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UV-B INDUCED CHANGES IN RABBIT MUSCLE ACTIN POLYMERIZATION

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ANITA RAY MUKHERJEE

SCHOOL OF ENVIRONMENTAL SCIENCES
JAWAHARLAL NEHRU UNIVERSITY
NEW DELHI - 110067 INDIA

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जवाहरलाल नेहरू विश्वविद्यालय
JAWAHARLAL NEHRU UNIVERSITY
NEW DELHI-110067

CERTIFICATE

This dissertation **UV-B INDUCED CHANGES IN RABBIT MUSCLE ACTIN POLYMERIZATION** has been carried out in the School of Environmental Sciences, Jawaharlal Nehru University, New Delhi. This work has not been submitted either in part or in full for any degree or diploma of any University.

Anita Ray Mukherjee
MRS. ANITA RAY (MUKHERJEE)
(Candidate)

DR. ARUN K. ATTRI
(Supervisor)

PROFESSOR P.S. RAMAKRISHNAN
(Dean)

GRAM : JAYENU TEL. : 667676, 667557 TELEX : 031-73167 JNU IN FAX : 91-011-6865886

Dedicated
To
My beloved Parents

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Anita Ray Mukherjee

Anita Ray Mukherjee

CONTENTS

ABSTRACT

Actin, is an essential and highly conserved protein for the survival of both plant and animal cells. In general, it constitutes more than 5 percent of total cellular protein. Distribution of actin is mainly confined under the cell membrane. Functionally the polymerized actin acts as an important platform for cellular reactions. In addition, the threadmilling of actin polymerization between actin monomers (G-actin) and filamentous actin (F-actin) plays an important role in locomotion and cell division. Any change in the external environment is likely to affect the polymerization characteristics.

The recent increasing concern related to the depletion of ozone in stratosphere has caused alarm worldwide as this has enhanced the UV-B part of solar radiation received on earth's surface. This increase is expected to significantly alter the existing life form on earth. We have used actin polymerization, one of the essential component of cell, as a model to asses the effect of UV-B. Our studies show that polymerization characteristics of actin from G-actin to F-actin are affected significantly. The observed,effects are, (i) at the level of polymerization rate, (ii) formation of significant amount of oligomer even when actin is in monomeric form and (iii) the increase in critical concentration on exposure to UV-B.

These observations clearly indicate that any increase in UV-B flux is likely to bring about significant alteration at molecular level in microbes, plants and animals. Changes are likely to affect overall functional traits at the level of cell. Further investigations are required to assess the effect of UV-B increase under living cell conditions.

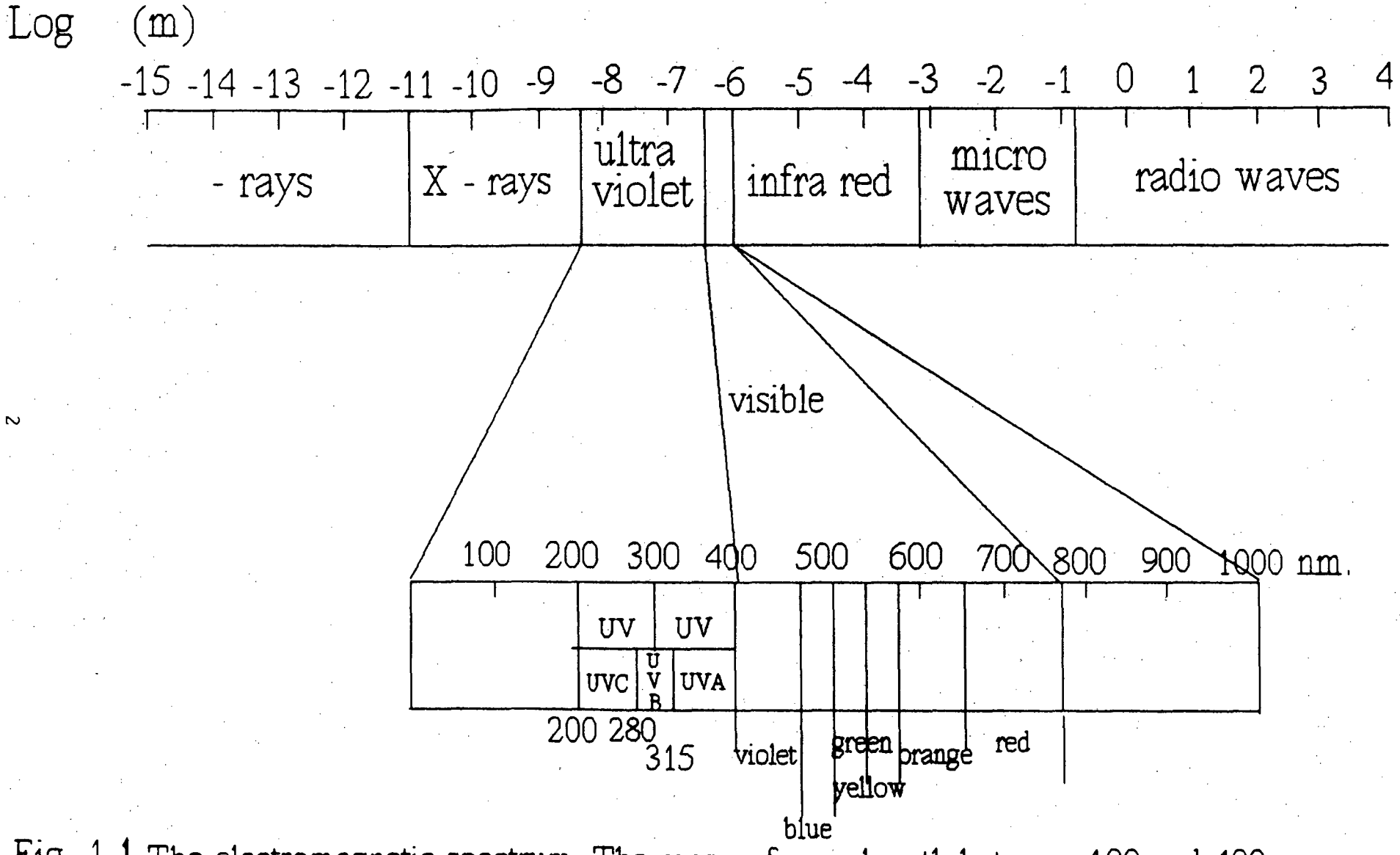


Fig. 1.1 The electromagnetic spectrum. The range of wavelength between 100 and 400 nm has been devided into the regions UVA (315-400 nm), UVB (280-315 nm) and UVC (100-280 nm) (after Rogger Philips in 1983)

INTRODUCTION

Incoming solar radiation is central to the survival and perpetuation of life on our planet. Solar radiation encompass wavelength range from 30 to $30x10^7$ nm (Sabins 1986). Wavelength range of 30 to 400 nm falls in ultraviolet region, 400 - 700 nm visible, 700 - 10,0000 nm covers infrared and 100 nm to 30 cm falls in microwave region. Less than 30 nm spectral range is known as γ-radiation and above 30 cm falls in radio wave region (Fig. 1.1). The visible part of the incoming solar radiation is responsible for survival and evolution of the life form. More energetic ultra violet(UV) radiation, most of it, is filtered by the earth's upper atmosphere (Larson and Weber, 1994, Fig. 1.2). This filtering is essential as it will be difficult to envisage life on earth otherwise. In fact the evolution of life on earth is closely linked to the formation of ozone shield in stratosphere, which primarily is responsible to keep away the biologically harmful UV radiation from earth's surface (Chaisson 1988).

However, rapid industrialization during this century has caused damage to the ozone shield which in turn has caused the increase of ultraviolet radiation reaching the earth's surface (Varshney and Attri 1995). The significant observed decrease in the ozone concentration is of considerable concern as stratospheric ozone is the primary attenuator of solar ultraviolet radiation. Ultraviolet radiation which ranges from 100 to 400 nm, for convenience, has been divided into three parts; UV-A (320-400 nm), UV-B (280-320 nm) and UV-C (less than 280 nm) (Coohil 1991). Much of the ultraviolet (UV) radiation is screened out by the atmosphere (Fig. 1.2). This is largely owed to the chemistry

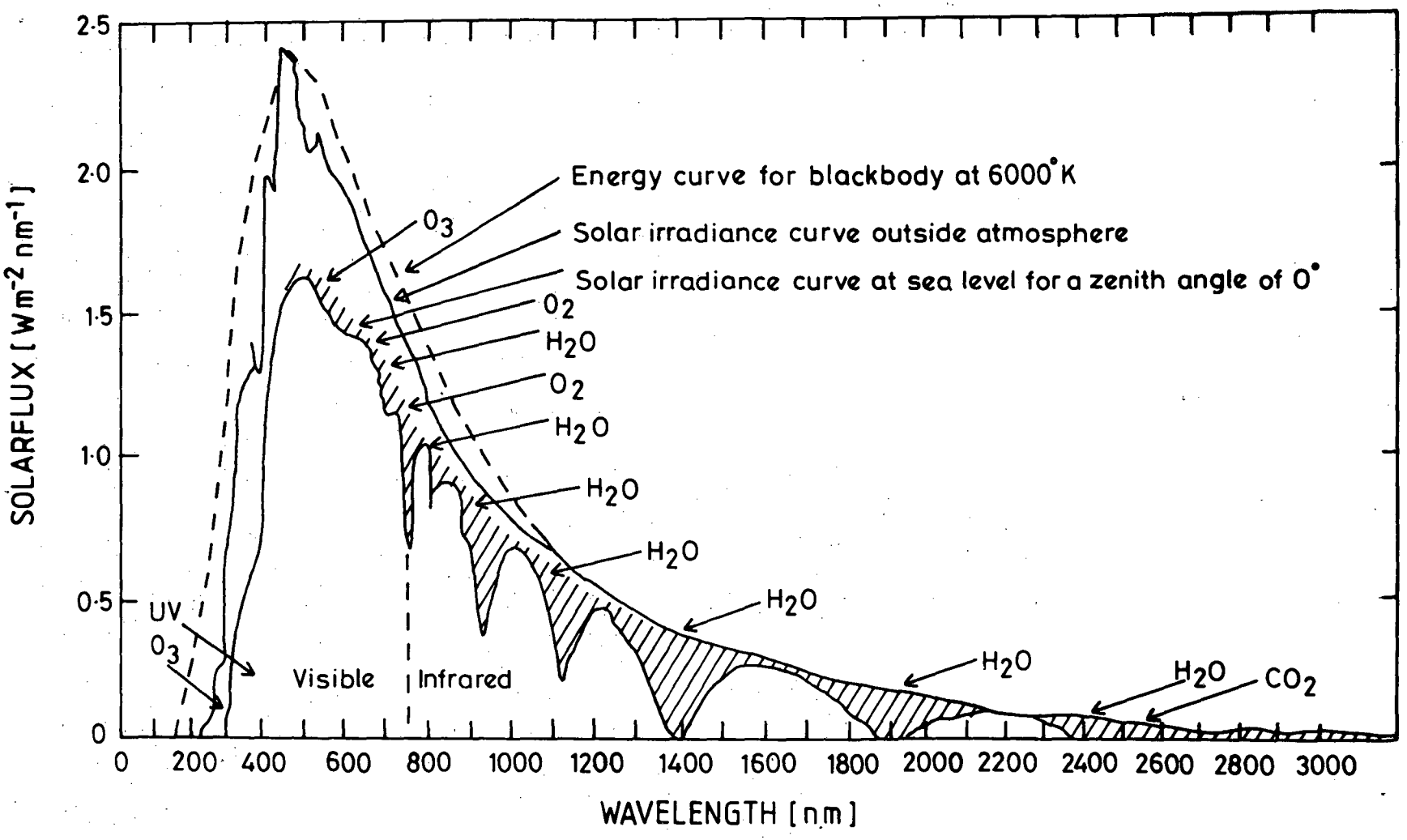


Fig.1·2: Energy flux (in watts per square meter) plotted against wavelength, calculated for a 6000° K blackbody radiator and compared to the observed solar flux at sea level and outside the earth's atmosphere. Reprinted from Chapter 16, "Thermal Radiation" in Handbook of Geophysics, U.S. Air Force Cambridge Research Center, 1960

prevailing in the stratosphere, which mainly involves oxygen (O_2). Solar radiation less than 245 nm photochemically reacts with O_2 and forms highly reactive atomic oxygen as shown below (Steinfeld 1989):

$$O_2 + h\nu\ (\lambda < 245\ nm) \text{ ---> } O + O \quad (1)$$

$$O + O_2 + M \text{ ------> } O_3 + M \quad (2)$$

The ozone formed in reaction (2), where M represents nitrogen or oxygen, in turn absorbs UV radiation greater than 245 nm and dissociates O_3 as follow:

$$O_3 + h\nu\ (\lambda > 245\ nm) \text{ ---> } O_2 + O \quad (3)$$

The absorption cross section of ozone for UV is a function of wavelength, therefore the anticipated absorption and the extent of UV radiation at a specific wavelength will occur differentially. For example, with decrease in the concentration of ozone one anticipates that more of the UV-B part will reach earth's surface (Caldwell et al., 1989a). This is apparent as in the wavelength region, 245 to 300 nm, absorption cross section of ozone for UV is lowered by 32 times (Molina and Molina 1986). Longer wavelength, UV-A, transmits further through the atmosphere as very small amount of it is absorbed by ozone, although it does get absorbed in lower atmosphere by particulates, water vapour and pollutants.

UV-B amount increases at a rate of 14-18% per 1000 m above sea level (Caldwell et al.,1980). Much larger changes in the amount of UV-B are observed with the latitudes. The amount of UV-B, also, is a function of solar zenith angles and declination (Larson and Weber, 1994). The distribution of ozone varies with latitude, being thinnest at poles and thickest at the equator (Caldwell et al.,1989b).

It is generally assumed that for a 10% ozone reduction, the flux of the biologically damaging UV-B radiation at earth's surface would increase by 20% (Mackie and Rycroft 1988). These expected changes in solar UV-B, however, will vary with latitude (Caldwell 1986). With substantial ozone reduction at temperate latitudes, the effective UV-B radiation flux may not exceed in comparison to that currently received in tropics (Caldwell et al., 1989b). It is implicit that even a small reduction in ozone layer might have a tremendous effect in tropics. In an estimate made by National Academy of Sciences (USA), ozone depletion during next century will range between 5 to 9% (Teramura 1983).

The main concern related to the increase in UV-B on the earth's surface arises from the fact that it is potentially mutagenic and dangerous to life form ranging from biomolecules to ecosystems. Overall impact due to the anticipated increase in the UV-B will be far reaching. The effects are expected to be on global climate (Lovelock 1987, Krause et al. 1989), living organisms, ecosystem dynamics and biogeochemical cycles (Green et al. 1980, Varshney and Attri, 1995).

The mechanism of UV effect in general, is related to the energies of photons which can raise atoms and molecules into electronically excited states. This leads to several different kinds of photo-chemical reactions. The excited electrons either returns to the ground state (**physical decay**) or will loose the excess energy by reacting chemically. Luminescence and photosensitization (**physical decay involving another molecule**) may also occur (Phillips 1983).

In addition, other processes like photolysis, physical quenching, dimerization, hydrogen abstraction, electron transfer etc may occur. All these phenomena will involve different biomolecules forcing alterations in the normal cellular functions. Some of these effects at the level of plant, animal and cell have been of great scientific importance.

1.1 Effect on Plants

About 200 species have been tested for UV-B sensitivity by Teramura (1986) and about two-third of them have been found to be sensitive. Broadly the observed effects are at the level of photosystem II (to a lesser extent photosystem I also), carboxylating enzymes, net photosynthesis, Hill reaction (Teramura 1983), ultrastructural damage in chloroplast (Allen et al. 1978) and change in total biomass (Teramura 1990a and b). Large increases in soluble leaf proteins and synthesis of aromatic acids also occurs (Esser 1980; Teveni et al., 1981). In addition, RuBPease activity (Steinback 1981), dark respiration (Brandle et al., 1977), stomatal resistance, increase in non-photosynthetic pigments like flavanoids (Teveni et al., 1991) are altered significantly. Chlorophyll concentration and calcium, phosphorus contents (Benedict 1934) and zinc-translocation (Ambler et al. 1975) changes significantly. Besides, UV-B increase affect cell division, cell elongation, seedling elongation, stem elongation, stem branching, leaf area (Dikson and Caldwell 1978), leaf thickness, reduction of shoot length (Ambler et al. 1978), culticular wax, flowering, pollen germination, pollen tube growth (Raue et al. 1988; Hart et al. 1975), morphology (Teramura et al. 1991) and pigmentation (Bornman and Vogelnann 1991). UV-B, have been reported to modify CO_2 induced increases in total biomass, seed yield and photosynthetic parameters (Teramura 1983). Plants showing above mentioned effects are pea, collard, soybean, barley, corn, bean, radish, tomato,

cucumber, oats, cabbage, wheat (*Tritium aestivum* L.), rice, cowpea [*Vigna unguiculata* (L.)], peanut, cotton, sunflower and few weeds and rye.

