ACTION OF CHLORPROMAZINE ON LOWER EUCARYOTIC CELLS

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TO MY PARENTS

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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CONTENTS

INTRODUCTION

Chlorpromazine hydrochloride (CPZ) was first introduced into psychiatry in 1953 and has been in extensive use since then. It has brought about a major evolution in the treatment of psychiatric diseases due to its ability to make hyperactive psychotic patients more manageable. Possibly CPZ is very widely used as a tranquilizer although there are numerous other tranquilizing agents. The drug is also a potent local anesthetic and suppresses the electrical activity of the neurones when applied directly (Bradley et al., 1966). It is a neuropharmacologically active drug which has profound effects on nervous tissues. However, the main usefulness of the drug is in the reduction and control of agitation, aggression and anxiety. It is used in the control of acute psychoses where it can diminish restlessness, anxiety, confusion, suicidal tendencies and aggression.

CPZ presents a wide spectrum of pharmacological and biochemical actions. The biochemical effects of CPZ and the related compounds have been studied extensively in a wide variety of animals ranging from the lowly developed unicellular protozoans upto the highly developed metazoans such as the mammals. The results of such studies indicate that

these agents inhibit a number of enzymes, interfere with co-enzymes, affect permeability and stability of biological and artificial membranes and modify the transport of neurotransmitters at the nerve terminals.

Charpentier and his co-workers (1951) synthesized CPZ which was initially employed as an ingredient in anesthesiology. Subsequently CPZ has been found to cure mental disorders because of its involvement in a number of neuronal and psychological phenomena. For instance, CPZ has a depressant action on the central nervous system where it blocks arousal produced by sensory stimulation (Bradley et al., 1966). Depression in the amplitude of polysynaptic reflex (PSR) in anesthetized spinal rats has been reported by Tsugutake et al. (1982). Courvisier et al. (1953) have suggested that hypothalamus of the Central Nervous System is affected by CPZ since temperature reduction (hypothermia) is seen in patients treated with CPZ (see, Bradley, 1963). Further CPZ has been found to reduce locomotor activity, increase sociability in cats and taming in Rhesus monkeys (Norton and Tamburro, 1957) and block conditioned response (Cook et al., 1957). Stressful response to a change in the environment is diminished by CPZ (Steinberg and Watson, 1959) which may be due to its action on the hypothalamus (Mohfouz and Ezz, 1958).

CPZ influences the endocrine system to some extent. Kornetsky (1976) has mentioned that CPZ reduces urinary levels of the gonadotrophins, estrogen and progesterone thereby blocking ovulation, suppressing estrus cycle and causing infertility. However, he has suggested that these actions of CPZ on the endocrine system are probably mediated via the hypothalmus which acts as a control for the pituitary gland which, in turn, controls secretory activity of other endocrine glands.

Besides its action on the nervous system, CPZ has also a number of miscellaneous actions on the nerves. Bulbring et al. (1954) have suggested a local anesthetic action for CPZ because it causes local anesthesia when infiltrated round the sciatic nerve in guineapigs. But if the concentration is too high, histological changes occur and there is some disorganisation of the myelin and cytoplasm.

It is true that CPZ has diverse effects on the central nervous system. But the precise way in which CPZ exerts its influence on the central nervous system is not clearly known and still remains a controversial issue. However, Iversen (1975) has suggested that CPZ might be acting by blocking dopamine receptors in the Central Nervous System.

According to Claude Bernard "An anesthetic is not a special poison for the Nervous System. It anesthetizes all the cells, benumbing all the tissues, and stopping temporarily their irritability.... we can study elsewhere than in the central nerve cells the phenomenon which causes this stoppage of action.... It is permissible to assume that something similar happens in the nerve cell" (see, Seeman, 1972).

These words of Bernard are indicative of the fact that many, if not all, of the membrane actions of anesthetics and tranquilizers occur in both excitable as well as nonexcitable membranes. Hence any attempt at studying excitable cells and nonexcitable cells as well to resolve the molecular pharmacology of CPZ is highly justified.

A number of studies have been done on the effect of CPZ on isolated single cells and unicellular animals. Yano et al. (1982) studied the effect of CPZ on isolated rat hepatocytes and have reported that oxygen consumption was elevated to a great extent at low concentration of CPZ. They have suggested this effect to be the consequence of membrane expansion or alteration of membrane fluidity which would enable a low molecular substance such as succinate to pass through the plasma membrane of hepatocytes.

Low concentration of CPZ was found to release small amount of 5-hydroxytryptamine with minor granular alterations in the mast cells. But at higher concentration the drug disrupted the cells, affected the nucleus and membranes to a high degree (Jansson, 1970).

Corps et al. (1982) have established that CPZ reduces lymphocyte ATP levels by greatly reducing the phosphorylation potential. Glycolytic flux in lymphocytes is increased when ATP level is lowered and it is the result of impairment of mitochondrial function.

The work of James and his colleague (1982) has indicated that local anesthetics including CPZ block junctional communication in the epidermal cells of the beetle Tenebrio molitor. Treatment of the epidermal cells with CPZ raised intercellular resistance two to three fold within 20 to 25 minutes; cell to cell passage of electrical current was abolished within 41 ± 5 minutes. The reaction was fully reversible, with normal electrotonic coupling being restored within 2-4 hours. They have suggested that the uncoupling potency of the drug correlates well with their known ability to inhibit calmodulin dependent phosphodiesterase activity. Altering the extracellular calcium concentrations did not affect the rate of uncoupling by CPZ while chelation of extracellular calcium with EGTA raised electrotonic coupling.

From time to time micro-organisms like Escherichia coli, Lactobacillus plantarum, Bacillus megaterium and protozoans like Tetrahymena pyriformis have been used as model systems to study the action of CPZ and related compounds. Klubes et al. (1971) have found that CPZ inhibits cell wall synthesis in Bacillus megaterium because cell wall precursors accumulated promptly upon addition of the drug. Growth, motility, glucose utilisation and phosphate uptake of Tetrahymena pyriformis have been reported to be inhibited by low concentrations of CPZ (Kusnicki et al., 1979). Blum (1980) has reported that exposure of Tetrahymena pyriformis to low concentration of CPZ inhibits its growth and nucleic acid synthesis. 17 hours exposure of stationary phase cultures to this drug caused marked alterations in the metabolism, including an almost complete loss of ability to decarboxylate L-(1-^{14}C)-Leucine and L-(1-^{14}C)-tyrosine. Weiss et al. (1980) have reported that CPZ has the ability to inhibit release of trichocyst in paramecium.

There are numerous reports which indicate that CPZ has the capacity to act on the cell membrane of neuronal and non-neuronal cells. Spirtes and Guth (1961) have pointed out that a large number of the effects of CPZ on biological systems may be due to its effects on semipermeable membranes. Seeman (1972) has said that the primary

actions of anesthetics and tranquilizers are on the cell membrane (plasmalemma) rather than on intracellular processes and the following observations support the assumption of Seeman.

(a) Axons, from which all axoplasm has been removed, are still readily blocked by anesthesia.
(b) Action potentials in artificial lipid bilayers are blocked by local anesthetics like CPZ.
(c) The concentration of anesthetics required to block neural transmission of impulses is lower than that required to inhibit metabolism and oxygen consumption.

Shanes (1958) has used the word "Stabilizers" for the anesthetics because they block membrane excitability without appreciably altering the resting membrane potentials. There are also reports that anesthetics may block various forms of hyperpolarization (see, Seeman, 1972). Trudell (1977) has suggested that anesthetics including CPZ act by fluidizing nerve membranes to a point where critical lipid regions no longer contain phase separations. As a result, the nerve membranes lose their capacity to facilitate the conformational changes in proteins that may be the basis for such membrane events as ion intake, synaptic transmitter release and transmitter binding to receptors. He has proposed that once the

phase separation behaviour of the membrane is modified, neural function may change by a combination of the following mechanisms :

(a) Inhibition of conformational changes of integral membrane proteins.
(b) Prevention of the association of protein subunits to form ion channels.
(c) Depression of transmitter release by preventing fusion of synaptic vesicles with the membrane of the pre-synaptic terminal.

Gruner and Narahasi (1972) have advocated that CPZ blocks excitability of neuronal membranes by abolition of the action potential mechanism directly. According to them, block is achieved by the inhibition of the mechanism of the transient sodium conductance and that of steady state potassium conductance after external drug application, whereas internal CPZ perfusion inhibits primarily the transient sodium conductance.

Seeman et al. (1969b) have proposed that anesthetics including CPZ act by expanding the membrane. Kwant et al. (1968) have reported that low concentration of CPZ protects human erythrocytes from hypotonic haemolysis. Seeman et al. (1969a) have suggested that the erythrocyte membrane expands by a number of possible mechanisms, the simplest of all being

that of a volume increase in the membrane as a result of "burying" of anesthetic molecules right into the membrane. According to them, CPZ can also displace some membrane associated component such as Ca^{2+} which normally keeps the membrane in a condensed state. They have shown that the membrane area of spherical erythrocyte ghosts expands by about 20% by CPZ at concentrations which cause local anesthesia. Seeman et al. (1971) have reported the decrease in passive influx of $^{22}Na^{+}$ into erythrocytes in presence of Ca^{2+} by CPZ. Earlier, Seeman (1966) has shown that high sub-lytic concentrations of CPZ over expand the membrane, which then buckles inwards, the cells showing apparently less expansion than the real expansion. He has also seen that the cationic form of CPZ as well as the neutral form have the membrane expanding effect on both the erythrocyte and the nerve membrane. Seeman (1972) has suggested that although anesthetics like CPZ electrically stabilize the membrane, they also fluidize and perturb the components within the membrane. As a result of the perturbation of membrane components, membrane associated enzymes and proteins can be either stimulated or inhibited. Occupation of Ca^{2+} sites on the membrane by the drug may interfere with the translocation rates and membrane bound Ca^{2+} is not available for excitation-contraction coupling and stimulus-secretion coupling. As a consequence nerve action potentials fail to

be generated since Ca^{2+} is a prerequisite for this phenomenon.

Manian et al. (1974) have commented that CPZ and related compounds have high affinity for the erythrocyte membrane. They have suggested that the drug is transported to possible receptor sites in the brain after being bound to erythrocyte membrane and is released there by a subsequent transfer mechanism to initiate pharmacological activity.

CPZ markedly inhibited K^+ loss and caused an equivalent increase in the K^+ contents of Staphylococcus aureus (Kristiansen et al., 1982) at low concentrations. But at high concentrations, CPZ inhibited the growth of this and other micro-organisms. They have concluded that the bacteriostatic action of CPZ is probably not related to its membrane stabilising effect only.

Not only membrane structures, but also membrane bound enzymes are affected by CPZ. Hyttel (1978) reported the inhibition of adenyl cyclase (a membrane bound enzyme) by CPZ. Wolff and Jones (1970) studied the effect of CPZ administration by employing cyclases of rat adrenal, rat liver and bovine thyroid membrane and have suggested that CPZ interferes with activation of the cyclase by various

hormones with little effect on the basal activity of the enzyme. Seppala et al. (1971) have proposed that CPZ binds to phospholipids and may exert its action on biological membranes by complexing with phospholipids and proteins.

CPZ and related compounds bring about changes in the stability of biological membranes which depend on the dose and some other factors. Low concentrations induce stabilisation and high ones labilisation. CPZ induces, in vitro, a direct disruption of the membranes when used in comparatively high concentrations. This is due to the surface active property of the drug. In living cells, the damage to biological membranes caused by moderate doses of CPZ is probably a secondary effect, the primary effect being disruption of bioenergetic reactions within the cell (Papov, 1974).

