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EFFECT OF UV-IRRADIATION ON SOME MEMBRANE RELATED FUNCTIONS IN AMOEBA

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CERTIFICATE

The research work embodied in this dissertation has been carried out in the School of Life Sciences, Jawaharlal Nehru University, New Delhi. This work is original and has not been submitted in part or full for any other degree or diploma of any university.

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INTRODUCTION

Effect of ultraviolet light (UV) on biological system has been an important problem since the beginning of this century. Though the studies on UV effects began with man in view, because of the complexities of the human and other higher organisms, such studies have been centred on simple systems like protozoans, blood cells, cells in tissue culture etc.

Ultraviolet spectrum can be divided into three classes, (i) near UV or long UV (310-340 nm) which is transmitted through glass and the intensity of this wave length of UV being maximum in sunlight, out of the three classes, (ii) the far UV (200-300 nm) is present in low intensity in sunlight. It has varied effects on biological systems, (iii) very short UV (15-200 nm) which is absorbed by glass is most harmful to living beings and has been least investigated.

The action of any part of the UV spectrum on any system depends on the capacity of the system to be able to absorb it. More the absorbance of UV, more is its effect. Similarly, at the cellular level, the effects are maximal in cellular organelles, which show more absorbance of UV. Macromolecules, like nucleic acids, proteins and lipids, the important functional entities

of the cells, show their characteristic absorption spectra which coincide with their biological action spectra. Photochemistry of these macromolecules has been studied in great detail (for Ref., see McLaren et al., 1964).

Nucleic acids absorb maximum UV at 260 nm and pyrimidine bases are the main components of nucleic acids that absorb UV. The photochemical changes due to UV-irradiation on nucleic acids, which is most studied, is mainly manifested in the formation of thymine dimers, that is, dimerization of neighbouring pairs of thymine rings to form cyclobutane thymine dimers. Most of these photochemical studies on UV-irradiated nucleic acids has been done in vitro.

Besides the direct effect on nucleic acid, some indirect effects have also been observed, especially, through the activity of free radicals.

Most biological systems have an in-built repair mechanism to undo the UV damage on nucleic acids. This phenomenon is called photoreactivation. Most commonly occurring photoreactivation is the direct photoreactivation, which is light dependent and involves an enzyme called photoreactivation enzyme. The reaction involves irreversible binding of the enzyme to the UV-irradiated

DNA, splitting of the dimers and release of the enzyme. There is no excision or insertion of the bases.

Absorption maximum for most proteins is 280 nm. In proteins, S-S bonds are one of the important bonds which maintain the integrity of their tertiary or quartenary structures. Photolysis of S-S bonds is one of the first reactions that occur during UV irradiation of proteins. Inactivation of most enzymes like trypsin and chymotrypsin is interpreted on this basis.

UV-irradiation of lipids induces peroxidation in an oxygen atmosphere. Polyunsaturated lipids undergo free radical auto-oxidation in the presence of oxygen (Farmer and Sulton, 1943). Final product of the oxidation is malonaldehyde, short chained aldehydes and acids.

Most of the UV damages on the biological systems studied are either due to effects on nucleic acids, proteins or via the effects UV has on the cell membrane. There has been a long standing belief that the membranes of the cells are the primary sites of radiation effects (including UV). But the development of a suitable model for the radiation damage on the membrane and its effects on the functioning of the cell has yet to be worked out. Much work has been done in this field and some drastic effects of UV on the

membranes have been observed. These observations do not restrict themselves to the living membranes, but are also observed in the artificial lipid or phospholipid biological membranes. In these cases, UV-irradiation affects the functional capabilities and physiochemical properties of the membranes.

Effect of UV on nerve cells was studied as early as in 1930's by Audiat (1931). As excitation of nerve is basically a membrane phenomenon, the effect of UV on the action potential can be viewed as an effect of the radiation on the membrane, though the earlier views said that some chemical damages of the axoplasm were responsible for the UV effect on the action potential.

Amplitude of the action potential of the nerve preparation is lowered and the threshold raised, thus, finally resulting in an impulse propagation block. Action spectrum for the above observed phenomenon shows a peak between 260-280 nm. Membrane resistance (Ohm^2 - Cm) is significantly lowered along with the potential. These could be secondary changes caused by alteration of K^+, Na^+ and Ca^{++}-membrane relationship as produced by 260 nm radiation.

Na^+-K^+ stumulated ATPase is directly related to K^+ and Na^+ fluxes. There is a decrease in its activity post-

irradiation in the nerve membrane (Lieberman, 1967). Conclusion that is drawn from this observation is that the structural and functional components of the membrane system, responsible for the excitibility, are hampered by UV-irradiation.

UV light induces structural transition in the erythrocyte membranes (Konev et al., 1968). The irradiated ghost shows a lower degree of solubilization in detergents like sodium dodecyl cholate and triton X - 100. The degree of solubilization decreases with increase in UV dose. The low solubility of membrane may be caused by a decrease in availability of noncovalent intermolecular bonds for detergent molecules, or by formation in membrane of new covalent bonds, such as protein-protein or protein-lipid cross links. Konev et al. (1968) consider the membrane as a wide set of interrelated photosensitive components. The photoprocesses lead to structural changes influencing the structural state of the biomolecules of the membrane, which is considered not to be affected directly by UV. Since the absorbance of the membrane is changed during UV-irradiation only to a small extent, one may deduce that the mechanism of UV effect is related to late alterations, that is, through some photoproduct.

Plant lectins, which bind specifically to some cell surface carbohydrates, are useful probes for assessing

some of the properties of the cell surface components, such as characterization and localization of carbohydrate containing receptor sites. Concanavalin A binds to α-D mannopyranose-like residues on the cell surface and causes agglutination. Intensity of reaction varies with the condition of the cell. It has been seen that UV-irradiated cells do not agglutinate when treated with Con.A, although Con.A does bind to the binding sites on the membrane (Esteve et al., 1978). It has been suggested that UV interferes with the mobility of Con.A receptors in the plane of the membrane, which seems essential for cell agglutinations. Thus, the low agglutination is observed in irradiated cells when they are treated with Con.A.

UV exerts a pronounced inhibitory influence on proteins (Setlow and Doyel, 1957). UV sensitivity of many enzymes has been shown to be controlled by the structural state of the proteins e.g. trypsin, aldolase, RNase etc. Acetylcholine esterase occurs in two states, free and membrane bound (Konev et al., 1978). Photosensitivity of the membrane bound acetylcholine estrase is under the control of the structural state of the membrane. UV-irradiation inactivates both free and membrane bound enzymes. But, inactivation is faster and more in the latter case. As mentioned earlier there is a sure alteration in physicochemical structure of the membrane by UV (shown

by solubility changes). This structural rearrangement in the membrane possibly changes the conformation of acetylcholine esterase. This membrane mediated conformational change of the enzyme leading to its functional incapability is called photochemical allotopy.

Lipid peroxidation of membrane lipids may be induced by UV-irradiation. Lipid peroxidation as an explanation for UV damage on membranes is becoming rather popular. But, it cannot solely explain all the observed effects of UV-irradiation, e.g. in case of Acetylcholine esterase inactivation, the effect is more in oxygen atmosphere as compared to nitrogen atmosphere. But the activity is not totally recovered in nitrogen conditions. Thus, only lipid peroxidation probably can not explain all the observed changes (Konev et al., 1978).

Besides the biological membranes, much work has been done on liposomal membranes. Phospholipid membranes, during its formation, is brought in contact with potassium chromate solution. The membrane is dialyzed against Tris-HCl buffer to release the chromate. If the membrane is irradiated with UV, the release or leakage of chromate is increased. The increase is linear with the UV-irradiation (Mandal et al., 1978). With the increase in dose of UV, there is an increase in the degree of oxidation of membrane. Therefore,

there seems to be a correlation between the oxidation and the leakage of chromate. Hence, this effect of UV can also be attributed to lipid peroxidation.

For Meffert et al. (1976) lipid peroxidation provided a suggestive concept by means of which the sunburn and other photopathological conditions of the skin can be explained. Malonaldehyde (a lipid peroxidation product) when incubated with glucose-6-dehydrogenase , the enzyme sub-units get covalently cross linked, resulting in diminished or no enzyme activity. This can be taken to some extent as an indirect evidence to correlate lipid peroxidation with damages to biostructure.