Studies have also been conducted to identify the compounds in plants that serve as biochemical markers of UV-B stress and/or protect the cell against UV-B damage (Kramer et al., 1992; Singh 1994). Activities of enzymes related to scavenging of free radicals such as super oxide dimutase, catalase and peroxidase have also been reported to change due to UV-B treatment (Krizek et al. 1993). Sensitivity of plants towards the UV-B radiation may depend on the abiotic and biotic (species and ciltivar characteristics). Effects on photosynthesis and changes in floristic composition is indirectly affecting the terrestrial ecosystem due to changes in evapotranspiration, nutrient cycling, and carbon cycle which in turn alters entire physiology of ecosystem (Varshney 1994).

1.2 Effect on Animals

Although most of the work of UV-B exposure is confined to plants but some work related to this aspect has also been done on animals. UV-B is known to be potent mutagenic agent for a long time. Molecular alteration in hereditary material, DNA causes genetic changes. The UV-B flux between 280-320 nm causes irreversible changes in lipid, steroids, melanin and uronic acid (Varshney and Attri 1995). These effects are seen in both plants as well as in animals. Effect of UV-B on human being is mainly due to changes it causes on protein synthesis and on DNA present in skin cells (Quevedo et al. 1985). Human skin undergoes premature ageing. Further, exposure can lead to the formation of skin cancer (Singh 1994). Indirect effects of UV-B includes genetic

disorders pellagra, Kwashiorkar, suppression of immune response, metabolic disorder (Varshney and Attri 1995), and eye problems (Hightower et al. 1994).

UV-B radiation poses a potential hazard for planktonic organisms (Worrest 1986) as they do not possess epidermal UV-absorbing layers unlike higher plants and animals (Häder and Worrest 1991). Due to high influence of UV-B, inhibition of orientation system (Häder 1993; Häder et al 1989, 1991) and nitrogen incorporation (Döhler et al. 1985, Döhler 1986; Döhler et al. 1987) gets affected along with few other changes which occurs in higher plants also. For example, chlorophyll bleaching (Häder et al., 1988; Nultsch and Agel 1986), disturbance in net biomass production as well as photosynthesis (Smith et al. 1980; Zündrof and Häder 1991). Besides, fish larvae and reproductive stages of many species have been found to be affected by UV radiation (Häder and Worrest 1991). Species composition is also found to change due to UV-B radiation (Bundestag 1986).

Effect on Motility of Microbes

Enhanced level of UV-B is found to impair the motility of microbes (which might be related to the effect on actin protein). In slime mold *Dictyostelium discoideum*, UV-B fluence of ≤ 100 Jm^{-2} has caused reduction in speed of movement by 50% and inhibited the photostatic orientation (Häder 1984). This effect has also been seen in other motile organism like green flagellate *Euglena* and blue green alga Phormidium. It was observed that *Euglena* became almost completely immobile when exposed to UV-B dose of ≥ 1600 Jcm^{-2}. When phormadium was exposed between 295nm-300 nm the motility was impaired immediately (Häder 1984).

1.3 Effect on biomolecules

Most of the work on assessment of damage by UV-B radiation has not touched molecular level changes resulting from the exposure. Only some work has been done on DNA.

UV induces changes in DNA at molecular level (Yamoto et al. 1983). Most common change at this level is the formation of thymine dimers (Beukers and Berends 1960). In 1974, Setlow reported intrastrand dimer formation between adjacent pyrimidine bases. In 1986, Attri and Minton and Love et al., have reported dimer formation which occurred interstrand when DNA is in Z-conformation. Absorption maxima of DNA is at 260 nm, however, UV-radiation corresponding to this wavelength does not reach the earth's surface. Proteins on the other hand are the largest UV-B absorbing component in a living cell having absorption maxima at 280 nm. As the absorption spectral profile of DNA and protein has a large spread, the incoming solar UV-B in the range of 280 nm and beyond will also be absorbed but to a lesser extent in comparison to their respective absorption maxima (Setlow 1974).

1.4 The Present Work

No systematic work so far has been done to address the effect of UV-B at the level of proteins. To undertake work in this direction, following points are important:

i) The chosen protein should be present in significantly large amount.

ii) It should form an important part of plant as well as animal cells.

iii) The presence of this protein should be vital for the survival of the cell.

Keeping in view these important pointers, we have selected **actin** as a model protein to assess the effect of UV-B. Actin constitutes highly conserved family of protein, found in all eukaryotes and it is an essential protein for the survival of the cell. Also, there are large group of proteins which shares very limited sequence homology but similar three dimensional molecular structures as actin are also expressed in prokaryotes (Sheterline 1994). Actin is abundant protein in most eukaryotic cells constituting 5% or more of total cell protein (Alberts et al., 1989).

Conventional actins have molecular weight of about 43,000 Dalton which shows conservation of sequence and amino acid number. This protein is predominantly found in cytoplasmic component of cell, but its presence in the nucleus is also reported (Nakayasu and Ueda, 1983). It is a major structural component of both muscle and cytoplasmic crontractile machinery of eukaryotic cells (Korn, 1982).

Like actins of other multicellular eukaryotes, plants actin are encoded by multigene families. Great diversity is found even within plant actin gene families (Baird and Meagher 1987). Soybean, maize, Arabidopsis and rice were reported to have three to ten genes (Meagher and Mclean 1990) whereas petunia has over 100 genes (Baird and Meagher 1987).

In plants, many epidermal cells and vascular parenchyma cells, actin is involved in vigorous cytoplasmic streaming (Lloyd in 1989). During cell division actin forms a concentrated band in the cytokinetic phragmoplast, the apparatus that is believed to fix the plane of division (Lloyd 1988) and to determine the boundary between daughter cells. Besides, actin plays an important role in the cell shape determination, tip growth (Heath

1987; Doonan et al. 1988), organelle movement, gravi precipitation, cell wall deposition, nucleus positioning (Katsuta et al., 1988) and karyotinesis (Meagher and Mclean 1990).

F-actin is double stranded helical polymer of actin molecule (Hanson 1963). Each actin monomer is arranged in two-stranded helical polymer, each monomer seems to be in an equivalent position with respect to its neighbouring monomers. The actin polymer also has polarity (Huxley 1963). Monomers in the two strands are oriented in the same direction along with the polymer axis but in the opposite direction in the perpendicular plane. Such an arrangement of monomers might be important for interaction of F-actin with other proteins, for example myosin and tropomyosin (Oosawa and Kasai 1971). Besides one mole of G-actin binds 1 mole of nucleotide as well as 1 mole of divalent cation. The binding of divalent cation stabilizes the binding of nucleotides (Asakura 1961).

In addition, recently extremely conserved primary and tertiary structure of actin has been explained as a requirement to accommodate the high number of specific interactions both with itself and with large variety of actin associated proteins (AAP). Presence of actin binding domains in large numbers of cellular protein also indicate the functional flexibility inherent in actin. In addition, combination of these domains with other functional entities by genetic exchange may lead to the formation of proteins with more complex functions (Vandekerckhove 1989). These includes actobindin which inhibits actin polymerization at an early stage by binding with G-actin (Lambooy and Korn 1988), cofilin (Matsuzaki et al. 1988), troponin (Heald and Hitchciote-De Greygori

1988), gelsolin (Boyer et al. 1987), dystrophin, α-actinin, villin, (Noegel et al. 1987), vinculin (Burn and Burger 1987), Fragmin, severise etc. (Vadekerckhove 1989).

Actin and associated proteins (AAP) are prominent part of cell cortex which gives mechanical support to the cell (Alberts et al., 1989). The most intensely studied function of actin filament is their role in providing the mechanochemical basis for contraction (Alberts et al., 1989). Besides, polymerized actin plays central role in mobile activities of all eukaryotic cells. For example acto-myosin dependent mechano-chemical activities of non-muscle cells that are thought to be fundamentally similar to the muscle contraction, are cell locomotion, cytokinesis, phagocytosis, platelet clot reaction, and ligand induced clustering of cell surface receptors (Korn 1978). Further, polymerized actin serve structural functions e.g. core bundles that support five cellular projections such as microvilli of the intestinal brush border and the sterocillia of the hair cells and colchea of the inner ear, the acrosomal process of sperm, and the cytoskeletal network that provides the structural organization and dynamic visco-elastic properties of cytoplasm, which are important for the coordination of the metabolic activities (Korn et al. 1987). Wegner (1982) has investigated tread milling of actin at physiological salt concentration.

In muscle, the polymerization process is important for providing and maintaining the filaments required for contractile activity. But in non-muscle cells extensive depolymerization and repolymerization of cytoskeleton are likely to be continuous as well as regulated process where actin filaments disappear and reappear at different times and places as they are needed for specific functions. Organisation state of actin in cell is

generally influenced by the interactions of G-actin and F-actin with other proteins, but the polymerization is a property of actin alone (Korn 1982). Oosawa and Kesai (1962) pointed out that actin polymerization is essentially unidirectional, therefore, it is not a pure condensation process and under appropriate experimental conditions, relatively high concentrations of oligomer can exist with monomers when total actin concentration is below and even slightly above the critical concentration (concentration at which monomers bind to the lenghening end of filaments with same rate as subunits are released) (Attri et al., 1991).

Correlation between actin polymerization and protrusion has been established (Small 1989) for the acrosomal reaction of sea cucumber. Subsequently, in leocycytes (Sheterline et al. 1986) and amoeba (Newell 1986) it was shown that a shift in the balance between the more or less equimolar monomeric and polymeric pools of actin, towards filamentous actin, accompanies the onset of active lomotion (Small, 1989). Massive mobilization of actin into likewise filamentous pool activate blood platelets also. These global effects in motile cells are reflected at cellular level by an increase in protusive activity in the form of F-actin rich lamellipodial extensions and membrane ruffles (Sheterline 1986).

In view of the fact that actin stands out as an important protein from plant cell to animal cells, to microbes and it is essential for their survival, to address the question related to the effect of increased UV-B on functional aspects of actin becomes very important issue. In addition, the proximity of actin distribution near plasma membrane probably makes it more vulnerable to UV-B absorption and subsequent chemical

modification. The problem and approach chosen in this work arises from these reasons. Presented work, only of its kind, is likely to raise unknown questions related to the molecular level changes in the cell due to anticipated enhanced UV-B flux.

MATERIALS AND METHODS

2.1 Chemicals used

Following chemical, Tris (Hydronymethyl) Aminomethane BRL, USA (Bethesda Research Laboratories), Ethanol (Bengal Chemicals), ATP (Disodium salt) (Sigma), Calcium chloride (Sarabhai M.Chemicals, Baroda), Sodium Azide (Fluka, Switzerland), Sodium dodecyl sulphate (Bio-Rad), Acrylamide (Sigma, Spectrochem), TEMED (SISCO Research Lab., Bombay), EDTA (Qualigens), ß-Mercapto ethanol (Sigma), Glycerol (Qualigens), Potassium chloride (Qualigens), Magnesium chloride (Qualigens), Pyrene Iodoacetamide (Molecular Probes (USA)), N,N Dimethyl Formamide (MERCK), Acetone (Glaxo Laboratories), Methanol (Qualigens), Ammonium Persulphate (Bio-Rad), Actin powder (Prepared in lab) and Double distilled water were used in the experimental work.

2.2 Method

2.2.1 Preparation of acetone powder

The protocol is based on method reported by Pardee and Spudich 1982. Ice chilled rabbit muscle was bought commercially which was washed with double distilled water and was minced at 4°C in a pre-chilled meat grinder. The mince was quickly extracted with stirring for 10 minute in 4 litres of ice cold 0.1M HCl, 0.15 M potassium phosphate, pH 6.5. Extract was filtered by squeezing through four layers of cheese cloth, which was boiled beforehand for approximately half an hour in double distilled water with a pinch of EDTA, and brought to 4°C.

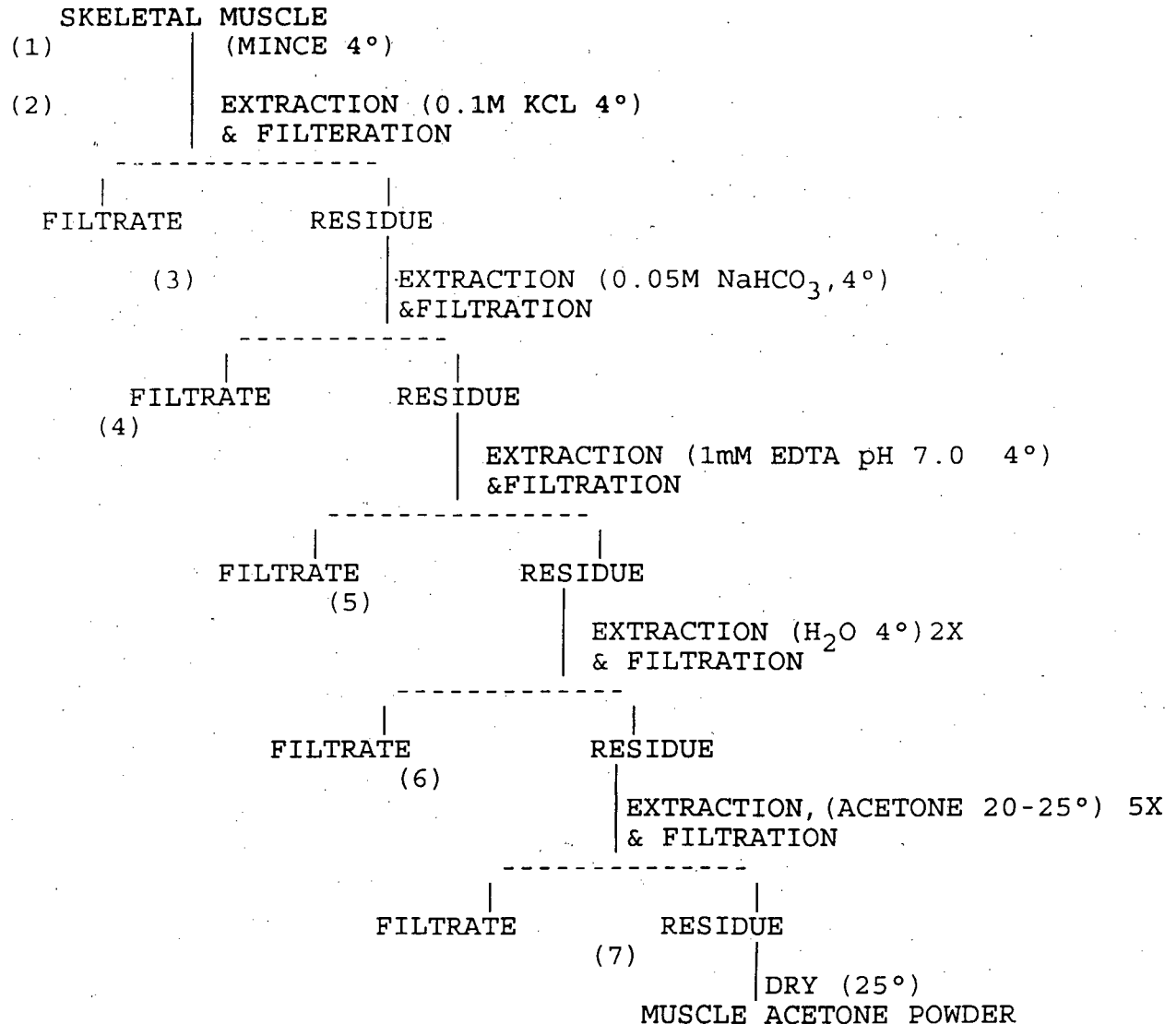


Fig 2.1 Flow diagram for preparation muscle acetone powder

The filtered muscle was extracted with constant stirring for 10 minutes at 4°C in 8 litres of pre-chilled 0.5 M $NaHCO_3$ and filtered. The filtered residue is extracted with 4 litres of 1mM EDTA at pH 7.0 by stirring for 10 minutes at 4°C. Further extraction were done with 8 litres of doubled distilled water for 5 minutes with stirring at 4°C.

The final extraction was done with 4 litres of acetone for 10 min each. Acetone extraction is done at 20-25°C. Acetone should be previously cooled to below 20°C. During each extraction clumpes of residues are broken and spread on filter paper sheets.

The filtered residues are placed in large glass evaporating dishes and air-dried overnight to obtain dried acetone powder in this form can be used for significantly long time if stored at -20°C.