Besides its actions on the membranes, there are evidences from other studies which indicate that the primary anesthetic action is on intracellular organelles such as mitochondria, microtubules, microsomes, lysosomes etc. Guth et al. (1965) have found that low concentration of CPZ causes a reduction in the spontaneous release of acid hydrolases from lysosomes and his finding supports the idea that the membranes of the subcellular organelles are also stabilized

or protected by low concentrations of drug. Popov (1974) has reported that CPZ protects the liver against necrosis caused by some toxic agents and decreases the leakage into plasma of lysosomal acid hydrolases normally associated with such injury. He has also found that CPZ prevents mitochondrial swelling and lysis. Beckel (1974) has reported that CPZ strongly binds to rat liver microsomes and mitochondria, whereas the binding to nuclei and soluble cell constituents is much weaker. The drug has been reported to uncouple oxidative phosphorylation in mitochondrial preparations from rat brain (Abood, 1955). Strecker (1958) has reported that this uncoupling action of CPZ in the respiratory chain is due to a non-specific effect on the enzyme system as a whole rather than a specific inhibitory effect on any one enzyme. Possibly the drug acts as a flavin antagonist since it is known to compete with flavin adenine dinucleotide (Yagi et al., 1956). Tsao et al. (1982) have reported the decrease of oxygen uptake ratio by higher concentrations of CPZ and they have suggested that this effect may be due to damage to the mitochondrial membrane of rat hepatocytes. Lee et al. (1976) has found the inhibition of calcium accumulation by CPZ in rat brain microsomes. The work of Carvalho (1968) has revealed that there is a local anesthetic induced reduction in the Ca^{2+} bound to the rabbit skeletal muscle sarcoplasmic reticulum.

In addition to the effects described earlier, CPZ is also capable of modifying a number of biochemical mechanisms. Protein synthesis of brain slices is depressed by CPZ and a 20% inhibition in the rate of glycine-1-^{14}C incorporation into rat brain protein occurs by CPZ (Lindan et al., 1957). Calmodulin, an ubiquitous heat stable calcium binding acidic protein has been reported to be inhibited by CPZ (Roufgalis, 1981). But he has suggested that antagonism of calmodulin is unrelated to its pharmacological specificity. Weiss et al. (1980) have given a list of calmodulin dependent processes that are inhibited by antipsychotics like CPZ. The processes that are inhibited are the activation of phosphodiesterase, adenylate cyclase, Ca^{2+} and Mg^{2+} ATPase, myosin light chain kinase, phospholipase A_2, tryptophan hydroxylase. They have also postulated that release of norepinephrine from nerve terminals, secretion of chloride in intestine, contraction of smooth muscle, glucose stimulated insulin release are inhibited by CPZ.

The works of Volpi and his colleagues (1981) have revealed that CPZ inhibits the specific calmodulin dependent stimulation of erythrocyte Ca^{2+}-ATPase and cyclic nucleotide phosphodiesterase from brain and heart. Increasing calmodulin, but not Ca^{2+} overcomes the inhibitory action. They have proposed that antagonism of calmodulin provides a molecular

mechanism that may explain the inhibition of many calcium dependent cellular processes such as Ca^{2+} transport, exocytosis, excitation contraction coupling, non-muscle cell motility etc. CPZ binds to specific sites on the Ca^{2+}-calmodulin complex. As a result, the ternary complex is rendered ineffective in activating enzymes. Hence, according to them many of the pharmacological actions of CPZ, which are directed against calcium dependent cellular processes, may be due to antagonism of calmodulin's effects on enzymes because both the pharmacological effects and the inhibition of calmodulin in vitro occur at similar concentrations of the drug. However they have suggested that CPZ acts not by a single mechanism, but a number of mechanisms may be associated with the diverse pharmacological effects. The mechanism by which CPZ inhibits cell processes that are not dependent on Ca^{2+} may not involve calmodulin. On the other hand, many of the other actions of CPZ directed against Ca^{2+} dependent processes may be due to its effect on the actions of calmodulin. West (1982) has commented that calmodulin acts as a receptor not only for Ca^{2+}, but also for drugs like CPZ which bind to it with very high affinity.

From the reports in literature, it seems that the exact site and mechanism of action of CPZ are not clear

till today owing to a number of non-specific effects of the drug. All that can be said is that the drug is a lipid soluble one which possesses surface active properties by virtue of which it can bring about a number of perturbations in the membrane architecture and its functions. Since most drugs have to cross the biological membranes prior to reaching their targets, a clear understanding of the mechanism of drug membrane interaction seems essential. Hence in the present piece of work, special emphasis has been laid to investigate the effect of the drug on some membrane - related phenomena. Investigators face a great deal of trouble in determining the precise region and the specific mode of action of chlorpromazine in the central Nervous System presumably because of the structural complexity of nervous system which stems primarily from the histological and functional heterogeneity of different cell types comprising the system. Hence investigations have been conducted in relatively simple systems and as a result, erythrocytes, hepatocytes, mast cells, unicellular animals like tetrahymena and procaryotic cells have been used as model systems to find out the possible biochemical mechanism of action of the drug. In the present study, a large free living species of amoeba (Amoeba proteus), has been chosen

as the experimental animal which provides a unique opportunity of visualising a number of cell membrane-related phenomena such as cell motility, adhesion, endocytotic processes etc. Moreover the structure, life-cycle and physiology of the animal as a whole, and the intricacies of these membrane linked processes in particular, are known in great details (see, Jeon, 1973). This study has been undertaken by us with a view to knowing more about the action of the drug, especially at the level of the cell surface. Besides, the action of this drug on some physiological processes has also been investigated. Hopefully, this line of work may be able to provide us an insight into some important yet unexplored aspects of interaction between this drug and the cells which are non-neuronal in origin.

MATERIAL AND METHODS

MATERIAL :

Organism

The experimental animal for the present work is Amoeba proteus. Amoeba proteus was originally obtained from King's College, London and is being cultured in our laboratory for many years. It is a large, free-living, uninucleate protozoa. Tetrahymena pyriformis, a free living ciliate was also cultured separately as a food for the amoebae.

METHODS :

(a) Cell Culture

Amoeba proteus was cultured at 22 ± 1°C following the methods described by Chatterjee and Rao (1974). The stock culture medium for amoebae contained : $CaHPO_4$, 50 mg; KCl, 60 mg and $MgSO_4$, 40 mg dissolved in 1000 ml of double distilled water. The working solution was prepared from this by mixing stock solution and distilled water in the ratio of 1:9. The pH of the final solution was adjusted at 6.8.

The amoeba cultures were fed on Tetrahymena pyriformis without the addition of any other nutrient. Tetrahymenae were cultured in conical flasks (250 ml) containing 100 ml of autoclaved 1.5% proteose peptone supplemented with a trace

amount of liver extract. Inoculation of the ciliates was carried out under sterile conditions. Everytime, prior to feeding, the tetrahymenae were harvested by alternate centrifugation and resuspension with large volume of amoeba medium. Care was taken to see that tetrahymenae were completely free from proteose peptone broth. The centrifugation was done in a clinical centrifuge at approximately 700 rpm for 2 minutes.

The amoebae were fed once a day for 4-5 hours, after which the amoeba medium along with the rest of the food organisms, was decanted. The culture dish was filled up again with fresh medium and a further wash was given with the medium ½ hour later.

(b) Chlorpromazine Hydrochloride (CPZ) Treatment :

CPZ (mol. wt. 355.3) a neuropharmacological drug, was dissolved in amoeba medium so as to prepare 0.028 mM (2.8 x 10^{-2}M) solution and the pH was adjusted between 6.6 to 6.7. Every time freshly prepared solution was used so as to avoid photoinduced decomposition of the drug. All experiments were carried out at 22 ± 1 ^{o}C on amoebae starved for about 36 hours. Different concentrations of CPZ were used in the initial attempts to find out a suitable dose. The amoebae were taken either in cavity blocks or in syracuse

watch glasses before they were exposed to CPZ. The amoeba medium was sucked off by means of a braking pipette and the amoebae were exposed to CPZ for the desired period of time. Immediately after the treatment, the amoebae were washed with amoeba medium for atleast three times before any further experimental manipulations. This experiment was repeated four times and each time 25 cells were used for the experiment unless stated otherwise. Adequate number of control cells were also kept for each experiment.

(c) Duration of Exposure to CPZ

The amoebae were taken in syracuse watch glasses and were treated with CPZ for 10 minutes, 20 minutes, 30 minutes, 40 minutes and 50 minutes and their survival was noted upto 3rd day of treatment with CPZ of 0.028 mM concentration. This experiment was conducted three times and 25 cells were taken for each observation.

(d) Effect of CPZ on Morphology, Attachment and Motility:

For these observations, cells were taken in syracuse watch glass in groups of 10 and also singly. They were observed for the above processes during the period of treatment and also immediately after treatment upto 5 hours at regular intervals. Cytological preparations were also done at different hours after CPZ administration. This

experiment was repeated for atleast three times.

(e) Effect of CPZ on Phagocytosis :

36 hours starved amoebae without any food vacuoles were taken and they were washed thoroughly with amoeba medium. Freshly harvested tetrahymenae were stained with Neutral red solution for 20 minutes to facilitate better quantification of phagosomes. For this 3 ml of tetrahymena suspension was taken in centrifuge tubes. To this was added 2 to 3 drops of 0.1% neutral red and it was left for 20 minutes. After 20 minutes, the excess stain was removed by repeated washing of the cells in amoeba medium. Sets of amoebae, control and CPZ treated, were taken in cavity blocks. About 30 cells were put in each block and were fed with a drop of stained tetrahymenae. Observations were carried out at an interval of ½ hour upto 2½ hours after feeding. Counting of phagosomes, seen as tetrahymenae in the food vacuoles, was done under a microscope at a magnification of X160. In this experiment, phagosomes from forty amoebae were counted in two separate experiments.

(f) Effect of CPZ on Pinocytosis :

Pinocytosis was quantitated by channel counting technique as described by Chatterjee and Ray (1975). Both

CPZ treated and control amoebae were taken in specially constructed chambers on slides separately. 0.125 M sodium chloride in phosphate buffer pH 6.4, was used as inducer of pinocytosis. The channels were counted at an interval of 5 minutes upto 35 minutes. To study the effect on the second pinocytotic cycle, the cells were recovered with sufficient amoeba medium after the induction of first pinocytotic cycle. They were thoroughly washed with amoeba medium and left for 5 hours. After 5 hours, again the cells were challenged with the inducer solution for pinocytosis and channels were counted in the same manner as in the first cycle. All the experiments on pinocytosis were repeated for atleast three times.

(6) Effect of CPZ on the Uptake of ^{3}H-Leucine :

For this study ^{3}H-Leucine (Specific activity, 3.3 Ci/mM) was used. About 100 cells, both CPZ treated and control, were taken separately in syracuse watch glasses. They were incubated for 20 minutes at room temperature in the labeled leucine diluted with sterile amoeba medium at a concentration of 100 µCi/ml. Immediately after incubation, the cells were thoroughly washed several times in excess of amoeba medium. Then amoebae, in batches of 15, were transferred to the lysing medium (SDS/EDTA solution, 0.3 ml) contained in the scintillation vials. 10 ml of Brey's

scintillation fluid was added to each vial and then all the vials were counted for 2 minutes each in a Beckman Liquid Scintillation Spectrometer. Bray's Scintillation fluid was prepared by taking 60 gms of Naphthalene (chromatographically purified for scintillation counting), 4 gms of PPO, 0.2 gm of POPOP, 100 ml of methanol, 20 ml of ethylene glycol and adding to it enough of 1,4-Dioxane so as to make the final volume 1000 ml. This mixture was taken in a bottle covered with aluminium foil and was stirred in cold room for ever night with a teflon coated magnetic bar. ^{3}H-leucine labelling was done in the following manner:

a. the isotope was administered simultaneously with CPZ treatment, and
b. immediately after and 5 hours after the CPZ treatment.

For each experiment 75 cells were taken in 5 vials, each vial containing fifteen cells and each experiment was repeated three times. Hence radioactivity of 225 cells were counted in three separate experiments. However, average counts of radioactivity of 15 cells has been represented in the data.

