ANALYSIS OF EARLY DEVELOPMENT IN
DICTYOSTELIUM DISCOIDEUM

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CERTIFICATE

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

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CONTENTS

ABBREVIATIONS USED

CPZ	Chlorpromazine hydrochloride
3,'5'cAMP	Cyclic adenosine 3,'5' monophosphate
5'AMP	5' adenosine monophosphate
cGMP	Cyclic guanosine 3,'5' monophosphate
Ax2	Axenic strain 2 of *D. discoideum*
PDE	Phosphodiesterase
ePDE	Extracellular phosphodiesterase
iPDE	Intercellular phosphodiesterase
PDI	Phosphodiesterase inhibitor
$CaCl_2$	Calcium Chloride
Ca^{+2}	Calcium ion
$MgSo_4$	Magnesium sulfate
EDTA	Ethylene diamine tetra acetic acid
TEA	Triethanolamine
$Na_2HPO_4 2H_2O$	Sodium phosphate (dibasic)
KH_2PO_4	Potassium dihydrogen phosphate (monobasic)
n-agar	Nutrient agar
p-agar	Phosphate agar
p-buffer	Phosphate buffer
kD	Kilo Dalton
csA	Contact Site A
M	Moles
mM	milli Moles
μM	micro Moles
μl	micro Litre
nm	Nanometer
S	Slug
Ag	Aggregate
St	Streaming
F.B	Fruiting Body

INTRODUCTION

Dictyostelium discoideum which is popularly known as cellular slime mould is a eucaryotic microbe which has long been used as a model system for the study of cell differentiation and pattern formation.

Dictyostelium discoideum cells begin their life cycle as free living amoebae which in turn are liberated from the spores. In nature, amoebae inhabit soil, humus and animal dung. The haploid amoebae feed on bacteria and divide by binary fission as long as the nutrition is available. Once the food is exhausted, a few cells start liberating out cyclic adenosine 3',5' monophosphate (cAMP) which is sensed by the distal cells and they in turn start moving towards the source of cAMP and start aggregating. As they migrate towards the center of aggregation the cells become adhesive and later aggregate to form a multicellular body called the pseudoplasmodium/slug. The migrating slug later differentiates into a fruiting body which consists of two types of cells; stalk and spore cells, in specific proportion. Unlike the higher plants and animals where growth and differentiation occurs simultaneously, in *D. discoideum* growth and differentiation are in separate existence (Bonner, 1982). This alone gives cellular slime moulds enormous advantages for the study of developmental process as we can study growth and differentiation separately.

Cellular slime moulds were first discovered by a German mycologist Brefeld in 1869, but the unique nature of their asexual life cycle was not fully understood until a study was made by the French mycologist, Ph.van Teighem (Bonner, 1982). Later important contributions in the field of cellular slime mould development was made by Harper at the Columbia University, U.S.A. (Bonner, 1982). Later axenic strains (Ax2 and Ax3) of *D. discoideum* were developed which could grow in minimal medium (Sussman and Sussman, 1967; Ashworth and Watts, 1970). A major breakthrough in the investigation of *D. discoideum* chemotaxis was the discovery of cAMP as the primary chemoattractant by Konijn and coworkers in 1967. Since then a lot of new information has accumulated in the past two decades on the life cycle of *D. discoideum*. Since the organism is a favourable material for the study of diverse biological processes the range of studies have continued to expand.

CLASSIFICATION

The question of where *Dictyostelids* should be placed in relation to other simple amoeboid organisms have been examined comprehensively by many workers. The debate is still on whether to classify *Dictyostelids* under protozoan or to classify them under fungi. The two systems of classification are, (Bonner, 1982):

1.	K.B. Raper			2.	L.S. Olive		
	Kingdom	:	Plantae		Kingdom	:	Plantae
	Sub Kingdom	:	Mycetae		Phylum	:	Gymnomyxe
	Division	:	Myxomycota		Sub Phylum	:	Mycetozoa
	Class	:	Acrasiomycetes		Class	:	Eumycetozoa
	Sub Class	:	Dictyostelidae		Sub Class	:	Dictyostelia.

DICTYOSTELIUM DISCOIDEUM AS A MODEL EUKARYOTIC SYSTEM FOR THE STUDY OF DEVELOPMENTAL PROCESS

To study the various problems related to cell biology, it is better to focus the investigation on one eukaryotic organism which is not very complicated, easy to handle and manipulate. Among eukaryotes one good candidate for intensive study is *Dictyostelium discoideum* due to its simple and well defined life cycle. One important aspect of *D. discoideum* is that the growth can be segregated from differentiation and this gives the organism enormous advantages for the study of developmental processes. *D. discoideum* presents a favourable material for the study of diverse biological processes related to development such as membrane shuttling, cell motility, cell recognition, chemotaxis and pattern formation. The advantages of studying *D. discoideum* as a model eukaryotic system are as follows:

1. *Dictyostelium* **cells can be easily grown (Spudich, 1987):**

   a) axenically in a well defined chemical medium under shaken conditions, and

   b) in association with bacteria on agar or in shaken suspension.

2. **Easy Handling of Cells:**

   Cells can be easily maintained as follows (Spudich, 1987; Sussman, 1987):

   a) lyophilized preparations can be stored in refrigerator,

   b) silica gel dessication : for storing the spores, and

   c) vegetative amoebae can be kept frozen with glycerol.

3. **Best Suited for Biochemical Studies:**

    Large amount of cells can be grown in axenic medium and a kilogram or more of wet cell pellet can be easily obtained for biochemical studies.

4. *D. discoideum* is well suited as an experimental system to study mechanisms of phagocytosis and cell motility and for both these processes pseudopod formation is essential. One good approach afforded by *D. discoideum* is the studies on the role of actin and myosin phosphorylation during cell motility (Kuczmarski and Spudich, 1980).

5. *D. discoideum* has become popular for investigation of molecules involved in cell adhesion, as the life cycle involves differentiation from a non associating vegetative state to an adhesive aggregated state initiated by starvation. Because this differentiation is fairly synchronous, large number of cells can be obtained with stage specific adhesive properties (Barondes, 1981).

6. *D. discoideum* **provides an excellent system for investigating developmental "timing"/"regulation":**

    Considerable evidence has accumulated to support the notion that many *D. discoideum* genes are expressed only at specific developmental stages. Thus *Dictyostelium* provides an extremely useful system for investigation of cellular differentiation and the mechanism with which developmental programmes are regulated (Chisolm *et al.*, 1984).

7.   Easy genetic manipulation:

Two attributes of the organism have substantially facilitated experimental genetics (Kessin, 1988):

i)   easy isolation of mutants:

as *D. discoideum* cells exist predominantly in haploid state the cells readily express the mutational abberations that may gain.

ii)   cloning of individual cells can be readily accomplished:

D. discoideum cells in association with bacteria lead to plaque formation, which in turn facilitates isolation and screening of mutants.

8.   *D. discoideum* is an outstanding system to study the molecular mechanism of signal transduction and pattern formation during morphogenesis. Signal transduction is mediated by cAMP through a membrane receptor and is similar to hormone action in higher eukaryotes (Devreotes, 1982; Robertson and Grutsch, 1981).

LIFE-CYCLE

D. discoideum is becoming increasingly recognized as a genetically and biochemically suitable system for the study of a number of developmental phenomenon because of its unusual developmental features during its life cycle. *D. discoideum* has two alternate life cycles: asexual and sexual.

1. ASEXUAL PHASE

Asexual life cycle includes both the unicellular and multicellular phases. It is characterized by transition of single-cell vegetative amoebae to a multicellular aggregate or pseudoplasmodium that differentiates into a fruiting body consisting of two cell types - stalk and spore cells. The asexual phase includes the following stages:

i) Vegetative Phase:

In the vegetative phase, haploid amoebae feed on bacteria. They grow and divide every three to four hours if food is abundant. The amoebae are 10μ to 15μ in size and pocesses 7 chromosomes. Chemotaxis to compounds released from bacteria i.e. folic acid is probably involved in food seeking (Pan et al., 1972). The amoebae can be cultured on agar plates or in suspension in association with food. Some axenic strains (Ax2 and Ax3) which can grow on semi-defined media have been developed (Sussman and Sussman, 1967; Ashworth and Watts, 1970) and these strains are useful for biochemical and genetic analysis.

ii) Aggregation Phase:

Upon the exhaustion of food the amoebae enters a quiescent state called interphase, following which they start aggregating (Bonner, 1971). Aggregation involves both chemotaxis and signal relay. Within six hours after the initiation of starvation a few cells secrete cAMP in the medium (Konijn et al., 1967; Alacantra and Monk, 1974). Stimulation of a cell by the secreted cAMP, causes not only local extension of pseudopods but

also a fast general contraction of the cell. This contraction is also called "cringing" and is transient, lasting about 25 seconds after cAMP stimulation (Futrelle et al., 1982). The exogenous cAMP binds to the cAMP receptor present on the cell surface and triggers a series of responses such as activation of adenylate cyclase, release of phosphodiesterase etc. which are essential for cAMP signalling. The chemotactic signal is propagated in a pulsatile wave like fashion from cell to cell (Cohen and Robertson, 1971). Within 8 to 10 hours of starvation, the amoebae become aggregation competent i.e. they are able to: (i) respond to cAMP chemotactically, (ii) relay the cAMP signal, (iii) initiate the signal autonomously; and (iv) form intercellular contacts (Gerisch, 1982). To ensure that chemotactic signals are detected as pulses, cells synthesize extracellular and membrane bound cAMP dependent phosphodiesterase (PDE) which regulate the level of cAMP by hydrolyzing 3'5'-cAMP to 5'AMP (Cohen, 1978). Stimulation of cells by cAMP also causes an increase in the adhesiveness of the cells with the expression of contact sites on cell surface (Gerisch et al., 1975). The net result of the signal relay is that, the cells start moving in the form of streams towards the aggregation center. Once the cells are collected in an aggregate, morphogenesis begins with the formation of the slug.

iii) Pseudoplasmodium/Slug :

The oscillating level of cAMP pulse results in bringing 10^5 to 10^6 aggregation competent cells into a single multicellular unit called the pseudoplasmodium/slug, which is enclosed within a cellulose sheath secreted by the aggregating cells. The migrating slug has distinct

anterior and posterior regions that can be visualized by histochemical and immunocytochemical methods (Takeuchi et al., 1986). The anterior half of slug contains cells that are predestined to become stalk cells and hence known as prestalk cells. The posterior half of the slug is constituted by the prespore cells (Loomis, 1985).

iv)   **Culmination Stage and Fruiting Body Formation :**

During culmination the slug tip stops the forward movement and points up while the posterior end continues to move so that all the cells are gathered under the tip. At this stage, formation of stalk cells occur near the tip by progressive vacuolization of the anterior cells which also deposit cellulose in the cell wall. Soon the stalk cells move down by pushing through the prespore zone. As a result of this downward movement of stalk cells, the whole cell mass lowers and bulges laterally and this is often called the "Mexican Hat" stage (Loomis, 1975). As the stalk cells continue to elongate the whole of the prespore mass rises into the air. This stage is called "Sorogen". On this upward voyage, the prespore mass begins to turn into fully differentiated spores encapsulated by a thick wall. The final fruiting body has a slender tapering stalk with a terminal, globular spore mass or "Sorus". When the spore wall bursts open, the spores are liberated. The spores are elliptical in shape and 5μ to 7μ in size. Spores are usually dormant and a large number of agents are capable of activating spores, such as heat shock, proteose peptone etc, thus initiating germination (Brisbin and O'Day, 1988). Following activation, the spore wall splits allowing the emergence of the amoeba. The whole life cycle of *D. discoideum* is depicted in Fig. 1 .

SPORES

SORUS
(containing spore cells)

STALK

BASAL DISC

FRUITING BODY
(SPOROPHYTE)

Spore germination

AMOEBAE
feeding and
proliferation

STARVATION

STREAMING

PSEUDO PLASMODIUM
(cell adhesion,
aggregation)

CULMINATION

MEXICAN HAT STAGE

MIGRATION

FINGER TIP STAGE

Prestalk cells

Prespore cells

SLUG / GREX
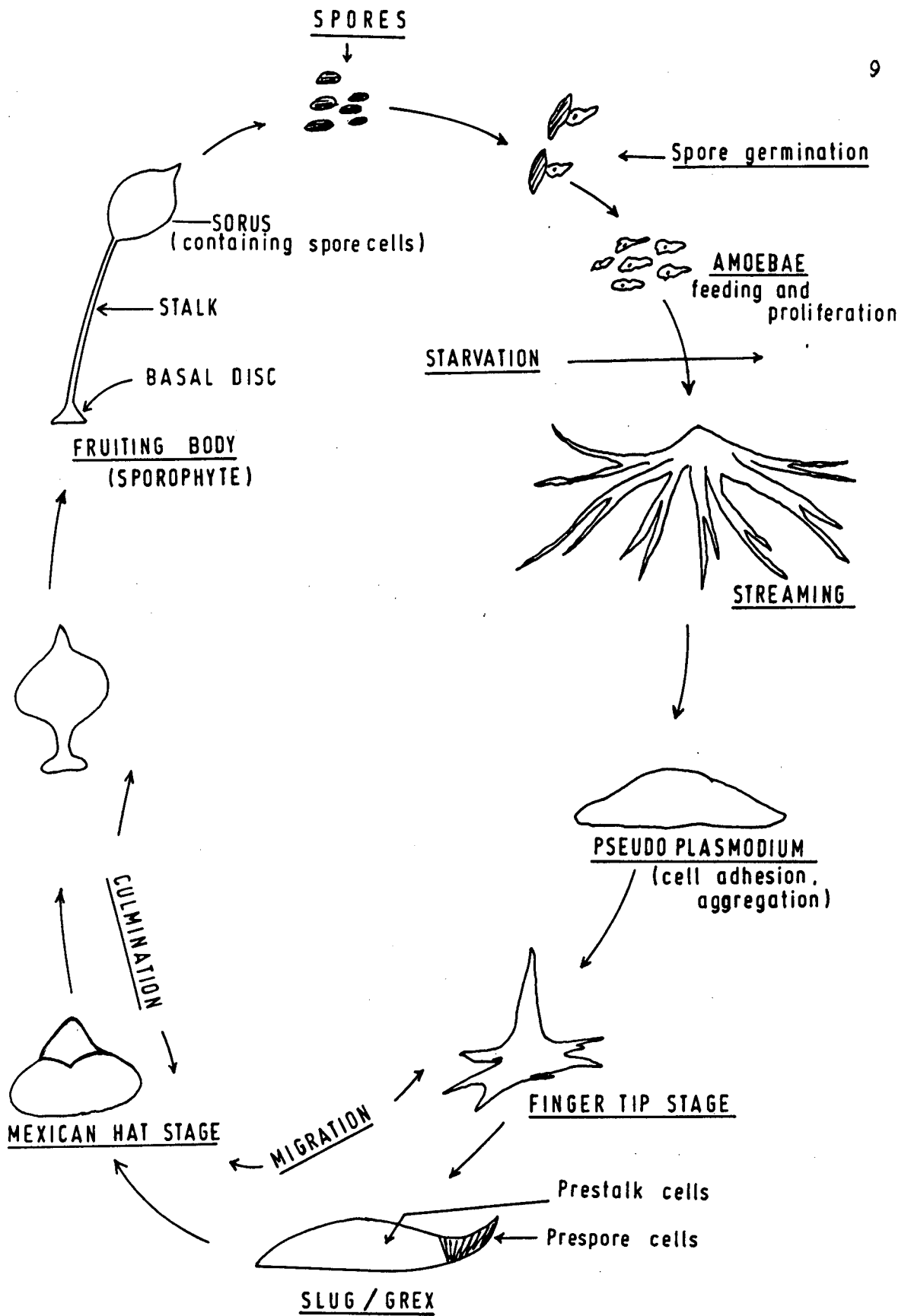
Fig.1: LIFE CYCLE OF DICTYOSTELIUM DISCOIDEUM
(not to the scale)

2.   SEXUAL PHASE

Macrocyst formation represents the sexual cycle in *D. discoideum*. Production of macrocyst requires particular conditions, such as the presence of cells of two complementary mating types, darkness, humidity and appropriate temperature   (Saga and Yanagisawa, 1982). Macrocyst formation consists of two steps:

i)   **Production of Giant Cells:**

During early stages of macrocyst formation large cells appear, around which amoebae subsequently aggregate. These large cells have been designated as giant cells and these are assumed to be zygotes formed by fusion of opposite mating cell types (O'Day, 1979).

ii)   **Induction of Macrocyst Development by Giant Cells.**

These giant cells engulf the surrounding cells, gradually increase in size and finally develop into macrocyst surrounded by a thick wall. The macrocyst is constituted by cells derived from the giant cell/zygote nucleus. When conditions are favourable the macrocyst bursts open to release the amoebae (O'Day, 1979).

EARLY *DICTYOSTELIUM DISCOIDEUM* DEVELOPMENT

Early development of *D. discoideum* begins soon after nutrient exhaustion during which an intercellular communication system appears

which coordinates the highly organized aggregation of several million cells. cAMP is the main factor that serves as a signal during the early development (Devreotes, 1982). The cell membrane plays an important role during the early development. Binding of cAMP to the membrane receptor leads to the sequential activation of different pathways essential for cell aggregation. The sequential events occuring during early development are as follows:

1) About 2 hrs after initiation of development a few cells start to secrete pulses of cAMP (Schapp and Wang, 1986).

2) Extracellular cAMP binds to the cell surface receptor and within 30 seconds after the activation of cell surface receptors the following changes are detected:

   a) Increase in cGMP concentration by activation of guanylate cyclase (Wurster *et al.*, 1977)

   b) Increase in cAMP concentration by activation of adenylate cyclase (Ross and Gerisch, 1976; Kelin, 1976; Klein *et al.*, 1985).

   c) Stimulation of calcium influx into the cells (Bumann *et al.*, 1984; Malchow *et al.*, 1982)

3) Coordinated movement of the cells towards the cAMP source within 3 to 5 hrs after initiation of development (Devreotes, 1982; Fontana, *et al.*, 1986)

4) Adaptation of cells to extracellular cAMP signals which results in the deactivation of adenylate cyclase (Van Haastert and Konijn, 1982).

5)   About 6 hrs of initiation of development, there is an increase in the activity of cAMP dependent phosphodiesterase that causes hydrolysis of cAMP and thus leads to the attenuation of the signal (Riedel and Gerisch, 1971; Panbacker and Bravard, 1972).

6)   Activation and deactivation of specific genes that produce the various components of cAMP signal system such as cell surface receptors, adenylate cyclase, guanylate cyclase, phosphodiesterase, 80 kilodalton (80 kD) protein etc. (Kessin, 1988; Newell, 1982).

7)   **Cell Adhesion**

Within 6 to 8 hrs of development, distinct EDTA stable contact sites appears on the cell surface which causes the cells to adhere to each other and thus leading to the formation of a multicellular mass (Gerisch, 1986). Also within 8 to 10 hrs of initiation of development an 80 kD glycoprotein/csA appears on the cell surface which is involved in cell adhesion.

The two responses which bring about the coordinated aggregation during early development are:

1.   Chemotaxis; and

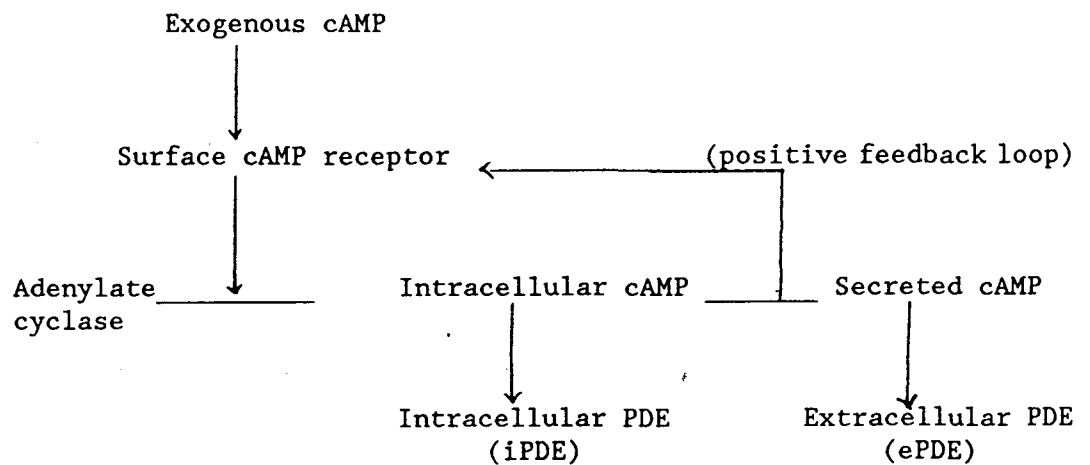2.   cAMP Signalling or Signal Relay. (Gerisch *et al.*, 1984).