Lipid peroxidation induced by UV also alters conductivity of bilayer phospholipid membrane (Putvinsky, 1979). Lipid peroxidation of unsaturated fatty acids in biomolecular lipid membrane, induced by UV, brings about an increase in conductivity. The precise mechanism of action of products of lipid peroxidation is still to be unravelled. The products are said to be negatively charged acidic substances.(Deev, 1976). Neutralization of this acidity, caused by the formation of this product, seems to render the membrane comparatively resistant to UV-radiation.

From all these observations, it can be seen that the effect of UV on the membrane might be as vital or important as the effect it has on nucleic acids.

A

Effect of UV on amoeba, in general, has been studied by several workers. In 1951, Mazia and Hirshfield studied the effect of UV in increasing dosages of whole amoebae and their fragments. Observing their survival, division rate and sterility they found that whole Amoeba is more reistant to radiation damage compared to anucleate or amputed amoeba fragments.

Using Amoeba as a generic term should always be in Capital and Underlines.

Synthetic capability of UV-irradiated amoeba has been investigated (Skreb et al., 1962). There is a decrease in RNA content post-irradiation which in time is increased again by the end of the fourth day after irradiation. This decrease is less in whole Amoeba than in amputated fragments. Similar results are obtained with proteins too, that is, a drop in protein contents immediately after irradiation and a recovery to almost normal level by 4th day post-irradiation.

both in anucleates & nucleates fragments

Length of starvation period preceeding UV-irradiation increases the sensitivity of the amoeba (Skreb and Errera, 1957). Cytochemical studies have shown that UV-irradiation results in a decrease in nuclear and cytoplasmic basophilia in nucleate and anucleate amoeba. It has been also noticed that, oxygen uptake drops off only in anucleate fragments, while in nucleate fragments or in whole amoebae it remains as high as the unirradiated control (Skreb, 1960).

Enzyme activity of acid phosphatase after UV-exposure has been studied in amoebae. Both nucleated and anucleated fragments show the same magnitude of inhibition post-irradiation unlike most other phenomena studied (Skreb, 1964).

These and other observations have been analysed to pinpoint the site of action of UV. Work has been done on flattened amoeba by Jagger et al. (1969). They have suggested that killing of amoeba, as an effect of UV, is due to both nuclear and cytoplasmic damages. Division delay is wholly due to cytoplasmic damage. There is not much interaction between nucleus and cytoplasm in this respect. The site of photoreactivation is mainly in cytoplasm. Since, it is almost certain that photoreactivation is restricted to nucleic acids, it is suggested that cytoplasmic nucleic acids, even RNA, may be affected by UV and are capable of photoreactivation.

Iverson, (1958) has tried to pinpoint the site of UV damage in amoeba. He has shown that the UV damage to the cytoplasm could be reversed by substitution of the irradiated nucleus with an unexposed nucleus, but the unexposed cytoplasm is not capable of undoing the UV damage to the nucleus.

Mitotic and early S-phase amoebae are more sensitive to UV as compared to mid-S, late-S and G_2 phase cells.

G_2 cytoplasm can reverse the damage to the exposed nucleus of early S-phase amoeba, the nucleus dies if transplanted into unirradiated S-phase cytoplasm. Therefore, there must be a repair system present in the G_2 phase cells (Chatterjee and Bhattacharjee, 1975).

The surface studies of amoeba has mostly been restricted to Amoeba proteus and Chaos (for ref., see Jeon, 1973). The surface is composed of a continuous trilaminar plasma membrane, 100 Å thick and an external filamentous coat. The plasma membrane differs from the rest of the cellular membranes of this organism in several ways. Plasma membrane is 100 Å thick unlike the endoplasmic reticulum membrane which is 60-70 Å thick. The three layers of the plasma membrane are not symmetrical in the sense, the inner denser lamina is thicker than the outer one.

The cell coat on the outside of the plasma membrane can be divided into two layers. The inner layers is a continuous amorphous layer of moderate electron density, of 150-300 Å thick. Outer layer is composed of numerous thin filamentous structures radiating outwards for a distance of 1500-2000 Å.

The cell surface chemically contains carbohydrates, proteins and lipids. It has neutral sugars and hexose

amines, major sugar being mannose, with a little galactose and glucose. Sialic acid and muramic acid are totally absent. Lipids are predominantly phospholipids. Aspartate, glutamate, serine, threonine and glycine are the major amino acids in the proteins of the surface. Amoeba surface, as all cell surfaces, has ionic groups. Unlike most cell surfaces, predominant ionic group is phosphate. As already mentioned, sialic acid and muramic acids, which are the major charged groups of most cell surfaces, are absent in amoeba.

The surface coat plays a big role in amoeba's membrane functions, especially, during endocytosis. The process of endocytosis shall be considered here briefly.

This process is assumed to include ingestion of anything between macromolecules and living food organisms. It basically involves invagination of the plasmalemma which then becomes lining membrane of the vacuole or channel enveloping the ingested material.

There are supposed to be two types of endocytosis-phagocytosis and pinocytosis. Phagocytosis is usually referred to as the endocytosis of light microscopically visible organisms and particles. Pinocytosis was defined by Lewis in 1931 as uptake of liquid droplets from the surrounding medium. But the recent definition by

Chapman-Andersen (1964) for pinocytosis is the uptake of material not visible under the light microscope. Phagocytosis is a natural mechanism for feeding, while pinocytosis is generally considered as a pure artificial phenomenon (Chapman-Andersen et al., 1956), although Rustad (1961) reported slight and occasional pinocytosis under normal culture conditions.

The phagocytic cycle of an amoeba as seen under a light microscope consists of the following events:

Food capture which involves the formation of the food vacuole around the prey. A locomoting or non-feeding amoeba adheres to the substrate over a small limited surface area and has longitudinal ridges over the posterior half of the cell. When such an amoeba is stimulated by prey animal, it flattens, usually losing its anterior position or polarity, and adheres to the substrate using a wider area of its surface (Jeon & Jeon, 1976). Phagocytic stimulus or initiation of phagocytosis solely depends on the interaction of the material to be phagocytosed and the cell surface of the amoeba. Both chemical and physical stimulus come to play at the prey-predator contact. Ciliates, the common food of these carnivorous amoeba, occasionally, brush against the amoeba in their rapid movement in the culture medium. This is suggested to result in partial clogging of a few cilia

that come in contact with the amoeba. This in turn, results in change in direction of its movement - it begins to circle back to the amoeba. This back and forth movement leads to more contact and more cilia damage. Thus, the prey slows down (Christiansen & Marshall, 1965). In a scanning electron micrographic study of Jeon et al. (1976) the Tetrahymena appears to be held immobile by the amoeba by means of a pseudopodial projection while the food vacuole is being formed. The contact between the prey and the amoeba also initiates the food vacuole formation. This process involves the evagination of the plasmalemma around the place of contact with Tetrahymena. This evaginated structure looks like a cup and thus, is called a phagocytic cup. The evagination continues around the prey till it completely surrounds it and the ends of the evaginations fuse. This leaves a vacuole surrounded by the cell membrane, with the prey inside. After the prey is captured, it is dehydrated resulting in the death of the prey. Lysosomal vesicles empty their contents (digestive enzymes) into the food vacuole leading to digestion and uptake of the digested material by the cytoplasm. The original food vacuoles are sub-divided into smaller vacuoles with more compact contents. After all the useful matter has been taken up by the cytoplasm, the vacuole may be referred to as defecation vacuole. The contents of these vacuoles are extruded from the cell

within 2-3 days by fusion of plasmalemma and incorporation of vacuole membrane into cell surface (exocytosis).

Pinocytosis is a mechanism used by the cell to engulf fluid through invaginations of plasma membrane. It can be induced by transferring the amoeba to inducer solution. They, then, respond to the presence of the solute (inducer) in the environment by formation of pinocytic channels.

The shape of the amoeba is altered when put in the inducer medium. Locomotion or cytoplasmic streaming becomes very sluggish. The contractile vacuole almost stops functioning when the pinocytosis is induced. Small pseudopodia are first formed in all directions giving the cell a rosette shape. The cell loses its attachment to the substratum. The pseudopodia lengthen and the tips of the pseudopodia invaginate to form the channels. The pseudopodia bearing the channels are composed of hyaline cytoplasm, containing few, if any, organelles. Finally the end of the channel starts forming vesicles of pinosomes. These vesicles are transported and the content processed in the cell (Chapman-Andersen, 1964).

Energy is consumed during pinocytosis and appears to be indispensable for channel formation. Metabolic inhibitions of diverse kinds inhibit channel formation in amoeba (Chapman-Andersen, 1965) and no pinocytosis occurs when temperature is lowered to $4^{\circ}C$ (Brandt, 1958).