(h) <u>Effect of CPZ treatment on Alkaline Phosphatase Activity</u> :

Modified Gomori's method for alkaline phosphatase

(EC 3.1.3.1) was used (see. Pearse. 1956) to study the effect of CPZ. The cells were squashed on the subbed slides and the excess of amoeba medium was sucked off. Immediately after flattening, the cells were fixed in chilled acetone for 2 hours at 4°C. After fixation they were passed through 40% acetone and distilled water. Then they were incubated in the incubation medium at 37°C for 45 minutes. The composition of the incubation medium was as follows:-

2%	Sodium β-glycerophosphate	- 25 ml
2%	Sodium barbitone	- 25 ml
2%	Calcium nitrate	- 05 ml
0.8%	Magnesium Chloride	- 05 ml
	Absolute acetone	- 40 ml

After incubation, the cells were treated with 40% acetone, then passed through 2% cobaltous nitrate in 40% acetone and treated with dilute yellow ammonium sulphide in 40% acetone (0.1 ml yellow ammonium sulphide was dissolved in 50 ml of 40% acetone). Then the slides were dehydrated and made permanent. Clean black deposits of cobalt sulphide were taken as the sites of enzyme activity.

(1) <u>Experiment on the Effect of CPZ on Generation Time of Amoebas Proteus</u>

The generation time of <u>Amoeba proteus</u> was determined

by observing well fed amoebae individually after selecting them at known points of cell cycle as described by Rao and Chatterjee (1974).

About 10 dividers (Mulberry shaped mitotic spheres) from well fed and flourishing amoebae culture were picked up with the aid of a braking pipette taking care so as not to damage the cells in any way. The dividers can be selected easily as they remain loosely attached to the substratum. The dividing cells were mechanically selected with the help of a braking pipette and these cells were kept singly in syracuse watch glasses till the division was completed. One daughter cell was transferred to another syracuse watch glass containing amoebae medium and it served as a control. It was fed with approximately 20-25 tetrahymenae and the time of its next division was also noted. The other daughter cell was treated with CPZ for 20 minutes and was then thoroughly washed with amoeba medium. Finally it was kept in a syracuse watch glass containing amoeba medium and approximately 20-25 tetrahymenae. The next division time of the treated cells was also recorded. Such procedures were carried on the dividers to study the effect on generation time.

(j) Cytological Preparations :

Both control and CPZ treated cells were flattened to give clarity in microscopic observations. The slides

were subbed in the subbing medium composed of chromic potassium sulfate and gelatin (Bacteriological). The cells were placed on the subbed slides with small amount of amoeba medium. Then excess of medium was removed with the aid of a fine braking pepette. The cells were then immediately covered with a cover glass (24 x 40 mm in size) containing a drop of 45% acetic acid for flattening the cells. The slide with the cover glass was immediately dipped into liquid nitrogen for about 5 to 10 seconds for freezing and withdrawn quickly. The coverglass was removed with the help of a razor blade and the slide was immediately fixed in acetic acid:ethanol (1:3) for 10 minutes. The slide was then passed through two changes of absolute C_2H_5OH and two changes of 90% C_2H_5OH for 10 minutes each and was air dried. Slides, thus prepared, were later stained with phosphate buffered giemsa solution and mounted on DPX for further microscopic analysis.

To study the surface morphology without flattening the cells, the amoebae were fixed with spreacyte fixative. Then the slides were stained by the usual procedure as described earlier.

Chemicals used :

CPS: anhydrous (mol. wt. 355.3), PPO, anhydrous (mol. wt. 221.3); POPOP, anhydrous (mol.wt. 364.4), hog liver

extract powder were purchased from M/s Sigma Chemicals Company, St. Louis, U.S.A.

Proteose peptone was obtained from Difco Chemical Company, U.S.A. Giemsa stain was purchased from British Drug House Limited, England and Neutral red was procured from George T. Gurr Limited, London. Spray-cyte was purchased from Clay Adams, Dickinson and Company, U.S.A. L-leucine T(G) was obtained from isotope division, Bhabha Atomic Research Centre, Bombay, India.

$CaHPO_4$, KH_2PO_4, C_2H_5OH, CH_3COOH, $CrK(SO_4)_2$, 12 H_2O, NaOH, HCl, EDTA, $Ca(NO_3)_2$, $4H_2O$, $MgCl_2$, $6H_2O$, PPO, POPOP CH_3OH, H_2SO_4, SDS, Diethylbarbituric acid (Bacteriological), acetone, 1,4-Dioxane (extrapure) DPX-mount, Naphthalene, ethylene glycol etc. were of "Guaranteed reagent AnalaR Grade from M/s BDH, India or M/s Sarabhai M. Chemicals, India.

Abbreviations used :

CPZ	-	Chlorpromazine hydrochloride
$CaHPO_4$	-	Calcium hydrogen orthophosphate
KH_2PO_4	-	Potassium dihydrogen phosphate
C_2H_5OH	-	Ethyl alcohol.
CH_3COOH	-	Acetic acid
NaOH	-	Sodium hydroxide
HCl	-	Hydrochloric acid

EDTA - Ethylene diamine tetra-acetic acid

$Ca(NO_3)_2.4H_2O$ - Calcium nitrate

$MgCl_2$ - Magnesium chloride

PPO - 2,5-Diphenyloxazole

POPOP - Phenyl-oxazolyl phenyl-oxazolyl phenyl

CH_3OH - Methyl alcohol

H_2SO_4 - Sulphuric acid

SDS - Sodium dodecyl sulfate

RESULTS AND OBSERVATIONS

Chlorpromazine hydrochloride (CPZ) was used in the present work to study the effect of this drug on Amoeba proteus, especially in relation to its membrane related activities.

Effects of CPZ on Amoebae

(a) Various Concentrations of CPZ :

Amoebae were exposed to various molar concentrations (0.28 mM; 0.14 mM; 0.056 mM and 0.028 mM) of CPZ in order to find out a suitable dose. After treatment, the survival of the amoebae was observed upto the third day of treatment. 0.028 mM CPZ was found to be the suitable dose for our experiments. Doses higher than 0.028 mM were found to cause lysis and cell death even if applied for smaller duration than 20 minutes. 0.14 mM CPZ and above were found to cause immediate cell lysis.

(b) Varying the Duration of CPZ Treatment :

For the determination of a suitable time period of treatment of 0.028 mM CPZ, the amoebae were exposed to CPZ for various durations (10 minutes, 20 minutes, 30 minutes, 40 minutes and 50 minutes). 20 minutes of treatment was found to be suitable for our experimental purpose since the percentage of cell survival at this duration of treatment was appreciably high (Fig. 1).

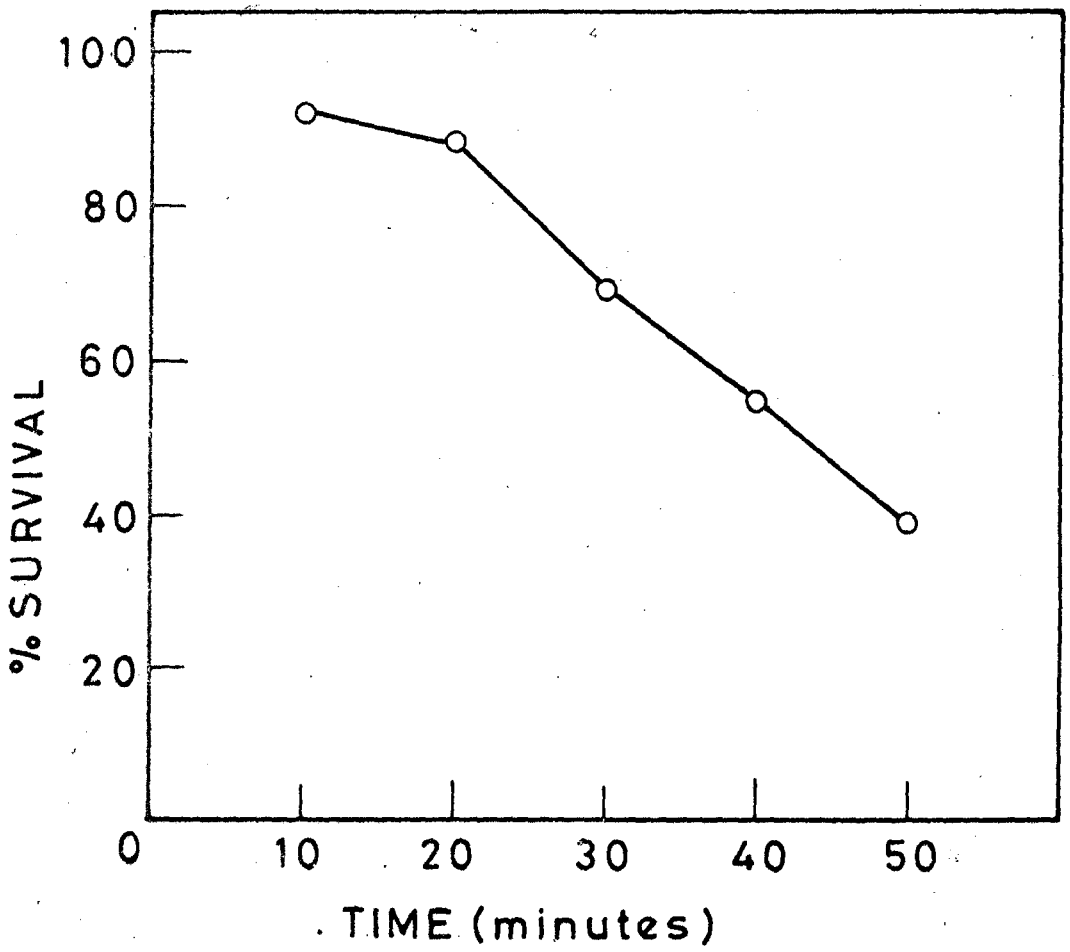


Fig. 1: Effect of varying duration of CPZ (0.028 mM) treatment on survival of *Amoeba proteus*.

(c) Effect of CPZ on Shape, Attachment and Motility of Amoebae

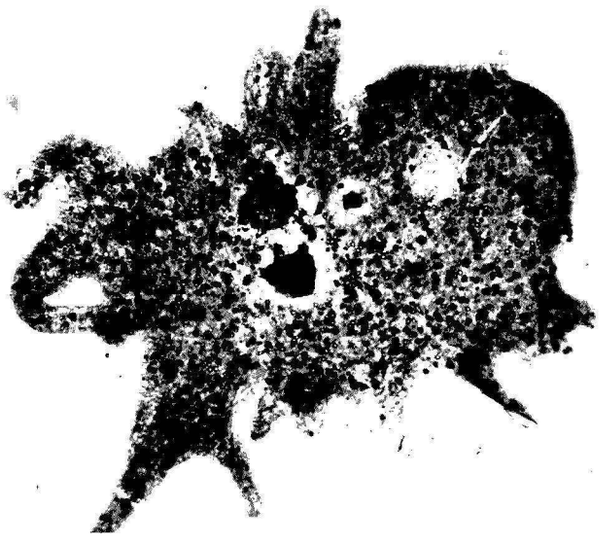
Within 5 to 10 minutes of treatment of CPZ, the amoebae assumed a rounded shape, indicating the loss of typical amoeboid shape. The amoebae were observed to remain in this rounded up condition as long as they were kept in the CPZ solution. Pseudopodia were withdrawn from all directions in most of the cells and new pseudopodia formation were stopped in those cells. However, a few cells exhibited some feeble pseudopodia formation. Hence motility was stopped in majority of the cells. Most of the cells were observed to be loosely attached to the substratum. The cells were washed with amoeba medium after CPZ treatment and they gradually regained their normal shape, became firmly attached to the substratum and showed normal motile behaviour around one hour after the drug exposure.

(d) Effect of CPZ on Morphology of Amoebae :

Granular inclusions in the cytoplasm of the treated amoebae were found to be concentrating in the rounded up cells. The treated cells looked darker than their corresponding control cells. A clear hyaline zone was noted at the periphery of the cells (Fig. 2 a & b). However, the cells regained their normal morphology within 4 to 5 hours post treatment (Fig. 3 a & b), (Fig. 4a & b).

