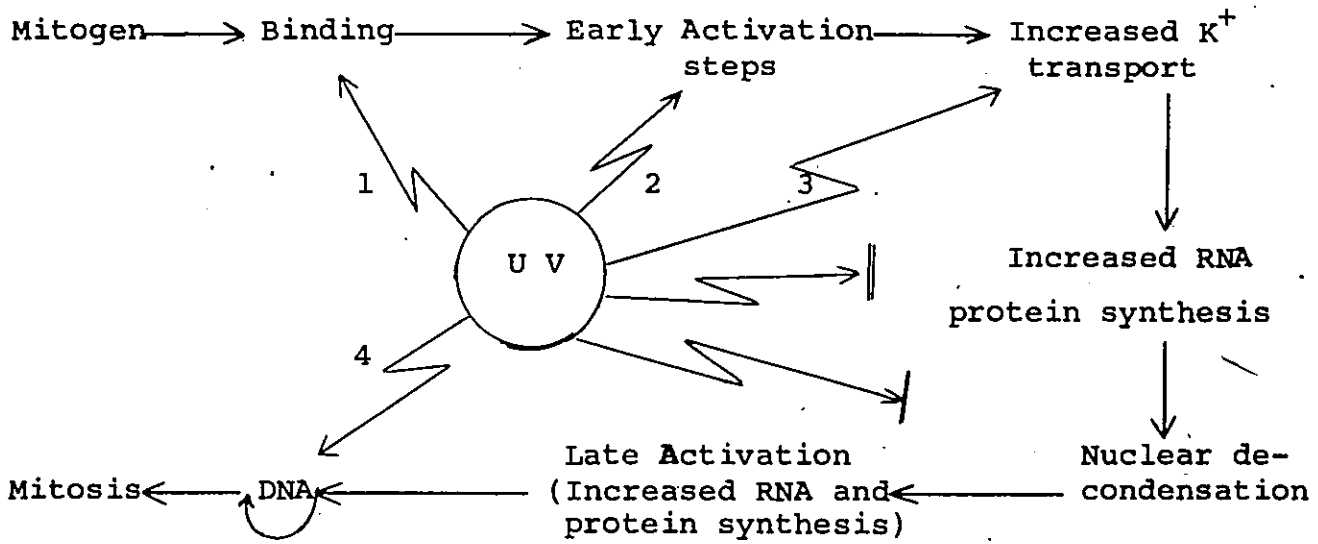
As shown, UV inhibits DNA synthesis, K^+ uptake, Con A binding, RNA and protein synthesis. Studies were undertaken to determine if UV might also prevent nuclear decondensation.

The results indicate that lymphocytes exposed to UV prior to addition of Con A did not show nuclear decondensation at 24 hours, whereas cells irradiated after being in the presence of Con A for 16 hr did show nuclear decondensation at 24 hours, as compared to stimulated controls.

One may conclude that UV inhibits decondensation if it is given to the cells before the process of decondensation has taken place but not if this process has begun.

It appears that UV irradiation affects an early event of cell activation by preventing nuclear decondensation. There are no reports published about the effect of UV on nuclear decondensation in lymphocytes.

Figure 4.1. MODEL SCHEME OF THE EFFECT OF UV LIGHT ON HUMAN PERIPHERAL LYMPHOCYTES.



4.C. MODEL:

Figure 4.1 represents a model scheme of the effect of UV light on human peripheral lymphocytes.

UV may have two sites of action on lymphocytes:

1. Cell membrane and/or cytoplasm, for early effects
2. Nuclear DNA, for late action of UV.

It was shown that when lymphocytes were irradiated prior to addition of Con A, the rate of Con A binding (an event that precedes lymphocyte stimulation) decreased. Whether or not this inhibition of Con A binding (1) may cause the observed inhibitions of all biochemical parameters of lymphocytes irradiated at 0 hr we cannot say.

When the effect of UV on protein and RNA synthesis was examined, irradiation at 0 hr had the strongest effect, whereas at later stages of the activation, there was little or no effect on protein or RNA synthesis. This indicated that UV was affecting some early event(s) of the activation process (2) leading to these syntheses. Possibly UV is directly affecting some essential protein(s). This has to be investigated.

K^+ influx was checked to investigate if UV affected its uptake. It was noted that irradiation inhibited this parameter as early as 3 hr.

Quastel and Kaplan (1970), showed that cyclohexamide and actinomycin D did not inhibited the increased transport of K^+ caused by mitogenic stimulation. UV is therefore affecting an event that does not require protein or RNA synthesis and is therefore completely independent of DNA. A direct effect of UV on DNA can therefore not explain this early effect.