2.2.2 Purification of Actin

G-buffer was prepared, which contained 2mM Tris HCl, 0.2mM Na_2ATP, 0.5 mM 2-mercaptoethanol, 0.1 mM $CaCl_2$, 0.003% sodium azide and kept in refrigerator at 4° C. The reagents are dissolved in double distilled water and titrated with hydrochloric acid to pH 8.0, then ß-merapto-ethanol (105 ul of 14.3 M) and NaN_3 where added. G buffer was added to the acetone powder (20 ml/gm acetone powder) with stirring and kept for half an hour, in an ice bath. The extract was filtered through eight layers of cheese cloth which was previously boiled in double distilled water with EDTA. The extract is centrifuged at 3000 rpm for twenty minutes at 4°C. The pellets were discarded and supernatant was used in subsequent steps. To the supernatant 0.3m M ATP, 0.7M KCl were added. To this 5mM of Magnesium chloride was added and the solution was further stirred at room temperature. The polymerized actin was transferred

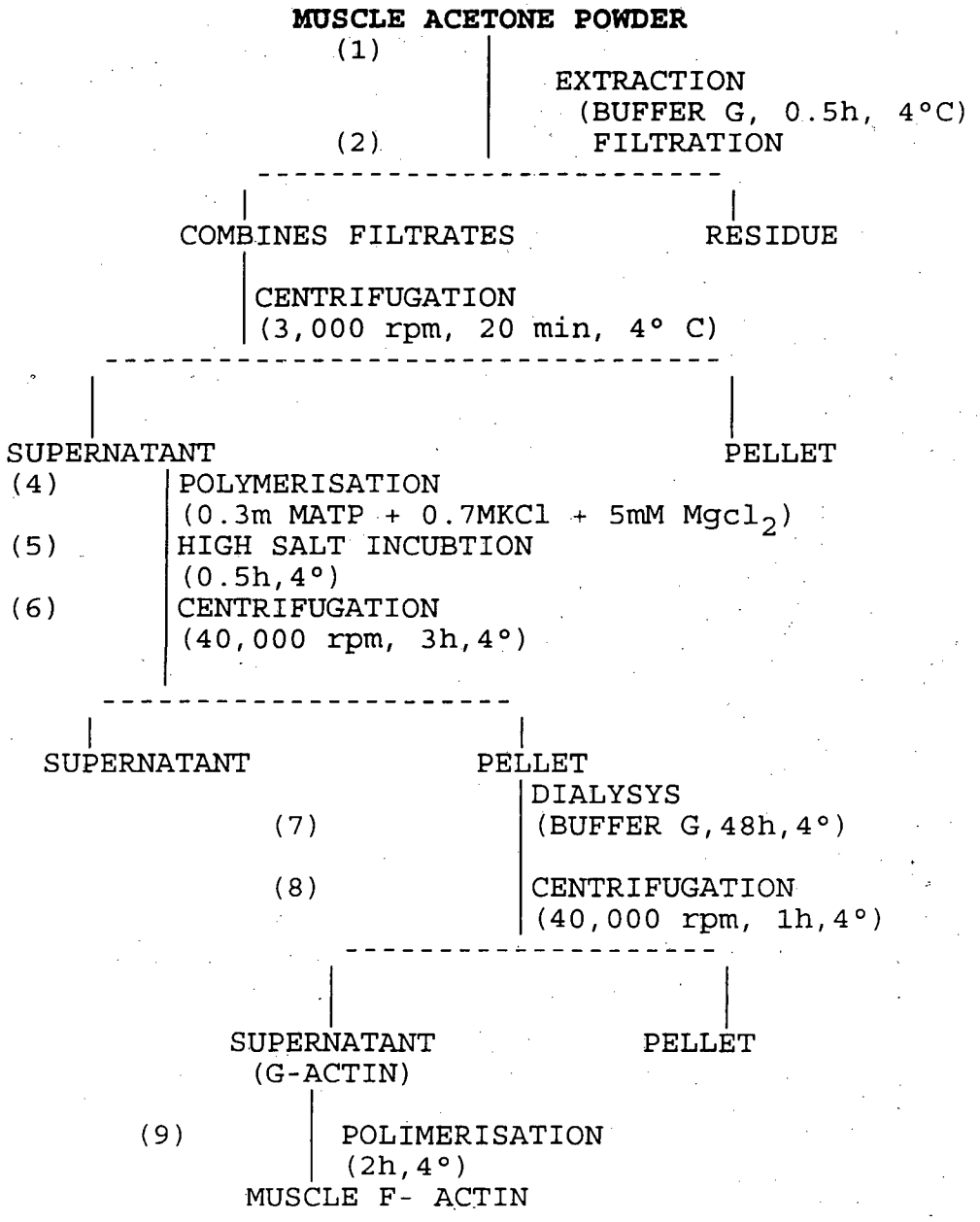


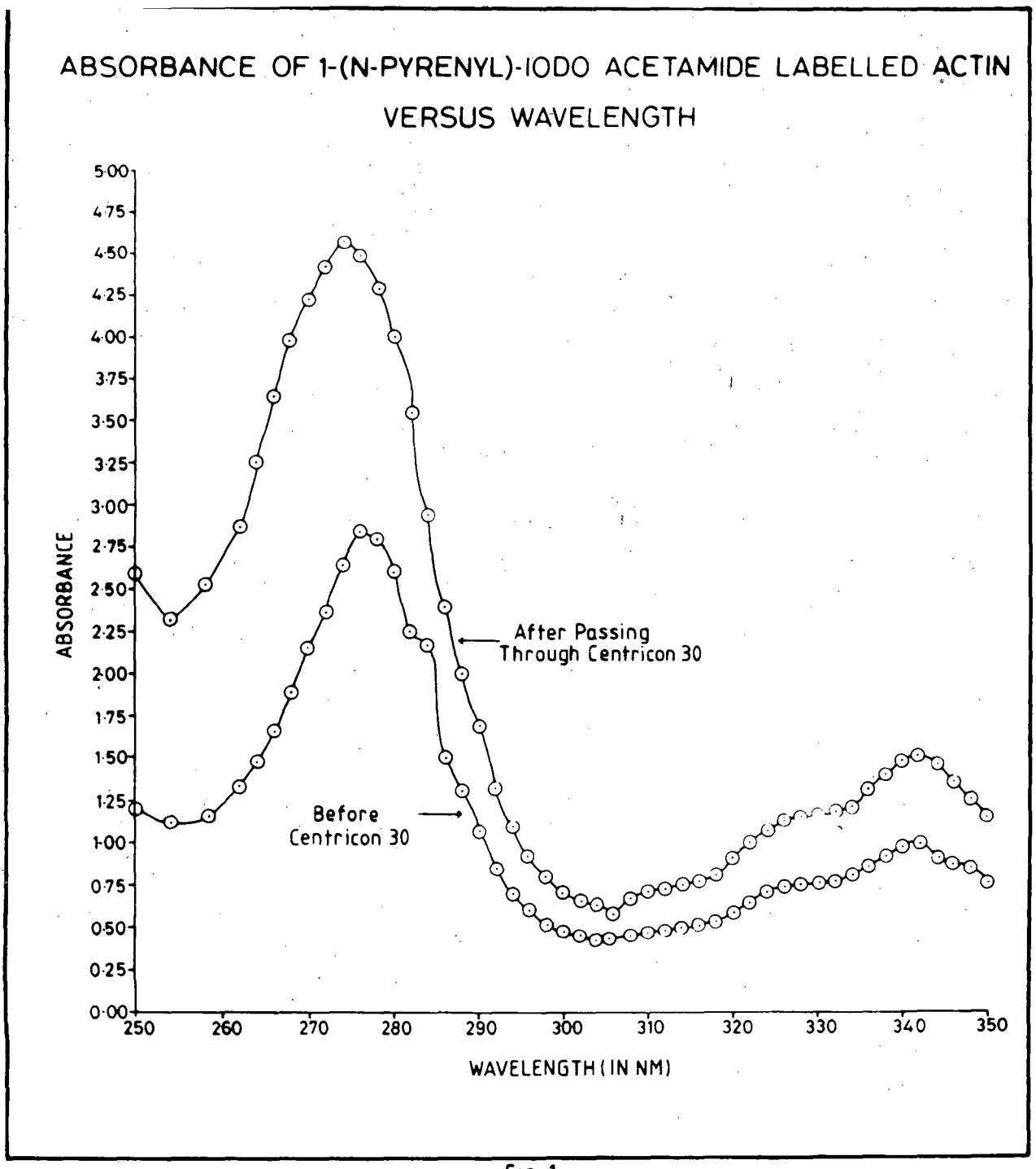
Fig-2.2 Flow diagram for isolation of muscle actin.

in Beckman tubes and centrifuged at 40,000 rpm for 3 hours at 4°C. Then the supernatant was discarded and pellets were washed and kept in 1 ml G-buffer overnight. Soften pellets were homogenized in G-buffer and dialyzed in G-buffer. Dialysis was carried by making four changes after every 12 hours. After dialysis the protein solution was centrifuged at 40,000 rpm at 1 hr. Polymerized contaminants settles down and are discarded. Supernatant which content G-actin was checked for purity in sodium dodecyl sulfate poly acrylamide gel electrophoresis (SDS PAGE). G-actin absorbance spectra was taken between 250-320 nm. Protein concentration was estimated by Lowry's method (Annexure III) and by measuring absorbance at 290 nm. Purified actin is always stored in the F-actin form at zero degree centigrade, on ice.

2.2.3 Labelling

Protein solution of 2 mg/ml was made for labelling with N-(1-pyrenyl) iodoacetamide which is an fluorescent label (Anexure I). This probe when attached to G-actin, on polymarization to F-actin shows an increase in intensity of more than 25 times (Kouyama and Mihashi, 1981).

0.2mM ATP, 0.1M KCl and 2mM of $MgCl_2$ were added with vigorous stirring to the monomeric actin (for polymerization). After 1 minute of stirring, 140 μl of Pyrene iodoacetamide (14 mg/ml DMF) was added to the polymerizing actin in two batches and stirred vigorously for 1 hour and kept for slow stirring overnight. 150 μml of ß-mercapoethanol (14.3 M) was added next day to quench the reaction. The solution was centrifuged at 5000 rpm for ten minutes. Excess pyrene iodoacetamide will settle down with ß-mercapto-ethanol. Pellet was discarded. Both labelled (supernatant after


ABSORBANCE OF 1-(N-PYRENYL)-IODO ACETAMIDE LABELLED ACTIN VERSUS WAVELENGTH
ABSORBANCE
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WAVELENGTH (IN NM)

Fig. 1

TH-7148

centrifugation at 5000 rpm) and unlabelled polymerized actin was centrifuged at 40K. Discarded the supernatant and kept pellets in G buffer (1ml for each pellet) overnight. Softend pellets were homogenized and dialyzed in G-buffer. Samples were centrifuged at 40 k for 1 hour and pellets were discarded. Spectra for labelled as well as unlabelled samples (supernants) was taken in the range of 250 to 390 nm. At this stage both labelled and unlabelled protein was passed through centricon 30, unlike previous authors who have used columns, which is very time consuming. We find that use of Centricon 30 is less vitiative way to remove impurity from G-actin in comparison with the use of Column chromatography (Fig.1).

Spectra of both the samples were taken between 250 to 390 nm. Concentration of both labelled as well as unlabelled samples were calculated after correcting for the absorbance of pyrene label (Cooper et al., 1983) by following formulas:

$$\text{mM actin} = (OD_{290} - 0.127\ OD_{344})/26.6\ mM^{-1}\ cm\text{-}1 \quad (2.1)$$

$$\text{pyrene/actin} = (OD_{344}/22.0)/\text{mM actin} \quad (2.2)$$

Mole to mole ratio of pyrene label and actin should be one, in our case also it is one.

2.3 Experiment Design

Stock solution was made of labelled and unlabelled samples in the ratio of 1:4, then seven sets were made of 1 ml each of ten different concentrations.

Each of these G-actin solution was exposed to 0.0237 mJ sec^{-1} cm^{-2} at 280 nm for 0, 5, 10, 15, 20, 25 and 30 minutes corresponding to total doses 0, 7.13, 14.14, 21.50, 28.78, 36.14, 43.58 mJ/cm^2 respectively. Doses were checked by chemical dosimetry (Annexure II) as well as by regression analysis.

Fluorescence was taken with molicules excited at 360 nm and emitted at 388 nm of zero minute exposure then added 0.1M KCl and 2mM $MgCl_2$ and taken the fluorescence intensity after every half minute upto 10 minutes. Repeated the same procedure for different exposed dose (5, 10, 15, 20, 25 and 30 minutes) for every concentration (total 70 sets). Plotted the relative floursence intensity versus time. Repeated the procedure for a 2 μM as well as 4 μM sample by exposing at different times (5, 10, 15, 20, 25, 30 minutes) at the same dose 0.0237 mW/cm^2 and noted the fluorescence (only base line without polymierizing) to check if the probe gets bleached during exposure (Table 4).

RESULTS AND DISCUSSION

3.1 Results

3.1.1 Polymerization

The polymerization of actin at zero UV-B dose is shown in Fig.3 (A), 4(A) and 5(A). The figures also indicate the onset of polymerization on addition of KCl (0.1M) and $MgCl_2$ (2mM). The relative fluorescence values shown are till 15 minutes (21.50 mJ/cm^2). After 24 hours, the steady state fluoresence values were recorded to estimate the extent of F-actin formation (Table II). Similar curves for samples, at fixed concentrations, are shown for different UV-B doses (Fig.3,4,5). Each figure shows the concentration and total dose at which the samples were exposed.

The main observation related to the changes in polymerization characteristics are:

i) UV-B exposed samples, in relation to the unexposed sample, systematically show lower fluorescence values,

ii) the rate of polymerization decreases with the increase in UV-B dose except in the case of concentration 15.16 μM.

These are two points observed for G-actin solutions at all the concentrations, the representative rates of polymerization are shown in Fig.6,7 and 8. The rates of all the sets are shown in Table 1. The polymerization rates were calculated by observing the fluoresence change in first 10 min for all the sets.

3.1.2 Steady state-fluorescence

The steady state fluroescence values decreased with the increase in dose as shown in Table II. In most of the cases, however, at higher concentrations, like 15.16 μM (Fig.10) no conclusive trend is noted. The plot of steady state of concentration 3.75 μM is given as a representative plot (Fig. 9).

The critical concentrations (Cc) were estimated by plotting the steady state fluorescence values at different concentrations but concentrations are shown in figures 11 to 18. Each figure indicates the critical concentration at the respective doses.

The results show that there is a significant increase in the critical concentration after dose increases more than 15 minutes. This correspond to the total dose of 21.50 mJ/cm^2. This trend is shown in Fig. 18.

3.2 Discussion

In most general case, of actin polymerization can be expressed at equilibrium as

$$C_o = C_1/(1-K_lC_1)^2 + \sigma C_1/(1-K_hC_1)^2 - (\sigma C_1 + 2\sigma K_hC_1) \qquad (3.1)$$

where

C_o = total actin concentration

C_1 = Monomer concentration

K_l = Association constant for linear polymer

σ = $\tau (K_l/K_h)^2$

where τ is related to the extra energy to convert linear oligomer into helical nucleus.

Kn = Association constant for the helical polymer

$C_1/(1-K_lC_1)^2$ represents formation of linear polymer

$\sigma C_1/(1-K_hC_1)^2$ and $(\sigma C_1 + 2\sigma K_hC_1)$ represents formation of helical polymer (Attri et al. 1991).

Oosawa and Kesai (1962) first developed theory of helical polymerization. In absence of helical polymerization, solution of first term of the equation 3.1 shows that for actin concentrations below K_l^{-1} i.e., $C_o/Kbl < 1$, more than 60% of actin subunit will be in short oligomer of average size 1.6. Under the conditions generally used to study actin polymerization, K_l is very much smaller than K_n and σ is very small, then the equation 3.1 reduces to-

$$C_o = C_1 + \sigma C_1/(1-K_hC_1)^2 \qquad (3.2)$$

During the polymerization, two types of dimers are formed longitudinal as well as diagonal dimer. Cooperative association, in which protein subunit is held simultaneously by two bonds, is enormously more favourable than association forming either bond alone (Erickson, 1989).

With the similar condition i.e. addition of 0.1M KCl and 2mM $MgCl_2$, the polymerization pattern of all the samples, native as well as UV-B exposed, is same. However, the magnitude is less for the exposed samples, which indicates that UV-B decreases the extent of F-actin formation. Though the initial decrease is seen for all the curves which indicates UV-B affects the oligomer formation. But since with increase in dose oligomer under this conditions samples formed may not be in right configuration reach the saturation point earlier than the zero dose exposed to samples. In other words, for monomer to F-actin formation, insertion of dimer and higher oligomer should be in helical configuration. If UV-B is promoting the formation of linear configuration

oligomer, they will affect the rate of F-actin formation and also will increase the critical concentration. Our results do support the above reasoning as we see the rise in critical concentration with UV-B dose and rate of polymerization goes down.