Chemotaxis

This involves the coordinated movement of vegetative cells towards the source of chemoattractant (Spudich and Spudich, 1982). In a field

of vegetative cells deprived of food for several hours, a few randomly distributed cells start secreting cAMP into the medium (Gerisch, 1987). A neighbouring cell senses the extracellular cAMP and initiates its movement towards the cAMP source (Cohen and Robertson, 1971; Cohen, 1978). Chemotaxis towards the cAMP source involves the pseudopod formation. Studies done by Spudich (1974) and Clarke *et al.*, (1975) shows that pseudopod formation involves an association of actin and myosin which are the primary components of the filamentous matrix that underlies the cell membrane.

cAMP-Signalling or Signal Relay

Signal relay involves the local propagation of the chemotactic signal over the field of amoebae. When cAMP binds to the surface cAMP receptor it activates adenylate cyclase and the activated cell in turn releases pulse of cAMP (Klein *et al.*, 1985). Thus when more and more cells are stimulated by cAMP, they synthesize and secrete additional cAMP into the medium, which then serves as a signal for more distal cells (Schaffer, 1975; Roos *et al.*, 1975). Thus a stable cAMP wave results from the cells capacity to relay the cAMP signals. One important aspect of signal relay is the occupancy of the cAMP membrane receptors which in turn leads to a cascade of reactions. This process is called "Transmembrane Signal Transduction" (Janssens and Van Hasstert, 1987). The various components essential in generation of cAMP signaling response can be diagrammatically represented as follows (Devreotes, 1982):

```
        Exogenous cAMP
               |
               ↓
     Surface cAMP receptor  ←_____  (positive feedback loop)
               |                                |
               ↓                                |
  Adenylate___↓____      Intracellular cAMP ___|__  Secreted cAMP
  cyclase                     |                              |
                              ↓                              ↓
                      Intracellular PDE              Extracellular PDE
                           (iPDE)                        (ePDE)
```

cAMP synthesis is not continuous, rather it is in the form of short pulses. Pulse shape of cAMP is due to:

i)   **Adaptation**: during which the cells will not respond to cAMP even though the extracellular signal (cAMP) is present. Adaptation causes deactivation of adenylate cyclase thus regulating the cAMP level (Devreotes, 1982).

ii)   **Presence of PDE**: PDE is secreted out and hydrolyses cAMP and thus causes the attenuation of the signal (Darmon *et al.*, 1978).

Within 10 hours of starvation most of the cells become aggregation competent and a periodic cAMP wave propagation begins from the aggregation centre. As the cAMP wave propagates outward, the cells start streaming inward towards the center. This coordinated cell movement can be visualized as bands that form concentric patterns (Alcantra and Monk, 1974). A critical cell density is essential for cellular aggregate to

form the slugs. In order to initiate normal morphogenesis approximately 5×10^6 cells should be present. Below this density cells are too far apart for signal propagation and as a result either too few fruiting bodies are formed or there is no morphogenesis at all.

Cell - Adhesion

During the development of *D. discoideum*, single cells aggregate to form a multicellular mass (Loomis, 1975). The change from single cell state to multicellularity is mediated by chemotactic cell movement and intercellular adhesion.

1. **EDTA stable and EDTA sensitive cell adhesion in aggregating**
   D. discoideum cells

   Growth phase cells of *D. discoideum* form only EDTA sensitive contacts, whereas aggregation competent cells form EDTA stable contacts, in addition to sensitive ones (Muller and Gerisch, 1978; Gerisch, 1986). At the time of this change in adhesiveness the cells acquire the capacity to aggregate into streams of end to end associated cells. The contact sites that develop are:

   a) **Contact site B/EDTA sensitive contact site**

   Both vegetative and aggregating cells show the presence of contact site B and is characterized by side to side adhesion. Adhesion through contact site B could be blocked by addition of 10 mM EDTA to the cells (Muller and Gerisch, 1978).

b) Contact site A/EDTA resistant contact site

It appears only on aggregation competent cells and is involved in the end to end intercellular contact. Adhesion established by contact site A (csA) is resistant to addition of 10 mM EDTA. Contact site A appears within 6 to 8 hrs after starvation on the cell surface (Beug *et al.*, 1973; Gerisch, 1982).

2. Cell surface glycoproteins

With the immunological approach, several molecules that appear to play a role in cell-cell adhesion have been identified. In *D. discoideum* evidence is accumulating for the role of three glycoproteins with molecular weights of 80,000, 95,000 and 150,000, in intercellular adhesion.

a) 80,000 dalton (80 kD) glycoprotein:

From membranes of aggregation competent cells an 80 kD glycoprotein has been purified. Antibodies raised against this protein is capable of blocking the EDTA-stable adhesion in aggregation competent cells (Muller and Gerisch, 1978; Gerisch, 1986). This 80 kD protein which is expressed in aggregating cells has been termed as contact site A (csA). The csA glycoprotein is modified by phosphorylation at serine residue and by fatty acid acylation. Contact site A has two types of carbohydrate residues: type 1 and type 2. Type 1 is characterized by its sulphation while Type 2 is highly immunogenic (Gerisch, 1986).

b)   95,000 dalton (95 kD) glycoprotein:

95 kD glycoprotein has been isolated from the membranes of slug stage cells and is probably involved in cell adhesion during the slug formation (Parish *et al.*, 1978).

c)   150,000 dalton (150 kD glycoprotein:

This appears both on vegetative and aggregating cells (Gletosky *et al.*, 1980). The 150 kD protein increases on the cell surface between 6 to 18 hours of development.

3.   Discoidins

Discoidins are carbohydrate binding proteins involved in cell to substrate adhesion. Two forms of discoidins have been purified from *D. discoideum*, discoidin I and discoidin II (Barondes *et al.*, 1982). Discoidins are virtually undetectable in extracts of vegetative cells but increase by 20-fold in aggregating cells within 6 to 12 hours of initiation of starvation (Barondes, 1981).

MOLECULAR COMPONENTS OF THE CHEMOSENSORY SYSTEM IN EARLY DEVELOPMENT OF *D. DISCOIDEUM*

1.   Surface cAMP Receptors

Starvation of *D. discoideum* cells triggers the appearance of cAMP receptors on the cell surface. The cAMP receptors number rises from a very low level to a maximum at aggregation competence i.e. about 8 to 10 hours after starvation (Gerisch, 1982). Kinetic studies of cAMP binding revealed receptor heterogenity and cooperativity in cAMP binding (Van Haastert and De Wit, 1984). The cAMP receptor is coupled to a protein

which is supposed to bind the cyclic nucleotide. This protein has been termed as G-protein. It is proposed that G-protein might have some regulatory role in the cascade of reactions which follows subsequent to cell activation (Leichtling *et al.*, 1981).

2. Adenylate Cyclase

Binding of cAMP to the membrane receptor leads to activation of adenylate cyclase involved in cAMP production. Periodic activation of adenylate cyclase has a central role in cAMP relay responses (Klein, 1976).

3. Guanylate cyclase

Guanylate cyclase activity is present in both vegetative and aggregating *D. discoideum* cells and is stimulated via cAMP receptors. Basal activity of guanylate cyclase is 20 fold higher in the aggregation competent cells than the vegetative cells (Janssen and Haastert, 1987). Europe-Finner and Newell (1987) recently obtained important evidence which suggests that guanylate cyclase is regulated via the inositol phosphate pathway.

4. cAMP dependent Phosphodiesterase

D. discoideum cell show the presence of three types of phosphodiesterase (PDE).

      i)   membrane bound PDE (mPDE).

    ii)   extracellular PDE (ePDE).

   iii)   intracellular PDE (iPDE).

Both mPDE and ePDE are glycoproteins whose polypeptide moieties appear to be coded by the same gene. PDE causes hydrolysis of 3',5' cAMP into 5' AMP and thus leads to the attenuation of the cAMP signal (Gerisch, 1987). Thus PDE is important to allow the cells to deadapt between two bursts of cAMP. Activity of PDE is down regulated by phosphodiesterase inhibitor (PDI) which is a heat stable glycoprotein (Franke and Kessin, 1981).

It is apparent from the foregoing discussion on *D. discoideum* development that a large number of membrane associated activities occur in a sequential manner during its morphogenesis. For instance, cell motility, cAMP mediated chemotaxis, development of aggregation competence leading to cell recognition and cell adhesion, appearance of membrane protein at specific developmental stage etc. These are all indicative of precise membrane interactions occuring during its development (Gerisch, 1982). Further, the liberation of extracellular cAMP as a chemical transducer, synthesis of membrane bound and extracellular phosphodiesterase and the attenuation of the chemotactic signal point out the tremendous importance of membrane related activities during morphogenesis (Murray, 1982). Thus, it seemed of great interest to analyze these wide ranging membrane functions and membrane interactions during *D. discoideum* development. In this respect a group of phenothiazine derivatives, popularly termed as "membrane anesthetics" can unravel a variety of mechanisms which involves membrane mediated cell-cell interactions. These groups of compounds have wide ranging

activities and we decided to employ one of the most potent membrane anesthetics viz., Chlorpromazine (CPZ) which is a well known neuropharmacological agent having diverse actions on membrane structure and functions (Seeman, 1972; Leterrier, et al., 1976; Luxnat and Galla, 1986). So far to our knowledge there is little or no studies on the action of this chemical on D. discoideum development which is greatly dependent on specific membrane interactions throughout its morphogenesis.

CHLORPROMAZINE AND MEMBRANE INTERACTIONS

Chlorpromazine is a neuropharmacologically active drug that shows a wide variety of biological activities. CPZ was first synthesized in 1950 by Charpentiar and later Labroit found it to have an amazing number of pharmacological actions, including striking CNS-depressant effects (Zirkle and Kaiser, 1968; Darcy, 1988). By 1953 CPZ was effectively used in treatment of various psychiatric disorders. This drug is also a potent local anesthetic as it suppresses the electrical activities of neurons (Bradley et al., 1966).

Actions and Uses of Chlorpromazine

CPZ has a wide range of actions arising from its CNS depressant, adrenergic blocking and weak anticholinergic actions (Byck, 1975; Siesjo, 1982). It inhibits dopamine, stimulates prolactin and alters endocrine functions. It is used in the control of acute psychosis where it can diminish restlessness, anxiety, confusion and aggression (Darcy et al., 1988).

Membrane Perturbation by Chlorpromazine

The primary action of anesthetics are on the cell membrane, though a few of the intracellular processes also get affected (Seeman, 1972). It has been widely accepted that anesthetics partition into membrane bilayer by interaction of lipid molecule with their ionic heads in the membrane surface (Sheetz et al., 1976; Zachowski and Durand 1988). Conrad and Singer (1981) questioned this concept and reported that anesthetics can also interact with the hydrophobic portions of the membrane protein.

Binding of anesthetics to the membrane perturb the biomembrane and results in membrane expansion, change in membrane fluidity and change in shape of biomembranes (Seeman, 1972, Sheetz et al., 1976; Luxnat, 1986).

1. **Membrane expansion**

Seeman, et al., (1969) proposed that anesthetics including CPZ act by expanding the membrane. All the evidence for such biomembrane expansion comes from the work on erythrocyte membrane, where it was seen that addition of CPZ to erythrocyte ghost enlarges the spheres and membrane expansion amounts to 1.5 per cent to 3 per cent increase in membrane area. One possible mechanism of membrane expansion can be that CPZ by binding to membrane displaces membrane bound Ca^{2+} which normally keeps the membrane rigid. As the membrane bound Ca^{2+} is displaced the membrane expands (Seeman, 1972).

2. **Membrane fluidization**

CPZ causes fluidization or disordering of the membrane as detected by ESR and NMR techniques (Seeman, 1972; Miller and Miller, 1975).

3.   Changes in membrane shape

Effect of CPZ and other anesthetics on cellular morphology have been extensively studied in erythrocytes (Sheetz, 1976; Salhab and Dujovne, 1986; Lieber *et al.*, 1984), rat hepatocytes (Salhab and Dujovne, 1986) and polymorphonuclear lymphocytes. CPZ imposed invaginations on red cell membrane (Sheetz *et al.*, 1976).

Microorganisms such as *Bacillus*, pathogenic fungi such as *Candida* and protozoans such as *Tetrahymena* have been used to study the action of CPZ and other related compounds. Klubes *et al.* (1971) have found that CPZ inhibits cell wall synthesis in *Bacillus megalirium*. Growth, motility, glucose utilization and phosphate uptake of *Tetrahymena pyriformis* have been reported to be inhibited by low concentrations of CPZ (Kuznicki *et al.*, 1979). CPZ has been found to have an antifungal activity. (Myskosen, *et al.*, 1987). Further, CPZ inhibits *invitro* growth of *Candida albicans* (Eelam *et al.*, 1987; Wood and Nugent, 1985). CPZ has also been found to act as an antiamoebic agent (Raul *et al.*, 1986).

Besides its action on membrane there are evidences from other studies that CPZ acts on intracellular organelles as well. These include:

1)   Mitochondria

CPZ leads to disintegration of inner membrane system of mitochondria of liver and brain (Takeichi *et al.*, 1987).

2)   Lysosomes

CPZ and other anesthetics causes disruption of lysosome membrane thus causing the lysosomal enzymes to leak out from the cells to the

surrounding medium (Salhab and Dujovne, 1986).

3)   Microfilaments and Microtubules

These subcellular structures are responsible for cellular movements. CPZ and its metabolites profoundly alters polymerization and gelation of F-actin leading to microfilament dysfunction (Elias and Boyer, 1978).

CPZ has been found to have a mutagenic activity (Ebor and Akerele, 1988).   Results indicated that CPZ required photoactivation for its mutagenic activity. CPZ was found to reverse some of the tester bacterial strains   from tryptophan and histidine dependence to independence respectively.

Not only membrane structure, but also a number of membrane bound enzymes are altered by CPZ. These include phosphodiesterase, adenylate cyclase, Na^+K^+ ATPase, Mg^{++} ATPase and cytochrome oxidase (Levin and Weiss, 1979; Seeman, 1972).

Effect of Anesthetics on Ca^{2+} and Calmodulin

Local anesthetic amine (CPZ) displaces Ca^{2+} from the membrane (Low et al., 1979; Corps et al., 1982). One membrane bound Ca^{2+} can compete with 2 amine molecules. At the same time cationic anesthetics e.g. CPZ inhibit the transmembrane influx of Ca^{2+} (Seeman, 1972). Calmodulin, an ubiquitous Ca^{2+} binding protein is inhibited by CPZ (Roufgalis, 1981). The Ca^{2+} calmodulin complex in turn binds to and activates a number of enzymes. Biological activity of calmodulin is strongly inhibited by CPZ which appears to bind to the Ca^{2+} calmodulin complex and thus prevents the

ternary complex from activating the enzymes (Volpi *et al.*, 1981). Hence the pharmacological actions of CPZ which are directed against Ca^{2+} dependent cellular processes may be due to its effect on calmodulin.

From the reports in the literature it is clear that CPZ is a lipid soluble drug which pocesses surface active properties by virtue of which it can bring about a number of perturbation in the membrane architecture and functions. So it is quite clear that interfering with membrane associated functions in early *D. discoideum* development using CPZ might help us in understanding the problems of cell-cell interaction and cell communication during development for which this organism provides an excellent model.

<center>MATERIAL AND METHODS</center>

MATERIAL

The experimental organism for the present work is *Dictyostelium discoideum* popularly known as "cellular slime mould". The axenic strain (Ax2) obtained from Dr. Robert Kay, Medical Research Council, Cambridge, U.K., was used.

METHODS

1. CULTURE CONDITIONS

D. discoideum cells can be grown under different culture conditions. The growing cells can be maintained in a liquid suspension and also on a solid agar substratum together with bacteria as food organisms.

(a) Preparation of Culture Medium (HL5 medium)

It has the following composition (Ashworth and Watts, 1970):

Proteose Peptone (Difco)	-	14.3 gm
Yeast extract (Difco)	-	7.5 gm
$Na_2HPO_4.2H_2O$ (BDH)	-	0.616 gm
$KH_2PO_4.2H_2O$ (BDH)	-	0.486 gm
Maltose (Merck)	-	16 gm

The above constituents were added to 1 litre of distilled H_2O and pH adjusted to 6.7. The HL5 medium (100ml) was then dispensed into 250 ml capacity Erlenmeyer flasks and autoclaved at 15 lbs for 20 minutes.

(b)   Phosphate Agar (P-agar)

0.017M Sorenson's phosphate buffer (pH 6.3) was made from the stock solutions in the following way:

a) $Na_2HPO_4.2H_2O$ ---------- 11.87 gm in 1000 ml distilled H_2O.

b) $KH_2PO_4.2H_2O$ ---------- 9.07 gm in 1000 ml distilled H_2O.

20 ml of (a) and 80 ml of (b) were mixed together to give 100 ml of p-buffer, pH 6.3.

10 gm of Difco agar was added to 500 ml of p-buffer to give a 2 % agar medium. 20ml of the autoclaved medium was then poured into sterile petridishes (100 mm) and allowed to solidify.

(c)   Nutrient Agar (N-agar)

Agar (Difco)   -   20 gm

Proteose Peptone (Difco)   -   1 gm

Glucose (Glaxo)   -   1 gm

The above constituents were added to 1 litre of Sorenson's buffer (pH 6.3) and autoclaved. 20ml of the medium was then poured into 100 mm petridishes and allowed to solidify.

(d)   Chlorpromazine Agar (CPZ-agar)

Chlorpromazine hydrochloride (Sigma) stock solution ($1x10^{-2}M$) was prepared in p-buffer and passed through sterile filter and stored in sterile vial. The stock solution was kept in dark. 2% p-agar was autoclaved and when it cooled down to 37°C and appropriate amount of CPZ

stock solution was added to it to give a specific concentration of CPZ agar. The agar medium was then mixed thoroughly and 20ml of agar was poured into petridishes (50 mm) and allowed to solidify. The plates were kept in dark.

From the 10^{-2}M CPZ stock solution the dilutions were made in the following way for 20 ml CPZ agar solution:

Dose of CPZ	2% agar solution (ml)	CPZ solution (μl)
1×10^{-3}M	18.0	2000
5×10^{-4}M	19.0	1000
1×10^{-4}M	19.8	200

(e)   CaCl$_2$ Agar

CaCl$_2$ was added to 100ml of p-buffer to give a final concentration of 500 mM and 1000 mM respectively. To this 2 gm of agar was added and autoclaved. 20ml of the CaCl$_2$ agar was poured into 50 mm petridishes and allowed to solidify.

2.   CELL CULTURE

(a)   Growth of Ax2 Cells in HL5 Medium

Spores of Ax2 cells were inoculated for germination into sterile HL5 medium contained in a 25ml capacity Erlenmeyer flask and maintained at 22^{0}C. After 3-4 days incubation at 22^{0}C on an orbital shaker at 150rpm, the pre-cultures which contained germinated amoebae were used to inoculate 30ml of HL5 medium. Serial transfers were made to HL5 medium thrice a week to provide a regular supply of Ax2 cells.

Multiwell test plates containing HL5 medium were also used for spore germination. This method provided a convenient way for the live observation of germinating amoebae, as the multiwell plate (containing amoebae) could be visualized under an inverted microscope. When amoebae grew upto certain density they were transferred from the multiwell plate into sterile 20ml of HL5 medium contained in an Erlenmeyer flask.

(b)   Growth and Differentiation of Ax2 Cells on Nutrient Agar

E.coli HB101 was grown overnight at 37^0C in 30ml of sterile Luria broth:

Luria broth Composition:

NaCl (Glaxo)	-	10 gm
Bactopeptone (Difco)	-	10 gm
Yeast extract (Difco)	-	5 gm

The above constituents were dissolved in 1 litre of distilled water and pH adjusted to 7.5 and autoclaved. Under sterile conditions, a loopfull of *E. coli* was transferred to the luria broth and allowed to grow at 37°C for overnight.

0.2ml of *E. coli* suspension was spread evenly with the glass spreader on the nutrient agar. 0.1ml of Ax2 cells were then uniformly spread on the bacterial lawn under sterile conditions. The plates were maintained at 22-23°C. Within 4 days the Ax2 cells differentiate into fruiting bodies.

(c)   Differentiation of Ax2 Cells on Phosphate Agar

D. discoideum cells gowing in exponential phase were harvested from HL5 medium by pelleting down at 1000 rpm at 4°C and after thorough washing in p-buffer, the cells were resuspended in p-buffer with a final cell density of 5x10^6 cells/ml. 300μl of the cell suspension was then spread uniformly on p-agar and were allowed to differentiate at 22°C.

(d)   Spore Collection and Inoculation

Spores were collected from the agar surface in the following ways:

(i)   5 ml of p-buffer was placed on the agar containing the fully differentiated fruiting bodies. The surface of the agar was then scraped off with a glass spreader and the contents were dispensed in sterile vials and stored at -20°C.

(ii)   Agar plates containing fruiting bodies were inverted over another plate containing p-buffer and gently tapped. The fallen spores were collected in p-buffer and stored in sterile vials.

-Before inoculation of the spores, the vials containing the spores were thawed. The spores were given a thorough wash with sterile phosphate buffer and resuspended at a density of 2x10^4 spores/ml. 0.3ml of the spore suspension was used to inoculate 20ml of sterile HL5 medium contained in an Erlenmeyer flask.