The formation of pinocytic channel is brought about by local contraction in the cytoplasm underneath the plasma-membrane. Fibrillar cytoplasmic structures have been observed in amoeba during pinocytosis (Nachmias, 1968). These fibrils contain contraclile proteins. Both actin-like thin filaments (Polard & Ito, 1970) and thick myosin like filaments are present in Amoeba proteus. Where the contractile apparatus is situated or how it produces invagination of the cell membrane is not yet known.

During pinocytosis much of the surface membrane is incorporated into the pinocytic vesicles in the cytoplasm. The ameeba is non-locomotive while pinocytosing. It is estimated that 70% of the cell membrane has been ingested during pinocytosis. Thus, the amoeba can no longer move or pinocytose at least for 4 hours, that is, till enough membrane has been regenerated (Chapman-Andersen, 1963). Very little new membrane is formed during pinocytosis. The imbalance between ingested surface membrane and restored surface membrane must be re-adjusted before amoeba can again locomote or before it can be re-induced for pinocytosis.

As in phagocytosis, initiation of pinocytosis occurs by the interaction of the inducer with the cell surface proteins. Substances which induce pinocytosis, first bind to the amoeba surface before invagination of the cell membrane occurs. This adsorption of the inducer on the

membrane is actually the trigger for the pinocytic cycle. With respect to the stability of the inducer-membrane complex the inducing cations have been grouped into: (1) 3 weakly bound amino acids, (2) inorganic cations (Sodium, potassium ions are the commonly used inducers) and (3) reversibly bound protein (bovine serum albumin, egg albumin etc.). Induction by protein is dependent upon the pI of the protein and the pH of the solution of the inorganic salts, monovalent cationic ones are effective to induce pinocytosis. Neutral or anionic salts do not induce at all (Chapman-Andersen, 1962).

The present investigation was undertaken to study the effect of UV irradiation on endocytic processes in a large, free living protozoa , Amoeba indica. The two important membrane related functions, viz., phagocytosis and pinocytosis, which can be easily visualized and quantitated in these organisms provide a suitable mode of approach to investigate some aspects of UV action on the membrane. These studies on UV-action on membrane functions are supported by the simultaneous assay of the treated cells' overall protein synthetic pattern in an attempt to correlate the altered endocytic process, which might occur, with the turnover and replenishment of the membrane proteins in amoeba.

MATERIALS AND METHODS

Amoeba used for the present study was Amoeba indica, originally collected from a pond in Bombay, India (Chatterjee and Rao, 1974). The amoebae were maintained at 22°C and fed with Tetrahymena pyriformis. The cultures were maintained at 12 hours light and 12 hours dark cycle.

All cultures were maintained in amoeba culture medium containing 5 mg $CaHPO_4$, 4 mg $MgSO_4$ and 6 mg KCl in each litre of the medium (pH 6.8). Tetrahymenae were cultured in 2% proteose peptone under sterile conditions. They were washed several times with amoeba medium and harvested by centrifugation before they were used to feed the amoebae.

The amoebae were fed once a day for 10-12 hours, after which, the medium with excess food were drained off and replaced with fresh medium. The petri plates were changed every 2-3 days.

Experiments reported here were all performed on 24 hours starved amoebae from tetrahymena fed cultures.

Irradiation of Amoebae:

For each experiment, about 10 cells were put into syracuse watch glasses using a braking pipette. Approximately

0.2-0.3 ml of medium was added to each of the watch glasses.

Watch glasses containing the cells were irradiated from above. UV source used was a germicidal lamp (Philips, Holland, 30W) with emission maximum at 260 nm. The intensity of radiation was checked with the help of a radiometer (Radiometer Company, Copenhagen), as 3.2 J/m^2, dose rate being 3.2 J/m^2.sec. Duration of exposure was 25 secs.

Morphological changes and lethality was checked for different durations of UV exposure (10-150 secs.) at the dose rate of 3.2 J/m^2.sec. The optimum duration which was found to be suitable for our studies was 25 secs. Thus, the total dose delivered was 80 J/m^2. At this dose all the cells were viable.

The medium was drained off after the UV exposure and fresh medium was added to the watch glasses. The cells were kept at 22°C under white light throughout the rest of the experiment.

QUANTITATING PHAGOCYTOSIS:

Staining of Tetrahymena - Tetrahymenae was stained with natural red to facilitiate counting of the phagosomes. Neutral red being a vital stain, the ciliates remained alive throughout the experiment.

Tetrahymenae were harvested from proteose peptone as described. The harvested cells were brought upto a specific concentration such that the percent transmission at 600 nm was 75%. 3 ml of this suspension was taken in a test tube to which equal volume of 2% neutral red stain was added. Thus, the total concentration of the stain was 1%. The cells were allowed to remain in the stain for 20 mins, after which, they were centrifuged down and stain decanted off. The pellet of stained tetrahymenae was resuspended in 3 ml of amoeba medium. The tetrahymenae were stained just before feeding the amoeba, that is, they were freshly stained for each feed.

A drop of tetrahymena suspension was put into a syracuse watch glass, containing the amoebae, immediately after irradiation. After two hours, when an appreciable number of phagosomes were formed, the amoebae were picked up and put on a slide. A cover slip was placed gently on it. The number of phagosomes visible as red vacuoles, in each amoebae, were counted under a microscope (Meopta, Czechoslovakia, Magnification - 80 X) and recorded. The control amoebae were similarly counted for their food vacuoles.

The counting was repeated with irradiated cells at 1, 2, 3, 4, 5, 6, 9 and 12 hours, post-irradiation. Each treated lot is run with a control set.

This whole procedure of feeding and counting was done in batches of 10 amoebae per reading for irradiated and control cells. It was difficult to handle more than 10 cells at a time. Thus, the experiment was repeated five times to get at least 50 cells for each hour.

QUANTITATING PINOCYTOSIS:

The method ~~that was~~ followed was according to that of Chapman-Andersen (1962).

15-20 amoebae were transferred into a cavity slide, which had been rinsed with the inducer medium. The amoeba medium was sucked off. A few drops of inducing medium (0.125 M NaCl in 0.01 M phosphate buffer, pH 6.4) was placed over them and a cover slip was gently placed.

The position of the first amoeba was noted on the microscope stage and the number of channels counted and recorded. The time was noted with the help of a stop watch. Similarly, the second amoeba was found, position noted and channels counted and so on, till 10 amoebae have been counted for pinocytic channels.

The amoebae were recounted. This continues for 3-4 times in the course of 30 mins. 30 mins was found to be the total duration of a pinocytic cycle (Microscope-Carl Zeiss, Jena, DDR, Magnification - 160 X).

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This sequence for counting channels was done every hour after irradiation till 6 hours. Control cells were also similarly counted for their pinocytic channels.

It is known that after a cycle of pinocytosis an amoeba can not again be induced to pinocytose immediately. This is because a lot of surface membrane is incorporated into the pinocytic vesicles in the cytoplasm. Thus, the membrane surface has to be regenerated before the cell can pinocytose again. This regeneration takes about 4-5 hours. To see if the UV has any effect on this lag period, the irradiated and control cells were challenged again with the inducer to pinocytose 4 hours and 6 hours after the first cycle. The channels were counted after the induction of the second pinocytic process, as described before, for both irradiated and control cells.

^{3}H-leucine incorporation:

50 cells were taken in 2 sets for each experiment. One set was irradiated and other served as the control. At each subsequent hour after irradiation till 6 hours, 50 cells were placed in 200 μCi/ml of ^{3}H-leucine (Specific activity: 7600 mCi/mMole). After an hour of incubation, the cells were washed and transferred to medium containing unlabelled leucine (2.6×10^{-3} mM/ml) for 5-10 mins. The cells were then transferred into a test tube with the lysing medium (1% SDS and 1 mM EDTA) at 60^{o}C for 30 mins.

After the cells have been lysed, 300 µg of carrier protein (BSA) was added followed by precipitation of protein by 10% TCA. Precipitation mixture was left in cold ($4^{o}C$) for at least 2 hours. The precipitate was collected on millipore filter and washed several times with 5% cold TCA. The filter paper was then placed in Bray's scintillation mixture (60 g naphthelene, 40 g PPO, 0.2 g POPOP, 100 ml methanol, 20 ml ethylene glycol, the volume finally made upto 1 litre with 1,4 dioxan) in a vial and counted in a Packard Scintillation Counter (Efficiency - 60% for ^{3}H).