Decrease in steady state fluorescence also supports that increase in dose does not favour formation of F-actin to the same extent as is seen for unexposed samples.

Critical concentration is found to be constant for the sample exposed to UV-B till 21.50 mJ/cm^2. But it increases 1.5 times more, when the dose is increased to 28.78 mJ/cm^2. Further critical concentration becomes double at 43.58 mJ/cm^2. It indicates that concentration of monomer, which are at equilibrium with F-actin significantly increases with the increase in dose above 21.50 mJ/cm^2.

It implies that at higher UV-B doses, the process of filament formation at cellular level will be retarded and consequently significant decrease in F-actin will occur. This will have a serious effect on the functioning of cell which might cause a decrease in the overall efficiency of the organism regarding motility and other actin related dynamic functions.

Average UV-B radiation coming in New Delhi is much more than doses used in our experiment. For instance, as per the data collected, in our laboratory, of UV-A, UV-B, UV-C flux of May 31st 1995, the total per minute irradiance at respective wavelengths is shown in Table (3) it is clear that much larger dose is encountered than used in our experiment. However, the probes used to collect this data has a spread of

± 12 nm. The actual value at 280 nm will be much less. We have exposed our samples at much lower dose of UV-B but at 280 nm.

Motility of slime-mold is found to be affected by ≥ 100 J/m^2 (Häder, 1984). We have used 71.3 J/m^2 (minimum), it shows that effect of UV on polymerization of actin might have direct link with effect on the motility. Actin polymerizes rapidly upon stimulation of neutrophils by the chemotactic peptide and there is enough evidence that this polymerization is essential for motility (Howard et al., 1990).

The effect of ultraviolet radiation has also been seen on ageing (Introduction). This also might be related to the effect on actin which is a part of cytoskeleton. Altered function of cytoskeleton may play a key role in age related changes observed in several cell types (Rao and Cohen 1990).

Effect of UV-B has been observed also on actin polymerization as this process governs many different functions, in both plants as well as animal cells. Approximately 50% of the total cellular actin molecules in most animal cells are unpolymerized. This means that they exist either as free monomers or as small complexes with other actin binding proteins. A dynamic equilibrium exist between pool of unpolymerized actin molecules and actin filaments, which helps many of the surface movement of the cell (Alberts et al., 1989).

Earlier pH and salt concentration, temperature pressure and organic solvents have also been found to affect the polymerization of actin (Oosawa and Kesai 1971). Hence, it is quite likely that UV-B photons can also affect the conversion of G-actin to F-actin.

3.3 Conclusion

It is concluded from the present work that polymerization of actin does get affected on exposure of actin monomers to UV-B radiation. This effect might be on account of denaturation or chemical modification. This is a significant observation as actin is a multifunctional protein, present in all cells in large amounts. The observed effects, as per our results, indicate that any increase in UV-B flux will seriously hamper the overall efficiency for the regions near equator as already much higher amount of UV-B reaches the surface of the earth in these regions.

The likely effects on the actin polymerization will be affecting at cellular level functions in following manner:

i) Decrease in the surface area required for the cellular reaction. As extent of F-actin will decrease on UV-B exposure.

ii) Decrease in the motility of the cell.

iii) Mechano-chemical basis for contraction of muscles.

iv) Acto-myosin dependent mechano-chemical activities of non muscle

v) Cell division, and

vi) treadmilling etc.

The major task before us is to extrapolate our present observations from in vitro conditions to the multifunctional dynamics of the cell in vivo. Inside the cell, there are many actin binding proteins which might be playing crucial a role in G-actin to F-actin formation. Since in our experiment no actin binding proteins were used, we can not precisely say whether these proteins protect actin from harmful ultraviolet radiation or not. Further research in this direction is needed to explore this new dimension.

Table-1

Rate of Polymerization versus total dose exposed

Total dose exposed (mJ/cm^2)	Rate of Polymersiation of different concentration (μM)									
	0.12	**0.25**	**0.49**	**0.98**	**1.97**	**3.95**	**7.91**	**3.75**	**7.58**	**15.16**
0	No	No	0.02	0.08	0.22	0.29	0.34	0.60	1.58	2.6
7.13	Poly	polym	0.03	0.08	0.20	0.35	0.47	0.55	1.50	2.4
14.14	mers	ersiati	0.03	0.05	0.18	0.30	0.29	0.51	1.42	2.6
21.50	ation	on	0.02	0.04	0.21	0.27	0.27	0.48	1.37	2.8

Table - 2

Steady state flurescence versus total dose exposed

Total dose exposed (mJ/cm^2)	Steady state flurescence of different concentration (μM)									
	0.12	**0.25**	**0.49**	**0.98**	**1.97**	**3.95**	**7.91**	**3.75**	**7.58**	**15.16**
0	No	No	2.2	2.4	11.7	12.2	37.0	21.2	35.0	90.3
7.13	Polym	poly	1.6	2.1	10.4	9.5	30.0	18.2	49.9	82.3
14.14	erisati	mers	1.2	1.6	8.6	7.7	24.1	16.9	34.3	92.2
21.50	on	ation	1.2	1.3	3.2	3.1	22.2	13.2	41.2	80.8

Table 3

UV-B Flux on May 31st, 1995 in School of Environmental Sciences, JNU

Time	Wavelength	Total Energy in 1 minute J/Cm^2
12.00 Noon	365	0.10864 J/Cm^2
12.00 Noon	312	0.05592 J/Cm^2
12.00 Noon	254	0.00192 J/Cm^2

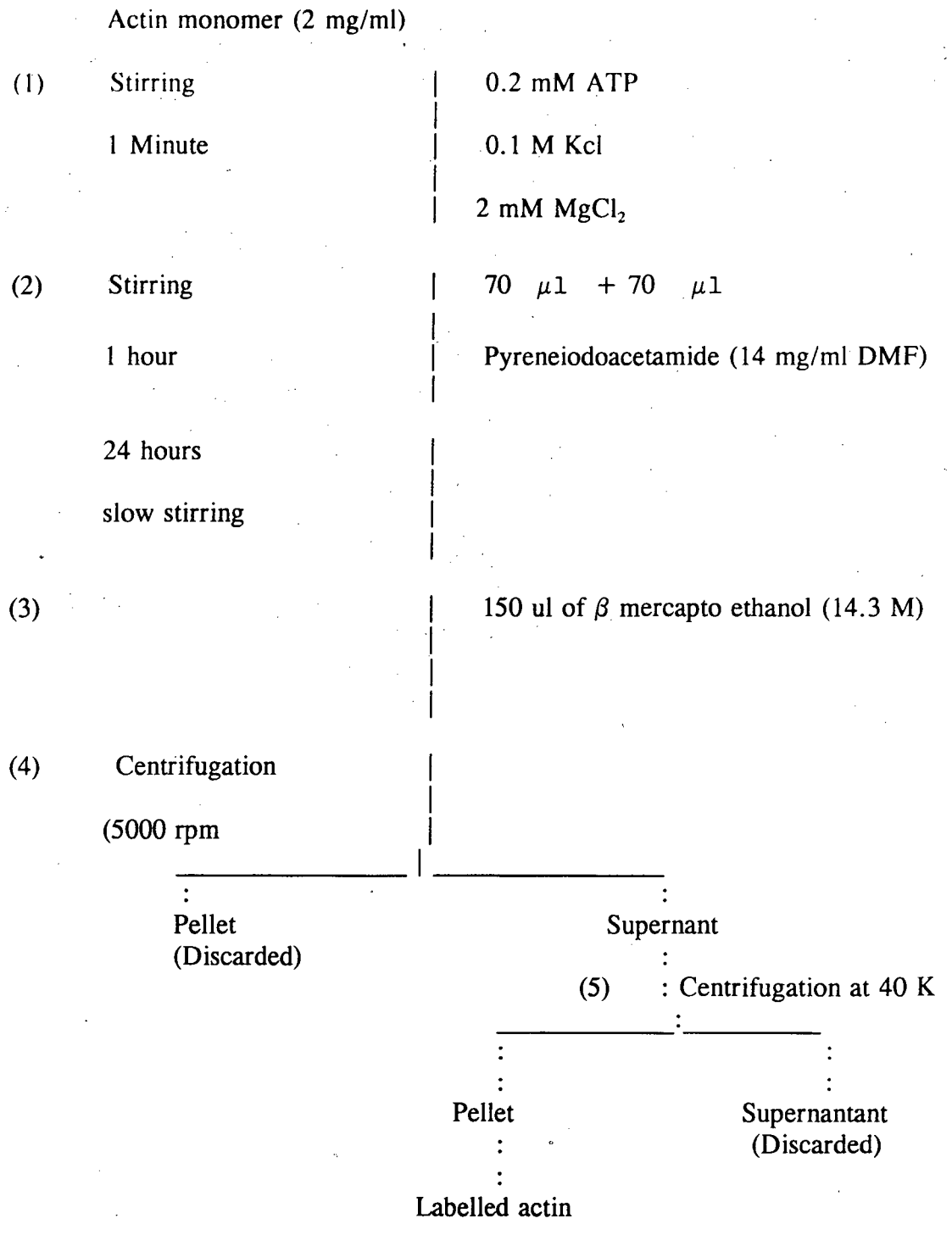
Table - 4

Baseline value of pyrene-idoacetamide on exposure of different dose

S.No	Total Dose Exposed (mJ/cm^2)	Florsence intensity	
		Concentration 2 μM	Concentration 4 μM
1	0	2.2	6.4
2	7.13	2.1	6.9
3	14.14	2.1	6.3
4	20.50	2.1	6.6
5	29.78	2.2	6.7
6	36.14	2.1	6.4
7	43.58	2.2	5.1

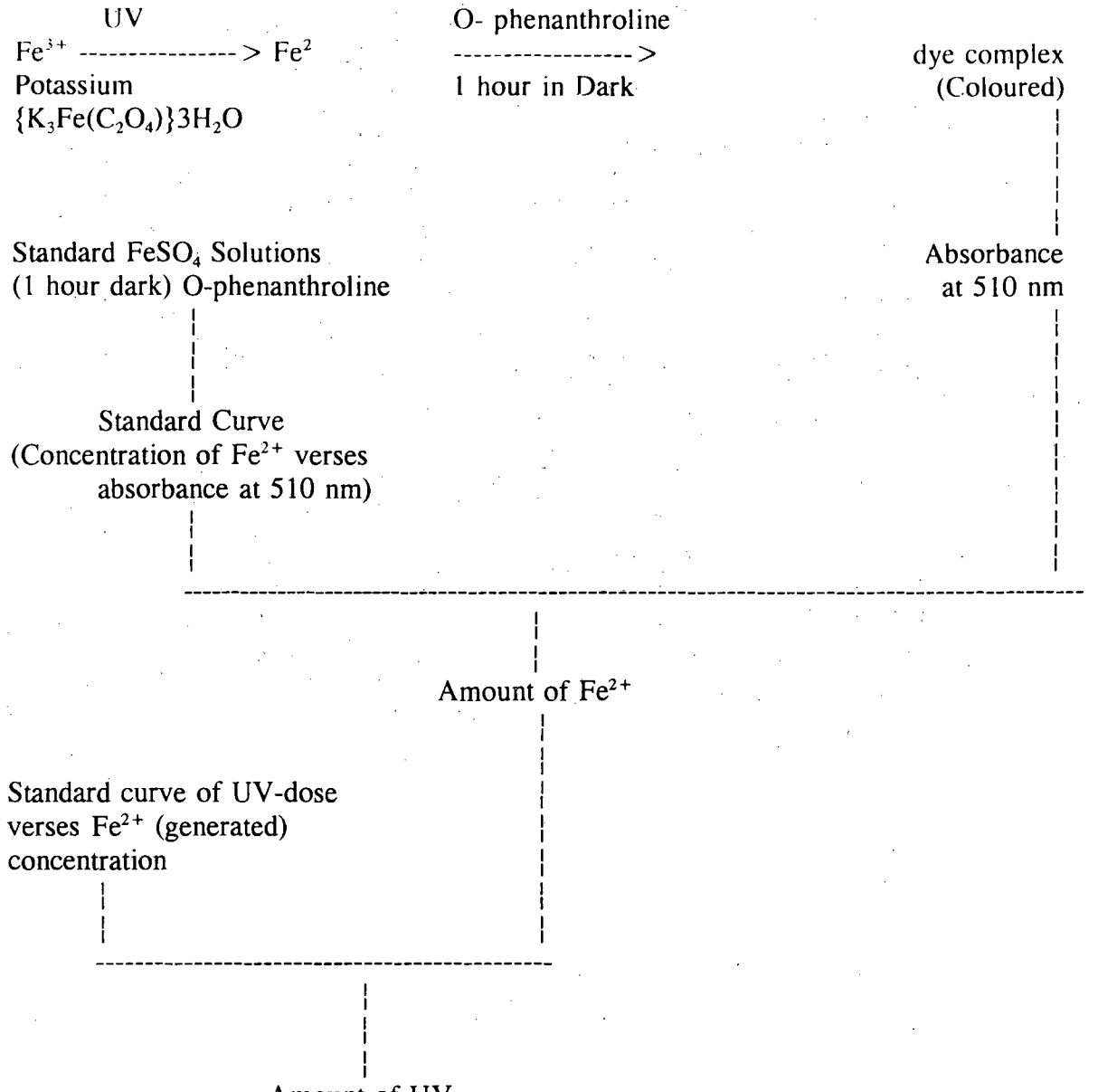
ANNEXURE I

Labelling of actin with Pyrene iodoacetamide



ANNEXURE II

Chemical dosimetry



Energy of photons (E) at 280nm wavelength,

$$\frac{hc}{\lambda} = \frac{6.55 \times 10^{-27} \times 3 \times 10^{10}}{280 \times 10^{-7}} - ergs$$

$$= 7.1 \times 10^{-12} \text{ ergs}$$

$$\text{or } E = 7.1 \times 10^{-12} \text{ ergs}$$

Where,

h = Planck's instant

c = velocity of light

λ = wavelength of UV=radiation

$$\text{Dose rate} = \frac{E \times 1.08 \times 10^{16} \times \Delta W \times V}{1.24 \times A \times t} \; ergs mm^{-2} \; sec^{-1}$$

$$= \frac{7.1 \times 10^{-12} \times 1.08 \times 10 \text{sup} 16 V \times \Delta W}{1.24 \times A \times t} mm^{-2} sec-1$$

$$= \frac{6.18 \times 10^{4} \times V \times \Delta W}{A \times t} ergs mm^{-2} sec-1$$

Where V = Volume of the solution irradiated

ΔW = Weight of Fe^{-2} formed

A = Surface area of total exposed material in mm^{-2}

t = time of exposure in seconds

(Saini in 1987)

ANNEXURE - III

Protein estimation by Follin-Lowry method

Standard solution of BSA = 1 mg/ml

Reagent A 2% Na_2CO_3 in 0.1N NaOH

Reagent B $CuSO_4$ + Sodium potassium tartarate

in 1:1 (volume to volume)
$CuSO_4$ 1g/100 ml (1%)
Sodium potassium tartarate 2g/100 (2%)

Lowry's Reagent: Reagent A + Reagent B in 50:1 ratio (volume to volume)

Follin's reagent: 1N

Table

Tube No.	BSA (μl)	Double distilled water	Lowry's	Follin's
1	0	500	2.5 ml to each tube and kept for 10 min	0.25 ml to each tube and kept for 30 min
2	10	490		
3	20	480		
4	30	470		
5	40	460		
6	50	450		
7	60	440		
8	70	430		
9	80	420		
10	100	400		
11	120	380		
12	460	340		

Taken the absorbance of each of the solutions at 660 nm and plotted the standard curve.