3.   CHLORPROMAZINE TREATMENT

Exponentially growing *D. discoideum* amebae were subjected to Chlorpromazine (CPZ) treatment in the following manner:

CPZ Solution

0.0148gm of Chlorprmazine hydrochloride was dissolved in 4ml of sterile p-buffer and pH adjusted to 6.3 so as to prepare a $1 \times 10^{-2}M$ CPZ stock solution. The stock solution was passed through a sterile filter and stored in a sterile vial in dark. The stock was diluted with p-buffer just before the start of the experiment so as to give different CPZ concentrations ($5 \times 10^{-4}M$, $1 \times 10^{-4}M$, $1 \times 10^{-3}M$ and $5 \times 10^{-3}M$).

The following experiments were conducted:

1. **SURVIVAL OF CPZ TREATED Ax2 CELLS IN P-BUFFER**

    (a) **Varying Concentrations of CPZ**

    Ax2 cells from mid log phase were harvested by pelleting down at 1000 rpm at 4°C and resuspended in CPZ solution to give a final cell density of 5×10^6 cells/ml. The cells were exposed to different concentrations of CPZ ($1 \times 10^{-2}M$, $5 \times 10^{-3}M$, $1 \times 10^{-3}M$, $5 \times 10^{-4}M$ and $1 \times 10^{-4}M$) for a desired period of time in dark. The cells were then washed four to five times with p-buffer. The cell count was taken with a haemocytometer and the final cell suspension was made in p-buffer to give a density of 5×10^6 cells/ml. The cells were kept shaken at 120 rpm. A survival curve was plotted by counting the number of cells using haemocytometer at different time intervals (t_0, t_4, t_6, t_8 and t_{24}).

    (b) **Varying Duration of CPZ Treatment**

    Exponentially growing *D. discoideum* cells from HL5 medium were

pelleted down at 1000 rpm and resuspended in CPZ solution at a density of 5×10^6 cells/ml. The cells were treated with a fixed dose of CPZ (1×10^{-4}M, 5×10^{-4}M, 1×10^{-3}M, 5×10^{-3}M and 1×10^{-2}M) in dark while varying the duration of CPZ treatment (5, 10, 15 and 20 min). The cells were washed four to five times with p-buffer and resuspended in p-buffer at a density of 5×10^6 cells/ml. The cell count was taken using a haemocytometer and a survival curve was plotted.

2. GROWTH AND SURVIVAL OF CPZ TREATED Ax2 CELLS IN HL5 MEDIUM

1×10^{-2}M stock solution of CPZ was made in HL5 medium and pH adjusted to 6.3. The CPZ stock solution was diluted to give different CPZ concentrations (1×10^{-4}M, 5×10^{-4}M, 1×10^{-3}M and 5×10^{-3}M). *D. discoideum* cells from mid log phase were harvested by pelleting down at 4°C and resuspended in CPZ solution at a density of 5×10^6 cells/ml. The cells were treated with CPZ in dark for 10 min and washed thoroughly four to five times with HL5 medium.

One lot of CPZ treated Ax2 cells and similar number of control (untreated) cells were put in a multiwell plate containing HL5 medium. The cells were observed at regular time intervals under an inverted microscope. In another set of experiment a survival curve was made by observing the survival upto the sixth day after inoculation of the CPZ treated and control cells into 15 ml of HL5 medium contained in an Erlenmeyer flask.

3. DIFFERENTIATION OF CPZ TREATED Ax2 CELLS ON NUTRIENT AGAR AND PHOSPHATE AGAR

In order to check for the differentiation of CPZ treated cells, they were plated both on n-agar and p-agar. For this, the Ax2 cells were pelleted down and exposed to different concentrations of CPZ ($1x10^{-3}$M, $5x10^{-3}$M, $5x10^{-4}$M $1x10^{-4}$M) for 10 minutes and washed thoroughly with p-buffer. Finally the cells were resuspended at a density, of $5x10^6$ cells/ml in p-buffer. The cells were kept shaken at 120 rpm and 200 ml of the suspension was plated at different time intervals (t_0 and t_6) both on p-agar and *E.coli* seeded n-agar and maintained at 22°C.

4. DIFFERENTIATION OF Ax2 CELLS ON CPZ AGAR

Ax2 cells were pelleted down from the exponential phase of their growth and resuspended in p-buffer (pH 6.3) at a density of $5x10^6$ cells/ml. 200μl of the cell suspension was then spread evenly on the CPZ ($5x10^{-3}$M, $1x10^{-3}$M, $5x10^{-4}$M, $1x10^{-4}$M) agar plates and kept at 22°C.

5. SPORE FORMATION IN CPZ TREATED Ax2 CELLS

Ax2 cells treated with CPZ ($1x10^{-4}$M and $5x10^{-4}$M) were plated both on p-agar and *E. coli* seeded n-agar plates. The plates were kept at 22°C and the cells were allowed to differentiate into fruiting bodies. Spores were scraped off from the agar surface and resuspended in p-buffer. The number of spores formed were counted using a haemocytometer.

6.   SPECIALIZED ASPECTS OF CPZ TREATMENT

(a)   Coculture Experiment

The cells were pelleted down and exposed to different doses of CPZ for 10 min. The cells were then resuspended in p-buffer at a density of 5×10^6 cells/ml. CPZ treated cells were mixed with untreated (control) cells in a 1:1 ratio. The cell suspension was kept shaken for 6 hrs and later plated on p-agar.

(b)   Supernatant Experiment:

The cells were treated with different doses of CPZ and kept shaken at 120rpm for 6 hrs in p-buffer. The cells were pelleted down and the supernatant was collected. The supernatant of untreated cells was also collected in a similar way and kept frozen at $-20^{\circ}C$. The following experiments were then carried out:

(i)   Exponentially growing Ax2 cells were pelleted down and resuspended in the supernatant of the CPZ treated cells. The cell suspension was kept shaken for 6 hrs and then plated on p-agar.

(ii)   Exponentially growing Ax2 cells were pelleted down and exposed to different doses of CPZ and resuspended in the supernatant collected from the control cells. The CPZ treated cells were kept shaken for 6 hrs and later plated on p-agar.

(c)   Development of CPZ Treated Ax2 Cells in Presence of $CaCl_2$

 i) Supplementation of $CaCl_2$ to the CPZ Treated Cell Suspension

  Cells were exposed to different doses of CPZ and washed three to four time with p-buffer. The cells were finally resuspended in p-buffer supplemented with $CaCl_2$ (100mM, 500mM and 1000mM) and kept shaken for 6 hrs. The cells were then plated on p-agar.

 ii) $CaCl_2$ Agar

  In another set of experiment Ax2 cells were pelleted down and treated with different doses of CPZ. The CPZ treated cells were washed with p-buffer and finally resuspended in p-buffer at a final cell density of 5×10^6 cells/ml. The CPZ treated cells were later plated on $CaCl_2$ (500mM and 1000mM) agar plates.

(d)   Development of CPZ Treated Ax2 Cells in Presence of cAMP

Ax2 cells were treated with different doses of CPZ and resuspended in p-buffer supplemented with 1 mM cAMP. The cells were kept shaken for 6 hrs and then plated on p-agar.

7. LIVE OBSERVATIONS AND LIGHT MICROSCOPIC OBSERVATIONS OF CPZ TREATED Ax2 CELLS

(a)   Morphology

Cell suspension of both CPZ (1×10^{-4}M, 5×10^{-4}M, 1×10^{-3}M and 5×10^{-3}M) treated and control cells were placed on a clean glass slide.

Extra fluid was drained off. The cells were fixed with spraycyte and stained with phosphate buffered Giemsa solution, air dried and mounted in DPX. The slides were photographed for permanent record.

(b)   **Cell Motility and Aggregation**

Cells treated with different CPZ doses were resuspended in p-buffer and HL5 medium separately and then were put in multiwell plates. The multiwell plates containing the CPZ treated cells were then observed under an inverted microscope.

(c)   **Check for EDTA Stable Cell Contacts**

Both the control and CPZ treated Ax2 cells were resuspended in p-buffer at a density of $1x10^7$ cells/ml and kept shaken for 6 hrs. $190\mu l$ of the cell suspension was taken and $10\mu l$ of 200 mM EDTA solution was added to it so as to give a final concentration of 10 mM EDTA. The cell suspension was kept shaken for 1 hr at 160 rpm. After 1 hr the cell suspension was put on haemocytometer and the number of cellular clumps/aggregate formed were counted. The EDTA treated cell suspension was also put on a clean glass slide and fixed with spraycyte fixative, stained with Giemsa and mounted in DPX. The slides were photographed.

(d)   **Chemotaxis**

Sterile 20ml of p-agar was poured in 50mm petridish and

allowed to solidify. Small wells were made in the agar by scooping out the agar. The wells were filled with 1×10^{-4} M cAMP solution. 6 hr old starved Ax2 cells (both control and CPZ treated) suspended in p-buffer were placed at a distance of 1cm away from the cAMP well (Konijn and Van Haastert, 1987). The chemotactic movement of the cells towards the cAMP well was checked after every hour.

7.   cAMP DEPENDENT EXTRACELLULAR PHOSPHODIESTERASE ASSAY

For determination of extracellular phosphodiesterase (ePDE) activity the Ax2 cells were pelleted down and treated with different doses of CPZ, washed and resuspended in p-buffer. The cell density was adjusted to 1×10^{7} cells/ml and the cell suspension was kept shaken. The cells were pelleted down at different time intervals t_6, t_8 and t_{12}. The supernatant was then collected and stored at -20°C. The PDE assay was done according to the method of Riedel and Gerisch (1971). To $50 \mu l$ of the supernatant, $250 \mu l$ of 0.1M. Triethanolamine (TEA) was added. To this $600 \mu l$ of assay mixture was added and incubated at 35°C for 10 minutes. The assay mixture contained 37.5ml of 0.1M TEA (pH 7.5), 1ml of 0.4M $MgSo_4$, 40ml of adenosine deaminase and 30ml of alkaline phosphatase. After incubation, $15 \mu l$ of 3mM cAMP was added and the change of extinction at 265nm was recorded with Shimadzu UV-260 spectrophotometer against a blank containing the complete reaction mixture except cAMP.

CHEMICALS USED

Chlorpromazine hydrochloride, EDTA, $CaCl_2$, cAMP, alkaline phosphatase and adenosine deaminase were purchased from M/s Sigma chemicals company, St. Louis, U.S.A.

TEA and Maltose were purchased from Merck company, Germany. Proteose peptone, Bactopeptone, Agar and Yeast extract were purchased from Difco Chemicals Company, U.S.A. Giemsa stain was procured from British Drug House Limited, England and Spraycyte was obtained from Clay Adams, Dickinson and Company, U.S.A.

Na_2HPO_4, KH_2PO_4, Glucose, NaCl, $MgSo_4$ etc. were of "Guranteed Reagent, AnalaR Grade" from M/s BDH, India, and M/s Glaxo, India.

OBSERVATIONS AND RESULTS

Chlorpromazine hydrochloride (CPZ) was used in the present work to study the effects of this drug on the early development of *D. discoideum*.

1.   SURVIVAL OF CPZ-TREATED Ax2 CELLS IN P-BUFFER

    a)   Varying the Concentration of CPZ

        Ax2 cells were exposed to different doses of CPZ for 10 min and the percentage survival was calculated. CPZ doses higher than $1x10^{-2}M$ were found to cause cell lysis and cell death. Approximately 15% survival was observed at $1x10^{-2}M$ CPZ treatment.   The survival was found to be highest at $1x10^{-4}M$ CPZ treatment (Fig.1).

    b)   Varying the Duration of CPZ Treatment

        Ax2 cells were treated with CPZ for different durations and the percentage survival was calculated. Ten minutes treatment was found to be suitable for our experimental purpose since the percentage survival of the cells were appreciably high as compared to other time durations (Fig. 2).

2.   GROWTH AND SURVIVAL OF CPZ TREATED Ax2 CELLS IN HL5 MEDIUM

        CPZ treated Ax2 cells were put back into HL5 medium and a survival curve was plotted. Cells treated with CPZ doses higher than $5x10^{-3}M$ showed negligible growth while the growth rate was slower in $1x10^{-4}M$ and $5x10^{-4}M$ CPZ treatments as compared to the control (Fig.3).

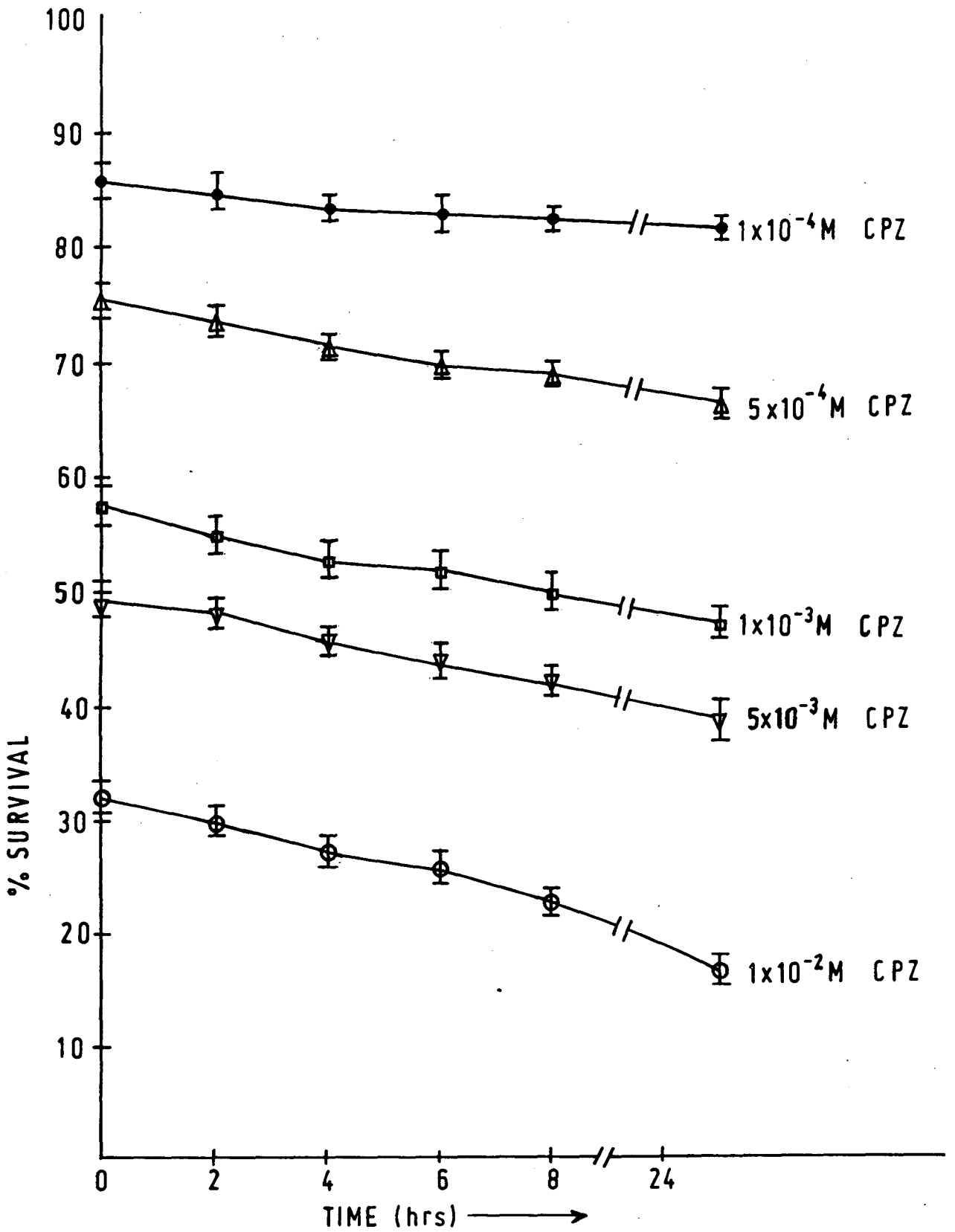Fig.1   Survival curve of *D. discoideum* cells at different CPZ concentrations in p-buffer.

Fig.1

Fig.2 · Survival curve of *D. discoideum* cells at varying duration of CPZ treatment in p-buffer.
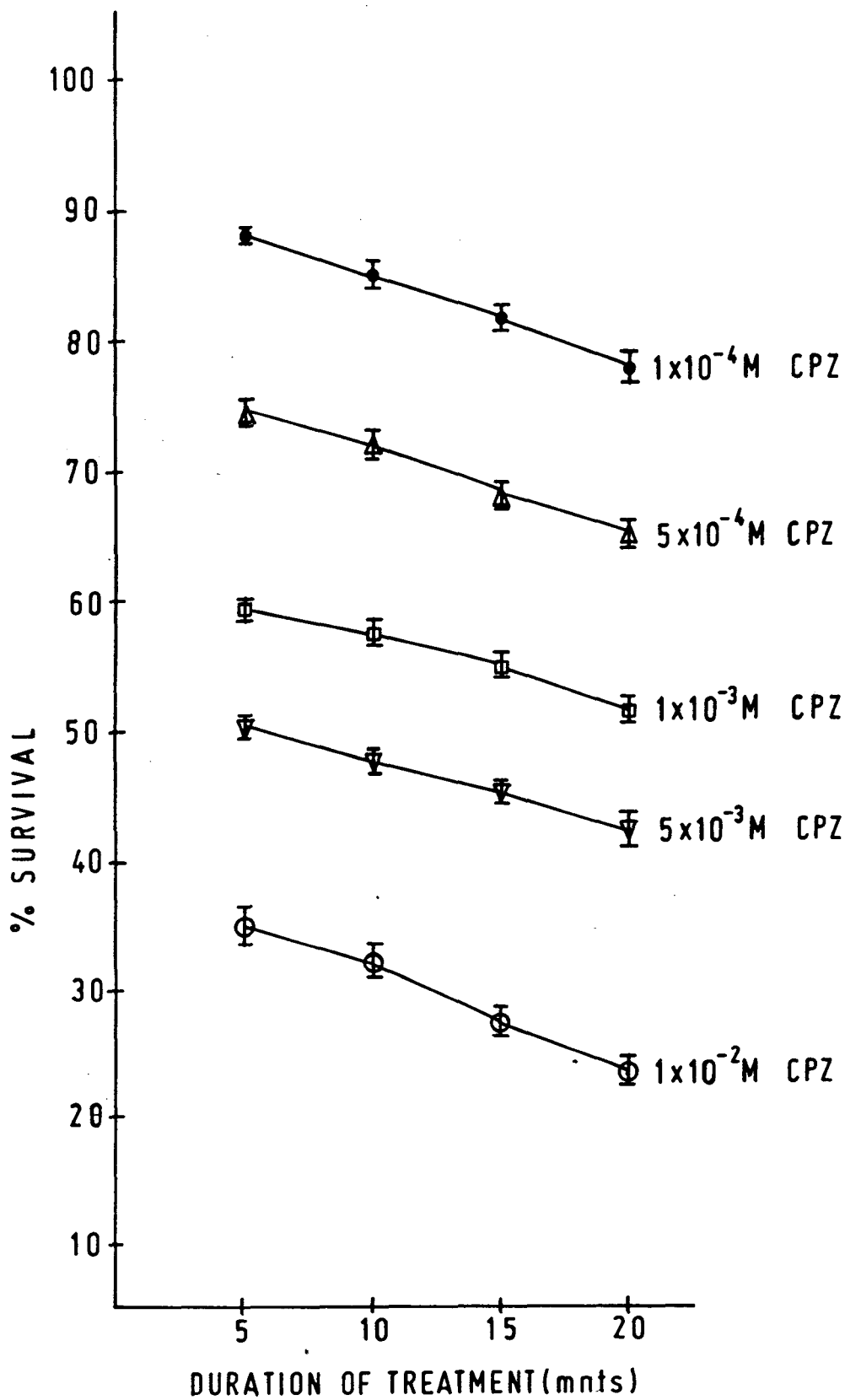
Fig. 2

41

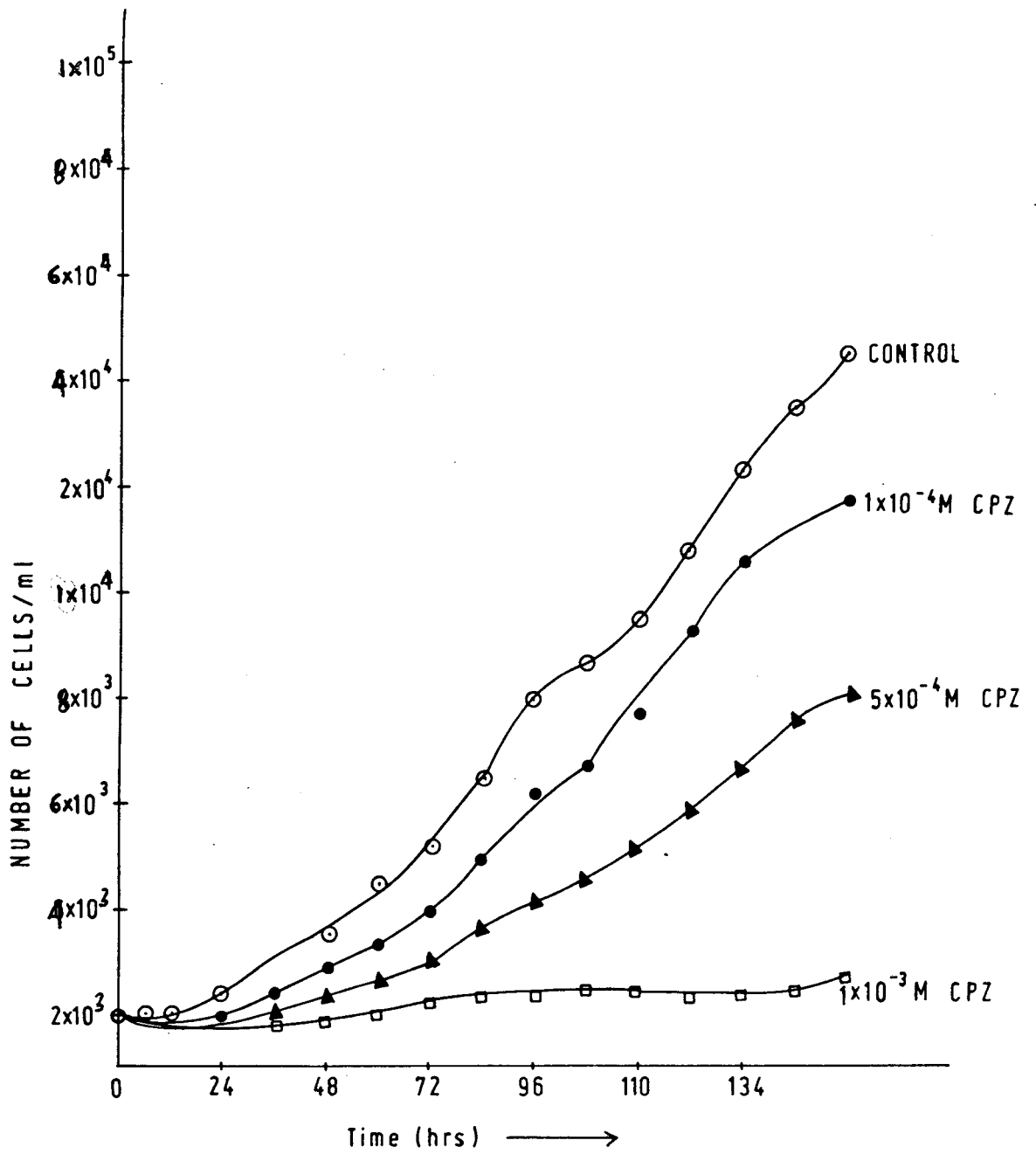Fig.3   Growth and survival of control and CPZ treated *D. discoideum* cells
in HL5 medium.