^{3}H-leucine uptake:

The same procedure as for the ^{3}H-leucine incorporation was followed till the amoebae were lysed in the lysing medium in test tubes. The content of the test tubes were emptied into a vial with Bray's scintillation mixture and counted.

Sources of Chemicals:

$CaHPO_4$, $MgSO_4$, KCl, NaCl, NaH_2PO_4, Methanol and 1,4 dioxan were obtained from B.D.H., India. Leucine, SDS, EDTA, BSA, PPO and POPOP were obtained from Sigma Chemical Company, USA. Neutral red was purchased from George T. Gurr, Ltd., London and Na_2HPO_4 from E. Merck, India. Ethylene glycol was from Sarabhai Chemicals Ltd., Napthelene from

Koch-Light Laboratories Ltd., TCA was supplied by Riedel-De Haen Ag Seeze-Hannover, Germany. ^{3}H-leucine was obtained from BARC, Bombay, India.

Abbreviations Used:

BSA	:	Bovine Serum Albumin,
$CaHPO_4$	:	Calcium Hydrogen Orthophosphate
EDTA	:	Ethylene diamine tetra acetic acid
KCl	:	Potassium chloride
$MgSO_4$	:	Magenisium sulphate.
NaCl	:	Sodium chloride
NaH_2PO_4	:	Sodium phosphate monobasic
Na_2HPO_4	:	Sodium phosphate dibasic
PPO	:	2,5-diphenyloxazole
POPOP	:	Phenyl-oxazolylphenyl-oxazolylphenyl.
SDS	:	Sodium dodecyl sulphate
TCA	:	Trichloroacetic acid.

RESULTS

Effect of UV on Phagocytosis:

As mentioned in Material and Methods, the effect of UV on phagocytosis was studied in five experiments with 10 cells in each. Table 1 shows the average counts of phagosomes in 10 cells at 0 hour and every subsequent hour post-irradiation till 6 hours. For 9 and 12 hours post-irradiation only three experiments are reported.

As can be seen from the Table 1, there is some variation in the number of phagosomes formed even in the control cells. This could be because the cells are randomly picked from an asynchronous culture. There was always a control run alongside each of the irradiated sets at each hour in each experiment. The data is expressed as percent of number of phagosomes formed in irradiated cells as compared to control, taking the control readings as 100%.

Fig. 1 shows the graphical representation of the effect of UV-irradiation on phagocytosis. It clearly shows that there is an inhibition of phagocytosis in irradiated cells. At 0 hour, on an average, there is a decline of only about 12% of the number of phagosomes formed as compared to control counts.

This is followed by a sharp decline to about 27% of the control at 1 hour post-irradiation. This effect is

gradually recovered. By about 6 hours after exposure, it is about 80% of the control. Recovery is completed by about 12 hours, when it is only 0.39% lower than the control.

Effect of UV on Pinocytosis :

The number of pinocytic channels were counted hourly after irradiation till 6 hours. Averages of 3 experiments (with ten cells in each) are given in Table 2.

The data on pinocytosis is represented graphically in Fig. 2. In control, there is a peak in the channel formation at 8 minutes, which declines thereafter. In all the hourly readings, except in 1 hour reading, the peak in irradiated cells is seen to be shifted to 14th minute of induction. But the number of pinocytic channels formed in both control and irradiated cells is almost the same, except in the 4 hour post-irradiated cells, where the number of channels formed are somewhat higher than control cells.

The irradiated cells were re-induced with the inducer to pinocytose 4 hours and 6 hours after the first pinocytic cycle. At 4 hours after the first pinocytic cycle, the control cells are able to form the normal number of pinocytic channels, with the peak at 8 minutes. But, as for the irradiated cells, the number of channels formed is very low. In the first hour post-irradiated cells there is no peak at all. In the others, there is some faint indication of a peak formation (Table 3 and Fig. 3).

At 6 hours after the first pinocytic cycle, both the control and the irradiated, cells are able to form normal number of pinocytic channels showing the peak at 8 minutes.

^{3}H-leucine Incorporation:

^{3}H-leucine incorporation studies were undertaken to see if there is a correlation between the effect of UV on phagocytosis and pinocytosis and protein synthesis in the irradiated cells. The result is shown in Table 4 and Fig. 4, and the counts per minute of irradiated cells are represented as percent count of that in control cells.

At the first hour post-irradiation, there seems to be a higher incorporation in treated cells than the control. There is a decline to about 20% of the control counts at 3rd hour post-irradiation, followed by a gradual recovery to about 60% by about 6th hour post-irradiation.

^{3}H-leucine Uptake:

The amino acid uptake pattern was investigated to assess the effect UV has on the permeability property of the amoeba membrane. In Table 5 and Fig. 5, the counts per minute of irradiated cells is expressed as percent of that of the control. At the first hour after irradiation, there seems to be a considerable stimulation in uptake of

^{3}H-leucine as compared to the control, which is followed by a decrease in uptake, At 3 and 4 hours post-irradiation the uptake is as low as 21 and 23% of the control. The precursor uptake after 5 hours post-irradiation shows an inhibition of about only 40% as compared to control.

TABLE 1

Expt. No.	Hours post-irradiation 0 C	0 T	1 C	1 T	2 C	2 T	3 C	3 T	4 C	4 T	5 C	5 T	6 C	6 T	9 C	9 T	12 C	12 T
1	10	7	11	4	9	4	6	3	10	4	5	3	11	9	8	8	10	10
2	11	9	11	3	9	6	12	5	6	5	5	4	10	8	9	8	5	6
3	10	7	9	3	11	5	15	8	18	10	10	7	9	7	8	7	9	7
4	3	4	10	2	17	9	9	5	15	10	6	5	6	4	-	-	-	-
5	5	4	11	3	10	6	6	5	11	8	7	5	4	4	-	-	-	-

Effect of UV-irradiation on phagocytosis of Amoeba indica. The treated and control amoebae were fed on tetrahymenae at different intervals for 2 hours and then phagosomes were counted in 10 amoebae. 9 sets of irradiated cells (T) were studied (0,1,2,3,4,5,6, 9 & 12) hours post-irradiation) each with a control set (C). Average number of phagosomes formed per control cell and irradiated cell in 5 different experiments has been shown. Only three experiments were done for 9 and 12 hours sets.

Figure 1: Effect of UV-irradiation on the phagocytosis of *Amoeba indica*. The amoebae fed on *tetrahymena* for two hours at different intervals after irradiation and the phagosomes counted. 9 sets of irradiated cells were studied (0, 1, 2, 3, 4, 5, 6, 7, 9 & 12 hrs post-irradiation) each with a control set. No. of phagosomes in each of the sets were expressed as percent of control value.