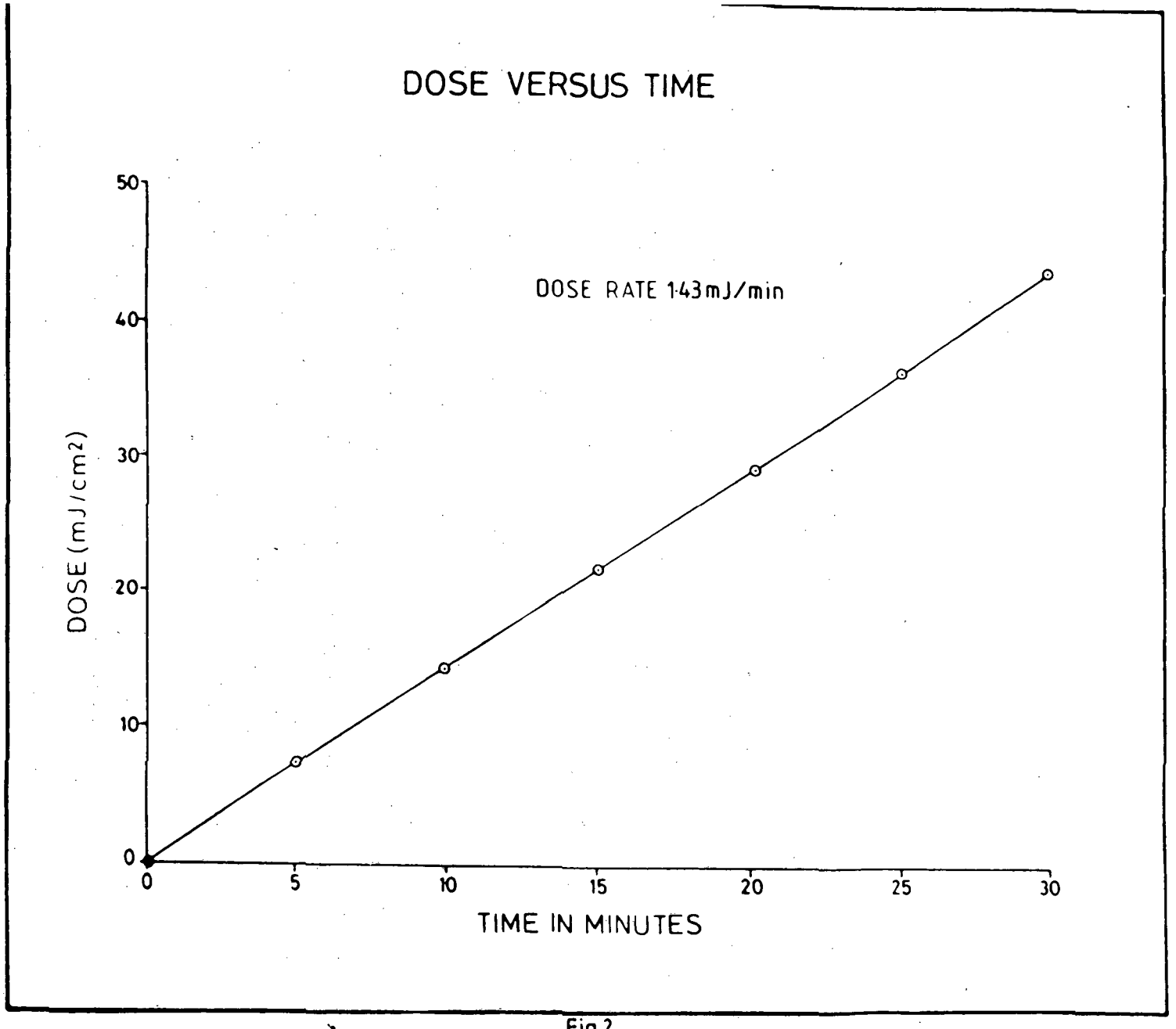

DOSE VERSUS TIME
DOSE RATE 1·43mJ/min
DOSE (mJ/cm²)
50
40
30
20
10
0
0
5
10
15
20
25
30
TIME IN MINUTES

Fig.2

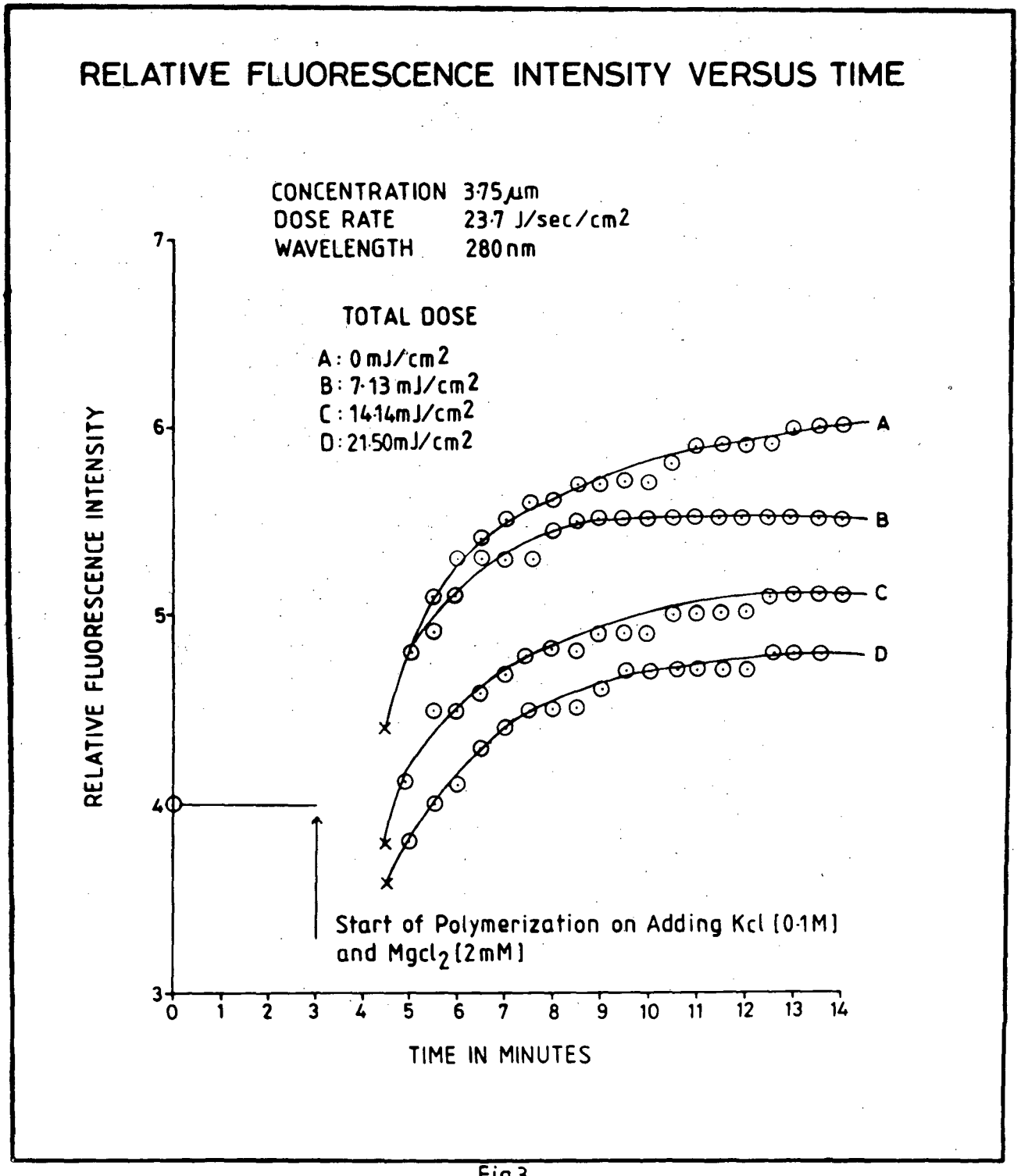

RELATIVE FLUORESCENCE INTENSITY VERSUS TIME
CONCENTRATION 3·75 µm
DOSE RATE 23·7 J/sec/cm2
WAVELENGTH 280 nm
TOTAL DOSE
A : 0 mJ/cm2
B : 7·13 mJ/cm2
C : 14·14 mJ/cm2
D : 21·50 mJ/cm2
RELATIVE FLUORESCENCE INTENSITY
7
6
5
4
3
A
B
C
D
Start of Polymerization on Adding Kcl [0·1M] and Mgcl2 [2mM]
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
TIME IN MINUTES

Fig.3

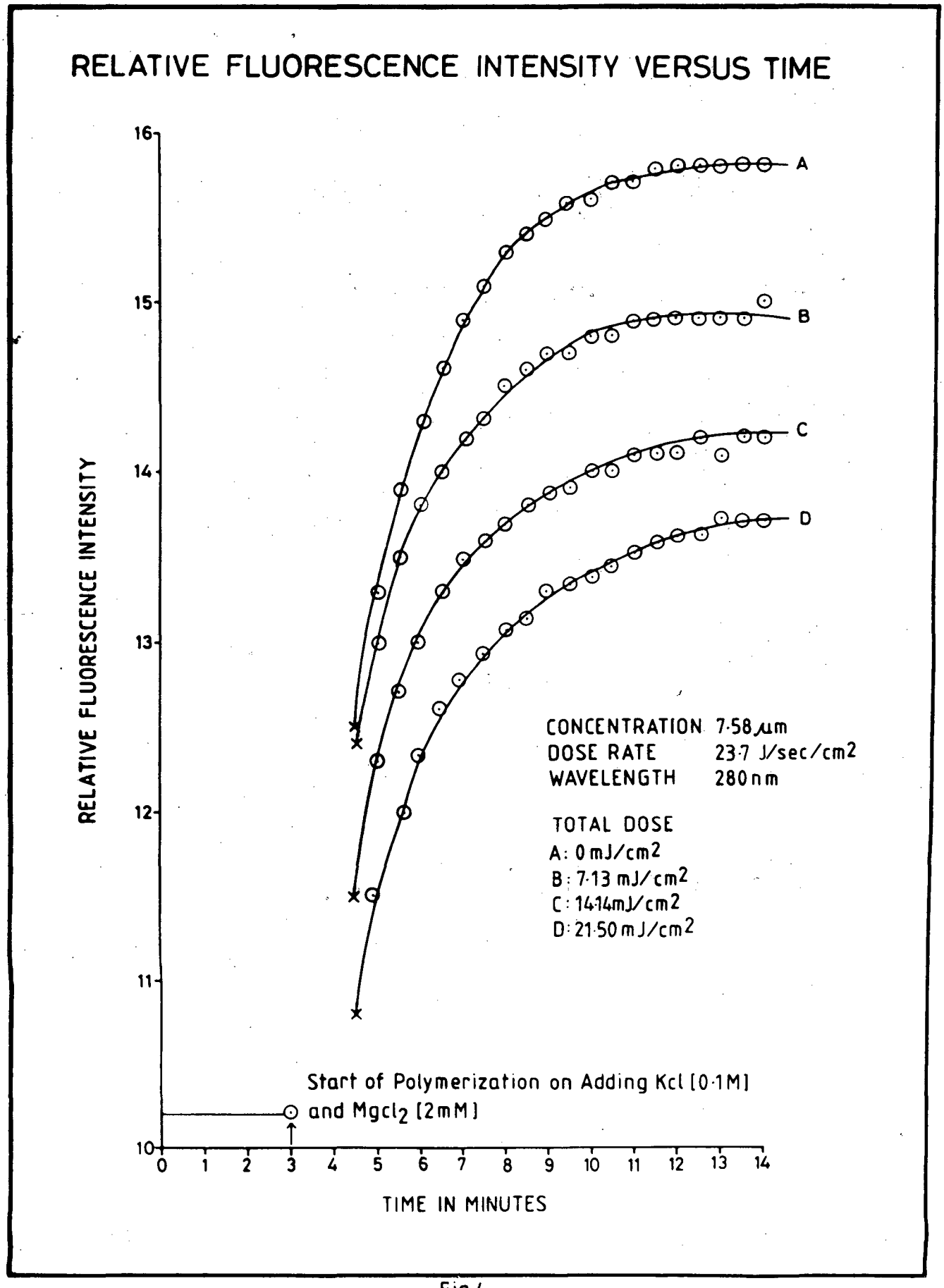

RELATIVE FLUORESCENCE INTENSITY VERSUS TIME
RELATIVE FLUORESCENCE INTENSITY
16
15
14
13
12
11
10
A
B
C
D
CONCENTRATION 7·58 µm
DOSE RATE 23·7 J/sec/cm2
WAVELENGTH 280 nm
TOTAL DOSE
A: 0 mJ/cm2
B: 7·13 mJ/cm2
C: 14·14 mJ/cm2
D: 21·50 mJ/cm2
Start of Polymerization on Adding Kcl [0·1M]
and Mgcl2 [2mM]
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
TIME IN MINUTES

Fig.4

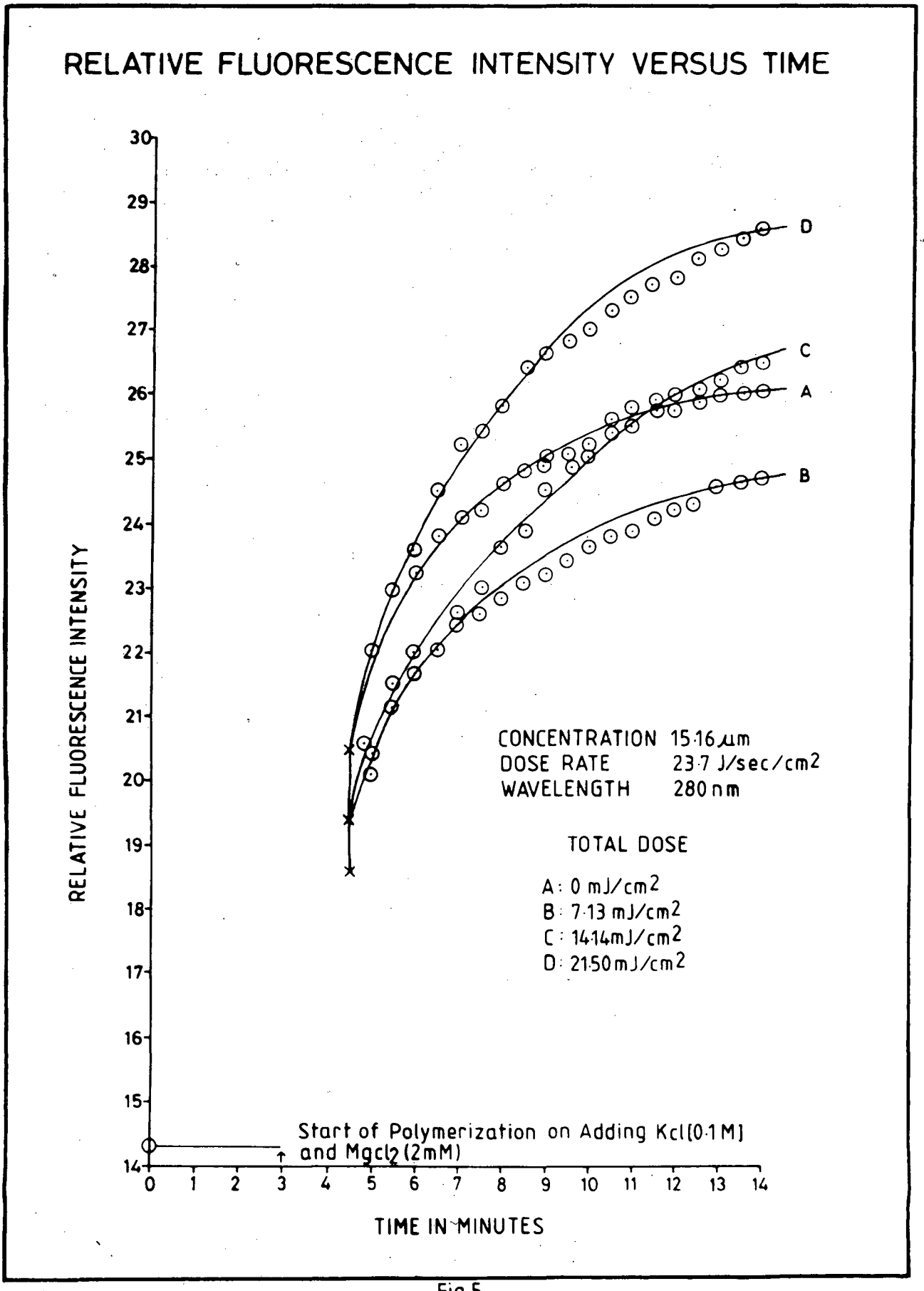

RELATIVE FLUORESCENCE INTENSITY VERSUS TIME
RELATIVE FLUORESCENCE INTENSITY
30
29
28
27
26
25
24
23
22
21
20
19
18
17
16
15
14
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
TIME IN MINUTES
D
C
A
B
CONCENTRATION 15·16 µm
DOSE RATE 23·7 J/sec/cm2
WAVELENGTH 280 nm
TOTAL DOSE
A: 0 mJ/cm2
B: 7·13 mJ/cm2
C: 14·14mJ/cm2
D: 21·50 mJ/cm2
Start of Polymerization on Adding Kcl(0·1M)
and Mgcl2 (2mM)