Fig. 3

3. DIFFERENTIATION OF Ax2 CELLS ON NUTRIENT AGAR

Growth and differentiation of CPZ treated cells was checked by plating the cells on n-agar seeded with *E.coli*. Control cells completed their morphogenesis within 50 hrs of plating. Cells treated with CPZ doses $1x10^{-3}M$ and above showed no morphogenesis when plated on n-agar, i.e. there was no plaque formation, streaming was absent and there was no fruiting body formation (Table 1). When Ax2 cells were treated with $1x10^{-4}M$ and $5x10^{-3}M$ CPZ, the morphogenesis was delayed by 18 hrs and 36 hrs respectively as compared to control (Fig. 4,5 and 6). Further the number of slugs and fruiting bodies formed were fewer in CPZ ($1x10^{-4}M$, $5x10^{-4}M$) treated cells as compared to the control (Fig. 7 and 8).

Table 1. Differentiation of CPZ Treated Ax2 Cells on Nutrient Agar.

	Development Stages				
	Plaque Formation	Streaming	Aggregate Formation	Slug stage	Fruiting body Formation
Control	+ (20-24 hrs)	+ (30-34 hrs)	+ (36-40 hrs)	+ (42-46 hrs)	+ (48-50 hrs)
$5x10^{-3}M$ CPZ	-	-	-	-	-
$1x10^{-3}M$ CPZ	-	-	-	-	-
$5x10^{-4}M$ CPZ	± (48-50 hrs)	± (52-54 hrs)	± (60-62 hrs)	± (72-78 hrs)	± (80-86 hrs)
$1x10^{-4}M$ CPZ	± (32-36 hrs)	± (40-46 hrs)	± (46-50 hrs)	± (56-58 hrs)	± (60-68 hrs)

+ present, - absent, ± partial and delayed. Numbers in parenthesis indicate the time of development.

Fig.4: (a) . Photomicrograph of control *D. discoideum* cells showing streaming (St) and aggregate (Ag) formation on n-agar (approx. 36 hrs of development). x 100.

(b) Photomicrograph of CPZ (1×10^{-4}M) treated *D. discoideum* cells showing plaque formation on n-agar. (approx. 36 hrs of development). x 100.
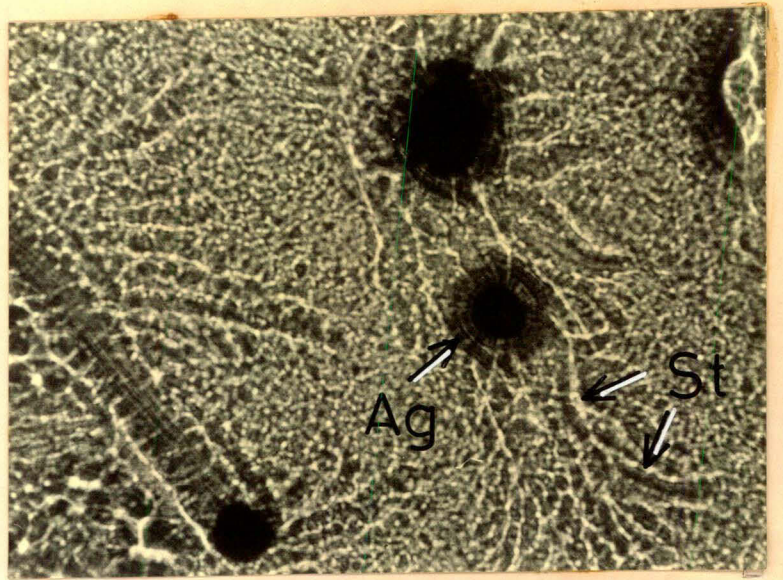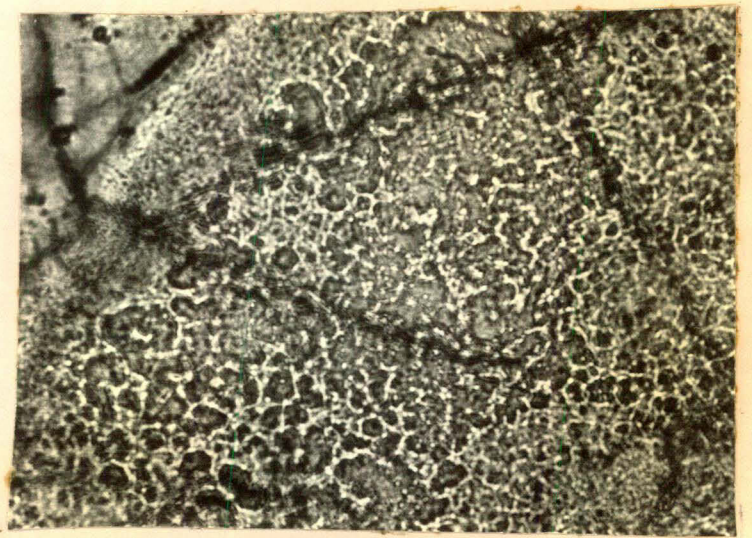
Fig. 4 (a)

Fig. 4 (b)

Fig.5: (a)   Photomicrograph of control *D. discoideum* cells showing the formation of slugs (S) on n-agar (approx. 46 hrs of development). x 100.

(b)   Photomicrograph of CPZ (1×10^{-4}M) treated cells showing aggregation and streaming (St) on n-agar (approx. 46 hrs of development). x 100.
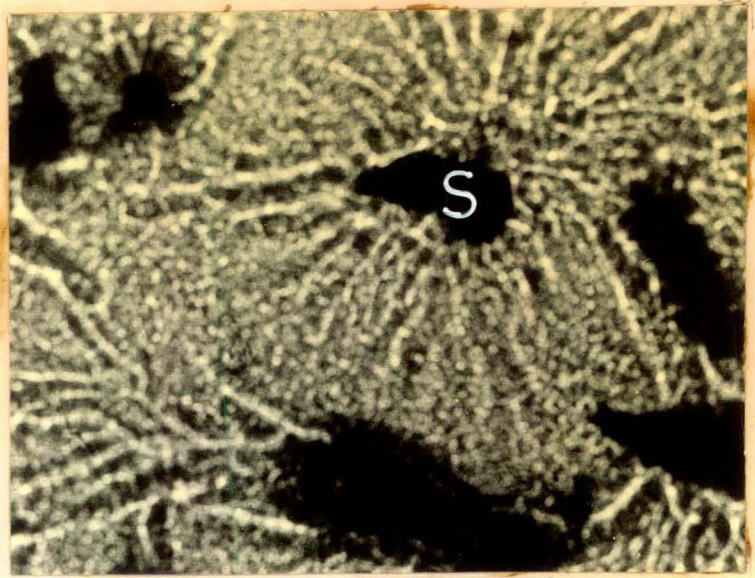
Fig. 5 (a)
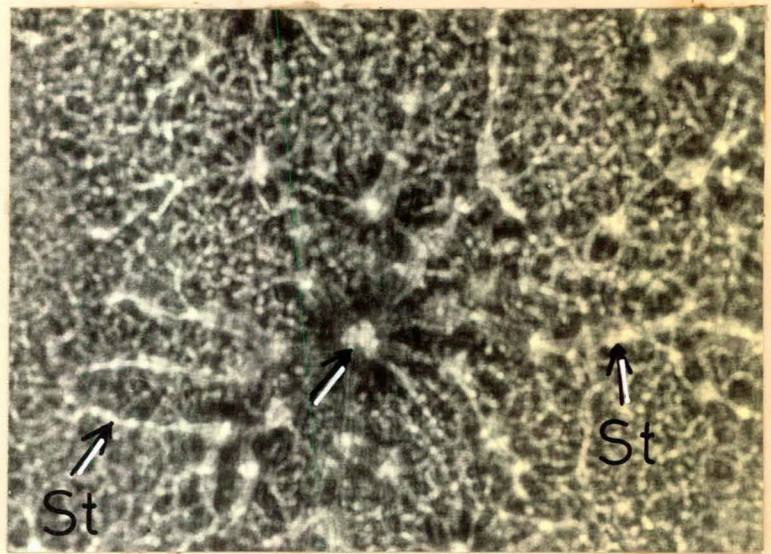
Fig. 5 (b)

Fig.6: (a)   Photomicrograph of a group of fruiting bodies (F.B) of control *D. discoideum* cells formed on n-agar (approx. 56 hrs of development). x 80.

(b)   Photomicrograph of *D. discoideum* cells showing the formation of slugs (S) on n-agar (approx. 56 hrs of development). x 100.
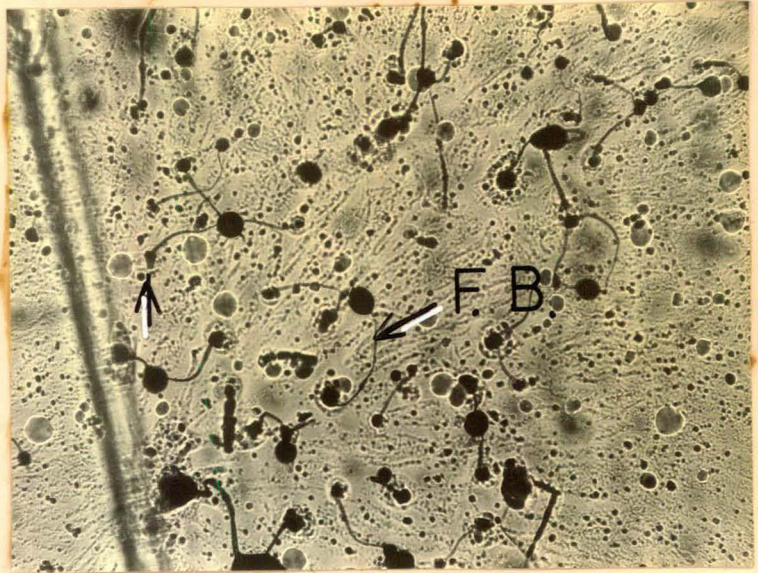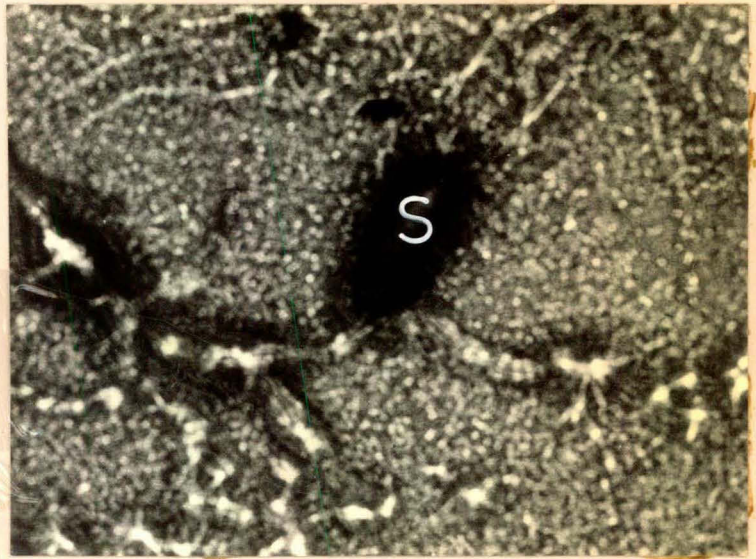
Fig. 6 (a)

Fig. 6 (b)

Fig.7   Photomicrographs of slugs (S) of *D. discoideum* formed on n-agar. Note the number of slugs (S) formed by control cells (7a) are more as compared to CPZ (1×10^{-4}M) treated cells (7b). x 100.
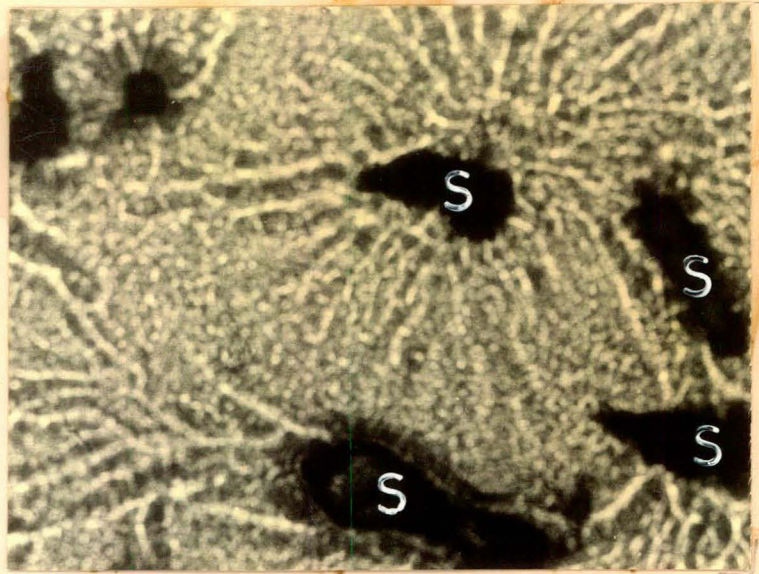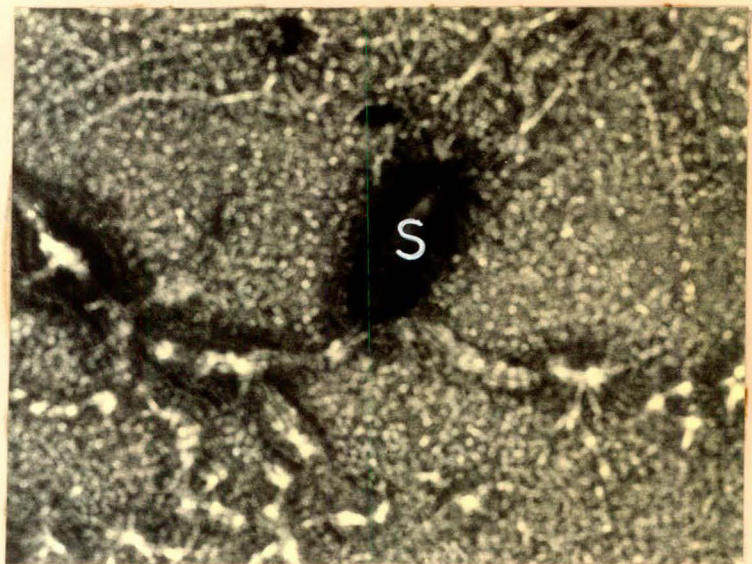
Fig. 7 (a)

Fig. 7 (b)

Fig.8   Photomicrograph of fruiting bodies (F.B) of *D. discoideum* formed on n-agar. Note the number of fruiting bodies formed by control cells (8a) are more as compared to CPZ (1×10^{-4}M) treated cells (8b). x 80.
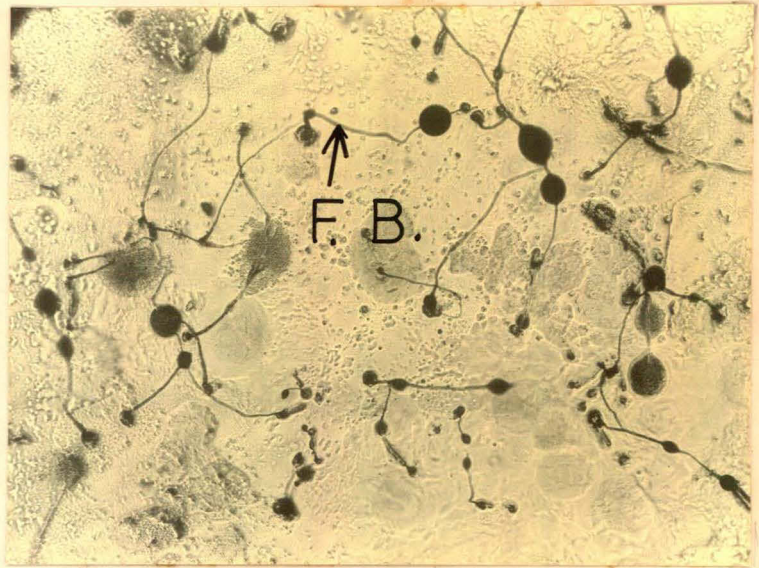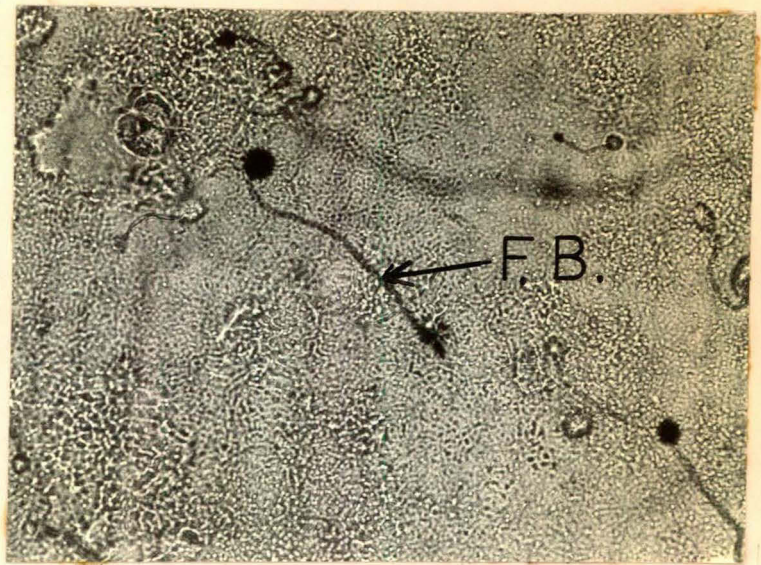
Fig. 8 (a)

Fig. 8 (b)

4. DIFFERENTIATION OF AX2 CELLS ON PHOSPHATE AGAR

Both the control and CPZ treated cells (5×10^6 cells/ml) were plated on p-agar. Control cells completed morphogenesis within 24hrs (Fig. 9a). Cells treated with CPZ doses, 1×10^{-3}M and above when plated on p-agar showed loose cell clumps but there was no streaming, slugs were absent and there was no fruiting body formation (Table 2; Fig. 9b and 10b). At lower doses of CPZ 1×10^{-4}M and 5×10^{-4}M the morphogenesis was completed by 48 hrs and 78 hrs respectively and this indicated that morphogenesis of CPZ treated cells was delayed as compared to the control. Further in comparison to control, the number of slugs and fruiting bodies formed were also less in CPZ (1×10^{-4}M and 5×10^{-4}M) treated cells (Fig. 10a and 10c).

Table 2: Differentiation of Control and CPZ Treated Ax2 Cells on Phosphate Agar

	Development Stages			
	Streaming	Aggregate Formation	Slug stage	Fruiting body Formation
Control	+ (8-10 hrs)	+ (12-16 hrs)	+ (18-20 hrs)	+ (24-30 hrs)
5×10^{-3}M CPZ	-	-	-	-
1×10^{-3}M CPZ	-	-	-	-
5×10^{-4}M CPZ	± (48-50 hrs)	± (52-54 hrs)	± (60-62 hrs)	± (72-78 hrs)
1×10^{-4}M CPZ	± (22-24 hrs)	± (28-30 hrs)	± (32-39 hrs)	± (44-48 hrs)

+ present, - absent, ± partial and also delayed. Numbers in parenthesis indicate the time of development.