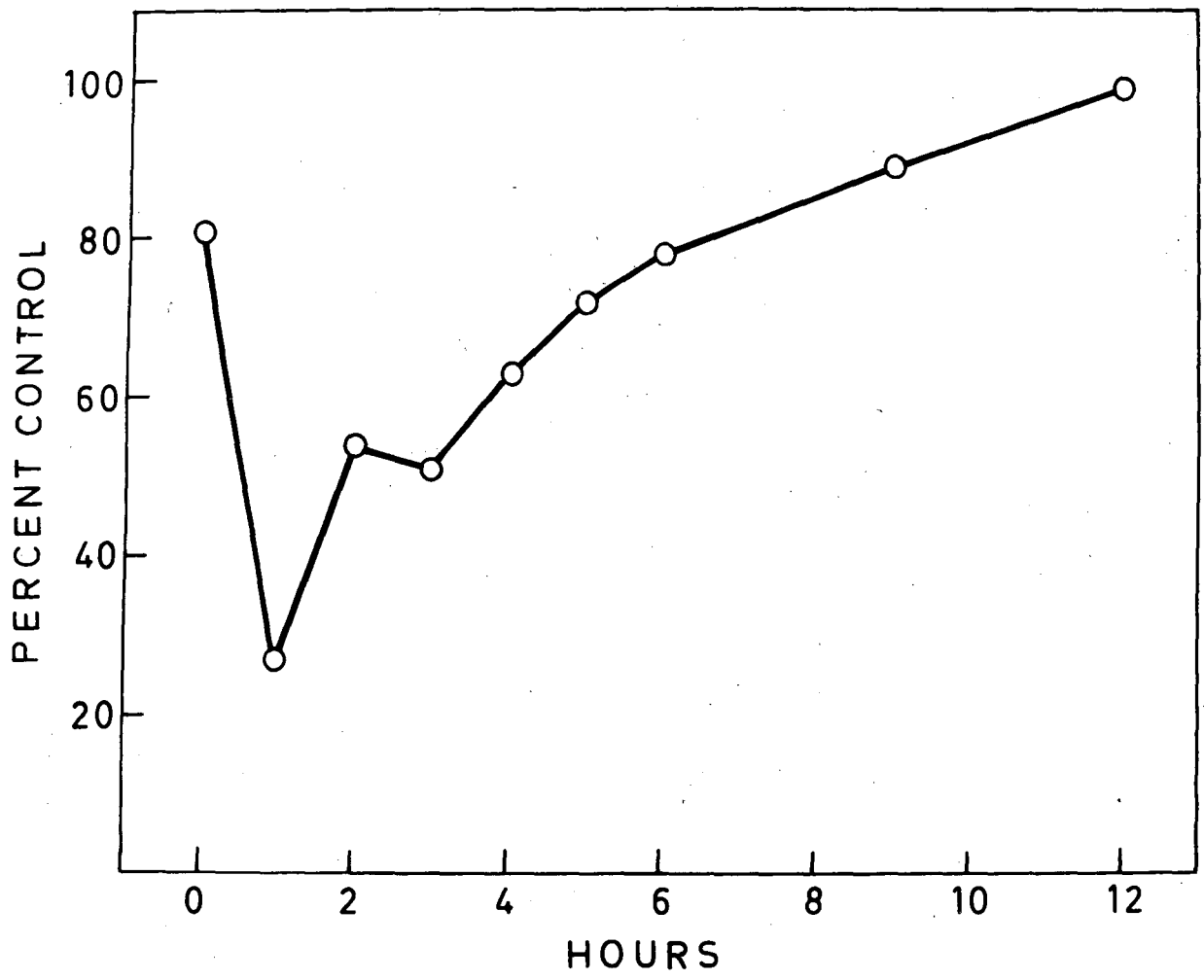

FIG.1 EFFECT OF UV ON PHAGOCYTOSIS

TABLE 2

	Mins. after Pinocytic induction			
	2	8	14	22
Control :	10 ± 0.5	14 ± 1	13 ± 1	9 ± 1
Treated ; (Hrs after irradiation)				
1	7 ± 1	12 ± 2	11 ± 1	8 ± 1
2	9 ± 1	11 ± 1	14 ± 1	9 ± 1
3	8 ± 1	13 ± 1	13 ± 1	9 ± 1
4	7 ± 1	19 ± 1	20 ± 1	11 ± 1
5	8 ± 1	14 ± 1	15 ± 1	8 ± 1
6	7 ± 1	11 ± 1	14 ± 2	9 ± 1

Effect of UV on the pinocytosis in Amoeba indica. The pinocytosis was induced by 0.125 M NaCl in 0.01M phosphate buffer, pH 6.4. No. of channels formed per cell were counted in controls and 6 sets (1-6 hours post-irradiation) of irradiated cells at 0,8,14 and 22 mins after induction of pinocytosis at one hour interval.

Figure 2: Effect of UV-irradiation on the pinocytosis in Amoeba indica. The pinocytosis was induced by 0.125 M NaCl in 0.01M sodium phosphate buffer, pH 6.4. No. of channels formed were counted at 2, 8, 14, 22 mins post-induction in irradiated and control cells. At 1 hr interval upto 6 hours after UV-exposure, the counts for both control (....) and irradiated (——) cells were plotted against time post-induction.

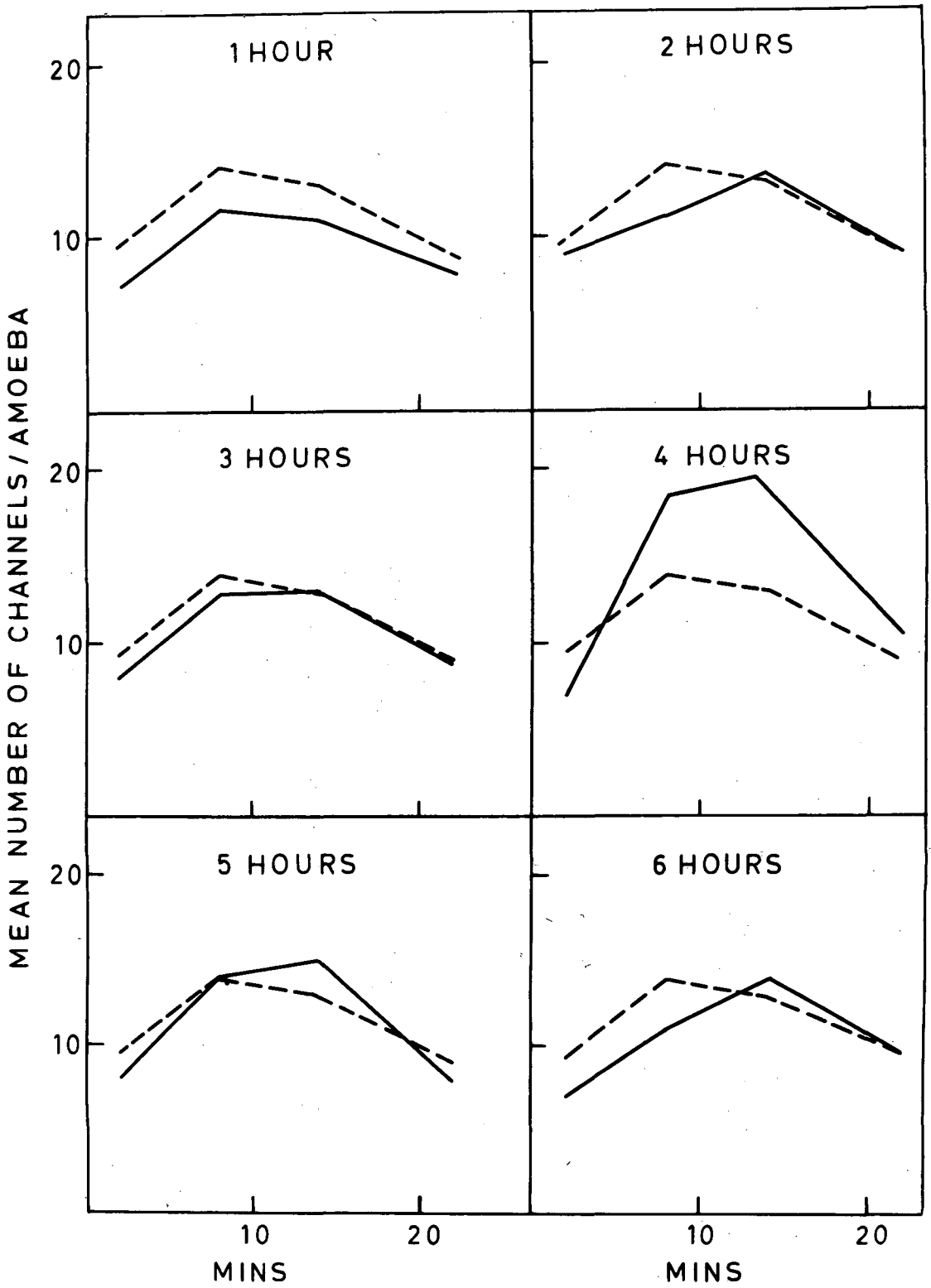

FIG. 2 EFFECT OF UV ON PINOCYTOSIS

TABLE 3

	Mins after Pinocytic induction			
	2	8	14	22
Control :	12 ± 1	15 ± 1.5	12 ± 2	6 ± 1
Treated : (Hrs after irradiation)				
1	6 ± 1	4 ± 0.5	2 ± 0.4	1 ± 0.3
2	5 ± 1	6 ± 1	4 ± 0.4	2 ± 1
3	5 ± 1	7 ± 1	5 ± 1	3 ± 0.4
4	5 ± 1	7 ± 1	3 ± 1	2 ± 0.4
5	5 ± 1	7 ± 1	5 ± 1	4 ± 1
6	5 ± 1	7 ± 1	5 ± 1	3 ± 0.3

Effect of UV-irradiation on re-induction of pinocytosis in Amoeba indica. After 4 hrs of the first pinocytic cycle, the cells were again put in the inducer medium (0.125M NaCl in 0.01M phosphate buffer, pH 6.4). No. of channels formed/cell were counted at 2,8,14 and 22 mins after induction of pinocytosis in control and irradiated cells (106 hours post-irradiation) at one hour interval.