Fig. 2(a): Photomicrograph of a Amoeba proteus showing the general morphology. X 480

(b) Photomicrograph of a CPZ treated Amoeba proteus immediately after treatment. Note the presence of a hyaline zone at the periphery and concentration of dense cytoplasmic material at the central region. X 480



2(a)

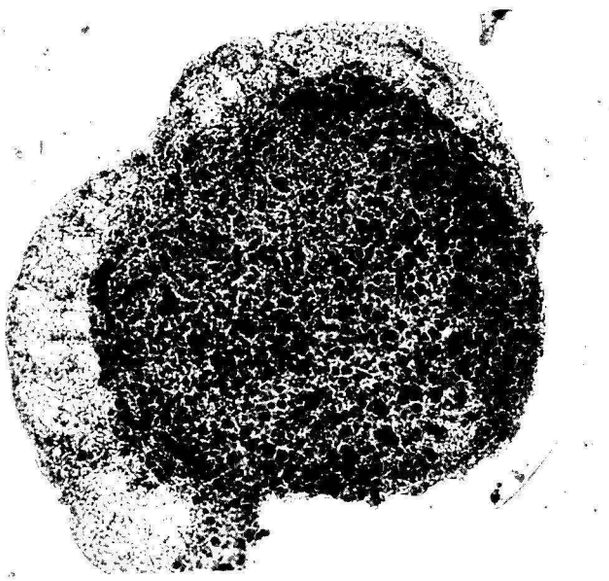
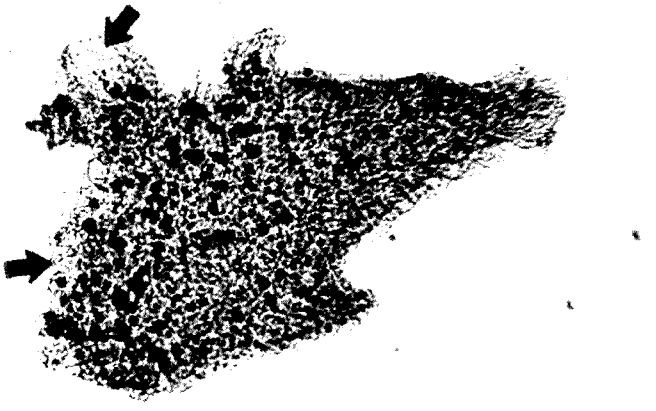


Fig. 3(a): Photomicrograph of a Amoeba proteus showing the general morphology. X 480

(b): Photomicrograph of a CPZ treated Amoeba proteus one hour after treatment. Note the presence of hyaline zone at the periphery of of the cell (⟶). X 400

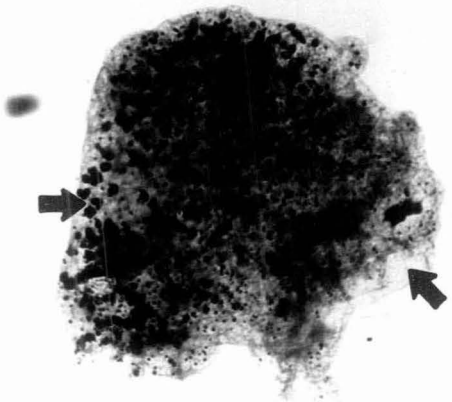
3(c)



3(b)

Fig. 4(a): Photomicrograph of a CPZ treated Amoeba proteus three ahours after treatment. Note the presence of hyaline zone in some peripheral region. (←→) X 400

(b): Photomicrograph of a CPZ treated Amoeba proteus four hours after treatment showing the restoration of almost normal morphology. X 400



4(a)

(e) Effect of CP2 on Endocytotic Activity of Amoebae

α - Effect on Phagocytotic Activity :

The treated cells were checked for their ability to capture food organisms, viz., Tetrahymena pyriformis. It was observed that the capacity to phagocytose the tetrahymenae has been inhibited to some extent and that this inhibition continued upto 2 hours post treatment after which it became almost normal as compared to the control cells. Thus there was only slight inhibition in the phagocytotic activity (Fig. 5) in the CP2 treated cells.

β - Effect of CP2 on Pinocytotic Activity of Amoebae.

Pinocytotic activity of the treated and control cells was studied by counting the channels formed after induction of pinocytosis. The following types of experiments were done :

(i) Pinocytosis was induced while the cells were in the drug.

In this experiment, the CP2 treatment was extended upto 35 minutes for the counting of pinocytotic channel. It was found that during the first 10 minutes of treatment, pinocytosis of treated cells was similar to that of the control group. But peak pinocytotic activity was greatly

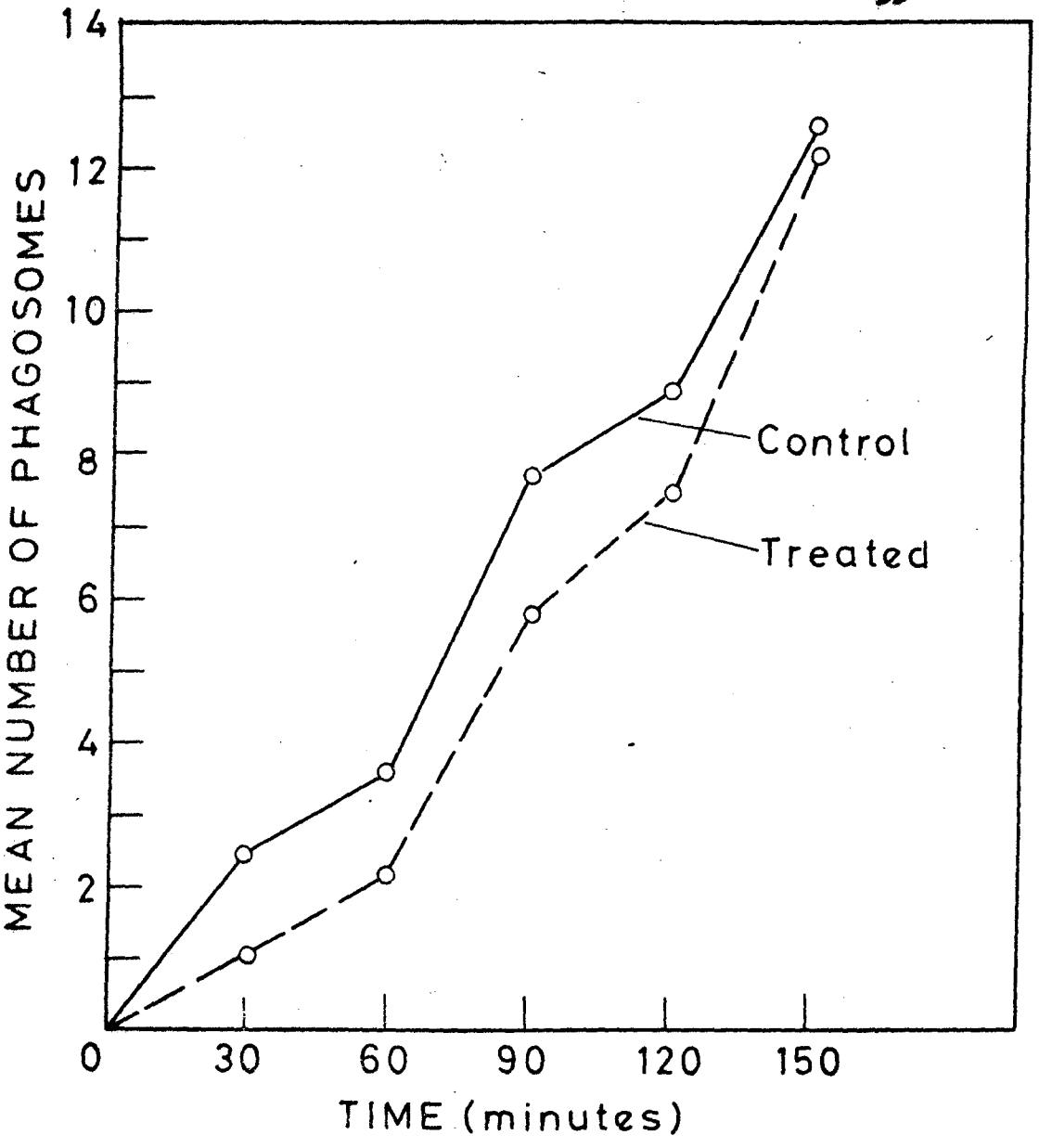


Fig. 5: Effect of CPZ on phagocytosis in Amoeba proteus. Each point represents an average count from forty amoebae from two separate experiments.

reduced in the treated cells when compared to the normal cells (Fig. 6).

(ii) Pinocytosis was induced immediately after treatment of CPZ.

It was observed that CPZ treatment results in pronounced depression of pinocytotic channel formation. There was a significant reduction in the pinocytotic activity during the peak period at 15 minutes and reduced activity also continued in the later period (Fig. 7).

(iii) Second pinocytotic cycle was induced five hours after first pinocytotic cycle.

First pinocytotic cycle of treated cells was found to be significantly inhibited as described earlier. In the second pinocytotic icycle of the treated cells, the peak number of channel formation was found at 20 minutes whereas the corresponding control cells showed the usual peak channel forming activity at 15 minutes. Thus there was a shift so far as the peak period of pinocytotic activity is concerned. The peak pinocytotic activity in the second cycle of the control cells was slightly decreased. The peak activity in the second cycle of the treated cells was also depressed as compared to the control group of that cycle (Fig. 8).

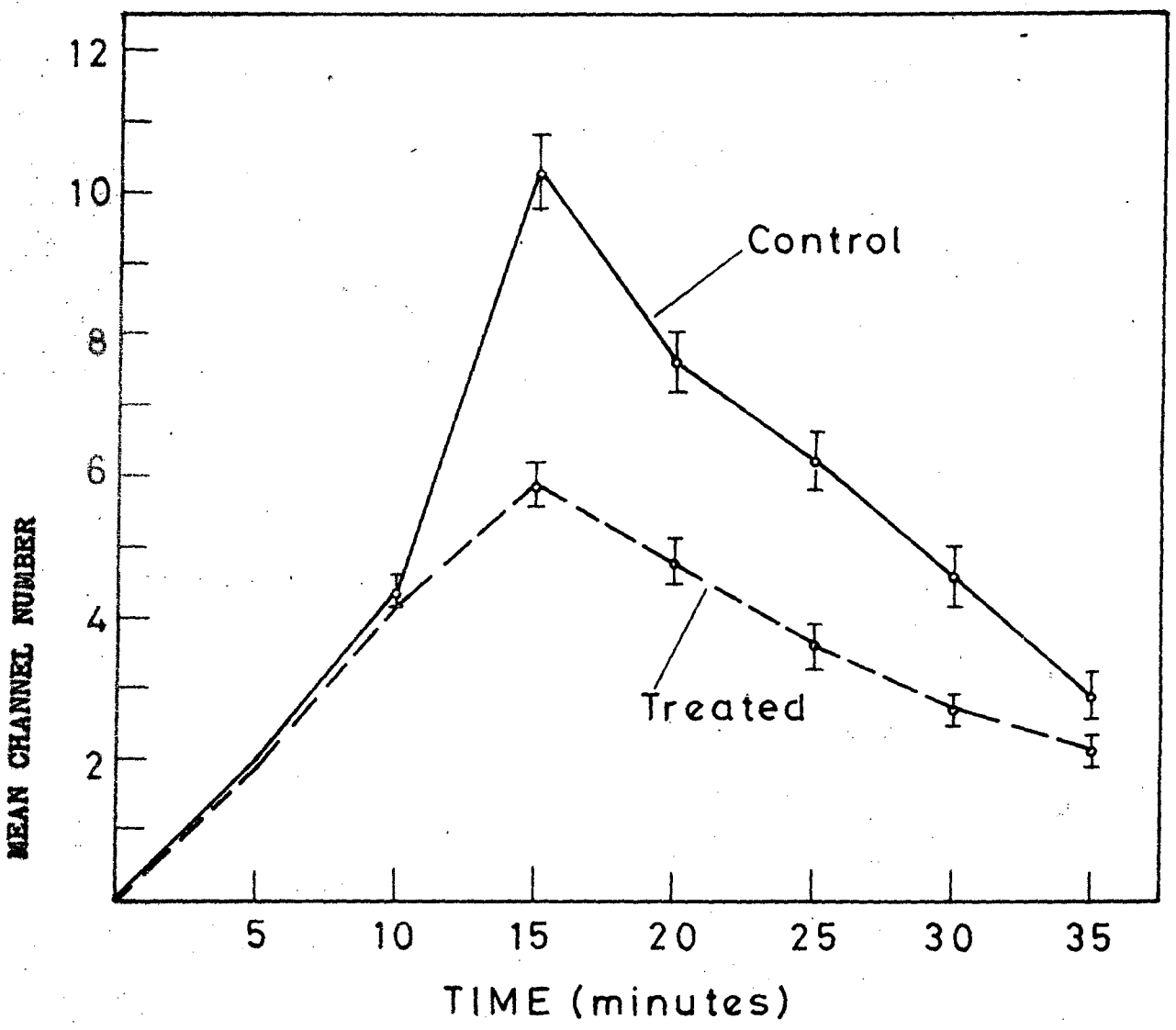


Fig. 6 : Summary of three experiments on the effect of CPZ on the pinocytotic activity of Amoeba proteus. Each point represents an average count from thirty amoebae. Vertical bars indicate mean ± S.E. The Experiment was conducted while the amoebae were in CPZ solution.