Experiments should be undertaken to determine whether or not UV is directly affecting K^+ uptake or whether it affects some other event leading to its activation. It should also be determined whether the K^+ influx, which is inhibited by UV, is ouabain-sensitive.

In cells irradiated at later stages of the activation process, it seems that UV directly affects DNA synthesis (4) by damaging the template of DNA and/or enzymes involved in replication.

B I B L I O G R A P H Y

-Agarwarl, S.S., and Loeb, L.A., 1972. Studies on the induction of DNA polymerase during transformation of human lymphocytes". Canter Res. 32:107.

-Anderson, J., Sjoberg, O. and Moller, G. 1972. "Mitogen as probes for immunocyte activation and cellular cooperation". Transplant Rev. 11:131-177.

-Averdunk, E. and Lauf P.K. 1975. "Effects of Mitogen on sodium-potassium transport, ^{3}H-Ouabain binding and adenosine triphosphatase activity in lymphocytes". Exp. Cell Res. 93, 331.

-Averdunk, R. 1972, " The Effect of phytohaemogglutinin and antilymphocyte serum on the transport of potassium ions, glucose and amino-acids in human lymphocytes". Hoppe-Seyler's Z. Physiol. Chem, 353:79-87.

-Benacerraf, B. and Germain, R.N. 1978 "The immune response genes of major histocompatibility complex". Immunol. Rev. 38:70-119.

-Boldt, D.H., Mac-Dermott, R.P. and Jorolan, E.P. 1975. "Interaction of plant lectins with purified human lymphocyte populations: binding characteristics and Kinetics of proliferation". J. Immunolog 114 (5): 1532-1536.

-Boldt,D., Skinner, A.M. and Kornfeld, S. 1972."Studies of two sub-populations of human lymphocytes differing in responsiveness to Concanavalin A". J. Chin. Invest. 51:3225-3234.

-Burg, K., Collins, A.R.S., and Johnson, R.T. 1977. "Effects of ultraviolet light on synchronized Chinese Hamster ovary cells: potentiation by hydroxyurea". J. Cell Sci. 28: 29-48.

-Cleaver, J.E. 1965. "Investigation into the effects of ultraviolet light on the rate of deoxyribonucleic acid synthesis in mammalian cell". Biochim. Biophys. Acta.108:42.

-Cleaver, J.E. 1966a "Inhibition of DNA synthesis by ultraviolet light under controlled temperature". Nature(Lond). 209:1317.

-Cleaver, J.E. 1966b. "Photoreactivation: A radiation repair mechanism absent from mammalian cells". Biochem. Biophys. Res. Commun. 24: 569.

-Cleaver, J.E. 1967. "The relationship between the rate of DNA synthesis and its inhibition by ultraviolet in mammalian cells". Radiat. Res. 30:795.

-Cleaver, J.E. 1968. "Defective repair replication of DNA on xeroderma pigmentosum". Nature. 218:652-656.

-Cleaver,J.E. 1977. "Nucleosome structure controls rates of excision repair in DNA of human cells". Nature. 270:451-454.

-Cleaver, J.E. "DNA damage, DNA repair and DNA replication in short term tests for exposure to mutagens". Proc. of Symposium on Population Monitoring and Genetic Risk Assessment. Ottawa,Ontario. Canada. 1980.

-Cleaver, J.E., Arutyunyan, R. , Sarkisian, T., Kaufmann, W.K., Greene, A.E., Coriell, L. (1980). "Similar defects in DNA repair and replication in the pigmented xerodermoid and xeroderma pigmentosum variant".Carcinogenesis 1 (in press).

-Domon, M. and Rauth A.M. 1968. "Ultraviolet irradiation of mouse L cells: Effects on DNA synthesis and progression through the cell cycle". Radiat. Res. 35:350.

-Downes, C.S., Collins A.R.S., and Johnson, R. T. 1979. "DNA damage in synchronized HeLa cells irradiated with ultraviolet. "Biophys. J. 25: 129-150.

-Edenberg, H.J. 1976. Biophysical Journal. 16:849.

-Edenberg, H.J., and Huberman, J.H. 1975."Eukariotic chromosome replication". Ann. Rev. Genet. 9:245.

-Ehman, U.K., Cook, K.H., and Friedberg, E. C. 1978. "Studies on the molecular mechanisms of nucleotide excision repair in UV-irradiated human cells in culture". In DNA repair mechanisms (Hanawalt, P.C., and Friedberg, E.C. eds). Academic Press.