Fig.5

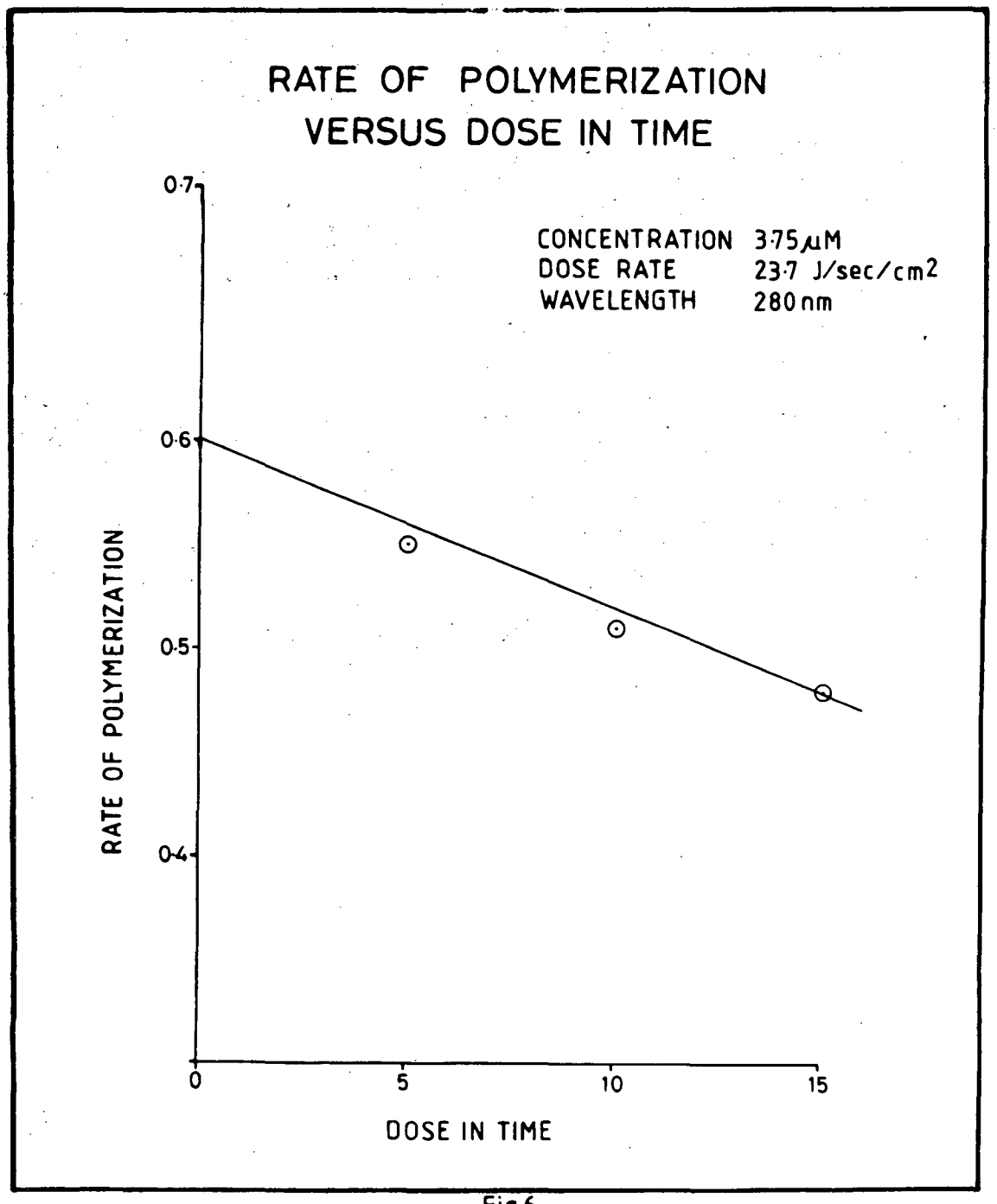

RATE OF POLYMERIZATION
VERSUS DOSE IN TIME
CONCENTRATION 3·75 μM
DOSE RATE 23·7 J/sec/cm2
WAVELENGTH 280 nm
RATE OF POLYMERIZATION
0·7
0·6
0·5
0·4
0
5
10
15
DOSE IN TIME

Fig.6

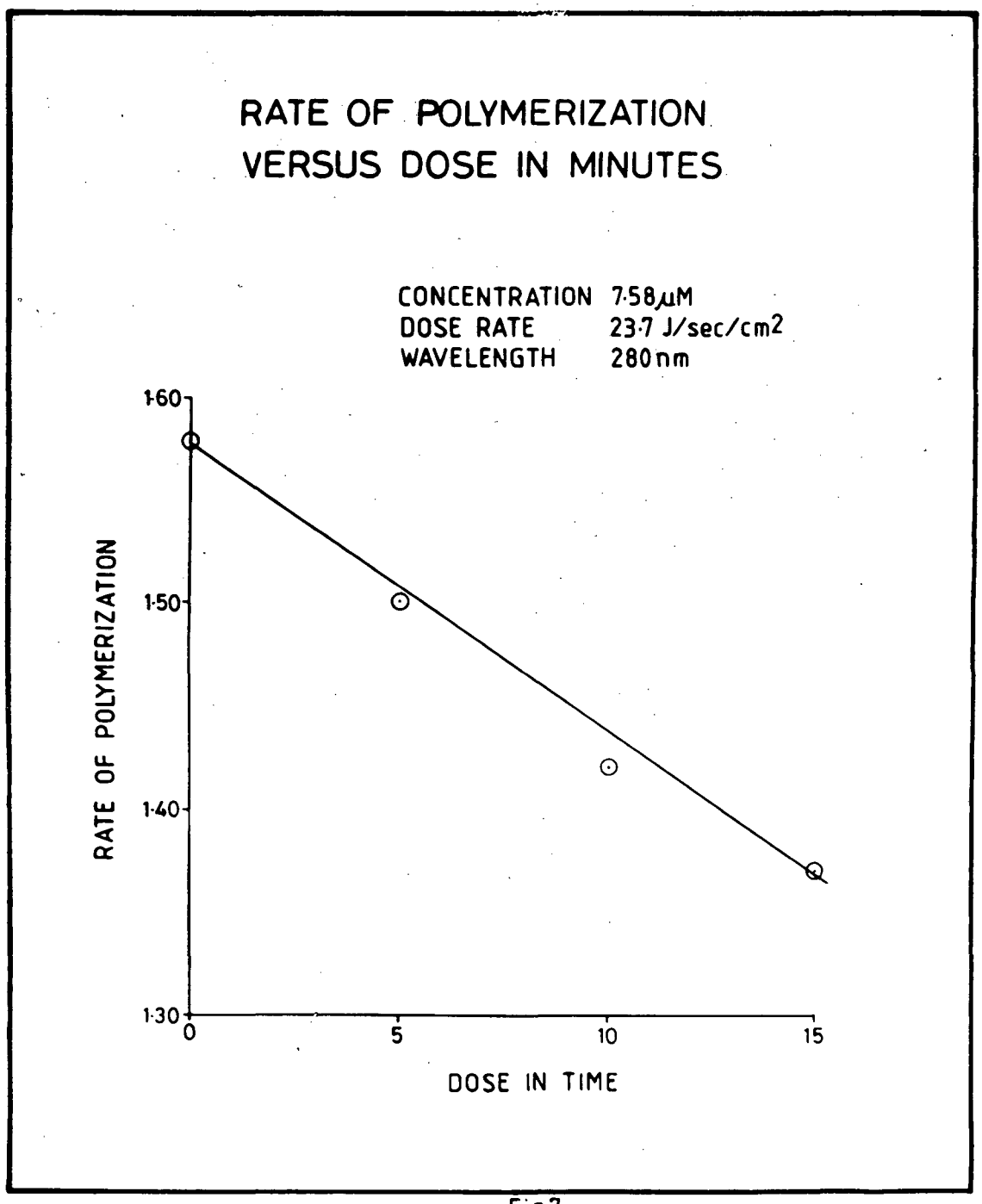

RATE OF POLYMERIZATION
VERSUS DOSE IN MINUTES
CONCENTRATION 7·58μM
DOSE RATE 23·7 J/sec/cm2
WAVELENGTH 280nm
RATE OF POLYMERIZATION
1·60
1·50
1·40
1·30
0
5
10
15
DOSE IN TIME

Fig.7

RATE OF POLYMERIZATION VERSUS TIME OF DOSE

CONCENTRATION 15·16 μM
DOSE RATE 23·7 J/sec/cm^2
WAVELENGTH 280 nm

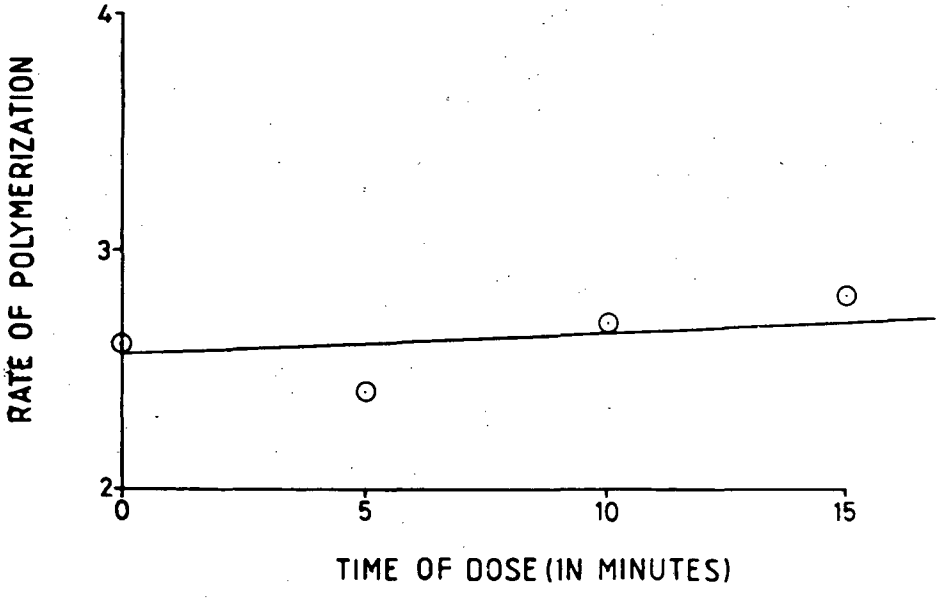


Fig.8

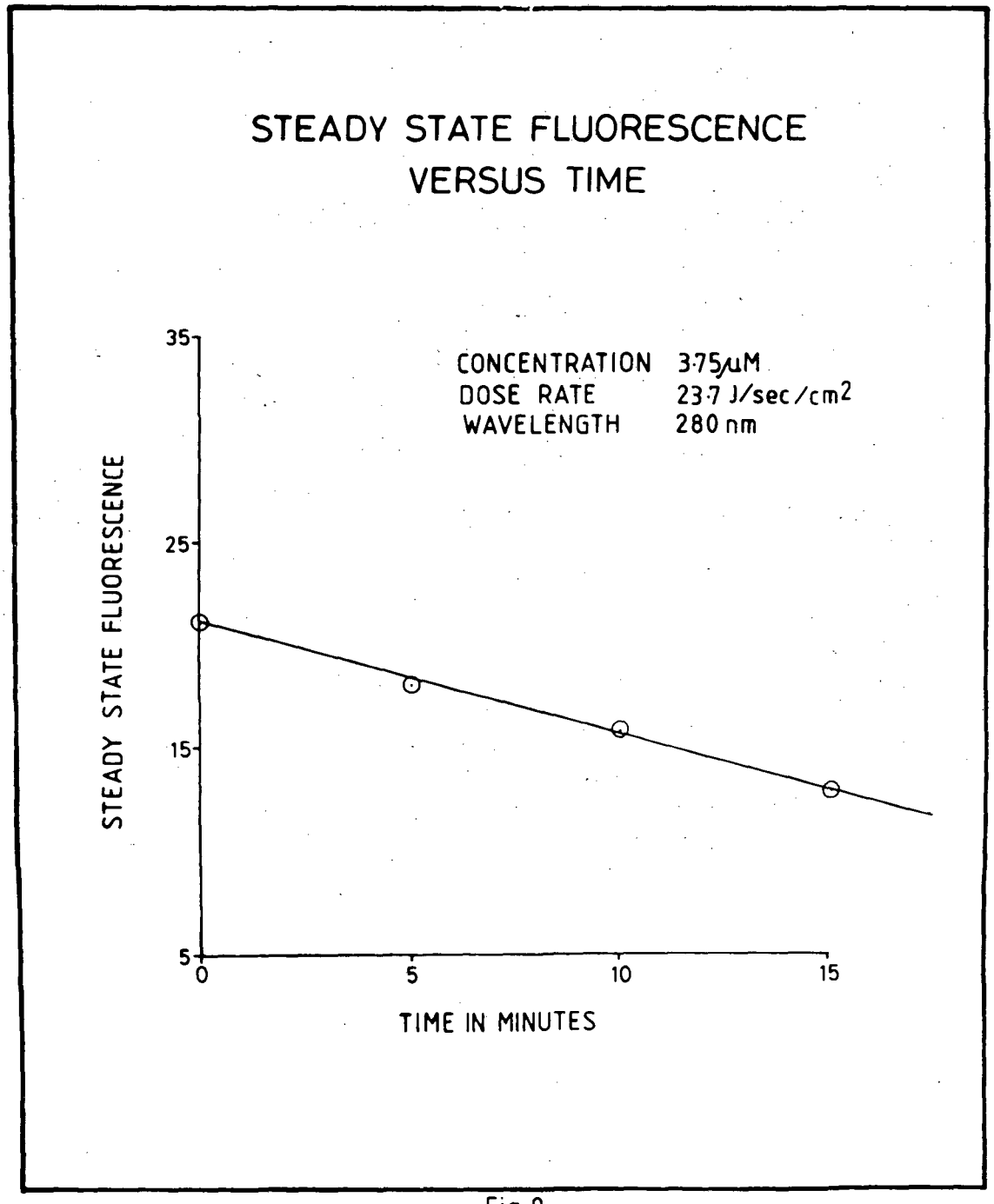

STEADY STATE FLUORESCENCE
VERSUS TIME
CONCENTRATION 3·75μM
DOSE RATE 23·7 J/sec/cm2
WAVELENGTH 280 nm
STEADY STATE FLUORESCENCE
35
25
15
5
0
5
10
15
TIME IN MINUTES

Fig. 9

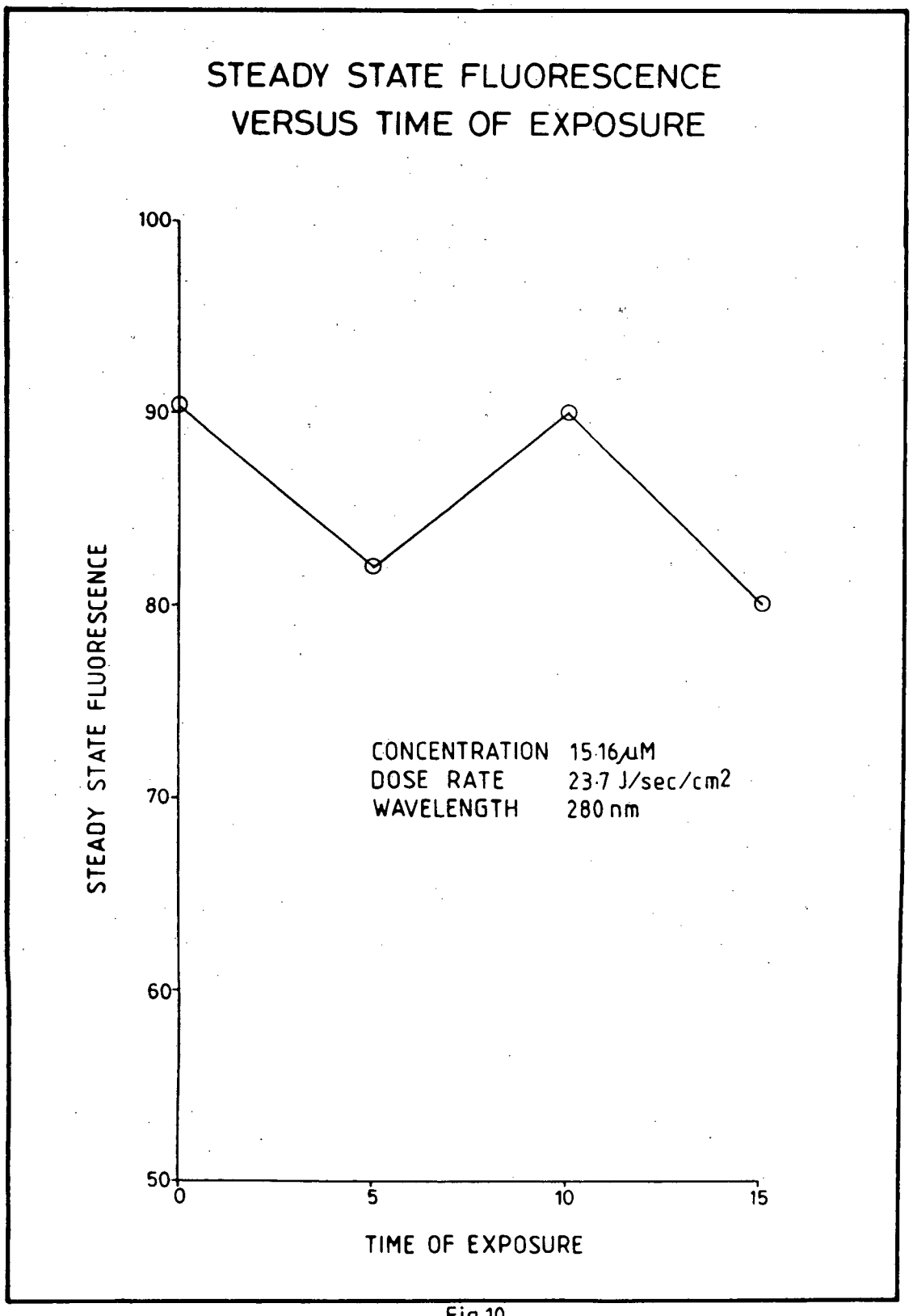

STEADY STATE FLUORESCENCE
VERSUS TIME OF EXPOSURE
100
90
80
70
60
50
STEADY STATE FLUORESCENCE
CONCENTRATION 15·16μM
DOSE RATE 23·7 J/sec/cm2
WAVELENGTH 280 nm
0
5
10
15
TIME OF EXPOSURE