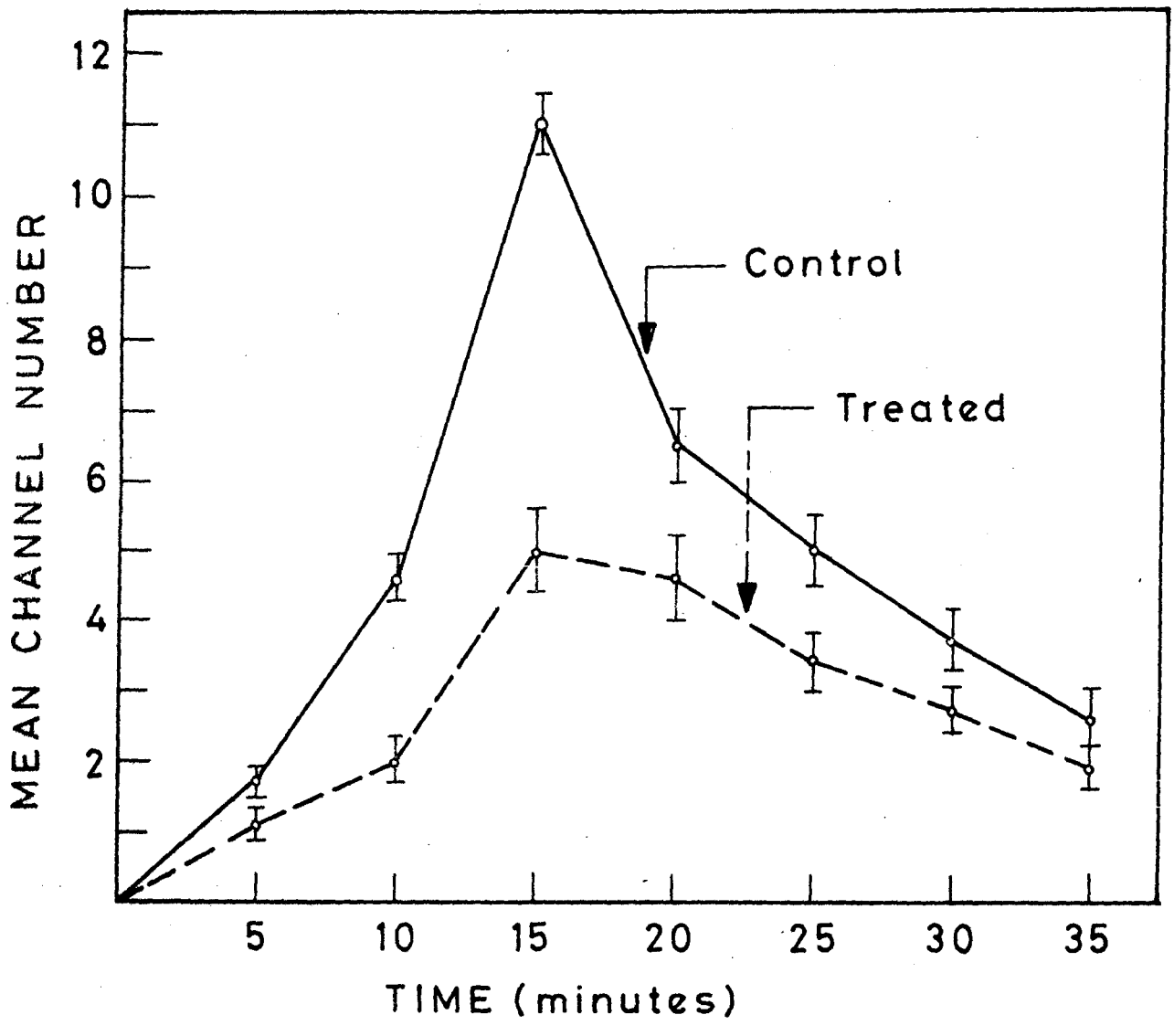


Fig. 7: Summary of six experiments on the effect of CPZ on the pinocytotic activity of Amoeba proteus. Each point represents an average count from sixty amoebae. Vertical bars indicate mean ± S.E.

Fig. 8. Summary of four experiments on the effect of CPZ on:

(a) First pinocytotic cycle

(b) Second pinocytotic cycle which was done five hours after the first cycle.

Each point represents an average count from forty amoebae. Vertical bars indicate mean ± S.E. Note the change in the peak in (b).

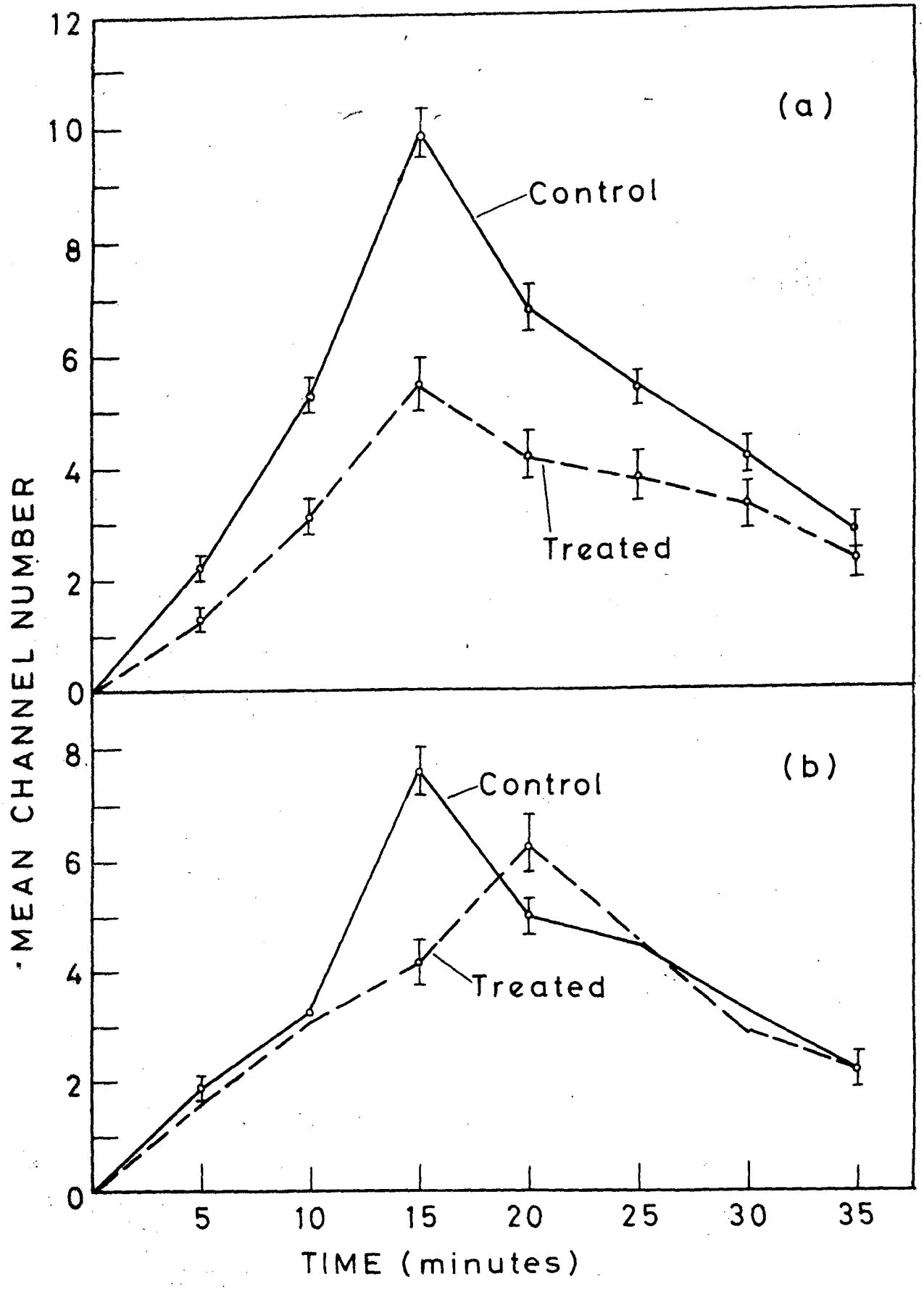

(a)
Control
Treated
(b)
Control
Treated
MEAN CHANNEL NUMBER
TIME (minutes)
0
2
4
6
8
10
12
5
10
15
20
25
30
35

Fig. 8.

Thus summarising the results from all the experiments, it can be concluded that CPZ treatment has brought about a depression of almost 50% in the peak pinocytotic activity of the amoebae. Also the ability of the treated cells to form maximum number of channels in the second pinocytotic cycle has been found to be less and delayed when compared with the control cells.

(f) Effect of CPZ Treatment on ^{3}H-Leucine Uptake:

To analyse further the action of CPZ on the amoebae cell membrane, ^{3}H-leucine uptake studies were undertaken. The ability of the treated amoeba's capacity for the uptake of tritiated leucine was tested while the cells were being incubated with the CPZ solution. It was found that the amoebae showed somewhat reduced radioactivity as compared to their normal counterparts. However, a dramatic inhibition in ^{3}H-leucine uptake was noted in the cells immediately after the CPZ treatment. There is more than 50% reduction in the ^{3}H-leucine uptake in the CPZ treated amoebae. This suppression of the ^{3}H-leucine uptake continued even five hours after the cell's exposure to CPZ (Fig. 9).