-Epstein, J.H., Fukuyama, K., and Fye, K. 1970. Photochemistry and Photobiology. 12:57-65.

-Erikson, R.L., and Szybalski, W. 1963. "Molecular radiobiology of mammalian cell lines. IV.Variation in ultraviolet light and X-ray sensitivity during the division cycle". Radiat. Res. 69:573-582.

-Friedberg, E.C., Cook, K.H., Duncan, J., and Mortelmans, K. 1977. "DNA repair enzymes in mammalian cells". In Photochemical and Photobiological Reviews. Vol. 2 (K.C.Smith, ed.). Plenum Press, New York. pp. 263-323.

-Giese, A.C. 1965. "Studies on ultraviolet radiation action upon animal cells". In Photophysiology. Vol 2 (A -C Giese, Ed.). Academic Press, New York. 203-243.

-Greene, M.L., M.S. sy., Kripke, M., and Benacerraf, B. 1979. "Impairment of antigen-presenting cell function by ultraviolet radiation". Proc. Natl. Acad. Sci. 76:6591.

-Hanawalt, P.C., Cooper, P.K., Ganesan, A.K., and Smith, C.A. 1979. "DNA repair in bacteria and mammalian cells". Ann. Rev. Biochem. 48:783-836.

-Haniszko, J., and Suskind, R.R. 1963. "The effect of ultraviolet radiation on experimental cutaneous sensitization in guinea pigs". J. Invest. Dermatol. 40: 183-191.

-Hart, R.W.,Hall, K.Y., and Bernard Daniel, F. 1978. "DNA repair and mutagenesis in mammalian cells". Photochem. and Photobiol. 28:131-155.

-Hart, R.W., and Setlow, R.B. 1974. Proc. Natl. Acad. Sci. U.S. 71:2169-2173.

-Hart, R.W., and Trosko, J.E. 1976. Interdiscipl. Topics Geront. 9:134-167.

-Haseltine, W.A., Gordon, L.K. Lindan, C.P., Grafstrom, R. H., Shaper, R.H., Grossman, L. 1980"Cleavage of Pyrimidine dimers in specific DNA sequences by a pyrimidine dimer DNA glycosylase of M. Luteus". Nature. 285:634-641.

-Hauser, H., Knippers, R. and Shaffer, K.P. 1978. "Increased rate of RNA-polyadenylation". Expt. Cell Res. 111:175-184.

-Horowitz, S., Cripps. D., and Hong R. 1974. "Selective T cell killing of human lymphocytes by ultraviolet radiation". Cell Immunol. 14:80-86.

-Huberman, J.A., and Riggs, A.D. 1968. "On the mechanisms of DNA replication in mammalian chromosomes". J. Mol. Biol. 32:327.

-Jessup, J.M., Hanna, N., Polaszynski, E., and Kripke, M.L. 1978. "Mechanisms of depressed radioactivity to dinitrochlorobenzene and ultraviolet-induced tumors during ultraviolet carcinogenesis in BALB/c mice". Cell Immunol. 38:105-115.

-Jones, G. 1973. "Reversible binding of phytomitogens to lymphocytes". Cell Immunol. 9:393-404.

-Kaplan, J.C., Kushner, S.R., and Grossman L. 1971 Biochemistry 10:3315-3324.

-Kaplan,J.G., Mitchell, M., and Bard, E. 1979. "Synthesis and proccessing of RNA in stimulated fibroblasts and lymphocytes". In Cell Biology and Immunology of Leukocyte Function, ed. M.R. Quastel, Academic Press (New York). pp. 87-105.

-Kay, J.E., Ahern, T., and Atkins, M. 1971. "Control of synthesis during the activation of lymphocytes by phytohaemogglutinin". Biochim. et Biophys. Acta. 247:322-334.

-Kay, J.E., Benzie, C.R., and Dieker, P. 1978. "Inhibition of protein synthesis in rabbit reticulocyte lysates by a factor present in lymphocyte cytoplasm". FERS. Letters 91 (1):40-44.

-Kay, J.E., Wallace, D.M., Benzie, C.R., and Jagus, R. 1979. "Regulation of protein synthesis during lymphocyte activation by phytohaemogglutinin". In Cell Biology and Immunology of Leukocyte Function, ed. M.R. Quastel. Academic Press (New York) pp. 107-114.

-Kirtikar, D.M., and Goldthwait, D.A. 1974. Proc. Natl. Acad. Sci. U.S. 71: 2022-2026.