Fig.10

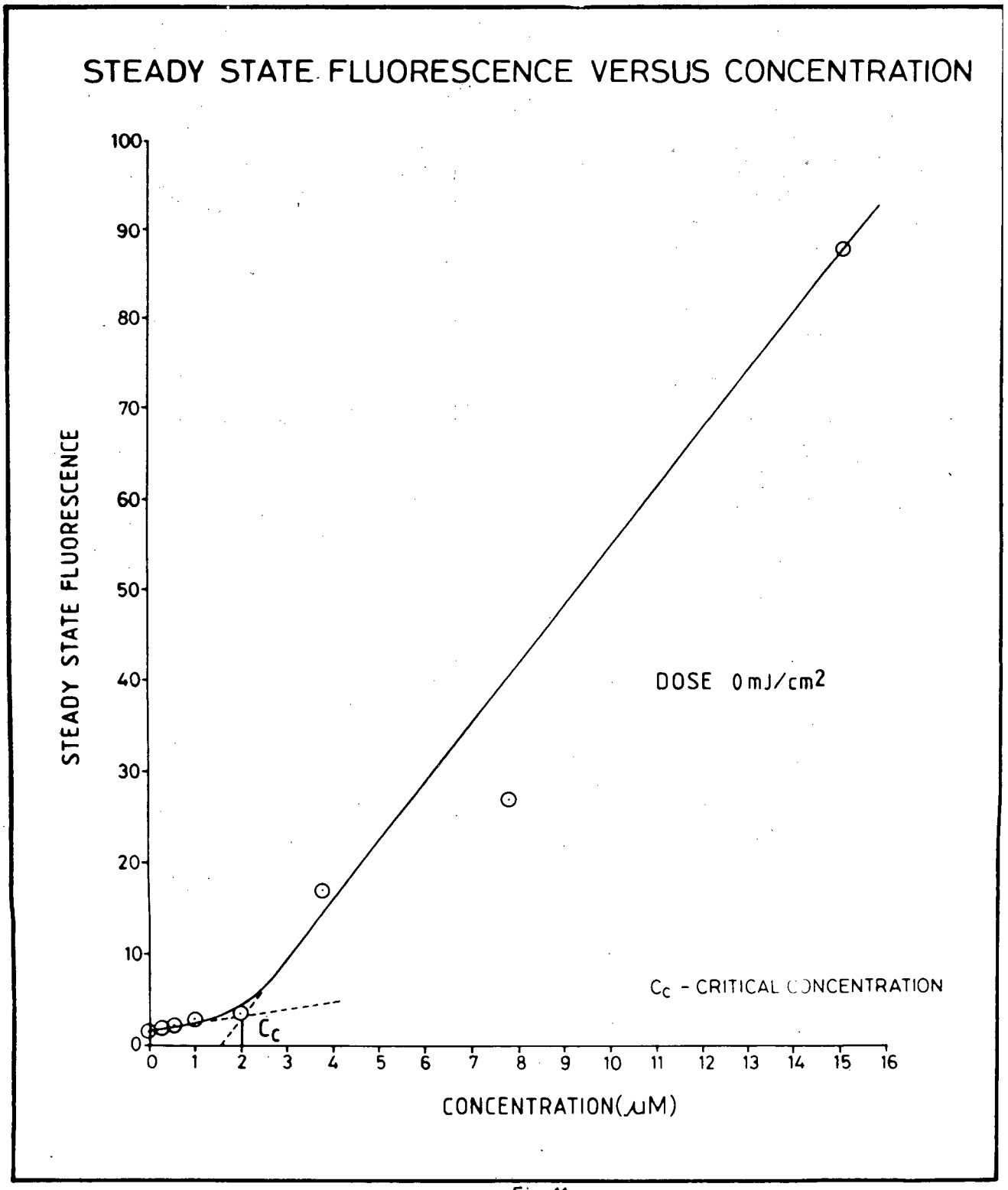

STEADY STATE FLUORESCENCE VERSUS CONCENTRATION
STEADY STATE FLUORESCENCE
100
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
CONCENTRATION(μM)
DOSE 0 mJ/cm2
Cc - CRITICAL CONCENTRATION
Cc

Fig.11

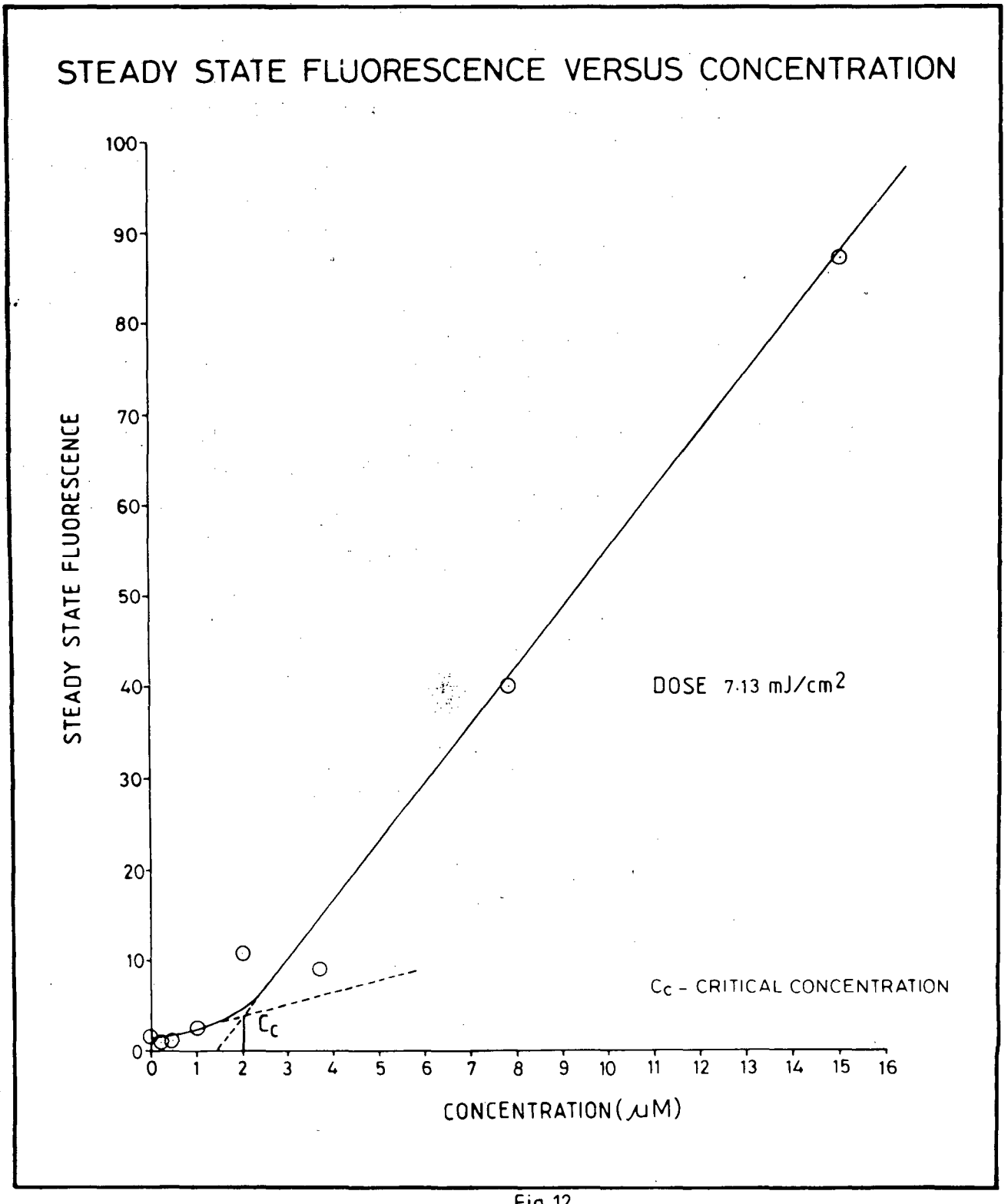

STEADY STATE FLUORESCENCE VERSUS CONCENTRATION
STEADY STATE FLUORESCENCE
100
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
CONCENTRATION (μM)
DOSE 7·13 mJ/cm2
Cc - CRITICAL CONCENTRATION
Cc

Fig.12

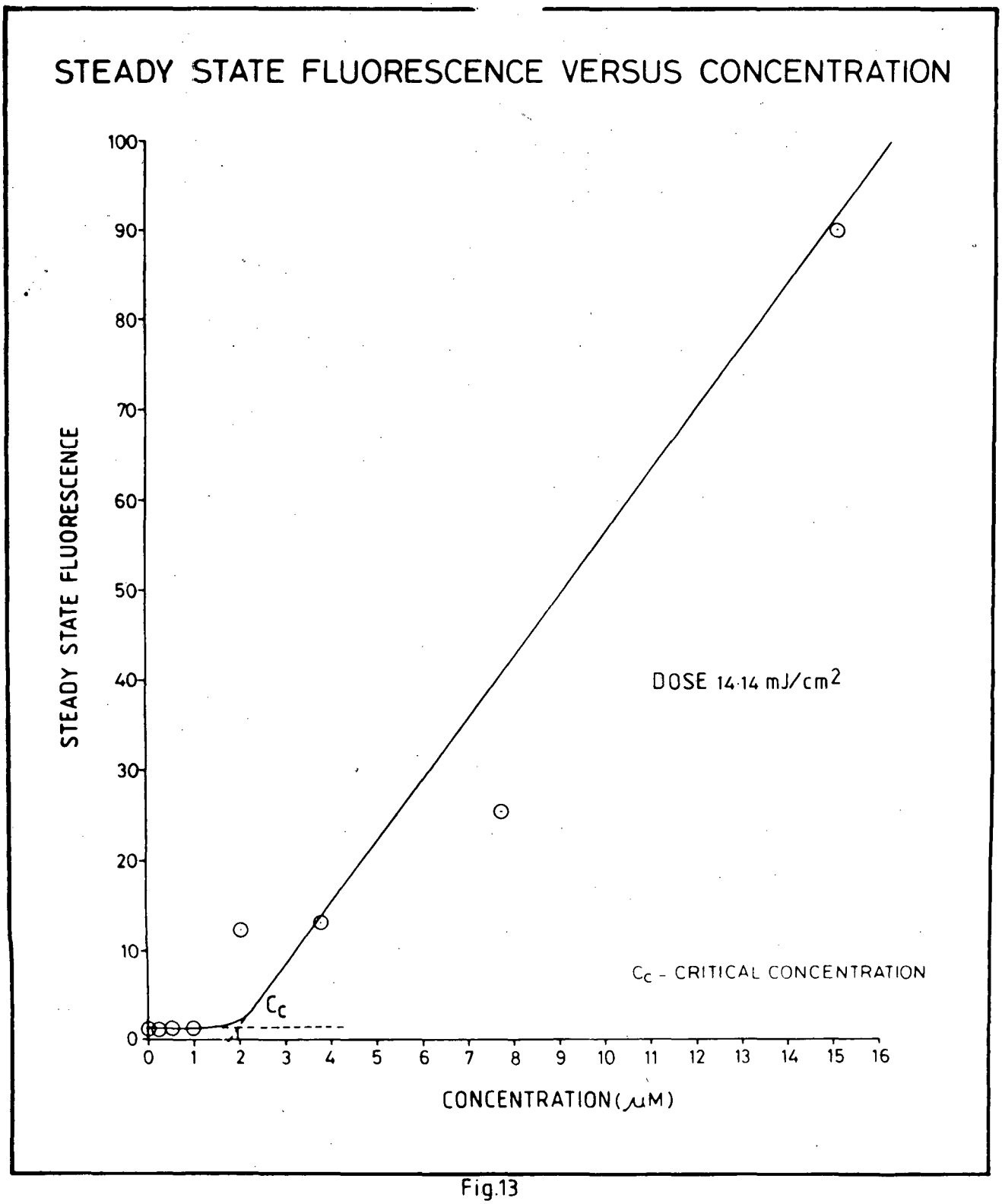

STEADY STATE FLUORESCENCE VERSUS CONCENTRATION
STEADY STATE FLUORESCENCE
100
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
CONCENTRATION (μM)
DOSE 14·14 mJ/cm²
Cc - CRITICAL CONCENTRATION
Cc

Fig.13

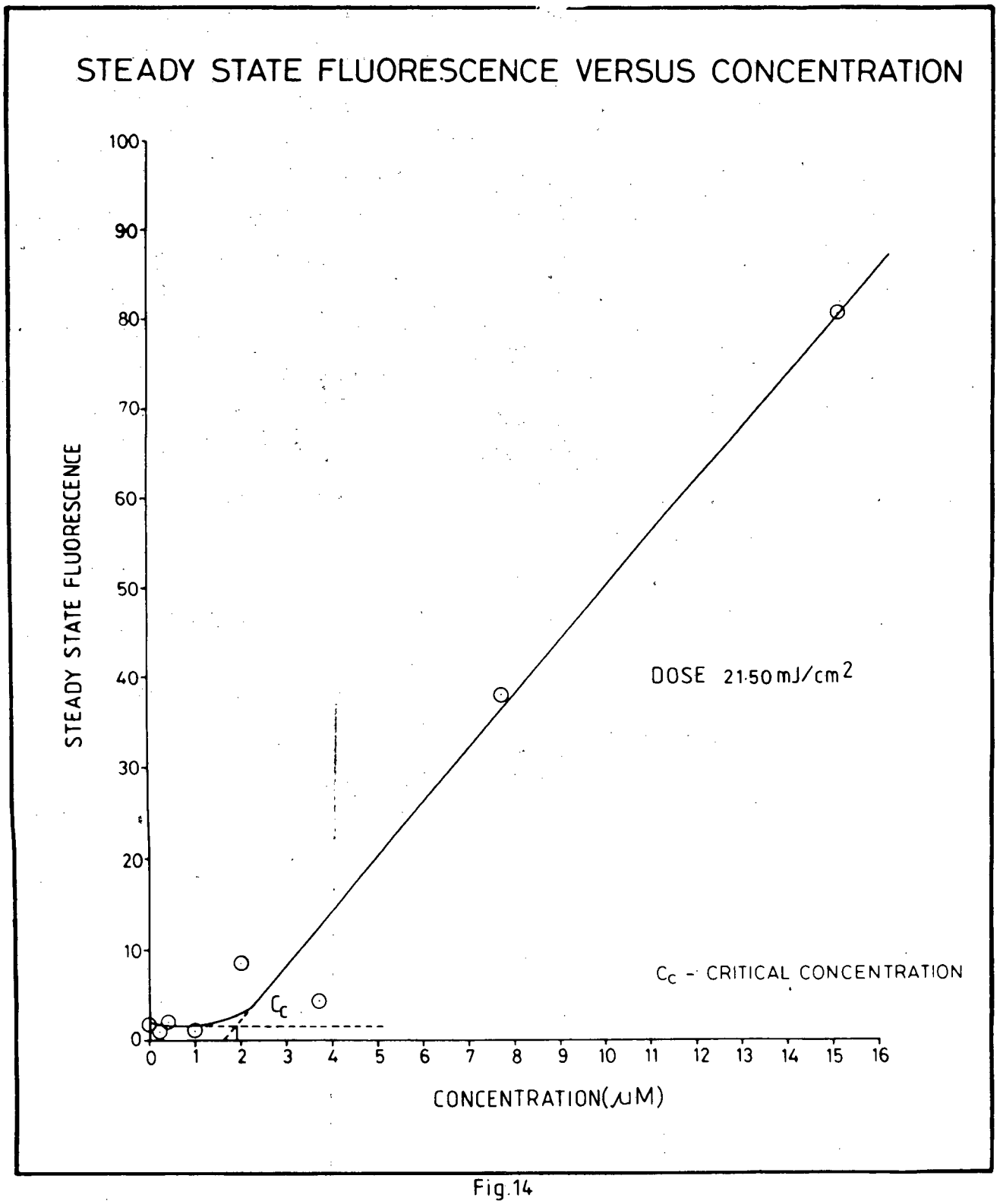

STEADY STATE FLUORESCENCE VERSUS CONCENTRATION
STEADY STATE FLUORESCENCE
100
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
CONCENTRATION(μM)
DOSE 21·50 mJ/cm2
Cc - CRITICAL CONCENTRATION
Cc