(g) Effect of CPZ Treatment on the Alkaline phosphatase Activity :

Changes in the alkaline phosphatase activity were

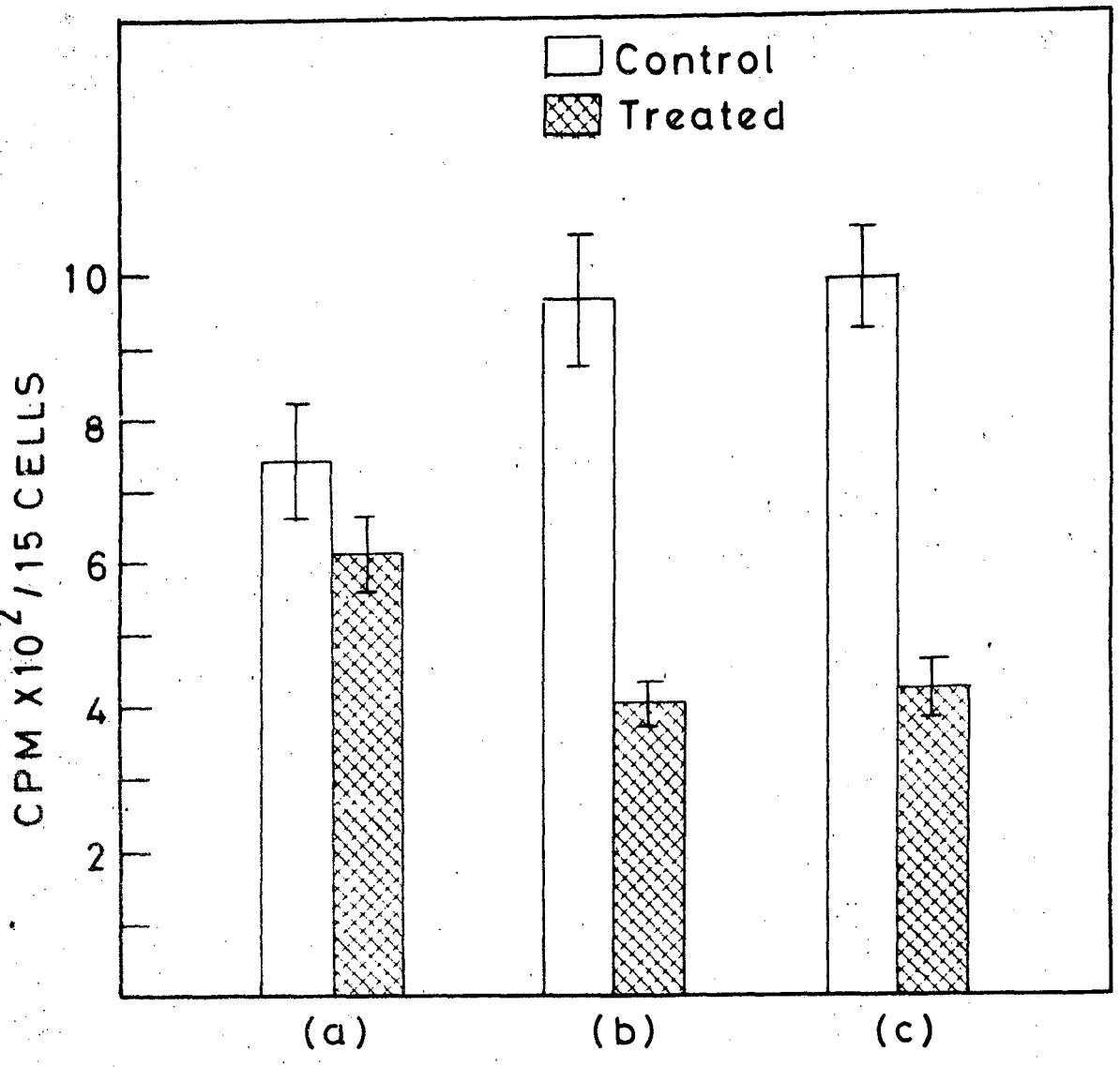


Fig. 9: ^{3}H-Leucine uptake by the CPZ treated *Amoeba proteus*, at different periods of time.

(a) While the cells are still in CPZ solution.

(b) Immediately after CPZ treatment.

(c) Five hours after CPZ treatment .

Each column represents the average radioactivty of fifteen cells. Vertical bars indicate standard error.

determined on the basis of the differences in the intensity of cytochemical staining of the CPZ exposed cells and their normal counterparts. It was found out that alkaline phosphatase activity in the treated amoebae was appreciably reduced following CPZ treatment. But the activity was found to increase gradually so as to comeback to the normal level within five hours after treatment of the drug (Fig. 10 a-d, 11a & b, 12 a-d).

(h) Effect of CPZ on the generation time of Amoeba Proteus.

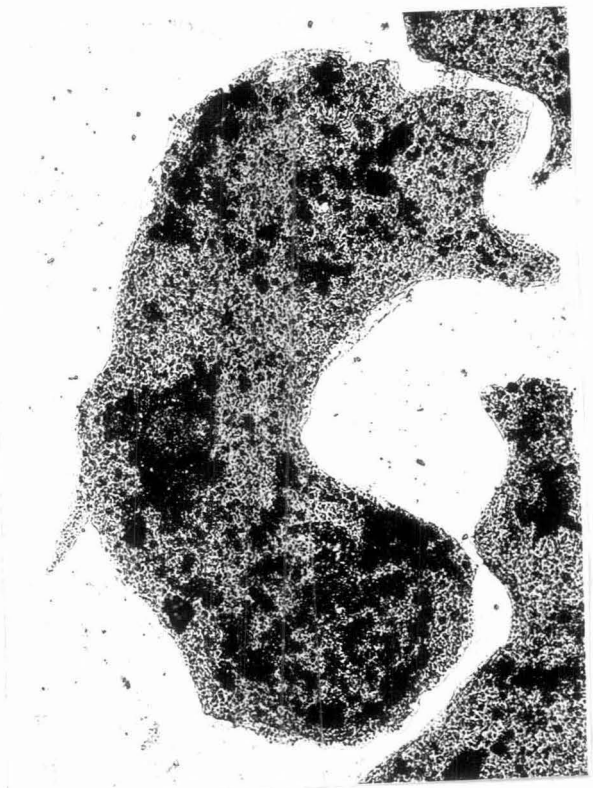
CPZ treatment was found to lengthen the generation time of amoebae. The generation time of the treated cells was found to be longer when compared to the control cells. Some preliminary experiments indicate that the average generation time of the control cells was found to be 49 ± 1 hours whereas that of the CPZ treated cells was 49 ± 2 hours. Thus, the generation time of the treated cells was delayed by about 6 hours as compared to the control cells.

Fig. 10(a) : Photomicrograph of a control Amoeba proteus showing alkaline phosphatase activity. Note the intensity of the activity of the enzyme. X 480

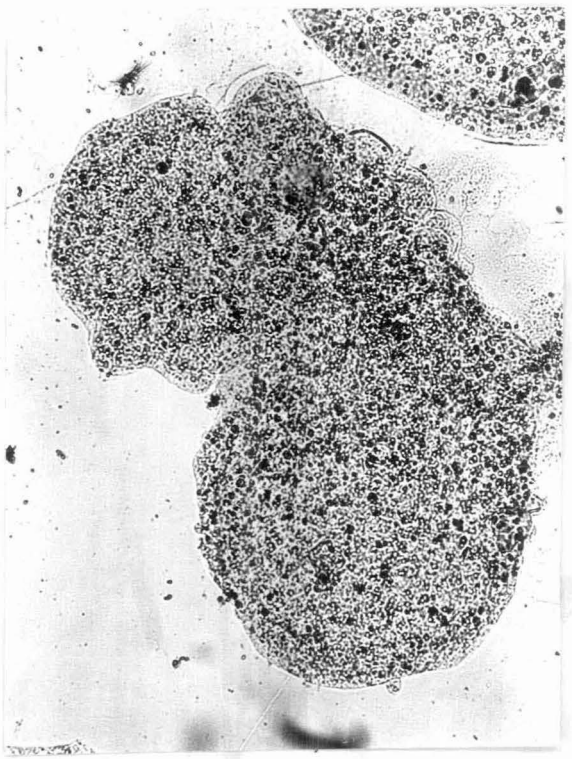
(b): Photomicrograph of a CPZ treated Amoeba proteus showing drastic reduction in alkaline phosphatase activity immediately after treatment. X 480

(c): Photomicrograph of a group of control Amoeba proteus showing alkaline phosphatase activity. X 189

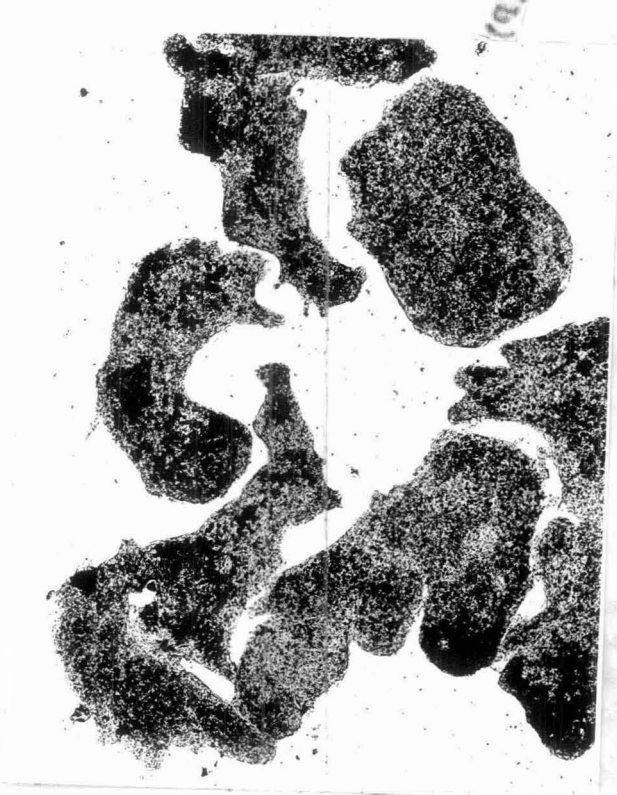
(d): Photomicrograph of a group of CPZ treated Amoeba proteus showing greatly inhibited alkaline phosphatase activity immediately after treatment. X 189



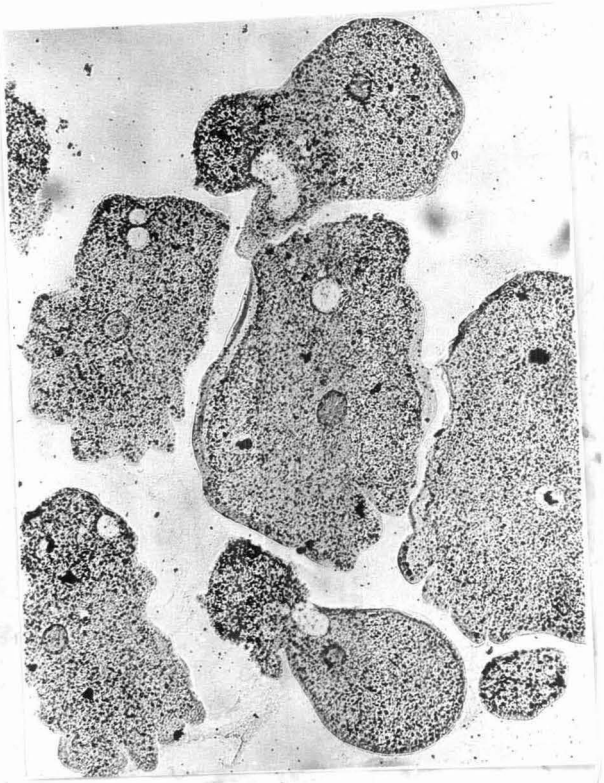
10(a)



10(b)



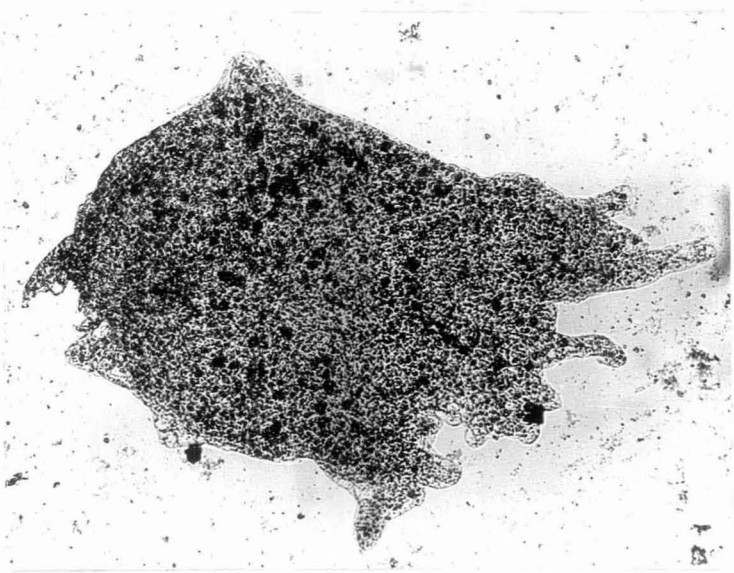
10(c)