Fig.9: (a)   Photomicrograph of control *D. discoideum* cells plated on p-agar. Note the streaming (St) and slug (S) formation (approx. 18hrs of development). x 40.

(b)   Photomicrograph of CPZ (1×10^{-4}M) treated *D. discoideum* cells plated on p-agar. Note the small loose clumps of cells and a few aggregates (approx. 18 hrs of development). x 40.
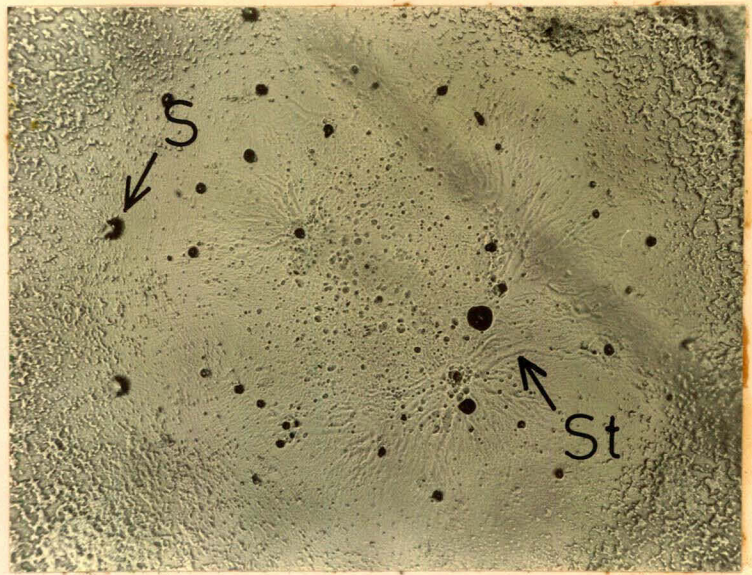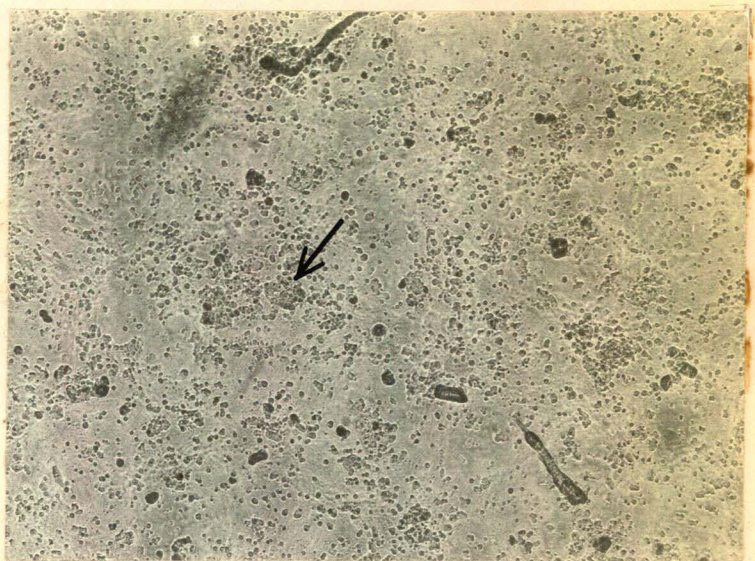
Fig. 9 (a)

Fig. 9 (b)

Fig.10: (a) Photomicrograph of fruiting bodies of *D. discoideum* formed plated on p-agar. ×10

(b) Photomicrograph of CPZ (5x10^{-3}M) treated *D. discoideum* cells plated on p-agar. Note the loose clumps of cells showing no differentiation. ×10.

(c) Photomicrograph of fruiting bodies formed by CPZ (1x10^{-4}M) treated *D. discoideum* cells. Note the number of fruiting bodies formed are less as compared to control cells (10a). ×10

Fig. 10 (a)

Fig. 10 (b)

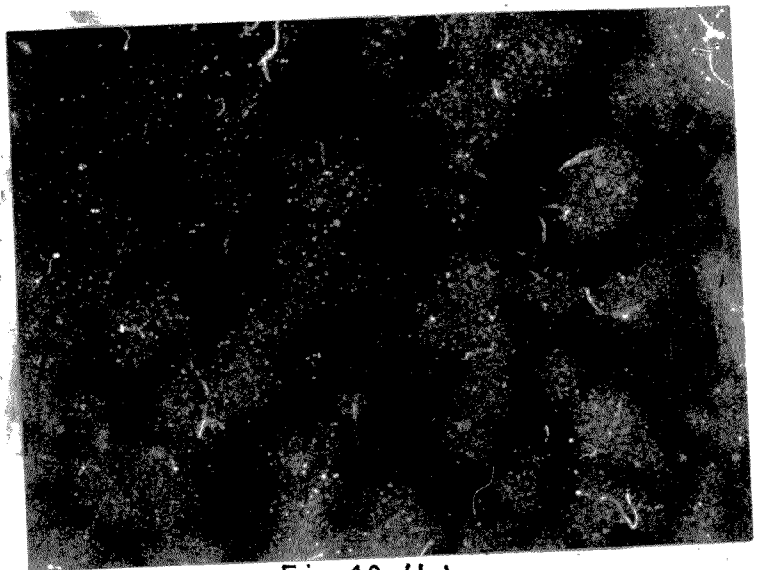Fig. 10 (c)

5. DIFFERENTIATION OF Ax2 CELLS ON CPZ AGAR

There was no morphogenesis when control Ax2 cells were plated on CPZ agar. The Ax2 cells formed loose clumps but did not form any slugs or fruiting bodies.

6. SPORE FORMATION IN CPZ TREATED Ax2 CELLS:

Spores formed by CPZ (1×10^{-4}M and 5×10^{-4}M) treated cells were scraped off from the agar surface, resuspended in p-buffer and counted using a haemocytometer. The following were observed:

a) The number of spores formed on n-agar was more as compared to the spores formed on p-agar. (Fig. 11)

b) The number of spores formed by CPZ (1×10^{-4}M and 5×10^{-4}M) treated cells were less as compared to the control cells (Fig.11). Further the number of spores formed by 5×10^{-4}M CPZ treated cells were less as compared to 1×10^{-4}M CPZ treated cells.

7. SPECIALIZED ASPECTS OF CPZ TREATMENT

a) Coculture Experiment

When CPZ treated cells and control cells were cocultured together on p-agar, it was observed that CPZ dose 5×10^{-3}M, inhibited the development of control cells (Fig. 12a). The development of control cells was delayed by 30 hrs and 12 hrs, when cocultured with 1×10^{-3}M and 5×10^{-4}M CPZ treated cells respectively. The morphogenesis occurred normally when the control cells were cocultured with 1×10^{-4}M CPZ treated cells (Table 3).

Fig.11   Spores formed by control and CPZ treated *D. discoideum* cells plated on p-agar and n-agar.
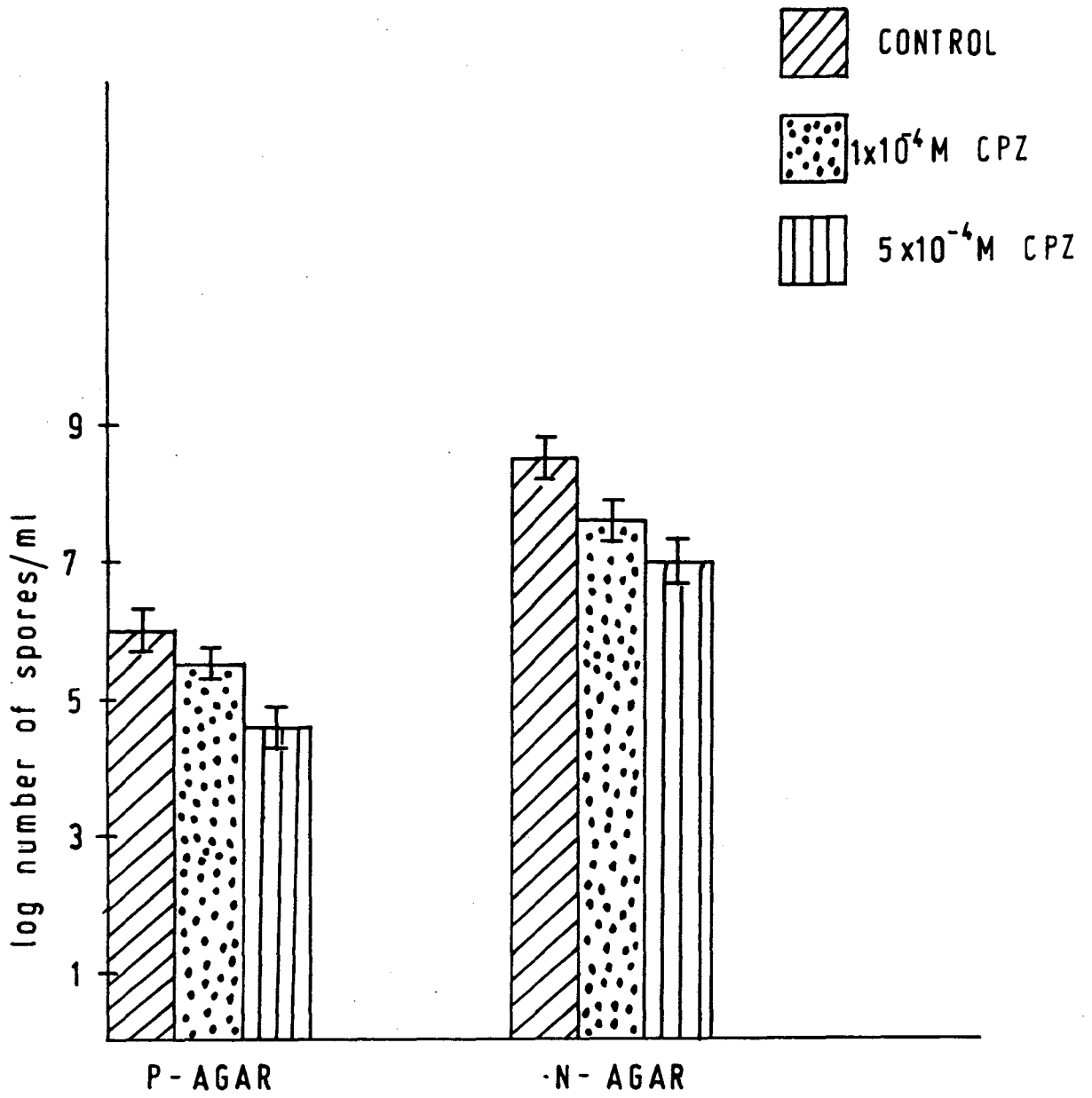
Fig. 11

Fig.12: (a) Photomicrograph of control and CPZ (5×10^{-3}M) treated *D. discoideum* cells cocultured together in 1:1 ratio on p-agar. Note the loose patches of cells showing no differentiation (approx. after 30 hrs of development) x 60.

(b) Photomicrograph of control *D. discoideum* cells showing fruiting bodies (F.B) formed on p-agar (approx. after 30 hrs of development). x 40.
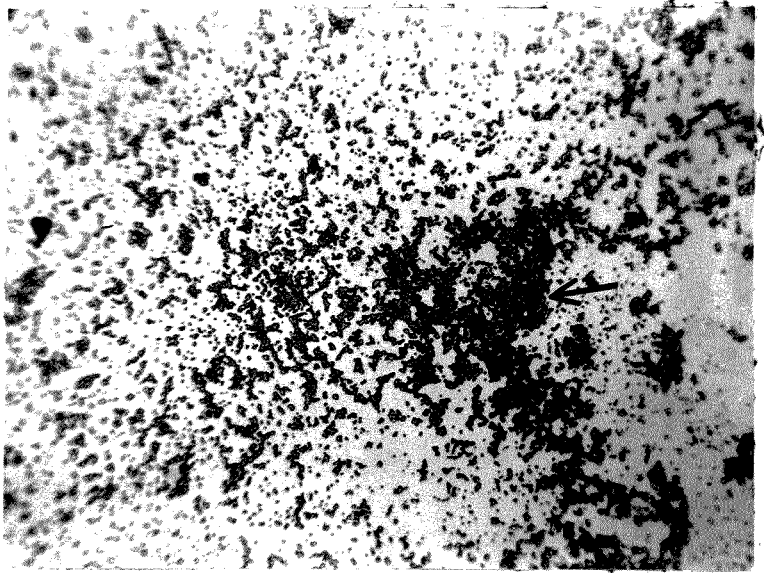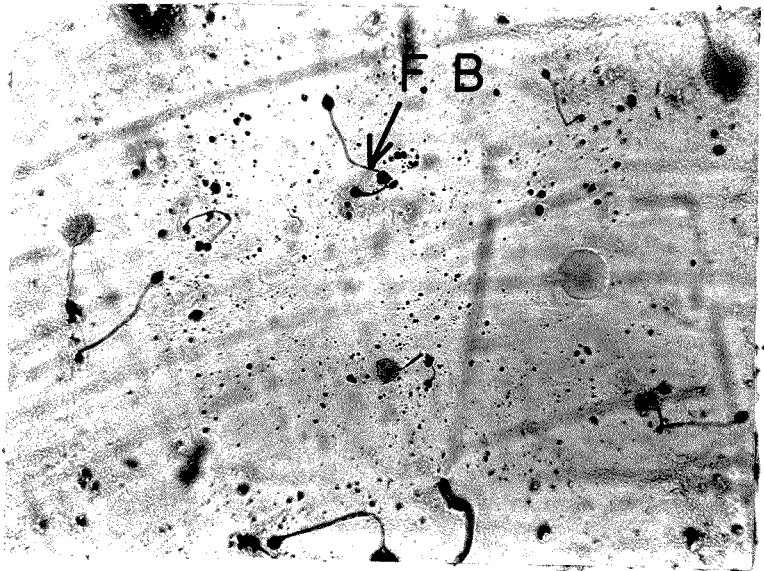
Fig. 12 (a)

Fig. 12 (b)

Table 3: Coculturing of Control and CPZ Treated Ax2 Cells on Phosphate Agar

Control Cell +	Development Stages			
	Streaming	Aggregate Formation	Slug stage	Fruiting body Formation
Control Cell	+ (8-12 hrs)	+ (12-16 hrs)	+ (18-20 hrs)	+ (24-30 hrs)
5x10^{-3} M CPZ	-	-	-	-
1x10^{-3}M CPZ	± (38-44 hrs)	± (44-46 hrs)	± (48-54 hrs)	± (56-60 hrs)
5x10^{-4}M CPZ	± (20-26 hrs)	± (26-30 hrs)	± (30-36 hrs)	± (38-42 hrs)
1x10^{-4}M CPZ	+ (10-12 hrs)	+ (12-17 hrs)	+ (18-20 hrs)	+ (24-30 hrs)

+ present, - absent, ± delayed. Numbers in parenthesis indicate the time of development

b)   **Supernatant Experiment**

i)   Differentiation of control Ax2 cells resuspended in 6hr old supernatant obtained from CPZ treated Cells

Ax2 cells resuspended in supernatant obtained from cells treated with CPZ doses 5x10^{-3}M and above, showed no morphogenesis. The morphogenesis was delayed by 10hrs and 20 hrs when the cells were resuspended in the supernatant obtained from 1x10^{-3}M and 5x10^{-3}M CPZ treated cells respectively (Table 4).

Table 4: Differentiation of Control Ax2 Cells Resuspended in Supernatant of CPZ Treated Cells.

Control Cells Suspended in Supernatant of	Development Stages			
	Streaming	Aggregate Formation	Slug stage	Fruiting body Formation
Control Cell	+ (8-10 hrs)	+ (12-16 hrs)	+ (18-20 hrs)	+ (24-30 hrs)
5x10^{-3}M CPZ	-	-	-	-
1x10^{-3}M CPZ	± (36-40 hrs)	± (42-44 hrs)	± (44-48 hrs)	± (48-50 hrs)
5x10^{-4}M CPZ	± (24-27 hrs)	± (27-30 hrs)	± (32-36 hrs)	± (38-40 hrs)
1x10^{-4}M CPZ	+ (8-10 hrs)	+ (12-16 hrs)	+ (18-20 hrs)	+ (24-30 hrs)

+ present, - absent, ± delayed. Number in parenthesis indicate the time of development

ii) **Differentiation of CPZ treated Ax2 cells resuspended in 6hr old supernatant obtained from control cell.**

Supernatant of control cells in no way promoted morphogenesis of CPZ treated cells i.e. cells treated with higher doses of CPZ 1x10^{-3}M and 5x10^{-3}M showed no morphogenesis even though resuspended in the supernatant obtained from control cells.

c) **Development of CPZ Treated Ax2 Cells in Presence of CaCl$_2$**

i) **Calcium agar**

CPZ treated (1x10^{-3}M and 5x10^{-3}M) and control cells were plated on CaCl$_2$ (500 mM & 1000 mM) agar. Control cells completed morphogenesis within 24 to 26 hrs. While the morphogenesis of CPZ treated cells was not augmented when plated on CaCl$_2$ agar.

ii) Supplementation of $CaCl_2$ to the Cell Suspension

CPZ treated cells and control cells were kept shaken for 6 hrs in p-buffer supplemented with $CaCl_2$ (100 mM, 500 mM and 1000 mM) and then plated on p-agar. Cells treated with higher doses of CPZ ($1x10^{-3}M$ and $5x10^{-3}M$) showed no morphogenesis, indicating that supplying $CaCl_2$ in no way augmented morphogenesis of CPZ treated cells.

d) **Development of CPZ Treated Ax2 Cells in Presence of cAMP**

Supplementation of 1mM cAMP to CPZ treated cells kept in shaken suspension did not promote their morphogenesis, while control cells completed their morphogenesis within approximately 24 hrs. This implied that suplementation of cAMP in no way augmented morphogenesis of CPZ treated cells.

LIVE OBSERVATIONS AND LIGHT MICROSCOPIC OBSERVATIONS OF CYTOLOGICAL PREPARATIONS OF CPZ TREATED CELLS

a) **Effect of CPZ on Cell Morphology, Cell Motility and Aggregate Formation**

Multiwell plates containing the cell suspension in p-buffer and HL5 medium were used for live observations of the *D.discoideum* cells. Fixed cytological preparations were also used to study the cellular morphology of both the control and CPZ treated cells. CPZ treated cells were spherical in shape, and did not form any pseudopodia and stained darker (Fig. 13b and 14b) than the corresponding control cells which were amoeboid in shape and actively motile (Fig. 13a and 14a). CPZ treated cells also showed blebs on their cell surface (Fig. 15c). Cellular

exudate could also be seen in the fixed preparations of the CPZ treated cells (Fig. 15a and 15b).   The number of cellular aggregates formed were also counted using haemocytometer. At higher doses of CPZ ($1\text{x}10^{-4}$M and $5\text{x}10^{-4}$ M) fewer cell aggregates were formed as compared to the control Ax2 cells (Table 5; Fig. 16a and 16b).

Table 5: Effect of CPZ on Cell Morphology, Motility and Aggregate Formation

	Shape	Cell motility	Cellular Aggregate formation
Control	Amoeboid shape with prominent pseudopodia	Actively motile	Aggregates could be seen 6 hrs after starvation
$5\text{x}10^{-3}$M CPZ	Spherical shape, blebs could be seen on the cell surface	Non-motile	No aggregate formation
$1\text{x}10^{-3}$M CPZ	Rounded/Spherical shape and without pseudopodia	Non-motile	A few aggregates could be seen
$5\text{x}10^{-4}$M CPZ	Rounded cells	Non-motile	Aggregates could be seen but fewer as compared to control
$1\text{x}10^{-4}$M CPZ	A few cells were amoeboid and the rest were spherical	Motile/Non-motile	Aggregates present, but fewer as compared to control

b)   EDTA Stable Cell Contact:

The CPZ treated cells suspended in p-buffer were checked for the development of EDTA stable cell contact at different time intervals during development.   Haemocytometer was used to count the number   of

Fig. 13: (a) Photomicrograph of control *D. discoideum* cells at t_4. Note the amoeboid shape of the cells. x 160.