Fig.14

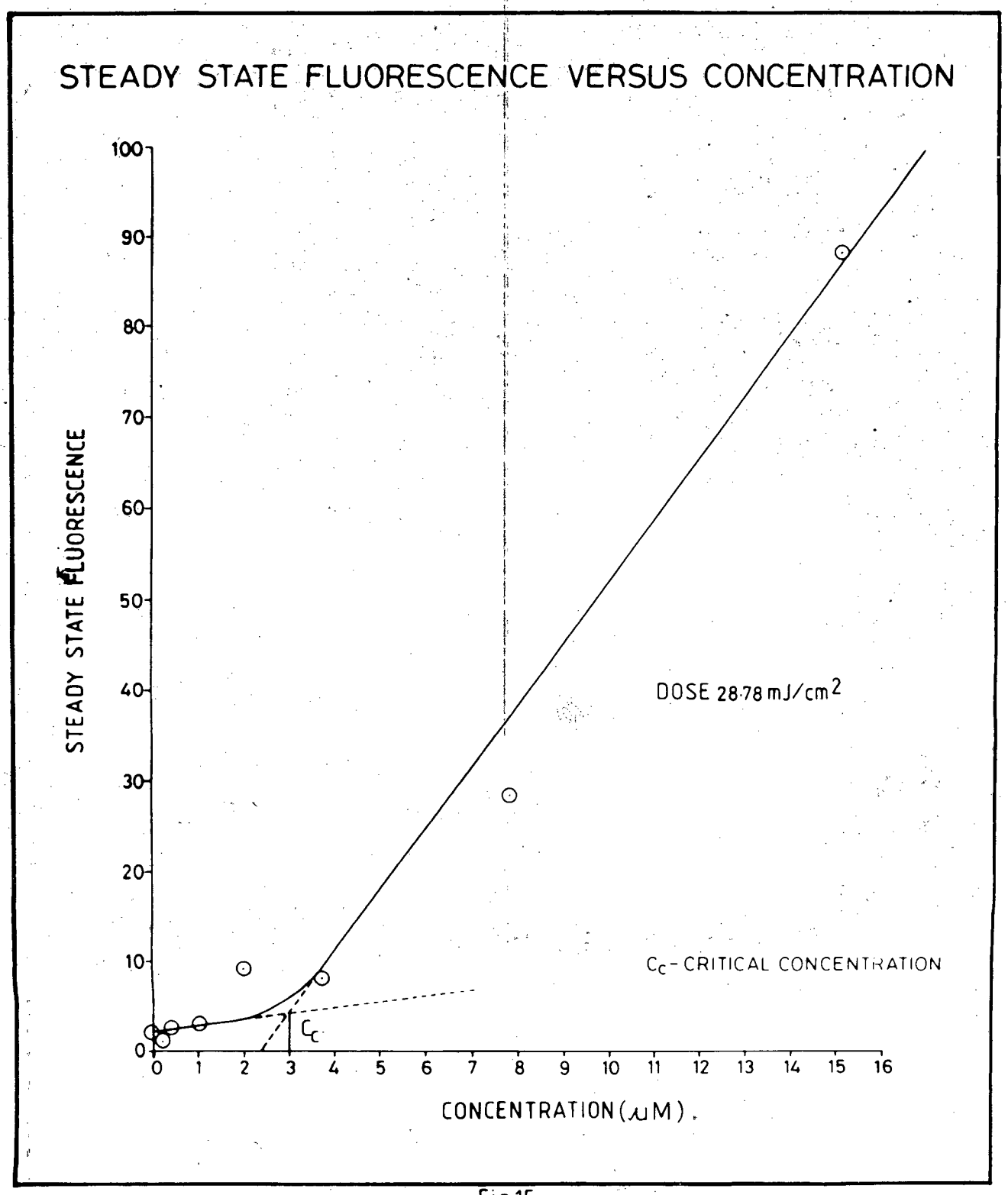

STEADY STATE FLUORESCENCE VERSUS CONCENTRATION
STEADY STATE FLUORESCENCE
100
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
CONCENTRATION (μM).
DOSE 28·78 mJ/cm²
Cc - CRITICAL CONCENTRATION
Cc

Fig.15

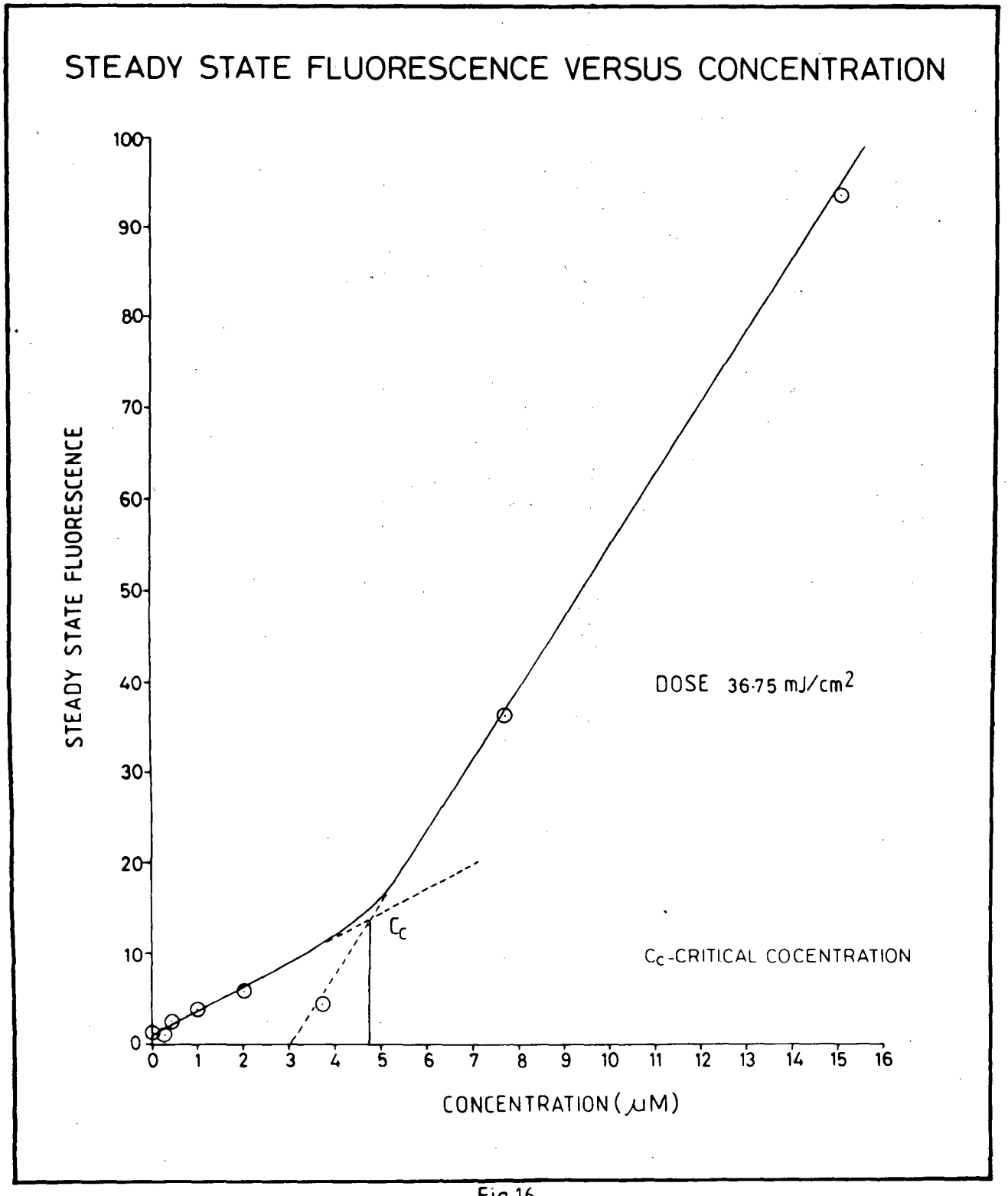

STEADY STATE FLUORESCENCE VERSUS CONCENTRATION
STEADY STATE FLUORESCENCE
100
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
CONCENTRATION (μM)
DOSE 36·75 mJ/cm2
Cc
Cc-CRITICAL COCENTRATION

Fig 16

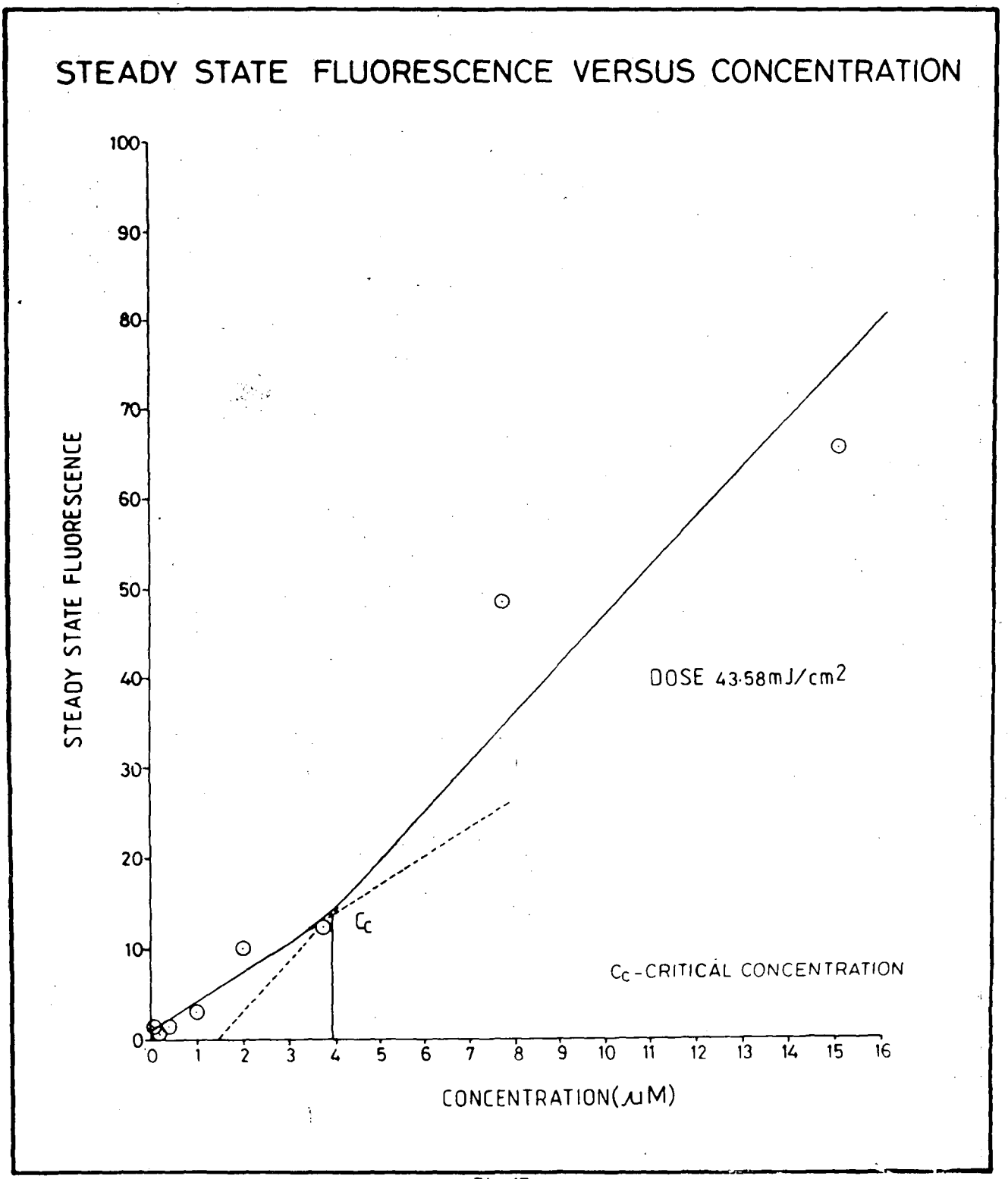

STEADY STATE FLUORESCENCE VERSUS CONCENTRATION
STEADY STATE FLUORESCENCE
100
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
CONCENTRATION(μM)
DOSE 43·58mJ/cm2
Cc
Cc-CRITICAL CONCENTRATION

Fig.17

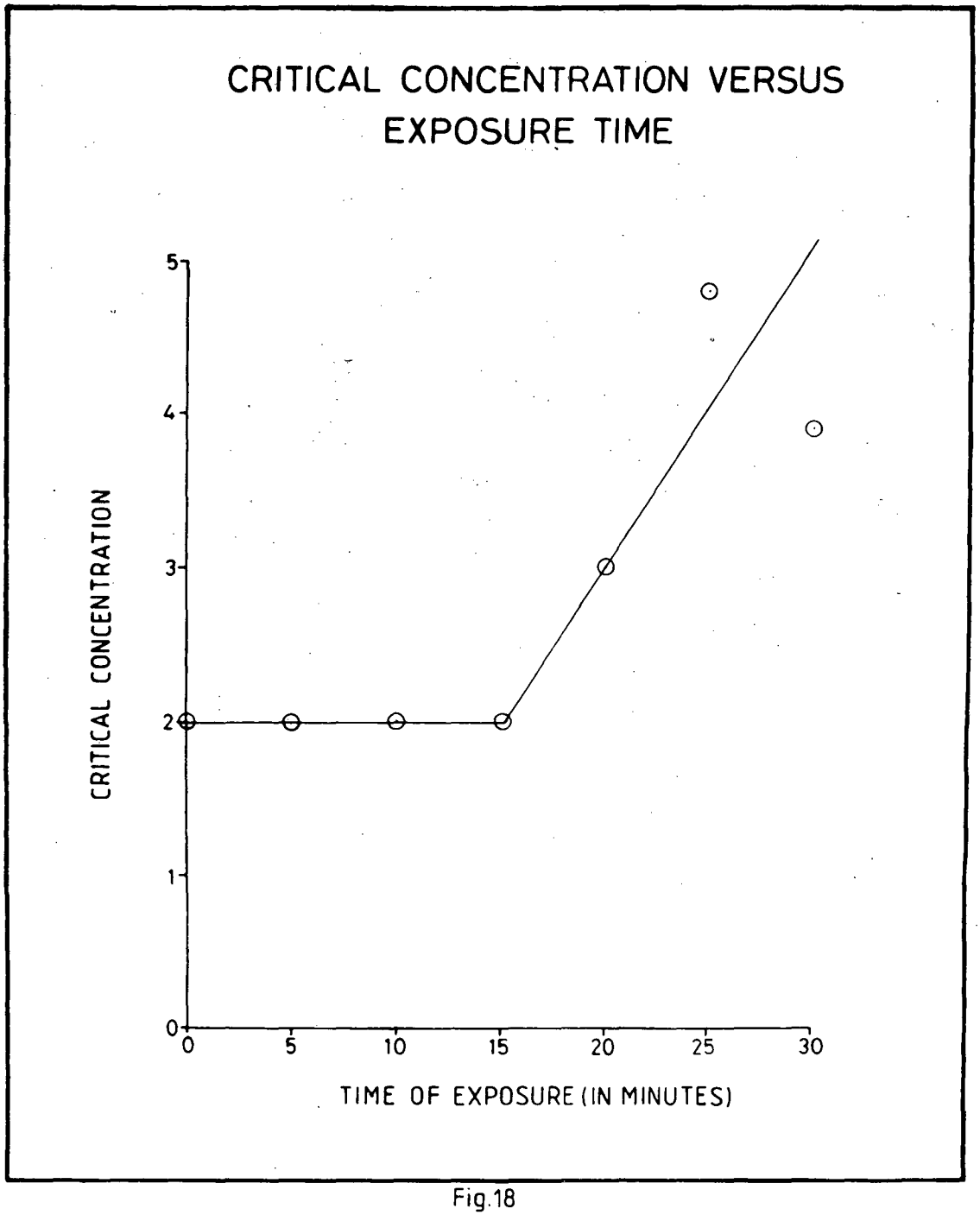

CRITICAL CONCENTRATION VERSUS
EXPOSURE TIME
CRITICAL CONCENTRATION
0
1
2
3
4
5
0
5
10
15
20
25
30
TIME OF EXPOSURE (IN MINUTES)

Fig.18

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