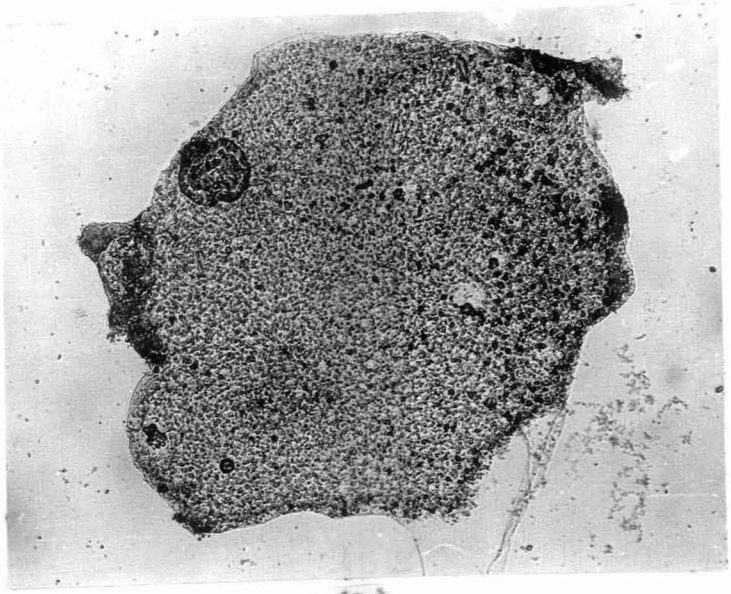
10(d)

Fig. 11(a) : Photomicrograph of a control *Amoeba proteus* showing alkaline phosphatase activity. X 480

(b) : Photomicrograph of a CPZ treated *Amoeba proteus* showing very feeble alkaline phosphatase activity one hour after treatment. X 480



11(a)



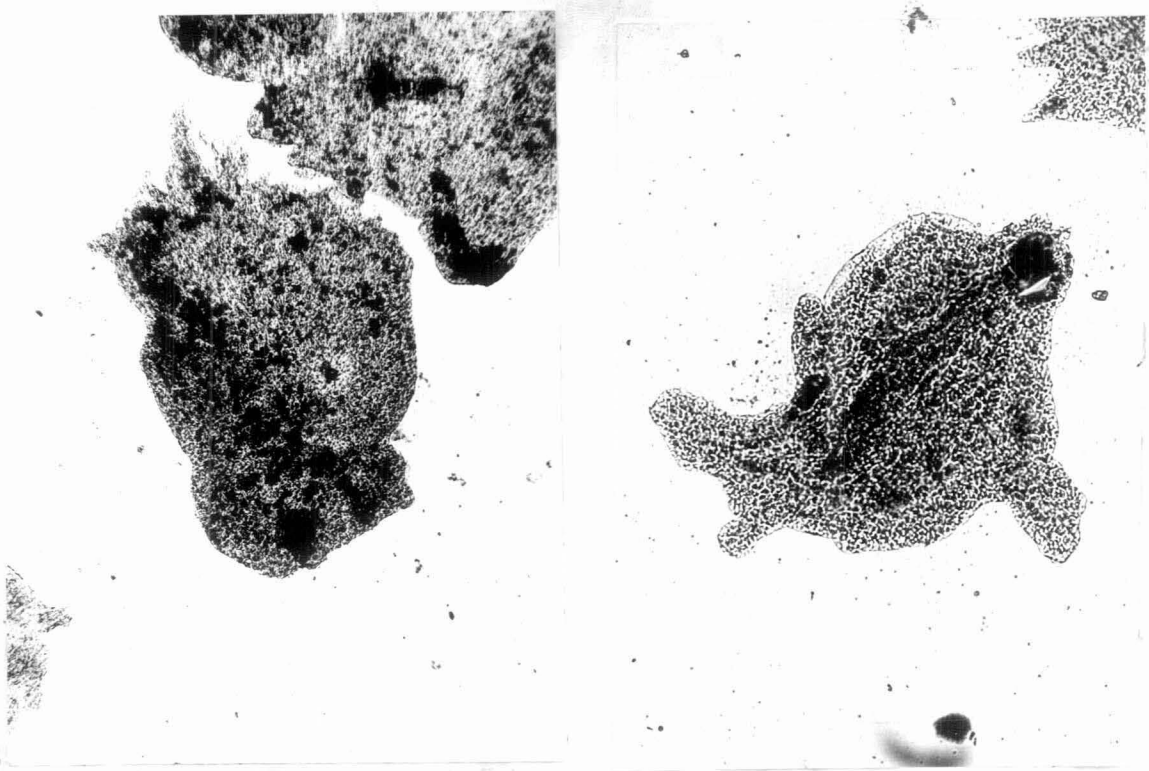
11(b)

Fig. 12(a): Photomicrograph of control *Amoeba proteus* showing alkaline phosphatase activity. X 300

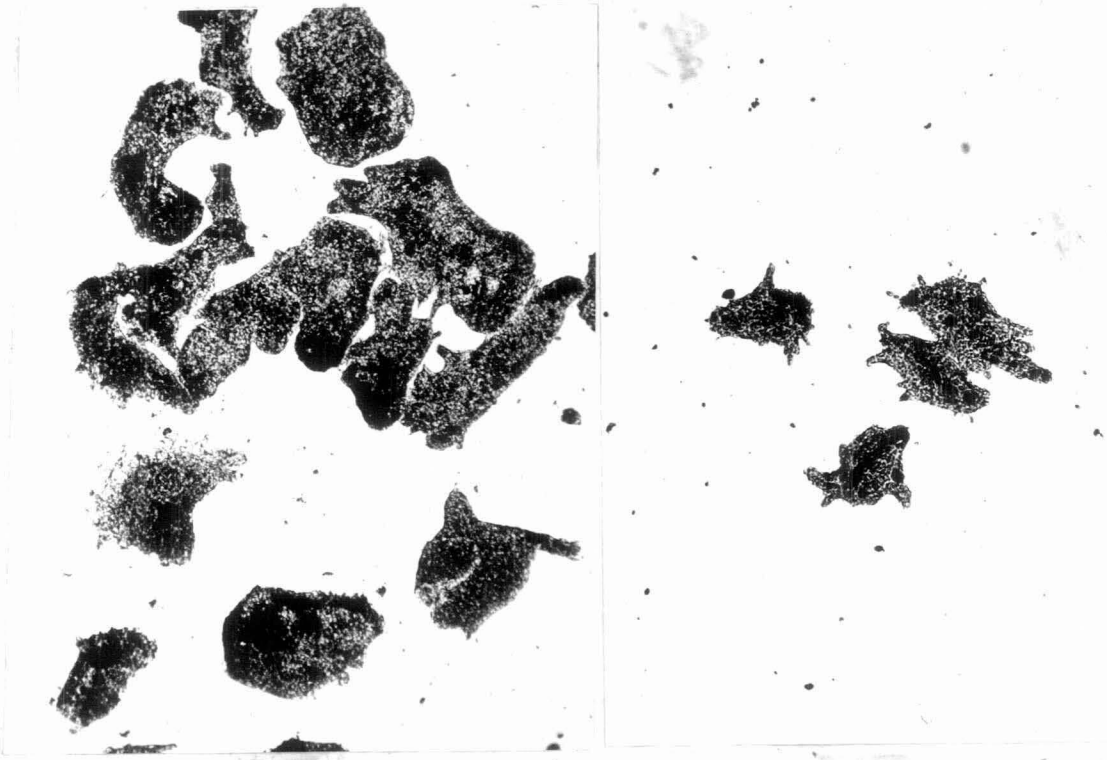
(b): Photomicrograph of a CPZ treated *Amoeba proteus* showing almost normal alkaline phosphatase activity five hours after treatment. X 300

(c): Photomicrograph of a group of control *Amoeba proteus* showing alkaline phosphatase activity. X 112

(d): Photomicrograph of a group of CPZ treated *Amoeba proteus* showing almost normal alkaline phosphatase activity five hours after treatment. X 112



12(a)



12(c)

DISCUSSION

The present study is aimed at investigating the effects of CPZ on amoeba, both at the structural as well as at the functional level. It is generally assumed that the primary actions of anesthetics and tranquilizers like CPZ occur in the plasmamembrane of cells rather than on intracellular processes (Seeman, 1972). There are a number of postulations regarding the mechanism of action of such surface active compounds. A survey of the literature suggests that the major actions of CPZ and related compounds are effected by one of the following mechanisms (see, Seeman, 1972).

a) Fluidising and disordering the membrane components.
b) Either by stimulating or by inhibiting membrane associated enzymes and proteins.
c) Displacing the membrane bound Ca^{2+} and inhibiting the transmembrane fluxes of Ca^{2+}.
d) Modifying the translocation of solutes and ions across the membranes.

However, there are also reports indicating the interference of CPZ on some intracellular processes like uncoupling of oxidative phosphorylation in the mitochondrial respiratory chain (Abood, 1955). Thus the exact site and also the mode of action of this drug at the cellular and molecular level are yet to be determined. Since we have

also shown inhibition in a number of membrane linked phenomena such as motility, phagocytosis, pinocytosis ^{3}H-leucine uptake etc., it is very likely that CPZ also effects the plasma membrane of amoebae to some considerable extent. That the most of the inhibited activities become normal with lapse of time has suggested the reversible nature of action of this drug and this finding has also justified its inclusion in the anesthetic group of chemicals.

Our experiment on the dose response of amoebae to various concentrations of CPZ has shown that they can tolerate only a narrow dose range. A dose higher than this range causes immediate cytolysis where as lower range does not. This finding is in total agreement with the finding of Seeman (1972) who has shown that all surface active compounds including CPZ can cause general biphasic effect of membrane protection at low concentrations and membrane lysis at higher concentrations.

Changes in the cell shape, inhibition of pseudopodia formation and consequent stoppage of motility, formation of a hyaline zone at the periphery have made it evident that the cell membrane of amoeba undergoes some drastic changes following treatment with CPZ. The inhibition of amoeboid movement by CPZ may be due to either one or both of the following reasons :

(i) The known capacity of the drug to reduce the influx of calcium thereby decreasing the cellular availability of the ion (see, Seeman, 1972).

(ii) Ability of thedrug to uncouple oxidative phosphorylation with a consequent reduction in the ATP molecules (Abood, 1955; Siesjo, 1982) in the cell.

The above possibilities seem to be likely because of the following findings :

(a) The sol-gel transformation during amoeboid movement uses ATP as the source of energy and ATP initiates the contraction of the gel network when brought into proper spatial relationship with the cytoplasm. (Zimmerman, 1959).

(b) Cytoplasmic extracts of Amoeba proteus move in a manner similar to the intact cell when warmed with ATP and during this movement there is an increase in apparent viscosity associated with the formation of thin actin filaments from precursors in the groundplasm (Pollard and Ito, 1970).

Carnivorous amoeba F actin is extremely unstable and might play an important role in the contractility of amoeba cytoplasm by polymerising in the presence of suprathreshold Ca^{2+} concentration and depolymerising in the presence of subthreshold Ca^{2+} concentration (Condeelis et al., 1976).

Hence it is very likely that in regions of the amoeba where the Ca^{2+} concentration is reduced the actin and myosin filaments might depolymerize, resulting in the inhibition of contractility (see, Condeelis, 1977).

The formation of a clear hyaline zone at the periphery of the cell has indicated that CPZ brings about a differentiation in the cytoplasm of amoeba as a result of which a clear peripheral hyaline zone and a dense central zone are formed. The differentiation into two zones might have occurred due to the action of CPZ on some structures in the cytoplasm in a similar manner as revealed by the work of Bruce and Christiansen (1965).