(b) Photomicrograph of CPZ (5×10^{-3}M) treated cells at t_4. Note the darkly stained spherical cells. x 160.
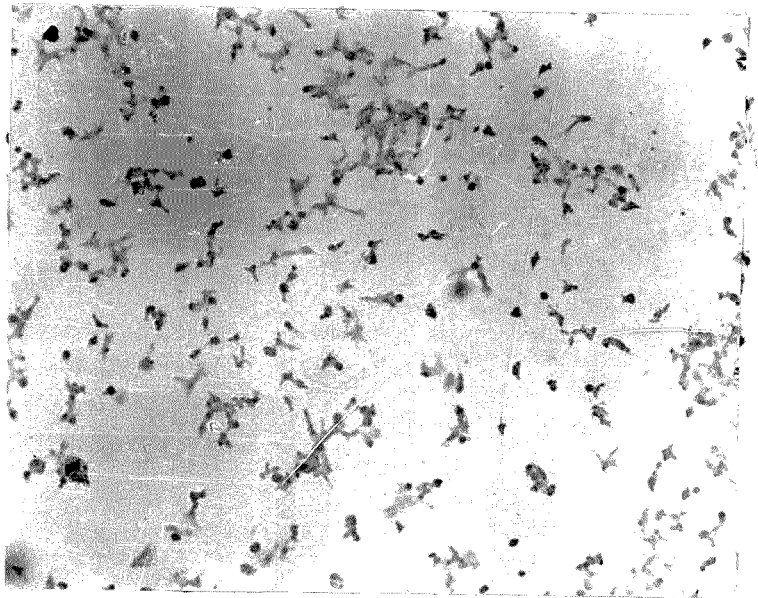
Fig. 13 (a)
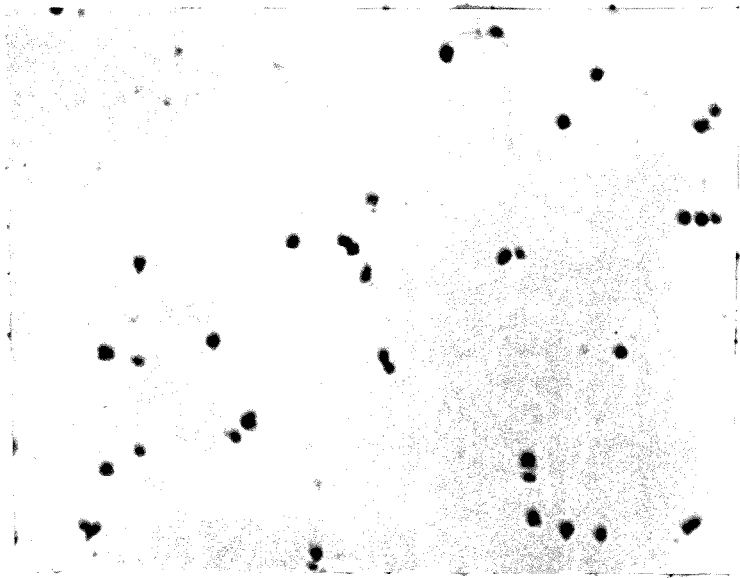
Fig. 13 (b)

Fig.14: (a)   Photomicrograph of control *D. discoideum* cells at t_4. Note the amoeboid cells with prominent pseudopodia. x 400.

(b)   Photomicrograph of *D. discoideum* cells treated with 5×10^{-3}M). Note the swollen spherical cells. x 400.
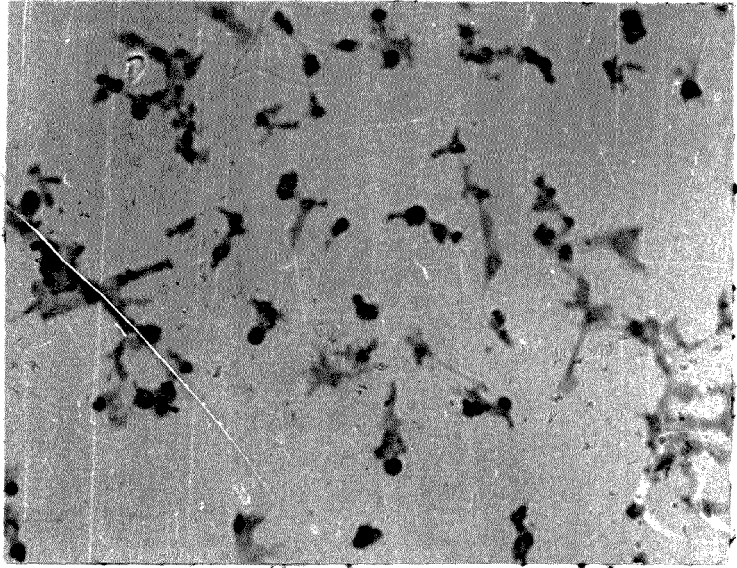
Fig.14 (a)
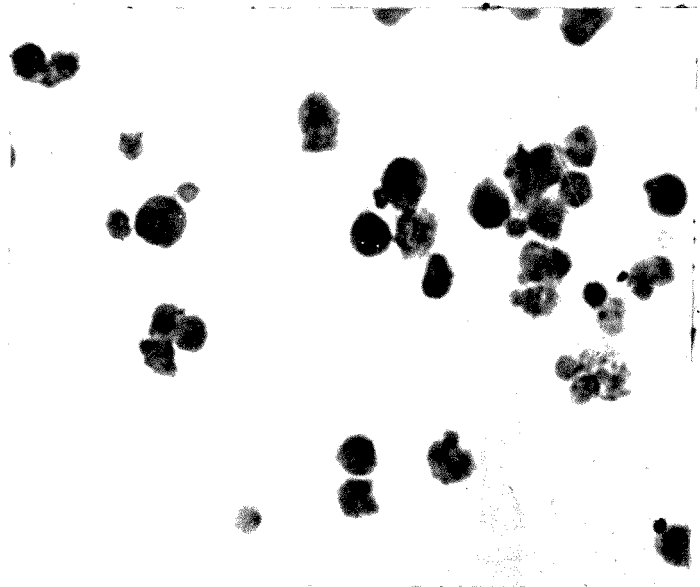
Fig 14 (b)

Fig.15   Photomicrographs of CPZ (5x10⁻³M) treated *D. discoideum* cells showing abberant cellular morphology. Note the cellular exudate (Ex) (15a and 15b) and blebs (B) in the CPZ treated cells (15c). x 300 and x 800
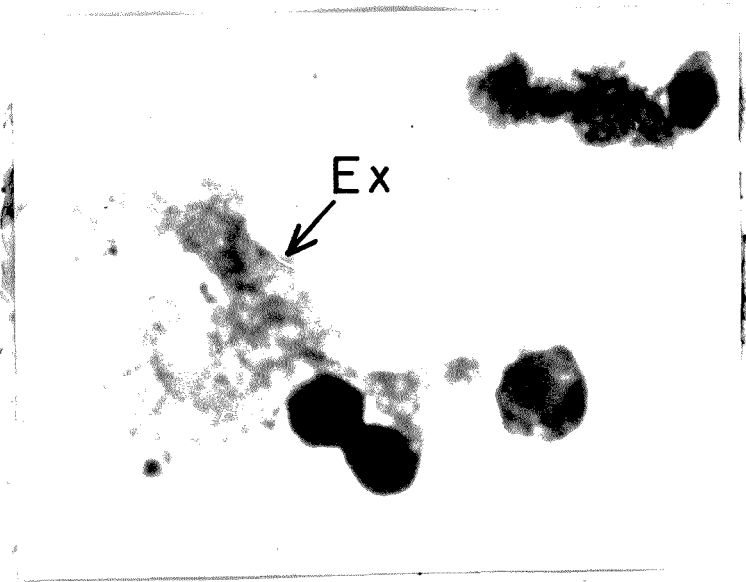
Fig. 15 (a)
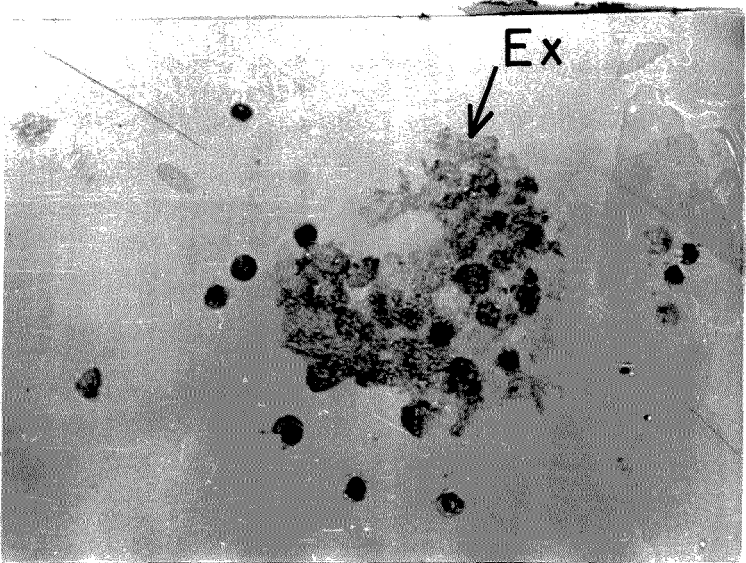
Fig. 15 (b)
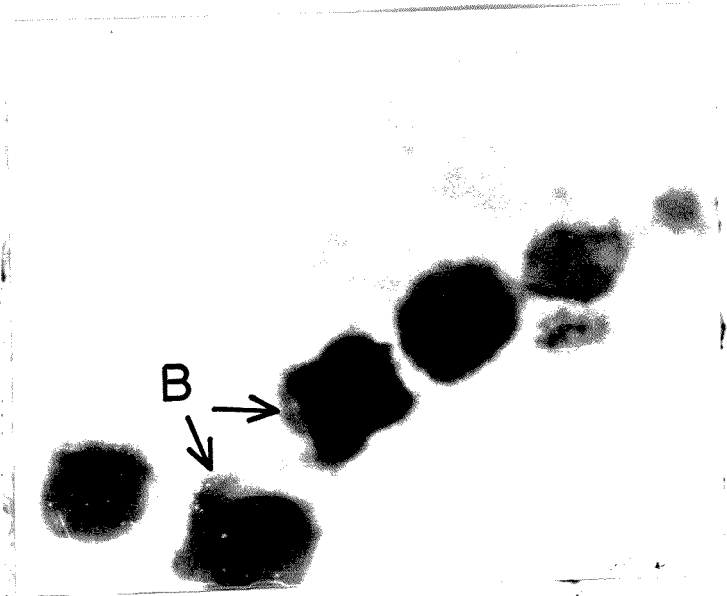
Fig. 15 (c)

Fig.16: (a)   Photomicrograph of control *D. discoideum* cells at t_6 showing intercellular adhesion (Ag). x 320.

(b)   Photomicrograph of CPZ ($5x10^{-3}M$) treated *D. discoideum* cells at t_6 showing individual spherical cells forming no cellular contacts. x 320.
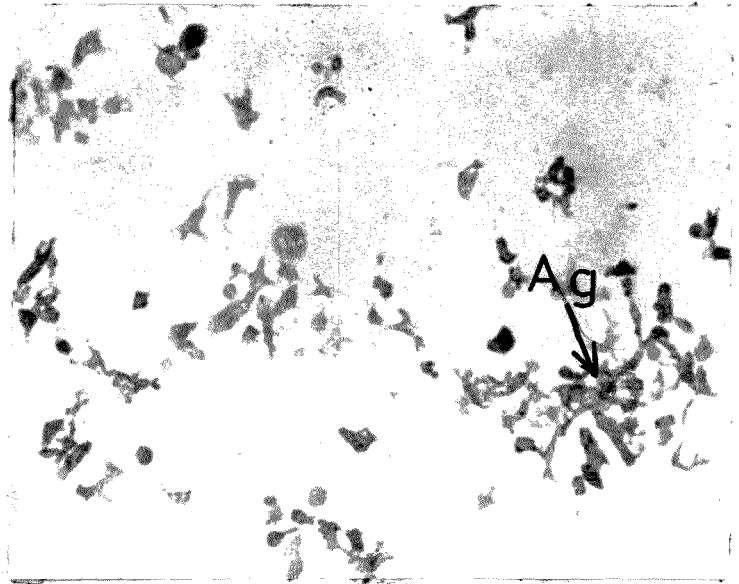
Fig. 16 (a)
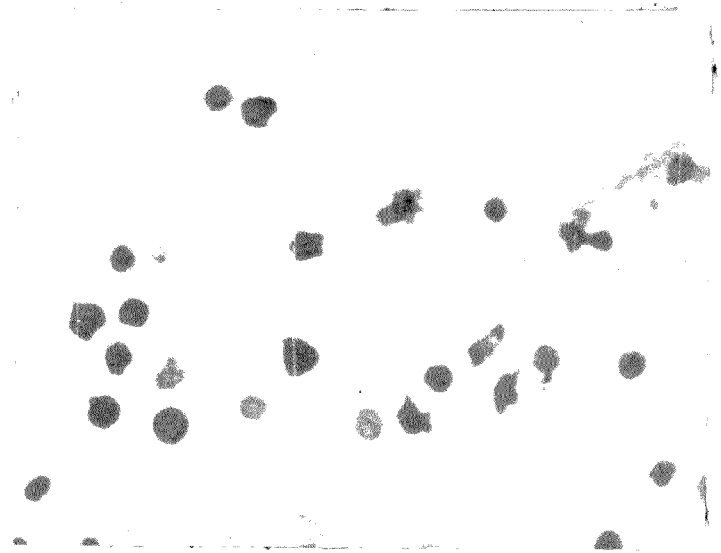
Fig. 16 (b)

aggregates formed during the development. Cells treated with CPZ doses, 5×10^{-3}M and above, remained single and did not form any aggregates. Ax2 cells treated with CPZ were characterized by:

i) fewer number of EDTA stable cellular aggregates, and

ii) smaller size of cellular aggregates. (Fig. 18a, 18b and 18c) EDTA stable cell contact developed within 6 hrs of starvation in control cells, while it took 10 hrs to develop EDTA stable contact for Ax2 cells treated with 1×10^{-4}M CPZ. Ax2 cells treated with 1×10^{-3}M and 5×10^{-4}M CPZ doses developed EDTA stable cell contact after 16hrs and 10hrs respectively. Further with the increase in CPZ dose the percentage of aggregates formed was also less as compared to the control (Fig.17).

c)   Chemotaxis

Control cells starved for six hours (t_6) show chemotactic movements toward cAMP source within 1 hours of plating on p-agar. While Ax2 cells treated with 1×10^{-4}M CPZ showed chemotaxis within 2 to 3 hrs of plating. Ax2 cells treated with CPZ doses, 1×10^{-3}M and above showed no chemotactic movement (Table 6).

Table 6: Chemotaxis of Ax2 Cells (t_6) Towards cAMP

Dose	Chemotaxis
Control	+ After 1 hr of plating on p-agar
1×10^{-4}M CPZ	± After 2-3 hrs of plating on p-agar
5×10^{-4}M CPZ	-
1×10^{-3}M CPZ	-
5×10^{-3}M CPZ	-

+ present, - absent, ± delayed.

Fig.17   Development of EDTA (10mM) stable cellular contacts in control and CPZ treated *D. discoideum* cells.
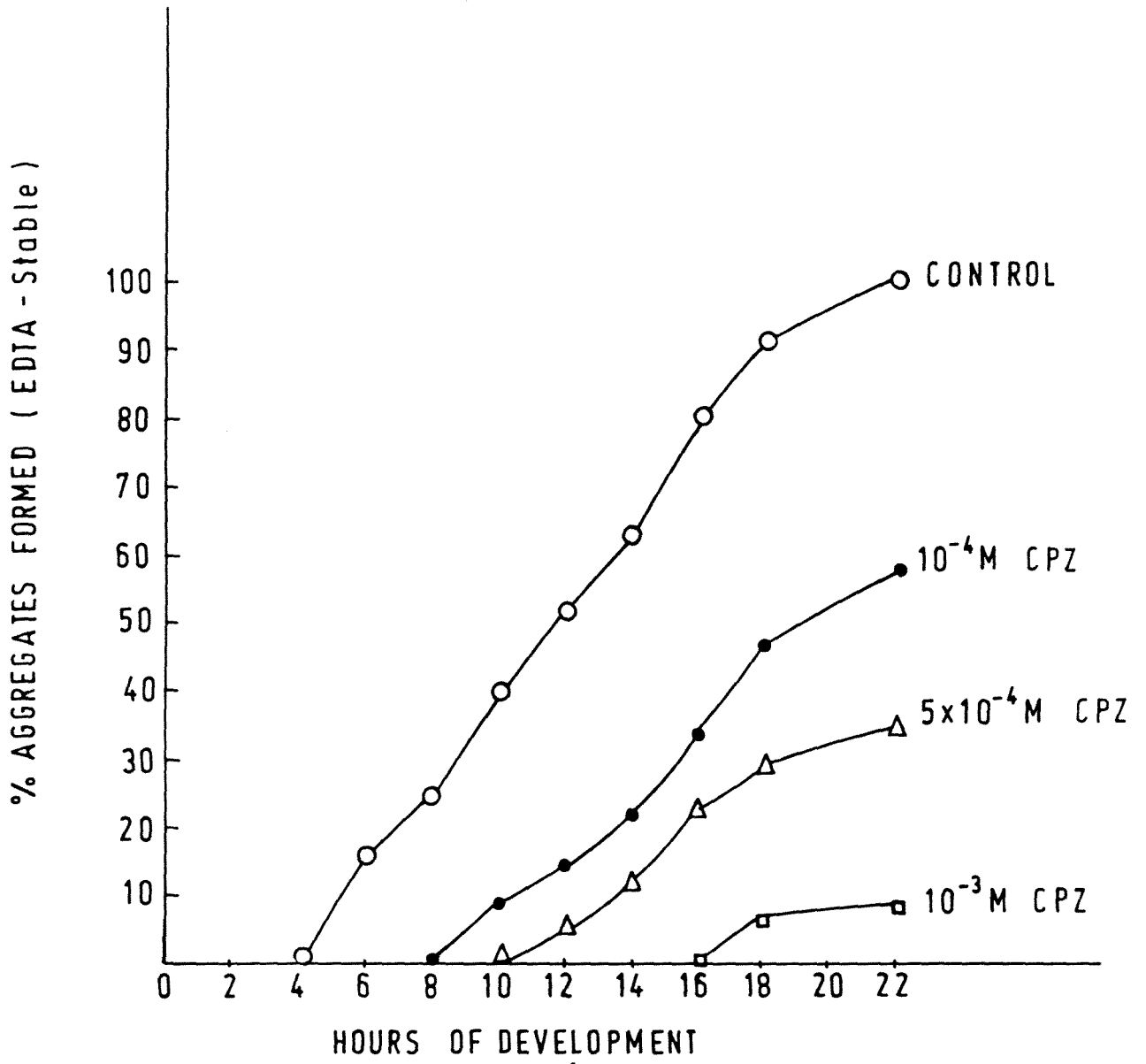
Fig. 17

Fig.18   Photomicrographs of control and CPZ treated cells for comparison of
          EDTA stable cell aggregates (Ag) at t_{16}. Note the number and size of
          EDTA stable cell aggregates is more in control cells (18a) as compared
          to CPZ (1×10^{-4}M) treated cells(18b). Cells treated with 5×10^{-3}M CPZ
          shows absence of cellular contacts (18c). x 200.
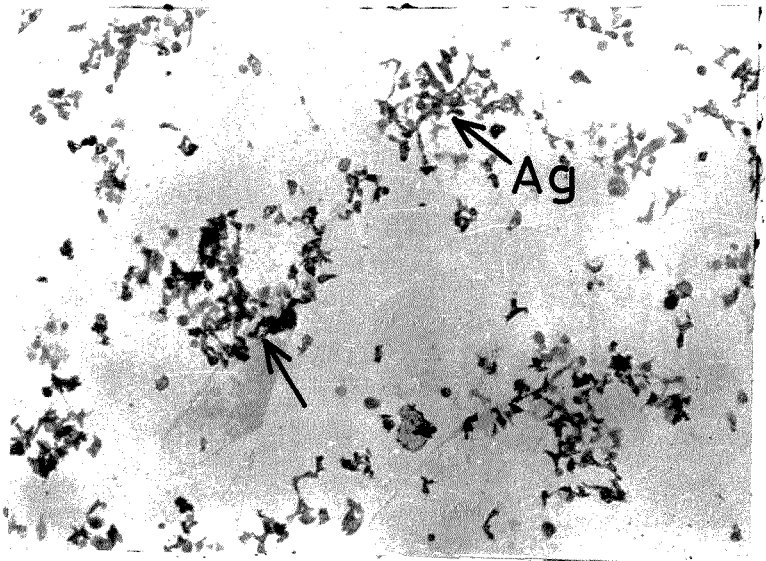
Fig.18 (a)
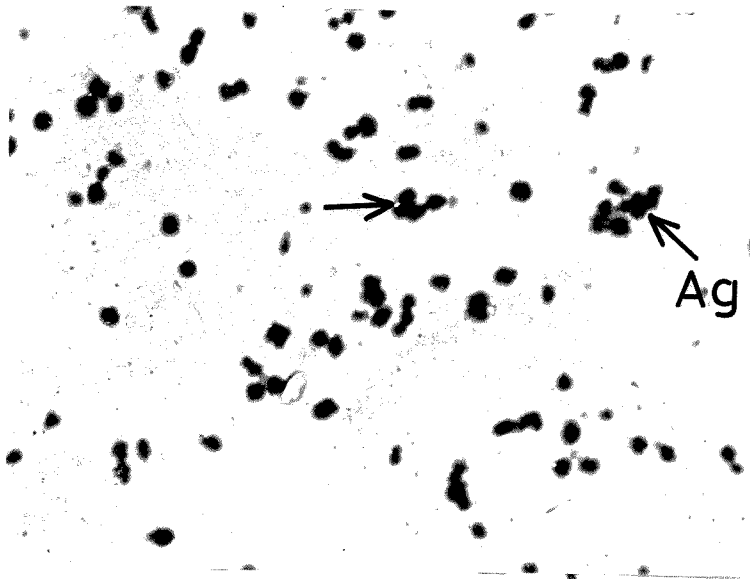
Fig.18 (b)
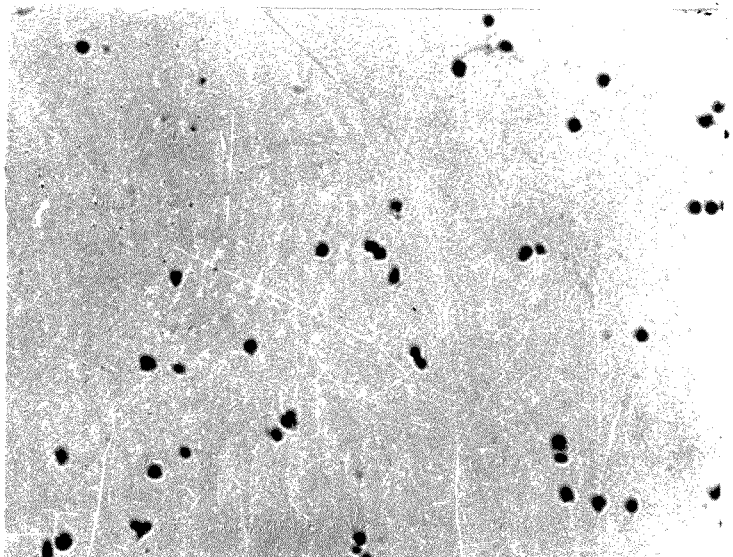
Fig.18 (c)

9. cAMP DEPENDENT EXTRACELLULAR PHOSPHODIESTERASE ASSAY

When the cellular supernatant was assayed for the cAMP dependent ePDE, the following were observed:

1) ePDE activity was 3 to 5 fold higher in CPZ treated Ax2 cells as compared to the control.