Figure 3: Effect of UV-irradiation on the reinitiation of pinocytosis in Amoeba indica. 4 hr after the first pinocytic cycle, the cells were again put in the inducer medium (0.125 M NaCl). No, of channels formed were counted at 2, 8, 14 & 22 mins. after the induction in control and irradiated cells (1, 2, 3, 4, 5, 6 hours post-irradiation). The counts for both control (....) and irradiated cells (——) were plotted against time.

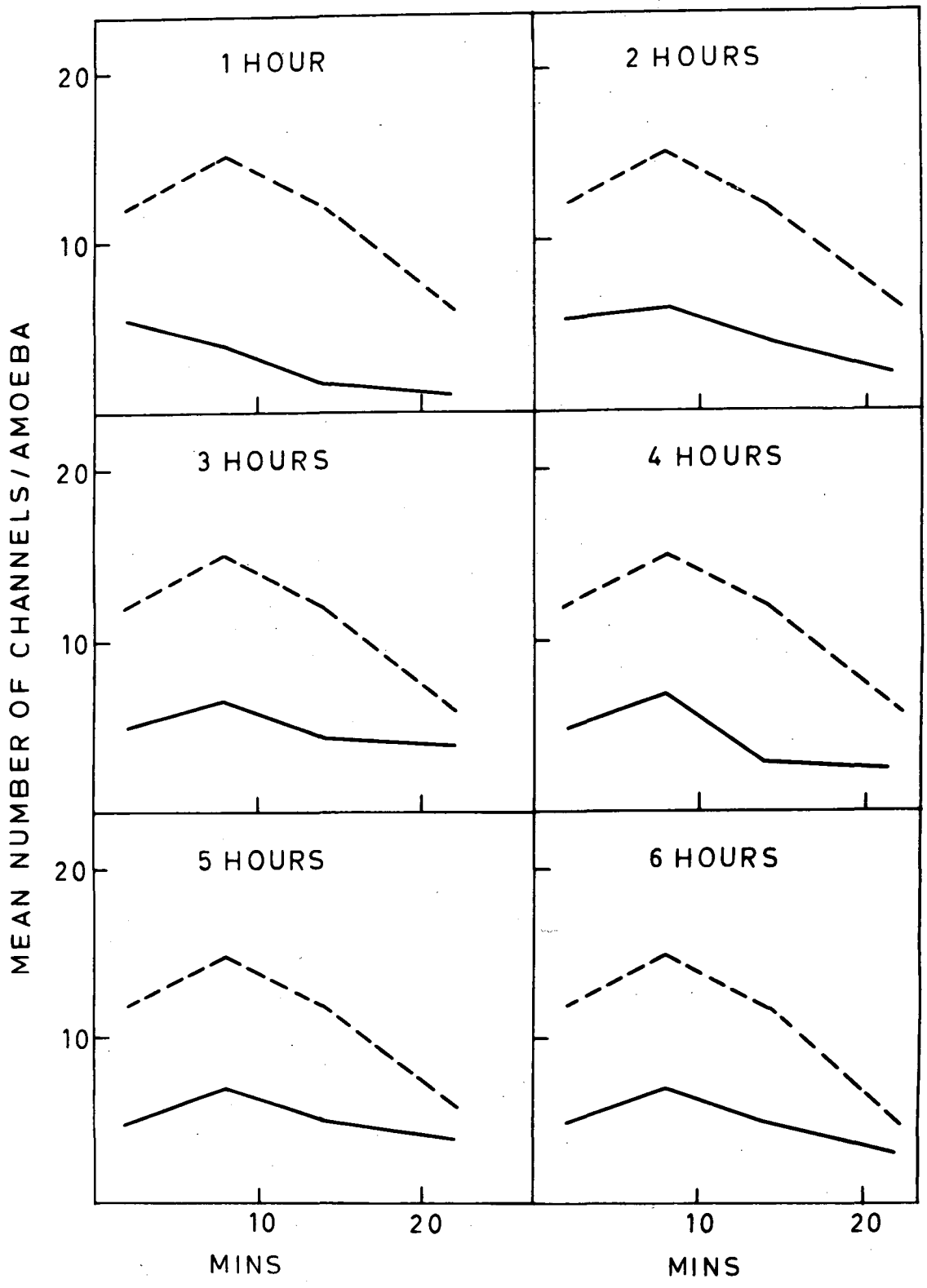

FIG. 3. EFFECT OF UV ON PINOCYTOSIS

TABLE 4

Hours post-irradiation	% of control counts
1	110.93
2	94.84
3	19.92
4	40.22
5	68.83
6	59.47

Effect of UV-irradiation in incorporation of ^{3}H-leucine in Amoeba indica. 6 sets of irradiated cells (1-6 hours post-irradiation) were incubated for 1 hour in ^{3}H-leucine (200 µCi/ml) each with a control set and counted in a scintillation counter. The counts of each irradiated set is expressed as percent of control value.

Figure 4 : Effect of UV-irradiation on incorporation of ^{3}H-leucine in *Amoeba indica*. 6 sets of irradiated cells (1,2,3, 4, 5 and 6 hours post-irradiation) were incubated for 1 hour in ^{3}H-leucine (200 µCi/ml), each with a control set and counted in a scintillation counter. The counts of each of the irradiated set was expressed as percent of control value.

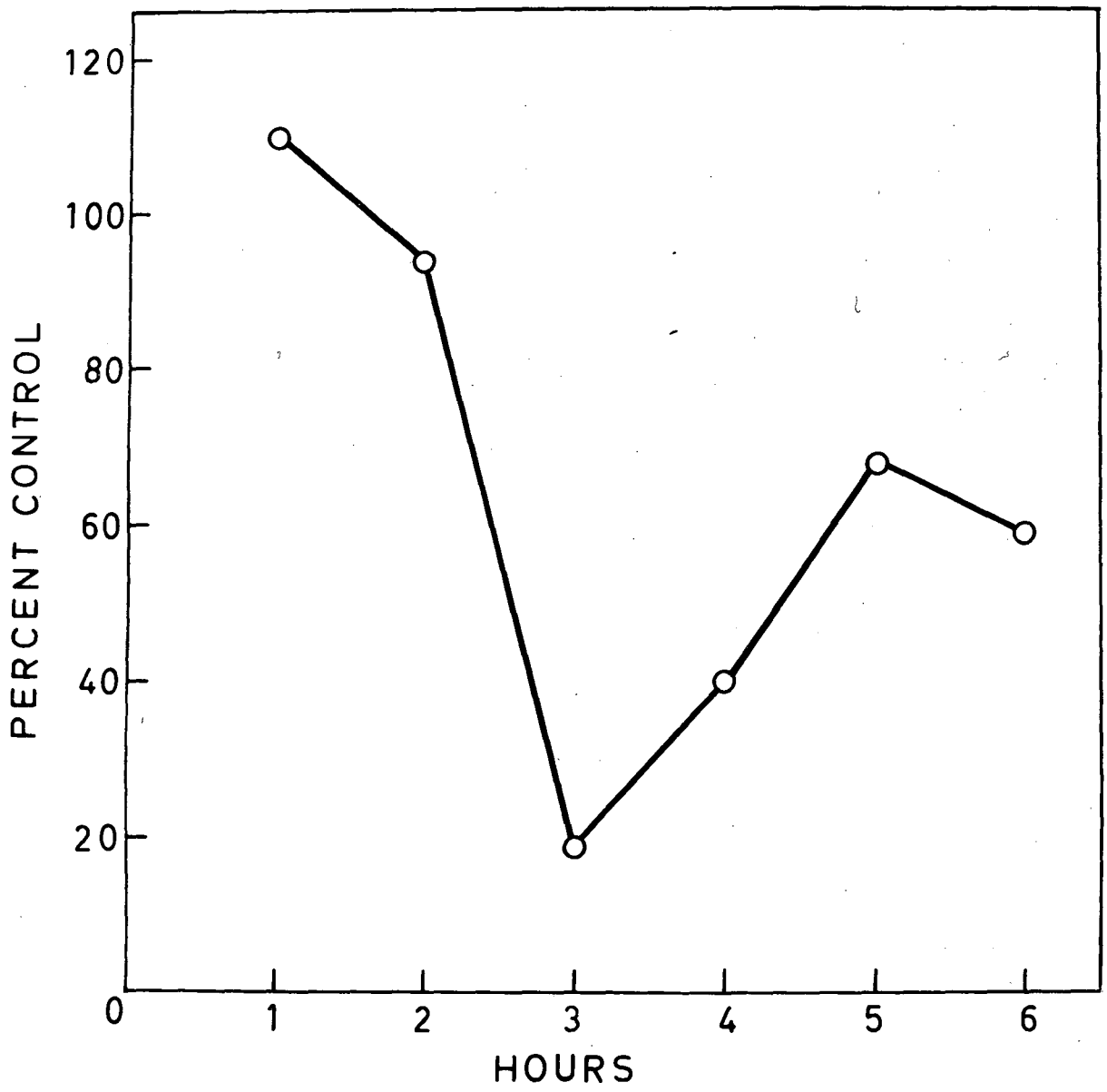

FIG. 4. ^{3}H-LEUCINE INCORPORATION.