Bruce and Christiansen (1965) have found the formation of two zones, a clear peripheral zone containing only small vesicles and a dense central zone containing all the large structures viz, fat bodies, nuclei, in the giant amoeba Chaos chaos following treatment with anesthetics like halothane and ether. They have hypothesized that differentiation into two zones involved the tight cross-linking of cytoplasmic colloids in the periphery of the cell, creating a meshwork, the interstices of which were sufficiently small to exclude the larger

particulate elements. In an analogous manner CPZ might be acting in the cytoplasmic structures of Amoeba proteus, but at this stage it would be premature to pinpoint such cytoplasmic structures that might be affected as well as the exact mechanism of such action.

Large free living amoebe like Amoeba proteus is particularly suitable for the study of endocytosis because phagocytosis is its normal mode of feeding and it readily exhibits pinocytosis in response to a wide variety of cationic chemicals. In addition, the species is fully aerobic and tolerates a wide range of hydrogen ion concentration. Since a great deal of information is available on the endocytotic activity, we have taken "endocytosis" as a parameter to study the effects of CPZ, a cationic form of amine anesthetic. It is well known that pinocytosis in amoeba is not an all-or-none response to an inducer. Fluctuations in concentrations of inducing solutes, calcium ions etc. can modify the response and the influence of calcium ions is especially interesting so far as pinocytosis is concerned (Josefsson, 1975; Stocken and Klein, 1979; Chapman-Andresen, 1977). Basically pinocytosis has two phases, the first phase consists of the binding of the inducing solute to the

mucous coat and the second phase is the active phase of channel formation (Chapman-Andresen, 1967).

According to Josefsson and Johnson (1979), formation of pinocytotic channels during induction of pinocytosis in Amoeba proteus requires Ca^{2+} and hence any agents which interfere with calcium metabolism of the amoeba would either inhibit or stimulate pinocytosis.

Stockem and Klein (1979) have recorded a significant increase in the size and number of Ca^{2+} binding sites at the cytoplasmic surface of the cell membrane in pinocytoting Amoeba proteus. This increase in the size and number of Ca^{2+} binding sites has been explained by them to be due to increased Ca^{2+} permeability of the plasma membrane during induced pinocytosis. Klein and Stockem (1979) have postulated that alterations in the intracellular Ca^{2+} concentration regulate the activation of actomyosin as a result of which the activity of the membrane associated filament layer is also influenced. They are of the opinion that contraction of the membrane associated filament layer is a sine qua non for the formation of pinocytotic channels and vacuoles.

An increased Ca^{2+} permeability of the plasma membrane of Amoeba proteus during induced pinocytosis

has also been reported by Chapman-Andresen (1973) who has found that initial changes in the physiological state of the cell membrane occur at the surface of the mucous layer. According to her, phosphate groups present in the mucous layer act as cation adsorber and under normal physiological conditions, inorganic ions, especially Ca^{2+}, remain bound to a certain extent at these phosphate groups. She has postulated that the induction of pinocytosis by various strongly cationic substances causes the release of Ca^{2+} from the cationic binding sites and in consequence the adsorption of large amounts of inducer in exchange for Ca^{2+} (see, Klein and Stocken, 1979). The adsorption of the inducer leading to changes in the electrostatic surface potential is always followed by an increased permeability of the plasma membrane and there also occurs a change in the molecular conformation of the membrane. Josefsson (1975) has postulated that as a consequence of conformational changes in the molecular structure of the membrane, Ca^{2+} and other ions can now enter the cell interior thereby altering the intracellular Ca^{2+} concentration.

In view of the above findings it appears attractive to speculate that pinocytotic activity of CPZ treated Amoeba proteus is decreased, as observed by us, presumably due to a reduction in the Ca^{2+} influx across the cell membrane. Compatible with this view are the recent

experiments on erythrocyte ghosts (Seeman, 1972) which indicate that local anesthetic amines (CPZ) readily adsorb to biomembranes, compete with and displace the membrane bound Ca^{2+} adsorbed to phospholipids. He has also shown that the transmembrane fluxes of Ca^{2+} are invariably inhibited by CPZ.

Josefsson (1975) has reported that local anesthetic amines, in their cationic form, inhibit pinocytosis by acting on anionic phospholipids in biological membranes and by preventing the binding of cations at those sites. According to him, pinocytosis induced by Na^{+} requires a considerable amount of Ca^{2+} in the cell surface and hence it is highly susceptible to inhibition by the local anesthetic amines. Hence, in our study of the effect of CPZ on pinocytosis, either observed in the presence of the drug or obtained by pretreatment with the drug, is secondary to variation of the Ca^{2+} concentration in the cell surface. Our view that the inhibitory effect of CPZ is due to inhibition of Ca^{2+} influx and displacement of membrane bound Ca^{2+} is supported by the finding of Josefsson (1975).

An indirect support to our assumption that CPZ inhibits pinocytotic activity in Amoeba proteus by reducing the influx of Ca^{2+} comes from the work of Stockem

and Klein (1979) who have shown that inhibition of the Ca^{2+} influx by the simultaneous application of antagonist (Verapamil) decreases the endocytotic activity, but does not stop it.

An alternative explanation for the inhibition of pinocytotic activity by CPZ may also be deduced from the studies on the influence of different metabolic inhibitors on this process in Amoeba proteus and from the known actions of CPZ on the energy metabolism of the cell. DeTerra and Rustad (1959) have suggested that inhibition of channel formation by metabolic inhibitors is presumably the result of a drop in the level of that energy which is normally made available to the cell through the functioning of the cytochrome system in respiration. Chapman-Andresen (1967) has indicated that channel formation is rapidly suppressed by inhibitors of respiration and glycolysis and more slowly by inhibitors of oxidative phosphorylation. Her studies have also shown that the surface binding of the inducer is not influenced, but the active process of channel formation is inhibited by various metabolic inhibitors. Therefore, in our study it can be inferred that the inhibition of pinocytotic activity by CPZ may be due to the capacity of the drug to uncouple oxidative

phosphorylation in the mitochondria of the organism thereby reducing the ATP level which is the energy source of the contractile system. This inference is supported by the view that CPZ uncouples oxidative phosphorylation in the mitochondrial preparations from rat brain (Abood, 1955) by the following probable mechanisms :

a. Due to a nonspecific effect on the respiratory enzyme system as a whole rather than a specific inhibitory effect on any one enzyme (Strecker, 1958).
b. Due to its antagonism with the flavo-enzymes (Yagi et al., 1965).

We can also suggest that endocytosis (both phagocytosis and pinocytosis) is inhibited by CPZ due to unavailability of required amount of ATP whose consumption may, in part, be related to the involvement of the contractile proteins in the endocytotic process.

From the experiments on morphological changes, we have found out that normalcy is restored within 4 to 5 hours of CPZ treatment. Hence the same CPZ treated amoebae which were challenged once with inducer solution immediately after treatment were again challenged with inducer solution five hours later. The shift in the peak

activity of the treated amoebae challenged with inducer after five hours of the first cycle is an indication of their delayed reponse. Moreover, the decreased pinocytotic activity of the treated group challenged for the second time with the inducer may be due to failure of the membrane components responsible for pinocytotic channel formation to become fully normal. However, almost normalcy has been found to be achieved which tend to indicate that the treated cells can presumably restore their normal organisation of the plasma membrane structure at a later time.

Inhibition of alkaline phosphatase (a predominantly membrane bound enzyme) activity immediately after CPZ treatment has been revealed by differences in the intensity of cytochemical staining between control and CPZ exposed cells. We presume that this inhibition may be due to a conformational change in the native enzyme structure which, in turn, may result form the perturbation of the membrane structure by CPZ. However, the activity of alkaline phosphatase has been found to be restored to the normal level at later hours thereby indicating a recovery of the treated cells.

The ability of CPZ treated cells for the uptake of tritiated leucine has been found to be affected only slightly

when the cells are incubated in labelled leucine along with CPZ. However, a dramatic inhibition in the uptake activity has been recorded immediately after CPZ treatment and this has been found to have improved only slightly at five hours post-treatment. Hence, it is very likely that recovery in the uptake capacity of ^{3}H-leucine may take place at a still later period. The impairment in the uptake ability may be attributed to the changes in the membrane architecture which, in turn, may alter the conformation of the leucine binding protein located in the cell membrane. This assumption may be supported by the finding of Penrose et al. (1970) who have reported reversible conformational changes in a leucine binding protein from Escherichia coli. According to them, most models of transport have the following three stages :

1. The first stage is the initial binding of the solute (A) to some macromolecule (X) in the membrane, thus forming a complex (AX).
2. The second stage involves a process in which the complex (AX) is taken from outside and is presented to the inside of the membrane. It is generally believed that the second stage includes reversible conformational changes within the macromolecule or membrane components with which it is associated.
3. The third step is the release of the solute at the inner surface of the membrane.

Oxender and Rahmanian (1972) have proposed that somehow at the inner surface of the membrane metabolic energy transformations are coupled to produce a modification of the binding protein that shows decreased binding activity, causing the solute molecule to be released inside. The modified binding protein can then orient itself to the outside of the membrane and spontaneously return to the thermodynamically stable high affinity form. Penrose et al. (1970) have concluded that amino acid binding site is associated with a unique protein conformation which can be reversibly altered.

In the impairment of leucine uptake ability in the CPZ treated amoebae, it is difficult, at present, to predict precisely the step that is affected following CPZ treatment. However, it can be presumed that CPZ might be involved in bringing about changes in the conformation of the leucine binding protein by acting either directly on it or through indirect actions on the other membrane components.

In conclusion, it can be said that there are several sites of interaction between anesthetics and membrane. One mechanism whereby anesthetics can lower excitability and induce a functional block is by reducing the membrane conductance to Na^+. It is easy to understand

that such an effect could cause generalised inhibition on neuronal circuits. However, other data suggest that some anesthetics function by reducing membrane conductance to calcium (see, Siesjo, 1982) and if this block occurs at presynaptic sites of neurones, there would be a reduction in the release of neurotransmitter. In the non-neural cells like Amoeba proteus, CPZ causes an inhibition in the mobility and pinocytotic activity probably by reducing the influx of Ca^{2+} and interfering with cellular energy productions. It was also found that CPZ markedly reduced the uptake of ^{3}H-leucine presumably by some changes in the membrane architecture which are difficult to pinpoint at this stage. However, restoration of normalcy about 5 hours after CPZ treatment clearly indicates that the treated cells can presumably restore the normal organisation of the altered membrane and other subcellular structures. The involvement of calmodulin in some of the processes studied can not be ruled out completely. But it would require more crucial experiments to firmly substantiate how CPZ and calmodulin interact at the biochemical level to bring about the observed effects of the drug.

SUMMARY

1. Large, free living, unicellular protozoa (*Amoeba proteus*) has been utilised for studying the effects of a neuropharmacological drug (CPZ) on some membrane related phenomena of this non-neural cell.

2. The cells have been found to tolerate only a narrow range of CPZ concentration. Increased concentration above the range causes lysis of the cells immediately after treatment.

3. The treated cells are found to be viable and capable of undergoing cell division.

4. The differentiation of the cell cytoplasm into a clear hyaline zone and a dense central zone has been found following CPZ treatment.

5. The drug treated amoebae assume spherical shape and become detached from the substratum.

6. The CPZ administration causes cessation in motility and some impairment of phagocytotic activity of the cell.

7. Pinocytotic ability has been found to be markedly reduced following treatment of the cells by CPZ.

8. The alkaline phosphatase activity, and the ability to take up ^{3}H-leucine have also been found to be inhibited

dramatically, presumably by some changes in the membrane components.

9. Many of the effects studied are found to be reversible which comeback to normal level about 5 hour after CPZ treatment.

10. Many of the observed inhibitory effects of CPZ on Amoeba proteus have been suggested to be due to the ability of the drug to reduce Ca^{2+} influx across the membrane and/or due to its ability to uncouple oxidative phosphorylation. But the evidence on which this conclusion is based bears further examination.

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