2) In the control Ax2 cells, the CPZ activity increased upto the eighth hour (t_8) of development following which it dropped down.

3) In the supernatant of Ax2 cells treated with higher doses of CPZ ($1 \times 10^{-3}M$ and $5 \times 10^{-3}M$) the ePDE activity remained consistently high during the development.

4) In the supernatant of Ax2 cells treated with lower doses of CPZ ($1 \times 10^{-4}M$ and $5 \times 10^{-4}M$) the ePDE activity gradually dropped down during the development (Fig. 19).

Fig.19   3',5' cAMP dependent extracellular phosphodiesterase (ePDE) profile during the development of control and CPZ treated Ax2 cells.

Fig. 19

The y-axis is labeled: 3',5' cAMP-PHOSPHODIESTERASE (n·moles cAMP hydrolysed ml^{-1})

The x-axis is labeled: HOURS IN DEVELOPMENT

Curve labels (top to bottom):
5×10^{-3} M CPZ
1×10^{-3} M CPZ
5×10^{-4} M CPZ
1×10^{-4} M CPZ
Control
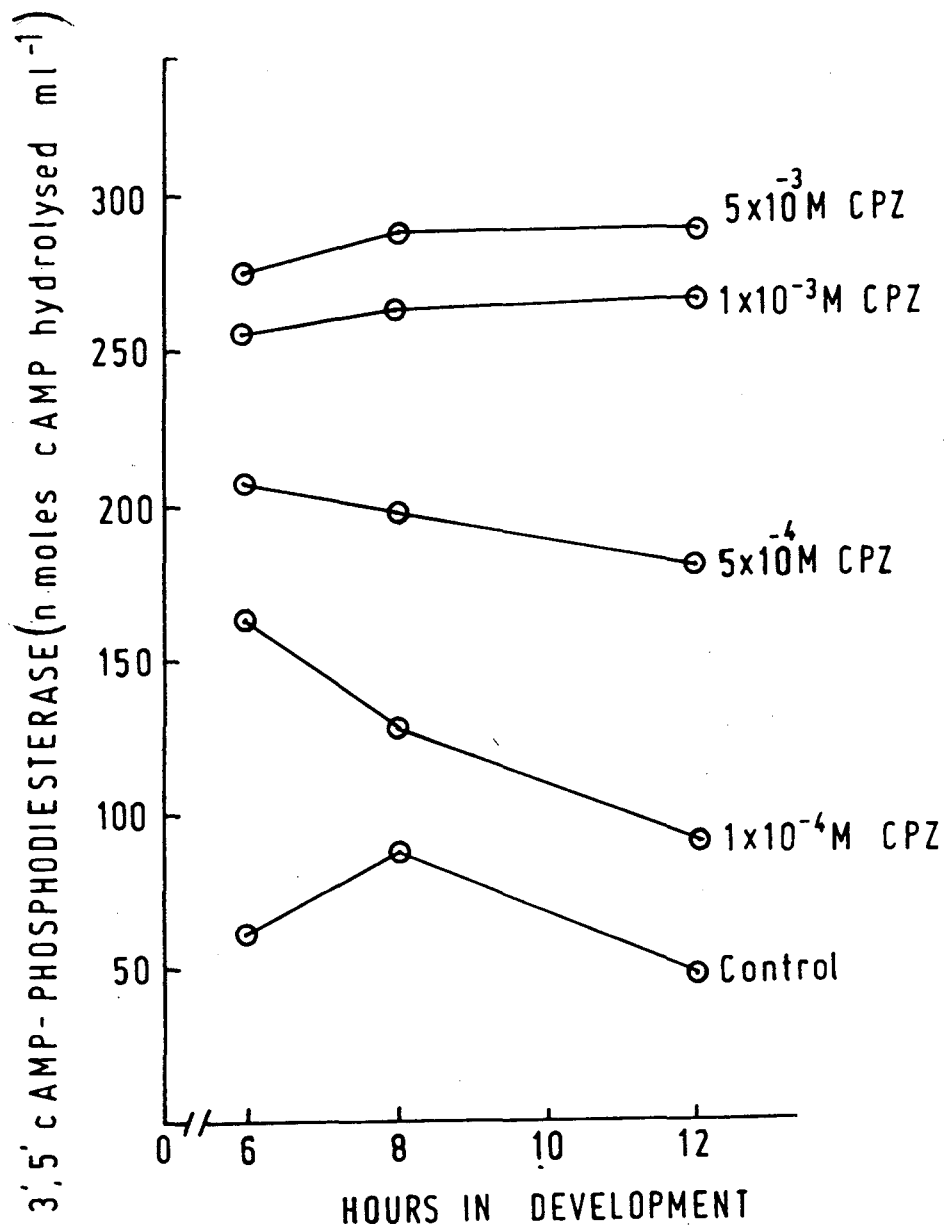
DISCUSSION

Chlorpromazine is an antipsychotic drug belonging to the class of phenothiazines. It modifies not only the neuronal processes but also a number of non-neuronal processes, such as exocytosis (Poste and Papahadjopoulous, 1978), membrane transport (Seeman *et al.*, 1971), cellular adhesion (Rabinovitch and Destefano, 1975) platelet aggregation and endocytosis (Rabinovitch and Destefano, 1974). All these effects are due to membrane perturbation caused by the interactions of CPZ with the plasmamembrane (Yamaguchi, *et al.*, 1985; Zachowski and Durand, 1988). In the present study, the mechanism of action of CPZ on some of the membrane related functions which initiate the early development of *D. discoideum* such as chemotaxis, cell motility, cell adhesion etc. were studied.

We have investigated the effect of various doses of CPZ (from $1x10^{-1}$ M to $1x10^{-4}$M) on growth and differentiation of *D. discoideum*. Experiments on dose response of the Ax2 cells, suspended in p-buffer after the treatment showed that cells could tolerate only a narrow dose range of CPZ. CPZ doses above $5x10^{-3}$M leads to cell lysis and the survival was only 15% at $1x10^{-2}$M CPZ, whereas at lower doses of CPZ ($1x10^{-4}$M) the survival was approximately 85% . This is in agreement with the findings of Seeman (1972) and Yamaguchi and coworkers (1985), that interactions of CPZ with the membranes is biphasic i.e., at lower doses, CPZ causes membrane stabilization whereas at higher doses it causes cell lysis by membrane disruption. Studies done by Lieber *et al.* (1984) on the interaction of CPZ with human erythrocyte membrane revealed that CPZ concentration above

1x10^{-4}M caused hemolysis. Further, they found that CPZ induced holes in the cell membrane. Freeze fractured electron microscopy of CPZ treated cells showed multiple round patches in the lipid bilayer and it was interpreted that hemolysis could be due to the openings within the CPZ treated membrane. (Lieber *et al.*, 1984).

In the axenic medium, the growth of Ax2 cells was almost negligible when treated with higher doses of CPZ (5x10^{-3}M). This indicates an antiproliferative effect of CPZ on *D. discoideum* cells. Further, the growth of Ax2 cells was slower when treated with lower doses of CPZ (1x10^{-4} M and 5x10^{-4}M) as compared to the control. CPZ has been shown to have an antifungal activity as CPZ inhibits the *invitro* growth of pathogenic yeast *Candida albicans*. (Eelam, *et al.*, 1987; Wood, *et al.*, 1985).

There was a total inhibition of morphogenesis when Ax2 cells treated with CPZ doses higher than 5x10^{-3}M were plated on agar while lower doses of CPZ (1x10^{-4}M and 5x10^{-4}M) caused a delay in the completion of morphogenesis. Ax2 cells plated on CPZ agar also show inhibition of morphogenesis. The major site of CPZ action is on the plasmamembrane (Seeman, 1972). In *D. discoideum* the important morphogenetic events are all membrane related. Thus it can be interpreted that at higher doses, CPZ causes membrane damage to such a great extent that cells cannot recover from this damage and hence the inhibition of morphogenesis. Whereas at lower doses of CPZ most of the inhibited activities become normal with a lapse of time suggesting a recovery or the reversible action of this drug with resultant delay in morphogenesis. Further, at lower doses of CPZ, the number of slugs and spores formed were fewer as compared

to control. Cytological preparation of CPZ treated cells showed a distroted cellular morphology, which included swollen cells, spherical shape and formation of blebs on the cell surface. These abberant cellular changes are all indicative of membrane disruption. Cellular exudate could also be seen in the fixed preparations of CPZ treated cells. Marked cytopathalogical changes due to CPZ toxicity has been reported in the hepatocytes where plasma membrane becomes granulated and exhibited multiple spiculae (Salhab and Dujovne, 1986). Sinusoidal blebs could be seen in isolated liver cells perfused with CPZ (Elias and Boyer, 1978). These reports indicate that cell membrane undergoes drastic changes following treatment with CPZ.

Ax2 cells plated on CPZ agar ($1x10^{-4}M$, $5x10^{-4}M$, $1x10^{-4}M$, $5x10^{-3}M$) showed no morphogenesis. Loose cellular patches could be seen on the CPZ agar which did not show streaming, slugs or fruiting body formation. As the cells were in continuous contact with the CPZ there was no morphogenesis even when plated on lower doses of CPZ ($1x10^{-4}M$, $5x10^{-4}M$) agar.

As CPZ was found to inhibit morphogenesis, we tried to investigate the effects of CPZ particularly on the well known developmental markers occurring during the early phase of differentiation. These included cell motility, cell adhesion, chemotaxis etc.

Live observations of CPZ treated Ax2 cells suspended in p-buffer showed that at higher doses of CPZ, the cells remained spherical while the control cells showed amoeboid movement when provided with a substratum. In D. discoideum motility is brought about by the formation of actin-

myosin complex which in turn leads to cellular contraction (Spudich and Spudich, 1982). The surface membrane perturbation by CPZ may be relayed to the submembranous actin and myosin matrix system, thereby presumably preventing the formation of actin myosin complex by one or more of the following mechanisms:

1. CPZ alters depolymerization and gelation of F-actin (Elias and Boyer, 1978) leading to microfilament dysfunction which in turn would cause inhibition of cell motility.

2. CPZ prevents Ca^{2+} influx (Low et al., 1979) and Ca^{2+} is important for

    a) sol. gel transformation, and

    b) actin myosin association.

3. CPZ causes uncoupling of oxidative phosphorylation (Siesjo, 1982). Since ATP is utilized during actin myosin association, a reduction in ATP level might lead to inhibition of chemotaxis.

There was a total inhibition of chemotactic movement of the aggregation competent cells towards the cAMP source when treated with CPZ doses higher than $5 \times 10^{-4}M$. These findings i.e., inhibition of pseudopod formation and chemotactic movement by CPZ treated cells are in support of the earlier reports where CPZ has been found to, (a) inhibit substrate adhesion and spreading of Sarcoma cells (Rabinovitch and Destefano, 1973 and 1975), (b) depress human neutrophil chemotactic response (Lohr et al.,

1984; Bjorksten and Quein, 1976). The phenothiazine induced depression of chemotaxis in *D. discoideum* could be due to the following factors:

1. CPZ is known to interact with membrane proteins, causing inactivation of the transmembrane proteins (Conrad and Singer, 1981; Bjorkstein and Quie, 1981). Binding of CPZ might lead to either inactivation of membrane bound cAMP receptor or alteration of the receptor binding affinity and thus preventing the chemotactic movement of the cells.

2. Phenothiazine interferes with cytoskeleton organisation, thus inhibiting cell motility (Elias and Boyer, 1978). Binding of chemoattractant to the receptor promotes assembly of microtubules and microfilaments which are required for cellular orientation and directed migration. Calmodulin enhances microtubule assembly, which may be indirectly inhibited by CPZ binding to calmodulin (Kohl *et al.*, 1989), thus inhibiting the cell motility.

Cells treated with higher doses of CPZ remained loosely attached when provided with a substratum, unlike the control cells which were firmly attached. During development from the growth phase to aggregation competent stage *D. discoideum* cells acquire the ability to form EDTA stable intercellular contacts within 6 hrs of starvation (Muller and Gerisch, 1978). Ax2 cells treated with higher doses of CPZ (5×10^{-3}M and 1×10^{-3}M) also showed loose cell clumps/aggregates which were sensitive to 10 mM EDTA treatment. Further at lower doses of CPZ (5×10^{-4}M and 1×10^{-4}M)

the EDTA stable contact was established quite late during development i.e. approximately at 16 hrs after starvation. The size and the number of the cellular clumps/aggregates formed with CPZ treatment were smaller as compared to the control cells. Prevention of substrate adhesion and aggregate formation by CPZ treatment could be due to the following reasons:

1) **Inactivation of Cell Surface Glycoproteins and Discoidins**

    Expression of cell surface glycoproteins and discoidins during development helps in intercellular and cell to substrate adhesion. As CPZ interacts preferentially with membrane proteins leading to their inactivation (Bjorkstein and Quie, 1976; Yamaguichi *et al.*, 1985; Conrad and Singer, 1981), it could be reasonable to assume that binding of CPZ to membrane might lead to conformational changes in the membrane glycoproteins and discoidins thus preventing the establishment of rigid inter-cellular contacts and cell to substrate adhesions.

(2) **Inhibition of Cell Motility**

    CPZ treatment might also prevent the *D.discoideum* cells from establishing the cellular contacts by inhibiting cell motility and thus preventing the cells from coming in contact with each other.

It was observed that even after 10 mM EDTA treatment, some clumps/aggregates could be seen in CPZ (1x10^{-3}M) treated Ax2 cells. Closer

examination under light microscope (Fig. 15a) revealed that these clumps were the cells enmeshed in cellular exudates of the CPZ treated cells and not that of EDTA-resistant cell aggregates.

Not only membrane structures, but also membrane bound enzymes such as phosphodiesterase, $Na^+K^+ATPase$, cytochrome oxidase etc. are effected by CPZ treatment (Seeman, 1972). The supernatant of the CPZ treated cells assayed for cAMP dependent ePDE, was found to have 3 to 5 fold increase in enzyme activity as compared to the control. The consistently higher ePDE activity would prevent the formation of a cAMP gradient essential for completion of morphogenesis. Electron micrographs of CPZ treated membranes revealed the presence of holes with a diameter of approximately 14Å in erythrocyte membrane, thus making the membrane leaky (Lieber, 1984). From this report it can be interpreted that formation of holes in the membranes of CPZ treated Ax2 cells might lead to leakage of the intracellular PDE (iPDE) into the medium which in turn might show a higher PDE activity in the supernatant of CPZ treated cells. Interactions of CPZ with the D. discoideum cell membrane could also possibly lead to the altered activity of membrane bound adenylate cyclase, thus leading to an alteration in the cAMP level which would further block the morphogenesis.

When control cells and CPZ treated cells were plated together or the control cells were resuspended in the supernatant of CPZ treated cells, the morphogenesis of control cells was either blocked at higher doses of CPZ ($5x10^{-3}M$) or gets delayed at lower doses of CPZ ($5x10^{-4}M$). This could be possible due to:

1)   Higher ePDE Activity

CPZ treated cells showed a 3 to 5 fold increase in extracellular 3',5'-cAMP phosphodiesterase (ePDE) activity as compared to the control cells. From the data obtained from our studies on ePDE activity in the supernatant of CPZ treated cells the following can be interpreted:

i)   consistently higher ePDE activity in 5×10^{-3}M, 1×10^{-3}M of CPZ treatment might cause inhibition of morphogenesis of the control cells.

ii)   at 5×10^{-4}M and 1×10^{-4}M of CPZ used, the ePDE activity in the supernatant was high during the beginning of the development, but decreased at later period of development. A higher ePDE activity during the initial phase of development might account for the delay in morphogenesis.

2)   Higher Lysosomal Enzyme Activity

A number of lysosomal enzymes are secreted from amoebae of *D. discoideum* during growth and development (Diamond *et al.*, 1981). A very high lysosomal enzyme level in the supernatant of the CPZ treated Ax2 cells might also interfere with the morphogenesis of control cells. This is supported by the fact that, supernatant of CPZ treated hepatocyte cells show higher activities of lactate dehydrogenase, a marker lysosomal enzyme (Salhab and Dujovne, 1986).

As it became apparent that CPZ blocks the early differentiation in *D. discoideum*, we tried to investigate if this block could be reversed. CPZ is a calcium antagonist (Low *et al.*, 1979), further Ca^{2+} plays an important role during early differentiation of *D. discoideum* cells (Malchow *et.al*, 1982; Bumann *et al.*, 1984). We tried to find out if the CPZ ($5x10^{-3}M, 1x10^{-3}M$) treated cells could show morphogenesis with Ca^{2+} supplementation. Besides Ca^{2+}, cAMP also plays a crucial role during early differentiation for acquisition of aggregation competence in *D.discoideum*. Chlorpromazine and other phenothiazines block cAMP accumulation in *D. discoideum* cells (unpublished observation of M. Brenner, cited by Devreotes, 1982). Thus, we checked for the completion of morphogenesis in CPZ treated cells in presence of Ca^{2+} (100mM, 500mM, 1000mM) and cAMP (1mM). It was observed that neither Ca^{2+} nor cAMP could reverse the block occuring during early differentiation under our experimental conditions. This suggested that CPZ must be acting at other sites as well during morphogenesis.

From our studies on the effect of CPZ on the main marker events of early development of *D. discoideum* it is very clear that CPZ affects both the responses of individual cells as seen by the distorted cellular morphology and also the response of groups of developing cells. Thus in conclusion it can be said that CPZ affects the development of *D. discoideum* cells in the following way:

1. Abberant cellular morphology of CPZ treated cells, such as round cells, presence of blebs, cell exudate etc. might interfere with the normal course of responses of *D. discoideum* cells initiated during early development.

2. CPZ alters actin myosin associations leading to microfilament dysfunction (Elias and Boyer, 1978) which in turn leads to inhibition of cell motility.

3. Inhibition of chemotactic movement towards cAMP source caused after CPZ treatment.

4. The absence of streaming in CPZ treated cells could be due to the failure of cells to establish any stable cell contact.

5. Size and the number of cellular aggregate was smaller in CPZ ($5x10^{-4}M$, $1x10^{-4}M$) treated cells as compared to the control and this could be the reason why the number of slugs and fruiting bodies developed were fewer when cells were treated with these CPZ doses.

6. Interaction of CPZ with membrane might lead to altered activities of membrane bound enzymes such as adenylate cyclase, phosphodiesterase etc. thus inhibiting cAMP wave propagation essential for aggregation.

D. *discoideum* cells treated with lower doses of CPZ ($5x10^{-4}M$ and $1x10^{-4}M$) showed a delayed morphogenesis as compared to control. This could be due to:

1. Establishment of EDTA Stable Contact Quite Late During the Development in CPZ Treated Cells:

    In control cells the EDTA stable cell contacts developed within approximately 6 to 8 hrs of initiation of development, while in CPZ treated cells it took 16 to 18 hrs for the stable cell contacts to be formed thereby delaying the morphogensis (Fig. 17).

2. Higher PDE Activity During Early Phase of Development.

    Supernatant of $5x10^{-4}M$ and $1x10^{-4}M$ treated cells showed a higher ePDE activity upto 8-10 hrs of development following which it dropped down. A higher ePDE activity during the early phase of development might account for the delay in the morphogenesis.

In conclusion, it can be said that CPZ causes extensive changes in cell membrane activities which are crucial determinants for smooth progression of developmental events in cellular slime moulds. Very high doses of CPZ prevents cell motility, chemotaxis and extracellular adhesion thereby preventing the stream formation. Further, an increase in ePDE activity also prevents the aggregate formation with CPZ treatment. Thus, CPZ block some of the key factor occuring during the early differentiation important for the acquisition of aggregation competence and hence the morphogenesis is blocked when cells are treated with chlorpromazine.