TABLE 5

Hours post-irradiation	% of control counts
1	230.4
2	46.26
3	21.04
4	23.67
5	62.06

Effect of UV-irradiation on uptake of ^{3}H-leucine by Amoeba indica. 5 sets of irradiated cells irradiated cells (1-5 hours post-irradiation) were incubated for 1 hour in ^{3}H-leucine (200 µCi/ml) each with a control set and counted in a scintillation counter. The counts of each irradiated set is expressed as per cent of control value.

Figure 5: Effect of UV-irradiation on uptake of ^{3}H-leucine by Amoeba indica. 5 sets of irradiated cells (1-5 hours post-irradiation) were incubated for 1 hour in ^{3}H-leucine (200 μCi/ml), each with a control set and counted in a scintillation counter. The counts of each irradiated set is expressed as percent of control values.

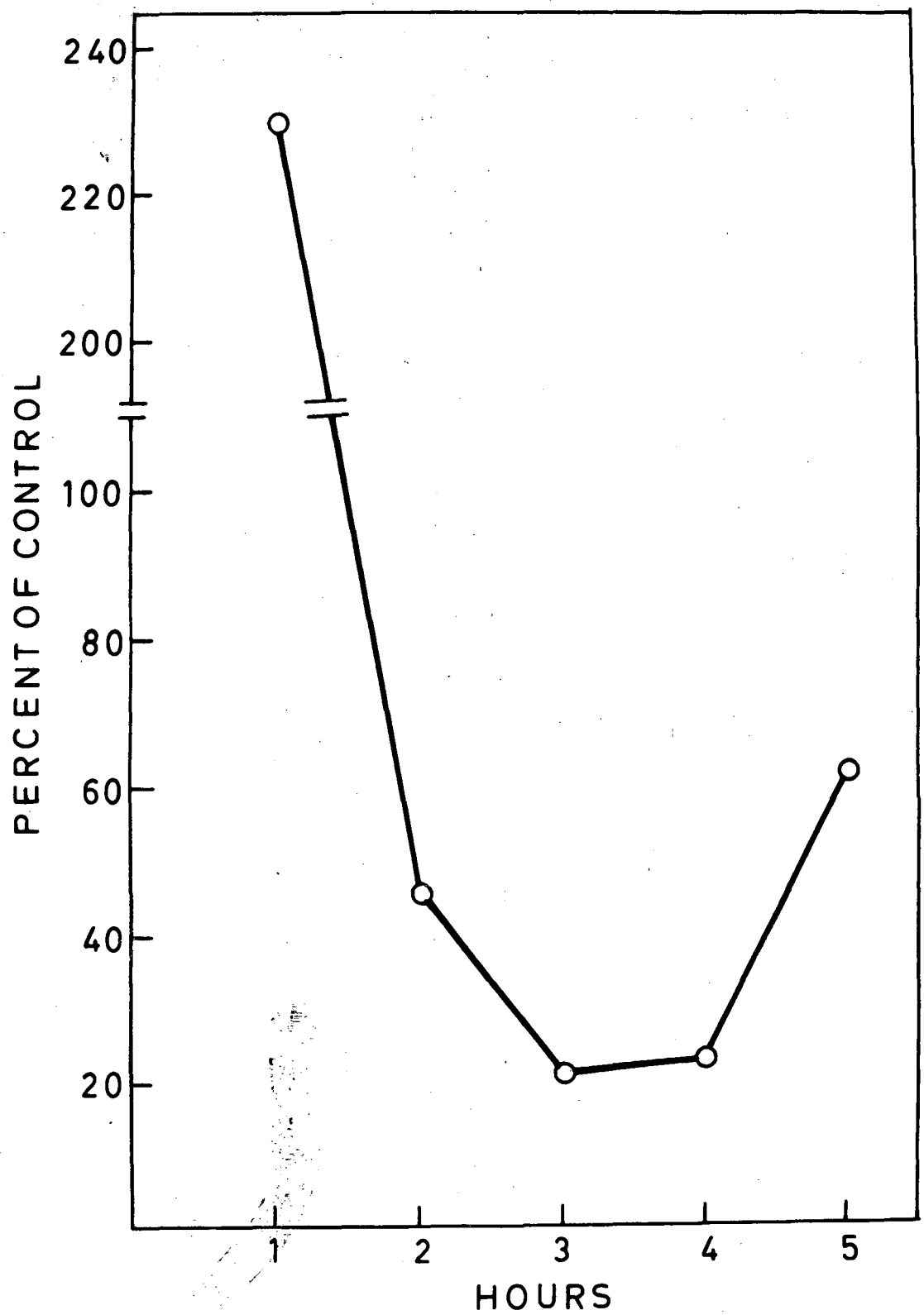

FIG. 5. UPTAKE OF ^{3}H-LEUCINE

DISCUSSION

We
~~Our foregoing experiments try to~~ investigated the irradiation
effects of UV exposure on the endocytic functions in amoeba, namely, phagocytosis and pinocytosis. It is known that both these membrane related functions are greatly dependent on membrane turnover and reassembly (Goodall and Thompson, 1971; Chapman-Andersen, 1972). Consequently, it was thought worthwhile to investigate also the UV-exposed cells' ability to synthesis proteins as measured by incorporation of radiolabelled amino acids. We shall describe the endocytic processes in UV-treated amoebae as compared to the unexposed cells and then try to correlate these functions with the treated cells' overall protein synthesis profile.

Effect of UV on Phagocytosis:

Our experiments clearly show that the UV has an inhibitory effect on phagocytosis but the effect is not immediate, that is, the inhibition at 0 hour is just about 12% to that of control value, which is followed by a sharp decline in phagocytosis showing an inhibition of about 70%. There is a slow recovery in phagocytic activity thereafter. By about 6 hours, the number of phagosomes formed by the irradiated cells is about 80% of that formed by the control. And 12 hours after irradiation

Statistical Analysis for Significance expressed as p values. This is important especially because of the Considerable Variation seen in control animals.

the number of phagosomes formed by the irradiated cells is almost same as that of the control cells.

UV-irradiated cells show this kind of inhibition of phagocytosis even though no visible difference was seen between the shape of the irradiated cells and their mobility as compared to the control cells.

There is an initial lag in the effect of UV-radiation on phagocytosis. It is, thus, probable that direct UV effect, if any, on the membrane is either repaired very fast so as not to show any immediate effect or the effect takes a long time before it can manifest itself. The UV effect on the membrane can also be a purely indirect one, with respect to phagocytosis in amoeba. In this case, UV might be impairing some other intracellular system(s) which, in turn, has its effect on the membrane and, thus, on the phagocytic activity of the membrane. Therefore, the probable indirect damage of UV, which may cause phagocytotic inhibition, takes about 1 hour to appear.

Effect of UV on pinocytosis:

Our present studies on pinocytic ability of the UV-irradiated cells show a number of interesting features. Control cells show a peak at 8 minutes after induction. For cells of 1 hour post-irradiation group, the pattern of the cycle is the same as that of the control, that is, the peak is at 8th minute, but the number of channels

formed is slightly lower than the control value. The rest of the irradiated sets (2,3,4,5 and 6 hours post-irradiation) show a shift in the peak from 8 to 14 minutes after induction. The number of channels formed are, however, almost the same as that formed in the control cells. That means, there occurs a slower rate of channel formation in the irradiated cells, although they are able to complete the pinocytic cycle within about 30 minutes, like the control amoebae. In 4 hours post-irradiation group of amoebae there seems to be an increase in number of pinocytic channels formed, though the peak is formed at 14 minutes after induction. The reason for the stimulation in channels forming activity is not known.