-Klaresko, L., Tjernlund, U.M., Forsum, U., and Peterson, P. A. 1977. "Epidermal Langerhans cells express Ia Antigens". Nature. 268-248-250.

-Kripke, M.L. 1980."Immunologic effects of UV radiation and their role in photocarcinogenesis". In Photochemical and Photobiological Reviews (Smith Kendric C. Ed.). Plenun Press, New York. 5:257-243.

-Krug, U., Hollenberg, M.D., and Cuatrecasas, P. 1973 "Changes in the binding of Concanavalin A and wheat germ agglutinin to human lymphocytes during in vitro transformation". Biochem. Biophys. Res. Commun. 52 (1): 305-312.

-Lafferty, K.J., Misko, I.S., and Cooley, M.A. 1974."Allogeneic stimulation modulates the in vitro responses of T cells to transplantation antigen "Nature. 249:275-276.

-Lawler, J.W., Chao, F.C., and Fang, P.H. 1976. "Effect of ultraviolet light on human platelet proteins." Federation Proc. Abstracts. 35(3):331.

-Letvin, N.L., Greene. M.I., Benacerraf, B. and Germain, R.N. 1980. "Immunologic effects of whole-body ultraviolet irradiation: Selective defect in in vitro splenic adherent cell function". Proc. Natl. Acad. Sei: 77:2881-2885.

-Letvin, N.L., Fox, I.J., Greene, M.I, Benacerraf, B., and Ronald N. 1980. "Immunologic effects of whole-body UV irradiation: II. Defect in Splenic Adherent Cell antigen presentation for stimulation of T cell proliferation". J. Immunologic. 125:1402-1404

-Klímek, M., and Vlăsínová. 1966."Thymidine and uracil-thymidine dimers and deoxyribonuclei acid synthesis in mammalian cells irradiated with ultraviolet light". Int. J. Radiat. Biol. 11:329.

-Lieberman, E.M. 1967. "Structural and functional sites of action of UV radiations in crab fibers". Exp. Cell Research 47: 508-517.

-Lindahl, T. 1976, Nature 259:64-66.

-Lindahl-Kiessling, K., and Safwenberg, J. 1971. "Inability of UV-irradiated lymphocytes to stimulate allogeneic cells in mixed lymphocyte culture". Int. Arch. Allergy. 41:670-678.

-Ling, N.R. and Kay, J.E.1975. "Lymphocyte stimulation". North - Holland Publishing Co., Amsterdam.

-Mitchell, M., Bard,E, L'Anglais, R. and Kaplan, J.G. 1978. Con. J. Biochem.. 56:659-666.

-Oliver, J.M. and Paterson, A. R. 1971. Can.J. Biochen 49:262-270.

-Ownes, T., and Kaplan, J.G. 1979. In the molecular Basis of Immune Cell Function (J.G. Kaplan, ed). Elsevier / North-Holland, Amsterdam. 402-405.

-Ownes, T. and Kaplan, J.G. 1980."Activation of lymphocytes of man and mouse: Monovalent cation fluxes". Can. J. Biochem. in press.

-Painter, R.B. 1970. "The action of ultraviolet on mammalian cells". In Photophysiology. (A.C.Giese, Ed). Academic Press, New York, 5: 169-189.

-Painter, R.B., Goodman, M.G., and Reisner, B.L. 1970. Photochemistry and Photobiology. 12:49-55.

-Parrish, J. A., Anderson, R. R., Urback, F., and Pitts, D. O. 1978. UV-A "Biological effects of ultraviolet radiation with emphasis on human responses to longwave ultraviolet". Plenum Press. New York.

-Perry, R. P., Hell, H., and Errera, M. 1961. Biochim. Biophys. Acta. 49:47-57.

-Quastel, M. R., and Kaplan, J. G. 1968. "Inihibition by Ouabain of human lymphocyte transformation induced by phytohaemagglutinin in vitro". Nature. 219:198-200.

-Quastel, M. R., and Kaplan, J. G. 1970a. "Lymphocyte stimulation: The effect of Ouabain on nucleic acid and protein synthesis". Exptl. Cell Res. 62:407-420.

-Quastel, M. R., and Kaplan, J. G. 1970b. "Early stimulation of potassium uptake in lymphocytes treated with PHA". Exptl. Cell Res. 63:230-233.

-Rauth, A. M., and Whitmore, G. F. 1966. "The survival of synchronized L cells after ultraviolet irradiation". Radiat. Res. 28:84-95.