SUMMARY

1. The cellular slime mould, *D. discoideum* has been utilized for studying the effects of the neuropharmacological drug chlorpromazine (CPZ) on some of the membrane associated events occurring during its early differentiation.

2. The cells can tolerate only a narrow range of CPZ concentration. Increasing the CPZ dose beyond $5x10^{-3}M$ causes an increase in cell death.

3. CPZ treated cells show distorted cellular morphology. The cells assume spherical shape, show blebs on the cell surface and also shows the presence of cellular exudate.

4. Cells treated with CPZ doses higher than $5x10^{-4}M$ show no morphogenesis when plated on agar surface, while at lower doses of CPZ, ($1x10^{-4}M$ and $5x10^{-4}M$) the morphogenesis gets delayed. Cells plated on CPZ agar ($1x10^{-4}M$, $5x10^{-4}M$, $1x10^{-3}M$, $5x10^{-3}M$) show no morphogenesis.

5. Cells treated with higher doses of CPZ ($1x10^{-3}M$ and above) do not show the formation of pseudopodia and hence are nonmotile.

6. Cells exposed to higher CPZ doses fail to show any chemotactic movement towards cAMP source.

7. CPZ treated cells show lesser number of EDTA stable cell clumps as compared to control cells during early phase of differentiation. Also the size of the clumps formed are smaller. At very high doses of CPZ $1x10^3M$ and above the cells fail to establish any cellular contacts.

8. Morphogenesis of control cells get inhibited or delayed when either:

   i) suspended in supernatant of CPZ treated cells, or

   ii) mixed with CPZ treated cells.

9. The extracellular phosphodiesterase activity was found to be high in the supernatant of the CPZ treated cells.

10. Supplementation of $CaCl_2$ or cAMP to CPZ ($1x10^{-3}M$, $5x10^{-3}M$) treated cells does not augment the morphogenesis of cellular slime moulds.

11. It can be concluded that CPZ interacts with the cell membrane of *D.discoideum* cells, thereby blocking some of the membrane related key events during early development such as chemotaxis, cell motility and intercellular adhesion, thus interfering with the normal course of morphogenesis.

BIBLIOGRAPHY

1. Alcantra, F. and Monk, M. (1974). Signal propagation in cellular slime mould *Dictyostelium discoideum*. *J. Gen. Microbio*. 85: 321-324.

2. Ashworth, J.M., and Watts, D.J. (1970). Metabolism of the slime mould *Dictyostelium discoideum* grown in axenic culture. *Biochem. Journal* 119:175-182

3. Barondes, S.M. (1981). Lectins: Their multiple endogenous cellular functions. *Ann. Rev. Biochem*. 50: 207-231.

4. Barondes, S.M., Springer, R.W., Cooper, D.N. (1982). Cell adhesion. p.195-226. In "The development of *Dictyostelium discoideum*" (Loomis ed). Academic press, New York.

5. Beug, H., Katz, F.E., and Gerisch, G. (1973). Dynamics of antigenic membrane sites relating to cell aggregation in *Dictyostelium discoideum*. *J. Cell Bio*. 56: 647-658.

6. Bjorkstein, B and Quie, P.G. (1976). Inhibition of human neutrophil chemotaxsis in vitro by phenothazine and related compounds. *Infection* and *Immunity* 14(4): 948-950.

7. Bonner, J.T. (1971). Aggregation and differentiation in cellular slime moulds. *Ann. Rev. Microbiol*. 25: 75-92.

8. Bonner, J.T. (1982). Comparitive biology of cellular slime moulds p. 32-38. In "The Development of *Dictyostelium discoideum*" (Loomis, ed.) Academic press, New York.

9. Bradley, P.B., Wolstencroft, J.H., Hosli, L. and Avanzino, G.L. (1966). Neuronal basis for central action of chlorpromazine. *Nature* (London) 212: 1425-1427.

10. Brisbin, J.N.R., and O'Day, D.H. (1988). Nuclear morphogensis during spore germination in the slime mould, *Dictyostelium discoideum*. *Trans. Am. Microsc. Soc*. 107 (2): 135-142.

11. Bumann, J., Wurster, B. and Malchow, D. (1984). Attractant induced changes in oscillations of extracellular Ca^{2+} concentration in suspensions of differentiating *Dictyostelium cells*. *J.Cell. Biol*. 98:173-178.

12. Byck, R. (1975). Drugs and treatment of psychiatric disorders. p.152-200. In "The pharmarological basis of therapeutics" (L.S. Goodman and A. Gilman, ed.) Macmillan Publishing Company.

13. Chisholm, R.L., Fontana D., Theibert, A., Lodish, H.F., and Devreotes, P. (1984). Development of *Dictyostelium discoideum* chemotaxis, cell-cell adhesion and gene expression. p. 219-264. *Microbial development* (R. Lo Sick and L. Shapiro, ed.) Cold Spring Harbor Laboratory, Cold spring harbor, New York.

14. Clarke, M., Schatten, G., Mazia, D., and Spudich, J.A. (1975). Visualization of actin fibres associated with cell membrane in amoebae of *Dictyostelium discoideum*. *Proc. Natl. Acad. Sci. U.S.A.* 72:1758-1762.

15. Cohen, M.H., and Robertson, A. (1971). Wave propagation in the early stages of aggregation of cellular slime moulds. *J. Theor. Biol.* 31:101-118.

16. Cohen, M.S. (1978). Cyclic AMP control system in development of *Dictyostelium discoideum*. *J. Theor. Biol.* 72:231-256.

17. Conrad, M.J. and Singer, S.J. (1981). Solubility of amphipathic molecules in biological membranes and lipid bilayers and its implications for membrane structure. *Biochemistry* 20:808-818.

18. Corps, A.N., Hesketh, T.R., and Metcalfe J.C. (1982). Local anesthetics as indicators of calmodulin function in intact cells. *FEBS Lett.* 138(2): 280-284.

19. Darcy, P.F.D., Speirs. C.J., Griffin, P., and Wright, C. (1988). p36-48. Manual of adverse drug interactions (4th ed.), Oxford University Press, London.

20. Darmon, M., Barra, J., and Brachet, P. (1978). The role of phosphodiesterase in aggregation of *Dictyostelium discoideum*. *J. Cell. Sci.* 31:233-244.

21. Devreotes, P.N. (1982). Chemotaxis. p.17-158. In "The development of *Dictyostelium discoideum*" (Loomis ed.) Academic Press, New York.

22. Diamond, R.L., Burns, R.A., and Jordan, K.B. (1981). Secretion of lysosomal enzymes in the cellular slime mould *Dictyostelium discoideum J. Biol. Chem.* 256: 6565-6572.

23. Ebor, S. and Akerele, B. (1988). Mutagenic activity of CPZ. *Mutation research* 208(1): 33-38.

24. Eelam, A., Yael. I., Polacheck and Gila Ben Gigi (1987). Chlorpromazine inhibited *invitro* growth of pathogenic yeasts. *Anti. Micro. Agents Chemoth.* 31(5): 834-836

25. Elias, F. and Boyer, J.L. (1975). Chlorpromazine and its metabolite alter polymerization and gelation of actin. *Science* 206: 1404-1406.

26. Europe-Finner, G.N., and Newell. P.C., (1987). Cyclic AMP stimulates accumulation of inositol triphosphate in *Dictyostelium discoideum*. *J. Cell. Sci.* 87:221-229.

27. Fontana, D., Wong, T.Y., Theibert, A., and Deverotes, P. (1986). Cell-cell interactions in the development of *Dictyostelium* p.261-281. In "Developmental biology: A comprehensive synthesis". Vol-3. The cell surface in development and cancer. (M.S. Steinberg ed.), Plenum Press, New York.

28. Franke, J., and Kessin, R. (1981). The cAMP phosphodiestrase inhibitory protein of *Dictyostelium discoideum*. Purification and characterization. *J. Biol. Chem.* 117; 213-218.

29. Futrelle, R.P., J. Traut, and Mckee, W.G. (1982). Cell behaviour in *Dictyostelium discoideum*. *J. Cell. Biol.* 98: 584-595.

30. Gerisch, G. (1982). Chemotaxis in *D. discoideum*. *Ann. Rev. Physio.* 44:535-552.

31. Gerisch, G. (1987). Cyclic AMP and other signals controlling cell development and differentiation in *D. discoideum*. *Ann. Rev. Biochem.* 56:853-879.

32. Gerisch, G., Fromm, H., Huesgen, A., and Wick, U. (1975). Control of cell contact sites by cAMP pulses in differentiating *D. discoideum* cells. Nature 255: 547-549.

33. Gerisch, G. (1986). Interrelation of cell adhesion and differentiation in *D. discoideum*. *J. Cell. Sci. Suppl.* 4:201-219.

34. Gerisch, G., Tsiomenko, A., Stadler, J., Claviz, M., Hulser, D and Rossier, C. (1984). Transduction of chemical signals in *Dictyostelium* cells. p.237-247. Information and Energy Transduction in Biological Membranes. Alan R. Liss, Inc., New York.

35. Gletosky, I.E., Birdwell, C.R., Weseman, J., and Berner, R.A. (1980). Glycoprotein involved in aggregation of *D. discoideum* is distributed on membrane surface in a nonrandom fashion favouring cell functions. *Cell* 21: 339-345.

36. Janssens, P.M.W and Van Haastert, P.J.M. (1987). Molecular biology of transmembrane signal transaction in *D. discoideum*. *Microb Rev.* 51(4): 396-418.

37. Kessin, R.H. (1988). Genetics of early *D. discoideum* development. *Microbio Rev.* 52(1): 29-49.

38. Klein, C. (1976). Adenylate cyclase activity in *D. discoideum* amoebae and its change during differentiation. *FEBS Lett.* 68:125-128.

39. Klein, P., Fontanna, D., Knox, B., Therbert, A. and Devreotes, P. (1985). cAMP receptors controlling cell-cell interactions in the development of *Dictyostelium*. *Cold Spring Harbor Symp.* 50: 787-799.

40. Klubes, P., Fay, P.J. and Cerna, I. (1971). Effects of chlorpromazine on cell wall biosynthesis and incorporation of orotic acid into nucleic acids in *Bacillus Megaterium*. *Biochem. Pharmacol.* 20:265-277.

41. Konijn, T.M., Van De Meene, J.G.C., Bonner, J.T. and Barkley, D.S. (1967). The acrasin activity of adenosine 3',5' cyclic phosphate *Proc. Natl. Acad. Sci.* U.S.A. 58: 1152-1154.

42. Konijn, T.M., and Van Haastert, J.M. (1987). Measurement of chemotaxis in *Dictyostelium*. *Methods in cell biology* (Spudich ed.) 28:283-296.

43. Kuczmarski, E.R., and Spudich J.A. (1980). Regulation of myosin self assembly: phosphorylation of *Dictyostelium* heavy chain inhibits thick filament formation. *Proc. Natl. Acad. Sci.* U.S.A. 72: 7292-7296.

44. Kuznicki, J., Kuznicki, L. and Drabikowski, W. (1979). Ca^{2+} Binding modulator proetin in Protozoa and myxomycetes. *Cell Biol. Intern. Reports*. 3(1): 17-23

45. Leichtling, B.H., Tihon, C., Spitz, E., and Rickenberg, H.V. (1981). A cAMP binding protein in *D. discoideum*. *Develop. Biol.* 92:150-157.

46. Leterrier, F., Mendyk, A., and Viret, J. (1976). Interaction of chlorpromazine with biological membranes. A Photochemical study using spin labels. *Bioch. Pharmac.* 25: 2469-2474.

47. Levin, R.M., and Weiss, B. (1979). Selective binding of antipsychotic agents to calcium-dependent activator of cyclic nucleotide phosphodiesterase. *J. Pharmacol. Exp. Ther.* 208: 454-459.

48. Lieber, M.R., Lange, Y., Weinstein, R.S., and Steck, T.L. (1984). Interaction of chlorpromazine with the human erythrocyte membrane. *J. Bio. Chem.*, 259: 9225-9235.

49. Lohr, K.M., Feix. B.M. and Kirth, C. (1984). Chlorpromazine inhibits neutrophil chemotaxis beyond the chemotactic receptor ligand interaction. *J. of Infec. Diseases*. 150(5): 112-119.

50. Loomis, W.F. (1975) Aggregation Stage. p.47-85. In *"D. discoideum a developmental system"* Academic press, New York.

51. Loomis, W.F. (1985) Regulation of cell type specific differentiation in *Dictyostelium* . *Cold Spring Harbor Symp*. 50:769-777.

52. Low, S.P., Lloyd, D.H., Stein, M.S. and Rogers. J.A. (1979). Calcium displacement by local anesthetics. *J. Bio. Chem.* 254(10): 4119-4215.

53. Luxnat, M. and Galla, H.J. (1986). Partition of chlorpromazine into lipid bilayer membranes: The effect of membrane structure and composition. *Biochim. Biophy. Acta.* 856: 274-282.

54. Malchow, D., Bohne, R. and Gras. U. (1982). On the role of calcium in chemotaxis and oscillations of *Dictyostelium* cells. *Bioph. Struct. Mech.* 9: 131-136.

55. Miller, J.C., and Miller, K.W. (1975). Approaches to the mechanism of action of general anesthetics. *Int. Rev. of Science. Biochem.* Series 12: 34-43.

56. Muller, K., and Gerisch. G. (1978). A specific glycoprotein as the target site of adhesion blocking fab in aggregating *Dictyostelium* cells. *Nature* (London) 274:445-449.

57. Murray, B.A. (1982). Membranes. p.71-103. In "The development of *Dictyostelium discoideum*". Academic press, New York.

58. Mykosen, A., Annarosa, V., Aleotti, A. and Vannin, G.L. (1987). Ultrastructural changes in pathogenic fungi treated in vitro with chlorpromazine. *Anti. Micro. Agents. Chemoth.* 30(5): 201-209.

59. Newell, P.C. (1982). Genetics p.35-65. In "The development of *Dictyostelium discoideum*". Academic press, New York.

60. O'Day, D.H. (1979). Aggregation during sexual development in *D. discoideum*. *Can. J. Microbio.* 25:1416-1426.

61. Pan, P., Hall, E., and Bonner, J.T. (1972). Folic acid as secondary chemotaxsis substance in the cellular slime moulds. *Nature New Biol.* 237:181-182.

62. Panbacker, R.G., and Bravard, L.J. (1972). Phosphodiestase in *D. discoideum* and the chemotactic response to cAMP. *Science* 175: 1014-1015.

63. Parish, R.W., Schmidlin, S., and Parish, C.R. (1978). Detection of developmentally controlled plasma membrane antigens of *Dictyostelium discoideum* cells in sodium dodecyl sulfate polyacramide gels. *FEBS Lett.* 95, 366-370.

64. Poste, G. and Papahadjopoulos, D. (1978). The influence of vesicle membrane properties on the interaction of lipid vesicles with cultured cells, *Ann. N.Y. Acad. Sci.* 308:164-184.

65. Rabinovitch, M., and Destefano, M.J. (1973). Cell to substrate adhesion and spreading inhibition by local anesthetics and tranquilizers. *J. Cell. Biol.* 59:276-280.

66. Rabinovitch, M., and Destefano, M. (1975). Cell to substrate adhesion and spreading: inhibition of cationic anesthetics. *J. Cell. Physio.* 85:189-194.

67. Raul, S.M., Imam, S.A. and Shukla, O.P. (1986). Phenothiazines as potential antiamoebic agents. *Curi. Sci.* (Banglaroe) 55(13): 616-617.

68. Riedel, V., Gerisch, G. (1971). Regulation of extracellular cyclic AMP phosphodiesterase activity during development of *Dictyostelium discoideum*. *Biochem. Biophys. Res. Commun.* 42:119-121.

69. Robertson, A.D., and Grutsch, J.F. (1981). Aggregation in *Dictyostelium discoideum*. *Cell* 24:603-611.

70. Ross, W., and Gerisch, G. (1976). Receptor mediated adenylate cyclase activation in *Dictyostelium discoideum*. *FEBS Lett*. 68: 170-172.

71. Ross, W., Nanjundiah, V., Malchow, D., and Gerisch, G. (1975). Amplification of cAMP signals in aggregating cells of *D. discoideum*. *FEBS Lett*. 53:139-142.

72. Roufogalis, B.D. (1981). Phenothiazine antagonism of calmodulin: A structurally non-specific interaction. *Biochem.Biophy.Res. Commu*. 98(3): 607-613.

73. Saga, Y. and Yanagisawa, K. (1982). Macrocyst development in *D. discoideum*. I. induction of synchronous development by giant cells and biochemical analysis. *J. Cell Sc*. 55: 341-352.

74. Salhab, A.S. and Dujovne, C.A. (1986). Toxicity of promazine and chlorpromazine to isolated rat hepatocytes and its modification by liposome entrapment. Pharmacology 33:311-321.

75. Schaffer, B.M. (1975) Scretion of cAMP induced by cAMP in the cellular slime mould, *Dictyostelium discoideum*. *Nature* (London) 255: 549-552.

76. Schaap, P., and Wang, M. (1986). The possible involvement of oscillatory cAMP signaling in multicellular morphogenesis of the cellular slime moulds. *Dev. Bio*. 105: 470-478.

77. Seeman, P. Kwant, W.O. and Argent, W. (1969). Membrane expansion of erythrocytes ghosts by tranaquilizers and anesthetics. *Biochim. Biophys. Acta*. 183: 499-511.

78. Seeman, P., (1972). The membrane effects of anesthetics and tranquilizers. *Pharmacol. Rev*. 24: 583-655

79. Sheetz P.M., Painter G.R., and Singer, S.J., (1976) Biological membranes as bilayer couples III. Compensatory shape changes induced in membranes. *J. Cell. Bio*. 70: 193-203.

80. Siesjo, B.K. (1982). Anesthesia and brain metabolism. *Acta. Anesth. Scand*. 26:652-653.

81. Spudich, J.A. (1974). Biochemical and structural studies of actomyosin - like proteins from non muscle cells. II purification, properties and membrane association of actin from amoebae of *Dictyostelium discoideum*. *J. Biol. Chem*., 249: 6013-6020.

82. Spudich, J.A. (1987). Introductory remarks and some biochemical considerations. *Methods in Cell Biology*. 28:3-8.

83. Spudich, J.A. and Spudich, A. (1982). Chemotaxis. p. 169-189. In "The development of *D. discoideum*" (Loomis ed.) Academic press, New York.

84. Sussman, M. (1987). Cultivation and synchronous morphogenesis of *Dictyostelium* under controlled experimental conditions. *Methods in cell biology.* 28:9-28.

85. Sussman, R.R., and Sussman, M. (1967). Cultivation of *Dictyostelium discoideum* in axenic medium. *Biochem. Biophy. Res. Commun.* 29:53-55.

86. Takeichi, Masashi, R. and Takeshi, S. (1987). Quantitative election microscopic investigations on changes of mitochondria in long term CPZ administration in rat brain, liver and heart. *Jpn. J. Psychyiatry Neurol.* 41(4): 749-754.

87. Takeuchi, I., Toshiaki Noce, and Masao Tasaka. (1986). Prestalk prespore differentiation during development of *Dictyostelium discoideum.* *Curr Top in Dev. Bio.* 20:(243-254).

88. Van Haastert, P.J.M., and Konijn, T.M. (1982). Signal Transduction in the Cellular Slime Moulds. *Mol. Cell. Endocrinol.* 26:1-17.

89. Van Haastert, P.J.M., and R.J.W. DeWit. (1984). Demonstration of receptor heteroginity and negative cooperativity by non-equilibrium binding experiments. The cell surface cAMP receptor of *Dictyostelium discoideum.* *J. Biol. Chem.* 259: 13321-13328.

90. Volpi, M, Shaafi, R.I., Epstein, P.M., Andrenyak, M., and Feinstein, M.B. (1981). Local anesthetics, mepacrine and propranolol are antagonists of calmodulin. *Proc. Natl. Acad. Sci.* USA 78(2): 795-799.

91. Wood, N.C. and Nugent, K.M. (1985). Inhibitory effects of chlorpromazine on *Candida* species. *Antimicrob. Agents Chemother.* 27: 692-694.

92. Wurster, B., Sehubiger, K., Wick, U., and Gerisch, G. (1977). Cyclic GMP in *Dictyostelium discoideum*: Oscillations and pulses in response to folic acid and cAMP signals. *FEBS Lett.* 76:141-144.

93. Yamaguchi, T., Watanabe, S, and Kimoto. E, (1985). ESR Spectral changes induced by chlorpromazine in spin labeled erythrocyte ghost membranes. *Biochim. Biophy. Acta* 820: 157-164.

94. Zachowski, A., and Durand, P. (1988). Biphasic nature of binding of cationic ampipaths with artificial and biological membranes. *Biochim. Biophy. Acta.* 337:411-416.

95. Zirkle, C. and Kaiser, C. (1968). *Antipsychotic agents.* p.1410-1411. In "Medicinal chemistry" (Alfred Burger ed.) McMillan publishing company.