Pinocytosis involves invagination and subsequent incorporation of cell membrane into the cytoplasm. After a cycle of pinocytosis an amoeba takes about 4-5 hours to be able to pinocytose again (Chapman-Andersen, 1963). Therefore, as expected the control cells followed the normal cycle of pinocytosis when they are induced again after about 4 hours of the first induction. But, the irradiated cells are not able to pinocytose normally. The number of channels formed is greatly reduced in UV-exposed cells. The cells from 1 hour post-irradiation set show no peak while the other groups show a feeble

indication of a peak. Reinduction at the 6th hour after the first cycle of pinocytosis show that by this time the irradiated cells are able to pinocytose like the control cells.

The above observation implies that the rate of restoration of the cell membrane in irradiated cells is lower than that of the control. Thus, UV irradiation probably impairs the synthesis of the membrane specific components and thus restore these components at a slower rate to the surface of the cells. It is obvious from Fig. 3 that 6 hours, after irradiation, the cells recover to some extent in their pinocytic ability as compared to those of 1 hour post-irradiation amoebae. Therefore, it can be concluded that there is probably a recovery in the rate of synthesis of membrane components by this time.

Effect of UV on pinocytosis has been studied by Rinaldi (1959). At a high dose of 254 nm (9888 ergs/mm^2) he has found the amoebae to pinocytose, even in the absence of any inducer in the medium. But the number of channels that were ~~been~~ formed were lower than the control. UV-induction of pinocytosis has also been observed in enucleate portions of the amoeba. This supports the view that there may be extranuclear damage resulting from UV-radiation which may also be responsible for the effect observed.

However, at the intensity we have tried (800 ergs/mm^2) no spontaneous induction of pinocytosis has been observed.

Examining the action spectrum of UV induced pinocytosis, Josefsson (1976) has indicated that proteins are the possible targets of the radiation especially those in or near the cell membrane. All effects of UV are abolished by reduced form of dithiothreitol (a radioprotector which form mixed di-sulphide bonds with free SH-groups of the proteins and thus preventing oxidation of SH group of the proteins by free radicals). Thus, the mode of UV action may involve disruption of these groups of the proteins. Similar mode of action of UV has also been observed in nerve fibres (Fox et al., 1975; Nakas et al., 1975).

Incorporation and uptake of ^{3}H-leucine in UV-exposed amoeba:

The pattern of incorporation of ^{3}H-leucine into total proteins shows an inhibition at 3 hours post-irradiation which is followed by a slow recovery to about 60-70% by about 6 hours. One thing that is worth noticing is the stimulation in uptake 1 hour after irradiation. The irradiated cells at 1 hour post-irradiation show an increase of about 10% incorporation of ^{3}H-leucine as compared to control.

This spurt in incorporation of ^{3}H-leucine is very well reflected in the uptake of the same by the cells. But the percent increase in uptake is much higher than that of control. As in case of incorporation, there is an inhibition of uptake of ^{3}H-leucine at 3 hours post-irradiation amoebae. The inhibition at the 3rd hour is continued into the 4th hour followed by a recovery to about 60% of the control value by the 5th hour of treatment.

Many other workers have reported a decrease or inhibition of uptake of various substances, like amino acids and sugars in various systems as an effect of irradiation of UV (Doyle et al., 1976; Sontag, 1977; Sprott et al., 1976; Sprott et al., 1977). Bhattacharjee and Chatterjee (unpublished observations) have found that near UV light at a sublethal fluence significantly inhibit the uptake of ^{3}H- bromo-uracil in Amoeba proteus. Near UV and visible light has been found to inactivate several membranes transport systems, making the cells "leaky" (Koch et al., 1976; Bhattacharjee and Chatterjee, 1977).

The increase in uptake of ^{3}H-leucine one hour after irradiation in our experiments can only be explained as a result of a direct injury of UV on the membrane which probably renders it leaky, thus more permeable to the amino acid administered.

UV-irradiation is known to inhibit protein synthesis. The action spectrum shows that 260 nm is the most effective in this respect (Muel et al., 1979). Many workers have reported that 254 nm radiation causes cross links between mRNA and protein components of ribosomes in E. coli (Gorelic, 1975; Gorelic, 1976; Ehresmann et al., 1975; Moller and Brimacombe, 1975). The photoinduced cross linking of rRNA and proteins in the ribosomes is thought to occur as a result of photoexcitation of the rRNA bases and amino acid components of ribosomal proteins. The action spectrum of UV with regards to RNA/protein cross linkage is similar to the action spectrum of UV for protein synthesis inhibition. Thus, the cross link formed between the two major components of the ribosomes (RNA and proteins) has been attributed as the cause for inhibition of protein synthesis in UV-irradiated cells.

One aspect that needs more attention is the similarity between the phagocytosis pattern and the ^{3}H-leucine uptake/incorporation pattern in irradiated cells. Both patterns have a lag before the inhibition of the respective activities. Although the length of lag period and the time of maximum inhibition do not precisely coincide, they still seem to be related. It probably shows that UV action on amoeba follows a lag-inhibition-recovery pattern.

As regards pinocytosis, the irradiated amoebae cannot be re-induced to pinocytose to the fullest extent 4 hours after the first pinocytosis. But the same was possible after 6 hours. This observations, as already discussed, shows that the rate of synthesis and restoration of membrane specific elements in irradiated cells is slower than the control cells which can pinocytose normally 4 hours after the first pinocytic cycle. This explanation is supported by the fact that the incorporation of ^{3}H-leucine is inhibited in irradiated cells. Synthesis of membranes is, obviously, dependent on the synthesis of proteins. Hence, inhibition of protein synthesis in irradiated cells does inhibit the synthesis of the membranes, too. Consequently, this impaired synthetic activity is reflected in the reduced pinocytic activity in irradiated amoeba.

The foregoing discussions clearly show that the endocytic processes are affected in UV treated amoebae. The phagocytic phenomenon shows a considerable inhibition after a lag period while pinocytosis shows only a slower rate of activity. However, the ability of the UV treated cells to pinocytose again is greatly impaired in the irradiated cells as compared to control cells.. The rate of synthesis of proteins as measured by the ^{3}H-leucine incorporation shows a definite decline and its uptake is

also greatly reduced in UV treated cells. This can probably be correlated with the impaired as well as the altered endocytic processes which is dependent on the protein synthetic activity of the cell. Our studies strongly suggest that probably there is considerable damage to the membrane structure in UV exposed amoebae which is reflected not only in their endocytic activities but also from the uptake of exogenous material into the cell.

A detailed study correlating the specific involvement of membrane proteins to altered and impaired endocytic processes in the UV treated amoebae will be a worthwhile subject for future investigations.

SUMMARY

1. Amoeba indica were irradiated with UV (260 nm, 80 J/m^2). Effect of UV-irradiation on the endocytic processes, viz., phagocytosis and pinocytosis were studied.

2. It was observed that UV exposure produced an inhibitory effect on the phagocytosis after an initial lag period. There was a maximum inhibition of about 80% at 1 hour after irradiation. The phagocytic activity was returned to normal by about 12 hours after irradiation.

3. The irradiated cells showed a slow rate of pinocytic channel formation, but they were able to finish the pinocytic cycle within 30 minutes after induction similar to unexposed cells. The ability of irradiated cells to pinocytose on reinduction after 4 and 6 hours of the first pinocytic cycle was checked. It was found that the UV-exposed cells showed a drastic reduction in pinocytic activity, 4 hours after the first cycle, as compared to the control. But they recovered to the normal level by about 6 hours after the first cycle.

4. Since the above mentioned membrane functions, which are dependent on protein synthesis, were impaired by UV-radiation, it was decided to check the radiation effect on amino acid (^{3}H-leucine) uptake and its incorporation into the total protein of the treated cells. ^{3}H-leucine incorporation studies showed an initial spurt of 10% in incorporation followed by a

steep decline to about 20% of the control value by 3 hours post-irradiation. This was followed by a slow recovery to 60% of the normal value by about 6 hours post-irradiation. To see if the decrease in incorporation of ^{3}H-leucine could be correlated with its uptake, the amino acid uptake was also studied. The profile of ^{3}H-leucine uptake was found to be parallel to the profile of its incorporation.

5. It was concluded that UV-irradiation could inhibit the membrane functions of amoeba, like phagocytosis and pinocytosis. These effects could be correlated with the inhibition in the protein synthesis activity (uptake and incorporation of ^{3}H-leucine) caused by the UV-irradiation in amoeba.

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