-Regan, J. D., Setlow, R. B., and Ley, R. D. 1971. Proc. Natl. Acad. Sciences. U.S.A. 68:708-712.

-Regan, J. D., and Setlow, R. B. 1974. Cancer Research. 34:3318-3325.

-Regan, J. D., and Setlow, R. B. 1975. "Repair of human DNA: Radiation and chemical damage in normal and Xeroderma pigmentosum cells". Abstract for the Symposium on the Biology of Radiation Carcinogenesis. Gatlinburg, Tennessee. April 7-10, 1975.

-Repacholi, M. H. 1980. Ph. D. "The effects of ultrasound on human lymphocytes:A search for dominants mechanisms of ultrasound actions". University of Ottawa,Ontario. Canada.

-Riott, I. M. 1974. "Essential Immunology". 2nd Ed., Blackwell Scientific Publications, Oxford.

-Röllinghoff, M., and Wagner, H. 1975. "Secondary cytotoxic allograft response in vitro. I. Antigenic requirements" Eur. J. Immunol. 5:875-879.

-Rudd, C. 1980. M. sc thesis. "Colchicine, Microtubules, Thymidine and Lymphocyte function". University of Ottawa Ontario. Canada.

-Sabovljev, A. 1976. "Importance of SH groups for maintaing cell me,brane integrity". Period. Biol. 78:133-136.

-Schafer, K. P., and Mitchell, M. 1979. "Workshop report: Nucleic acid and protein synthesis". In The Molecular Basis of Immune Cell Function., ed. J. G. Kaplan, Elsevier-North Holland Biomedical Press(Amsterdam). pp. 725-727.

-Segel, G. B., Gordon, B. R., Lichtman, M. A., Hollander, M. M., and Klemperer, M. R. 1976. J. Cell Physiol. 87:337-344.

-Segel, G. B., and Lichtman, M. A. 1976. In Program and Abstracts Eleventh Leukocyte Culture Conference. # 44, Sept. 19-23. 1976. p. 29.

-Setlow, R. B., and Carrier, W. L. 1966. "Pyrimidine dimers in ultraviolet-irradiated DNA's". J. Mol. Biol. 17:237-254.

-Setlow, R. B., and Pollard, E. C. 1962. Addison-Wesley Publ. Co., Inc. 277-278.

-Siegel, S. 1956. Non parametric statistics for the behavioural sciences. McGraw Hill (New York).

-Sinclair, W. A., and Morton, R. A. 1965. "X-ray and ultraviolet sensitivity of synchronized Chinese hamster cells at various stages of the cell cycle". Biophys. J. 5:1-25.

-Smith, K. C. 1974. "Molecular changes in the nucleic acids and visible radiation". In Sunlight and Man:Normal and Abnormal Photobiologic Responses. (M. A. Pathak, L. C. Harber, M. Seiji, A. Kukita, Eds. T.B. Fitzpatrick Consulting Ed.) University of Tokyo. Tokyo Press. 57-66.

-Stingl, G., Katz, S. I., Shevach, E. M., Wolffschreiner, E., and Green, I. 1978. "Detection of Ia antigen on Langerhans cells in guinea pig skin". J. Immunol. 121:1920-1928.

-Strauss, P., Sheehan, J., and Kashket, E. 1976. "Membrane transport by murine lymphocytes. I.A rapid sampling tecnique as applied to the adenosine and thymidine systems". J. Exp. Med. 144:1009.

-Stubo, J. D., Rosenthal, A. S., and Paul, W.E. 1972. "Functional heterogenecity of murine lymphoid cells. I.Responsiveness to and surface binding of Concanavalin A and phytohaemagglutinin". J. Immunol. 108:1-17.

-Sutherland, R.M., Rothstein, A., and Weed, R. I. 1967. "Erythrocyte membrane sulphydyl groups and cation permeability". J. Cell Physiol. 69:185.

-Vansteveninck, J., Weed, R. I., and Rothstein, A. 1965. "Localisation of erythrocyte membrane sulphydyl groups essential for glucose transport". J. Gen. Physiol. 84: 617.

-Wagner, H., and Röllinghoff, M. 1976. "Secondary cytotoxic allograft responses in vitro. II.Differentiation of memory T cells into T lymphocytes in the absence of cell proliferation". J. Immunol. 6:15-21.

-Wands, J. R., Podolsky, D. K., and Isselbacher, K. J. 1976. "Mechanisms of human lymphocyte stimulation by Concanavalin A:Role of valence and surface binding sites". Proc. Natl. Acad. Sci.(USA). 73(6):2118-2122.